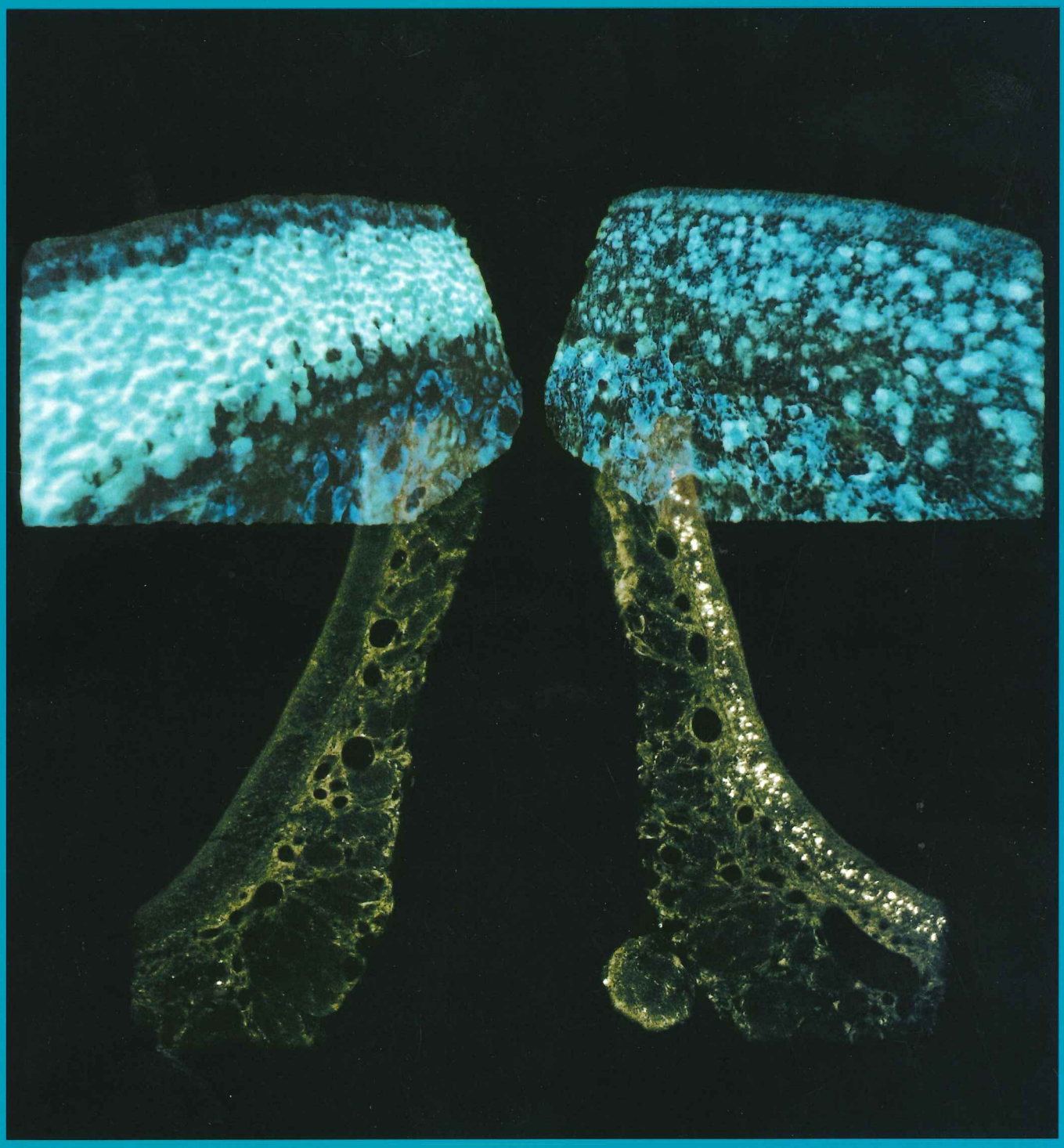
OWEN



CALTECH Biology Annual Report 2003

Legend for the front cover illustration

Sylvian Bauer and Professor of Biology Paul H. Patterson

Apoptosis-committed olfactory sensory neurons, as evidenced in only one side of the nasal septum by strong TUNEL staining present in the olfactory epithelium two days after unilateral axotomy (black and white picture), were shown by *in situ* RT-PCR to express the cytokine LIF mRNA (right blue picture) as soon as eight hours post-lesion. Mature olfactory sensory neurons were identified on the adjacent section by *in situ* hybridization for olfactory marker protein (OMP, left blue picture).

Legend for the back cover illustration

Farshad Moradi and Professor of Biology Christof Koch

Human brain activation following visual stimulation by photographs of faces versus scenes and non-face objects. Color map indicates voxels that showed a significant BOLD signal change during exposure (red: faces, blue: scenes. See color key at the middle right side), overlaid on T1-weighted anatomical images (top: axial, middle: sagittal, bottom: coronal slices). The experiment was conducted in the 3.0-Tesla human magnet at Caltech's new Brain Imaging Center (3.3 x 3.3 x 3 mm^3 voxels, TR = 2 s, TE = 30 ms).

Division of Biology

California Institute of Technology

Pasadena, CA

Annual Report 2002 - 2003

BIOLOGY - 2003

Yolanda Duron, Annual Report Coordinator

Research Reports

Biological research summarized in this report covers the time period from June, 2002, through July, 2003. The annual report is not intended to serve as an official forum, since some portions of the research listed in this report have not yet been published. When referring to an individual abstract(s), special permission must be obtained from the investigator.

References to published papers cited throughout the report are listed at the end of each individual research report.

TABLE OF CONTENTS

INTRODUCTION

DEVELOPMENTAL AND REGULATORY BIOLOGY

ERIC H. DAVIDSON, PH.D.

MICHAEL H. DICKINSON, PH.D.

SCOTT F. FRASER, PH.D.

BRUCE A. HAY, PH.D.

EDWARD B. LEWIS, PH.D.

ELLIOT M. MEYEROWITZ, PH.D.

ELLEN V. ROTHENBERG, PH.D.

ELLEN V. ROTHENBERG, PH.D.

MELVIN I. SIMON, PH.D.

PAUL W. STERNBERG, PH.D.

MOLECULAR, CELLULAR, AND INTEGRATIVE NEUROSCIENCE

STRUCTURAL, MOLECULAR AND CELL BIOLOGY

Introduction

75, 50 and 25 Years Ago

1928, 75 years ago, was the date of founding of the present Division of Biology. The 50th anniversary was marked in the 1978 Biology Annual Report by a brief history of the Division written by Charles Brokaw, including the following excerpt:

"As his initial faculty, Morgan recruited Alfred H. Sturtevant from Columbia to head his group in genetics, and Sterling Emerson and Ernest G. Anderson from the University of Michigan. All four became Caltech faculty members on July 1, 1928, but the summer of 1928 was spent at Woods Hole while the first increment of the Kerckhoff Laboratories was being completed in Pasadena… Morgan, Sturtevant, Emerson and Anderson moved into their new laboratories in the autumn of 1928, along with two research fellows (Yoshitaka Imai and Theodosius Dobzhansky) and three graduate students from Columbia who were employed as teaching fellows (Albert Tyler, Russell Biddle, and William Hetherington). Morgan also brought Calvin Bridges from Columbia…"

Biology 1953 at the California Institute of Technology

"During the 1952-53 academic year the research, teaching and other activities of the Division of Biology were carried on by a staff of 95 members holding academic titles, 36 graduate students, and 148 research assistants and non-academic staff members… These workers have attacked various problems in the broad field of the biological sciences… One has investigated how desert plants can store in the soil and later utilize the life-giving water condensed on their leaves as dew; another has attempted to find the particular area of the rat's cerebral cortex that is associated with the instinct to hoard food pellets… One group has been working to determine the kinds and number of breaks and rearrangements in the chromosomes of fruit flies exposed to radiation from an A-bomb set off at the Nevada test sites in the spring of 1953…"

"Several lines of evidence point to desoxyribonucleic acid as an important gene component or perhaps even as the gene itself…"

"Three lectures at the Institute were sponsored by the Hixon Fund. On November 24 Dr. Marthe Vogt, F.R.S. (Edinburgh), spoke on 'Brain Sympathin.' Two lectures were given by Professor Roger W. Sperry, of the University of Chicago. On January 6 his subject was 'Prefunctional Organization of Brain Patterns' and on January 8 'Recent Studies on Neural Mechanisms of Visual Perception.'"

"With the death of Lilian V. Morgan (Mrs. Thomas Hunt Morgan) in December, 1952, members of the Division of Biology lost a respected colleague and a friend to whom they were devoted. For twenty-four years Mrs. Morgan worked in Kerckhoff on various phases of the genetics of *Drosophila*…"

"Gifts and bequests by the late Norman W. Church have provided approximately a million dollars to be used for the construction of a new laboratory to be known as the Norman W. Church Laboratory for Chemical Biology… Preliminary plans are complete, but it has not yet been decided when construction will begin."

Caltech Biology Annual Report 1978

"It is thus increasingly clear that the final act of the turning-on of gene expression is caused by histone acetylation. The only remaining questions are what directs the histone acetylase, a chromosomal protein, to acetylate the histones complexed with particular sequences of DNA?"
"The problems of the control of gene expression in higher eukaryotes appear to be too complex to approach on the level of the entire genome…"
from the Report of Prof. James Bonner

"We have obtained evidence that the bithorax gene complex (BX-C) is under negative control. That is, the BX-C genes which normally are repressed in the head and first two thoracic segments become derepressed in those segments when another gene outside the complex, Polycomb (discovered by P.H. Lewis) is inactivated by mutation.
from the Report of Prof. Edward B. Lewis

"Most remarkable during the year has been the emergence of our laboratory at the leading edge of the soft-pellet world. Suggested originally by Watership Down, this excursion into the fascinating domain of coprophagy has sniffed out immunological territory still to be charted…"
from the Report of Prof. Ray D. Owen

Michael Elowitz

Professor Michael Elowitz joined the Division of Biology in June, 2003 as our first faculty hire targeted in computational biology. A major focus of his work has been the use of synthetic gene regulatory networks, constructed in *E. coli* using the green fluorescent protein (GFP) and its color variants, to study the underlying logic of genetic regulatory systems.

Professor Elowitz's fundamental approach is to use synthetic networks as simpler counterparts to the complex networks found in nature, built out of a small number of well-characterized genetic elements borrowed from other systems. Such synthetic networks can be built up successively from well-understood subunits, and used as "*in vivo* models" of natural cellular programs to test the accuracy of our understanding of the behavior of simple elements inside cells. Their behavior can be used to "probe," or report, the characteristics of other elements that act upon them within cells. His methodology integrates mathematical modeling with direct measurements in living cells, complements and extends existing biological techniques.

Professor Elowitz received his B.A. in Physics from the University of California at Berkeley, and his M.A. and Ph.D. degrees in Physics from Princeton for his thesis work with Dr. Stanislas Leibler. His graduate work showed the feasibility of constructing simple genetic networks for the study of gene regulation. He went on to perform postdoctoral research at the Rockefeller University in the lab of Dr. Arnold Levine, analyzing the noise intrinsic to genetic networks. In recognition of his excellent work and promise, he was awarded a Burroughs-Wellcome Career Award to support the final years of his postdoctoral research and the first years of his work as an assistant professor.

HONORS AND AWARDS - 2003

Anderson Zhou Bjorkman

Qiao Zhou is the winner of the Ferguson Award for the 2002-2003 academic year. This award goes to the student who is judged by the faculty to have produced the best Ph.D. thesis over the past year. Dr. Zhou performed his graduate studies in the laboratory of Dr. David J. Anderson.

His thesis work focused on mechanisms that control cell fate determination in the developing vertebrate central nervous system (CNS). Neural stem or progenitor cells have to make a series of developmental decisions critical to cell type diversification in the CNS. For example, they must decide whether to generate neurons or glia; they must decide what subtype(s) of neuron to generate; and they must decide what subtype(s) of glia (astrocyte or oligodendrocyte) to generate. Prior to Dr. Zhou's work, the extent to which these decisions were coupled, or independent of one another, was not clear. Dr. Zhou discovered a novel family of basic helix-loop-helix (bHLH) transcription factors, called the "Olig" genes. Through a series of gain- and loss-of function experiments performed in chick embryos and mice, Dr. Zhou established that these genes link a choice between two alternative neuronal subtypes (motoneuron vs. interneuron), to a choice between two alternative glial subtypes (oligodendrocyte vs. astrocyte).

PROFESSORIAL AWARDS 2003

Professor of Biology, David J. Anderson, was named a Fellow of the American Association for the Advancement of Science, 2002.

President and Nobel Laureate, Professor David Baltimore received the following awards: Achievement Award in Basic Biotechnology from The Biotechnology Center at New York University; and the American Medical Association Scientific Achievement Award.

Professor Pamela J. Bjorkman, Executive Officer for Biology, and Full Investigator, HHMI, was the recipient of the following awards: University of Oregon Department of Chemistry Alumni Achievement Award; Richard M. Furlaud Distinguished Lecturer Award at the Rockefeller University; and the Paul E. Lacy Lecture at Washington University in St. Louis.

Albert Billings Ruddock Professor of Biology, Marianne Bronner-Fraser, received the following awards: the Javits Awards-NINDS; and the Scientific Advisory Board-March of Dimes.

Norman Chandler Professor of Cell Biology, Eric H. Davidson, was awarded the Alexander Kovalevsky Medal for outstanding achievements in the field of evolutionary embryology.

George W. Beadle Professor of Biology, Elliot Meyerowitz, gave the 2002 Bateson Lecture at the John Innes Institute in Norwich, U.K. in November.

Professor Paul H. Patterson received the 2002/2003 McKnight Foundation Neuroscience of Brain Disorders Award.

Professor Shinsuke Shimojo, was awarded the 2003 Tokizane Award by the Japanese Society of Neuroscience. The award was provided for his work on the discovery of new perceptual phenomena related to visual contours and surfaces, and understanding of underlying neural processes.

Assistant Professor Athanassios G. Siapas was awarded an Alfred P. Sloan Fellowship.

Howard and Gwen Laurie Smits Professor of Cell Biology Alexander Varshavsky has received the 2003 ICRF Award for Excellence in Cancer Research from the Israel Fund for Cancer Research. In 2003, Varshavsky gave keynote lecture at the NIH Symposium on the Ubiquitin System in Health and Disease, in Bethesda, MD.

OTHER AWARDS

Gary Hathaway, Director of the Protein Micro Analytical Laboratory, received the following award: The Association of Biomolecular Resource Facilities (ABRF) first annual best oral presentation award was given to the Protein/Peptide MicroAnalytical Laboratory at the ABRF '03: Translating Biology Using Proteomics and Functional Genomics, February 10-13, 2003, Denver, Colorado.

In 2003, the Biology Division was host to the following Moore Distinguished Scholars in Biology:

Cornelia Isabella Bargmann, Professor
Department of Anatomy and of Biochemistry and Biophysics
The University of California, San Francisco

Ioan Negrutiu, Professor
Department of Life and Earth Sciences
Ecole Normale Superieure de Lyon, France

Ottoline Leyser
Reader, Department of Biology
The Plant Laboratory, University of York, England

In 2003, the Biology Division hosted the following Wiersma Visiting Faculty in the last year:

Frances Ashcroft – January 22, 2003
Department of Physiology
Oxford University, UK
"The Ying and Yang of the KATP Channel: Diabetes and Congenital Hyperinsulinism"

Marina De Biasi – February 4, 2003
Division of Neurosciences
Baylor College of Medicine
"Autonomic Function in Neuronal Nicotinic Receptor Mutant Mice"

John Dani – February 5, 2003
Division of Neurosciences
Baylor College of Medicine
"Nicotinic Cholinergic Mechanisms in the Central Nervous System"

Kristen Harris – March 12, 2003
Institute for Molecular Medicine and Genetics
Medical College of Georgia
"Ultrastructure of LTP"

Barry Keverne
Department of Zoology
Sub-Department of Animal Behavior
University of Cambridge, UK
April 22, 2003, "Olfactory Learning in Mammals"
April 23, 2003, "Impact of Imprinting on Brain and Behavior"

Ivor Mason – July 23, 2003
Department of Developmental Neurobiology
MRC Brain Development Program
UMDS Guys Hospital, London
"Deployment and Redeployment of Fibroblast Growth Factors to Instruct Vertebrate Head Development"

In 2003, special named lectures were presented:

WEIGLE LECTURE – JANUARY 22, 2003
Stanley Falkow
Department of Microbiology and Immunology
Stanford University
"How might *Heliobacter pylori* cause cancer?"

NORMAN DAVIDSON LECTURE – APRIL 7, 2002
James C. Wang
Department of Molecular and Cellular Biology
Harvard University
"The type 1A DNA topoisomerases: Omnipresence and role in genome stability"

NORMAN HOROWITZ LECTURE – APRIL 14, 2003
James Crow
Department of Genetics
University of Wisconsin
"Weinberg's heritage: Mutation and paternal age"

Photos from Broad Center Magnet delivery on April 8, 2003 by Bob Paz

Photos from the Biology Division Retreat at El Capitan Canyon, Oct 17-19, 2003 taken by Elliot Meyerowitz

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George Beadle Professor of Biology

Seymour Benzer, Ph.D., D.Sc.h.c. Crafoord Laureate
James G. Boswell Professor of Neuroscience (Active)

Charles J. Brokaw, Ph.D., *Biology*

John J. Hopfield, Ph.D.
Roscoe G. Dickinson Professor of Chemistry and Biology

Norman H. Horowitz, Ph.D., *Biology*

Edward B. Lewis, Ph.D. Nobel Laureate
Thomas Hunt Morgan Professor of Biology (Active)

Ray D. Owen, Ph.D., Sc.D.h.c., *Biology*

Senior Research Associate Emeriti

Roy J. Britten, Ph.D.
Distinguished Carnegie Senior Research Associate in Biology, Emeritus

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Frank P. Hixon Professor of Neurobiology

Richard A. Andersen, Ph.D.
James G. Boswell Professor of Neuroscience

David J. Anderson, Ph.D.[1]
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Grace C. Steele Professor of Molecular Biology

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[1]*Joint appointment with Howard Hughes Medical Institute*
[2]*Undergraduate Student Advisor*

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[1]Joint appointment with Howard Hughes Medical Institute

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Summary: We are interested in studying the intracellular signaling pathways that regulate cell fate determination during T cell development. Each developing T cell expresses a unique receptor (T Cell Receptor) that interacts with molecules of the major histocompatibility complex (MHC) associated with short peptides. The TCR determines the antigen specificity of the T cell and is the most important element in the regulation of T cell development and function. Ligand occupancy of the TCR in immature CD4+CD8+ thymocytes may result in activation-induced apoptosis (negative selection), or in their survival (positive selection) and further differentiation into either CD4 or CD8 mature T cells (CD4/CD8 lineage commitment). Though the existence of these selective events has been documented extensively, it remains puzzling that signals transmitted through the same receptor in seemingly identical cells may provoke either death or survival. Hence, the biochemical signals elicited by ligand occupancy of the TCR must either vary with the stimulating antigen, or must be interpreted flexibly in the context of other receptor-mediated signaling processes.

Our research is focused on the signaling requirements during thymocyte positive selection and lineage commitment. Our previous work has shown that the strength of the signals derived from the tyrosine kinase Lck determine whether a DP develops into the CD4 or the CD8 lineage, and we have recently identified GATA-3, as a downstream effector of Lck in this developmental decision. Work in the lab is aimed at understanding the molecular basis of this process using biochemical and genetic approaches. We are also very interested in the role of Ras and its downstream effectors in the regulation of positive selection, since previous experiments showed that this pathway is required for positive selection of T cells. The projects in the lab address the regulation of Ras activation during T cell development, as well as the downstream effectors of Ras involved in positive selection.

The long term aim of our research is to understand better the signaling networks that regulate cell fate determination during T cell development, and to unravel the genetic programs that give rise to different types of mature T cells.

1. Gata-3 expression is regulated by TCR signals and controls CD8 and CD8 T-cell development

G. Hernandez-Hoyos, M.K. Anderson, C. Wang

GATA-3 is a member of the GATA family of zinc-finger transcription factors. Within the hematopietic tissues, GATA-3 is exclusively expressed in the T-cell lineage. Expression of this transcription factor is already present in the earliest precursors in the thymus -the organ where T cells develop. Expression of GATA-3 is initiated from embryonic day 12.5 – when T-cell development begins - and is maintained throughout T-cell development and in peripheral T cells. We found that its expression is tightly regulated during development where it is upregulated during positive selection of CD4 but not CD8 T cells in the thymus. Subsequently, in peripheral CD4 T cells GATA-3 is upregulated in T helper 2 (Th2) but not T helper 1 (Th1) cells, which are two mutually exclusive developmental fates of CD4 cells that regulate different types of immune responses. In Th1/Th2 differentiation, GATA-3 is an essential positive regulator of the latter and a negative regulator of the first. We decided to address the significance of the differential expression of GATA-3 during CD4 and CD8 development, expecting that it would operate as a CD4/CD8 regulator in the way it regulates T h1/Th2 development. Due to the early lethality of the GATA-3 knockout mice (E12.5) and the requirement for GATA-3 expression at the earliest (double-negative) stages of T cell development, the role of GATA-3 during positive selection of CD4 and CD8 T cells had not been possible to address.

We have previously shown that strong T cell receptor/Lck (TCR/Lck) with the signals induce CD4 development while weak TCR/Lck signals induce CD8 development; TCR/Lck is the receptor/tyrosine kinase complex that initiates T-cell responses. First our studies determined that upregulation of GATA-3 expression in DP thymocytes is triggered by TCR stimulation, and the extent of upregulation correlates with the strength of the TCR/Lck signal. These results indicated that differences in the strength of the TCR/Lck signal can translate into different levels of GATA-3 expression. Then we performed gain-of-function and loss-of-function studies using retroviral transfer of wildtype and mutant GATA-3 constructs as well as small interference RNAs into fetal T cell precursors, and then analyzed their development in reaggregate fetal thymic cultures (RgFTOC). This approach allowed us to bypass the early blocks in T-cell development induced by alteration of GATA-3 expression. These studies showed that overexpression of GATA-3 or a partial GATA-3 agonist during positive selection inhibits CD8 SP cell development. Conversely, expression of the GATA-3 antagonist ROG or of a GATA-3 siRNA hairpin markedly enhances development of CD8 SP cells and reduces CD4 SP development. Our results show that inhibition of Gata-3 function enhances CD8 development at the expense of CD4 development, while increased GATA-3 expression has the reciprocal effect. Together with the observation that Gata-3 is naturally upregulated during positive selection of CD4 but not during selection

of CD8 cells, our data indicate that the role of Gata-3 in positive selection is to inhibit CD8 development and promote CD4 development. We propose that GATA-3 contributes to linking the TCR signal strength to the differentiation program of CD4 and CD8 thymocytes.

2. Elucidating the role of the Ras/MAP kinase pathway in lineage determination of thymocytes using a lentiviral transgenesis model

H. Green

Signaling initiated by T cell receptor (TCR) engagement is mediated through the tyrosine kinase p56lck and is transduced to a number of distinct signaling pathways. Previous work in our laboratory has shown a pivotal role for Lck in positive selection and CD4/CD8 lineage determination in thymocytes. In addition, the Ras/MAPK pathway is required for positive selection of thymocytes, but its relevance for lineage commitment has been controversial. Here we are attempting to determine if the Ras/MAPK pathway plays a role downstream of Lck in CD4/CD8 lineage determination.

Using a method of lentiviral-mediated transgenesis developed by the Baltimore laboratory here at Caltech, we are creating several lines of mice expressing a kinase-dead, dominant-negative form of the MAPKK Mek1 (dMek) or the dominant-negative form of Ras (dnRas) under a thymocyte-specific promoter. These mice also express the AND transgenic TCR, an MHC class II-restricted TCR that promotes the development of mature CD4 single positive thymocytes. Given the nature of this method of lentiviral transgenesis, each pup in a litter of mice will have received a different number of copies of the transgene and will have different protein expression levels of dMek or dnRas. Therefore, each litter will act as a dose response experiment for the given transgene. We hope then to correlate this dose response to the development of AND+/CD8+ mature single positive thymocytes, indicating whether the Ras/MAPK pathway plays a role downstream of p56lck. By extending this experiment to other class II-restricted TCRs, we hope to determine whether alterations in the Ras pathway can change the developmental program of T cells.

3. Analysis of the role of Ras signaling during T cell development

M. Laurent

The Ras family of GTP-binding proteins is important in the regulation of many different activities within many diverse cell types, including choices made by T cells during their development. In this laboratory, it was previously shown that the expression of a dominant-negative form of Ras (dnRas) within developing murine thymocytes is sufficient to block positive selection, such that the number of mature T cells is significantly reduced. Downstream of Ras, the Erk family of MAPK is also required for positive selection, but genetic analysis suggest that their activation is not sufficient to rescue a dominant negative Ras phenotype. We are trying to determine which additional Ras downstream effectors are important in mediating Ras effects during positive selection.

To address this issue, we are utilizing a series of Ras effector mutants that contain point mutations made within the effector loop region of a constitutively active form of Ras. Each of these mutants stimulates only a specific subset of pathways downstream of Ras, thereby providing us with a method to study the effects of activating only particular signaling pathways. Using retroviral-mediated gene transfer, these mutants were introduced into fetal thymocytes derived from animals expressing the dnRas transgene in which positive selection is significantly reduced. Infected thymocytes are allowed to develop in fetal thymic organ culture (Ftoc) for approximately two weeks and subsequently analyzed for their ability to rescue the effects of a dominant-negative form of Ras.

One of the mutants that has been tested enhances T cell development in the dnRas background. Its described effects suggest that the activation of Ral signaling assists Raf-MEK-ERK signaling in promoting T cell maturation. Future experiments will address the molecular interactions of Ral in during Ras-mediated positive selection.

4. KSR connects Ras to the MAPK cascade

M. Laurent

It is well established that the Ras-Raf-MEK-ERK cascade is necessary for proper thymocyte development. However, the details regarding the regulation of this cascade are incomplete, particularly in regards to the molecular associations made during and following Ras signal activation. Three novel molecules, the Kinase Suppressor of Ras (KSR), Connector Enhancer of KSR (CNK), and the Suppressor of Ras 8 (SUR-8), were identified based upon their ability to regulate Ras signaling in invertebrate developmental model systems. To determine if these molecules play a similar role during murine T cell development, these molecules were tested in a Ftoc assay. Of these molecules, only the over-expression of KSR affects fetal thymocyte development, eliciting a block in the number of mature thymocytes, a phenotype consistent with the inhibition of Ras signaling.

To determine the structural elements of KSR necessary for these results, a series of KSR mutants have been generated and tested in this assay. This analysis reveals that the ability of KSR to interact with MEK is necessary for its observed effects in thymocytes. The other KSR domains tested appear to be dispensable for this effect suggesting that the ability of KSR to couple Ras to the MAPK cascade is critical for its effect on T cell development. In agreement with this observation, KSR over-expression in a thymoma cell line inhibits ERK activation, while CNK and SUR-8 had no effect in this assay. These results suggest that KSR may function as the main scaffolding protein connecting Ras to the MAPK cascade, while SUR-8 and CNK may connect Ras to additional pathways. Currently, the effects of the over-expression of KSR and a dominant-negative form of MEK

on fetal thymocyte development are being compared to assess the ability of KSR to affect thymocyte development.

5. EGR transcription factors as mediators of Ras signaling during thymocyte selection

E. Tse

Each developing thymocyte expresses a unique T cell receptor (TCR), whose ligation triggers either apoptosis (negative selection) or survival and further differentiation (positive selection), and the process by which such antithetical fates are adopted in response to signals from a single receptor has remained a key question for immunologists. It has been previously shown that the Ras-ERK MAPK cascade plays a critical role in positive selection, but is dispensable for negative selection. Furthermore, recent studies have shown that activation of this cascade in thymocytes undergoing positive selection results in decreased DNA binding activity by the basic helix-loop-helix (bHLH) proteins E12 and E47; this reduction is effected by the upregulation of the helix-loop-helix (HLH) protein Id3, an inhibitor of E12 and E47. My studies address the role of the early growth response (Egr) family of immediate early genes as a potential downstream mediator of the Ras-ERK MAPK cascade in Id3 induction and, accordingly, in positive selection.

Our lab has generated a transgenic line of mice overexpressing NAB2, a suppressor of Egr activity, under the control of a T cell-specific promoter. NAB2 expression elicits a mild reduction in the appearance of mature, positively selected T cells in the thymus; this reduction approaches 50% in OVA mice, whose exogenous expression of a specific class-I restricted TCR allows the study of a more uniform developing T cell population. The ongoing characterization of these mice should reveal whether the expression of the NAB2 transgene is able to impair TCR-ligation-induced Id3 upregulation and, correspondingly, positive selection.

I have also begun the study the effects of disrupting Egr activity with two other dominant negative Egr proteins, WT1:Egr1 and Egr1; both retain the wildtype DNA-binding domain of Egr1 but lack the transactivation domain, and WT1:Egr1 also bears the transcriptional repressor domain of Wilms tumor-associated protein. In fetal thymic organ cultures (FTOCs), an *ex vivo* model of thymocyte development, thymocyte populations transduced with WT1:Egr1 show reduced numbers of CD4+CD8- cells with no significant change in the numbers of CD8+CD4- cells; thymocyte populations transduced with Egr1 show a similar but weaker phenotype, consistent with its lack of exogenous repressor function. Currently, FTOCs with fetal thymocytes expressing transgenic TCRs are being performed to further characterize the effects of disrupting Egr function. These experiments should provide additional insight into the role of Egr family members as mediators of the Ras-ERK MAPK cascade in positive selection.

6. Regulation of T-cell development by Notch: Proliferation vs. differentiation

G. Hernandez-Hoyos, E. Tse, C. Wang

Notch is a family of transmembrane receptors of which there are four genes in mammals - Notch1 to 4 - all of which are expressed during T cell development. Binding of Notch to its ligands induces proteolytic cleavage that frees the intracellular domain (ICN or activated Notch). This fragment translocates into the nucleus where it associates with the transcription factor CSL to positively regulate transcription of a number of genes. Activation of Notch is involved in different cellular processes including regulation of cell fate determination, differentiation and cell survival in a variety of tissues. Work from various groups suggests that the Notch cascade is involved in regulating many aspects of T cell development, including the T/B, / and CD4/CD8 lineage decisions. Nevertheless the mechanisms by which Notch exerts its effects are not well understood, and in some cases the physiologic role is unclear. During positive selection of T cells, overexpression of activated Notch signaling can block CD4, or both CD4 and CD8 development, depending on which domains of the Notch intracellular fragment are overexpressed. As a result it was recently suggested that activation of Notch serves to downregulate TCR-mediated responses during positive selection in the thymus. Pre-TCR selection is another TCR-mediated event during T cell development that occurs prior to positive selection. Pre-TCR selection leads to significant proliferation at the DN4 and immature CD8 SP (CD8 ISP) stages and to subsequent differentiation into quiescent DP cells. The latter cells are then subjected to positive and negative selection events. Interestingly, although both pre-TCR and positive selection signals are transduced by similar signaling pathways, Notch was not reported to inhibit the first.

We have undertaken a more detailed analysis of the role of Notch during both pre-TCR selection and positive selection. First we determined if Notch inhibited positive selection by acting on the Ras/MAPK pathway, as had been suggested. The Ras/MAPK pathway is downstream of the TCR signal, is activated early and necessary for positive selection. Using bicistronic retroviral vectors co-expressing GFP and Notch and primary DP thymocytes as well as a DP cell line we tested whether overexpression of activated Notch affects the MAPK cascade, by measuring phosphorylation of the MAPK ERK1,2. Although activated Notch reproducibly inhibits activation, as determined by inhibition of expression of the surface markers CD69 and CD5, it does not inhibit ERK phosphorylation. We concluded that Notch does not significantly inhibit activation of the MAPK pathway and the block in positive selection must be induced by other factors. Second, using fetal thymic organ culture and a recently described *in vitro* T cell differentiation system we are currently exploring the role of Notch in pre-TCR selection. So far our results indicate that activated Notch increases the CD8 ISP cell population and the fraction of cells undergoing mitosis. These results

are most consistent with Notch enhancing proliferation following pre-TCR selection. The increase in proliferation is so dramatic that cells enter the DP cell stage (normally a quiescent stage) as blasts. As described by others, DP cells undergo positive selection quite inefficiently in the presence of activated Notch. We think that the inefficient selection results as least in part from the abnormally activated state of the DP cells. We are currently trying to determine if the proliferation is dependent on CSL or on a CSL-independent pathway by employing RNA interference to eliminate CSL expression. This loss-of-function approach should yield information relevant to the physiological role of Notch during both pre-TCR selection and positive selection events. We also plan to address molecular aspects of the Notch-induced proliferation by determining if Notch alters the expression of cell cycle regulators. In conclusion, our results suggest that instead of inhibiting TCR-mediated events, Notch is a T cell growth factor that operates during or following pre-TCR selection.

7. Real-time detection of MAPK activity using fluorescence resonance energy transfer

H. Green

Previous work has shown that the activation of the Ras/MAP Kinase (MAPK) signal transduction pathway is necessary for positive selection of thymocytes. Positive selection is a process by which developing thymocytes receive signals through the T Cell Receptor (TCR) and determine whether they are viable and will live, or not viable and will die. Our purpose is to study the biochemical nature of Erk1/2 MAPK signaling within these thymocytes to gain insight into the mechanisms of positive selection. In particular, we wish to examine whether the cascade acts as a signaling rheostat, in which increasing exogenous signal begets increasing internal signaling in a linear fashion, or as an all-or-none response, in which internal signal is begotten only by reaching a threshold of exogenous signal. In an analogous fate determination system, data from *Xenopus* oocytes has implicated that an all-or-none mechanism of MAPK activation controls oocyte maturation induced by progesterone.

To perform these experiments we must analyze MAPK activity on a single-cell basis. Using the cyan and yellow variants of Green Fluorescent Protein (CFP and YFP, respectively), we are developing an assay that can detect MAPK activity on a single-cell level using single- and two-photon confocal microscopy and flow cytometry. Utilizing Fluorescence Resonance Energy Transfer (FRET), a phenomenon by which two fluorophores come into close proximity and the emission of one fluorophore (donor) causes the excitation of the other fluorophore (acceptor), we hope to be able to create a highly sensitive fluorescent protein substrate sensitive to Erk MAPK activation. Using short peptide sequences taken from known Erk substrates, we have created several gene fusion constructs containing CFP and YFP, with these peptides acting as phosphorylation sensitive substrates between the two fluorescent proteins. Although these constructs have been successfully tested *in vitro*, they are not sensitive enough for *in vivo* use. We are currently trying to improve their function.

8. Role of phosphatidylinositol 3-kinase plays during T cell development

S.D. Barbee

Phosphatidylinositol 3-kinase (PI3K) has been described in a diverse array of roles in multiple cell types, including lymphocytes. The best-characterized of these are the proliferative and survival effects mediated by the class IA family members; PI3K is responsive to a variety of stimuli in T cells including stimulatory signals (CD3, CD28), inhibitory receptors (CTLA-4), and growth factors and cytokines (IL-2, -4, -7). A heterodimer, PI3K activity causes the accumulation of phosphorylated inositol lipids on the inner surface of the cell membrane, thus recruiting and thereby activating two principal effector molecules, Akt and Itk. Akt appears to be a bifurcation point that mediates many of PI3K's functions, but the best-characterized role of Akt is the regulation of cell survival via multiple downstream effectors including Bad/Bcl-XL, NF-kB, GSK-3β, and forkhead transcription factors. Itk is a T cell specific member of the Tec family of kinases, which have been shown to modulate Ca++ signaling pathways. The most famous Tec kinase is Btk: the immune deficiency diseases XLA in humans and *xid* in mice have been mapped to defects in Btk signaling. Genetic alterations of PI3K effectors including Akt and Itk have yielded a variety of phenotypes in mice that suggest a crucial role for PI3K in thymocyte development.

We have generated several lines of mice expressing p110ABD, the domain of the class IA PI3K p110a that mediates binding to the adaptor subunit, under the control of the *lck* proximal promoter. Expression of p110ABD sequesters the regulatory adaptor subunit p85, thus endogenous p110 exists as a highly unstable but catalytically active monomer. As a result, there is a constitutive PI3K activity in p110ABD thymocytes. Kinase assays reveal constitutive Akt activity in resting transgenic thymocytes, and p110ABD expression potentiates Ca++ flux responses to TCR crosslinking, likely reflecting Itk potentiation.

Phenotypically, the thymii of p110ABD animals appear largely normal but with slight increases in the percentages and numbers the mature single positive thymocyte subsets. Experiments with the AND TCR transgenic line suggests that positive selection is specifically improved, as AND+ p110ABD CD4 SP thymocytes efficiently develop in a partially-selecting H-2b/d background. The enhanced development of AND thymocytes in the presence of p110ABD expression is reminiscent of that observed with the hypersensitive Erk *sevenmake*r mutant, but we observe no potentiation of Erk signaling in p110ABD thymocytes. The effects upon positive selection are highly specific. We do not observe any alterations in the life span of DP thymocytes nor defects in negative selection, as assayed both *in vitro*

(using OT-1 deletion assays) and *in vivo* (superantigens). Thymocytes doubly transgenic for p110ABD and the ovalbumin-specific TCR OT-1 express a single clonal TCR that recognizes the peptide SIINFEKL; co-culture of these thymocytes with an antigen-presenting cell and the SIINFEKL peptide provides an *in vitro* simulation of negative selection. The p110ABD transgene does not offer statistically significant protection from deletion in these assays. Likewise, endogenous vSAG7-mediated deletion of TCR Vβ6+ thymocytes is unaltered in DBA2/J x p110ABD mice.

However, PI3K modulates more than just positive selection in thymocyte development. The Class IB PI3K isoform is associated with the bg subunits of G-protein coupled receptors (GPCR). Thymocyte development is a highly refined process, exquisitely choreographed to place thymocytes within the appropriate thymic architectural niches as differentiation progresses. This process appears to be scripted by a coordinated system of chemokine and integrin signals that direct thymocyte migration through the cortical and medullar compartments appropriately. By competitively transferring a mixture of p110ABD bone marrow and wildtype bone marrow into lethally irradiated host animals, we observe that despite parallel recolonization of the thymus and B cell compartments, p110ABD-derived T cells take much longer to appear in the periphery. These results are confirmed by a delayed appearance of peripheral T cells in neonatal p110ABD mice compared to NLC. This suggests that our disruption of class IA PI3K signaling has had an effect upon the activity of class IB PI3K downstream of the chemokine GPCRs that are involved in the final stages of thymocyte maturation.

9. Chemical-genetic analysis of the role of Lck in T cell signaling

D. Leopoldt

One of the first biochemical events after TCR stimulation is the activation of the src-family protein tyrosine kinase Lck. Studies from many laboratories, including ours, have shown that Lck plays a major role in T cell development and activation. However, the exact contribution of Lck to many of these processes remains badly defined, due in part to the limitations of the genetic and pharmacological approaches used so far. We are using a recently developed chemical-genetic approach to engineer a mutant Lck protein (Lck-T316G) that is supersensitive to highly specific low-molecular weight kinase inhibitors. These cell-permeable drugs were specifically designed to inhibit Lck-T316G, but not wildtype Lck or any other protein kinase. Their use will allow us to perform a complete or partial functional knock-out of Lck kinase activity at different stages of T cell development or immune responses.

Using the Cre/loxP-based gene targeting strategy the T316G mutation has been knocked-in exon 8 of the murine lck gene by homologous recombination in embryonic stem (ES) cells. In order to isolate a genomic lck clone from the target ES cell line we have screened a 129SVJ mouse library. Two partial clones were isolated and characterized. Clone 7-42 containing the first 8 exons of the lck gene was used for the left (short) arm of the replacement vector after introducing the point mutation by site-directed mutagenesis. Additionally, a silent mutation introducing a *Bam*HI site in exon 8 was performed. Clone 7-111 covering intron 8 and downstream sequences including exon 12 was used for the right (long) arm of the replacement vector. Pluripotent murine ES cells were transfected with the *Not*I-linearized targeting vector for homologous recombination. We have identified one correctly targeted ES cell clone by Southern blot analysis. Currently we are waiting for germline transmission of the targeted allele.

Thymocytes and peripheral T cells from wildtype and homozygous T316G mutant Lck mice will be harvested at discrete times during differentiation, treated *ex vivo* with different doses of selective and potent kinase inhibitors and tested for possible Lck-mediated signal transduction pathways. Generation of these mutant Lck-T316G mice will later allow us to test the hypothesis that Lck activity controls CD4/CD8 lineage commitment, i.e., the decision of a T cell to become either a CD4+ helper T cell or a CD8+ cytotoxic T cell.

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Summary: This laboratory is concerned with analyzing the cellular and molecular events underlying the formation, cell lineage decisions and migration of neural crest cells. The neural crest is comprised of multipotent stem-cell-like precursor cells that migrate extensively and give rise to an amazingly diverse set of derivatives. As important precursors of the peripheral nervous system, neural crest cells have been of interest to the neuroscience community for decades. In addition to their specific neuronal and glial derivatives, neural crest cells can also form melanocytes, craniofacial bone and cartilage and smooth muscle. This plasticity makes the neural crest an exciting system in which to investigate the processes by which cells make decisions about their fate.

The neural crest is induced to form in the ectoderm at the junction between the neural plate and the prospective epidermis. Following neural tube closure, these cells leave the neural tube and migrate to diverse regions throughout the embryo. The pathways along which neural crest cells migrate are well characterized, and these cells are accessible to both surgical and molecular manipulations throughout their development. As a result, in addition to being an excellent system for investigating cell fate decisions, the neural crest has become an important model system for studying cell movement and behavior.

Our laboratory concentrates on studying the cellular and molecular mechanisms underlying the induction and early development of the neural crest. This research addresses fundamental questions concerning cell commitment, migration and differentiation using a combination of techniques ranging from experimental embryology to genomic approaches to novel gene discovery. These studies shed important light on the mechanisms of neural crest formation and migration. In addition, the neural crest is a cell population unique to vertebrates. In studying the evolution of this unique population, we hope to better understand the origin of vertebrates.

Because neural crest cells are involved in a variety of birth defects and cancers such as neurofibromatosis, melanoma, neuroblastoma, our results on the normal mechanisms of neural crest development provide important clues regarding the mistakes that may lead to abnormal development or loss of the differentiated state.

10. Wnt in the ectoderm functions as a neural crest inducer

Martin Garcia-Castro, Christophe Marcelle, Marianne Bronner-Fraser

Neural crest cells, which generate most of the vertebrate peripheral nervous system and facial skeleton, arise at the border of the ectoderm and neural plate by an apparent inductive interaction between these tissues. We find that Wnts function as neural crest inducers in the trunk region of avian embryos. Wnt6 has the proper distribution pattern in the ectoderm at the time of neural crest induction, and inhibition of Wnt signaling *in vivo* perturbs neural crest formation. Wnt molecules induce neural crest from naïve neural plate tissue *in vitro* a defined medium in the absence of added factors; in contrast, BMPs, which were previously proposed to induce neural crest, do so only in a complex medium. Taken together, these data suggest that Wnt molecules are both necessary and sufficient to induce neural crest cells in avian embryos.

11. Genomic analysis of neural crest induction

Laura Gammill, Marianne Bronner-Fraser

Neural crest cells are multipotent stem cells that migrate from the developing central nervous system to generate diverse structures throughout vertebrate embryos. Because premigratory neural crest cells cannot be isolated as a pure population, the events that cause certain neural cells to become different from their neighbors and migrate are unknown. By screening an arrayed library with subtracted probe, we have identified 83 genes that represent a gene expression profile of a newly induced neural crest cell. Surprisingly, we find that changes associated with migrating cells appear before movement is initiated, suggesting that migratory potential is established

in a pool of cells from which a subset are activated to migrate. The results reveal a sequential "migration activation" process, with different categories of gene expression reflecting stages in the transition to a migratory neural crest cell. In addition, we uncover previously unrecognized similarities between neural crest cells and endothelial cells, another migratory cell population. Finally, this work identifies numerous new markers of premigratory neural crest cells, as well as candidate regulatory molecules of their development. We describe for the first time the consequences of an embryonic inductive event at a genomic level and, hence, provide a molecular profile of a premigratory neural crest cell that changes the way we view the process of neural crest migration. This approach opens the door for future testing of the developmental function of both known and novel molecules.

12. Id gene expression in amphioxus and lamprey highlights the role of genetic co-option during neural crest evolution

Dan Meulemans, David McCauley, Marianne Bronner-Fraser

Neural crest cells generate many of the adult structures that differentiate them from their closest invertebrate relatives, the cephalochordates. Id genes are robust markers of neural crest cells at all stages of development. We compared Id gene expression in amphioxus and lamprey and asked if cephalochordates deploy Id genes at the neural plate border and dorsal neural tube in a manner similar to vertebrates. Furthermore, we examined whether Id expression in these cells is a basal vertebrate trait or a derived feature of gnathostomes. We found that while expression of Id genes in the mesoderm and endoderm is conserved between amphioxus and vertebrates, expression in the lateral neural plate border and dorsal neural tube is a vertebrate novelty. Furthermore, expression of lamprey Id implies that recruitment of Id genes to these cells occurred very early in the vertebrate lineage. Based on expression in amphioxus, we postulate that Id co-option conferred sensory cell progenitor-like properties upon the lateral neurectoderm, and pharyngeal mesoderm-like properties upon cranial neural crest. Amphioxus Id expression also supports an evolutionary relationship between the anterior neurectoderm of amphioxus and the presumptive placodal ectoderm of vertebrates. We relate these observations to previous studies and propose that neural crest evolution was driven in large part by cooption of multi-purpose transcriptional regulators from other tissues and cell types.

13. Neural crest contributions to the lamprey head

David McCauley, Marianne Bronner-Fraser

We have performed focal DiI injection into the cranial neural tube of lampreys in order to follow the migratory pathways of discrete groups of cells from origin to destination and to compare neural crest migratory pathways in a basal vertebrate to those of gnathostomes. The results show that the general pathways of cranial neural crest migration are conserved throughout the vertebrates, with cells migrating in streams analogous to the mandibular and hyoid streams, and branchial neural crest cells migrating ventrally as a sheet of cells from the hindbrain and super-pharyngeal region of the neural tube. These neural crest cells form a cylinder surrounding a core of mesoderm in each pharyngeal arch similar to that seen in zebrafish and axolotl. In addition to these similarities, we uncovered differences as well. Neural crest cell migration into the presumptive branchial arches of the lamprey involves both rostral and caudal movements of cells that has not been described in gnathostomes, suggesting that barriers that constrain rostrocaudal movement of cranial neural crest cells may have arisen during vertebrate evolution after the agnathan/gnathostome split. Accordingly, we found contribution of cells from the same axial level to multiple arches and extensive mixing amongst populations. Furthermore, there was no apparent filling of neural crest derivatives in a ventral to dorsal order that is observed in higher vertebrates. Finally, we found no evidence of a neural crest contribution to cranial sensory ganglia. These seem to be entirely derived from the ectodermal placodes.

14. Excess FoxG1 causes overgrowth of the neural tube

Sara Ahlgren, Peter Vogt, Marianne Bronner-Fraser

The winged helix transcription factor *FoxG1* (*Bf-1, qin*) plays multiple roles in the development of the brain, with different parts of the protein affecting either proliferation and differentiation. To probe the mechanisms by which it FoxG1 achieves these disparate effects, we have examined the consequences of over-expression of either a full-length or DNA binding-defective forms. The results show that excess full-length *FoxG1* results in a decrease in the rate of neuroepithelial apoptosis, thickening of the neuroepithelium, and ultimately large outgrowths of the telencephalon and mesencephalon. In contrast, the myelencephalon appeared unaffected, exhibiting normal apoptosis and growth characteristics. These results demonstrate a previously unrecognized role for winged helix factors in the regulation of neural cell apoptosis.

15. Dual function of Slit2 in repulsion and enhanced migration of trunk but not vagal neural crest

Maria Elena deBellard, Yi Rao, Marianne Bronner-Fraser

Neural crest precursors to the autonomic nervous system form different derivatives depending upon their axial level of origin; for example, vagal but not trunk neural crest cells form the enteric ganglia of the gut. We find Slit2 is expressed at the entrance of the gut, which is selectively invaded by vagal, but not trunk, neural crest. Only trunk neural crest cells express Robo receptors. *In vivo* and *in vitro* experiments demonstrate that trunk, not vagal crest avoid membrane-bound Slit2, thereby

contributing to the differential ability of neural crest populations to invade and innervate the gut. Conversely, exposure to soluble Slit2 dramatically increases the speed and the distance traversed by trunk neural crest cells. These results suggest that Slit2 can act bifunctionally, both repulsing and stimulating motility of trunk neural crest cells.

16. Neuronal differentiation from post-mitotic precursors in the ciliary ganglion

Vivian Lee, Jack Sechrist, Marianne Bronner-Fraser, Rae Nishi

Neurogenesis is typically thought to occur independently of programmed cell death. However, in the early ciliary ganglion, dying neurons are replaced so that neuronal number is kept constant between St.29-St.34 (E6-E8). We characterized the source of neurogenic cells in the ganglion as post-mitotic but undifferentiated neural crest cells. At St. 29 nearly all cells, neurons and non-neuronal, expressed the neural crest markers HNK-1 and $p75^{NTR}$. Over 50% of the cells were neurons at St. 29; of the non-neuronal cells, a small population expressed glial markers whereas the majority was undifferentiated. When placed in culture, non-neuronal cells acquired immunoreactivity for HuD, suggesting that they had commenced neuronal differentiation. The newly differentiated neurons arose from non-dividing precursors, as they did not incorporate bromodeoxyuridine. To test whether these precursors could undergo neural differentiation *in vivo*, purified non-neuronal cells from St. 29 quail ganglia were transplanted into chick embryos at St. 9-14. Subsequently, quail cells expressing neural markers were found in the chick ciliary ganglion. The existence of this precursor pool was transient because non-neuronal cells isolated from St. 38 ganglia failed to form neurons. These results suggest that there are post-mitotic precursors in the developing ciliary ganglion and they can differentiate into neurons in the appropriate environment.

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Summary: The major focus of research in our laboratory is on gene regulatory networks (GRNs) that control development and their evolution. Our areas of research include the transcriptional mechanisms by which specification of embryonic blastomeres occurs early in development; structure/function relationships in developmental *cis*-regulatory systems; sea urchin genomics; and regulatory evolution in the bilaterians. Most of our work is carried out on sea urchin embryos, which provide key experimental advantages. Among these are an easy gene transfer technology; virtually unlimited availability of embryonic material, necessary for isolation of rare molecules such as transcription factors; an optically clear, easily handled embryo that is remarkably able to withstand micromanipulations, and blastomere recombination and disaggregation procedures; a very well understood and relatively simple embryonic process; and in-house egg-to-egg culture of the species we work with, *Strongylocentrotus purpuratu*s (in a special culture system we have developed located at Caltech's Kerckhoff Marine Laboratory). At this point there is also an unusually rich collection of arrayed cDNA and genomic libraries for sea urchins, a fair amount of EST and a growing avalanche of genomic sequence. The sequence is being done at HGSC (Baylor) and is expected to be finished this coming year. A very extensive repertoire of effective molecular technologies for experimentation on sea urchin gene regulatory systems has evolved.

We pursue an integrated, "vertical" mode of experimental analysis, in that our experiments are directed at all levels of biological organization, extending from the transcription factor-DNA interactions that control spatial and temporal expression of specific genes to the system level analysis of large regulatory networks. But one thing that has become apparent is that the only level of analysis from which explanations of major developmental phenomena directly emerge is the system level represented by the sea urchin GRN.

The main initiatives at the present time are as follows: ***i. Analysis of the gene regulatory network underlying endomesodermal specification in S. purpuratus embryos***: At present about 50 genes have been linked into this GRN. The architecture of the network is emerging from an interdisciplinary approach in which computational analysis is applied to data from gene expression knockouts, genomic sequence, and gene discovery projects, combined with experimental embryology. Regulatory and downstream genes required for skeletogenesis and for territorial specification have been isolated utilizing high-density arrayed cDNA libraries. A predictive model which indicates expected inputs and outputs of key *cis*-regulatory elements has been built and is being verified as the relevant *cis*-regulatory systems are found. Many direct experimental tests that can be carried out by altering gene expression in given embryonic cells can now be deduced from the architecture of the model. Most of the individual projects reported below are contributing to understanding of this network. ***ii. Testing the cis-regulatory predictions of the GRN:*** The GRN is constructed essentially by integrating the results of a massive perturbation analysis of expression of individual genes, with spatial and temporal expression data for these genes. It predicts all the required specific regulatory inputs and outputs linking the genes within the GRN. These predictions are subject to direct test, and correction, if need be. At present for the following key genes in the GRN, we have the relevant *cis*-regulatory elements in hand and we are determining at the DNA level their inputs, and their response to upstream perturbations: *wnt8, gatae, krox1, foxa, foxb, delta, otx* (several *cis*-regulatory modules), *cyclophylin, pmar1, gcm, brachury*. We will soon have similar ongoing investigations of *snail, alx, ets1, tbr*. For some regions of the GRN the analysis is approaching completion, in that it extends convincingly from maternal inputs to differentiation, e.g., that GRN subregion determining skeletogenic micromere specification. The subnetwork determining the stability of the endodermal specification state is also well established at the *cis*-regulatory level. Overall, the results of these experiments promise to convert the GRN into a map of the

hard-wired genomic control logic for this portion of development. ***iii. Completion of the repertoire of regulatory genes engaged in the endomesoderm GRN.*** We are using the trace data emerging from the genome sequence project to identify and assemble computationally all gene sequences that encode transcription factors. The temporal patterns of expression of these genes are determined, and for those possibly relevant to the GRN, the spatial patterns as well. Those genes that evidently play a role in endomesoderm specification will then be linked into the GRN by perturbation and cis-regulatory analysis. ***iv. Computational representation of core GRN linkages:*** A mathematical representation of the transcription factor target site preferences for key regulatory genes of the GRN will depend on accurate knowledge of these preferences, and these are being measured *in vitro*. Methods can then be developed for "reading" the GRN architecture from the genomic sequence, using the established GRN as a test bed. ***v. Evolution, viewed as a process of change in GRN architecture:*** Starfish and sea urchins shared a last common ancestor about 500 million years ago, and so analysis of the GRN controlling similar developmental events in the starfish embryo will reveal both the nature of functional change in the GRN, and conservation of features that are so essential that they have resisted alteration for half a billion years. We have already seen examples of both. The underlying processes are of course change, or conservation, of functional *cis*-regulatory features. To study this we are examining starfish/sea urchin GRN differences at the *cis*-regulatory level, and also carrying out a large scale study of 12 different *cis*-regulatory elements from the GRN in genomes of five sea urchin species ranging from 15 to 250 million years since divergence. ***vi. Oral ectoderm GRN:*** We have begun work on the GRN that controls oral ectoderm specification. Three parts of this process can be distinguished, initial specification, maintenance of oral ectoderm state, and oral ectoderm regionalization (i.e., into the neurogenic ciliary band and apical plate, and the central columnar epithelium). The GRN so far concerns only the maintenance parts of the process. Nonetheless, maintenance alone is shown to involve complex feedback loops and repression, as also seen in the endomesodermal GRN. ***vii. Computational and experimental kinetic cis-regulatory model:*** To build a logic model of the information processing functions of a *cis*-regulatory element that relates the input kinetics (i.e., the temporal changes in relevant transcription factor levels) to its output, we have returned to the cyIIIa gene. The logic model of the regulatory system of this gene is being completed by additional mutational gene transfer experiments, and input kinetics are being measured. ***viii. Various technological explorations:*** As always we are trying to expand the repertoire of available technologies for analysis of sea urchin GRNs and sea urchin genomes. Current projects include first attempts to "reengineer" the process of embryonic development, by installing regulatory subcircuits in novel spatial domains; tests of new reagents for perturbing gene expression, *viz* PNAs; development of new methods for gene transfer; application of quantitative imaging methods to assessment of expression construct function; and a project to obtain a physical map of the sea urchin genome, using the inbred lines in our system to measure assortment of microsatellite markers. ***xi. Computational approaches to regulatory gene network analysis:*** Regulatory gene networks for development cannot be informatively treated as equilibrium or steady-state systems. With Hamid Bolouri of the Institute for Systems Biology, Seattle, WA, a new approach to mathematical description of these developmental regulatory systems is being developed, with which to describe its unidirectional progression through successive spatial regulatory states. In addition, several genome analysis tools are being development and a new project to build probability models of target sites for all known transcription factors in the endomesoderm network is under way. Many additional computational genomics and other projects are summarized below.

The Center for Computational Regulatory Genomics CCRG

The Center for Computational Regulatory Biology and its established subsidiary, the Genomics Technology Facility, is a newly constituted research unit and genomics facility that grew out of the Transcription Factor Resource Center. The goal of the new Center is to develop, refine and test computational approaches in genomics broadly and *cis*-regulatory analysis specifically. The primary focus for the latter is the elucidation of gene regulatory networks in development. The Center interacts with the wider research community in several ways: it provides open source software for use by academic research groups; it provides web-based servers for genomic analysis using software developed locally; and it maintains databases fundamental to the Sea Urchin Genome Project, an initiative that began in the Davidson laboratory and at the Genomics Technology Facility. The Facility provides to the Caltech and external scientific community upon request services and materials stemming from the macroarray libraries and arraying equipment that we maintain. The Center and the Facility are both under the direction of R. Andrew Cameron. Oversight for the Genomics Technology Facility is shared by the PI of this Center (Eric Davidson) and the PI of the Genomics Research Center (Mel Simon).

Genomics Technology Facility

The operation of the Facility centers on the Genetix Arraying Robot, a large flatbed robotic arm with video camera used to produce bacterial macroarray libraries and filters. Ancillary equipment in support of robot library construction, including automated medium handling equipment and an automated DNA preparation unit, are also housed in the Facility. An additional robotic DNA preparation machine is situated in the PI's laboratory and is available for Facility high-throughput use. We currently maintain in -80°C freezers 19 different

echinoderm libraries comprising a total of approximately 1.8 million arrayed clones. In addition to providing these materials to academic research groups, we also offer the opportunity for outside groups from Caltech and elsewhere to array and spot their own libraries.

In the past year we have prepared and arrayed a series of large BAC genomic libraries for use in a comparative genomics effort (see below) and several new cDNA libraries for the gene regulatory network (GRN) project. Highlights to be noted are the completion of a series of libraries from the sea star, *Asterina miniata*. These include an oocyte cDNA library, a combined blastula-gastrula embryo cDNA library and two genomic DNA BAC libraries, one with large inserts and one with small. In order to ascertain the role of G-protein-coupled receptors in the maturation of sea star oocytes by 1-methyladenine, a sea star adenosine receptor was isolated and characterized from our libraries. A study is currently underway to identify elements of the sea star endomesoderm specification pathway from these resources. The results constitute the first demonstration of the evolution of gene regulatory networks in development.

Personnel: The staff at the facility are: Ted Biondi, Julie Hahn and Eve Helguero.

Research Center

Computational aspects of gene regulatory network project. The description and simulation of gene regulatory networks can only be accomplished with computational tools specific to the task. We have locally developed several software tools that are in constant use by our laboratory investigating sea urchin development, as well as over 110 users working in a variety of other systems (see below). These tools were specifically designed to aid the experimentalist working at the bench and using iterative cycles of experimentation and computation. The software tools include: BioArray, a program that uses macroarray spot data from phosphoimagers to manage intensity and position information; SUGAR, a system to perform, display and correlate large-BAC sequence analyses to aid the experimentalist with the functional analysis of *cis*-regulatory elements in genomic DNA; SeqComp and FamilyRelations, programs for comparative sequence analysis; and NetBuilder, an environment for creating and analyzing models of gene networks.

In order to make the sequence analysis programs convenient we have installed a web-based facility, the Cartwheel Project, that allows the user to have complete control over the process. Within the circumscribed domain of genomic sequence-based information, Cartwheel provides facilities to organize, analyze, and curate information on the level of individual labs. The analyses are then viewable by programs such as FamilyRelations. The Cartwheel Project is the umbrella term for a bioinformatics infrastructure first developed by C. Titus Brown and now maintained and extended by the computational staff of the Center.

The equipment that supports the computational efforts of the Center includes two 18 unit Beowulf clusters, a web server for the Sea Urchin Genome Project (SUGP) and several dual processor development machines used by the staff for software construction, testing and maintenance.

The utility of these comparative sequence analysis facilities is reflected in the user population. At present, the Caltech Cartwheel server, Woodward, has 60 active users who have used the server in the last two months out of 111 total registered users in 31 lab groups. A total of 8896 jobs have run in the last year for a total of 48 CPU days. The majority of these are Seqcomp and Blast analyses.

The sea urchin genome project. The Sea Urchin Genome Project web site (http://sugp.caltech.edu/) is the distribution center for sequence and annotation information related to the sea urchin genome and the macroarray libraries. We have maintained and extended this part of the facility that is housed in the Center over the last year in preparation for the incoming whole genome sequence.

As genomic BAC sequence from the *S. purpuratus*, *L. variegatus* and other echinoderm genomes accumulates, it is annotated using the software package SUGAR, and is now being rewritten to be useful with the Cartwheel queueing system. These programs also reside on the SUGP machines and efficiently interdigitate with the existing packages.

The most unique, information-rich component of the Sea Urchin Genome Project web site is the database of macroarray filter information. Here all of the sequence mentioned above and the gene annotation information collected in the process of screening these library filters, for whatever purpose, are stored. This includes sequence collections from complex probe screens such as those used for the identification of genes in the endomesoderm specification pathway, the results of homology screening strategies, and random EST projects. Because the data is coupled to a filter location that contains an individual clone from the library, the clone is immediately recoverable. As more clones are characterized in a library, that library becomes more valuable. Eventually, the several well-characterized libraries can be used to confirm *ab initio* gene predictions and confirm gene catalogs for the sea urchin.

Personnel: The staff who support the computational aspects of the Center are: Kevin Berney, C. Titus Brown, Ramon Cendejas and Ian Lipsky.

17. Further analysis of early zygotic regulators in the endomesoderm network

Andrew Ransick

Analyses continue of several interesting transcription factors that are likely to play essential roles in regulating early endomesoderm specification. *Speve*, *Spgcm* and *Spfoxc* are all currently the subject of analyses to determine the complete mRNA expression profiles, determine what other genes in the Enodmesoderm Network the proteins interact with and what role they play

in specification. Using morpholino antisense oligonucleotide (MASO) microinjection in combination with quantitative PCR expression assays for endomesodermal marker genes and whole mount *in situ* hybridization, it has been found that *Spgcm* expression is downstream of Delta/Notch signaling and upstream of pigment cells differentiation.

These findings are entirely consistent with its expression profile, which initiates at 12 h in a subset of veg2 lineage cells immediately adjacent to *Delta* expressing large micromere progeny. *Spgcm* is then expressed in the subset of the secondary mesenchyme precursors that give rise to the pigment cells. Additional MASO experiments have shown that *Spgcm* upregulates several pigment cell-specific genes and plays a role regulating *Spgatac*, another gene expressed in the early secondary mesenchyme domain. On the other hand, *Spfoxa* may repress *Spgcm*, as supernumerary pigment cells are present in *Spfoxa* MASO-injected embryos.

A comparison of the genomic sequence of *Spgcm* and *Lytechinus variegatus gcm* (*Lvgcm*) revealed three conserved noncoding DNA segments within 15 kilobases of the coding exons. Reporter constructs made by combining these conserved noncoding regions have given expression profiles resembling endogenous Spgcm mRNA. Of particular interest is the finding of consensus six-binding sites for Suppressor of Hairless (SuH) in these conserved noncoding regions, as this transcription factor is a known effector of Notch signaling. A paired-site SuH element is contained in a relatively short fragment of DNA that confers a dramatic improvement on the spatial expression on GFP reporter constructs. Site directed mutagenesis of all the SuH sites is being carried out to test their role in controlling correct spatiotemporal expression of *Spgcm*.

18. Micromere-PMC GRN

Paola Oliveri, Leah Vega, Katherine Gora, David R. McClay[*]

During sea urchin development, micromeres segregate at the vegetal pole of the embryo by 4th division. These four cells and their immediate descendants (Primary Mesenchyme Cells) are the initial source of two signaling events essential for the entire endomesoderm specification and later will form the larval skeleton.

Thus far, it has been shown that a key component of the zygotic regulatory gene network that accounts for micromere specification and subsequent differentiation is the homeodomain factor *pmar1*. Expression of *pmar1* is zygotic and transient during cleavage stages. Recent studies have shown that ectopic *pmar1* expression is sufficient to activate the micromere-PMC specification and the differentiation regulatory cascade events in every cell of the embryo, and also to rescue all of the micromere features knocked down by blocking β-catenin nuclearization (Oliveri *et al*., 2003). Thus, *pmar1* is the main transducer of maternal cues (Otx and β-catenin/Tcf) to the downstream micromere specification regulatory apparatus and its transducer action is exerted through a double-negative regulatory interaction.

Lately our attention has focused on the studies of the functional interrelations among regulatory genes immediately downstream of *pmar1*. Six transcription factors have been identified and generally classified in two main classes: 1) *alx, tbr* and *ets1* transcription factors, in which micromere restricted expression starts a few cell divisions after the *pmar1* appearance in micromeres and remains on only in PMC cell lineage until late gastrula; and 2) *dri, gsc* and *foxB* factors, in which expression is transient, starts at late cleavage-early blastula stage and ends after PMC ingression. Uniquely, *foxB* expression stays on in PMCs until midgastrula stage. The *alx1, tbr* and *ets* function has been knocked down by the injection of specific morpholino antisense oligos. The absence of each of these factors prevents synthesis of spicules at larval stage, and the phenotypes are dissimilar during development. The effects of the knockdowns are quantified by QPCR for all of the potential target genes. A series of interregulatory interactions are so far uncovered, which explain the setting up of a new regulatory status of the micromere descendant cells. The new regulatory arrangement directly activates the skeletogenic genes and does not require *pmar1* presence anymore.

[*]*Duke University*

19. Mapping the functional elements in the Otx endodermal specific modules and the interrelationship of the inputs of Krox, GATA (e) and Otx

Chiou-Hwa Yuh, Elizabeth R. Dorman

Earlier study on the promoter of the *otx* gene of *Strongylocentrotus purpuratus* identified four separate *cis*-endomesodermal expression. In this study, we tried to answer the question of whether those DNA fragments contain the information for regulating correct spatiotemporal expression elements, and specifically which DNA sequence contains which information.

According to QPCR results, Otxβ1/2 transcripts are affected by microinjections of GATA (e)-Morpholino, cadherin mRNA and Engrailed-Otx mRNA. However, the Otxa transcript is not affected. To determine how those functional perturbations map on the *cis*-regulatory system of *otx* gene, we applied the co-injection experiments. We found a construct (Otx11-CAT) that is sensitive to all three perturbations at 20 hour, 30 hour and 48 hour stages. The activity of two other constructs (Otx14-CAT and Otx15-CAT) decreased in the Engrailed-Otx mRNA and Gata-e morpholino co-injection. Otx17-CAT is not affected by those perturbations, which corresponds to the results of perturbations on the endogenous Otxa transcript.

A detailed search for the Gata, Otx, Krox and Tcf 1-binding sites was done on the genomic DNA sequence immediately upstream of the Otxβ1/2 transcription unit. Multiple sites for each transcription factor were found in the region. Site-directed mutageneses were done to mutate those sites, and tested by co-injection experiments. Mutation on the five Gata putative-binding sites, three Otx-binding sites, and two Krox-binding sites

all showed decrease in the transcription activity of the promoter. Deletion of the three Tcf1-binding sites also decreased the promoter activity. Morpholino antisense RNA was introduced with the wild type and mutant constructs. Mutation on the binding sites made the promoter no longer response to those morpholino. Those data strongly suggests that the Krox, Gata-e and Otx inputs are working through those conserved binding sites.

Searching for the modules required to repress the ectopic expression of the Otx15-CAT has been carried out. Otx12 and Otx13 by themselves have no positive function at all and cannot serve as repressor modules either. Nor can the repeat sequences, which are highly conserved between species. The results suggest it will not be possible to depend on FamilyRelations comparison to find the repressor. Interestingly, a 1 kb sequence immediately upstream repressed the ectodermal activity. This nonconserved region contains two CREB-binding sites, which has been shown to have repression function on *Endo16* promoter.

20. Identify the negative elements regulate the endo16 promoter and developing a useful tool for identified the unknown negative module in the sea urchin genome

Chiou-Hwa Yuh, Jane Wyllie

Study of the transcriptional regulation of *endo16*, which is an early vegetal plate marker gene, can help us understand the endomesoderm specification. Detailed analyses have been done for the positive modules of the *endo16* gene. There are three positive modules (A, B and G) that regulate *endo16* gene expression in the vegetal plate/gut territory. There also are three-negative modules. The function of F, E and DC modules is to repress the ectopic expression on ectoderm (F, E) and skeletogenic mesenchyme cells (DC). Sites within module A and module B have been mutated and each of the important sites within those modules is thoroughly understood. But, for the negative modules, little is known in terms of the importance of particular binding sites. This study focuses on mutation of those binding sites, and also on more advanced testing using those modules in constructs of other promoters to see if a negative module can serve as a general repressor. In the end, we hope to develop a useful tool with which we can assay nonhomologous DNA sequence for possible repression functions.

Using site directed mutagenesis of the E and F modules of the endo16 promoter, we found out all the binding sites identified from gel-shift assays are important elements for the negative module to function. For example, mutation of UC (CREB site) on the F module increased the boundary ectoderm cell expression, while mutation of repeat-1 sequence (immediately upstream of F module), increased the overall expression in the ectoderm. The effect is stronger than mutation of the CREB site itself. Mutation of UD, CY and CB sites together caused the decreasing of CAT activity indicating an active function for those elements. Mutation of UE, UF and CG, CY sites together caused less effect, but slightly increased the ectoderm expression. Mutation of repeat-2 sequence caused increasing CAT activity.

To test the idea of whether those negative modules from *endo16* gene served as general repressor elements, we used the ectodermal specific modules from *otx* gene. Putting the negative module in front of the ectodermal expression module reduced expression in CAT assays and shut off the expression only at the boundary ectoderm territory when assayed by *in situ* hybridization assay.

From the study of many genes by FamilyRelations, we and others have noticed that the negative modules usually are not conserved DNA sequence. We believed that single repressive module could not be identified with the FamilyRelations strategy adapted so far. So, it is important to develop a new tool for testing the nonconserved regions for possible repressors. A good way is to use dominant repressors controlled by negative modules, and co-injection with a pan-expression. We found that EF module of Endo16-derived dominant repressor (Eng-Ets) represses the HE-GFP on the boundary ectoderm. This type of system will serve as a useful tool to search for repression modules in the genome, because of the easily identified pattern alterations and the amplifier effect of the co-injection system.

21. Transcriptional control of the sea urchin ***brachyury*** gene

R. Andrew Cameron, William Chiu, Jane Wyllie, Paola Oliveri

Over the past two years we have characterized many aspects of the transcriptional regulation of the *brachyury* gene, a participant in the endomesoderm specification pathway and the founding member of T-box family of transcription factors. A region 4 kb long just 5' of and including the transcription start site can activate a reporter gene on the approximate temporal pattern of the endogenous gene: expression begins in the blastula, continues through gastrulation and decreases in the pluteus stage. A region about 1000 bp that occupies most of the intron between the 6th and 7th exons of this transcription unit provides good temporal control beginning in gastrula stage. However, the early spatial control is still uncharacterized. The endogenous gene is obviously localized to the vegetal plate as seen by *in situ* hybridization in the blastula stage. To discover the source of this early phase we are employing three strategies. 1) We continue to identify, clone and test sequences around the *brachyury* gene that are conserved between the purple sea urchin and the Atlantic variegated sea urchin, two species with a common ancestor 50 million years old. Using the FamilyRelations software we have identified several conserved tracts of sequence not yet tested. One in particular lies 5' of the ubiquitous enhancer near the transcription start site and contains a sequence identical to the Smad inhibiting protein (SIP) binding site found in the upstream region of the *Xenopus* '*blimp*' gene, a ortholog of sea urchin *brachyury*. 2) We are employing a BAC

recombination approach to clone GFP into a sequenced BAC containing the *brachyury* gene. This will provide a test of the sufficiency of the surrounding sequence to properly control expression. 3) We are testing conserved sequences that give no expression by themselves to see if they will function in a repressor transactivation assay-based on a strong GFP reporter and an *ets-engrailed* fusion carrying the putative repressor sequence. We expect that the results of these approaches will bring the sequences governing the expression of *brachyury* into focus.

22. Notch signaling role in endomesoderm specification in sea urchin

Cristina Calestani

In sea urchin the endomesoderm is specified from a vegetal embryonic territory, the vegetal plate. The central disc of cells of the vegetal plate is specified as Secondary Mesenchyme Cells (SMCs), while the ring of cells around it is specified as the endodermal progenitor cells. SMCs subsequently differentiate into four cell types: pigment cells; blastocoelar cells; muscle cells; and coelomic sac cells. A Delta-Notch signaling at the blastula stage has been proven to be necessary for the differential specification of presumptive SMC and endodermal territories. The Notch ligand, Delta, is expressed in the micromeres, localized at the vegetal pole, and it activates the Notch receptor in the surrounding SMC precursors. A block of the Notch signaling drastically reduces SMC formation and it causes an expansion of the endodermal territory into the presumptive SMC territory.

In order to identify genes involved in the endomesodermal specification process that act downstream of N signaling, the expression of endoderm- and mesoderm-specific genes was studied in embryos in which Notch signaling was blocked. The gene expression level was tested by QPCR on normal and Notch knockdown embryos at blastula, early gastrula and late gastrula stages.

A total of 11 genes expressed in the SMCs and 12 genes expressed in the endoderm were tested. These included transcription factor, cell signaling and differentiation genes. In general, Notch signaling showed a positive regulation of several genes expressed specifically in the SMCs. The transcription factor *gcm* and the differentiation genes *pks*, *fvmo-1,-2,-3, sult* and *dpt*, which are expressed in pigment cells, were downregulated in Notch knockdown embryos in all developmental stages tested. The same result was observed for the transcription factor *foxC*, which is expressed in the precursors of the coelomic pouches. The transcription factor *gataC*, which is expressed in blastocoelar cell precursors, is positively regulated by Notch at blastula stage, but not at later stages. Similarly, the transcription factor *gataE*, which is expressed in SMC precursors at blastula stage and in both SMC and endodermal precursors at later stages, is regulated by Notch only at the blastula stage. The block of Notch signaling did not affect the transcript abundance of endodermally-expressed genes, as measured by QPCR. Additionally, *delta* gene expression was tested and it was not affected in Notch knockdown embryos.

Overall these results show that Notch signaling positively regulates genes involved in SMC specification, while it does not seem to regulate genes expressed in the endoderm. The approach used in this work, though, does not exclude the possibility of an ectopic expression of endodermal genes caused by the block in Notch signaling.

23. Refinement of a ***cis***-regulatory model for ***cyIIIa***

C. Titus Brown, Chiou-Hwa Yuh

The *cyIIIa cis*-regulatory region directs expression of the *cyIIIa* gene in the aboral ectoderm of *S. purpuratus* throughout development. This region was first isolated in 1985, and a variety of studies have identified approximately 20 distinct binding sites for nine distinct transcription factors. All but one of these factors has been cloned, and the role of all of these factors in the regulation of *cyIIIa* is known. In recent work, we have determined the precise time course of the expression directed by three of the transcription factors, and we have also started to systematically characterize the time courses of all five of the positive-acting transcription factors; this latter project depends upon nuclear extracts made from a set of staged embryo cultures at the Kerckhoff Marine Lab. We have also started to use QPCR and WMISH to characterize the temporal and spatial expression patterns of these factors across the same time points, with the end goal of building a causal kinetic model linking input transcription factor presence and prevalence to the output of the *cyIIIa cis*-regulatory region.

24. ***cis***-Regulatory analysis of the sea urchin ***delta*** gene

Roger Revilla

The *delta* gene has been suggested to play two different roles in the specification of the endomesoderm of the sea urchin embryo. Each one of these roles requires Delta to be localized in a specific territory of the embryo. It is first required in the micromeres to serve as a signal that is necessary to segregate the mesodermal and endodermal fates of the surrounding cells. It is later localized in the prospective SMCs, and its function there is still under investigation. The goal of this project is to analyze the *cis*-regulatory system that localizes the expression of Delta in the right place and the right time to serve its roles in the specification of the endomesoderm. It has already been suggested that the early localization of Delta in the micromeres depends on activator(s) that are present ubiquitously, and a repressor that is present everywhere except in the micromeres. Comparison of genomic DNA of *Strongylocentrotus purpuratus* containing *delta* gene with the orthologous region of *Lytechinus variegatus* genome has been used to identify conserved patches of sequence that might contain *cis*-regulatory elements. A sequence element has been found that is able to localize expression of a reporter gene in the regions where Delta is localized, and evidence has been produced that suggests that this element contains

binding sites for activator(s) ubiquitously present and binding sites for the repressor that localizes *delta* in the micromeres. Future work will identify the sites in the DNA that bind these factors, and it will also elucidate the binding sites of the key factors that are responsible for localizing Delta in the prospective SMCs. Finally, we also hope to be able to identify the transcription factor that acts as a repressor of *delta* everywhere in the embryo except the micromeres, which has been suggested to play a key role in the installation of the skeletogenic program of gene expression.

25. SpKrox1 and the endomesoderm network
Carolina Becker Livi

The sea urchin zinc-finger transcription factor SpKrox1 is a regulator involved in the specification of endomesoderm in echinoderms. Initial expression of SpKrox1 starts early, during cleavage stages, and continues in a subset of vegetal blastomeres and its descendants. SpKrox1 function was blocked by two different mechanisms and these perturbations were found to disrupt endomesoderm territory specification and gastrulation. Analysis of results from these revealed that SpKrox1 acts both as an activator (e.g., SpOtx) and as a repressor (e.g., SpKrox1 itself). Furthermore, SpKrox1 affects multiple downstream genes, though many of these connections are likely to be indirect and therefore mediated through other transcriptional regulators in the endomesodermal network. SpKrox1 functions to integrate upstream signaling and transcriptional events in the vegetal plate to a series of downstream transcriptional gene batteries. To do this it primarily interacts with SpOtx, another early transcription factor, upregulating Otx expression in the vegetal plate territory. In turn, SpOtx also increases the expression of SpKrox1, securing their expression in the endomesoderm. The function of SpKrox1 includes establishing the boundaries of new territories. Initially it delineates the vegetal plate and subsequently it distinguishes the endodermal portion of veg1 from the surrounding ectoderm. This second function again involves a cross-regulatory loop, this time involving the orthologue of the fly *evenskipped* gene in sea urchin (*Speve*). SpKrox1 activates SpEve expression and vice versa. From work by other members of the Davidson laboratory, we predict we will find inputs into the *cis*-regulatory region of SpKrox1 from Otx and Tcf/Lef. Conserved noncoding regions between *Strongylocentrotus purpuratus* and *Lytechinus variegatus* that are shown to drive expression in the correct spatial territory contain several OTX- and TCF-binding sites that might be responsible for their activity. Mutation of these binding sites and subsequent functional analysis *in vivo* will confirm the connections predicted by the downstream analysis described in the endomesoderm network model.

26. ***cis*-Regulatory analysis of *Spgata-e***
Pei Yun Lee

SpGata-e is the *S. purpuratus* ortholog to vertebrate *gata* genes *4/5/6*. The expression of *SpGata-e* is first detected in the presumptive secondary mesenchyme cells (SMCs) during the hatching blastula stage. Its expression expands to include both the future SMCs and the endoderm in the mesenchyme blastula. In the gastrula, *SpGata-e* is expressed in the tip of the archenteron and hindgut. By the end of embryogenesis, *SpGata-e* is expressed in the midgut and coelomic pouches.

An analysis of the *cis*-regulatory region of *SpGata-e* will verify the connections between *SpGata-e* and other genes in the endomesoderm network at the DNA level and further refine the network model. Comparison of the *S. purpuratus* and *L. variegatus Gata-e* genomic regions using FamilyRelations identified 28 conserved regions. Twenty-one of these regions have been cloned into GFP reporters and tested by injection into sea urchin eggs and observation of GFP expression. Of all the tested reporter constructs, a 600 bp DNA sequence (fragment 10) in intron 1 is the *cis*-regulatory element responsible for *SpGata-e* expression in the vegetal plate at the hatching and mesenchyme blastula stages.

A search in the sequence of fragment 10 for putative DNA-binding sites of transcription factors known to be upstream of SpGata-e identified three putative *SpOtx*-binding sites. Co-injection of Otx-en mRNA and the fragment 10:reporter was able to abolish the GFP expression from the injection of fragment 10:reporter alone. Mutation of the *SpOtx*-binding sites abolishes the effect of injection of Otx-en mRNA on the expression of the fragment 10:reporter construct. Therefore, the *SpOtx*-binding sites in fragment 10 are functional and are capable of activating the early vegetal expression in the sea urchin.

27. Snail and Twist and their role in mesoderm specification in sea urchin
Stefan Materna

Snail and Twist are two transcription factors that are thought to play an important role in the early specification of ventral mesoderm. Both are highly conserved over a wide range of organisms from *Drosophila* to humans.

Twist, a bHLH transcription factor, is necessary for activation of mesoderm-specific genes in *Drosophila*. Snail, a member of the zinc-finger family, represses the mesoderm anlage genes that are otherwise expressed in the adjacent ectodermal regions. Both genes are necessary for proper gastrulation.

In addition to its function in mesoderm specification, Snail seems to be involved in formation of the neural crest. It does this by triggering the epithelial-mesenchymal transition, a process that allows cells to separate from their neighbors and migrate to different regions in the embryo.

Both genes are present in *S. purpuratus* as was shown by PCR amplification of specific fragments. With the Snail fragment a presumable full-length cDNA could be retrieved from a 30 h cDNA library.

Analysis of the spatial and temporal expression pattern will shed light on the function of Snail in sea

urchin and will help in evaluating a potential position in the network for endomesoderm specification.

28. ***cis*-Regulatory analysis of *SpWnt8***

Takuya Minokawa, Athula Wikramanayake[*]

SpWnt8 is a signaling molecule that is involved in the early specification of endomesoderm of sea urchin development. Perturbation experiments using a dominant-negative form of *wnt8* or overexpressing *wnt8* mRNA strongly suggest that this gene is responsible for the early endomesoderm specification process (Wikramanayake *et al.*, unpublished).

SpWnt8 is first expressed in the 16 cell-stage embryo in the micromeres. The expression extends to veg2 descendants by late cleavage stage. At the mesenchyme blastula stage, expression shifts towards the veg1 region. This expression pattern coincides with the region where various cell-cell interaction events are known to occur, strongly suggesting a role for this signaling molecules in this process.

The purpose of this project is to determine how the gene expression of *SpWnt8* is controlled. A newly developed software analysis tool, "FamilyRelations," was used to find candidate *cis*-regulatory elements. We identified two highly conserved regions in the flanking region of *SpWnt8* exons by this analysis. One exists just 5' of the first exon, and is approximately 700 bp (Fragment A). Another exists in the second intron, and is approximately 400 bp long (Fragment B). Using a CAT-reporter vector system, we examined the function of these two conserved regions. Fragment A-CAT construct exclusively expresses in the micromere and its descendants at 16- to 32-cell stages, indicating that the Fragment A is responsible for the correct expression of *SpWnt8* in the cleavage-stage embryos. Fragment B-CAT construct, on the other hand, does not show any expression at 16- to 32-cell stage.

Fragment A contains many putative transcription factor-binding sites. Five putative TCF-binding sites are estimated from the sequence data. We co-injected both Fragment A-CAT construct and mRNA encoding cadherin intracellular domain (Cad) to trap the β-catenin, which is a cofactor for TCF transcription factor. As a result, the transcription of CAT mRNA was totally suppressed by the overexpression of Cad. This indicates that at least one of the TCF-binding sites in Fragment A is functional, and responsible for the expression of *SpWnt8* in the micromere lineage in the cleavage stages.

One of the primary goals of this project is to find *cis*-regulatory elements to control the complex gene expression patterns. We are analyzing the expression patterns of Fragment A-CAT and Fragment B-CAT in the relatively later developmental stages (early blastula, and mesenchyme blastula stages) when the expression pattern of this gene changes drastically.

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29. Expression patterns of various genes that play parts in the gene regulatory network for early development of the sea urchin ***Strongylocentrotus purpuratus***

Takuya Minokawa, Cristina Calestani, Gabriel Amore, Jonathan Rast[*]*, Cesar Arenas-Mena*[**]*, Sagar Damle, Christopher Franco*

The expression profiles of genes are the basic and essential preliminary information required for an understanding of gene regulatory networks. Details of spatial and temporal expression patterns are needed to build a reliable gene regulatory network structure.

We have been studying the gene regulatory network (GRN) in the early developmental process of the sea urchin *Stronglylocentrotus purpuratus*. More than 40 genes in the endomesoderm gene regulatory network have been identified. The GRN has been constructed according to information from functional perturbation experiments, *cis*-regulatory analyses, and gene expression patterns. The expression patterns for most of the genes in these GRNs have been reported so far, and confirmed repeatedly in many independent experiments. On the other hand, the expression patterns of some genes have not been reported because they have been isolated and identified only fairly recently. One of the purposes of this project is to describe the expression patterns for these genes.

The other purpose of this project is to re-visit and add several important pieces of information to our knowledge of gene expression patterns. Recently, a new, quite sensitive whole mount *in situ* hybridization (WMISH) protocol was developed (C. Arenas-Mena, unpublished data). Using this protocol, we have re-examined most of the gene expression patterns reported so far. We have found several genes that have loci of expression that have not been reported previously. This may have been due to low transcript abundance of these genes that are hard to detect by the less sensitive, conventional WMISH method, or to the inadequate treatment of the embryos resulting nonspecific staining. The expression pattern of newly isolated genes (*SpHES*, *SpNK1*, and *SpNrl-like*) is examined in parallel with a detailed re-examination of the expression patterns of *SpFoxB* (formerly *Spfkh1*), *SpGsc*, *SpKrl*, *SpHmx*, *SpSoxB1*, and *SpWnt8*.

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30. Functions of SpSoxB1 and SpKrl in sea urchin development

Lili Chen, Lynne Angerer[*]

S. purparatus SoxB1 (SpSoxB1), a member of Sox (SRY-related box) gene family, is a maternally expressed transcription factor involved in the ectodermal specification (Kenny *et al.*, 1999). SpKrl (*S. purparatus* Kruppel-like factor) is an early zygotic transcription repressor that was reported to be a direct target of the β-catenin/Tcf signaling pathway (Howard *et al.*, 2001), which is essential for endomesoderm specification. This project is to elucidate the function of SpSoxB1 and SpKrl in the genetic network of early sea urchin development.

Using the whole mount *in situ* hybridization

method, we studied the expression pattern of SpSoxB1 and SpKrl. The mRNA SpSoxB1 is first transcribed in the entire embryo and starts to be cleared from the vegetal territory at early cleavage stage (about 12 h after fertilization). SpSoxB1 mRNA is only detected in the ectoderm with a sharp boundary at the middle mesenchyme blastula stage (24 h embryos). SpKrl starts to be expressed in the micromeres at 16-cell stage. The expression is then shifted to veg2 region at late cleavage/early blastula stage and to veg1 region at mid-mesenchyme blastula stage. The transcription of SpKrl disappears after the onset of gastrulation. The spatial and temporal coincidence of SpSoxB1 clearance and SpKrl expression raises the possibility that SpKrl functions to suppress the expression of SpSoxB1 in vegetal territory thereby specifying endomesoderm.

To understand the function of SpSoxB1 and SpKrl in the endomesoderm gene network, different disturbance techniques were used to interfere with the normal expression pattern of these genes. The effect on possible downstream genes was assessed using real-time quantitative PCR. The ectopic expression of SpSoxB1 mRNA leads to the downregulation of gene batteries essential for endomesoderm specification. Transcription factors essential for endomesoderm specification including *gcm, gataC, gataE, foxA, eve*, and *bra*, were downregulated by overexpression of SpSoxB1. Ectopic expression of SpSoxB1 in micromeres strongly inhibits expression of micromere-specific transcription factors such as *pmar1, dri,* and *gsc*, as well as a set of skeletogenic differentiation genes, like *sm130, sm50, pm27, and cyclophillin*. SpKrl is also downregulated by SpSoxB1. These results support that the clearance of SpSoxB1 from vegetal region is critical for correct endomesodermal specification. To study the function of SpKrl, SpKrl morpholino antisense oligonucleotide (MASO), which blocks the translation of SpKrl protein by binding to the translation initiation site of SpKrl mRNA, was injected into one-cell embryos. The SpKrl MASO did not prevent gastrulation as previously reported and had minimal effects on more than the 20 tested genes, including SpSoxB1. To test whether the SpKrl MASO is effective to inhibit SpKrl translation, 5' UTR and initial coding region of SpKrl were linked to a GFP reporter gene in frame (making a Krl-GFP construct). Coinjection of SpKrl MASO and Krl-GFP mRNA demonstrated that SpKrl MASO was effective to inhibit the translation of Krl-GFP mRNA. It is possible, however, that SpKrl MASO is less efficient to block endogenous SpKrl mRNA translation due to the presence of binding proteins. Alternatively, different splicing forms may exist, which cannot be eliminated by the SpKrl MASO. Further, to develop other techniques to knock out specific genes, we are exploring the possibility to use RNA interference, a conserved mechanism found in plants, flies, worms, and mammals, that can lead to specific degradation of a RNA sequence homologous to a dsRNA.

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31. ***Spdeadringer***, a sea urchin embryo gene required separately in skeletogenic and oral ectoderm gene regulatory networks

Gabriele Amore, Robert Yavrouian[*], Andrew Ransick, Kevin J. Peterson[**], David R. McClay[***]

Spdri is the first e-ARID-class transcription factor ever found in euchinoderms. In the sea urchin embryo *Spdri* plays a key role in two separate developmental gene regulatory networks (GRNs): the PMC GRN (where *Spdri* is expressed from 12 h until ingression) and the oral ectoderm GRN (form about 24 h on). In both territories the periods of *Spdri* expression follow prior territorial specification events. The functional significance of each phase of expression was assessed by determining the effect of an α*Spdri* morpholino antisense oligonucleotide (MASO) and that of an *engrailed* fusion mRNA on the expression of a number of different endomesodermal and oral ectoderm genes. These effects were measured by quantitative PCR, supplemented by whole mount *in situ* hybridization (WMISH) and morphological observations. In the micromere descendants *Spdri* is shown to act in the pathways that result in the expression of batteries of terminal differentiation genes. Interference with *Spdri* function results in the abolishment of proper PMC patterning and in the loss of the embryonic skeleton. In the oral ectoderm *Spdri* is linked with several other genes encoding transcriptional regulators that are expressed specifically in various regions of the oral ectoderm. In this territory, *Spdri* participates in the central GRN that controls oral ectoderm identity. If its expression is blocked, oral-specific features disappear and expression of the aboral ectoderm marker *spec1* encompasses the whole of the ectoderm. In addition to these autonomous effects, a failure of gastrulation is observed. To further analyze these phenotypes chimeric embryos were constructed consisting of two labeled micromeres combined with micromere-less 4th cleavage host embryos; either the micromeres or the hosts contained α*Spdri* MASO. These experiments showed that while *Spdri* expression is required autonomously for expression of skeletogenic genes prior to ingression, complete skeletogenesis also requires the expression of oral ectoderm patterning information. Presentation of this information on the oral side of the blastocoel in turn depends on *Spdri* expression in the oral ectoderm. Failure of gastrulation is not due to indirect interference with endomesodermal specification per se, since all endomesodermal genes tested function normally in α*Spdri* MASO embryos. Part of its cause is interference by α*Spdri* MASO with a late signaling function on the part of the micromere descendants that is needed to complete clearance of the Soxb1 repressor of gastrulation from the prospective endoderm, but in addition there is a nonautonomous oral ectoderm effect.

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32. ***SpCyclophillin***: A downstream target of ***Spdri*** in the PMC territory

Gabriele Amore

SpCyclophilin is a member of the peptidyl prolyl *cis-trans* isomerase (PPI) protein family found in sea urchins. Proteins of this family, usually take part into signal transduction events.

The sequence encoding the full-length *Spcyclophilin* cDNA has been isolated and sequenced. Whole mount *in situ* experiments have been performed showing that the gene is expressed in the PMC precursors at around 15 h (before these cells ingress into the blastocoel) and it remains expressed in these cells after they ingress until at least 48 h. An initial analysis was performed using qPCR to measure the amount of *Spcyclophilin* mRNA in development. Two peaks of expression were found: right before ingression and at the time spiculogenesis is initiated. In a different study, *Spcyclophilin* was shown to be activated by *Spdri*. Blockade of *Spdri* produces embryos with ingressed PMCs that are unable to pattern correctly and organize a skeleton. PMC patterning requires extensive cross talk between cells. The possibility for *Spcyclophilin* to be one of the genes involved in these processes, remains to be explored.

At present, the genomic sequences for both the *S. purpuratus* and the *L. variegates* genes have been recovered form BAC libraries. The comparative analysis of the DNA surrounding the two genes has been initiated. Evolutionarily conserved stretches of DNA between the two genomic clones will be functionally tested to reveal relevant *cis*-regulatory elements necessary to ensure the proper pattern of expression of *Spcyclophilin.*

33. Hepatocyte nuclear factor-6: A positively acting regulatory gene of the sea urchin embryo

*Ochan Otim, Gabrielle Amore, Takuya Minokawa, Chiou-Hwa Yuh, David R. McClay**

We have cloned and functionally characterized the *Strongylocentrotus purpuratus hepatocyte nuclear factor-6* (*Sphnf-6*) gene, the homologue of the mammalian *hnf-6*. The *Sphnf-6* gene contains the information required for synthesizing a new member of the ONECUT family of transcription factors. The temporal and spatial domains of expression of *Sphnf-6* mRNA in a developing *S. purpuratus* embryo is biphasic. At first, *Sphnf-6* is expressed everywhere prior to gastrulation (30 h) as revealed by whole mount *in situ* hybridization. Thereafter, *Sphnf-6* transcripts are localized in the ciliated band. During the transition from the global expression domain to the ciliated band, the activity of *Sphnf-6* is never extinguished completely at any time as discovered by quantitative PCR, although fewer mRNA molecules are present at the interphase of transition.

The role of *Sphnf-6* in development was investigated mainly by perturbation of *Sphnf-6* expression through morpholino antisense oligonucleotide injections and by micromeres transplantation. The loss of *Sphnf-6* expression causes a radialization of the embryo. This phenotype suggests a major role for SpHNF-6 in the oral/aboral specification. The radialization of the embryo is accompanied by a dramatic increase in the number of primary mesenchyme cells. In the details, *Sphnf-6* functions variously in all the embryonic territories as an activator of genes, both terminal and positive-acting transcription factors, as well as repressors. Specifically, *Sphnf-6* plays three vital roles in development, two of which occur prior to gastrulation. First, *Sphnf-6* is utilized in the activation of the PMC terminal genes such as *sm50*, *sm30*, *pm27* and *msp130*. Secondly, *Sphnf-6* is used to specify and maintain the regulatory state of the oral ectoderm. This second function is primarily a direct consequence of the initial unrestricted expression of *Sphnf-6* at the early-stage in development. And lastly, *Sphnf-6* appears to be involved in the patterning of the embryonic skeleton through the activation of the *Spotp* gene. Later on when *Sphnf-6* is localized only in the ciliated band, there is no clear evidence that *Sphnf-6* is influencing any other specification or differentiation events.

Given the role of the *Sphnf-6* gene in both the PMC and specification of the oral/aboral axis, it was of interest to us to determine if the gene was in anyway involved in the micromere signaling. Micromere transplantation experiments show that *Sphnf-6* is downstream of the micromere signaling systems.

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34. Purification of the spatial control factors of the ***sm50*** gene and the possible mechanism of DNA-protein and protein-protein interactions at the C-element

Ochan Otim, Chiou-Hwa Yuh

Developmental genes interpret their temporal and spatial positions in development through specific recognitions of *cis*-regulatory target sites by transcription factor assemblies. This work is part of a demonstration on how to resolve and purify components of such a transcription complex for a gene expressed exclusively in the skeletogenic precursor and the daughter cells of a developing sea urchin embryo. For this work we have chosen the *sm50* gene, a molecular marker for the skeletogenic lineages. The *sm50* is a gene turned on autonomously during cleavage in the precursors of the skeletogenic cells at the vegetal pole. The *cis*-regulatory system of the *sm50* is confined in a single module. The module uses multiple activators, namely, the positively-acting sequences A and D (two copies), and an indispensable spatial control element C. This *cis*-regulatory system contains no repression system.

Our overall objective in this work is to define the mechanism by which the 25 bp C-element of the *cis*-regulatory region of the *sm50* gene exercises its

primary spatial control function on the accurate expression of *sm50* in the skeletogenic lineages. We have so far resolved the components of the activation complex assembly that targets this element and devised the means of purifying the diverse members of this complex. At present, we are actively pursuing the molecular identities of the members of the complex.

In the course of this work, we have discovered a cluster of target sites by site-specific mutagenesis of the C-element. Not only does the C-element contain the core-binding site of the assembly, but also two additional DNA sequences recognized specifically by factors within a 6 h and 22 h embryonic nuclear extract.

35. AmHNF-6: New member of the ONECUT class of transcription factor expressed in starfish embryos

Ochan Otim, Veronica Hinman

In our effort to understand how gene batteries in a regulatory network evolve, a number of the starfish *Asterina miniata* (*A. miniata*) transcription factor (TF) homologues of the sea urchin *Strongylocentrotus purpuratus* known to be involved in the endomesodermal specification in starfish have been cloned recently. This report presents one such transcription factor, the AmHNF-6 protein, a member of the ONECUT family of TFs. In sea urchin, HNF6 regulates PMC terminal genes and oral ectoderm specification (see report #17). Since the starfish *A. miniata* does not have PMC, the question we are asking is, what is then the role of AmHNF6 early in the development of *A. miniata*?

So far, two variants of the gene have been isolated: AmHNF-6α and β. The fraction of the Amhnf-6 mRNA molecules present in the population of the total mRNA molecules expressed at any given time in *A. miniata* was found to be extremely small. Out of the screen of six filters containing arrayed cDNA from three-day embryos, only nine clones were identified as AmHF6 cDNA molecules (AmHNF-6α, 5; AmHNF-6β, 1; and cDNA clones missing the identifying linker sequence of HNF6, 3). A filter is a 22-cm^2 membrane that carries 18,432 clones.

36. Whole-genome search for forkhead-box-containing transcription factors

Paola Oliveri, C. Titus Brown, Leah Vega

The forkhead-box ('fox') family of transcription factors is ancestral to the bilaterians. Members are involved in developmental processes in many model organisms. Only three members of this family have previously been characterized in sea urchin.

We have conducted a systematic search for forkhead-box factors in the newly available shotgun reads from the *S. purpuratus* whole-genome sequencing effort being carried out at the Baylor HGSC. A total of 20 novel fox factors have been identified from the currently available 2x coverage, and we are doing a QPCR analysis of temporal expression profiles across embryogenesis for each of them. QPCR has been carried out on nine of the novel factors, and all but one of them are present within the first 72 h of development, as are the three previously characterized factors. This suggests that most, if not all, of the 23 fox factors now known will be present during embryogenesis.

We are also developing software to extend the individual traces (averaging 600 bp each) into larger contiguous sequences, and we will use these larger contigs to design probes for whole mount *in situ* hybridization. Once we obtain expression patterns for these factors, we will place them in the Endomesoderm Network and begin to investigate their causal roles in early development.

37. ***cis***-Regulatory analysis of genes at the nodes of endomesoderm GRN

Paola Oliveri, Jonathan P. Rast[*]*, Sangeeta Bardhan*

The key elements of the GRN are comprised of DNA sequences encoding regulatory genes and their respective *cis*-regulatory apparatuses. Such elements constitute the nodes of the network, which in turn receive a series of inputs (DNA-regulatory protein interactions) that are integrated and transferred to the new regulatory protein as specific expression patterns. The network architecture is built as functional linkages connecting *cis*-regulatory elements. Logic studies of the regulatory apparatuses at the nodal regions of each gene have been carried out so as to understand the *cis*-regulatory system. Three genes have been chosen as key nodes in three different locations of the GRN: *pmar1, gataC* and *foxA*. The approaches used for studying their regulatory sequences are different in each case and similar in the employment of GFP as reporter gene.

pmar1 is the key transducer of maternal inputs to zygotic gene expression in micromeres. A 15 kb genomic fragment containing the *pmar1* gene was scanned for its functionality using *Mbo*I partial digestion and random cloning in GFP constructs containing the Endo16 basal promoter. This resulted in a series of overlapping fragments that were injected into the zygote and tested by functional assay. An element of roughly 2 kb which directs the expression of the reporter gene transiently in the vegetal cells has been thus isolated.

gataC starts its expression in a subset of SMC at the blastula stage. During gastrulation the *gataC* expression pattern is dynamic, showing transient expression in the aboral domain of PMC and eventually in coelomic pouches. The regulatory apparatus of this gene has been preliminarily analyzed for its ability to correctly drive the expression of the GFP using a bacterial homologous recombination procedure in which the first exon of the *gataC* gene present in a 140 kb BAC clone was replaced with the GFP coding sequence. The whole BAC recombinant then was reintroduced into embryos and found to recapitulate the dynamic expression pattern of the *gataC* endogenous gene.

foxA is an endoderm-specific transcription factor which is activated in the oral ectoderm domain at mid gastrulation. The whole *foxA* gene is contained in a single

BAC clone. Typically, only one exon contains the entire coding sequence. The approach used in the *foxA cis*-regulatory analysis involved the identification of conserved patches of noncoding sequences *via* comparison with the *Lytechinus variegatus* genomic sequence. Eighteen conserved regions have been identified in the comparison of 75 kb of sequence and six of these patches have been tested for functionality. As of this date, an oral-specific regulatory sequence and a general ubiquitous regulatory sequence have been identified.

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38. The conservation of ***cis***-regulatory information in lower deuterostomes

Jonathan Rast[*], Ping Dong, Julie Hahn, R. Andrew Cameron

An effective method of locating individual *cis*-regulatory elements is comparative sequence analysis conducted at appropriate divergence times to reveal conserved elements. Current results show that this approach may yield a more than 10-fold increase in rate of experimental *cis*-regulatory element discovery, compared to the most efficient "blind" search methods. However, general rules for carrying out such analyses are not yet known, and initial work has shown very different degrees of similarity between genomic regions surrounding different genes in a single pair of sea urchin species. We have launched a project to determine the rules for efficient *cis*-regulatory sequence prediction by interspecific sequence analysis. We are analyzing and then testing by gene transfer putative *cis*-regulatory elements identified in the vicinity of about 20 different genes. The candidate genes come from several different echinoderm species that display a range of phylogenetic relatedness. We have constructed BAC libraries for three species of sea urchins, and for more distant comparisons, a sea star and a hemichordate. We are currently screening the libraries to obtain BACs containing the candidate genes. As the BACs are identified they are put in the pipeline for sequencing. We will then identify putative *cis*-regulatory regions for the candidate genes selected, as indicated by interspecific sequence comparison at diverse distances. These DNA fragments will be tested for *cis*-regulatory capability by gene transfer. Not only will this approach reveal rules for computational *cis*-regulatory analysis, but it is also supporting the extension of the current repertoire of BAC libraries, improving computational tools, and generating more efficient laboratory methods for this essential research area.

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39. Regulatory gene network evolution: A comparison of endomesoderm specification in starfish and sea urchins

Veronica F. Hinman, Albert Nguyen

We are undertaking an evolutionary comparison of the regulatory network of transcription factors underlying the specification of endomesoderm in sea urchins and starfish. The recent extensive analysis of this network in sea urchins has provided a unique opportunity for a comparative investigation to elucidate mechanism of evolution at this level. We would like to answer questions such as, which components of such a regulatory system are conserved, how are changes incorporated into a network, and importantly, how do these changes relate to the evolution of morphology? The starfish *Asterina miniata* has been developed as an ideal experimental model for this analysis. Gametes are readily available and gene transfer and perturbation of gene products have been performed. Starfish last shared a common ancestor with sea urchins around 500 million years ago. While many aspects of their early embryonic development are conserved, there are some key differences that will provide for meaningful evolutionary comparisons of their underlying developmental processes.

We have isolated starfish cDNA orthologs to some of the transcription factors known to be part of the specification network in sea urchins (i.e., *otx*, *krox*, *gatae*, *foxA*, *brachyury*, and *tbrain*). The spatial and temporal expression patterns of each of these genes has been established in the starfish. The function of Krox, Gatae, FoxA and Otx, have been perturbed using morpholino-substituted antisense oligonucleotides and/or a dominant engrailed repressor strategy. The effects on the other genes of perturbing the expression of each were then determined. This has allowed us to construct a regulatory network of these genes in the starfish. This analysis has shown that many regulatory connections are conserved between these two taxa, including some early interactions that are thought to initiate and stabilize the endoderm specification network. Among the differences, we have noted that *tbrain* is incorporated into the endoderm-specification network in starfish, while it is involved in primary mesenchyme cell specification in sea urchins. This is reflected in the difference in spatial expression of this gene in the two animals.

We know from this comparative network analysis that the *brachyury* (*bra*) gene in starfish and sea urchins is similarly regulated, yet comparative sequence analyses using "FamilyRelations" fail to find any significant patches of conservation in the approximately 90 kb of surrounding DNA. In order to understand how the sequence of a *cis*-regulatory region has evolved whilst maintaining a conserved function we are analyzing, in detail, the *cis*-regulatory region of the starfish *bra* gene and comparing it to that regulating sea urchin *bra* (see report by R.A. Cameron). We currently have an approximately 500 bp intronic region of AmBra DNA that drives correct reporter gene expression in pregastrular starfish embryos. Further computational analyses and trans-species experiments are

planned.

40. ***cis*-Regulatory analysis of the starfish *delta* gene**

Feng Gao, Veronica F. Hinman, Kirsten Welge

It is well known that signaling ligand, Delta, has two different roles during endomesodermal specification in the sea urchin embryo. Each of these roles requires *delta* to be expressed in a specific territory of the embryo, first in the micromeres and later in the prospective SMCs. The aim of the current project is to analyze the *cis*-regulatory system of the *delta* gene from the starfish *Asterina miniata* and to compare its structure to the known *cis*-regulatory element controlling *delta* expression in *S. purpuratus* (i.e., R11; see report by R. Revilla). Sea urchins and starfish last shared a common ancestor around 500 mya, so this analysis will provide insights into the evolutionary mechanisms operating on *cis*-regulatory machinery over this immense period of time.

A cDNA encoding for the *A. miniata delta* orthologue (*AmDelta*) was obtained by library screening and RT-PCR. Alignment of the predicted amino acid sequence of *AmDelta* with other Delta homologues showed that it has the characteristic domain structure of Delta family members. In order to determine the expression levels of *AmDelta*, real-time quantitative RT-PCR (QPCR) was performed on samples of developmental stages throughout embryogenesis (from egg to 72 h larva). *AmDelta* is expressed at low levels throughout cleavage with a distinct peak in the mid-blastula stage (18 h) and increasing transcript abundance throughout gastrulation. Whole mount *in situ* hybridization (WMISH) was performed on post-hatching *A. miniata* embryos. Specific staining was difficult to distinguish as *delta* is expressed at low levels. Signal intensity was improved with the addition of polyvinyl alcohol (PVA) of high molecular weight (70-100 KD) to the BCIP-NBT detection buffer. PVA functions to enhance the alkaline phosphatase reaction and prevent diffusion of reaction intermediates. *AmDelta* transcripts are detected in the center of the vegetal plate during blastula stage. In order to determine the spatial distribution of *delta* in pre-hatching embryos, techniques are being developed to remove or permealize the fertilization envelope. Two methods, including acid and mercaptoacetate treatments have been tried with good success with probes for other, more abundant transcripts.

We are now in the process of obtaining a BAC clone for the *AmDelta* gene by screening an *A. miniata* BAC genomic library. A three-way sequence comparison of the DNA sequence surrounding the sea urchin (*S. purpuratus* and *Lytechinus variegatus*) and *A. miniata delta* genes will be used to identify conserved patches of sequence. These will be tested for functionality using a GFP reporter system already developed for *A. miniata*. We will also determine if the *S. purpuratus delta cis*-regulatory element R11 has any function in *A. miniata* by injecting the R11-GFP construct into *A. miniata* eggs.

41. ***Amdri*: Initial characterization of a starfish ARID transcription factor**

Gabriele Amore, Charlotte Guo, Veronica Hinman

The *Spdeadringer* (*Spdri*) gene encodes the first extended-ARID (e-ARID)-class transcription factor found in echinoderms. ARID transcription factors are classified into three subgroups on the basis of the number of α-helices present in their DNA binding domain (DBD). Members of the e-ARID subfamily feature eight α-helices in their DBD, have highly specific DNA-binding target sequences and have been shown to play vital roles in several developmental systems. In the sea urchin embryo, *Spdri* plays essential roles in the specification and differentiation of the primary mesenchyme cells (PMCs) and oral ectoderm cells. Blockade of *Spdri* function leads to developmental abnormality, namely the absence of an embryonic skeleton (normally elaborated by the PMCs) and the failed specification of the oral ectoderm. Recently, we determined some of the regulatory interactions of *Spdri* in the two gene regulatory networks (GRNs) that control the specification of the PMCs and the oral ectoderm territory.

The developmental emergence of PMCs is not a general feature of all echinoderm embryos. Several groups (e.g., asteroids and holoturoids) do not form an embryonic skeleton at all and the production of skeletal elements only happens in the adult. For this reason, the employment of *Spdri* in the sea urchin PMCs GRN has to be looked at as a case of cooption and not as a shared developmental feature of the whole echinoderm group.

To gain insights on what may be a more general (and ancestral) role of ARID proteins in echinoderm development, we looked for a *Spdri* ortholog in the starfish *Asterina miniata.* We partially reconstructed the cDNA sequence of *Amdri*, the *Asterina miniata* orthologue of *Spdri,* by aligning overlapping cDNA clones. The translation of the putative DBD encoded in these clones, features eight α-helices. The alignment of this sequence with that of *Spdri*, as well as that of other ARID proteins found in several other organisms, confirms that *Amdri* is an e-ARID protein. Time-course experiments have been performed to measure the amount of the *Amdri* mRNA during development. Low levels of expression are present throughout development, but as the embryos start to gastrulate, *Amdri* levels begin to rise. From this time on, until the completion of gastrulation, *Amdri* mRNA levels increase 50-100 times. At this time, the expression of *Amdri* is localized in the mesoderm that tops the gut and in a patch of ectodermal cells that is the site where the mouth will form.

At the moment we are completing the sequencing of the *Amdri* cDNA and we are producing an mRNA for an *engrailed-dri* fusion protein to interfere with the function of the *Amdri* in the embryos. Morphological observation and marker analysis expression will follow to assess the role of the endogenous *Amdri*.

42. Understanding gene regulation through motif analysis

Meredith Howard

The developmental program directing the growth of a single-cell fertilized egg into an sea urchin embryo is encoded in the organism's genomic DNA. The essence of this program is a network of genes encoding transcription factors and the *cis*-regulatory modules controlling the expression of those genes. Each module can receive multiple inputs at various sequence-specific target sites for other transcription factors in the network, and these signals are integrated into a single output resulting in the gene being turned on or off in different areas of the developing organism at different points in time. Understanding the developmental process, therefore, requires finding the functional linkages of the network – connecting the output of regulatory genes to the genomic target sites to which those products bind to activate further rounds of specification. This task is made challenging by our incomplete understanding of how transcription factors discern functional target sites from the vast population of nonfunctional sites with the same sequence in the genome.

In order to study how functional target sites within *cis*-regulatory modules differ from nonfunctional sites scattered throughout the genome, we propose assembling a target site database of transcription factors known to be active in the gene regulatory networks that control early development of the sea urchin. This data will then be incorporated into a *cis*-regulatory prediction algorithm that maps onto a sequence the most probable positions of binding sites and background. Possible *cis*-regulatory modules can then be identified by choosing a window size and threshold of binding sites per window.

Since the quality of data available on transcription factor-binding sites in the literature is highly variable, the SELEX method (systematic evolution of ligands by exponential enrichment) will be used to generate libraries of sequences that bind specifically to *S. purpuratus* endomesodermal transcription factors. In this way we will avoid introducing biases into our data as a result of the dearth or excess of information available in the literature for any one transcription factor. We plan to acquire data for a substantial fraction of the genes in the sea urchin endomesoderm model, since the aim of the project is not only to deepen our understanding of the current model, but also to extend the model by finding more co-regulated genes as more data becomes available from the sea urchin genome-sequencing project. The proteins we intend to characterize initially include SpKrox-1, SpEve, SpGataE, SpFoxA, SpFoxB, SpBra, SpGataC, SpTbr, SpGcm, and SpPmar1.

43. A genome-wide survey of sea urchin transcription factors

Meredith Howard, Lili Chen, C. Titus Brown

The sea urchin genome sequencing project now underway presents us with the opportunity to do a definitive survey of transcription factors involved in the organism's development. The goal is to create a DNA chip that can be used to take a snapshot of the state of the developmental gene regulatory network at any time point. Such a chip will be very useful for conducting perturbation analyses.

The project will proceed in several phases. The first step will be to obtain a non-redundant set of sea urchin transcription factors, which we estimate to be on the order of 5% of a predicted 20,000 total genes. As traces are released, they will be BLASTed against NCBI's non-redundant protein database and sorted using a combination of automated screening and human annotation. QPCR studies will then be undertaken to identify which of the identified transcription factors are expressed during development and when. From this data a decision will be made as to which sequences should be incorporated into the chip.

To best suit our needs, the DNA chip should allow for rapid screening in a small volume of nuclear extract, and be sufficiently sensitive to detect a small number of transcripts per embryo. A very promising technology allowing exponential self-amplification of small DNA sequences has been developed by David Galas[*]. Typically, the amplified oligos are 8-16 bases, sufficiently long that each oligo can in theory uniquely target one transcript in sea urchin embryonic nuclear extract. Oligos can be arrayed on a chip for simultaneous detection of multiple transcripts in a complex mixture. The reactions take place isothermally in a volume of only 25 microliters in approximately ten minutes, and the method is sensitive enough to give a positive response given ~6000 copies of a transcript. In collaboration with the Galas lab, we will evaluate the suitability of this approach for assaying biological samples.

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44. Computational analysis of the emerging sea urchin genome sequence

Kevin Berney, C. Titus Brown, Ramon Cendejas, Ian Lipsky, R. Andrew Cameron

A most prominent event of the past year is the initiation of the sequencing of the purple sea urchin genome by the Baylor College of Medicine, Human Genome Sequencing Center with support from HGRI. The Sea Urchin Genome Project housed in the Center for Computational Regulatory Genomics provided the materials including libraries for the project. The Director of the Center acts as the coordinator for the project and liaison to the scientific community. The Sea Urchin Genome Project (SUGP) web site serves as an information exchange for the research community, while the staff of the Center have adopted the responsibility of supporting a 'first pass' analysis of the data as they emerge. At the time of this writing, the sequence in the public databases consists of 2,192,990, which was primarily from a whole-genome shotgun (WGS) project. This is sufficient to provide a 96% coverage of the genome of the purple sea urchin. The WGS project will be supplemented with a BAC matrix project in the coming months. Because of the

nature of the BAC matrix approach we do not expect to see assembled sea urchin genomic contigs at Genbank for some time. In the meantime, the Sea Urchin Genome Project will provide a simple analysis in order to facilitate gene discovery and future annotation of the genomic sequences. We perform a Blast search of the translated trace sequences against the Genbank non-redundant protein database. The Blast e-value cutoff is set to approximately 1.0e-10 because this value was empirically shown to yield an estimated 50,000 hits per genome for sequences the size of the traces. Although this works out to probably two to three times as many genes as the sea urchin genome contains, we expect that most of the false positives are eliminated and many divergent protein matches are retained. The information thus obtained is provided as a list that is searchable at the web site.

The computational staff of the Center is continuously conducting other sorts of analyses on the available trace sequences as well. Quality control and distribution measurements of the trace collection are derived from comparison to other data sets of sequences collected in the past, *viz* the BAC-end STC sequences determined at the High-throughput Sequencing Center at the University of Washington and posted on the web site. A pipeline for small database searches using curated sets for particular classes of genes is being developed. Preliminary results emerging for immune function genes demonstrate that this is a feasible approach well within the computing power we have available.

45. Expansion and refinement of comparative sequence analysis tools

C. Titus Brown, Yuan Xie, Kevin Berney, Ian Lipsky, R. Andrew Cameron

We have continued to work on our software suite for comparative sequence analysis, Cartwheet/FamilyRelations. This set of tools provides a simple, easy-to-use system for analyzing and annotating genomic sequence. The tools were developed for the purpose of identifying potential regulatory regions near to genes central to the Endomesoderm Network; they are also being used by many other labs both inside and outside Caltech.

Recent improvements include: extensions to Cartwheel to permit remote access from several different programming languages; a complete rewrite of the Java-based FamilyRelations graphical interface in C++ ('FRII'), with extraordinary increases in speed; the development of several libraries for parsing the variety of data produced by our analysis programs; and the development of a motif-searching library, named 'motility,' that enables flexible and customized searching for sequence motifs in large regions of DNA.

All software is available through the associated web sites, http://cartwheel.caltech.edu/ and http://family.caltech.edu/. A tutorial is available at http://family.caltech.edu/tutorial.

46. FACE: Field Accelerated Capillary Electrophoresis

Ochan Otim

During the purification of transcription factors by affinity chromatography, we are always confronted with the challenge of resolving the complex components of purified nuclear extracts at successive levels of purification to determine which fraction is being enriched. SDS-PAGE has been the traditional choice for this kind of analysis. However, from our experience, SDS-PAGE does not always achieve the high degree required to permit accurate and unequivocal location of the protein (complex) of interest.

We are therefore in the process of developing a novel mode of capillary electrophoresis to address this problem. We choose to call this method Field Accelerated Capillary Electrophoresis (FACE). FACE is base on an understanding that however slim the molecular or ionic differences amongst biomolecules are, the overall sum of the biophysical nature of each molecule is uniquely defined for each of the molecules. A gradually changing electric field strength should, therefore, be able to resolve completely any complex mixture of biomolecules however closely related their properties are.

Additional benefits of FACE over SDS-PAGE are many. For example, FACE does not require rigid separation gels, instead it uses polymer solutions that are easy to prepare and store in-house. Performing an analysis using FACE can take anywhere from a few minutes up to half an hour to complete, a time much shorter than that required for SDS-PAGE. FACE can be automated if the need arises. Quantifying the exact amount of analyte of interest is possible if internal standards are included in samples. Relative amounts of the components, a measure of enrichment, can be determined without the need of an internal standard. All these are possible because the area under a specific signal in an electropherogram can be determined very accurately, and is directly proportional to the amount of the analyte in a sample. The analyte in FACE can be protein, DNA or RNA.

47. Biophysical nature of polymer-biomolecular interactions under electric field

Ochan Otim

We have extended the study of the mechanism by which bimolecular solutes migrate and separate in the polymer matrices under electric field in capillary electrophoresis. We are currently focusing on the Tris-base/hydroxyethyl cellulose (HEC)/EDTA buffer systems containing urea. The use of HEC in capillary electrophoresis is well documented. Pertinent to this line of study is the fact we have shown earlier that urea raises the viscosity of HEC below its entanglement threshold in direct proportion to the amount of HEC in solution. More importantly, we have also discovered that the concentration of urea can be fixed at any value without affecting the viscosity of HEC for separations that require amounts of HEC above the entanglement threshold of HEC. These observations provide a wide spectrum from

which to optimize the concentrations of urea and HEC for capillary electrophoresis of biomolecules.

48. Precambrian life

Paola Oliveri, Feng Gao, Eric Davidson, Hiroto Kimura[1], John Eiler[1], David Bottjer[2], Steve Dornbos[2], Alexander Tsapin[3], Chia-Wei Lee[4], Jun-Yuan Chen[5]

We have set up a collaborative interdisciplinary consortium to explore by multiple technologies one of the world's most interesting paleontological deposits, the Doushantuo phosphorites of SW China. This formation dates to about 590 +/-10 million years ago, i.e., 50 +/-10 million years before the beginning of the Cambrian. Certain Doushantuo strata contain extremely well preserved microscopic animal fossils. The level of detail visible in some specimens is amazing, and in thin rock sections individual cells can sometimes be made out. No macroscopic animal organisms existed at this time, so far as is known, but several years ago well-preserved cleavage-stage eggs were reported in scanning EM's of acid macerates from Doushantuo rocks. Many of these are apparently sponge eggs, and microscopic sponges were prevalent in the relatively shallow benthic environment represented in the Doushantuo rocks. However, in the last couple of years our team has also identified a variety of cnidarian forms, including embryonic stages, planula larvae, and some adult cnidarian-like body plans. Among these are radial polyp-like structures with septa similar to those of modern anthozoa, and a stem group form covered with grooves lined with what appear to be sensory projections also similar to those of modern forms; however, no indications of cnidoblasts are ever seen in these microscopic cnidarian-like animals. Phylogenetic considerations and various logical arguments indicate that there should also have been present animals of the bilaterian evolutionary lineage, and a major focus of our effort is to discover fossil evidence of their existence. Following earlier indications of bilaterian-like embryonic forms, evidence of adult bilaterian body plans has indeed just been uncovered, predating the earliest previously reported fossil bilaterian by at least 30 million years. The microfossils that we study are observed by transmitted light in thin rock sections, and also by SEM, but we are now exploring the use of X-ray tomography to recover details of internal structure in three-dimensional microfossils as well. To illuminate the mystery of why all animal forms so far discovered in the Doushantuo are less than 1 mm in size, and to relate what the fossil record holds to the environmental circumstances in which these animals lived, and were preserved on death, we are carrying out a suite of additional studies. Samples of rock have been collected from top to bottom of the Doushantuo formation, which in the region of our investigation is 60 m deep, and have been subjected to extensive chemical and isotopic analysis. The most dramatic finding is that there was a sharp shift from quite anoxic to a much more oxygenated environment at just the level at which complex animal microfossils appear. A major objective is to bring physical methods to bear on the fossils themselves. Recently it was discovered that amino acids could be extracted from acid-residue fossil eggs, and so the way is now open for investigation of informative trace compounds that may be present within the fossilized remains. Taphonomic and other related studies are also being carried out to distinguish regions of decay prior to fossilization, and aspects of the fossils that have been generated by secondary mineralization processes, and we will attempt in the lab to reproduce the initial stages of the fossilization process.

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Postdoctoral Scholars: Doug Altshuler, William Dickson, Mark Frye, Debbie Liang
Graduate Students: John Bender, Seth Budick, Gwyneth Card, Mary Dunlop, Sean Humbert, Michael Reiser, Jasper Simon
Undergraduate Students: Robert Bailey, Xiao Wang
Research and Laboratory Staff: Rosalyn Sayaman, Amber Steele

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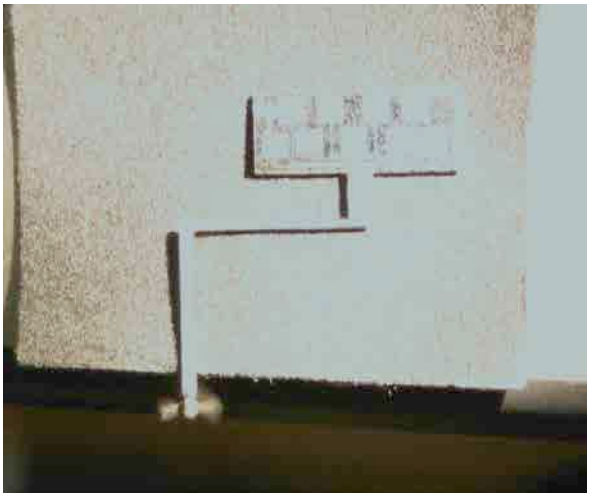
Summary:

1.) We have constructed and tested a prototype MEMS-based flight balance for fruit flies that will have use in many future experiments.
2.) We have been able to map the aerodynamic consequence of the several key steering muscles.
3.) We have demonstrated the changes in flow structure and force production around the wing as a function of Reynolds number.
4.) We have succeeded preliminary *in vivo* measurements of changes in the calcium concentration within power muscles during flight.
5.) We have characterized the interaction between olfactory and visual reflexes during flight, and have mapped the spatial tuning of visual motion reflexes.
6.) We have characterized the interaction between visual and mechanosensory stabilization reflexes.
7.) We have developed a control theory model that replicates the salient features of a male's tracking behavior during courtship.

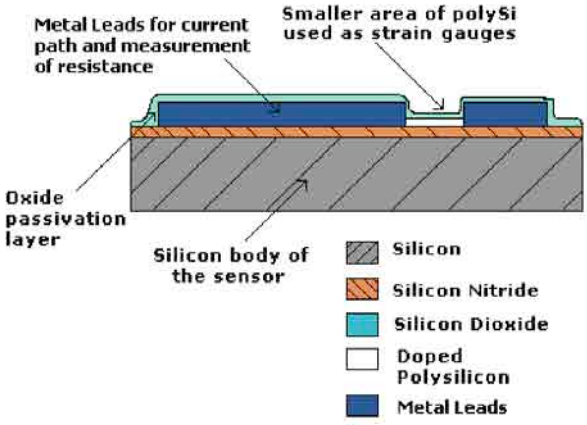
49. Multidirectional force and torque sensor

Mark Frye

During the last year, A MEMS micro-force sensor has been fabricated that can simultaneously measure force and torque of a tethered fly. Currently the device has been designed to measure the yaw or turning force as well as the thrust and lift generated by the fly. The device has four microfabricated strain gauges made out of piezoresistive material. The first generation sensor is shown in Figure 1A. It consists of two arms connected to a thick base. A pair of strain gauges is microfabricated on each arm of the sensor. When the tethered fly responses to visual input the beams will bend and the strain across each structure and attached strain gauges will change. The piezoelectric response changes the gauge resistance that provides direct measurement of force. The strain gauge pair on the vertical arm detects pitch and thrust, while the strain gauges on the horizontal arm provide yaw and lift. Two generations of sensors have been developed. In the first generation of sensor design, the electrical leads were fabricated out of polysilicon that had too high a resistance increasing the signal-to-noise of the device. In the current design the material for the leads has been changed to silver which is more complicated to fabricate but will improve device performance by two orders of magnitude. The fabrication details are shown in Figure 1B. The first generation prototypes have been fabricated, tested, and modified in tests with flies. Fabrication of the second generation sensor is currently being completed.



(A)



(B)

50. Comparing forces and fluid flow at low and high Reynolds numbers

William Dickson

One of the challenges with developing a comprehensive theory of flight in biological systems is to understand how aerodynamic mechanisms change with body size. In recent years, a large body of work has emerged focusing on flows generated by a variety of animals, ranging in size from hawkmoths and butterflies to bats and birds. The body size of a flying animal determines, for the large part, the Reynolds number (Re; the ratio of inertial to viscous forces) at which its wings operate. While prior research has studied animals that operate at Re > 2000, there has been little work at Re <200, the fluid regime of most insects.

Over the past year, we have performed several experiments to describe and quantify the flows and forces that insects experience across an order-of-magnitude change in Reynolds number and body size. These experiments utilized a dynamically-scaled robotic wing flapping in two tons of mineral oil. At the base of the wing is a force sensor that measures lift and drag. To visualize flow, we have used Digital Particle Image Velocimetry (DPIV). By grabbing pictures at identical times in a stroke, but at different positions along the wing, we can reconstruct the three-dimensional flow of fluid in a region that encompasses the wing. Both force and flow experiments were performed with an identical wing and wing kinematics, but in two oils with different viscosities. Thus, any differences in force or flow structure can be attributed solely to the changed Reynolds number.

When we flapped the wing thorough a 270º arc, forces were remarkably stable (Figure 2A). Mean coefficient values during 1/3 of translation (dotted lines in Figure 2A) are plotted in the aerodynamic polars in Figure 2B. Except at very low angles of attack, the net force coefficients were higher at Re 1400 (Figure 2C). In side view at mid-downstroke, the flow structure around the wing shows two areas of opposite vorticity (Figure 2D). Above the leading edge and spreading rearward over the upper side of the wing is a large area of CW vorticity indicative of the leading edge vortex (LEV). Along the undersurface of the wing there exists a region of vorticity of the opposite sense (CCW) that we call the under-wing vorticity layer (UWV). Qualitative inspection of the vorticity plots shows a region of comparatively greater vorticity near the core of the LEV at Re 1400, a result consistent with the force measurements. Integrating vorticity values over the entire panel provides an approximation of the local circulation around the wing (Figure 2E). Measured along the wing from the base to the tip, local circulation shows a general increase at both Reynolds numbers until approximately 0.6R, where it decreases due to separation and formation of the tip vortex.

In biological fluid mechanics, predicting forces from flow structures is often difficult because the requisite pressure information on and around the wing is missing. Mathematical manipulations of formulae with these pressure terms can create equations that require only flow velocity and vorticity information, information we have gathered through DPIV. Our force calculations from the flow structures gathered with DPIV are within 15% of those measured by the robot, a remarkable agreement given the DPIV and formulae utilize 2-D data.

A significant discovery was that, along with the differences in circulation, the flow structure within the LEV was different at both low and high Re. Most of the axial flow at low Re moved over the surface of the wing toward the wingtip, behind the LEV. At high Re there was this surface flow, but also flow within the LEV that often reached 150% the tip velocity (Figure 2F). DPIV showed that the structure of this flow consisted of a tightly wound spiral, unlike the smooth flow seen at low Re. These results suggest that the transport of vorticity from the leading edge to the wake takes different forms at different Reynolds numbers. We have yet to determine whether the appearance of the spiral flow within the core, or some other feature of the higher Re flow, such as less viscous dissipation accounts for the circulation differences at these two Reynolds numbers.

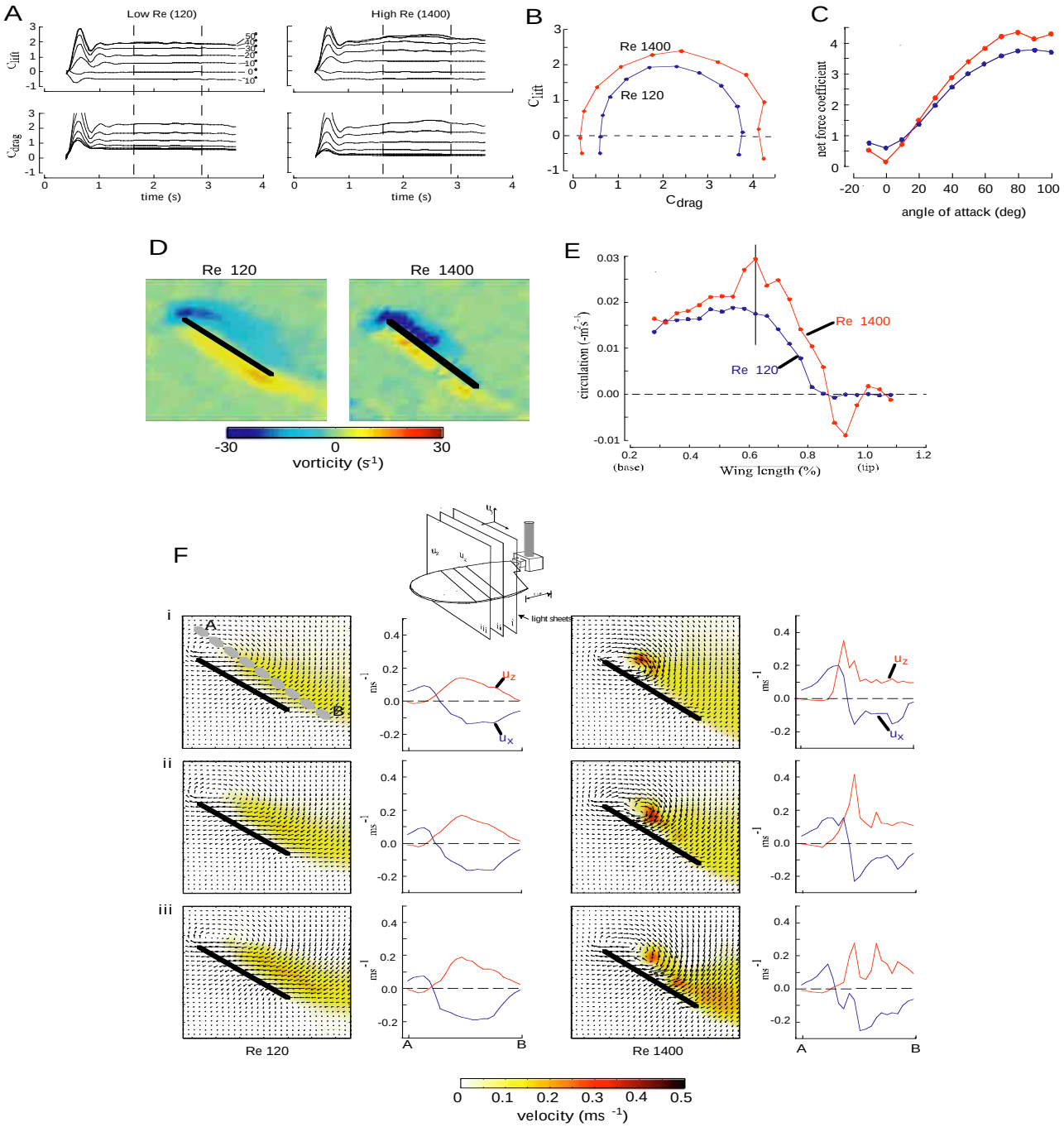


Figure 2. Comparison of translation force coefficients at Re 120 and 1400. (A) We rapidly accelerated the wing from rest and increased the angle of attach between trials by 10 increments. At both Reynolds numbers, an initial transient peak was followed by stable force generation. (C) C_L and C_D values averaged between the dashed lines in A. (C) Net force coefficients increase with angle of attack, with greater increases at high Reynolds numbers. (D) Side views of wing at 0.65R (R-length of one wing) at mid-downstroke. Wing is moving to left. Note the stronger and larger leading edge ortex at higher Reynolds number. (E) Circulation around the wing as a function of wing length. The vertical line at 0.65R represents the position if the pseudocolor plots in A. The area of greatest vorticity shifts slightly toward the wingtips at high Reynolds numbers, occurring at 0.65R versus 0.49R at low Re. (F) The magnitude and distribution of axial flow is dependent on Reynolds number. Top cartoon shows the position of the three side view panels (0.45R, 0.55R, and 0.65R) at each Reynolds number. Flows are captured from a wing at mid-downstroke starting from rest. Columns 1 and 3 show the sectional velocity field as arrows (u_y and u_x) superimposed over a pseudo-color plot of axial velocity (u_z). Next to each column are plotted the u_y and u_x values along the gray dashed transect from A to B shown in (i). The left two columns show flow atl ow Re. Note how the maximum axial flow (u_z) at low Re occurs farther behind the vortex center than at high Re. Also, at high Re, flow near the leading edge is much more complicated, with a stronger axial flow component.

51. Visuo-olfactory sensory fusion for flight behavior in flies

Mark Frye

Over the past year we have used the support of this grant to study the neurobiological basis of multisensory flight control in flies. We have specifically focused on vision and olfaction and how feedback from these sensory modalities is integrated to coordinate complex spatiotemporal dynamics of search behaviors. Using a state-of-the-art stereo video system, we tracked freely flying flies within different sensory landscapes and found that visual expansion cues generated as flies approach vertical edges is required for odor localization (Figure 3A). Using a 'virtual reality' tethered flight simulator, we examined the fine scale motor responses to visual expansion, odor, and both presented simultaneously. Our results show that during flight sensorimotor responses to odor are linearly superimposed upon visual responses (Figure 3B). This is a remarkable finding because it suggests that – from an engineering perspective - the underlying neural processing for tracking multiple sensory cues is relatively simple. A parallel sensory-to-motor control architecture may be an evolutionary adaptation that imparts both the extraordinary flexibility and robustness exhibited by flies in diverse sensory landscapes. These results have culminated in one publication, presentations at two international meetings, and two more manuscripts to be submitted for publication this month.

Our recent results have shown that during odor search, *Drosophila* more closely approach visual features near an invisible odor source. Computer simulations based on free flight statistics showed that this vision-odor interaction is sufficient to enable flies to localize the odor. Quantitative analyses of animals' responses to different visual patterns revealed that the motion of vertical edges is the salient visual cue that interacts with olfactory feedback (Figure 3A).

We further examined the fine scale of visuo-olfactory reflexes using a flight simulator in which we could manipulate a fly's visual and olfactory environment. Flies modulate wingbeat frequency and amplitude in response to visual and olfactory stimuli. Responses to both cues presented simultaneously represent the linear superposition of responses to stimuli presented in isolation for the onset and duration of odor delivery (Figure 3B). This suggests that odor does not alter the time course or magnitude of visual reflexes. Visual feedback does, however, alter the time course of odor-off responses. Based on the physiology of the flight motor system and recent free-flight analyses, we have posited a model to account for multisensory integration for flight control, which suggests that visual and olfactory signals are selectively targeted to separate groups of flight muscles. A simple parallel hierarchy could produce complex flight maneuvers while preserving the sensitivity of each modality.

We have also examined how complex patterns of optic flow affect visual stabilization reflexes in flies. We found that flies are more sensitive to patterns of visual translation than patterns of rotation. Until now, the classic view of flight control had been founded on a linear model for the stabilization of image rotation. Our new findings suggest an alternate model that takes into account the complex patterns of optic flow experienced by animals in natural sensory landscapes.

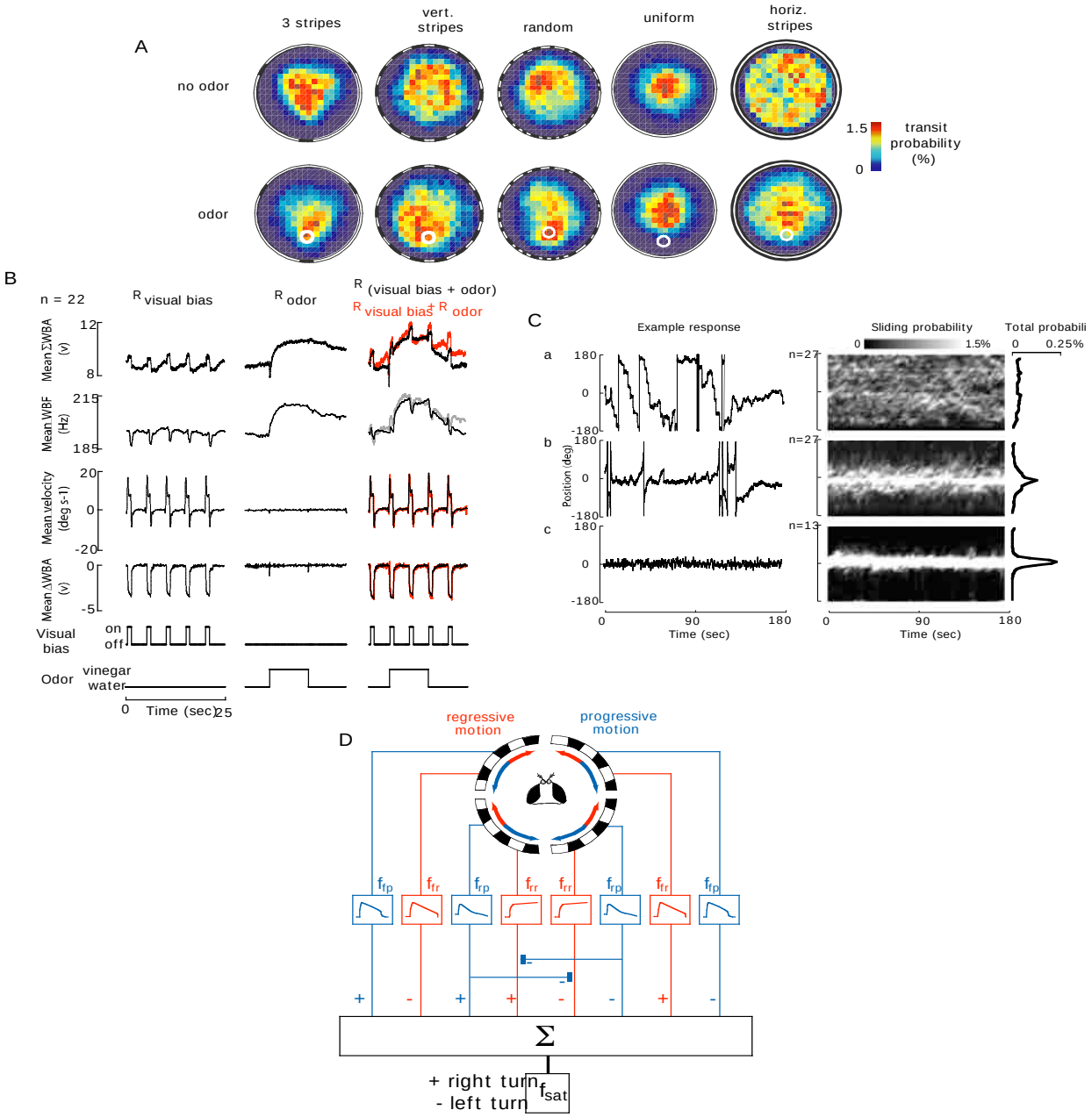


Figure 3. Flies fail to localize the horizontal position of an attractive odor source in the absence of visual backgrounds containing vertical edges. Imbedding a vial of apple cider vinegar in the floor of the arena (location indicated by white circle) resulted in biased flight trajectories for animals flying within the vertical stripe, three-stripe, and random checker backgrounds, but not for those within the uniform treatment. (B) During the duration of an odor pulse, motor responses to visual and olfactory cues represent the linear superposition of responses to each stimulus presented alone. Data sets are suggested into responses to visual bias during cold water vapor delivery (left column), odor in the absence of visual bias (center column), both stimuli simultaneously (indicated by black lines, right column), and the sum of responses to each stimulus presented alone (indicated by red line, right column). ΣWBA indicates sum of right and left wing beat amplitude, WBF indicates wing beat frequency, and ΔWBA indicates differences in amplitude between the right and left wings. (C) In closed-loop conditions, flies show a powerful steady-state expansion avoidance reflex. By adjusting the difference between the right and left wing stroke amplitude, flies control the horizontal position of (a) a random checkerboard pattern, (b) a single vertical stripe, and (c) the poles of a constantly expanding/contracting pattern of vertical stripes. For each experimental treatment, example responses are plotted in the left column, time series averages are plotted in the center column (indicated in grayscale pseudocolor), and total probability distributions are plotted in the right column. For the pseudocolor plots are white area indicates that flies maintain the rotating pattern in that particular position. On average, flies do not show preference of any single element of the random checkerboard pattern, whereas they tend to fixate the vertical stripe in front (0 degrees). Flies show even more robust fixation of the poles of expansion/contraction. There is less variability in fly's tendency to stabilize the poles of the expanding pattern in the rear field of view, thus the pole of contraction is fixed frontally. (D) Model of visual reflexes.

52. Interaction between vision and equilibrium

John Bender

In flies, both the visual system and small gyroscopic hind wings called halteres can detect angular velocity of the body during flight. To study the interaction of these sensory systems, we used a specialized flight simulator capable of delivering separate and concurrent visual and mechanical stimuli. My results have shown that these two sensory modalities have distinct and complementary operating regions. By analyzing the behavioral responses to concurrent visual and mechanical stimulation, we were able to quantify the contribution of each feedback channel during flight reflexes. These results show that input from both systems are combined in a weighted sum, and furthermore, that haltere feedback is weighted more heavily than visual information. In summary, these results present a framework for describing the integration of two sensory systems in the control of compensatory flight reflexes.

Using the flight simulator, we determined the dynamics of each sensory modality by measuring the frequency response separately for the compound eyes and the halteres. We found that the visual system is more sensitive to slow rotations, whereas the haltere-mediated wingbeat response increases with increasing rotational velocity. Thus, by using both sensory modalities the flight control system can achieve greater bandwidth without sacrificing sensitivity.

Once each system has been characterized individually, the next step is to determine how the animal integrates feedback from both sensory modalities simultaneously. Using the simulator, we measured the flies' response to concurrent visual and mechanical oscillations that vary in their phase, amplitude, and axis of rotation. We found that the flight control system uses both sensory channels when available, such that the response to complimentary concurrent stimuli is larger than the response elicited by exciting just one modality. The peak response occurs when visual and mechanical stimuli are phase offset by 180°, the phase that would occur when a freely flying animal rotates in space (Figure 4A). The flight control system integrates these sensory inputs in a manner that can be modeled by weighted sum, in which haltere feedback is given preference over visual information (Figure 4B). The results of these experiments provide a quantitative model of multi-sensory feedback integration in the control of complex motor behaviors.

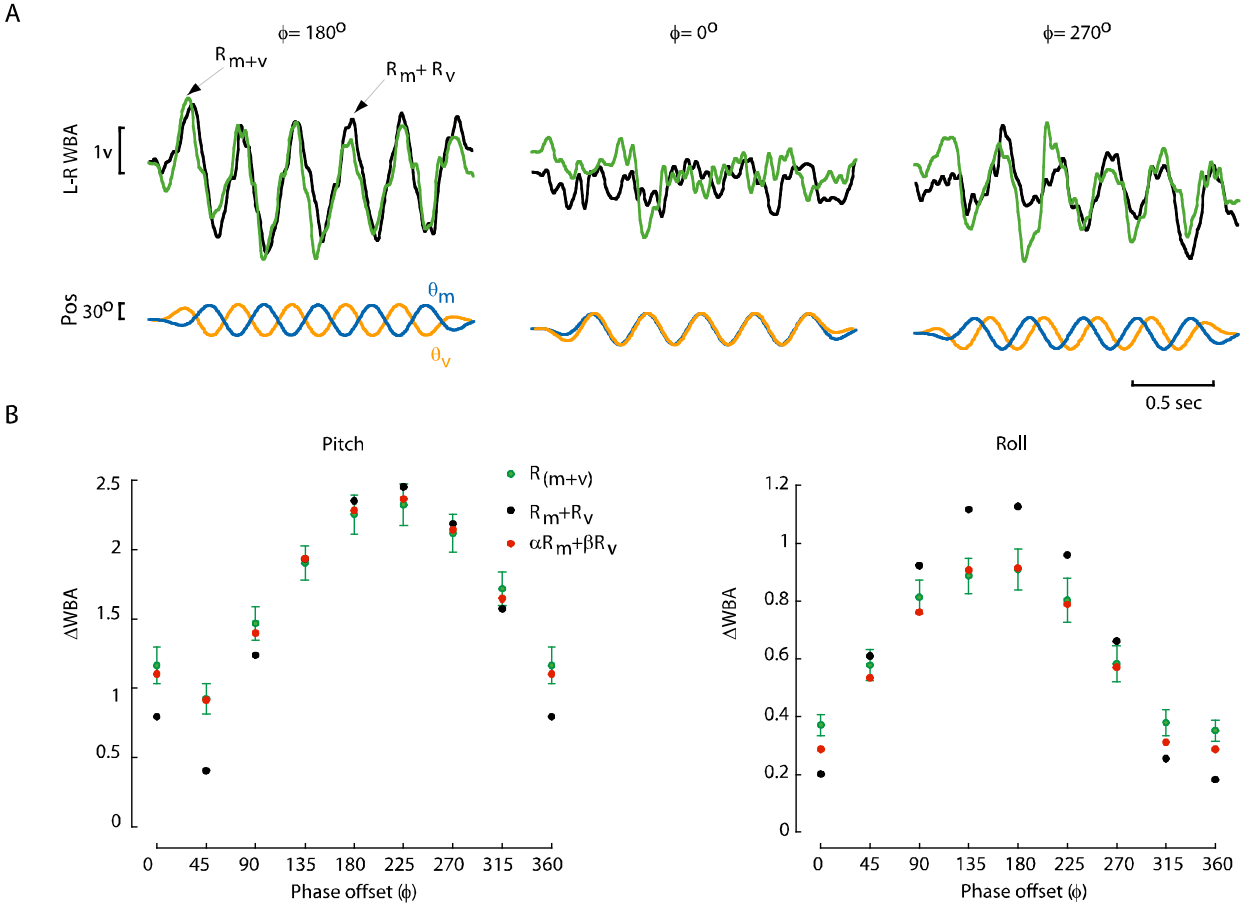


Figure 4. Reponses of flight motor to simultaneous input from visual and mechanosensory systems. (A) Wing beat amplitude response to simultaneous presentation (Rm+v), compared to linear sum of separate responses (Rm+Rv) for three different stimulus phases. (B) Summary of results across phase for both pitch and roll stimuli. See text for details.

53. Fly turing test

Mary Dunlop

To fully model the behavior of courting flies, we developed an arena that allows us to track freely-courting flies. Images are captured at 30 frames per second (fps) and processed in real-time to record the position (x, y) and orientation (θ) of each fly along with their body length and area. This captured data was used to create and evaluate a multi-dimensional model for the freely-courting male. The model simulates the male's trajectory using the captured female data as the input and contains a different controller for each of the three above-mentioned velocities. One proportional-integral (PI) controller acts to directly control the male's forward velocity based on the discrepancy between the azimuthal distance and the desired trailing distance. This controller includes the addition of an integral term necessary for attaining a constant tracking distance. A second PI controller modulates the angular velocity based on the target's angular location in the field of view. This follows the angular velocity control scheme proposed by Collett for hoverflies. A third controller modulates sideways velocity and supports data showing occasional lateral translation by the male flies. The third controller is also necessary for full controllability, especially at close distance to the target. A non-linear controller based on the relative angles of the flies was therefore used to describe this lateral velocity.

The model's performance over multiple data sets was analyzed in an attempt to determine the source of the model error. This revealed that the predicted position is far more accurate than predicted body angle. The analysis was done by checking the cross-correlation of the model inputs to residuals (modeled outputs – actual outputs) as well as the autocorrelation of the residuals themselves. The error for the predicted position was slightly over the acceptable amount for 3σ (97%) accuracy; however, the error for the predicted angle was significantly over the acceptable level. This indicates that a more complex model is necessary to completely capture the fly's behavior. Additional work focusing on the modeling of their angular movements may be key to reducing model error.

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Summary: Our laboratory has worked for several years to develop the tools that will allow *in vivo* tests of the cellular and molecular mechanisms proposed to guide developmental patterning. The majority of our work employs biological imaging tools to follow molecular, cellular and tissue level events in the intact system so that we can ask which of the events proposed from *in vitro* analyses might play roles *in vivo*; in short, we hope to move forward from what might happen to what does happen in the natural biological context. The challenges of such *in vivo* tests involve the tagging of cells or molecules so that they can be followed in the intact system, the visualization of the tagged structures, and the interpretation of the time-varying events these images represent.

Recent work in our laboratory has moved forward on all of these fronts. For example, high resolution MRI and light microscopic techniques have allowed the construction of three-dimensional atlases of developing embryos ranging from frogs to mice. These atlases provide unprecedented views of the morphology of the cells and tissues at key stages of development. By combining these technologies with molecular approaches, we are now able to ask questions about the exact timing, position and nature of the signals that pattern the embryo. Parallel refinements in microscopic techniques have resulted in multispectral imaging tools. These allow a multitude of labels to be employed in the same preparation, offering the opportunity to perform a direct analysis of cell interactions and responses within developing tissues. Finally, through a collaboration with laboratories in the Beckman Institute (J. Solomon), the PMA (M. Roukes, M. Cross) and EAS (R. Phillips) Divisions, we are making dramatic progress on the development of nanomechanical array detectors able to sense very small numbers of RNAs and proteins, offering the potential of a systems analysis performed at the level of the single cell. Combined, these advances offer the exciting prospect of asking detailed questions of single cells as they execute their developmental programs.

54. Regeneration of the cerebellar anlage in zebrafish larvae requires FGF-mediated re-patterning of the anterior hindbrain

Reinhard W. Köster, Scott E. Fraser

We recently showed that neuronal migration from the upper rhombic lip is an important feature of cerebellar development during zebrafish embryogenesis (1). To understand whether zebrafish larvae can recover from cerebellar lesions involving neuronal migration, we have established an *in vivo* survival surgery that allows removal of the anlage of the forming cerebellum during stages at which neuronal migration occurs. Using a stable transgenic zebrafish line that expresses the Green Fluorescent Protein (GFP) in migratory neuronal precursors from the rhombic lip, we found that cerebellar neurons regenerate very efficiently after this ablation over a time period of three to six days. *In vivo* time-lapse studies show that these regenerating neurons are derived

from the anterior hindbrain migrating along routes reminiscent to the embryonic program, thereby repopulating the missing cerebellar region.

A significant increase of proliferation in the anterior hindbrain could not be observed after ablation of the cerebellar anlage. Instead, downregulation of the anterior expression of *hox*a2, marking rhombomere 2, occurs simultaneously with *de novo* expression of rhombic lip marker genes close to the new mid-hindbrain boundary (MHB) during stages when first regenerating neurons appear. These findings argue for a re-patterning of anterior hindbrain tissue from rhombomere 2 fate to a cerebellar cell fate.

During late gastrulation, FGF8-signaling emanating from the MHB has been shown to suppress *hox*a2 expression in the anterior hindbrain in order to create a cerebellar territory (2). Fgf8 is still expressed in cells of the posterior midbrain after surgical ablation of the cerebellar anlage making it a candidate to initiate regeneration by suppressing *hox*a2 expression in rhombomere 2 which after the ablation process abuts the new MHB. Using a chemical inhibitor of FGF signaling we found that migrating GFP-expressing neuronal precursors derived from the cerebellar rhombic lip are not induced by FGF signaling. When this inhibitor is administered subsequent to the ablation of the developing cerebellum *hox*a2, repression cannot be observed any longer. Furthermore, regeneration of the cerebellar anlage is severely impaired. This indicates that FGF signaling, likely from the new MHB, mediates the regeneration of the ablated cerebellar anlage in zebrafish larvae by re-patterning the anterior hindbrain through the suppression of *hox*a2 expression.

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55. Analysis of intracellular non-viral vector motion using correlation spectroscopy

Rajan Kulkarni, Scott E. Fraser

Recently, there has been much interest in developing alternatives to viral-based gene therapy due to safety and efficacy concerns. Non-viral gene vectors hold the promise of delivering DNA to cells in a more controllable fashion than through traditional viral vectors. However, less is known about the intracellular dynamics and kinetics of such novel vectors. Of particular interest are a group of synthetic cyclodextrin vectors that have been previously shown to be taken up by different cell lines. By fluorescently labeling DNA and complexing it with cyclodextrin vectors, these particles can be tracked within the cell upon internalization. Once inside the cells, these vectors display varied dynamic behavior ranging from subdiffusion through directed transport. This latter behavior is the most interesting, because it suggests that the particles might be able to engage the intracellular transport machinery (microtubules and motor proteins). The mechanism for this is still unclear, but likely involves transport of the particles while they are enclosed in endosomes. Image correlation spectroscopy (ICS), which uses spatial and temporal information encoded within an image series, was used to analyze the fluorescence fluctuations of the particles to determine biophysical parameters of the system. ICS relies on correlating the fluctuation of the signal between different temporal and spatial points with the average intensity of the system. Diffusion coefficients for entire cells, as well as for subsets were measured using ICS and were compared to theoretical Stokes-Einstein diffusion values. However, such analyses give ensemble average measurements and do not yield information on individual entities. Both single particle tracking analysis and higher order autocorrelation are being explored to address the stochasticity and dynamics of individual particle motion.

56. Cranial neural crest migration in the avian embryo: Getting around a road block

Carole Lu, Scott E. Fraser

Cranial neural crest cells are a migratory multipotent population of cells that form most of the craniofacial structures, as well as some PNS derivatives, in the vertebrate embryo. In the chick hindbrain, these cells migrate out from the dorsal neural tube in three discrete streams from rhombomere(r) 2, r4, and r6. Previous work in our lab has been directed at understanding the migratory behavior and pathways of these cells *in vivo*. In order to visualize the migrating neural crest cells, we pressure inject lipophilic dyes or electroporate reporter constructs expressing GFP or RFP. We use an explant culture system developed in the lab and time lapse the embryos on an inverted confocal microscope for up to 24 hr. My own work is currently directed at understanding the importance of cell-cell interactions, as well as the potential role of several cell adhesion and extracellular matrix molecules in the migratory process.

Thus far, we have assessed the cell mixing of cranial neural crest arising from different rhombomeres in the same migratory stream and the regulative capacity of the r4 stream of migrating crest in overcoming foil barriers in their path to branchial arch 2. Despite a sizeable impermeable barrier, we found that neural crest cells are very plastic in their migratory pathway and 85% of placed barriers were ineffective at blocking their migration. With time lapse analysis, we found that cells at the front of the migration tend to explore the barrier more than following cells, and can be blocked. Later cells are able to interact with those cells and migrate around them and the barrier, hugging the barrier as they migrate into repulsive, neural crest-free, zones in order to get around it. We observed a reduction in the denseness of the migratory streams but found that the cells still interacted with each other and tended to migrate as a community.

Current work is directed at genetic manipulations of cell adhesion and signaling molecules and extracellular matrix blocking antibodies to get at more biologically relevant players in the migratory process.

This work was done with Paul Kulesa, who is now at the Stowers Institute.

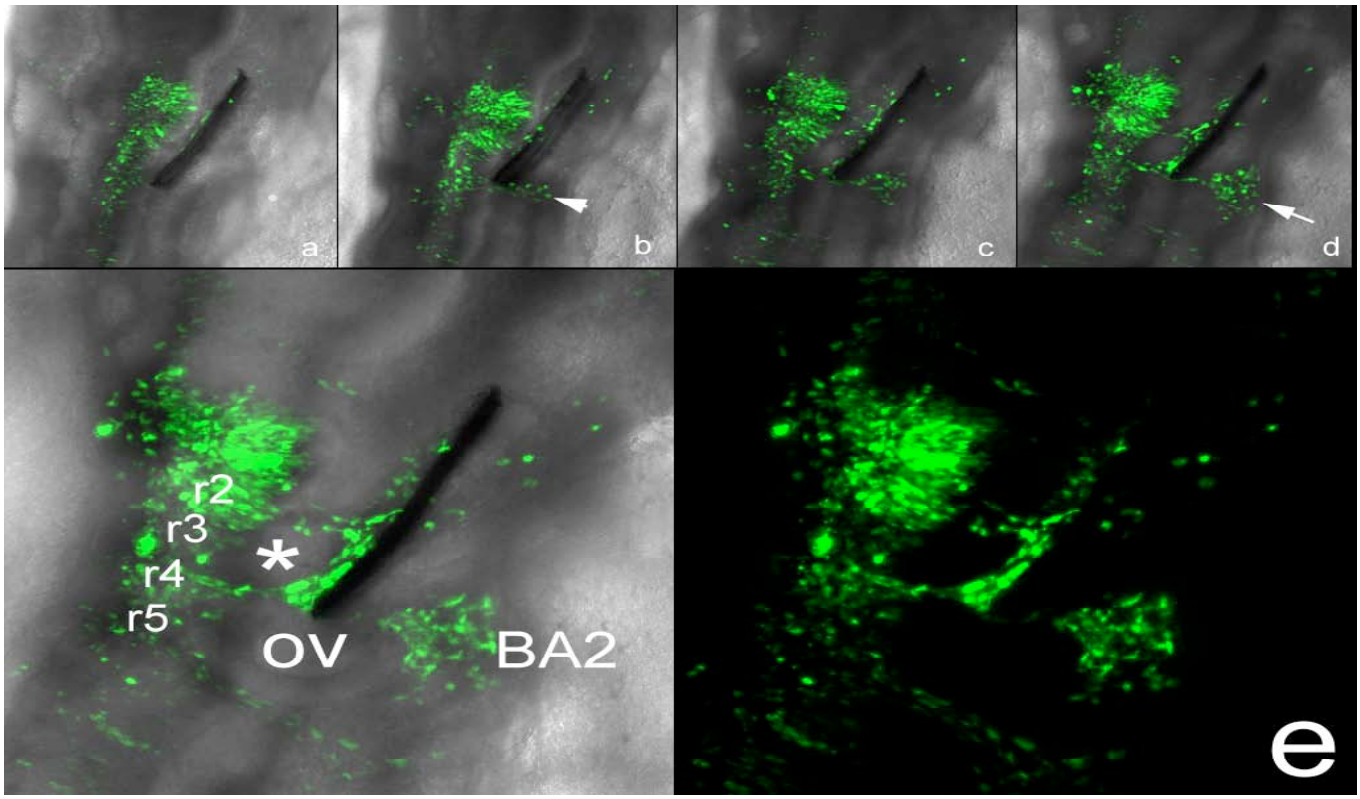

Figure 1: Panels from a 16-hr time lapse showing the migrating neural crest as they migrate around or get trapped behind a permeable barrier. A. At the start of the movie, the permeable barrier can be seen extending from the midbrain/hindbrain boundary to the anterior edge of r4. B. As the movie progresses a distinctive r4 stream is formed by migrating neural crest just posterior of the barrier (arrowhead). C. The front of the stream have arrived in BA2 and some cells are now trapped behind the barrier. In D, the neural crest cells have arrived at BA2 and are spreading out (arrow). E. Many neural crest cells are now trapped behind the permeable barrier. Two distinct r2 and r4 streams are separated by a crest-free zone (asterisk) next to r3. ov, otic vesicle; BA2, branchial arch 2; r2, rhombomere2.

57. Intermolecular double quantum coherence (i-DQC) Imaging of the mouse brain ***in vivo***

P.T. Narasimhan, S. Sendhil Velan, Benoit Boulat, Russell E. Jacobs

Magnetic Resonance Imaging (MRI) offers a variety of contrast mechanisms that can be utilized to highlight different aspects of tissue structure. Apart from the well-known contrast mechanisms based on T_1, T_2, spin density and diffusion, the contrast generated by intermolecular multiple quantum coherence (i-MQC's) has aroused considerable recent interest. These coherences are generated from distant dipolar fields of magnetic dipoles such as protons located on different water molecules separated by distances larger than the diffusion length of a few microns. The i-MQC's are not directly observable but can be converted into single quantum coherences (SQC's) that are observables and thus, provide images with unique contrast. A simple two-pulse sequence of the type: $90\text{-}G_1\text{-}t_1\text{-}\theta\text{-}G_2\text{-}t_2$-echo can be used to observe i-MQC echoes. The double quantum version (i-DQC) utilizes $G_1{:}G_2 = 1{:}2$ and the signal is maximum with $\theta = 120^\circ$. In order to generate the images we have adopted two different phase encoding schemes [Velan *et al.* (2001) *J. Magn. Res*.]. In one, the i-DQF method, we phase encode the SQC's filtered from the i-DQC's after conversion by the θ pulse. In the other method, the i-DQP method, we phase encode the i-DQC's directly, during their evolution period, t_1. These two ways of phase encoding lead to two kinds of i-DQC images. We have utilized these i-DQC techniques to obtain images of the mouse brain *in vivo*.

Both 2D and 3D i-DQC imaging pulse sequences were developed and implemented on a 500MHz (11.7T) Bruker DRX Avance spectrometer running Paravision software for image acquisition. Figure 1 shows the 3D i-DQC pulse sequence using standard nomenclature. Phase encoding gradients G_P are utilized for i-DQP imaging while the gradients G_P, are utilized for i-DQF imaging. The images (see Figure 2) were obtained using

an actively shielded gradient employing a 38mm RF birdcage coil. The temperature of the live mouse was maintained at 36˚C. Figure 2 shows 2D coronal slices obtained from the respective 3D data sets (128x64x64; FOV: 3x3x3cm). The 2D slices correspond to data size of 64x64. Figure 2a displays the i-DQP image while Figure 2b is the i-DQF image. For the same FOV the i-DQP image employed G_P values that were only 50% of the G_P, values of DQF due to the double quantum nature of i-DQP phase encoding. Thus, the phase encode values were similar for i-DQF and spin-echo (SE) imaging with same FOV. The SE image (Figure 2c) obtained with a SE sequence and with TR and TE values comparable to those employed for i-DQC imaging. The high contrast between brain areas and muscle tissue outside the brain in the i-DQC images (Fig. 2a and Fig. 2b) is noteworthy.

i-DQC signals are weak in comparison to SE signals, being typically about 10-15% of SE. This results in longer imaging time for obtaining i-DQC images. We are at present exploring the possibility of integrating fast spin echo imaging sequences with i-MQC sequences to reduce imaging time significantly and thus, facilitate biological i-MQC imaging.

Figure 1.

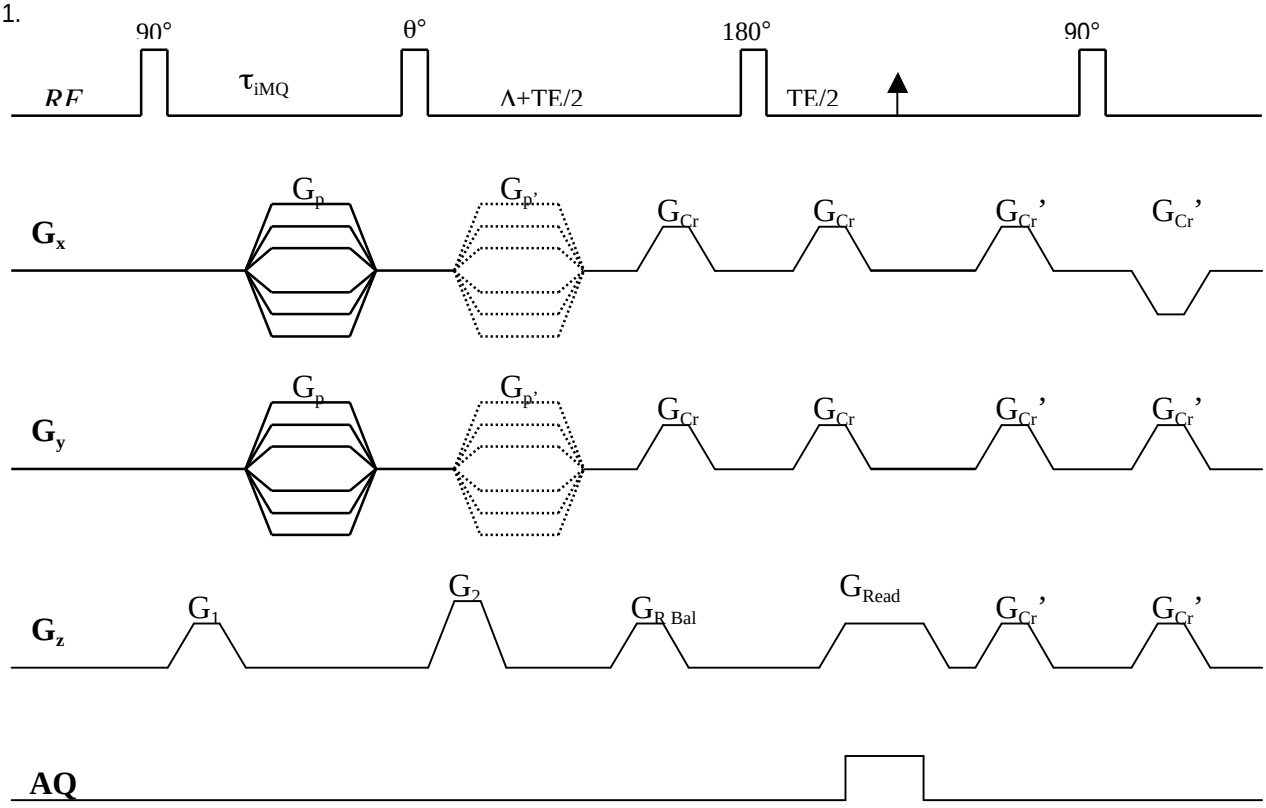

Fig. 1. 3D pulse sequence for i-DQP and i-DQF imaging. Phase encoding gradients G_P are utilized for i-DQP imaging while gradients $G_{P'}$ are utilized for i-DQP imaging. The arrow shows the position of the echo. The portion of the pulse sequence following the echo is a module for suppressing stimulated echoes.

Figure 2

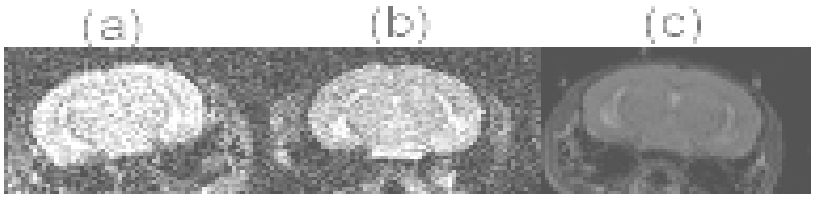

Fig. 2. i-DQC and SE mouse brain images. 2D coronal slices obtained from 3D data sets are shown. The coherence selection gradients used for the i-DQC imaging were G_1 = 7 G/cm and G_2 = 14 G/cm)
3D data set size: 128x64x64; 2D slice dimension: 64x64; FOV: 3.0x3.0x3.0cm; 2a: i-DQP image, NS = 4, TR = 2s; 2b: i-DQF image, NS = 4, TR = 2s; 2c: SE image, NS = 2, TR = 2s.

58. 3D time-lapse analysis of early ***Xenopus*** development using μMRI

Cyrus Papan, Benoit Boulat, S. Sendhil Velan, Scott E. Fraser, Russell E. Jacobs

Studies focusing on the amphibian morphogenesis and tissue relationships are severely hampered by the almost complete optical opacity of their early embryos. As a consequence, observations related to morphogenesis of the embryo remain indirect interpretations that rely on either fixed and sectioned material, on the observation of the external movements or on tissue explants.

To directly examine the development of the live frog embryo, we are using microscopic magnetic resonance imaging (μMRI). The initial feasibility of observing the development of the early frog embryo has been demonstrated earlier [Jacobs and Fraser, (1994) *Science* 263 {5147}:681-684.] Here we explore the method in greater detail for the live examination of early *Xenopus* development in a true 3D time-lapse analysis.

By using water-proton or fat-proton imaging, several embryonic regions can be distinguished by intrinsic contrast (Figure 1): the vegetal cell mass, the animal cap tissue, the blastocoel and the archenteron. A voxel size of $(39\ \mu m)^3$ and an imaging time of 55 min yields sufficient spatial and temporal resolution to visualize changes of the morphology over time with great detail. By injecting T1-or T2-contrast agents into early blastomeres, we are able to follow the development of the labeled clones within the context of the whole embryo.

A main function of Spemann organizer in *Xenopus laevis* is neural induction. However, models about the tissue relationships during neural induction are controversial. Because of the total opacity of the *Xenopus* embryo, it is not possible to track morphogenetic movements by optical methods. Thus, none of these relationships have been observed *in vivo*.

By labeling the C1-blastomere alone, which gives rise to most of the organizer tissue, or together with the C4 blastomere, our imaging experiments allow us to identify the relationship between the organizer and ectodermal and endodermal tissue during blastula and gastrula stages (Figure 2). In summary our results show that the axial mesoderm, which is considered to be in a planar configuration to the presumptive neuroectoderm, is actually vertically underlying the ectoderm throughout blastula and gastrula stages, much earlier than previously thought. Vertical interactions between mesoderm and presumptive neuroectoderm therefore do not depend on involution movements. This finding explains the long lasting controversy between the planar and vertical induction model by integrating the early timing of inductive signals from the planar model with the vertical configuration of the vertical model.

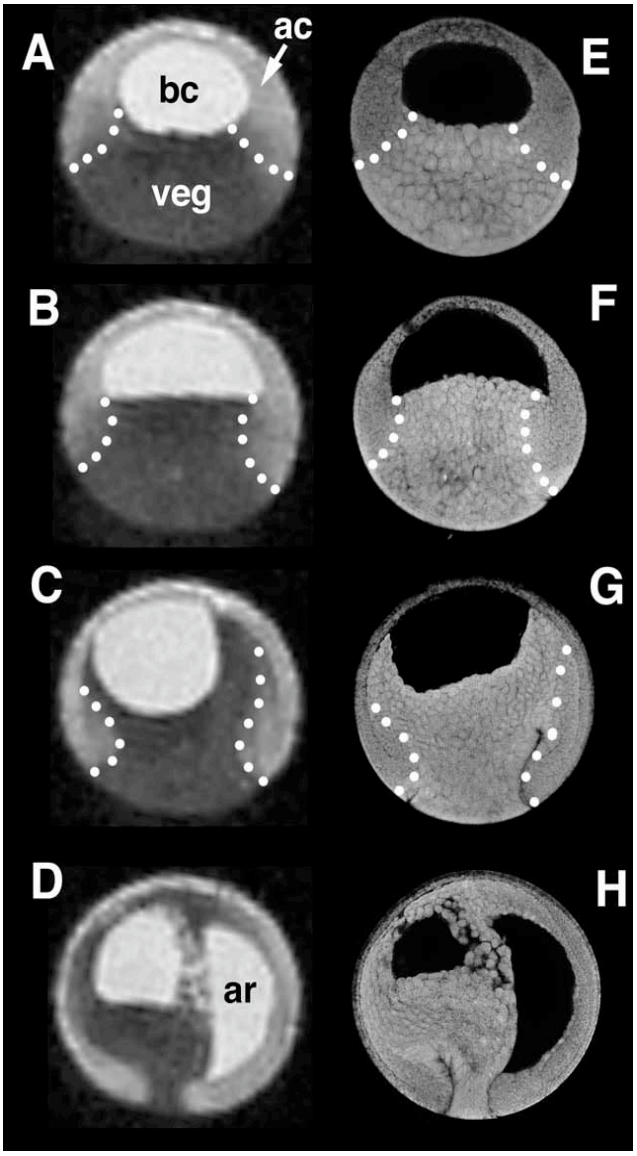

Figure 1: Time series of blastula and gastrula stages of *Xenopus laevis*. A-D MR-images, E-H histological images of sibling embryos fixed at the time of the corresponding scans of A-D. A,E: stage 9; B,F: stage 10; C,G: stage 11; D,H: stage 12. The intrinsic contrast in the MR-images allows to distinguish the major tissue regions of the early embryo: ac:animal cap (intermediate intensity); bc: blastocoel and ar:archenteron (high intensity); veg:vegetal cell mass (lowest intensity). The boundary between the animal cap and the vegetal cell mass is highlighted with whithe dots. It corresponds to the boundary of small cells in the animal cap and larger cells of the vegetal cell mass in the histological images.

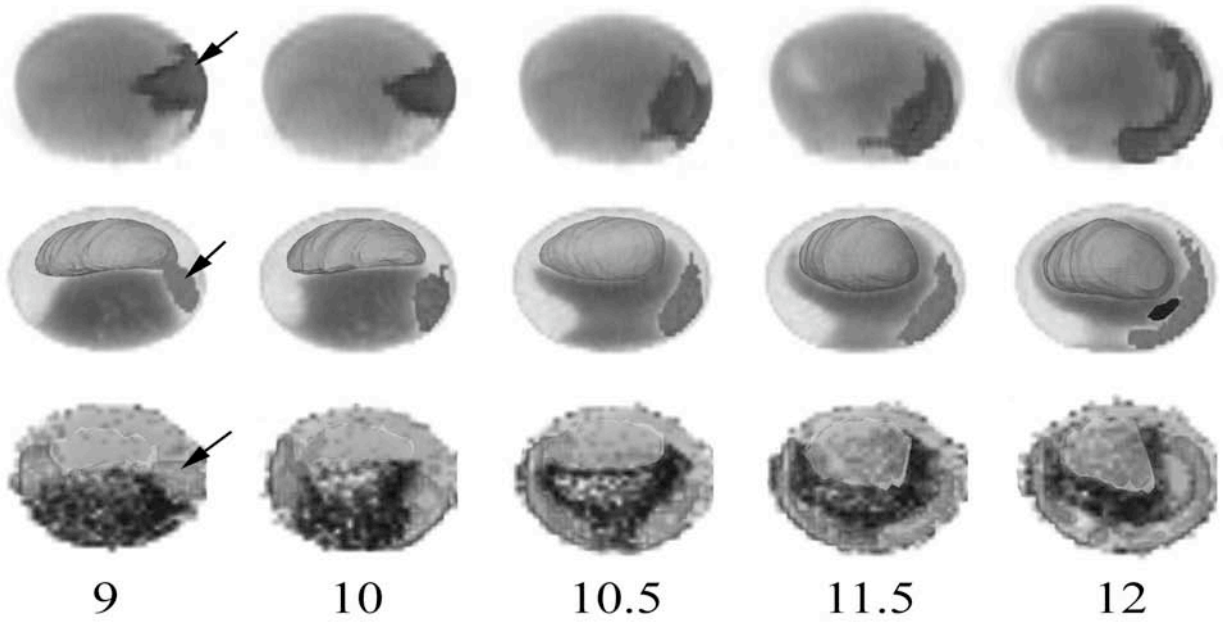

Figure 2: Dynamics of the C1-clone (arrow) during gastrulation in *X. laevis*. Numbers denote the embryonic stages. At stage 9, the clones extend deep into the embryo. The inner part is presumptive mesoderm which during gastrulation extends along the dorsal aspect of the embryo. The outer part is presumptive ectoderm which after gastrulation is located externally at the dorsal side of the embryo and will thus, form neural tissue. Thus, the deep presumptive mesodermal and the superficial presumptive neuroectodermal parts of the clones are in vertical contact well before gastrulation begins.

59. Diffusion anisotropy microscopy of the ***shiverer*** mouse mutant

J. Michael Tyszka, Carol W. Readhead, Robia G. Pautler, Timothy Hiltner, Russell E. Jacobs

Magnetic resonance imaging is one of very few non-invasive techniques capable of measuring the directional anisotropy of water diffusion in opaque biological tissues. Having refined magnetic resonance diffusion tensor imaging (DTI) (1-4) and anisotropy measurement techniques in the Biological Imaging Center, we are now applying these optimized methods to the investigation of neuroarchitectural differences between wild-type and *shiverer* mutant mice.

shiverer mice exhibit a mutation if myelin basic protein with incomplete myelination in the CNS. The *shiverer* mouse is a popular model system for study of dysmyelination in the absence of inflammation and axonal injury. Recent studies with DTI in live adult *shiverer* mice by *Song et al.* (5) suggest that although the principle diffusive component along the axon remains unchanged in the presence of dysmyelination, the radial component increases in the CNS white matter tracts of *shiverer* mice.

We are extending the previous *in vivo* study with complementary high-resolution (115μm isotropic voxel) diffusion tensor microscopy of perfusion fixed brains from five PM114 wild-type controls and five *shi/shi* PM114 mutants. MRI *ex vivo* has the advantage of allowing significantly higher spatial resolution data to be acquired in the absence of confounding physiological motion artifacts, and as such can be thought of as 3D non-invasive histology. DTI images from both groups were co-registered using polynomial spatial warping to allow within group and between group comparisons. Initial results reveal many differences in the diffusion microenvironment in both the white matter tracts and non-white matter parenchyma not limited to those suggested by the *in vivo* study. In addition to differences in the tensor trace, fractional anisotropy, perpendicular and parallel eigenvalue components, subtle neuroarchitectural differences have also come to light that are yet to be explained by simple dysmyelination. We are currently exploring more sophisticated spatial analysis of the DTI data, which in the broader scheme will be applicable to any image-based phenotyping of rodent models.

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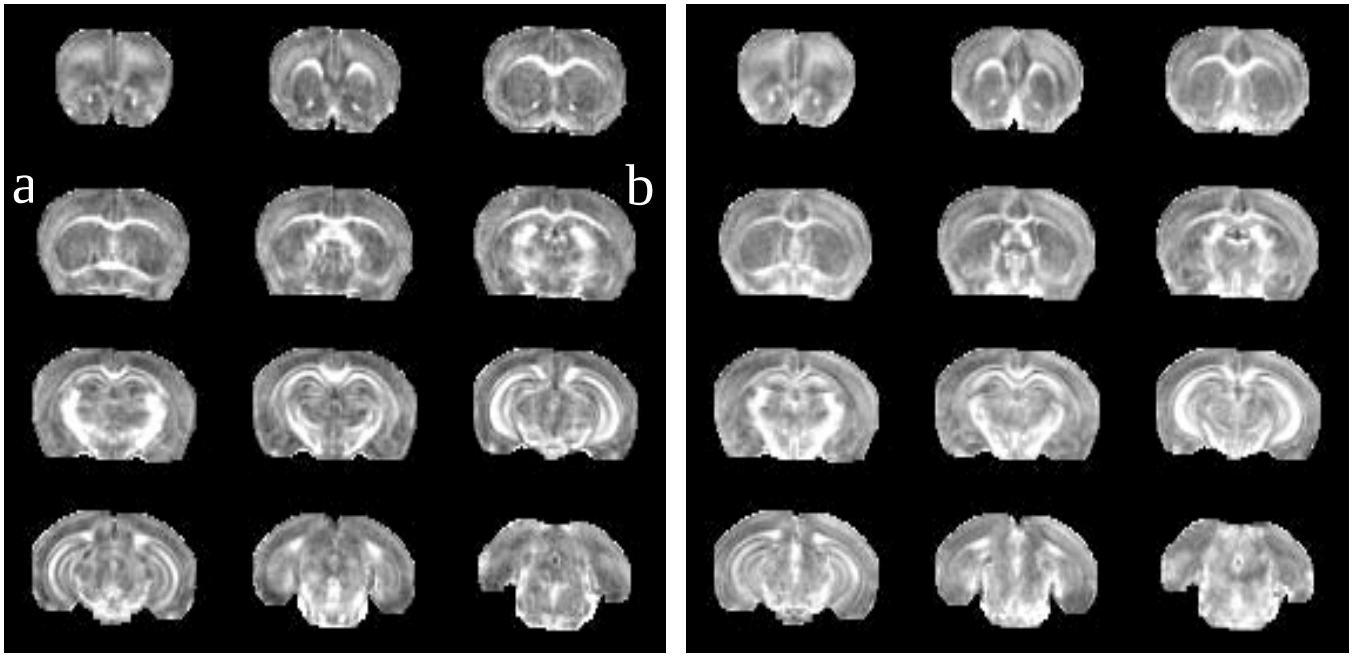

Figure 1. Registered mean fractional anisotropy maps from fixed brains of (a) PM114 controls (n=5) and (b) PM114 *shiverer* mutants (n=5), demonstrating multiple differences in the external capsule and other white matter tracts. Parenchymal anisotropy appears to be greater in the *shiver* mouse. Images are transverse sections of the brain from cranial (top left) to caudal (bottom right).

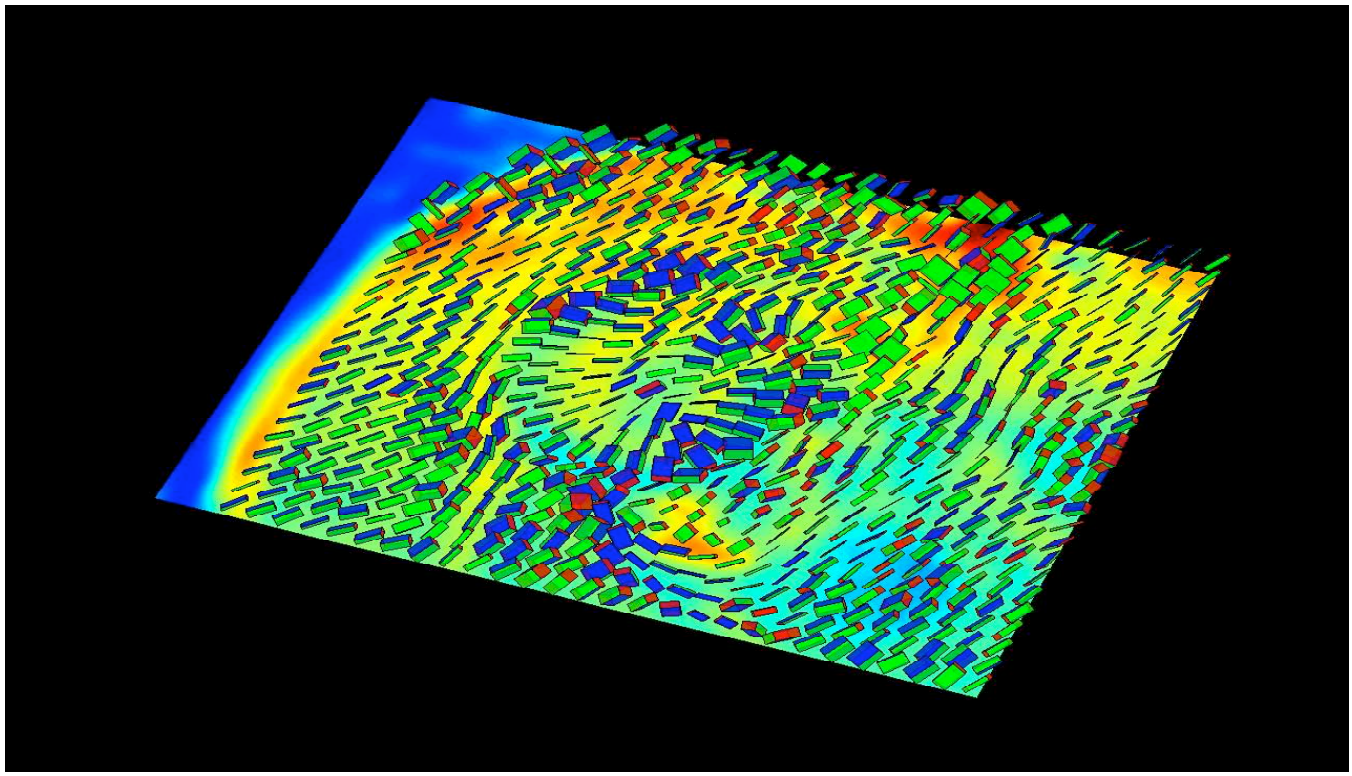

Figure 2. Diffusion tensor visualization using cuboids with axes corresponding to the eigenvalue weighted eigenvectors of the tensor in each voxel. This image shows DTI data from the dentate gyrus of the hippocampus of a single wild-type mouse.

60. Differences in midline kinetics of forebrain commissural axons ***in vivo***

Magdalena Bak, Scott E. Fraser

We are interested in studying the early phases of forebrain circuitry development. We would like to understand how the neuronal scaffold is formed, first by studying axon behaviors during commissure formation and second by gaining insight into molecular signals that guide this process.

Early neuronal scaffold development studies suggest that initial neurons and their axons serve as guides for later neurons and their processes. While this arrangement might aid axon navigation, the specific consequence(s) of such interactions are unknown *in vivo*. Recently, we followed forebrain commissure formation in living zebrafish embryos using time-lapse fluorescence microscopy to quantitatively examine commissural axon kinetics at the midline: a place where axon interactions might be important (Bak and Fraser, 2003). While it is commonly accepted that commissural axons slow down at the midline, our data showed this is only true for leader axons. Follower axons do not show this behavior. However, when the leading axon is ablated, follower axons change their midline kinetics and behave as leaders. Similarly, contralateral leader axons change their midline kinetics when they grow along the opposite leading axon across the midline. These data suggest a simple model where the level of growth cone exposure to midline cues and presence of other axons as a substrate shape the midline kinetics of commissural axons.

Our demonstration that axon kinetics are shaped by an ongoing interaction between leader and follower axons, in addition to any midline cues, highlights the importance of investigating the molecular underpinnings of midline crossing in vertebrates *in vivo*. We are currently in the process of testing the role of *netrin* and *Fgf* family of molecules at the midline.

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61. Cell dynamics during somite boundary formation revealed by time-lapse analysis

Paul M. Kulesa, Scott E. Fraser

We follow somite segmentation in living chick embryos and find that somitogenesis occurs in a precise spatiotemporal order tissue separation, cell movements and integration of cells at both the anterior and posterior somite. 3D imaging of bodipy-ceramide-labeled live embryos and fixed tissue shows that a somite pulls apart from the segmental plate. Time-lapse analyses reveal this ball and socket tissue separation is followed by a series of complex movements in which cells move across the presumptive somite boundary. The movement of cells across the presumptive somite boundary is not foreshadowed by gene expression boundaries. The expression of two key genes (assumed to correlate with the site of somite boundary formation), are not expressed at the correct site to play their proposed roles. The shaping process is not a simple periodic slicing of tissue blocks but a much more carefully choreographed separation in which the somite pulls apart from the segmental plate. Similarly, cells do not appear to be preassigned to a given somite as they leave the node. Our findings demonstrate that the sculpting of somites in chick is a more involved process than previously thought, violating current models of somite segmentation. Somite separation is not a simple, straightforward slicing. Our results motivate a model for somite formation in which both dynamic gene expression and cell motions pattern and sculpt the presomitic mesoderm into somites.

62. Wnt signaling components in the chicken intestinal tract

Helen J. McBride, Bastian Fatke[*], Scott E. Fraser

Wnt signal transduction has emerged as an increasingly complex pathway due to the numerous ligands, receptors, and modulators identified in multiple developmental systems. Wnt signaling has been implicated in the renewal of the intestinal epithelium within adult animals and the progression of cancer in the colon. The Wnt family, however, has not been explored for function during embryonic gut development. Thus, to dissect the role of Wnt signaling in the developing gastrointestinal tract, it is necessary to first obtain a complete picture of the spatiotemporal expression of the Wnt signaling factors with respect to the different tissue layers of the gut. Here, we offer an in depth *in situ* gene expression study of Wnt ligands, *frizzled* receptors, and *frizzled*-related modulators over several days of chicken gut development. These data show some expected locations of Wnt signaling, as well as a surprising lack of expression of factors in the hindgut. This paper describes the first comprehensive characterization of the dynamic expression of Wnt signaling molecules during gut development. These data form the basis for future studies to determine the role of Wnt signaling in the developing gastrointestinal tract.

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63. Distinct modes of floor plate induction in the chick embryo

Iain Patten[1], Paul Kulesa, Michael M. Shen[2], Scott Fraser, Marysia Placzek[1]

To begin to reconcile models of floor plate formation in the vertebrate neural tube, we have performed experiments aimed at understanding the development of the early floor plate in the chick embryo. Using real-time analyses of cell behavior, we provide evidence that the principal contributor to the early neural midline, the future anterior floor plate, exists as a separate population of floor plate precursor cells in the epiblast of the gastrula-stage embryo, and does not share a lineage with axial mesoderm. Analysis of the tissue interactions associated with differentiation of these cells to a floor-plate fate reveals a

role for the nascent prechordal mesoderm, indicating that more than one inductive event is associated with floor plate formation along the length of the neuraxis. We show that *Nr1,* a chick *nodal* homologue, is expressed in the nascent prechordal mesoderm and we provide evidence that Nodal signaling can cooperate with Shh to induce the epiblast precursors to a floor-plate fate. These results indicate that a shared lineage with axial mesoderm cells is not a prerequisite for floor-plate differentiation and suggest parallels between the development of the floor plate in amniote and anamniote embryos.

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Summary: We are interested in how cell fate choice is regulated and carried out. A large focus of our work is directed towards understanding the genetic and molecular mechanisms that regulate and bring about cell death. Specifically, we are using *Drosophila melanogaster* as a model system to identify genes that function to regulate cell death, and to identify important roles that cell death plays in normal development. Important cellular regulatory pathways are evolutionarily conserved; thus, molecules identified as important regulators of cell death in *Drosophila* are likely to have homologs in vertebrates and the pathways that link these molecules are likely to be regulated similarly. A second set of goals is to take the molecules and pathways uncovered in *Drosophila* and apply this information to the study of cell death in vertebrates, with the ultimate goal of determining the role that aberrations in this process play in human pathologies. In this context we see *Drosophila* as a powerful tool for uncovering conserved components and modes of death regulation.

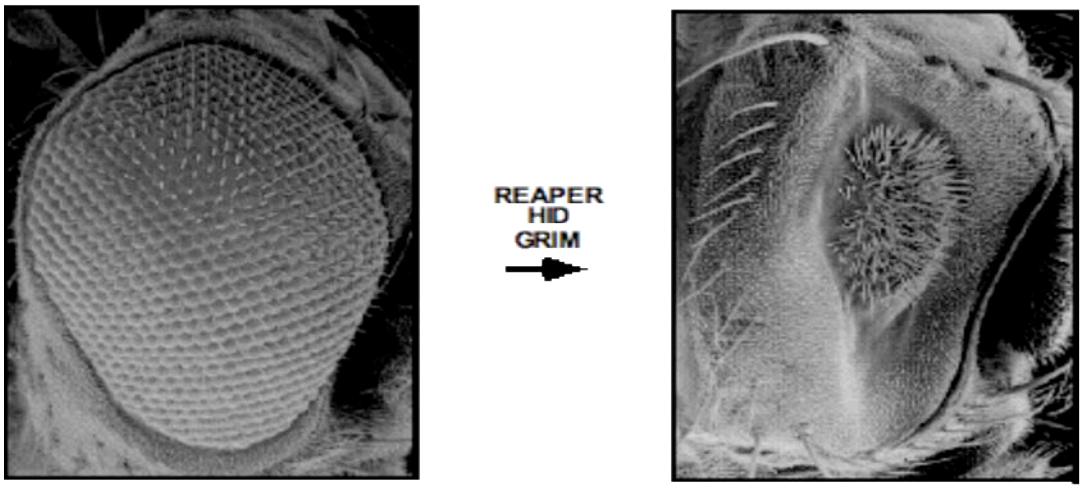

Figure 1. Overexpression of REAPER, HID or GRIM in the fly eye leads to excess cell death, which is manifested as flies with small eyes.

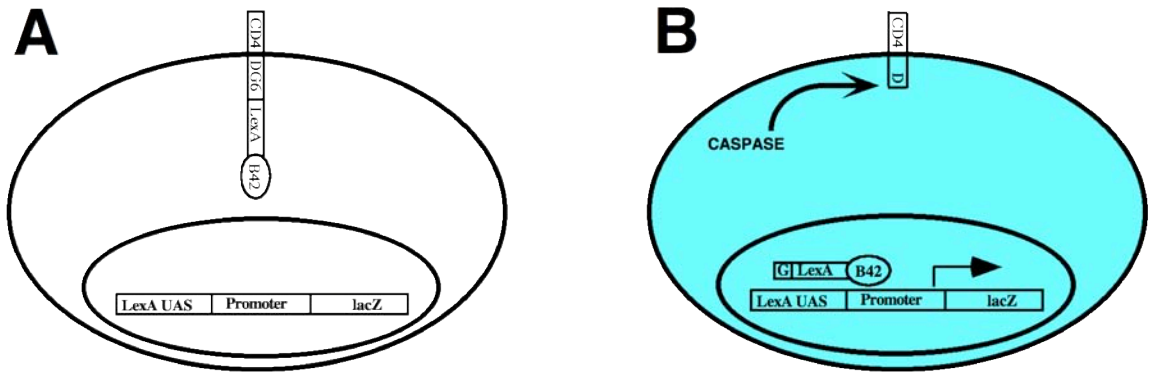

Figure 2. A yeast strain that acts as a reporter for caspase activity. (A) Six caspase cleavage sites are placed between an N-terminal fragment of CD4 and a C-terminal LexA-B42 transcription factor. (B) Caspase cleavage results in release of the LexA-B42 protein, and activation of *lacZ* transcription.

64. Yeast and fly based screens for proteases that can cleave in a transmembrane environment

Ming Guo, Hong Yu, Yile Ding, Bruce A. Hay

Alzheimer's disease is genetically heterogeneous, but it is invariantly associated with the accumulation in the brains of affected individuals of senile plaques consisting largely of amyloid beta peptide (A-beta), which is derived by proteolytic processing from the amyloid precursor protein (APP). A large body of evidence suggests that A-beta deposition is a cause rather than a consequence of Alzheimer's disease. Thus, blocking A-beta deposition is an important therapeutic goal. APP is initially translated as a type 1 transmembrane protein, but it can be processed by different pathways (see Figure 2). In the major pathway, alpha secretase releases the APP N-terminal ectodomain into the lumenal and extracellular space. Alpha secretase cleaves in the middle of the sequence that could give rise to the A-beta peptide, thus precluding its formation. In an alternative pathway A-beta peptides are formed through the action of beta and gamma secretases. Gamma secretase activity (which may consist of distinct proteases) cleaves in the transmembrane region of APP to generate, in conjunction with beta secretase, two major A-beta species of 40 or 42-43 residues in length, differing in the length of their C-termini. The longer forms of A-beta aggregate and are thought to seed the formation of amyloid plaques. The molecular nature of the gamma secretase(s) is unknown.

We have developed two screens to search for proteins that either are gamma secretase activity, or that regulate its activity. The first screen, diagrammed above, is a simple variant of the caspase reporter system in yeast, in which lacZ expression is the readout. We generated a form of APP in which a cleavable signal sequence lies just N-terminal to the APP beta secretase cleavage site. APP C-terminal sequences are then followed by the transcription factor LexA-B42. We are using yeast expressing this construct, as well as one of the presenilins, as a background in which to screen for proteins that show potential gamma secretase activity: cleavage-dependent reporter activation.

We have also set up a related screen in *Drosophila*, in which a similar APP fusion protein (in which the transcription factor is GAL4) is expressed in the eye in flies that carry a UAS-*rpr* construct (Guo *et al.*, 2003). In this system release of GAL4 from the membrane, as a result of gamma secretase activity, creates a cell death signal, and thus, flies with small eyes. This readout is very convenient for us because we can compare modifiers identified in GMR-*rpr* screens with those identified in GMR-APP-GAL4 screens. Those modifiers that are specific for GMR-APP-GAL4 are potentially interesting in terms of identifying genes that regulate APP cleavage. At this point we have carried out several large screen for enhancers and suppressors and have identified a modest number of interesting loci that are being pursued. Importantly, mutations that alter the levels of components of gamma secretase - presenilin and nicastrin - alter the reporter eye phenotype in the expected way. These observations give us confidence that the screen is likely to be pulling out interesting loci.

Doing a screen for modifiers of gamma secretase activity in a higher eukaryote is also important for the following reason. While gamma secretase is of course critical for cleavage of APP, it is also likely to be important for the cleavage of other transmembrane signaling proteins such as Notch. Thus, drugs targeted directly at gamma secretase may have pleiotropic effects. A genetic approach that focuses more generally on identifying modifiers of this activity may point towards new ways of modifying its activity or specificity in ways that more specifically affect APP processing.

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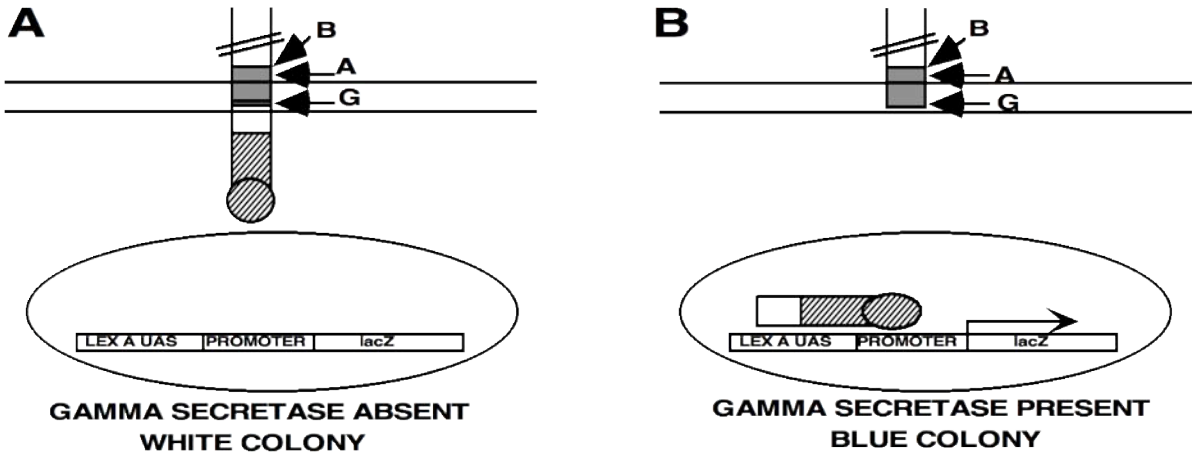

Figure 3. Generation of a yeast strain that acts as a reporter for gamma secretase activity. APP is made as a type 1 transmembrane protein. In (A) APP (open and shaded boxes) is shown with LexA-B42 (diagonal lined domains) fused to its C-terminus. Cleavage by alpha secretase (A) results in release of an N-terminal ectodomain. Alpha secretase cleaves within the A-beta peptide sequence (shaded box). Alternatively, cleavage by beta- (B) and gamma secretase (G) results in formation of the A-beta peptide 40 and 42-43 forms and release of a C-terminal fragment consisting of the APP cytoplasmic domain. (B) In the presence of gamma secretase the APP cytoplasmic domain-LexA-B42 fusion is released from the membrane and translocates to the nucleus where it activates transcription of lacZ.

65. Gene activation screens for cell death regulators: MicroRNAs, small non-coding RNAs, define a new family of cell death regulator

Peizhang Xu, Chun Hong Chen

The GMREP vector contains an eye-specific promoter near one P-element end, as well as sequences sufficient for plasmid rescue of genomic DNA flanking the site of P-element insertion. When this P element inserts near the 5' end of a gene it causes the gene to be misexpressed at high levels in the developing eye (Hay *et al*., 1997). We have mobilized this P element throughout the genome and are characterizing the insertions that act as cell death regulators. We first score the lines for dominant phenotypes that may be due to increased cell death (a small eye) or decreased cell death (a large, rough eye). We then cross these insertions to lines of flies that express the cell death activators REAPER, HID or GRIM specifically in the eye (GMR-rpr, GMR-hid, or GMR-grim flies), and that thus, have small eyes. The progeny of these crosses are then scored for the ability of the GMREP insertion to alter the GMR-*rpr*-, GMR-*hid*- or GMR-*grim*-dependent small eye phenotype. These modifiers identify new cell death regulators. Genomic DNA that is likely to contain a portion of the gene being overexpressed can be quickly isolated using plasmid rescue. The *Drosophila* genome is now finished. Thus, a small sequence tag from the end of the P element serves to tell us exactly where in the genome our insertion is. Because GMREP-dependent phenotypes are primarily due to insertions near the transcription start site, and because GMREP carries the dominant eye color marker *white*, imprecise P-element excision using a genomic source of transposase or X-rays can be carried out to rapidly generate deletions that create loss-of-function phenotypes for the overexpressed gene.

We generated and screened 8,000 transposon insertions for their ability to suppress *rpr- hid-*, or *grim*-dependent cell death and identified a modest number of new loci (about ten) that specifically suppress death due to overexpression of one or the other, or all of these genes in the eye. We are in the process of characterizing some of these lines. One death suppressor encodes the large ubiquitin conjugation protein Bruce (Vernooy *et al*., 2002) (we didn't name it), while four others encode cell death-inhibiting microRNAs (Xu *et al*., 2003).

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66. IAPs, cell death and ubiquitination

Soon Ji Yoo, Jun Huh, H. Arno J. Müller

DIAP1, as with many IAPs, also shows E3 ubiquitin-protein ligase activity. The function of this activity *in vivo* is unclear. One possibility is that this activity simply constitutes a mechanism for conferring a short half life to the IAP, thus serving a proapoptotic function. Alternatively, ubiquitination of IAP-bound proapoptotic proteins may provide a prosurvival mechanism by which IAPs can catalytically remove these molecules. IAPs may also engage in substrate choice, preferentially degrading themselves when not bound to proapoptotic molecules, but degrading-binding partners

when the opportunity arises. In this way IAPs could serve to create, through a posttranscriptional mechanism, a relative balance between pro and antiapoptotic proteins, at different levels of proapoptotic proteins. Finally, binding of proapoptotic proteins to DIAP1 may promote DIAP1 degradation, thereby promoting apoptosis. Over the last year we have obtained evidence for all of these activities. An important goal for the future is to understand how these diverse activities of DIAP1 are regulated in the contexts of developmental cues and environmental stresses.

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67. Identification of DIAP1-interacting proteins

Soon Ji Yoo, Hong Yu, Jeff Copeland

DIAP1 is required for cell survival in *Drosophila*. This suggests that its activity is likely to be regulated through interactions with other proteins. Genetic screens for cell death regulators provide one approach to identifying proteins that may interact with DIAP1. However, a more direct approach to identifying proteins that regulate DIAP1 function involves identifying proteins that physically bind DIAP1 in living *Drosophila*. We are using multiply-tagged versions of DIAP1 as bait to immunoprecipitate, identify and characterize associated proteins from healthy cells, as well as from cells exposed to various death stimuli.

68. How does ***Drosophila*** activate caspase-dependent cell death?

Soon Ji Yoo, H.-A.J. Müller, Jun Huh

An important set of questions is how different cell death signals, which in many cases are initiated through distinct signal transduction pathways, converge to activate a set of common downstream effector pathways. In *Drosophila* many different death signals lead to the transcriptional activation of one or more of three genes, *reaper* (*rpr*), *hid* and *grim*. The function of these genes is required for most normally occuring and induced cell death in *Drosophila*. Thus, their transcriptional activation acts as a point of death signaling convergence. One of our primary goals has been to identify mechanisms by which these different proteins activate cell death. Three important facts are known about these proteins that suggested testable mechanisms of action. These are: 1) that death induced by their expression requires caspase activity; 2) that their death-promoting activity is suppressed in a dose-dependent manner by DIAP1; and 3) that REAPER (RPR), HID and GRIM bind to DIAP1 in insect cells. These results have suggested several models of how DIAP1, caspases and RPR, HID and GRIM might interact to regulate cell death. In one model, death-activating proteins such as REAPER, HID or GRIM activate caspases through an IAP-independent pathway. This model postulates that *Drosophila* IAPs act at two different points to suppress apoptosis: by acting as a sink for death activators such as REAPER, HID or GRIM, preventing them from interacting with their normal targets, and by inhibiting the caspase activity initiated by their action. In a second model, DIAP1 is proposed to function primarily as a caspase inhibitor, and REAPER, HID and/or GRIM initiate caspase-dependent cell death by preventing IAPs from productively interacting with caspases, thereby promoting their activity, and ultimately cell death.

Susan Wang, a former graduate student, and Christine Hawkins, a former postdoc, carried out experiments in *Drosophila*, yeast and *in vitro* to test the idea that RPR, HID and GRIM promote apoptosis by blocking DIAP1's ability to inhibit caspase activity (Wang *et al*., 1999). They found that all three proteins, while nontoxic on their own, killed yeast coexpressing DCP-1 or drICE, and DIAP1, suggesting that they were blocking DIAP1's ability to function as a caspase inhibitor. They pursued the basis for this activity further with HID and found, both in yeast and *in vitro*, that proteins containing the N-terminal 37 residues of HID, which are sufficient to induce apoptosis in insect cells, suppressed DIAP1's ability to inhibit DCP-1 activity.

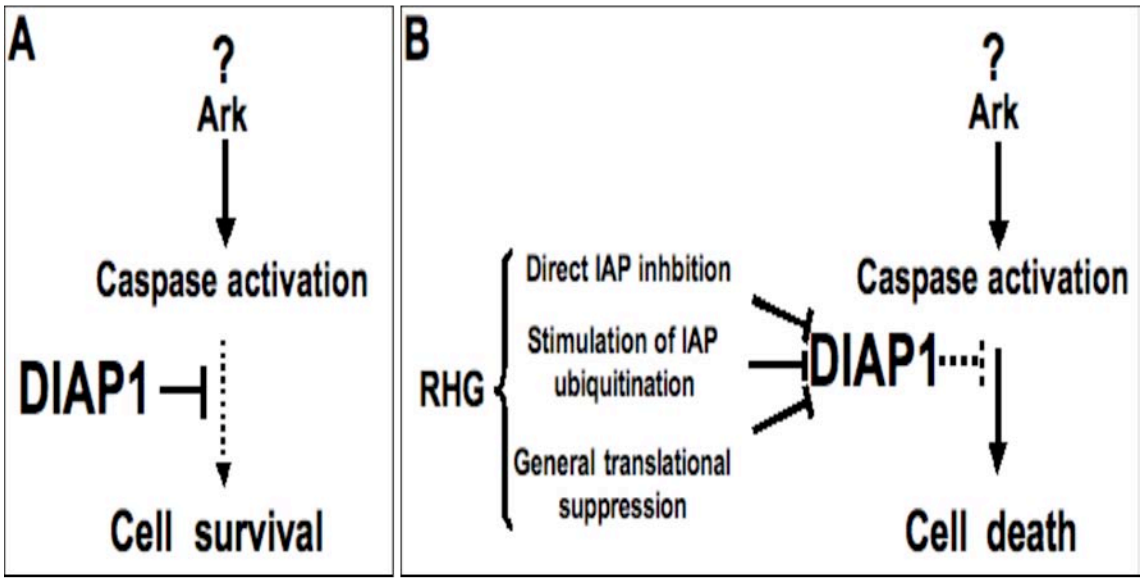
These results are consistent with a model in which RPR, HID and GRIM act through DIAP1 to promote death-inducing caspase activity. This model predicts that DIAP1 should be essential for cell survival, and that a loss of DIAP1 function should result in an increase in DIAP1-inhibitable caspase activity. To test this idea they carried out a second set of experiments in which we characterized the phenotype of a DIAP1 loss-of-function mutation, as well as the phenotype of a double mutant that removed DIAP1, as well as *rpr*, *hid* and *grim*. They found that the DIAP1 loss-of-function phenotype consists of an embryo-wide set of cellular changes reminiscent of apoptotic cell death, and that these were associated with the activation of DIAP1-inhibitable caspase activity. Furthermore, double mutants that remove zygotic *rpr*, *hid*, *grim*, and DIAP1 function showed phenotypes similar to those of the DIAP1 loss-of-function mutant alone (Wang *et al*., 1999).

Together, the above observations suggest that a principal function of DIAP1 is to promote cell survival by blocking caspase activity, and that at least one mechanism by which REAPER, HID and GRIM promote apoptosis is by disrupting IAP-caspase interactions. These early studies left several important questions unanswered: 1) how does RPR, HID or GRIM binding to DIAP1 suppress DIAP1's ability to inhibit caspase activity. 2) Do RPR, HID and GRIM regulate DIAP1 function through other mechanisms? Domain analysis of RPR and GRIM suggests that these proteins have apoptotic domains distinct from their N-terminal DIAP1-binding motifs. One question we are interested in is whether these other

domains regulate DIAP1 through other mechanisms. 3) Finally, it is interesting to ask if there exist other proteins that function similarly to RPR, HID and GRIM. However, the yeast survival-based assay we used to show that these proteins disrupt IAP-caspase interactions provides a straightforward approach to screening for such molecules. Importantly, because such a screen is a function-based screen, and does not rely on identifying candidates based on sequence homology, we may identify proteins that disrupt IAP-caspase interactions even if they have only minimal homology to RPR, HID or GRIM.

Finally, The DIAP1 loss-of-function phenotype, the caspase-dependent death of all cells in the embryo, raises an important question? What is the source of the activity DIAP1 fights against to maintain cell survival. The generation of double mutants in cell culture using RNAi of Dronc or Ark, and DIAP1 (Muro *et al*., 2002), provides a clear answer - Ark and Dronc-dependent caspase activation. An important implication of these observations is that cells that normally live experience a chronic Ark-activating death signal. This promotes the continuous activation of Dronc. DIAP1 promotes cell survival, at least in part, by suppressing this Dronc activity (Figure 1).

An important unsolved question is the source of the signal that activates Ark. In mammals Apaf-1 is activated by cytochrome *c*, released from mitochondria, and ATP or dATP. The role of mitochondria and cytochrome *c* release in *Drosophila* is still unclear. Exposure of apoptosis-specific cytochrome *c* epitopes has been observed by the Abrams lab, and cytochrome *c* can be found associated with a high molecular weight apoptosome-like complex in cell extracts (Dorstyn *et al*., 2002). However, depletion of cytochrome *c* in cell culture using RNAi has failed to demonstrate any involvement of this protein in Ark-dependent cell death [Zimmermann *et al*., *J. Cell Biol.* (2002) 156:1077]. This latter observation raises the intriguing possibility that Ark activation in flies (and by implication perhaps in other organisms, as well) can be regulated through associations with other molecules. We have designed several screens and biochemical approaches to address this question.



69. Bruce, cell death, caspases and spermatogenesis
Jun Huh

As mentioned above, one of the potent cell death suppressors we identified is the Bruce gene. Bruce mutants are viable, but they are male sterile. In examining this we discovered several interesting facts: 1) Bruce mutants are blocked in a late aspect of spermatogenesis known as individualization, in which spermatids (which develop in a common cytoplasm) eventually become enclosed in individual plasma membranes; 2) During the process of individualization spermatids have very high levels of active caspases. But these cells do not die. Together these observations suggest that spermatids use caspase activity for nonapoptotic purposes, during differentiation. Several questions are of interest to us: 1) What are the sources of the caspase activity (what are the upstream signals); 2) What are the nonapoptotic targets that facilitate differentiation; and 3) how is cell death prevented in the face of high levels of caspase activity that would normally be associated with cell death?

70. Autophagic cell death, caspase inhibition in ***C. elegans***, and the ***echinus*** mutant
Jeffrey Copeland

While much cell death is apoptotic, a number of cell deaths share features with a process known as autophagy, which has been described in some molecular detail in yeast. In yeast, starvation leads to a cellular response in which double membrane bound vesicles are

formed that take up and hydrolyze organelles as well as bulk cytoplasm. This process of autodigestion provides the cell with nutrients, allowing survival under starvation conditions. It has been clear for some time that there are a number of situations in which cell death in animals shows morphological features similar to those of autophagy rather than apoptosis. However, the molecular mechanisms that mediate these deaths has remained unexplored. *Drosophila* homologs are available for many of the yeast proteins involved in autophagy. The goal of my project is to explore the molecular mechanisms underlying autophagic cell death in *Drosophila*.

In *C. elegans*, in contrast to the situation in flies and mammals, caspase inhibitors have not been identified. I used the yeast screens described above to identify several potential *C. elegans* caspase inhibitors. One of these is highly evolutionarily conserved. We are currently focusing our characterization on the *Drosophila* counterpart of this gene.

Echinus is a *Drosophila* mutant that lacks normally occurring cell death in the eye. I have generated multiple new alleles of *echinus* and cloned the gene. Characterization of its activities is in progress.

Death related Hay lab publications

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Summary: Our first report involves a study of the evolution of the homeobox complex (HOX-C) from mosquitoes, represented by *Anopheles gambiae*, to fruit flies of the genus *Drosophila*. The HOX-C is a gene cluster that controls the body plan of all higher animals. It evolved over 500 million years ago and in most animals has remained intact until the present time. Moreover the genes of the HOX-C have been retained in the chromosome in the order in which they are expressed along the longitudinal axis of the embryo. Ironically, in *Drosophila* where the genes of the complex were first discovered the HOX-C has proved exceptional in being split into two, known as the bithorax (BX-C) and Antennapedia (ANT-C) complexes. That this is a relatively recent event became evident when sequencing of the genome of *A. gambiae* showed it retains the ancient intact HOX cluster. In contrast, *D. melanogaster* has the complex split between the genes *Ultrabithorax* (*Ubx*) and *Antennapedia* (*Antp*). In another species, *D. virilis*, it was discovered in 1996 that the complex is split between *Ubx* and the *abdominal-A* gene (*abd-A*). Since that species has yet to have its genome sequenced, we decided to determine the genomic organization of the *Ubx* and *Antp* regions in *D. virilis* and we report on our findings.

In our second report, we describe work in which we are attempting to dissect the epigenetic and *cis*-regulatory network that controls expression of genes in the BX-C. In two related projects, we are examining the functional role of non-coding RNAs and a novel *cis*-sequence, termed the promoter-tethering element (PTE).

71. Evolution of the HOX-C in the ***Diptera***

Edward B. Lewis, Barret Peiffer[1], David Mathog[2], Susan Celniker[1]

In a collaborative effort the *Antp* and *Ubx* genes of *D. virilis* have been sequenced during the period of this report in order to determine whether they have remained in proximity as in *A. gambiae* or have separated as in *D. melanogaster*. The answer is an unusual one. In both *D. melanogaster* and *D. virilis* there is actually an insertion of a gene, *CG31217*, between *Ubx* and *Antp* that is not found in *A. gambiae*, where the gene exists but is remotely located from its HOX complex. Evidently, two branches occurred in the evolution of *Diptera*, or two-winged insects. One branch led to an intact HOX-containing chromosome as in *A. gambiae* and the other to a hypothetical "vir-mel" chromosome in which the *CG31217* gene became inserted between *Ubx* and *Antp*. From the latter, *D. virilis* arose when a split occurred between *Ubx* and *abd-A*, while *D. melanogaster* arose when the split occurred between *Ubx* and *Antp*. In the latter case *Ubx* and *CG31217* are now next to a *chaperonin-containing gamma gene* (*Cctg*) while *Antp* is next to a *sorbitol dehydrogenase* (*Sodh*) gene. Evidently, *Ccctg* and *Sodh* were closely linked in the "vir-mel" chromosome. A weak prediction is that they should still be closely linked in *D. virilis* and a search is underway for them in that species. Why are such splits so rare among animals that have been sequenced so far? We believe it is related to several factors: (1) the enormous fecundity of *Drosophila*; (2) transposons that induce a high frequency of inversions which cause the splits; and (3) inversions do not reduce fertility in *Drosophila*. Thus far the only other organisms that we are aware of that have such splits in their HOX-C are the nematode, *C. elegans*, and the chordate, *Ciona*. Those organisms would be expected to have even greater fecundity than that of *Drosophila*.

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Publication

Lewis, E.B. (2003) C.B. Bridges' repeat hypothesis and the nature of the gene. *Genetics* 164:427-431.
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72. Epigenetic and ***cis***-regulation of gene expression in the ***Drosophila*** BX-C

Robert A. Drewell, Esther Bae, Cory E. Olsen, Mitzi Shpak, Edward B. Lewis

The bithorax complex (BX-C) consists of over 300 kb of DNA, but encodes only three homeotic genes. The extensive intergenic *infrabdominal* (*iab*) region is known to harbor *cis*-regulatory elements capable of directing the segment-specific expression patterns of the homeotic genes. However, it is unclear how the *cis*-elements are primed for activity early in embryogenesis. Using *in situ* hybridization we showed that the control regions are themselves transcribed early in development. As with the homeotic genes of the BX-C, the transcription patterns of the RNAs from the *iab* regions demonstrate colinearity, both with the sequence of the *iab* regions along the chromosome and the domains in the embryo under the control of the specific *iab* regions. Our results suggest that intergenic transcription is required early in embryogenesis to initiate the activation of the BX-C and define the domains of activity for the *iab* *cis*-regulatory elements.

In a related project we have been examining the mechanisms that regulate promoter-enhancer interactions at the BX-C. There are many examples within gene complexes of shared transcriptional enhancers interacting

only with a subset of target promoters. Promoter competition and insulators are thought to play a role in regulating these interactions. In the bithorax complex of *Drosophila*, the IAB5 enhancer is located 55 kb 3' of the *Abdominal-B* promoter and 48 kb 5' of the *abdominal-A* promoter. Although roughly equidistant from the two promoters, IAB5 specifically interacts only with the *Abdominal-B* promoter, even though the enhancer and promoter are separated by at least two insulators. Core promoter elements cannot account for IAB5 specificity as neither *Abd-B* nor *abd-A* contains a TATA element or consensus initiator or downstream promoter elements. Our experiments demonstrate that a novel 254 bp *cis*-regulatory element located 40 bp 5' of the *Abd-B* transcriptional start site is able to tether IAB5 to the *Abd-B* promoter in transgenic embryo assays. Furthermore, the tethering element is sufficient to direct IAB5 to an ectopic site in competition assays. These results provide *in vivo* evidence that specific long-range enhancer-promoter interactions in the bithorax complex are regulated by a tethering element 5' of the *Abd-B* promoter and suggest that tethering elements might regulate enhancer-promoter specificity at other gene complexes.

Publications

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Summary: We continue to identify and clone genes whose mutant phenotypes indicate that their products play important regulatory roles in shoot growth and in flower development in the laboratory plant *Arabidopsis thaliana*. Such work has in the past has led to the current model for organ specification in flower development, to the view of a shoot apical meristem as a collection cells communicating via extracellular protein ligands and transmembrane receptor serine-threonine kinases, and to an understanding of perception of the plant hormone ethylene. Among the recently cloned genes undergoing analysis are *SHREDDIE*, *EARLY EXTRA PETALS*, and *HANABA TARANU*, all involved in flower development; and *TOPLESS* and *BIG TOP*, engaged in axis specification in embryos.

In addition to this sort of "forward genetic" approach, we are also taking three new approaches as a coherent approach to achieving a deeper understanding of plant development than is afforded by a gene-by-gene analysis. The first is the use of whole-genome microarrays, representing all of the known genes of *Arabidopsis* as a set of over 26,000 oligonucleotides on a glass substrate, for assay of RNA populations from developing flowers of a number of different genotypes. The arrays read out the population of gene transcripts present in any tissue and developmental stage. In particular we are performing time-course experiments after activation of a variety of floral development regulatory genes, to obtain a dynamic and causal understanding of gene expression changes in each floral whorl during formation of each type of organ. These experiments are supplemented by bioinformatic approaches to identify regulatory regions responsive to each of the floral regulators, and should soon be accompanied by chromatin immunoprecipitation experiments to directly assess the genomic binding sites of those of the regulatory proteins that are transcription factors.

The second new approach is an attempt to understand the dynamics of cell division and gene expression changes during normal and mutant development, especially after induced changes in the expression of known molecules involved in cell-cell communication. We have achieved a real-time three-dimensional visualization of all of the cells in growing shoot apical meristems, labeled with fluorescent proteins expressed from transgenes that indicate in one wavelength the location of plasma membranes or nuclei. In another differentiable wavelength, we can simultaneously observe the activation of genes such as *CLAVATA3*, which codes for a ligand involved in cell-cell communication in growing shoots, or the location of proteins such as PIN1, which regulates the flow of the plant hormone auxin. For the first time we can now see all of the cell divisions and changes in gene expression that accompany normal development of shoots, and also the alterations in these parameters that result from changes in cellular communication.

Finally, we are engaged in a collaboration with colleagues at the Jet Propulsion Laboratory, the University of California at Irvine, and Lund University in Sweden, to begin computational modeling of cell division, auxin concentration and gene expression behavior in growing shoot apical meristems. We do this to analyze the results of the dynamic visualization experiments, and to start towards a long-term goal of complete modeling of plant development *in silico.*

73. Analysis of spatial gene expression in ***Arabidopsis*** flowers

Frank Wellmer, Jose Luis Riechmann, Marcio Alves-Ferreira*

For a better understanding of *Arabidopsis* flower development we have generated an expression map for genes that are specifically transcribed in one of the four different types of floral organs: sepals, petals, stamens, and carpels. For this, we have compared the gene expression profiles of wild-type inflorescences with those of the floral homeotic mutants *apetala1*, *apetala2*, *apetela3*, *pistillata*, and *agamous* using two different types of microarrays: a cDNA microarray whose elements are enriched for flower and inflorescence specific transcripts and oligonucleotide-based 'whole genome' arrays. In the homeotic mutants certain types of floral organs are absent or are replaced by other types of organs. By combining the data sets obtained from these experiments we were able to identify groups of genes that are predicted to be specifically expressed or whose transcripts are strongly enriched in one type of floral organ. The vast majority of these genes is predicted to be expressed in stamens or carpels, whereas only a few genes are likely expressed specifically in sepals and petals. We have analyzed the

expression patterns of several genes by *in situ* hybridization and found that they match the predictions that were made based on the microarray experiments. Moreover, genes with known floral organ specific expression patterns were correctly assigned by our analysis. Our findings suggest that the spatially limited expression of a relatively large subset of the genome is required for the formation of reproductive floral organs whereas only few genes are specifically expressed in the perianth. Furthermore, our data provide a rich source for reverse genetics approaches and for the generation of floral organ specific or tissue specific markers.

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74. Identification of target genes of MADS-box factors involved in flower development

Pradeep Das, Frank Wellmer, Jose Luis Riechmann[*], Toshiro Ito, Marcio Alves-Ferreira

The patterning of the floral meristem into the different floral organs is mainly regulated by transcription factors that contain the so-called MADS-box domain. These factors, including APETALA1 (AP1), APETALA3 (AP3), PISTILLATA (PI), AGAMOUS (AG) and the three functionally redundant SEPALLATA (SEP*)* proteins act in different combinations to specify each type of floral organ. Hence sepal fate is specified by the action of AP1, petal fate by AP1, AP3, PI and the SEPs, stamen fate by AP3, PI, AG and the SEPs, and carpel fate by AG and the SEPs. It has been demonstrated that some of these factors interact physically, suggesting that they form regulatory complexes that directly control genes involved in organ formation.

Despite our increasing knowledge of the mechanisms regulating flower development, it is unclear precisely which genes are regulated by the MADS-box factors themselves, and what these genes do. We are currently trying to identify the targets of these transcription factors using whole-genome microarrays. To this end, we have created plants expressing fusion proteins of the MADS-box factors with a fragment of the glucocorticoid receptor (GR). These fusion proteins can be specifically activated by treating the transgenic plants with dexamethasone (dex), a steroid hormone. This system allows us to do time-course experiments and observe changes in gene expression that occur shortly after activation of the transcription factors, as well as later changes that are presumably downstream of those primary events.

The GR fusion proteins rescue the missing floral structures in their appropriate mutant backgrounds, demonstrating that our system works as expected. We have generated plants carrying the gene combinations required for different floral organ fates to be achieved. Current experiments focus on activating the fusion proteins with dex and determining the gene expression changes in inflorescences. However, in order to avoid the effects of endogenously expressed MADS-domain genes, we will also perform these experiments in seedlings and leaves. This should allow us to make a clear distinction between the target genes required for organ identity determination in each whorl.

This work is being performed in collaboration with Pedro Robles and Martin Yanofsky at UC-San Diego.

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75. Identification of LFY and AP1 target genes in ***Arabidopsis thaliana***

Annick Dubois, Frank Wellmer, Toshiro Ito

Flowering in higher plants involves the transition of a vegetative meristem, producing leaves and stems, into an inflorescence meristem, producing flowers. LEAFY (LFY) and APETALA1(AP1) are transcriptional regulators that play a key role in the initiation of floral development in the plant *Arabidopsis thaliana*. The *AP1* and *LFY* genes are primarily expressed in the whole meristem and activate floral homeotic genes following a whorl-specific pattern. Later on, AP1 is required for the specification of sepal and petal identity. The first part of this work is to identify and study the genes that are controlled by *AP1* and *LFY* using mainly whole-genome microarray analyses. Following this work, will perform chromatin immunoprecipitation (ChIP) experiments coupled to promoter-array analysis. For the ChIP experiments, we have obtained plants expressing the LFY protein fused to the GR and to multiple c-Myc or HA tags (LFY-GR-Tag), providing respectively post-translational inducible activity and good epitopes for immunoprecipitation. We are now preparing the plants expressing AP1-GR-Tag constructs for the same purpose. To enhance the sensitivity of the assay, the ChIP experiments will be coupled to promoter-array hybridization. Together with the transcriptional analysis, these experiments should lead to a better view of the genetic network acting downstream of *LFY* and *AP1*.

76. Looking for genes involved in the AP1 pathway

Annick Dubois, Carolyn Ohno

I am interested in understanding how AP1 functions in the determination of floral organ identity and meristem identity. A very weak allele of *AP1* (*ap1-511*) has been identified that does not resemble any previously characterized alleles of the gene. The mutant plants have an enlarged inflorescence, petaloid sepals, and sometimes supernumerary petals. In contrast to *ap1-511*, strong loss-of-function *ap1* alleles produce bract-like sepals in the first whorl, reduced petal number in the second whorl of the flower, and secondary flowers that initiate in the axils of the first whorl organs. Similar to other *ap1* alleles, the *ap1-511* allele also interacts with *LEAFY*. In *ap1-511 lfy-5* double mutants, plants produce leaf-like and carpelloid organs in the most affected flowers, and also stamens in the less affected flowers. Thus, this phenotype appears less severe than in *ap1-4 lfy-5* double mutants (*ap1-4* being an intermediate allele of *AP1*). The *ap1-511* EMS-induced mutation results in an amino-acid substitution in the K domain of AP1, a domain which was shown to be involved in the interaction with the SEP3 transcription factor, and the B function transcription factors AP3 and PI. To better understand the AP1 pathway, and to identify

potential partners of AP1, I have started an EMS mutagenesis screen to identify enhancers and suppressors of this mutant phenotype. I will also use an activation-tagging strategy to identify other genes implicated in the pathway.

77. Regulation of gene expression in the shoot apical meristem

Frank Wellmer

During the postembryonic development of plants, all above ground tissues originate from a group of undifferentiated cells called the shoot apical meristem. In order to function as a site of continuous organ formation the meristem must maintain its cell population by tightly controlled cell division patterns. Past work in our laboratory, as well as from other groups, has identified several key components that are involved in the regulation of meristem development. Among the well-characterized meristem regulatory genes are *CLAVATA3 (CLV3)*, coding for a small-secreted protein that likely functions as a ligand for the membrane bound receptor-like kinase CLAVATA1, and *WUSCHEL (WUS)* and *SHOOT MERISTEMLESS (STM)* which code for homeodomain containing transcription factors.

Although the genetic interactions of these genes have been studied in great detail the molecular mechanisms by which these regulators control meristem development remain largely unknown. In order to understand their function in more detail we are currently studying their effects on gene expression in the meristem. For this we have generated inducible systems that allow the specific activation of STM, WUS, and CLV3 in responsive tissues. For example, the induction of *CLV3* expression in the enlarged meristems of a strong *clv3* loss-of-function allele leads to a rescue of the mutant phenotype. Since CLV3 is thought to trigger a signaling pathway regulating meristem size, the induction of *CLV3* expression should allow us to monitor changes in the expression of those genes that are controlled by the pathway. For the detection of changes in gene expression whole genome microarrays are used.

78. Using reverse genetics to analyze five genes in the ***CLE*** family

Catherine Baker

CLAVATA3 is a small, secreted protein which acts to reduce cell proliferation in the shoot meristem by activating CLAVATA1, a leucine-rich repeat receptor-like kinase. Twenty-four putative genes similar to *CLV3* have been identified in *Arabidopsis*; each member of this family is small (less than 400 bp) and is predicted to encode a protein with an N-terminal signal sequence. In addition, the predicted proteins share homology within a small C-terminal region, containing an invariant histidine, an invariant arginine, and a conserved glycine. This group of genes has been termed the *CLV3/ESR*-like (*CLE*) family, because of additional resemblance to embryo-surrounding region proteins in maize.[1] We are interested in determining whether some of these predicted secreted proteins might act as ligands for one or more of the numerous receptor kinases in *Arabidopsis*. In collaboration with Jennifer Fletcher's lab at the Plant Gene Expression Center, we are investigating the function of six of these genes (*CLE11*, *12*, *13*, *16*, *17*, *18*). Overexpressing *CLV3*, *CLE11*, and *CLE12* from the constitutive cauliflower mosaic virus promoter *35S* results in very similar phenotypes: early termination of the shoot meristem (with axillary meristems making few leaves and terminating early), and occasional flowers with reduced or absent stamens and carpels. *35S::CLE2* and *35S::CLE40* also have this phenotype,[2,3] suggesting that several of the *CLE* genes are capable of mimicking *CLV3* when overexpressed. *35S::CLE12*, like *35S::CLV3*, requires wild-type CLV1 and CLV2 for its phenotype, suggesting when present at high levels, it is signaling predominantly through the CLV1/CLV2 receptor complex. We are currently using RNA interference and promoter-GUS reporter lines to elucidate the *in vivo* function and expression pattern of these CLE proteins.

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79. A floral homeotic gene product AGAMOUS directly controls ***NOZZLE,*** a gene involved in ovule pattern formation and early sporogenesis in ***Arabidopsis thaliana***

Toshiro Ito, Frank Wellmer, Hao Yu, Pradeep Das, Natsuko Ito[1], Marcio Alves Ferreira, Jose-Luis Riechmann[2]

The floral homeotic selector gene *AGAMOUS* (*AG*) together with other members of the same class of floral homeotic genes is sufficient to trigger male and female reproductive organ (stamen and carpel) development. The *AG* gene, which encodes a member of the MADS box family of DNA binding transcription factors, is expressed in stamen and carpel primordia and is thought to control developmental pathways by regulating subordinate target genes responsible for stamen and carpel identity. Although *AG* is the most studied example of the floral homeotic selector genes, the mechanism by which the floral organ primordia of whorl 3 and whorl 4 respond to the genetic cascade initiated by AG and differentiate into stamens and carpels are largely unknown. There are two extreme possible hypotheses about how AG functions. One is that AG triggers a few genes at the top of the hierarchy to determine reproductive organ identity, and in turn these AG targets regulate other genes. The second possibility is that AG directly regulates expression of the genes with different functions acting in concert to form the reproductive organs.

To find if either of the hypotheses describes AG action in flower development, we first needed to reveal the nature and function of the target genes of AG. We have

been doing cDNA and full genome microarray screening using transgenic plants with an inducible AG activity. A strain was created that is homozygous for *ag-1* and transgenic for the construct *35S::AG-GR*, that is, virus 35S protein promoter-driven *AG* gene with a carboxyl-terminal fusion to the steroid binding domain of the rat glucocorticoid receptor. In a time-course experiment after induction of AG activity, inflorescences from *35S::AG-GR ag-1* mutant plants were collected at 0, 4, 8, 10, 12 and 16 hours after mock- or DEX-treatment. The RNA was isolated from each time sample and used as a probe for microarray screening. Thus, we identified several target genes of AG, including *SPOROCYTELESS (SPL)/NOZZLE (NZZ*) (Schiefthaler *et al*., 1999; Yang *et al*., 1999; Balasubramanian and Schneitz, 2002)**.** The *SPL/NZZ* gene is involved in ovule pattern formation and early microsporogenesis. The induction of *SPL/NZZ* gene was observed after 4 hours and until 16 hours after the DEX-treatment. The expression of *SPL/NZZ* gene was also induced by AG in the presence of cycloheximide, an inhibitor of protein synthesis, indicating that *SPL/NZZ* is directly regulated by AG. These results suggest that AG directly controls not only the establishment of stamen and carpel identity, but also sporogenesis in later organ development.

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80. ***AGT2ATH,*** a direct target of AGAMOUS, patterns gynoecium tissue by controlling ***ETTIN*** expression in ***Arabidopsis***

Toshiro Ito, Natsuko Ito[1], Hao Yu

We have been adopting a bioinformatics approach in order to identify direct target genes regulated by the floral homeotic protein AGAMOUS (AG). The *AGT2ATH* gene was identified as a gene adjacent to putative AG binding sites. The *AGT2ATH* gene encodes a member of the AT hook-type DNA binding protein, and is expressed throughout wild-type floral primordia at stages 1 through 5. From stage 6, *AGT2ATH* expression is localized to the stamen and carpel primordia, and the expression continues at the adaxial side of the pistil. In a loss-of-function *ag-1* mutant background, *AGT2ATH* expression is only detected in the early-stage floral buds, and the late-stage expression after stage 5 disappears gradually. In *35S::AG-GR ag-1* plants, the *AGT2ATH* gene was induced after 4 hours in the presence of cycloheximide. These results suggest that the *AGT2ATH* expression after stage 5 is directly controlled by AG.

To examine the developmental function of the *AGT2ATH* gene, we ectopically expressed *AGT2ATH* in wild-type plants, as well as in *ag-1* mutant plants. The strong expression of *AGT2ATH* in the wild-type plants resulted in distorted gynoecium patterning with increased stigmatic cells and short valve tissues, which is reminiscent of the loss-of-function phenotype of the *ettin (ett)* mutant (Sessions *et al.,* 1997; Nemhauser *et al*., 2000). Consistent with this observation, *ETT* expression was reduced significantly in the overexpression line of *AGT2ATH*. Furthermore, the expression patterns of *AGT2ATH* and *ETT* are mutually exclusive in stamen, carpel and ovule primordia. These results indicate that AGT2ATH is a negative transcriptional regulator of *ETT*, which controls the polarity of the gynoecium. The overexpression of *AGT2ATH* in *ag-1* showed partial rescue of the *ag-1* phenotypes of the fusion of internal sepals and presence of ovule-like projections, while downregulation of *AGT2ATH* by double-strand RNA silencing resulted in the underdevelopment of the tapetums of stamens. These results suggest that AGT2ATH as a direct target of AG, and controls stamen and carpel development at later flower development stages, including the negative regulation of *ETT* cascades in gynoecium development.

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81. ***Arabidopsis*** SUPERMAN directly regulates ***INNER NO OUTER*** and ***WALL-ASSOCIATED KINASE2*** expression in ovule development

Toshiro Ito, Frank Wellmer, Annick Dubois, Pradeep Das, Natsuko Ito[1], Marcio Alves Ferreira, Jose-Luis Riechmann[2]

We have shown that the *SUPERMAN* (*SUP*) gene, which encodes a C2H2-type zinc-finger transcription factor, is involved in cell proliferation control in floral primordia of whorl 3 and whorl 4. Furthermore, we demonstrated that SUP functions in whorl 4 non-cell autonomously. SUP protein continues to be expressed in the funiculus of the ovules and affects cell proliferation in the outer integuments of the ovules in a non-cell autonomous manner. In order to reveal the molecular nature of the downstream activities of SUP, we used a system providing inducible SUP activity and searched for induced/repressed genes by using cDNA microarrays. As a result of microarray hybridization, we only found several downregulated genes after SUP induction, indicating that SUP functions mainly as a transcriptional repressor. The *INNER NO OUTER* (*INO*) gene is one of the downregulated genes. Previous work showed that *ino* mutants are epistatic to *sup* in ovule development and that SUP attenuates the autoregulation of *INO* [(Villanueva *et al*., 1999; Meister *et al*., 2002). In the *35S::SUP-GR* inducible system, the expression of *INO* is repressed by

DEX treatment in the presence of cycloheximide, strongly suggesting that SUP directly affects the autoregulation of *INO*.

We also identified *Wall Associated Protein Kinase 2* as another direct target of SUP. Cell-Wall Associated Protein Kinases (WAKs) are one of the receptor kinase families that physically link the extracellular matrix (ECM) to the plasma membrane and may facilitate communication between the ECM and cytoplasm (Wagner and Kohorn, 2001). Previous work has showed that *WAK2* is expressed in floral buds, in addition to a strong expression in seedlings, shoot apical meristems and young leaves, and that antisense expression of *WAK2* inhibits cell elongation and reduces plant size (Wagner and Kohorn, 2001). Our work has demonstrated that ectopic SUP expression reduces plant and flower size by inhibiting cell elongation. Thus, the repression of the *WAK2* gene by SUP may be one of the causes of the dwarf phenotype of 35S::SUP plants. We are confirming this possibility by introducing an estradiol-inducible promoter::*WAK2* construct into *35S::SUP-GR* transgenic plants, and then checking if WAK2 expression can rescue the dwarf phenotype induced by ectopic SUP expression. In our *in situ* hybridization experiments, the expression of *WAK2* is detected in developing ovules, suggesting that SUP may regulate *WAK2* expression during ovule development.

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82. Downstream cascades of AGAMOUS and SUPERMAN in early flower development in ***Arabidopsis***

Toshiro Ito, Annick Dubois, Pradeep Das, Frank Wellmer, Jose-Luis Riechmann[*]

AGAMOUS (*AG*) acts as a C function gene (specifying stamens with B function genes, and on its own, carpels), but is also required for providing determinacy in the center of the flower after carpels are specified. *AG* starts to be expressed in stage 3 floral primordia. Stamen and carpel primordia appear at stage 5 and 6, respectively. Thus, the organ identity function, as well as the meristem determinacy function should have been achieved by *AG* prior to stage 5 or 6. Furthermore, *AG* continues to be expressed in specific regions of stamen and carpels at later stages to induce genes with various functions in the subsequent development.

SUPERMAN (SUP) acts to prevent the cell proliferation of floral primordia of whorl 3 and to promote differentiation of the whorl 4 floral meristem. SUP starts to express at the adaxial side of the stamen primordia at stage 3, and the SUP function for floral meristem control is also thought to be achieved at stages 3-5, before the stamen and carpel primordia appear. SUP continues to be expressed in specific regions of ovules and is involved in the control of cell proliferation in outer integuments of the ovules.

We have been doing cDNA and full genome microarray screening using transgenic plants with inducible AG and SUP activities to search for the target genes of AG and SUP. Previously, the floral samples were collected by the naked eye and they contained floral buds from stage 1 thorough stage 10 or 11. Because most target genes we identified from these samples turned out to be middle-stage or late-stage targets of AG or SUP, we reviewed our sample preparation method. We calculated the volume of floral buds at each stage based on SEM images of inflorescences and reasoned that genes that are expressed only at early stages were likely to be under-represented. For example, a floral bud at stage 10 is about 400 times larger than one at stage 3. In inflorescence samples containing stage 1-10 floral buds, the ratio of stage 3-5 floral buds is estimated to stand only 1.2 % (V/V). This estimation clearly suggested that target genes induced only at early stages are not detectable.

To identify the early-stage targets of AG and SUP, which are involved in the control of organ identity and floral meristem, we started to collect floral samples of stage 1-5 under the dissecting microscope. This time-consuming step greatly enhanced the ratio of stage 3/4 floral buds to ~30 % (V/V), which would make it possible to detect the up- or down-regulation of early targets. For example, 10-fold induction of one gene at stage 3 and 4 could be detected as 2-3-fold in these samples, which is clearly over the detection sensitivity of microarray hybridization. Due to the limited sample volume, we performed double-step *in vitro* transcription and showed that RNA samples obtained from one or two inflorescence (~10 ng) were enough for microarray probe preparation. To prevent biological variability, we have been collecting 5-10 inflorescences as time-course samples. Repeated sample preparation and microarray hybridization are under way.

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83. Genome-wide binding site analysis of AG by chromatin immunoprecipitation (ChIP) in ***Arabidopsis***

Toshiro Ito

In order to identify target genes directly controlled by floral regulators, we have been doing chromatin immunoprecipitation (ChIP) analysis of genome-wide binding sites of AGAMOUS (AG). Although this method has been successfully applied to a single-celled eukaryote (*S. cerevisiae*), it has a technical limitation of its sensitivity in a multicellular organism with a relatively large genome size. In order to overcome the limits of the application of ChIP to a multicellular plant system, we have been using homogeneous populations of cultured cells with both inducible gene activity and

efficient epitope tags. We also established an efficient DNA amplification method, followed by an intergenic microarray screening.

We generated transgenic plants in which GR-epitope (HA or c-myc) fusion proteins with AG, SEP3, AP3 or PI are ectopically expressed. We first produced callus culture overexpressing AG and SEP3, which are sufficient to induce carpel identity, and then performed phenotypic analysis after mock- or dex-treatment. The callus tissue was treated with mock- or DEX-solution for 24 hours and transferred on to SIM (Shoot Inducing Medium). The callus treated with DEX produced a shoot with terminated multiple carpelloid organs, while mock-treated callus produced a wild-type shoot. We also confirmed that one of the AG targets, *AGT4HOX* is induced 4 hours after DEX treatment in the callus tissue. From these results, we were reasonably convinced that these callus cultures are useful for identifying at least a subset of the target genes of AG. We are also planning to use floral buds of *35S::AG-GR*-epitope tag in the *ag-1* mutant background for ChIP experiments.

To perform the intergenic microarray hybridization, we needed to improve the DNA amplification method applied for immuno-enriched DNA in yeast, especially because *Arabidopsis* has a genome about ten times larger than yeast and contains more repetitive sequences. To develop an efficient and unbiased DNA amplification method, we synthesized a new inker with a T7 RNA polymerase-binding site and tested it for the first round of PCR followed by the subsequent *in vitro* transcription reaction. By this method, we successfully amplified DNA from 10 pg – 0.1 ng up to 3 μg – 30 μg. These DNA samples were less biased than the ones simply amplified by PCR.

84. Dynamics of cell behavior in developing shoot apical meristems

G. Venugopala Reddy, Marcus G. Heisler

A tight coordination between stem-cell division and displacement of their progeny has been proposed to be a major factor in regulating the size of stem-cell domain and in generating a radial pattern of the shoot apex. Thus far the studies related to analyzing cell behavior in the meristem are based on cytological staining methods on tissue sections. The studies so far have shown that the cells in the central zone (CZ) divide at a slower rate than cells in the peripheral zone (PZ). But these methods do not reveal the cell division and displacement dynamics. Therefore generating a precise map of cell division and displacement patterns is central to understanding both cell type specification and morphogenesis in developing meristems.

We have utilized a combination of live imaging techniques and cell division markers to establish a spatio-temporal map of cell division and displacement in the developing shoot apex. Cell division is monitored through a Histone2B-Yellow Fluorescent Protein (H2b-mYFP) fusion protein transgene. This protein complex has been shown to dynamically localize to chromatin during M phase of cell cycle, thus facilitating the detection of mitotic cells (Boisnard-Lorig *et al*., 2001). In the shoot apex the mitotic stages in M phase are transient and last for approximately 1 hour. We have followed SAMs expressing H2b-mYFP for 4-5 days at 1 hour intervals by confocal microscopy. Our studies have revealed that cell division in the SAM is organized into temporal waves and division is temporally coordinated across three clonally distinct layers of cells. Analysis of the progression of mitotic wave reveals a local spatio-temporal coordination implying a role for local signals in influencing cell division. The mitotic wave does not follow incipient primordia in time and thus can be temporally uncoupled from primordium formation. However, the analysis of cell cycle length has revealed that a specific set of cells in the peripheral zone cycle more rapidly than others and are spatially coordinated across layers. Our current analysis is aimed at examining whether or not these cells ultimately become part of the primordia. The integrated 4-D movie reveals that the initial outgrowth of primordia is primarily due to coordinated cell expansion and the initial growth occurs in two dimensions. The stage at which the floral meristem resumes rapid cell divisions coincides with the appearance of a groove, which separates each floral meristem from the inflorescence meristem. The detailed description of cell behavior should form a basis for future studies aimed at understanding the cellular basis of meristem maintenance and morphogenesis.

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85. Fluorescent cell type-specific markers for shoot apical meristems (SAMs)

G. Venugopala Reddy

The shoot apical meristem (SAM) is a collection of distinct cell types located in specific positions with specialized functions. The central zone (CZ) cells harbor initials or stem cells, while the cells at specialized regions in the peripheral zone (PZ) differentiate into organ primordia. The cells in the rib zone are incorporated into the developing stem. All these cell types are located in a dynamic environment and hence they have to constantly assess their position in order to retain their identity. The dynamics of cell fate specification can be best studied by combining cell type-specific markers and cell behavior in a single study. In this context, I am developing a set of promoter constructs to direct the expression of fluorescent proteins in distinct cell types. I have designed promoter-fluorescent protein constructs for *CLAVATA3 (CLV3), UNUSUAL FLORAL ORGANS (UFO), LEAFY (LFY)* and *WUSCHEL(WUS)* to mark cells in CZ, PZ, incipient primordia and part of the rib zone, respectively. These constructs are currently being tested in transgenic plants.

86. Cell behavior, cell types and meristem maintenance

G. Venugopala Reddy

Genetic studies have revealed some of the signaling mechanisms involved in meristem maintenance. Mutations in *CLAVATA* genes (*CLV1, CLV2* and *CLV3*) result in bigger meristems, while mutations in *WUSCHEL (WUS)* result in a failure to maintain a functional meristem. The nature of gene products, expression domains, genetic interactions and mis-expression studies have contributed to an elegant model involving positive and negative feedback loops operating on each other to maintain meristem size. The function of WUS is required to maintain a constant stem-cell pool in the central zone of the shoot apical meristem and at the same time CLV genes function to repress the WUS expression domain. However, studies aimed at understanding the cell behavior and analyzing cell types in these mutant contexts are restricted to single time-point observations and mostly restricted to inflorescence meristem, which represents a terminal phenotype. The function of these gene products can be best understood by analyzing the effects of their transient dysfunction, in real time, on both cell types and cell behavior. Such studies might yield new insights into the kinetics of reorganization of cell types in SAM in relation to the altered cell behavior. Such a system can also be employed to study the effect of altered cell types on cell division patterns in relation to cell position. I am utilizing hormone inducible (both dexamethasone- and estrogen-based) constructs, which result in mis/overexpression of WUS and CLV3 to perturb meristem maintenance function (constructs are provided by Frank Wellmer). I am are currently combining these constructs with fluorescent cell type-specific and cell division markers to assess cell types and cell behavior in real time.

87. Auxin patterning and organ initiation in the ***Arabidopsis*** shoot apical meristem

Marcus Heisler, Carolyn Ohno

A fundamental developmental process in plants and animals is the positioning of lateral organs. In plants, recent experiments have implicated the hormone auxin in controlling this process. When polar transport of auxin is disrupted organs fail to initiate and this can be rescued by exogenous auxin at the site of application.

In order to gain insight into auxin dynamics in the shoot meristem we, together with Jeff Long, have constructed a translational fusion between the auxin efflux carrier PIN1 and GFP that is capable of complementing the *pin1* mutant. Imaging of PIN1:GFP in vibratome sections and meristem whole mounts reveals that PIN1:GFP is localized to the lateral sides of cells in the meristem epidermis and to the basal sides of cells in developing vasculature. Interestingly, at sites predicted to form organ primordia, PIN1:GFP signal is up-regulated and polarized towards the developing anlagen. At later stages PIN1:GFP reverses polarity and now faces towards developing anlagen in adjacent regions. Furthermore, *in situ* expression analysis shows that the auxin influx carrier *AUX1* is expressed in primordia anlagen. These results strongly suggest that the auxin transporter proteins PIN1 and AUX1 function to directly transport auxin to sites of primordia emergence in order to bring about their initiation.

Microarray experiments have also indicated that expression of *PIN1* and *AUX1* are themselves regulated by auxin, and this has been confirmed by RT-PCR. In order to analyze this response at the cellular level, we have used time-lapse confocal imaging of PIN1:GFP in living meristems. This reveals a dynamic pattern of gradual increase in signal at sites predicted to form primordia, followed by a gradual restriction of this signal to the developing vasculature. In meristems treated with NAA, a form of auxin able to diffuse into cells, PIN1:GFP signal appears throughout the meristem. Delocalization of signal also results when auxin transport activity is disrupted by the addition of NPA.

Taken together, these data suggest that in the apical meristem a positive feedback loop operates through the transcription and localization of auxin transporter proteins to concentrate auxin in small domains in order to determine primordia position.

88. Gene expression dynamics during axillary meristem activation

Ottoline Leyser[1], Sally Ward[2]

Shoot axillary meristems are laid down in the axil of each leaf produced by the primary shoot apical meristem. The meristems may become dormant, or they may activate to produce a lateral branch. Hence the regulation of the activity of axillary meristems plays a major role in determining shoot system architecture. In order to understand this process better, we have collected a series of mutants with defects in the regulation of axillary meristem activity, as well as a set of genes whose expression is characteristic of either active or inactive axillary meristems. Analysis of the expression of these marker genes in the mutant axillary meristems indicates that their expression is deregulated in these backgrounds. For example, the bushy mutants *max1-max4* define a genetic pathway required to suppress axillary meristem activity, with mutant axillary buds activating early and ectopically (1,2,3). However, the transcriptome of these over-active buds resembles that of wild-type dormant buds. In order to assess in more detail the dynamics of this marker gene deregulation, we are seeking to develop 4D image analysis for axillary meristems, similar to the methods recently established for the primary shoot apical meristem.

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89. Genetic and molecular dissection of early and late SUP functions – from SupMan to SupWoman

This project is a collaborative effort between the Plant Reproduction and Development Laboratory, ENS Lyon, France (Stephanie Broyer, Patrice Morel, Ioan Negrutiu, Christophe Trehin) and the Meyerowitz laboratory (Toshiro Ito, Frank Wellmer)

SUPERMAN (SUP) is one of the most surprising genes controlling flower development. By acting to separate the female from the male territories within the hermaphrodite flower, *SUP* must have played a key role in the evolution of the angiosperm flower. The structure of the gene itself is unique (Ito *et al*., 2003), several regulatory elements for early stamen function being located in transcribed and translated regions. The so far assigned roles of the gene reveal critical *SUP* functions for the reproductive success of the species. These functions (cadastral, floral determinacy, ovule integument differentiation) are based essentially on the systematic analysis of one allele, *sup1* (Bowman *et al.*, 1992; Sakai *et al*., 1995 and 2000). They do not allow one to fully understand the range of phenotypes observed in the allelic series, which vary from flowers with excess stamens and (almost) no female organs (superMan) to flowers with excess carpels (superWoman) or to flowers with excess of both stamens and carpels (superSex). In addition, it is not clear so far, mainly due to the absence of effective SUP antibodies, which of the allelic classes contain reference null allele(s). Furthermore, the formation of numerous and variable chimeric structures between stamens and carpels raises questions on their origin and precise nature.

In this context, we wanted to advance our understanding of this gene by: (1) making a comparative and comprehensive genetical, morphological, cellular and molecular analysis of the main classes of allelic forms; and (2) dissecting the multiple functions of the gene in its early and late components. To do so, we performed crosses between allelic classes, between *sup* alleles and mutations affecting flower identity/determinacy (B function mutations, *clv* mutations) and reporter genes for GUS or GFP behind *AP3, AG* and *WUS* promoters, as well as *in situ* hybridization with some of these genes. While experiments are still in progress, we can anticipate and extend the range of *SUP* functions to intra-whorl organ separation and stamen identity and number.

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90. Misexpression of ***SHREDDIE(SDI)*** results in ectopic growth of leaf-like tissues

Carolyn Ohno, G. Venugopala Reddy

In plants, leaf and floral organ morphogenesis involves coordination of cell division and differentiation. We have identified a recessive mutant *shreddie (sdi)* in which organ morphogenesis is abnormal and that results in narrow organs with defects in cell shape and size. The gene encodes a single C2H2 zinc-finger protein and is a member of a large gene family in *Arabidopsis* that also includes the *SUPERMAN* gene. Since normal expression of the *SDI* gene is restricted to vegetative and floral organ primordia, *SDI* was misexpressed with a constitutive CaMV 35S promoter in order to uncover additional functions of the gene. *SDI* constitutive expression results in fusion of cotyledons in the seedling, and fusion of vegetative leaves. *SDI* constitutive expression also results in bract formation at the base of some flowers and ectopic development of leaf-like tissues on the stem, stipules, pedicels and sepals. Thus, *SDI* appears to be sufficient for growth of leaf tissues and specification of leaf cell identity. To determine the consequence of misexpression in the flower, the *SDI* gene was expressed under the control of the *AP1* promoter. It is likely that the *AP1* promoter is more highly expressed in the flower in comparison to the 35S promoter. Surprisingly, flowers were replaced by leaf-like organs and floral organs were never produced.

To determine the subcellular localization of the SDI protein, a translational fusion of the SDI gene to VENUS (a rapidly maturing variant of YFP) was introduced into *Arabidopsis*. This construct fully rescued *sdi* mutant plants. Confocal live imaging of intact inflorescences reveals that the pattern of expression of the transgene is consistent with RNA *in situ* hybridization patterns. The SDI:VENUS fusion protein is localized to nuclei in sepal primordia. Thus, *SDI* functions in the nucleus as a putative transcription factor.

91. Searching for genes acting redundantly with SDI

Carolyn Ohno

Gain-of-function mutant analysis suggests that the *SDI* gene is sufficient to induce ectopic leaf growth, however the *sdi* mutant phenotype indicates that it alone is not absolutely necessary for leaf development. *sdi* loss-of-function mutants show development of narrow floral organs and leaves. This mild phenotype suggests that *SDI* may be redundant with another gene. Blast searches of the *Arabidopsis* whole genome reveal a single closely related SDI-like gene that exists on chromosome I along with *SDI*. Overall the two related C2H2 zinc-finger encoding genes share 47% amino acid identity. *In situ* hybridization with gene-specific probes reveals the expression pattern of

SDI-like is similar to and overlapping with that of *SDI*. In seedling sections, the *SDI*-like RNA transcripts are detectable in leaf primordia but are absent from meristem. Antisense/RNAi strategies are underway to obtain a loss-of-function *SDI*-like mutant phenotype. In a separate attempt to genetically identify genes that function redundantly with *SDI*, an EMS mutagenesis screen in the *sdi*-1 mutant background has been initiated to identify second site mutations that enhance the *sdi* phenotype.

92. HANABA TARANU is a GATA transcription factor that regulates shoot apical meristem and flower development

Yuanxiang Zhao, Kazuaki Ohashi[1], Leonard Medrano[2], Hajime Sakai[3]

GATA transcription factors are known to play important and diverse roles in animals and fungi. However, their function in plants is scarcely known. We isolated a new mutant, *hanaba taranu* (*han*), which affects both flower development and shoot apical meristem (SAM) development in *Arabidopsis thaliana*. Mutants have fused sepals, reduced organ numbers in the 2nd (petal) and 3rd (stamen) whorls, as well as carpel fusion defects. In addition, the *han* mutation interacts strongly with the *clavata* (*clv*) mutations (*clv1*, *clv2* and *clv3*), resulting in more fasciated SAMs and more severe floral defects. Cloning the *HAN* gene revealed that it encodes a GATA-3 like transcription factor with a single zinc-finger domain (C-x2-C-x18-C-x2-C). Consistent with its role in promoting organ separation and organ identities, *HAN* is transcribed in the boundaries between the meristem and its newly initiated organ primordia, and also in the boundaries between different floral whorls. It is also expressed in the vascular tissues, developing ovules and the embryo. Ectopic expression of HAN causes growth retardation and loss of meristem activity, further supporting its roles in restricting stem cell activity and in regulating cell growth/differentiation during normal development. Both genetic and molecular studies suggest that HAN normally acts as a negative regulator of *WUS* expression. CLV3 has been shown to restrict *WUS* expression in the SAM, and examination of *HAN* expression in *clv3-2* mutants revealed *HAN* being ectopically expressed along the expanded apical layers in the SAM. This suggests that HAN and CLV3 may function both dependently and independently to regulate *WUS* expression.

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93. Using TOPLESS to identify genes involved in root/shoot formation during embryogenesis

Jeff A. Long

Arabidopsis embryos develop with a distinct apical/ basal polarity. Two groups of stem cells, the shoot apical meristem and the root apical meristem, form at opposite poles of the embryo (the apical and basal poles, respectively). *topless-1* (*tpl-1*) is a single, temperature-sensitive mutation that transforms the shoot pole of the embryo into a second root pole. *tpl-1*, which was originally identified by Dr. Kathryn Barton, is the only *Arabidopsis* mutation identified to date that can cause homeotic transformation of the embryo.

TPL encodes an 1131 amino acid protein containing 14 predicted WD40 repeats and a proline rich domain. These domains are also found in the TUP1/GROUCHO family of transcriptional corepressors. Four other predicted proteins that share at least 74% amino acid similarity with TPL are found in the *Arabidopsis* genome. Genetic and molecular evidence suggest that the original *tpl-1* allele is a dominant-negative mutation that affects the function of multiple TPL-like proteins. The wild-type role of TPL would then be to suppress root fate in the apical half of the embryo.

Our next goal is to identify the genes that are regulated by TPL during embryogenesis. To achieve this aim, we have generated lines where either the wild-type or dominant-negative version of the *TPL* gene is under the control of an inducible promoter. We will induce both forms of the gene and collect tissue at distinct time points after induction. RNA from this induced tissue will be compared to RNA from uninduced controls by microarray analysis. This technique should allow us to identify targets of TPL action and identify those genes involved in establishing the root/shoot system during embryogenesis.

94. ***AtGCN5*** is necessary for shoot to root transformations in ***topless-1*** mutant embryos

Jeff A. Long

In order to gain insight into the function of the TOPLESS (TPL) protein, we took advantage of the temperature sensitivity of the *tpl-1* allele. At the restrictive temperature, the majority of *tpl-1* embryos display a transformation of the shoot into a second root. We mutagenized *tpl-1* homozygous seeds with EMS and allowed the plants to set seed at the restrictive temperature. We then screened the resulting population for plants that had a wild-type appearance.

In this screen, one strong, recessive suppressor was isolated and named *big top* (*bgt*). *bgt* plants carry a mutation in the *Arabidopsis* homologue of the yeast GCN5 protein, a histone acetyltransferase. In yeast, GCN5 is recruited to chromatin by DNA binding transcription factors, where it can act as a transcriptional coactivator. This result indicates that genes necessary to form the apical root in *tpl-1* embryos are under the control of AtGCN5 and implicates chromatin remodeling in embryonic polarity decisions. This result also further supports our model for TPL as a transcriptional corepressor.

To identify putative targets of BGT, we have generated lines with a dexamethasone-inducible version of the protein. Microarray analysis of RNA isolated from induced and uninduced controls will identify some of the genes controlled by GCN5 in *Arabidopsis*. The validity of these genes as targets will then be determined by

chromatin immunoprecipitation using an antibody for acetylated histones.

95. Floral meristem organization and floral primordium size

Patrick Sieber, Robert W. Williams[1], Mark P. Running[2], Jennifer C. Fletcher[3]

Meristems of plants serve two main purposes. They produce organs from their flanks and they sustain themselves. Meristems keep a constant size by maintaining a slowly dividing stem cell population in the center and allowing more quickly dividing cells at the periphery to differentiate, and to be incorporated into organs. Floral meristems produce a determined number of organs in a whorled pattern. Thus, the whole floral meristem is consumed during flower formation and the floral primordium is subsequently divided into four types of floral organs that are arranged in four whorls. From the outermost whorl to the center four sepals, six petals, four stamens, and two carpels are initiated. The organ primordia within a single whorl are of approximately equal size and mutations, which cause enlarged floral meristems often lead to an increase in primordium number but not to bigger floral organs (Clark *et al*., 1993; Running *et al*., 1998). This could indicate that, if a critical number of cells is reached at the periphery of the meristem, these cells might subsequently become incorporated into initiating primordia. Mutations in the *SNOWBALL* gene cause the formation of flowers with 4-5 sepals and 3-5 petals in most flowers, but do not lead to an increase in meristem size (Running *et al*., 1998). The sepals and petals of *sno* flowers are usually smaller than the corresponding wild-type organs. So far only few mutants have been described which form flowers that produce aberrant numbers of organs without affecting the size of the meristem, and without affecting organ identity. Mutants that are affected in the basic process of partitioning cells into organ primordia can help to further our knowledge on how meristems are organized. We are working on the phenotypic characterization of *sno* mutants and we are interested to identify the molecular nature of the *SNO* gene in order to understand better the processes underlying meristem organization and floral organ primordium initiation.

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96. ***early extra petals* (*eep*) affects petal number in *Arabidopsis thaliana***

Catherine Baker, Frank Wellmer

In wild-type *Arabidopsis thaliana*, floral organ numbers are largely invariant, with each flower containing four sepals, four petals, six stamens, and two carpels. Various mutations that increase the size of the floral meristem (*clavata1*, *clavata3*, *wiggum*, *ultrapetala*) or cause the floral meristem to be smaller than wild type (*wuschel*) show an increase or decrease, respectively, in the number of organs in two or more whorls. Floral organ numbers can vary, however, in the absence of a dramatic defect in meristem size, as demonstrated by the *perianthia*, *pinoid* and *pinformed* mutations. Plants homozygous for these mutations have an increased number of petals, and the latter two mutants show additional phenotypes associated with disruptions of auxin signaling and/or transport.

In the interest of further uncovering what governs the number and position of floral organs within a whorl, we are studying a mutant called *early extra petals* (*eep*). *eep1 -1* plants have a variable number of extra petals in the first ten flowers, without affecting the organ numbers in other whorls (unlike *clv1*, *clv3*, *wig*, *ult*, and *pan*). In addition, they lack the pleiotropic auxin-related phenotypes associated with *pinoid* and *pinformed*. However, in double mutants, *eep1-1* strongly enhances the *pinoid* phenotype and partially suppresses the delay of flower production in the *pinformed* mutant, suggesting that EEP1 may function in auxin transport and/or signaling.

Using a map-based approach, we have localized the *eep1-1* mutation to 50 kb on chromosome 5. We have successfully rescued the *eep1-1* phenotype with a 12-kb genomic subclone and have discovered an *Arabidopsis* transposon present in this region of *eep1-1* but not in the wild-type background (L-*er*). As the transposon insertion site is not within an annotated gene, we are in the process of identifying putative open-reading frames and/or transcribed sequences that may be disrupted by this insertion.

97. Clarification of ***AGL24*** function in the control of flowering time

Hao Yu, Toshiro Ito

The flowering-time genes *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*), together with the floral meristem identity gene *LEAFY* (*LFY*), are three essential regulators integrating floral signals from multiple flowering promotion pathways. Our previous studies showed that part of the crosstalk among these genes is mediated by a putative transcription factor, *AGAMOUS-LIKE 24* (*AGL24*), which is a dosage-dependent flowering promoter regulated in several floral inductive pathways.

Although genetic analyses of epistasis has described the scenario in which *AGL24* acts downstream of *SOC1* and upstream of *LFY* in the control of flowering time, the concrete relationship between these genes is not clear. To clarify this point, we have applied a

glucocorticoid (GR) inducible system to generate *35S-SOC1-GR* transgenic plants. Upon treatment with Dexamethasone (DEX), *35S-SOC1-GR* can almost rescue the late flowering phenotype of *soc1*, indicating that this inducible system is efficient to provide *SOC1* activity in the endogenous context. By using this inducible system, some preliminary experiments have suggested that *SOC1* can rapidly promote *AGL24* expression in the flowering pathway, which is consistent with our previous genetic analyses of their epistatic relationship.

Furthermore, our recent studies also show that *AGL24* and *TERMINAL FLOWER 1* (*TFL1*) have a potential relationship in flowering time control. *In situ* hybridization of *35S::AGL24* revealed that *TFL1* expression is ectopically expanded into the tunica region of the inflorescence meristem (IM), while its expression is much reduced in the IM. Consistent with this finding, we also observed similar early flowering in *tfl1-1* and *35S::AGL24,* as well as similar late flowering in 35S*::TFL1* and *agl24*. These data raise the possible regulatory hierarchy, in which *AGL24* may negatively regulate *TFL1*, that in turn antagonizes the activity of *LFY* in the control of flowering time.

We are now testing the *35S-AGL24-GR* inducible system, which will be subsequently used for the investigation of the relationship between *AGL24* and *TFL1*. Also, we have made genetic crosses between *AGL24* and *TFL1* loss-of-function and gain-of-function plants, which will further help to clarify the genetic epistasis between these two genes. Taken together, these data will eventually provide a framework of flowering time control covering the networks from *SOC1 (*the integrator of flowering signals) to *LFY* (the meristem identity gene), in which *AGL24* functions as an essential intermediate.

98. ***AGL24*** mediates inflorescence meristem development

Hao Yu, Toshiro Ito, Frank Wellmer

The initiation and maintenance of floral meristems (FM) in the inflorescence meristem (IM) is a subtle and dynamic process promoted by several floral meristem identity genes, including *LFY* and *AP1*. In contrast to the establishment of FM identity, little is known about its maintenance. Our recent work clearly demonstrated that *AGL24* is a negative regulator of the transition from IM to FM.

The function of *AGL24* in the IM was initially revealed by the flower phenotypes of overexpression of *AGL24*. First, the flowers in *35S-AGL24* transgenic plants develop bract-like organs in the position of whorl 1 and frequently generate secondary flowers in the axils of the first and fourth whorls. Second, floral organs in *35S-AGL24*, including sepals, petals, and carpels, are partially transformed into leaf-like structures. These phenotypes are reminiscent of the strong *ap1-1* and *lfy-6* mutants. *In situ* hybridization further revealed that *AGL24* is ectopically expressed in the whole zone of the FM in *lfy-6* and *ap1-1*, which is in contrast to its expression only in the tunica region of the FM in wild-type plants. These results indicate that *LFY* and *AP1* may negatively regulate *AGL24*.

Two lines of genetic evidence strongly support the above suggestion. First, *ap1-1 agl24* double mutants show the significant reduction of secondary flowers in the axil of the first whorl as compared with *ap1-1*. Second, compared with *lfy-6, lfy-6 agl24* double mutants show a dramatic change of inflorescence structure. The flowers subtended by cauline leaves in *lfy-6* are transformed back into single flowers in *lfy-6 agl24*. Also, floral organ formation in *lfy-6* is intermediate between the whorled mode and the spiral mode, which is mostly rescued back to the whorled mode in *lfy-6 agl24*. This genetic evidence demonstrates that *AGL24* is responsible for the increased inflorescence character in *lfy-6* and *ap1-1*. By using both the *35S-LFY-GR* and *35S-AP1-GR* inducible systems, we have clearly observed the downregulation of *AGL24* transcripts just within 4 hours of Dex treatment, which further proves that both *LFY* and *AP1* can transcriptionally repress *AGL24* rapidly.

It is noteworthy that *in situ* results show that the initiation and maintenance of ABC genes expression in floral organs are almost not altered in both *35S-AGL24* and *agl24*, which indicates that *AGL24* cannot interfere the transcriptional regulation of ABC genes.

In general, this study has established a new genetic model, in which both *LFY* and *AP1* need to repress *AGL24* expression to secure the maintenance and normal development of floral meristems, which is independent of the regulation of ABC genes by *LFY* and *AP1*.

99. Involvement of the gibberellin (GA) signaling pathway in flower development

Hao Yu, Toshiro Ito

It has been recently proposed that that gibberellin (GA) may regulate plant developmental processes including floral development via the function of DELLA domain-containing GRAS regulatory proteins (GAI, RGA, RGL1, RGL2, and RGL3) in the signaling pathway, although the molecular genetic mechanism of these procedures is so far unclear (1).

Our recent studies have provided important information on the concrete function of the GA signaling pathway in flower development. Particularly, the genetic combination of *rga rgl2* in the *ga1-3* background can mostly rescue the underdeveloped flowers of *ga1-3* to relatively normal flowers, which is not observed in the combination of other GA signaling mutants. In this process, *RGA* should play a major role, since *rga ga1-3* has already shown a partially rescued flower phenotype, however, which is not clearly observed in *rgl2 ga1-3*.

In addition, *in situ* hybridization shows that *AP1*, *AP3* and *AG* expression is significantly reduced in flowers of the GA-deficient *ga1-3* mutant compared with that in wild-type plants, although the expression patterns of these genes are not changed. This kind of reduction is observed uniformly from the initiation of floral organ primordia to the development of mature flowers, which indicates that

GA may promote flower development in part by maintaining the expression levels of ABC gene at a reasonably high level. This effect may be more critical for later stages of flower development because *ga1-3* mutants only produced underdeveloped flowers with non-functional stamens and carpels.

Now it is clear that RGA and RGL2 are two critical GA signaling genes involved in flower development. There are two critical questions. First, what is the exact function of the ABC genes in the later stages of flower development? By using a *35S-AG-GR* system, we have found that the maintenance of *AG* expression level is important for the late development of stamens and carpel, although their identities have been set up at an early stage. Second, is the RGA function related with the regulation of ABC genes? To address this question, we have created a *35S-RGA-GR* inducible system in the background of *rga ga1-3,* which is now being applied to investigate if the expression of ABC genes is associated with RGA function.

The current investigation is expected to produce insights into the connection between the GA signaling pathway and flower development, and will possibly, at the same time, address the function of ABC genes in late-stage flower development.

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100. Investigation of the gibberellin (GA) signaling pathway in the control of flowering time

Hao Yu

GA plays a major role in the control of flowering time, especially in the short days (SDs) conditions. One demonstration of this is that GA deficiency (as in *ga1-3* mutants) causes complete arrest at the vegetative stage in SDs. Previous studies have demonstrated that the gibberellin response genes, GAI and RGA, may play critical roles in the control of flowering time in SDs because *rga gai ga1-3* mutants develop with a normal flowering time just as wild-type plants in SDs condition. RGA seems to play a major role in this process, since *rga ga1-3* has already shown a clearly rescued phenotype in terms of flowering time as compared with that in *gai ga1-3*.

It is clear that GA will affect *LFY* activity in the control of flowering time. Similarly, the expression of some other flowering-time genes, such as *SOC1* and *AGL24*, also responds to GA treatment. To identify the potential downstream genes of RGA and GAI in the control of flowering time in SDs, I have established both *35S-RGA-GR* and *35S-GAI-GR* inducible systems in a *rga gai ga1-3* genetic background. Application of both inducible systems will be helpful to elucidate the mode of RGA and GAI function in control of flowering time in SDs. In the meantime, the effect of overexpression of some potential downstream genes, such as *LFY*, *SOC1*, and *AGL24* in a *ga1-3* genetic background is under investigation in SDs. These studies will lead to the clarification of the mechanism by which the GA signaling pathway is integrated into the complex networks that control flowering time.

101. Exploring floral organ number specification

Pradeep Das

While much is known about some aspects of flower development, such as floral meristem or floral organ identity, others, such as organ number specification, remain largely unclear. We are using mutations in the *PERIANTHIA* (*PAN*) gene, which encodes a bZIP transcription factor, to intensively study this process. PAN is one of the few genes known to affect only floral organ number without altering meristem size. While the wild-type *Arabidopsis* flower is tetramerous (four sepals, four petals, six stamens, two carpels), flowers of *pan* mutant plants are largely pentamerous (five sepals, five petals, five stamens, two carpels). Interestingly, ancestral members of the *Brassicaceae* family, to which *Arabidopsis thaliana* belongs, are pentamerous, suggesting that it may be a recently acquired activity of *PAN* that has brought about this change.

We are following several lines of investigation to determine the function of PAN in the regulation of organ number. Firstly, we are performing gene expression studies via whole-genome microarray analyses. By fusing *PAN* to a portion of the glucocorticoid receptor (GR), we have produced a construct that can be activated by addition of the steroid hormone dexamethasone. Time-course experiments with this system are expected to yield both genes that are directly activated by PAN as well as secondary targets of those factors. Analysis of these genes should shed some light on the processes downstream of *PAN* expression.

Secondly, we are performing directed expression studies of PAN in a *pan* mutant background to better understand what role, if any, it plays in initiation of organ primordia. Further, these experiments should also elucidate the effects, if any, of organ number in one whorl on organ number in an adjacent whorl. We will use the LhG4-6xOp system to express *PAN* in specific domains of the floral meristem and assay its effects on organ number by examining both the adult flowers as well as through markers for organ primordia.

Thirdly, we are performing genetic modifier screens to identify other loci that might partner with PAN to specify organ number. Characterizing the roles of those loci through classical genetics and through methods similar to those outlined above will in turn contribute to our understanding of organ number determination.

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Summary: The Rothenberg group seeks to define the molecular mechanisms that control commitment of hematopoietic precursors to develop into T lymphocytes. Lineage choice in hematopoiesis is a combinatorial function of the activities of multiple transcription factors (1,2). At the same time, the set of permitted outcomes available to a given precursor is restricted by inputs from signaling molecules in the microenvironment. Thus, whereas any particular lineage choice (e.g., "T vs. B lymphocyte") can be seen to be controlled by one of these factors, an understanding of the full lineage commitment process ("T vs. anything else") must take into account the activities of multiple factors, and the timing and microenvironmental contexts of their actions. In the past several years, new cellular and molecular approaches have begun to make such an undertaking approachable. Individual projects in the lab dissect particular components of the process, addressing the gene expression changes associated with each stage of the commitment process and the regulatory influences that control them. As an overarching goal, we are seeking to develop better models for the relationships between subsystems, to be able to analyze the results of all these studies in terms of the operation of the developmental system as a whole.

T-lineage commitment is protracted in time, perhaps more obviously than any other hematopoietic lineage decision. It involves both positive gene regulation changes, "specification," and negative regulation of alternatives, "commitment." The process can be seen to begin in the bone marrow or fetal liver, then continues through up to ten cell divisions in the thymus, before it is complete. There are excellent cell surface marker phenotypes that have been defined to distinguish stages in this process, making it an ideal system in which to dissect temporally distinct components of a lineage choice mechanism. As precursors winnow down their choices to develop into T cells, it is clear that the most persistent alternatives to T-lineage fate are development into B cells, into natural killer cells (NK), or into non-lymphoid components of the innate immune system known as dendritic cells (DC). Over the past few years, studies in this lab and several others have identified the transcription factors PU.1, Id2, and GATA-3, and microenvironmental stimuli from activated Notch and cytokine receptors, as potent influences in these choices. One large group of projects in the Rothenberg group is focusing on the relationships between the roles of these factors, and the crucial molecular mechanisms through which they operate, as they negotiate the fate of the cells among these different outcomes (abstracts 102-108). A central insight that has emerged from our studies is the stage dependence of all of these effects. Thus, T-cell commitment appears to be choreographed as a specific sequence of regulatory events, rather than a simple response to a one-time trigger.

The best-known candidates for positive regulators of T-cell specification are GATA-3, bHLH type I factors (e.g., E2A and HEB), and Notch1/Deltalike 1 signaling. However, none of these are specific to T-cell development except GATA-3, and our most recent studies emphasize that GATA-3 is not "read" as a T-cell specification signal when it is expressed precociously by uncommitted hematopoietic precursors (abstract 102). This suggests that other factors remain to be found that could guide the initial events of T-cell specification. Thus, an ongoing project in the lab is also to use gene discovery approaches to identify new candidate transcription factors that show T-cell specific expression (abstracts 109, 110). This has resulted in the identification of several new T-lineage-associated factors, which can now be tested functionally by perturbation studies. The impact of this work can also be enhanced by another new project, developing new technology to monitor expression levels of a large set of combinatorial regulators simultaneously, in small numbers of cells (abstract 111). This would allow the most rigorous correlations to be made between developmental potential of a cell and the instantaneous state of its transcriptional regulatory network. We are also seeking new T-cell specification/commitment functions through a "fishing" approach, using as "bait" the *cis*-regulatory sequences of genes that must undergo changes in expression status in the early stages of the commitment process. One of these genes is IL-2 (abstracts 114, 115), which apparently becomes open for expression in the earliest T cells or in a T/NK/DC precursor. The other of these genes is PU.1 itself, which is shut off specifically as the cells complete T-lineage commitment (abstracts 112, 113).

T-lineage commitment is apparently complete only when the cells finish several days of T-cell gene expression and proliferation, and pause before the β-selection checkpoint. This is when, for the first time, the cells are tested for success in undergoing a T-cell receptor gene rearrangement. It is possible that the enforcement of the checkpoint may be intimately linked with the mechanisms that complete commitment. It is therefore interesting to examine mutations that affect progression to β-selection and the rare cases in which cells can bypass the β-selection checkpoint without a rearranged T-cell receptor

gene (abstracts 116, 117, 118). One of these cases is found in the autoimmunity-prone NOD mouse. We have determined a unique pattern of gene expression perturbations that are correlated with the NOD-specific form of checkpoint violation (abstract 117).

Finally, the complexity of the T-cell developmental program could be a reflection of its evolutionary history. T and B lymphocytes appear to have emerged as a cell type only in the base of the vertebrate radiation. There are small round cells in the jawless vertebrates, the lampreys, but they do not have the complex gene rearrangement program that confers recognition specificity on the lymphocytes of jawed vertebrates. In contrast, even the cartilaginous fish, the most anciently divergent group of jawed vertebrates, have well-defined B and T lymphocytes and lymphoid organs with properties similar to those of birds and mammals. For the past several years, therefore, we have examined the transcriptional regulatory profiles of hematopoietic tissues in cartilaginous fish and have begun to compare them with those of lampreys (abstract 119). The skate, a cartilaginous fish, demonstrates that the transcription factor usage seen in mammals is strikingly well conserved in skate hematopoietic cells, with a few alterations in detail. This is now providing the launching pad for our assessment of the lamprey, which possesses representatives of most of the same transcription factor families but appears to use them in markedly different combinations.

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102. Roles of GATA-3 expression in prethymic vs. intrathymic hematopoietic choices

Alexandra Arias, Tom Taghon

T-cell lineage development occurs within the framework of blood cell development (hematopoiesis) that results in not only the generation of T cells but also at least nine other blood cell types. This process originates with the hematopoietic stem cell that developmentally progresses through progenitor stages that ultimately gives rise to a multitude of blood cell types which include T cells. The Rothenberg lab is interested in understanding the mechanisms involved in generating T cells from these hematopoietic progenitors, and in particular, what transcription factors are involved in regulating T-cell lineage specification and commitment as distinct from delineating other blood lineage choices.

An important transcription factor in this process is GATA-3. GATA-3 is in the family of zinc-finger transcription factors defined by GATA as their DNA binding motif. Currently there are six family members, three of them (GATA-1, GATA-2, and GATA-3) with important roles in hematopoiesis. Each of the hematopoietic GATA factors has been implicated as essential in distinct stages of blood cell lineage decisions. In particular, GATA-3 is an essential T-cell factor with its expression maintained throughout T-cell development into the late mature stages. Much focus is on further understanding the role of GATA-3 in T-cell development. Its role in other aspects of hematopoietic development, however, is less clear. Nevertheless there is evidence for GATA-3 involvement in prethymic development, as its expression is also found in hematopoietic stem cell populations.

Because of this implied importance in hematopoietic development we are interested in understanding the role of GATA-3 in hematopoietic precursors as it pertains to T-cell development. Our approach is to analyze perturbations of lineage choice caused by overexpressing GATA-3 in various progenitor populations. Progenitor populations are localized in the mouse according to developmental age. At embryonic day 14, the majority of multipotent precursors are localized in the fetal liver with populations of mainly lymphoid-restricted progenitors in the thymus. Both organs serve as sources for isolating and enriching the hematopoietic cells of interest. These cells are then transduced with retroviral vectors that direct GATA-3 expression. Various *ex vivo* culture systems (both short and long term) are used to evaluate the transduced populations' developmental potential. The cells can then be evaluated, for example, through flow cytometric analysis of surface marker expression or hematological stains. Thus, we gain a survey of hematopoietic development beginning with the hematopoietic stem cell through T-cell development. We use this to monitor the progression of our GATA-3 transduced cells. This allows for comparisons of context dependent perturbations caused by GATA-3 at specific developmental stages.

Through these types of studies, our lab has shown that over expressing GATA-3 in fetal liver derived populations causes a very early prethymic inhibition of T-cell development. This early effect has obscured attempts at understanding the role GATA-3 in the later transitioning of precursors into the T-cell lineage. As first described last year, to compensate for this block, our experiments now include a system that allows for the conditional activation of GATA-3. We fused the coding sequences of GATA-3 and a mutant estrogen receptor hormone-binding domain to generate a tamoxifen inducible chimeric protein. Not only is GATA-3 over expressed, it is quantitatively and temporally controlled. This has become a valuable system in evaluating the effects of GATA-3 expression in hematopoietic progenitors in conjunction with our hematopoietic assays.

So far, our studies have confirmed that high levels of GATA-3 over expression in fetal liver derived progenitors are detrimental to nearly all progenitor cell populations; thus, no mature cells of any lineage develop. However, low levels of GATA-3 over expression in fetal liver derived progenitors allow for an expansion of very early progenitor populations and are more selectively inhibitory, blocking B-cell development but permitting

other lineage choices such as myeloid development. The block caused by GATA-3 over expression that results in inhibition of T-cell development may be due to an early loss of lymphoid progenitors, distinct from the negative perturbation at the early stem cell stage. For example, these hematopoietic progenitors do not develop into B cells but can progress to other hematopoietic stages. However, when overexpressed in fetal thymocytes, GATA-3 over expression can enhance thymocyte developmental progression, although early pre T-cell populations can still be partially inhibited. Thus, we are now beginning to see stage-specific perturbations that differ among populations of particular significance to the questions being addressed in our lab.

103. Transcriptional changes induced by Notch signaling and a transcriptional and kinetic analysis of B-and T-cell development

Tom Taghon

B and T lymphocytes are blood cells that belong to the adaptive immune system and are characterized by receptors on the cell surface that are responsible for recognizing pathogens and eventually their elimination from the body. Besides these similar characteristics, B and T lymphocytes have many different features, one of them being the place in the body were they originate. While B cells, like other blood cell types, develop in the bone marrow, T lymphocytes require a specific niche for their maturation, provided by on organ that is called the thymus. As a result of this specific requirement, studying the development of T lymphocytes has been seriously hampered. B cells can easily be generated *in vitro* by culturing hematopoietic progenitor cells onto OP9 cells, a bone marrow derived stromal cell line. Recently, a cell culture system was reported by a Canadian research group that also allows T-cell development in the absence of the thymus. To accomplish this, they stably introduced Delta-like ligand, which interacts with Notch, into OP9 cells. Notch signaling is critical for T-cell development and continuous activation of this pathway inhibits B-cell differentiation. As a result, developing hematopoietic cells can no longer develop into B cells on the OP9 stromal cells that express the Delta-like ligand but instead differentiate along the T-cell pathway. We will now use this tool to carefully monitor the kinetics of and the transcriptional changes during the development of hematopoietic progenitor cells into B and T cells, starting from the same progenitor cells. Early data show that Notch-specific target genes are already upregulated after 24 h of culture in progenitor cells cultured on OP9 cells that express Delta-like ligand. These genes, however are not necessarily the T cell-specific genes that indicate that the cells become specified towards the T-cell lineage. Known genes for both T-and B-cell specification seem to appear only after 72 h of culture. However, this system also enables us to identify novel genes that indicate the development of progenitor cells in one or both lymphoid pathways. Also, by performing transfer experiments of hematopoietic progenitor cells from normal OP9 cells onto OP9 cells that express the Delta-like ligand, it seems that precursor cells have a longer time frame to adopt a T-cell fate instead of a B-cell fate.

Furthermore, we can now easily investigate and identify the downstream target genes of Notch and further explore their specific roles during the process of T-cell development. Two of those genes are the HES-related repressor proteins 1 and 2, HERP-1 and –2. We observed that both genes were immediately upregulated as a result of Notch signaling and analysis of their expression pattern during the early stages of T-cell development shows a unique expression at the DN3 stage. Using retroviral overexpression, we are currently investigating their role during T-cell development and try to identify their target genes. Also, as these proteins interact with the HES proteins, we will perform double infections of hematopoietic progenitor cells with HES- and HERP-encoding retroviruses to investigate if both protein families are required to mimic Notch signaling or if each gene family has a distinct role on its own.

104. The precise role of GATA-3 during early T-cell development and its relation to Notch

Tom Taghon, Alexandra Arias

Like Notch1, GATA-3 is essential for T-cell development. Recent studies that performed overexpression of this transcription factor have shown that cells can only tolerate GATA-3 overexpression at precise stages of T-cell development. Overexpression in mouse fetal liver progenitors results in a drastic reduction of GATA-3-infected cells and almost complete arrest of T-cell development at the earliest stage, showing how critical it is to tightly control the expression of this transcription factor (see abstract 102). However, some cells escaped this developmental block and were apparently able to differentiate further. These data, combined with evidence from overexpression studies with human thymocytes, seem to suggest that the developing T lymphocytes show different sensitivities to high level GATA-3 expression at different stages of their development. Despite the knowledge that this gene is required, we currently still do not know what its function is or what genes it regulates during T-cell development. We will further investigate this by retroviral overexpression in cells at different stages of T-cell differentiation and by eliminating GATA-3 expression using RNAi constructs. Given the fact that both Notch1 and GATA-3 are both required for T-cell development to occur, we want to analyze how both factors interact, if at all, by culturing GATA-3-infected progenitor cells on the OP9 stromal cell line, in the presence or absence of Delta-like ligand signaling, and also by performing transfer experiment from normal OP9 cells to those that express Delta-like ligand and vice versa at different time points during the culture period. Also, gene expression analysis of cells taken at different time points will give us information if both factors can act together to activate or repress certain genes that specify progenitor into the T-cell pathway.

105. A precise characterization of the earliest thymocyte subsets

Tom Taghon, Mary Yui, Angela Weiss

Developing T cells progress though a series of developmental stages in the thymus that can be identified through their expression of cell surface markers. The classical identification of the most immature subsets, termed "double-negative" (DN) thymocytes because they lack the expression of both CD4 and CD8, relies upon the use of the markers CD44 and CD25. The most immature thymocytes (DN1) start off as $CD44^+$ and $CD25^-$. In their first change, the cells acquire CD25 to become $CD44^+CD25^+$ thymocytes (DN2), and then they down regulate the expression of CD44 to become $CD44^-CD25^+$ (DN3). These DN3 thymocytes are undergoing extensive rearrangements of the T-cell receptor β-chain encoding genes and if successful, the cells will progress through a process called β-selection to become $CD44^-CD25^-$ (DN4) thymocytes. These different subsets are still heterogeneous populations and recently, c-kit was shown to be important as an additional marker to identify the 'true' DN1 and DN2 subsets. We are currently investigating the use of CD27 as an additional marker to subdivide the different DN subsets even further. Early results indicate that the DN1 cells that express c-kit virtually all express relatively high levels of CD27 and as the cells progress, the expression of this marker is slightly reduced. Furthermore, CD27 enables us for the first time to phenotypically subdivide the DN3 subset into two different cell populations of which we are currently analyzing their developmental status by analyzing both their developmental capacities and their gene expression profile. Based on previous reports, it seems that this marker would allows us to distinguish cells before and after β-selection and this would enable us to do a more precise study of the events that occur during this important process of T-cell development.

These results are particularly valuable in light of the detailed expression patterns of c-kit and IL-7Rα in the DN1, DN2, and DN3 cells. While useful as stage markers, these molecules are primarily important to the T-cell precursors as growth factor receptors. Their expression must change, therefore, in accord with the homeostatic mechanisms that regulate thymocyte proliferation and the checkpoint function that shifts newly committed cells from dependence on cytokines to dependence on pre-TCR signals. We have recently found that c-kit and IL-7Rα are also sensitive to the kinetic perturbations of DN1 to DN2 and β-selection checkpoint control that occur in several mutant mouse strains, including $RAG2^{-/-}$, Fos-deficient (see abstract 116), and NOD-background mice (see abstract 117). Initial results indicate that in at least one of these models, the regulation of c-kit and CD27 becomes uncoupled. We are therefore testing how closely CD27 expression is correlated to the maintenance of lymphoid developmental potential.

106. Role of PU.1 during early T-cell development

Angela Weiss, Michele K. Anderson

PU.1, a member of the Spi subfamily of ETS domain DNA-binding proteins, is a powerful transcription factor that is known to be essential for the development of macrophages, granulocytes, B cells, and fetal T cells. It is expressed during the earliest stages of thymocyte precursor development but is sharply downregulated just during the stages when developing thymocytes commit to the T-cell lineage. As we reported last year, the downregulation of PU.1 at this particular point during T-cell development is essential for T cells to progress beyond commitment and β-selection into the double-positive stage. Both of the effects exhibited by forced expression of PU.1 (reduction in cell number and stop of T-cell development just before β-selection) depend on the DNA binding activities of PU.1. Selective mutations in other domains of PU.1, either deletion of the Q-rich transactivation domain (Δ75-100) or mutation of a critical Ser residue in a protein interaction domain (S148), modify these effects but do not eliminate them. However, the Δ75-100 mutant form of PU.1 was reproducibly less inhibitory for T-cell precursor generation and proliferation than either of the other two forms, even though it too imposed a substantial block to progression beyond the β-selection checkpoint.

In addition to the long-term effects of PU.1 on developing hematopoietic stem cells (HSC) in a thymic environment, we also investigated the immediate effects on gene expression of these cells. Wild-type, Δ75-100, and S148 mutant forms of PU.1 all caused dramatic developmental changes in fetal liver-derived precursor cells, but the observed perturbations in the gene expression pattern were surprisingly selective. Certain responses, such as the effects on the variant bHLH transcription factor HEB-alt, were positive in fetal liver cells, whereas they were negative in thymocytes. These results indicated subtle differences in the gene-specific transactivation potential of the different PU.1 isoforms and potent effects of developmental context on the readout of PU.1 overexpression.

Some effects could have seemed minor because the cell population investigated was too heterogeneous. Among our starting population of fetal liver cells enriched for HSC are not only true stem cells but also a large fraction of cells that are not stem cells anymore, although they do not yet express any lineage markers. By staining our enriched HSC population we could find the fraction of cells that is most affected by overexpression of PU.1. This subpopulation is characterized by the expression of the cell surface marker CD27, which has been described as a marker of those cells among the HSC that already are on route to becoming lymphoid cells (see abstract 105). We are currently investigating long-term effects of PU.1 overexpression in this subpopulation in our developmental FTOC assay as well as short-term effects of PU.1 and PU.1 mutants on gene expression in these cells.

107. A potential cell line model for T-lineage commitment

Christopher Dionne, Kevin Tse

Discovering the signals and choices that lead to a pluripotent precursor in the thymus to adopt a T-lineage fate has been hampered by the lack of a cell line model system for assaying these steps of commitment. Spontaneous clones of the SCID.adh thymic cell line have arisen that present a system for assaying the late stages of commitment to the T lineage. As developing cells commit to the T lineage they lose the ability to develop along other lines (such as B or NK), as well as the ability to respond to factors that may direct them in a different direction (like cytokine receptors that can support/induce myeloid development). PU.1 is a transcription factor that is important for the development of multiple lineages, and while PU.1 is essential for early stages of precursor development, its extended expression inhibits T development and promotes either myeloid-like development or death. SCID.adh cells in many respects resemble thymocytes in the later stages of lineage commitment, and they do not express PU.1 normally. However, when retroviral transduction is used to introduce PU.1 into the cells, a fraction of the stable transductants shifts to turn on myeloid markers and to shut off lymphoid gene expression in an all-or-none response.

Subcloned SCID.adh cells yielded multiple cell lines, two of which, P2C2 and P6D4, are representative of groups of clones that are profoundly different in their responses to PU.1. P2C2 is highly responsive and shifts gene expression programs in a large proportion of the cells, whereas P6D4 appears virtually unresponsive. This raises the possibility that the clonal difference may mimic an *in vivo* step in commitment to the T lineage. Analysis of the lines demonstrates an increased susceptibility to death in the P6D4 when they are transduced with PU.1, which contributes to the inability of the cells to respond. If the P6D4s are provided with Ca^{2+} + protein kinase C stimulation as well as PU.1, however, they are able to make a transient myeloid shift response. We are currently assaying different cytokines and T-cell receptor signaling pathway components in the context of PU.1 expression to determine what stimulation events may be playing a role in this life and death choice. We are also using microarray technology to detect families of genes and novel genes that are differentially expressed between P2C2 and P6D4 cells that may be important.

The differences between the P2C2 and P6D4 lines may lie in each cell line's ability to signal through the pathways that respond to correct pre-TCR assembly or TCR/ligand interaction in normal T-cell development. These pathways often give the cell-specific signals either to proliferate or to die. We hypothesize that the combined effects of PU.1 overexpression and each cell line's unique ability (or inability) to signal may allow the P2C2 cells to be responsive to PU.1 and survive while the P6D4 cells die. Recent research has indicated that the organization and concentration of lipid rafts on the cell surface can affect the cell's ability to signal through the Ras, Fas, and other important pathways. Therefore, in searching for this possible difference in signaling aptitude, we examined the lipid raft content of P2C2 and P6D4 cell lines. We stained the surface of cells from each line with fluorescent cholera toxin subunit β (CTB), which is a marker that localizes on lipid rafts, and used confocal microscopy to view individual cells and their lipid raft concentrations. No major difference in CTB staining patterns was found. Currently we are analyzing CTB levels to determine if they are significantly different between P2C2 and P6D4.

Microarray analysis revealed that the two cell lines differed in their levels of expression of interferon (IFN) responsive genes. To assess the role of IFN signaling in the line, we carried out the PU.1 response assay in the presence of different IFN family members. Infection of cell lines with a viral vector carrying PU.1 normally results in upregulation of the macrophage marker Mac-1. IFN β and γ have opposite effects on this upregulation, with IFN β enhancing the upregulation of Mac-1 and IFN γ repressing the upregulation. We are further investigating this observation. It is interesting to speculate that PU.1 transduction, along with signals from interferon β, may enable the immature cell line to mimic an *in vivo* response to host infection that may cause the host organism to produce more cells of the innate immune system quickly, rather than wait for the adaptive immune system to make mature T cells.

Although every cell line is unnatural in some way, these clones are an excellent model for primary DN cell populations, without the heterogeneity that can make it difficult to define the target genes of PU.1 in the transcriptional networks that control cell fates (see previous abstract). By sorting populations of cloned SCID.adh cells that are responding to PU.1 or stimulation (or both), cells in a known developmental state responding synchronously to the perturbation can be compared rigorously with appropriate controls. Using this system, we have been looking at transcription factors that are known to play roles in hematopoietic development and are examining groups that correspond to PU.1 and/or stimulation. Many of the effects observed confirm effects also seen in normal immature thymocytes, illuminating the regulatory networks important to T- or non-T-developmental pathways.

108. New developmental systems for analysis of regulatory function in T-cell development

Deirdre D. Scripture-Adams

Changes in the expression patterns of a number of transcription factors are critical to the proper development of T and B cells. It has been demonstrated by the Rothenberg group that PU.1, for example, must be downregulated at the stage of T-lineage commitment. Other transcription factors must be downregulated or upregulated at different developmental stages.

Our group has successfully employed over expression techniques to determine whether the overexpression of a particular factor is inhibitory to development at certain developmental stages. The

opposite question, however, that of which genes *must* be expressed during particular developmental stages, cannot be appropriately addressed using overexpression techniques. Thus, I am constructing a series of tetracycline regulatable retroviral vectors encoding siRNA sequences that will allow us to specifically inactivate particular transcription factors of interest to T-cell development. This will allow us to determine which specific factors are required at each distinct developmental stage for survival and continued developmental progression.

In order to study the timing of expression of these developmentally regulated factors, an *in vitro* system that is as physiologically relevant as possible is necessary. Fetal thymus organ culture can be limited by the logistical difficulty and expense of obtaining suitable host organs as well as experimentally treated donor (hematopoietic precursor) cells. These limit the number of perturbations that can be tested in parallel and the numbers of precursors of defined cell types that can be included in the assays. I am currently developing new *in vitro* T-cell culture systems to further these goals. I have obtained progression to the DN2/3 stage of development of $RAG^{-/-}$ bcl-2 transgenic thymocyte precursors, using the OP-9-delta stromal cell culture method. I have also obtained development to the CD4/CD8 DP stage using adult and neonatal murine thymic fragments repopulated with fetal liver derived precursors. While the fragment culture system still requires considerable optimization for consistency, it should allow us to track development of T cells within a relatively intact thymic environment.

109. New transcription factors from early T-cell precursors: Expression patterns of new candidate regulators

Elizabeth-Sharon David

T-cell development is ultimately controlled by the rise and fall of transcription factors that work in concert to turn genes on and off from one stage to another. Factors like PU.1 and GATA-3 seem to provide permissive conditions for T-cell development, but they do not appear to cause a stem cell to choose the T-cell fate in the first place. None of the T-cell transcription factors already known have yet shown any evidence of being able to initiate the T-cell development process. To obtain a more complete picture of the transcription factors present during early T-cell development, we have identified several new candidate transcription factor genes from an early thymocyte cDNA library and are now attempting to characterize their expression patterns in detail. Our strategy is to focus primarily on genes that we have previously recovered from the arrayed cDNA library in screens for transcription factor family conserved-DNA-binding-domains. Selected clones have been rearrayed for sequence analysis and additional expression screens (Annual Report 2002). Based on sequence analysis, at least 200 clones encoding transcription factor genes are identified in the rearrays.

Genes showing broad differences in expression from non-T to T-lineage hematopoietic cells were chosen from the rearrays for closer study by performing differential expression screens using complex probes from "stem cell like" or "immature T-cell like" cell lines and immature thymocytes (Annual Report 2001). Alternately, we chose to study genes already known for a regulatory role in some aspect of haematopoietic function. Expression of these genes was then measured by quantitative real-time Polymerase Chain Reactions (qPCR) in cDNA samples from hematopoietic and nonhematopoietic mouse adult tissues. Any transcription factors that seem to be present at greater levels in these early DN thymocyte subsets was further tested by qPCR through samples of cDNA from actual DN1-DN4 FACS-sorted cells. Genes that have already been or are currently under study in the laboratory showed patterns of expression validating our assays, e.g., PU.1 that is highest in bone marrow and spleen and present in thymus as well, and Tcf7 (a.k.a. TCF-1) that is present predominantly in thymus, and at particularly high levels.

These criteria led us to focus attention on 32 genes. Some genes were found to be expressed broadly in haematopoietic cells, such as those encoding sHKR3 (similar to *Homo sapiens* Kruppel Related 3), Mta1l1 (Metastasis activator 1-like-1), Tle3 (*Mus musculus* groucho), Dimp (cyclin-D interacting myb-like protein), DSC43, LRF (lymphoid related factor), mybl2 (myb-like 2), an HMG-box protein, NaspC, similar to cut-like, mKr5 (mouse Kruppel 5), and a novel gene.

Other gene products were present in thymus at substantially higher levels than the other tissues tested, such as T-bet, FoxP4, IRF, KAB-1, Pou6f1, Rbak and Period. GCMa is present in thymus and also in kidney, while GCMb is present in bone marrow and thymic cells. Both GCMs, however, are only expressed in these tissues at very low levels. The *GCMa* and *GCMb* genes were initially of interest because in other model systems, sea urchin embryo and in some *Drosophila* tissues, *gcm* is activated by Notch signaling, and Notch signaling has a known vital role in the initiation of T-cell development. However, our subsequent measurements do not establish either of the GCMs as being downstream of Notch signaling in hematopoietic precursors.

The genes remaining most interesting show distinctive, specific expression patterns in the earliest stages of thymocyte differentiation. T-bet and FoxP4 appear to be expressed in complementary patterns, while the two *GCM* genes are also expressed noncoordinately. FoxP4, GCMb, and T-bet are all present in the earliest stages of thymocyte development, making them candidates for early pre-commitment specification functions.

110. Global differential expression screen for early T lineage-specific genes

C. Chace Tydell, Elizabeth-Sharon D. David

The Rothenberg lab is primarily interested in the question, "How are hematopoietic precursor cells guided to a T-lymphocyte fate?" One avenue of inquiry is the identification of all the transcription factors that are specifically upregulated when hematopoietic precursors

initiate T-cell development (see previous abstract). Common techniques for gene discovery are made problematic by the low copy numbers of mRNAs encoding most transcription factors. As an alternative, we are employing a subtraction technique developed in the Davidson lab to compare mRNA from two cell populations. The screening technique we are using subtracts message found in one cell population from that of a second population. The subtraction depletes message that the two cell populations have in common and relatively enriches message found more abundantly in the target population, enhancing visualization of low transcript genes. Subtractively hybridized message is used to probe a robotically arrayed cDNA library of >70,000 clones, generated from thymocytes in the stages immediately leading to commitment. To help concentrate the search on genes coding for transcription factors that may regulate T-cell development, degenerate molecular probes, designed to recognize conserved domains in selected protein families, have been utilized in a pre-screen to recognize ~20 families of transcription factors (see E.-S. David, *et al.,* abstract in 2002 Annual Report). The resultant clones have been rearrayed by the Q-Bot facility and sequenced by the Institute for Systems Biology. The subtracted probes will be assayed on the transcription factor-enriched subarrays as well as the whole master library.

From the first subtraction we will identify genes that are upregulated during T-cell specification as opposed to myeloid-lineage specification. These results will help us determine what transcription factors could be involved in the positive regulation of lymphoid cell development over the myeloid arm of haematopoiesis. On the other hand, the second subtraction set should show us what gene products are correlated with the split in developmental programming between the T- and B-lymphoid lineages. Such transcription factors that we isolate as playing a role in T-cell specification and commitment will be studied by expression analyses as well as for their functional role in development.

The cell populations that we have purified for these subtraction experiments are DN3 thymocytes, pre-B cells and pre-myeloid cells. DN3 thymocytes are harvested from the thymi of B6-Rag2 knockout mice for use as the target cell population. Thymocyte development in the B6-Rag2 null mouse arrests at the DN3 stage, resulting in a thymocyte population greatly enriched in DN3 cells. The same strain of mice has also provided the subtracting cell populations. The first subtracting population, pre-myeloid cells, were generated by culturing FACS-purified Lin^- (granulocyte, macrophage, natural killer cell, B-cell, RBC and DP/SP T-cell marker negative) c-kit^+ stem cells for two days with IL-3, SCF, and IL-6 before harvest. In order to generate these pre-myeloid cells from the c-kit^+ stem cells, a number of experiments were performed to determine the culture-conditions under which the cells would give rise to cells that had not terminally differentiated, but which retain their capacity to divide further and generate myeloid cells. Morphological observation was used to ascertain that the cells had not differentiated, while a colony forming methyl-cellulose culture assay was used to verify the colony-forming, proliferative potential of the (pre-myeloid) cells. Pre-B cells (Lin^-CD19^+), purified by fluorescence-activated cell sorting, will be used as a second subtracting population. Subtraction of DN3 thymocytes against themselves will provide an experimental control.

We predict that several transcription factor genes known to be involved in T-lymphopoiesis will be identified, and these can be used to monitor the efficiency of the enrichment. The most interesting clones to be identified will be the few novel ones that were previously unknown or unlinked to T-cell development. These will be added to the repertoire of transcription factors studied by the laboratory.

111. Exploring the state-space of transcriptional regulatory networks

Luigi A. Warren

There is growing appreciation of the role of combinatorial gene action in the unfolding of developmental programs and the encoding of differentiation states. The shift in focus from individual actors and shallow cascades towards larger networks of cross-acting transcriptional regulators is a logical extension of traditional genetic pathway analysis. It is in the nature of such analysis that the number of lines of influence and the set of kinetic parameters governing the "genetic circuits" so delineated grows rapidly with network size. The difficulties in characterizing, representing, and thinking about network-level behavior using this "circuit diagram" paradigm prompt the question of whether there might be alternative approaches which obviate the need for a detailed causal explanation of the activity of such networks. "State-space"-oriented network analysis offers one possible short cut. The state-space view attempts to characterize stable modes of the network in an n-space whose axes correspond to the transcriptional activity of the genes. The stability landscape derived from such analysis might provoke insights and inform hypotheses concerning permitted trajectories through developmental space, the response of the network to perturbing inputs (natural or artificial) and the role of stochasticity in lineage plasticity. In principle, this type of inquiry could proceed straightforwardly from a database of expression profile readings. However, the low abundance of transcription factor mRNAs, the requirement for quantitative, multiplexed analysis, and the practical and theoretical premium on profiling small numbers of cells set up formidable technical hurdles to acquiring the necessary data.

My thesis project is directed toward developing technology to facilitate the readout of transcriptional regulatory network state from individual cells. If this proves feasible, a detailed mapping of the stability landscape of the networks involved in thymocyte development could follow in short order, offering a test case for the fruitfulness of the state-space paradigm.

112. Identification of regulatory elements for PU.1 expression in hematopoiesis

Jing Chen

The ETS-family transcription factor PU.1 plays a pivotal role for development of hematopoietic cells at all stages of hematopoiesis. Recent studies have demonstrated that its expression level is also a critical element for erythroid, lymphoid and myeloid cell differentiation. However, how PU.1 itself is regulated in hematopoiesis is still not quite understood. Using the DNase hypersensitivity assay, our previous studies have identified eight potential sites of DNA/protein interactions in those hematopietic cells expressing PU.1. Four of those sites were shared by non-PU.1 expressing hematopoietic cell lines, and two of those sites were also shared by non-hematopoietic cells. To search for the transcription factors regulating PU.1 expression, we first focused on a 2 kb region in the proximal promoter region. Using the luciferase reporter assay in transient transfection, we found c-Myb and GATA-1 are strong activators for the PU.1 promoter activity, but this activation can be completely repressed by GATA-3 in co-transfection assay. We identified two potential sites in the 2 kb region which are responsible for c-Myb binding, while GATA binding sites are abundant. Two of the GATA sites tested have shown that they can be bound by GATA-1 or GATA-3 proteins with very similar efficiences. Both GATA-1 and GATA-3 can be co-immunoprecipitated with c-Myb *in vivo*. We propose that competitive interactions between c-Myb and different GATA family members may play a critical role for PU.1 expression and thus, determine the fate of different lineages of hematopietic cells.

113. Identifying PU.1 regulatory mechanisms in developing T-cells

Mark Zarnegar

PU.1 is an Ets family transcription factor expressed in early thymic precursors (DN1, DN2) and downregulated at the time of T-cell lineage commitment (DN3). During the previous year, we sought to understand how PU.1 expression is controlled in the hematopoietic system with the ultimate goal of learning how it is downregulated upon T-cell commitment. DNase hypersensitivity (see previous abstract), genomic sequence comparisons, and chromatin immuno-precipitation assays led to the identification of several potential upstream regulatory domains that may control the expression of PU.1.

Further characterization of these potential regulatory elements continues. Much effort has gone into identifying the transcription factors that bind and thus, regulate the activity of the regulatory elements. Previous electrophoretic mobility shift assays implicated two important transcription factor families, Ets and Runx, which may contribute to the regulated expression of PU.1. Members of these families bind an important conserved region 14 kb upstream of the transcription initiation site. This region is being examined by *in vivo* footprinting to determine the occupancy of important sites in PU.1 expressing and non-expressing cells. The results of the footprinting will supplement the previous gel shifts and also provide clues as to what other factors and bindings sites are being employed by this potential regulatory element. Our results with the gel shifts and footprinting should provide us with insight to begin appropriate functional analysis.

The regulatory functions of the -14 kb upstream domain and other regions will be examined. Two approaches are being developed. The first approach will utilize transgenic animals. By creating a "knock-in" mouse, with GFP replacing PU.1, we will be able to follow GFP expression patterns in a developing embryo as controlled by normal PU.1 regulatory elements. By creating animal strains with deletions for specific regulatory regions or carrying mutations in the binding sites of relevant transcription factors, we can monitor the changes in GFP expression versus the normal knock-in mouse. The second approach will exploit episomes for studying PU.1 expression.

Episomally replicating vectors containing an OriP/EBNA-1/Hygromycin cassette have been developed for stable long-term expression of transgenes. We have obtained an HSV-1 based episomal system called Infectious BAC (iBAC) technology and a BAC clone with the entire PU.1 locus. The iBAC system was designed for the single-step conversion of a BAC clone into HSV amplicons for infectious genomic delivery. All BAC constructs contain a loxP site. By co-transforming the HSV retrofitting vector, also with a loxP site, and a Cre recombinase expressing plasmid, into bacteria harboring the BAC, an HSV-BAC plasmid is produced. The HSV-BAC plasmid is co-transfected into the packaging cell line with helper plasmids to produce infectious virions. We will use an *E. coli* based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA to introduce mutations and/or deletions into the PU.1 regulatory domains for functional characterization of the conserved elements we have identified. A luciferase/IRES/DsRed2 bicistronic element will replace the PU.1 coding domain for easier monitoring and visualization of expression controlled by the PU.1 regulatory domains.

114. Preferential activation of an interleukin-2 (IL-2) regulatory sequence transgene in TCR$\gamma\delta$ cells: Subset-specific differences in IL-2 regulation

Mary A. Yui

The IL-2 gene has stringent requirements for transcription, being expressed only in T-lineage cells and in response to specific developmental and activating signals. We previously derived and characterized a set of transgenic mice using a construct with 8.4 kb of regulatory sequence from the murine IL-2 gene, which drives consistent expression of a Green Fluorescent Protein (GFP) reporter gene in all cell types that normally express IL-2. Quantitative analysis of this expression shows that different T-cell subsets within the same animal show

divergent abilities to express the transgenes as compared to endogenous IL-2 genes. Peripheral TCRγδ cells show faster transgenic GFP RNA accumulation than endogenous IL-2 RNA accumulation upon stimulation, whereas TCRαβ cells express more IL-2 than GFP RNA. In TCRγδ cells, IL-2-producing cells are a subset of the GFP-expressing cells, whereas in TCRαβ cells, endogenous IL-2 is more likely to be expressed without GFP. TCRγδ cells also show much higher background expression of the transgene *in vivo* than TCRαβ cells. These results are seen in several independent transgenic lines and thus, reflect functional properties of the transgene sequences themselves, rather than copy number or integration site effects. The high ratio of transgene to endogenous IL-2 gene expression in TCRgd cells may be explained in part by the accelerated kinetics of endogenous IL-2 RNA degradation in TCRγδ cells. These results also suggest that additional subset-specific regulatory elements for IL-2 expression may map outside of the 8.4 kb regulatory region (see next abstract). High percentages of TCRγδcells and NKT cells express the transgene during intrathymic development, indicating that transgene expression in our IL-2-GFP transgenic mice may prove to be valuable tracers for immature cells of these lineages.

115. Biochemical and functional studies of distal regulatory sequences of the murine ***IL-2*** gene

Satoko Adachi

Transgenic experiments with the IL-2 regulatory sequences driving reporter genes have shown that the sequences that are optimal for transient expression are insufficient to promote efficient expression of transgenes. Critical additional sequences appear to be located between –2 kb and –8.4 kb with respect to the transcriptional start site, a region that also includes some DNase hypersensitive sites that are specific for T cells. We are currently exploring the regulatory functions that may be mapped to these sites, with particular interest in any protein-DNA interactions that may provide T cell-specific marking functions that make the IL-2 locus inducible.

To assess whether the additional 6.4-kb region works for chromatin remodeling or to provide an auxiliary enhancer function, we made stable transfectants of Jurkat cells by 2 kb and 8.4-kb IL-2 promoter GFP constructs and a deletion mutant of the 8.4-kb construct which lucks -5.0 to -8.4 kb (including predicted nuclear matrix attachment region). At least in a culture of multiple clones of transfectants, GFP expression level was same between these constructs. We made three new constructs for further study; they are the IL-2 minimal promoter (300 bp), and minimal promoter or 2 kb promoter with attached -7.8 to 8.0 kb region (the boundary of histone acetylation, and the acetylation of this region is greatly enhanced after stimulation with PMA and A23187). No difference was observed between these constructs when they were transfected transiently. In a culture of multiple clones of stable transfectants, the -7.8 kb to -8.0 kb region appeared to enhance GFP expression to the 300 bp minimal promoter while it decreased GFP expression to the 2 kb promoter. However, this result was not confirmed in single-clone transfectants. Thus the new functions responsible for efficient transgene expression give no consistent evidence of acting as conventional enhancers.

Histone acetylation is usually associated with open chromatin, thought to be induced by sequence-specific protein/DNA interactions. Therefore, chromatin immune precipitation assays mapping the sequences involved with acetylated histones are useful to detect sequences where novel regulatory proteins might have their target sites. Accordingly, most genes regulated by this mechanism have their expression increased by the histone deacetylase inhibitor Trichostatin A (TSA). However, several groups have reported that TSA actually decreases IL-2 gene expression in human T cells, instead of increasing it. This raised the question of whether the activity of the 8.4 kb IL-2 regulatory region is correlated with histone acetylation or with histone deacetylation. We tested the effect of TSA in EL4 cells and in the IL-2 promoter-GFP stable transfectants. TSA decreased the IL-2 mRNA level dose dependently (3.5-, 4.9-, and 6.1-fold decrease were observed when 10, 100, and 1000 nM TSA each were added). However, in the IL-2 promoter GFP stable transfectants, TSA did not change the GFP expression levels in either 2 kb or 8.4 kb regulatory region transfectants. Thus, any sequences that may mediate repression in response to histone acetylation are likely to reside outside the 8.4 kb regulatory region that contains the transgene expression function we have found, and histone acetylation assays are still helpful for identifying the basis of this function.

Previously we showed that around the -8.0 kb region seems to be a sharp transition point for histone acetylation of the mouse IL-2 gene. Sequences closer to the promoter than –8.0 kb were predominantly associated with acetylated H3 and H4 in T cells, but not in non-T cells, whereas sequences immediately upstream were associated with deacetylated histones in all cell types. With new real-time PCR quantitation, we have mapped a reproducible contour of histone acetylation across the region from -8.4 to -2 kb in EL4 cells. Histone acetylation of these regions was not observed in NIH3T3 cells. These results confirm that peak levels of H3 and H4 acetylation are associated with a region that is not conserved between murine and human IL-2, especially upon stimulation. The degree of histone acetylation in this region greatly exceeds the level in the well-studied proximal promoter/enhancer of the IL-2 gene, with or without stimulation. This region correlates also with the preferential sites of DNase hypersensitivity seen in resting and activated T cells. Sequences from this region also appear to form some complexes with nuclear proteins from EL4 T cells but not from 32Dcl5 pre-mast cells. We are now seeking to determine the nature and expression of the proteins responsible.

116. Thymocyte development in c-Fos- and FosB-deficient mice

Angela Weiss, Fei Chen, C. Chace Tydell, Ruben Bayon, Rochelle Diamond

The expression of Fos family proteins, c-Fos and FosB, which are components of AP-1 heterodimers, is subject to sharp regulation at two check points during thymocyte development. Both can be induced in $CD4^-$ $CD8^-$ double-negative (DN) and in mature $CD4^+$ and $CD8^+$ single-positive (SP) cells, but not in cortical $CD4^+/CD8^+$ cells. AP-1 activation is an essential aspect of all known effector responses of mature T-cells and is likely to be a component of the TCR and pre-TCR signaling cascades at most stages of development. C-Fos and FosB are therefore thought to play an important role in the activation events involved in thymocyte development and function.

As we described last year (Annual Report 2002), mutation of either the *c-fos* or *fosB* gene alone allows roughly normal T-cell development, but in early experiments with double-knockout mice we could show a gene dosage-dependent, age-dependent inhibition of T-cell development. The block appeared in an unexpected stage, with an apparent failure to enter the $CD44^+CD25^+$ (DN2) stage. Cells with the $CD25^+CD44^-$ or $CD25^-CD44^-$ phenotypes (DN3 and DN4, respectively) were greatly reduced, while the more primitive DN2 cells were spared and the most primitive CD25-CD44+ cells were dramatically enriched to 70-85%. In addition, the total number of thymocytes was decreased by 100 fold. This was surprising because the DN1 to DN2 transition is not known to be associated with a specific requirement for Fos/AP-1 activating signaling cascades. The double-knockout mice are badly stunted in growth and toothless, and poor fecundity made it difficult to obtain enough individual pups to perform detailed characterization of the arrested cells. However, the thymocyte populations thought to affected by stress artifacts are only the later, $CD4^+$ $CD8^+$ ("double-positive") cells, and their vulnerability to stress appears only as a result of β-selection. Thus, the novel phenotype of the *c-fos*$^{-/-}$ *fosB*$^{-/-}$ thymocytes seemed to indicate that Fos function was required at a new, early checkpoint involving the survival of DN2 cells or their transition to the DN3 stage.

Since both transcription factors are expressed in thymocytes as well as in thymic stromal cells, the question arose whether the loss of these transcription factors is more important in one or the other of these populations. To answer this question we created bone marrow chimeras and did thymus transplantations under the kidney capsule. During the course of these experiments we found that the thymus size in the double-knock out donor mice used in these experiments was nearly normal (two-fold decrease in cell number compared to wild-type mice) compared to 100-fold smaller thymuses seen in previous experiments. Most dramatically, the abnormalities that had been seen previously in subset composition of the thymus were no longer detectable in most of the mutant mice. Since genotyping confirmed that the investigated animals were indeed *c-fos*, *FosB* double-knockout mice we looked at other explanations for this dramatic change in phenotype. At the same time, with extended breeding and improved husbandry, the animals stayed healthier and survived longer than before. One possible explanation we are currently investigating is whether stress-triggered glucocorticoid exposure can act as a co-factor with Fos deficiency to cause arrest at the DN2 stage. However, under optimal conditions, it now appears that T-cell development can proceed essentially normally in spite of a profound loss of AP-1 function.

117. β-selection checkpoint breakthrough in immunodeficient strains of NOD mice

Mary A. Yui

Non-obese diabetic (NOD) mice exhibit many defects in T-cell functions, which have been postulated to contribute to diabetes susceptibility in this strain. NOD mice with the *scid* mutation and *Rag1*-deficiency were used for an analysis of the earliest stages of T-cell development in the NOD genetic background. These mutations block normal T-cell receptor (TCRβ) rearrangements and cause a developmental arrest in the double-negative $CD4^-CD8^-$ stage at the β-selection checkpoint in the thymus. Both strains were found to have T-cell development typical of all immunodeficient strains until 5-8 weeks of age when $CD4^+$ and $CD4^+CD8^+$ double-positive cells appear, spontaneously breaking through the β-selection checkpoint. These breakthrough cells do not result from a spontaneous TCR-β rearrangement. Although they differentiate and undergo some proliferation, the breakthrough cells fail to restore thymic cellularity. The breakthrough cells were found to undergo some of the major changes that occur at β-selection. Unlike normal developing T cells, the NOD breakthrough cells do not completely downregulate transcription and surface expression of the stem cell factor (SCF) receptor, c-kit (CD117). However, these cells do downregulate Bcl2 and upregulate $BclX_L$ as do normal cells after undergoing normal β-selection. The breakthrough cells fail to downregulate HEB-alt and Spi-B, two genes normally shut off in β-selection, but they do upregulate the bHLH inhibitor Id3 like normal thymocytes undergoing β-selection. Thus, the *scid* mutation and *Rag* deficiency reveal a unique NOD genetic defect in checkpoint control wherein the cells exhibit some, but not all, of the normal outcomes of pre-TCR signaling. This developmental defect observed in immunodeficient NOD mice may also affect the T cell responsiveness of wild-type NOD mice. Furthermore, these breakthrough cells may include precursors to the thymomas that develop at very high frequency in both NOD-*scid* and –*Rag1*^{null} mice.

Current studies are focusing on identifying the molecular mechanisms of the checkpoint failure and determining whether the defect is T cell intrinsic or due to interactions between T cells and thymic stroma, using bone marrow chimeras and *in vitro* culture assays.

118. Genetic analysis of the NOD-***scid/scid*** β-selection checkpoint breakthrough

*Mary Yui, Rachel Cota**

Type 1 diabetes in the NOD mouse is a genetically and phenotypically complex disease, mediated by autoreactive T cells. At least 18 genes contributing to disease have been mapped in genetic crosses with this strain. We have identified a defect in T-cell development and checkpoint control in NOD- *scid/scid* and NOD-*Rag*$^{-/-}$ mice, which may have an impact on T-cell development and responsiveness in NOD mice (see previous abstract). This defect appears to be the source of a high incidence of thymomas in the immunodeficient strains of NOD mice and such a T-cell developmental defect may also contribute to diabetes in that strain. To map this trait genetically and determine whether this defect maps to one of the diabetogenic loci, some time ago we initiated a genetic cross (NOD-*scid/scid* X B6-*scid/scid*) and found, as reported previously, that the NOD trait is recessive as F1 thymocytes arrest normally at the β-selection checkpoint. After backcrossing the F1 to NOD-*scid/scid* mice and phenotyping 58 backcross mice we found that approximately half of these mice had some significant breakthrough to ISP or DP at 6.5-8 weeks of age. We are continuing to use microsatellite marker (simple sequence repeat polymorphism)-based PCR to compare genotypes for markers that are closely linked to the most important NOD diabetogenic loci to the phenotypes of the 58 backcross mice. So far, 15 microsatellite markers on seven different chromosomes have been typed for 48 of the backcross mice. Based on single-locus analyses, a few weak correlations between mapping and phenotypic data have been detected requiring additional linked markers and individuals to be typed. Ultimately, a multi-locus analysis will be performed when a more complete data set is available.

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119. Transcriptional regulators as probes for the phylogeny of lymphocyte development

Rashmi Pant, Michele K. Anderson

T and B lymphocytes are an evolutionary innovation within the chordate lineage. The emergence of lymphocytes has been placed early in vertebrate evolution on the basis of recognition structures, cellular morphology, and tissue architecture. No lymphocyte-like cells have been described outside the vertebrates. The jawless vertebrates, such as lampreys and hagfish, have cells in several different tissues that morphologically resemble lymphocytes but apparently lack rearranging antigen receptor genes. Furthermore, they lack any recognizable spleen or thymus, which are major lymphoid organs in all jawed vertebrates. In sharp contrast, even the most basally divergent group of jawed vertebrates, the cartilaginous fish, are fully armed with the capability for mammalian-like antigen recognition and mammalian-like spleen and thymus. We are seeking to understand the functions that allowed the unique lymphocyte developmental program to emerge.

Transcription factor genes can be used as powerful probes for evolutionary relationships over long times. In mammals, knockout studies and analysis of expression patterns have defined essential roles for many different transcription factors in lymphocyte development. For example, GATA-3, Pax-5 and EBF are needed for hematopoietic cells to take a T- or B-lymphoid pathway. GATA-3 is essential for T-cell development from the earliest stages. B-cell development requires a transcriptional cascade involving the transcription factors E2A, EBF-1, and Pax-5, in which the requirements for EBF-1 and Pax-5 are unique to B cells. Other factors, such as Runx, PU.1, and Ikaros family members, also contribute critical functions in mammalian T-or B-cell development, though they also have shared roles in other hematopoietic lineages.

The question we have addressed first is: How much of the mammalian pattern of transcription factor usage is conserved throughout all forms of true lymphocyte development? We have tested this by examining the skate, a representative of the cartilaginous fish, which is the most deeply divergent group from mammals that still possesses lymphocytes. We have identified homologs of transcription factors with roles in mammalian lymphopoiesis, and conducted an integrated analysis of the expression patterns of those factors in skate embryos and adults. The results demonstrate that major components of the T-cell and B-cell gene regulatory networks observed in mammals are ancestral to the jawed vertebrates.

Additional work is now underway to describe and identify key lymphoid/pre-lymphoid transcription factor genes in lampreys. So far, we have recovered one PU.1, one GATA and two Ikaros like family members from lamprey. These genes show conserved DNA binding domains, but divergent protein-protein interaction domains. Furthermore, in initial surveys, the expression patterns of these factors show signs that they are not used as coordinately as they are in skate or mammal. This suggests that both the coding and regulatory regions underwent modifications during recruitment to the lymphocyte lineage. Additional work is underway to identify and describe genes from lamprey and other basal taxa, to further define the origins and eventual diversification of these transcriptional networks.

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Summary: A large fraction of our laboratory is devoted to working as part of the Alliance for Cellular Signaling. This is a consortium that is devoted to understanding the mechanisms involved in information processing by mammalian cellular systems. We are primarily focused on understanding the pathways that transduce information from the environment into intracellular metabolic changes. Thus, there is a great focus on the response of cells to specific ligands. Our laboratory represents the molecular biology effort of the Alliance and in the last three years we have focused primarily on developing the tools necessary for probing, in a high throughput fashion, the complex responses to stimulation of cells by single and multiple ligands in a variety of combinatorial protocols. To do this, we have established a collaboration with other groups both academic and commercial including Myriad Genetics and Isis Pharmaceuticals. Together with Myriad Genetics we have instituted a program of yeast two-hybrid screening of genes that are primarily involved in signaling. Myriad technology is used in an attempt to understand the kinds of interactions that are possible between subdomains of signaling proteins and other proteins that are involved in generating intracellular signaling circuits. With Isis Pharmaceuticals we have developed a series of antisense probes that can be used to eliminate translation of specific gene products to test their involvement in signaling pathways. In addition, we have spent a good part of the last year developing methods to introduce RNAi into mammalian cells. We have successfully developed a variety of targeting systems and now have available appropriate double-stranded oligonucleotide sequences for some of the pathways that the Alliance is interested in interrogating. We have also continued to clone and generate expression vectors and fusion-tagged vectors for expressing specific signaling gene products and fluorescent tagging them for cellular localization. We have developed methods, together with the cell laboratory group at Stanford, to study protein translocation in response to ligand stimulation using microscopy and single-cell assays. Many of the plasmids that we have generated and the systems for moving genes around, as well as the sequence verified mouse full-length clones that we have built, are available through the American Type Culture Collection. Together with the informatics group in San Diego, we have developed a database that makes these vectors not only accessible but also annotates them so that they can be readily used by groups around the country. Finally, we have continued to develop our ability to do transcript analysis using specific DNA microarrays. Together with Agilent Technologies we have developed a cDNA chip using the RIKEN cDNA collection. The chip has 16,000 elements and we have been using it to probe the responses of cells to stimulation by specific ligands. We have completed a series of experiments testing 32 different ligands against primary mouse B cells and the data that resulted from this >500 microarray chip analysis is currently available as is all of our other data and protocols on the Alliance for Cellular Signaling website (www.afcs.org). We are continuing to develop these tools and others. In the next year we will be applying them not only to the B-cell system but also to RAW macrophages. Together with the other groups in the Alliance, we hope that by the end of this coming year we will get a comprehensive picture of the signaling properties of the RAW cells and that we will be able to generate the circuitry that is involved in many of the signaling pathways, particularly the interaction between signaling circuits that is required in order to maintain the integrity of the information processing system.

In addition to the Alliance for Cellular Signaling, our laboratory continues to study both the G-protein signaling systems in mammalian cells and on bacterial chemotaxis. With respect to bacterial chemotaxis we have focused primarily on the biochemistry of phosphate transfer. We are trying to understand the molecular complex that is the primary chemotaxis intracellular signaling complex. It includes the intracellular portion of the cell-surface receptor, the CheA signal-generating molecule, the CheY second messenger, the CheW controlling molecule and the CheB feedback element. Our work has focused on the dynamics of the CheA protein and its interaction with some of these other components. We are using a variety of techniques including x-ray crystal structure analysis, spin labeling, and EPR spectra analysis. We use mutagenesis and biochemistry to understand the events, intramolecular and intermolecular, that drive the dynamics necessary for generating and suppressing signaling in this system.

We are also continuing to study the function and role of Gα12 and Gα13 during mouse development. In addition, we are applying the techniques of RNAi to specific systems involved in signaling in cells in the

hematopoetic system in order to resolve the nature of the requirement for different combinations of G proteins in the specificity of the response to ligands and receptors. The hypothesis is that a specific combination of beta and gamma subunits or a specific combination of alpha, beta, and gamma subunits is required to generate a response efficiently for different subsets of receptors. This can now be tested in a very definitive fashion by using RNAi to eliminate specific genes. This approach is being applied both by using bone marrow transplantation and by studies in specific cells in tissue culture. We are also continuing to try to understand signaling in the nervous system and our collaboration with David Anderson is ongoing. We have made great progress in understanding the roles both of the intermediate signaling and of the Mrg pathway. These Mrg receptors are G-protein coupled receptors and a subset of them are involved in the signaling process in neurons that innervate the skin from the dorsal root ganglion. In our collaboration with David Anderson we have begun to define some of the ligands that bind to these receptors and the nature of the components involved in the signaling pathway, as well as the phenotypes of mutants that are deficient in these GPCRs.

Finally, our work in the visual system has focused primarily on defining the nature of the specific genes that are involved in generating the differentiated function of different subsets of neurons in the retinal ganglion layer. This work is still in an early phase and we are beginning to have a clearer picture of the markers that can be used to identify specific subsets of ganglion cells.

120. Molecular biology laboratory of the Alliance for Cellular Signaling: RNAi projects

Iain D.C. Fraser, Joelle R. Zavzavadjian, Jamie Liu, Pam Eversole-Cire, Dan Allen, Mei Wang, Melvin I. Simon

Much of the past year has been spent developing techniques to apply RNA interference to the WEHI231 B cell line. In preliminary studies, we found that chemically synthesized siRNA duplexes could not be introduced into WEHIs at a sufficiently high efficiency to mediate significant knockdown of target genes. We therefore developed vector-based RNAi technology with the goal of introducing interfering RNA into WEHIs using viruses. We chose to take advantage of the gateway-cloning system to allow us to test and validate siRNA hairpin sequences in small, easily manipulated vectors, and then to transfer validated siRNA cassettes into a variety of viral vectors by site-specific recombination. Our approach was to design four siRNAs targeted against different regions of each target gene, and to test the efficacy of these hairpins against a co-expressed YFP-tagged expression construct. Using this method we have screened hairpins for approximately 25 genes, and identified effective reagents for 20. Most of our hairpin constructs use the promoter for the small RNase P, H1, but we have also made vectors that express hairpins from the promoter for the small nuclear RNA, U6. We have developed both retroviral and lentiviral vectors for siRNA expression, which permit co-expression of several combinations of selection markers and reporters. WEHI cells were infected with lentivirus carrying siRNAs directed against either G alpha i2, Syk or CXCR5. Co-expression of GFP and puromicin resistance allowed the selection of stable cell populations, and we confirmed integration of the siRNA cassette into the WEHI line in each case by genomic PCR and sequencing. Despite this, we were unable to knockdown any of the target genes mentioned above. Parallel experiments have shown the same G alpha i2 lentivirus to be highly effective in reducing G alpha i2 protein levels in J774A.1 macrophages and NIH 3T3 fibroblasts. These results led us to question the applicability of RNAi technology to the WEHI cell line. To compare the RNAi capacity of WEHIs to other cell lines, we used a reporter assay involving co-expression of a lacZ reporter with a potent anti-lacZ siRNA driven by either an H1 or U6 promoter. We have observed efficient levels of lacZ knockdown with HEK293, RAW264.7, N1E-115, 3T3-L1 and IC-21 cells. We observed no lacZ knockdown whatsoever with WEHI cells using either H1 or U6 promoter-driven siRNA. We expect the approach and vector systems we have developed for RNAi to be very efficient for the application of this technology to a tractable cell system. These will now be applied to the new model cell for the AfCS consortium, the RAW264.7 mouse macrophage cell line.

121. Molecular biology laboratory of the Alliance for Cellular Signaling: Protein-protein interaction projects

Iain D.C. Fraser, Joelle R. Zavzavadjian, Sam Couture, Melvin I. Simon

In collaboration with Myriad Genetics, the AfCS yeast two-hybrid screens have been generating data since April, 2002. Of 163 bait proteins submitted, 55 have produced data from screens of B cell and cardiac myocyte-specific libraries. All 472 reported interactions are available to the research community on our website: (http://www.signaling-gateway.org/data/Y2H/cgi-bin/y2h.cgi), and an automatic data transfer system is in place to ensure immediate release of data as we receive it from Myriad. We have published a brief communication on the yeast two-hybrid project in the reports section of the AfCS website (http://www.signaling-gateway.org/reports/v1/DA0002/DA0002.pdf). Of the interaction data reported, some of the "hits" are verified in the literature while others are novel interactions, suggesting involvement in known signaling pathways. The adapter protein Ruk 1 is a good case in point. Earlier AfCS screens have pulled the Ruk1 protein (a.k.a. CIN85) out as a bait with PLC gamma 2, BLNK and Grb2. The literature suggests that Ruk 1 is an adapter protein that regulates PI3 kinase activity, and has been shown to influence apoptotic pathways in neuronal cells. A recent paper [*J. Biol. Chem.* (2002) 277(42):39666-39672] shows that Ruk1/CIN85 binds to the tyrosine kinase Cbl-b. This ties in nicely with our yeast-two-hybrid data as we have pulled out Cbl-b as a prey with Grb2 (which also binds Ruk1-see above). These multiple protein-protein interactions involving Ruk1

provide clues to an expanded protein interaction network.

As reported last year, we have also been involved in the development of retroviral vectors for use in immunoprecipitation protein-protein interaction studies in the AfCS Cell and Protein labs at UT Southwestern. Further constructs have been prepared this year in an effort to optimize the combination of promoter, selection method and affinity purification tag to facilitate the identification of protein complexes by co-IP and mass spectrometry. These vectors systems are now being now be applied to the new model cell for the AfCS consortium, the RAW264.7 mouse macrophage cell line.

122. Molecular biology laboratory of the Alliance for Cellular Signaling: cDNA cloning projects

Joelle R. Zavzavadjian, Sam Couture, Kavitha Dhandapani, Jamie Liu, Melvin I. Simon, Iain D.C. Fraser

We have continued to clone full-length mouse cDNAs for expression of proteins in a variety of experimental contexts. The gateway system greatly facilitates the movement of cloned genes into multiple expression systems. We find a mixture of brain and testis cDNA provides a good general source material for PCR amplification of signaling genes, while spleen cDNA is better for hematopoietic-specific targets. Amplification of two forms of each target gene (with and without a termination codon) allows for N- and C-terminal tagging for expression studies, and also allows us to differentiate between PCR-introduced sequence mutations and Genbank discrepancies (which are very common). We have now cloned almost 600 fully verified sequences into gateway entry vectors, and have built over 100 gateway-ready expression vector backbones, ranging from fluorescent protein-tagging vectors to viral vectors for co-immunoprecipitation studies and RNAi. From these cloned genes and parent vectors we have generated approximately 1500 expression constructs. The distribution of these plasmids to the ATCC is now underway and they are expected to be available to the research community by July/August. Many of the constructs expressing fluorescently-tagged proteins have been used for microscopy analysis at Stanford, and a brief communication on the utility of a set of subcellular marker constructs has been published (http://www.signaling-gateway.org/reports/v1/DA0002/DA0002.pdf). In collaboration with the AfCS Bioinformatics and Microscopy labs, we have developed a sophisticated plasmid database (http://www.signaling-gateway.org/data/plasmid/plasmid.html) that barcodes each plasmid construct and records details such as location of features, diagnostic restriction digests and complete construct sequence. We have also implemented a local batch BLAST server at Caltech that allows us to screen new gene sets against our current inventory. This has proven extremely useful in preventing redundancy between overlapping cloning projects.

123. The activities of transcription analysis group in Caltech as a part of the Alliance for Cellular Signaling

Sangdun Choi, Rebecca Hart, Mi Sook Chang, Sun Young Lee, Jong-Woo Kim, Yun Anna Cao, Xiaocui Zhu, Melvin I. Simon

As a part of the Alliance for Cellular Signaling (http://www.signaling-gateway.org/), the transcription analysis group at Caltech performed several projects. First, transcript analysis of mouse B cell single-ligand screen was completed using cDNA microarrays. B cells were treated with 32 different ligands at four different time points (30 min, 1 h, 2 h, and 4 h) and tested for gene expression using Caltech's 16K cDNA microarrays. Analysis of these arrays revealed some common and differentiating regulatory patterns were found among AIG, IL4, CD40, LPS, CpG, and ligands that are known to activate B cells. The influence of these ligands on distinct patterns observed with the activating ligands should be informative. Double-ligand screens were also performed with a limited subset to find the relationships between the ligands and the data is being analyzed.

The level of gene expression changes in Bcl-$2^{+/TGN}$ B cells were followed using 16K cDNA microarrays over three days (day 0, 1, and 3) with or without AIG. The data analysis between control groups revealed no major gene expression changes among Bcl-$2^{+/TGN}$ B cells cultured for various days, indicating that these cells are relatively stable during culture. In addition, the gene expression pattern of Bcl-$2^{+/TGN}$ B cells treated with AIG for two hours after different days (day 0, 1, and 3) of incubation showed that a robust subset of genes was coordinately regulated by AIG treatment, independent of the culture period. This suggests that these cells can be used for long-term studies of ligand effects.

To generate part lists of mouse macrophage and gene expression profiles for various cell lines, Affymetrix GeneChip experiments were performed with BMDM (bone marrow-derived macrophage), RAW 264.7, J774A.1, AtT-20 and B cells. From these data, chemotaxis and other signaling maps were created using the GenMAPP program to visualize gene expression data on maps representing biological pathways and groupings of genes. Receptors expressed in RAW and J774 were also examined to predict an initial list of signaling molecules to aid in the identification of potentially useful ligands.

To evaluate the effects of RNAi and its delivery systems, the expression pattern of J774 cells infected with lentivirus containing potent Gi2 or Gi3 siRNA was examined using Caltech 16K oligo and cDNA arrays (with Dr. J.I. Hwang). The data analysis reveals that siRNA is specific for targeted gene knockdown but the regulation of transcripts related to the genes that process siRNA-mediated gene knockdown remains to be addressed.

124. Current issues of DNA microarrays: Platform comparison, double-linear amplification, and universal standard

Sangdun Choi, Yun Anna Cao, Sun Young Lee, Jong-Woo Kim, Mi Sook Chang, Melvin I. Simon

DNA microarray technology has been widely used to simultaneously determine the expression levels of thousands of genes. A variety of approaches have been taken to apply DNA microarray technology to the measurement of transcript levels in cells. However, the existence of alternative array platforms, which may differ in probe preparation methods and array surface chemistry, raises the question of cross-platform agreement in gene expression measurements. In this study, we present reliable and sensitive comparisons of several microarray platforms and methods used for DNA microarray target preparation. We included inkjet-deposited arrays of pre-synthesized cDNAs or oligonucleotides, as well as Affymetrix GeneChips in our comparison, measuring transcript differences in mouse spleen and liver total RNA. The high correlation of the QRT-PCR results with the Affymetrix GeneChip, cDNA and Operon oligo microarray results demonstrated the reliability of the three arrays. We have also developed and tested a double-amplification method which allows the use of small amounts of starting material. The second added round of amplification produced highly reproducible result as compare to the arrays hybridized with single-round amplified target. Our result from the double-amplification data suggests that this method can be used reliably for future microarray experiments, especially when the amount of total RNA is scarce. Currently, a consensus has not been reached regarding the type of RNA reference sample that is best for two-color microarray experiments. To see if a standard for microarray RNA reference can be applied to research practice, a universal RNA reference for microarrays was tested. The results obtained from using a universal RNA reference suggested that multiple comparisons of experimental conditions by using the same control could be accurate and could enhance sharing of array information within the research communities.

125. Inhibition of gene expression using antisense oligonucleotides

Pamela Eversole-Cire, Mei Wang, Iain Fraser

One of the four major areas of research to be conducted in the Alliance for Cellular Signaling Molecular Biology Laboratory directed by Melvin I. Simon, Ph.D., is the nucleic acid-based inhibition of gene expression project. Antisense, as well as RNA interference approaches, are used to selectively inhibit the expression of genes in a macrophage-like cell line (RAW 264.7) in order to elucidate various signaling pathways in a spatial and temporal context. Experiments are being performed to test the effectiveness of antisense reagents received from Isis Pharmaceuticals in inhibiting specific gene expression in RAW 264.7 cells. Target mRNAs are chosen based on transcript profile data from cDNA-based microarray analysis of RAW 264.7 cells. Antisense oligos targeting the mRNA sequences of expressed genes such as PTEN and Galpha i2 were delivered to RAW 264.7 cells using various lipid-based delivery methods. Various concentrations of either a control antisense oligo or gene-specific oligo (0.05, 0.2, and 0.4 μM) were used to determine if the antisense oligo was effective in knocking down the expression of the target proteins in RAW 264.7 cells. Gene inhibition was assessed using RT-PCR and protein immunoblot analysis. Results indicate that inefficient delivery of the antisense oligos into RAW 264.7 cells may be responsible for the moderate knockdown in protein expression observed for some of the target genes. Therefore, co-transfection with a CD4 selectable marker has been performed and has yielded promising results. Selected CD4+ positive cells appear to have an increased level of knockdown of the targeted genes. Attempts are currently being made to optimize transfection efficiencies so that a significant population of cells with protein knockdown can be obtained to perform functional assays in order to assess the effect of inhibiting the expression of various genes in known signaling pathways.

126. Investigation of protein dynamics in ***Thermotoga Maritima*** CheA

Bryan Beel

CheA is the histidine kinase controlling phosphotransfer in the chemotaxis systems of many species of prokaryotes. CheA's kinase activity is controlled by the inputs from a set of homologous, membrane-bound MCPs, or methyl-accepting chemotaxis proteins. The kinase activity of CheA is controlled such that it can vary 500-fold or more in response to activating and inactivating input signals. It was previously observed for *E. coli* CheA, that two inactive mutant CheA homodimers could regain activity by exchanging partners and forming two functional heterodimers. We are investigating this phenomenon in *Thermotoga maritima* CheA. We have observed the exchange of monomers between dimers of CheA at high temperature, but not at low temperature. Using spin-labeled CheA proteins and EPR spectroscopy, we have begun to characterize the requirements for and determinants of monomer exchange and the topology of the chemotaxis-signaling complex. The data suggest the involvement of an interaction surface between the dimerization domain of CheA and either its own domain P5 or CheW. The EPR spectra of spin labels placed in the dimerization domain clearly show signs of exchange and conformational changes at high temperature; the presence of the CheW coupling protein and MCP fragments affect these changes. Finally, the spectra of "buried" dimerization-domain residues show anomalously high mobilities during the exchange of CheA monomers.

127. Histidine phosphorylation in bacterial chemotaxis

Cindy Quezada, Alexandrine Bilwes[1], Brian Crane[1], Cristan Gradinaru[1],Damon Hamel[2], Frederick Dahlquist[2], Melvin I. Simon

Bacterial chemotaxis, the directed movement of bacteria in a chemical environment, represents one of the best biochemically and structurally characterized signal transduction pathways. The histidine kinase CheA is a central player in this two-component regulatory system. Its active site is spread across two domains: the histidine phosphotransfer domain (P1) and the kinase domain (P4). Our efforts focus on elucidating the mechanistic contribution of P1 residues to the autophosphorylation reaction.

An atomic resolution structure (0.98 Å) of the *Thermotoga maritima* CheA histidine phosphotransfer domain was obtained, affording a unique opportunity to view the environment surrounding His45, the phosphoaccepting histidine, in detail. His45 participates in a hydrogen bonding network including three other residues (Glu67, Lys48, and His64), which are conserved in CheA. Employing a combination of site-directed mutagenesis studies, protein crystallography, and 2 D heteronuclear NMR techniques, we explored the functional roles of these residues involved in the largely conserved hydrogen bonding network.

Our experiments revealed that the P1 domain provides the nucleophile for phosphate transfer (His45) and the activating glutamate (Glu67) completing a catalytic center observed in the GHL family of ATPases. Glu67 tunes the reactivity of His45 through a hydrogen bond. This interaction activates His45 to the normally unfavored $N^{-1}H$ tautomeric state. As a result, His45 possesses an altered pKa. Upon mutation of Glu67 to a Gln, the chemical properties of His45 change. When existing in the predominantly $N^{-2}H$ tautomeric state, its pKa is similar to that of a solvent exposed histidine and its phosphorylation is dramatically reduced *in vitro* and *in vivo*.

Hence, the phosphoaccepting histidine must exist in the normally unfavored $N^{-1}H$ tautomeric state in order for CheA autophosphorylation to occur. The other two residues, Lys48 and His64, do not affect the reactivity of His45. Instead they contribute towards the structural integrity of the P1 active site. The results obtained in this thesis provide a solid structural and biochemical basis for further understanding the CheA phosphotransfer mechanism and may provide critical insight for the development of novel antibiotic agents.

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128. Lack of G alpha(13) changes cell destination during mouse development

Lingjie Gu, Yosuke Mukouyama, Shirley Pease, David J. Anderson, Melvin I. Simon

Lack of G alpha(13) leads to degenerative vascular structure and embryonic lethality around day ten of mouse development. In an effort to examine the role of G alpha(13) in vascular development, we first examined vessel formation during different stages of embryo development. Staining of embryos around E8.25, when the endothelial cells start to form the primitive vascular structures including dorsal aorta, revealed that G alpha(13)-/- embryos form at least the same amount of PECAM-1 positive vessels as the wild-type littermates. At E9.25, however, G alpha(13)-/- embryos showed little, if any staining of vascular smooth muscle actin around dorsa aorta compared with a clear positive staining in the wild-type littermates. Further, in an *in vitro* system where we culture embryonic stem (ES) cells from inner cell mass and then differentiate them into endothelial cells and smooth muscle cells, ES cells from G alpha(13)-/- mouse showed a significant increase in the percentage of cells differentiated into PECAM-1 positive cells compared to ES cells from the wild type. This difference is also observed in pooled wild-type and mutant embryos at E9.25. Activated G alpha(13) has recently been shown to replace beta-catenin binding to E-cadherin, leading to increased beta-catenin nuclear translocation, as well as decreased cell adhesion (1,2). A comparison of beta-catenin localization showed increased cytoplasmic localization and decreased nuclear localization in the G alpha(13)-/- ES cells versus the wild-type cells, indicating that beta-catenin may be the effector of G alpha(13) in changing the cell fate. A possible Wnt-pathway connection is being investigated.

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129. Isotype-specific roles of Gβ subunits in chemokine-dependent cellular responses

Jong-Ik Hwang, Iain D.C. Fraser, Xiao-Feng Qin, Melvin I. Simon

Heterotrimeric G proteins are membrane-attached signaling components that mediate cellular responses to a variety of extracellular stimuli. They undergo a cycle of guanine nucleotide exchange and GTP hydrolysis, while they dissociate into Gα and Gβγ subunits that separately activate intracellular effector molecules. The specific roles of Gα subunits in these processes have been largely established. On the other hand, the β and γ subunits composed of 5 and 12 isotypes each was thought to be less diverse and less specific in signal transduction. Recently the subunits have been understood as signal transduction molecules in their own and they function in the regulation of various intracellular molecules, including ion channels,

adenylate cyclases, phospholipase C, and PI3 kinase.

The Gβ subunits are made up of N-terminal α-helix structure and seven WD repeat regions that are involved in interaction with Gα, Gγ subunits and other effectors. In spite of high homology for each other, different expression patterns of individual Gβ subunits are found within an organism and specific interaction with Gγ subunits suggests subtype-unique roles in signal transduction. Based on this hypothesis, we have developed lentivirus-based siRNA for each Gβ subunit to elucidate subtype-specific roles in the differential coupling of G proteins to various GPCRs. First, we applied them to J774A.1, mouse monocytic cell line, which expresses Gβ1,2, and four subtypes, then established cell lines lacking each subtype. The cells express C5aR, a complement receptor, and migrate through a gradient of C5a concentration in a pertussis toxin-sensitive manner. However, Gβ2 KO cells lost the ability to migrate toward C5a. When the human Gβ2 gene was introduced it was expressed because it does not have target sequences found in mouse Gβ2 siRNA, then a migration defect was recovered. These results indicate that Gβ2 plays a specific role in chemokine-dependent migration and suggest that each Gβ subunit may mediate different cellular responses to extracellular stimuli.

130. The physiological role of G-protein signaling in nociception

Sang-Kyou Han, Melvin I. Simon

Pain is the most common reason individuals seek medical care and more than a third of the world's population suffers from a chronic pain condition at some point during life. Little is known about the cellular and molecular mechanisms by which heat, cold and force are initially transduced into receptor potentials at the sensory nerve terminal. G-protein signaling is known to be implicated in vertebrate and invertebrate sensory function such as vision, olfaction, chemosensation or nociception. First, we will investigate how the signaling of Gq family members is implicated in pain perception or development of sensory neurons. Our results demonstrate that the main mode of signaling of Mrg receptors is through the Gq/11 signaling pathway in heterologous cellular systems. Our expression analysis showed that Gq, G11 and G14 but not G15 are expressed in DRG neuron. Importantly, we found that G14 is expressed in specific subpopulation of DRG neurons, is co-expressed with MrgA1+ neurons in neonatal mice. This suggests that G14 has a specific role in cutaneous nociceptive neurons. However, receptors activating Gq family members do not seem to discriminate between Gq isoforms, although there is evidence that Gq, but not G11, has functional specificity in central nervous system. To avoid such functional redundancy as much as possible, we will perform pain behavior test for Gq-/-G14-/- or G11-/-G14-/- littermates. We will determine if Gq class signaling can modulate specific pain modality in PNS. Also, we will further examine to see if the resultant phenotype originates from G-protein signaling defects in sensory neurons because Gq may participate in regulating pain sensation at the level of CNS rather than PNS. Next, we will investigate whether specificity exists in signaling through selective coupling to the down stream effector, PLC. Gi/o coupled receptors also can activate PLC through βγ or G14. Thus, we will further analyze significance of PLC-Ca2+/PKC signaling in the pain responses by generating Gq class KO plus PLC KO mice through different combinatorial crossing.

131. Pharmacology of mrg receptors and their functional consequences in sensory neurons

Sang-Kyou Han, Melvin I. Simon

In mice, mrgA1 appears to be co-expressed with mrgC11 or other mrgAs, while mrgD is partially co-expressed with mrgA1 at the neonatal stage. In rat, mrgA and mrgD are co-expressed. Thus, individual neurons typically express more than one mrg gene, raising the question of whether multiple mrgs in the same neuron are redundant or whether they interact for greater functional diversity. Moreover, our functional assays showed that mrg A1, A4, MrgC11, and MAS1 are distinctively activated by Rfamide-related peptides. Interestingly, each mrg receptor was activated with a different spectrum of specificity plus promiscuity against RFamide-related peptides. This suggests that the direct physical association between mrgs is likely to regulate ligand specificity or G-protein coupling, thus adding a novel and unexpected layer of complexity. Otherwise, the diversity of Mrg receptors might reflect different affinities for a single or small number of ligands, rather than different specificities that discriminate amongst a large number of mrgs.

First, we are investigating whether mrgs can be associated as dimers, both as homodimers or heterodimers, with other members of mrgs using coimmunoprecipitation method. Also, we are investigating whether ligand-induced or constitutive dimerization occurs using fluorescence energy transfer approaches and whether dimerization can alter the functional properties of the receptor such as ligand-binding affinity, signaling, and agonist-induced regulation. If we find alteration of ligand specificity and/or G-protein coupling specificity in heterologous cellular systems, we will further examine the resultant functional consequences on both DRG neurons and heterologous cellular system by a combination of calcium imaging and electrophysiology. Alternatively, differential pharmacology and/or responses may result from interactions between mrg receptors and other interacting molecules. Or, the accessory molecules may be required for providing necessary subcellular targeting or modifying the conformation of the receptor to ensure proper transport and stability of a given receptor.

To identify mrg interacting molecules, we will pull-down using mrgA1- or mrgD-specific antibody from DRG extract derived from wild-type and mutant mice. The resultant precipitates will be analyzed in one or 2D gel electrophoresis and then specific bands will be identified using tandem mass analysis.

132. Identification of retinal ganglion cell subset-specific genes

Kum-Joo Shin, Melvin I. Simon

Retinal ganglion cells (RGCs) are the output neurons that transmit visual information to the brain. Intensive anatomical and physiological studies have shown that there are many subsets of RGCs that have distinct functions and morphologies, with underlying molecular mechanisms unknown. Therefore, we are searching for specific genes expressed in subsets of RGCs. RGCs were isolated using antibody for thy-1 (reported as a marker for these cells) and magnetic-associated cell sorting (MACS) from embryonic and postnatal mice. We are identifying specific genes using subtractive hybridization and microarray analysis. For the candidate genes, we will confirm their expression in subsets of RGCs with *in situ* hybridization.

133. Characterizing pheromone-induced cell polarization in yeast

Tau-Mu Yi[1,2], Kiroaki Kitano[2], Melvin I. Simon

The movement (taxis) or growth (tropism) in the direction of some chemical signal is fundamental to all living organisms. Budding yeast cells respond to pheromone gradients by executing polarized growth in the direction of the pheromone source (e.g., mating partner). This process involves both heterotrimeric and small G-protein signaling. We are interested in achieving a quantitative understand of this system through a combination of experiments and modeling.

In previous work, we quantitatively characterized the yeast heterotrimeric G-protein cycle using FRET to monitor *in vivo* G-protein activation (1). The goal of the current project is to construct a rough-draft model of pheromone-induced cell polarization in yeast that describes quantitatively the pathway from the production of free $G\beta\gamma$ to the formation of the *shmoo*. The data for the model has been collected by monitoring the spatial and temporal dynamics of 20 proteins in the pathway-tagged with XFP (GFP/CFP/YFP) after the addition of pheromone. Cells containing one or two XFP-tagged proteins were imaged by confocal microscopy in wild-type and mutant strain backgrounds. We developed software to convert the fluorescent images into quantitative information, which was used to fit the model. A frequently observed localization pattern for the tagged proteins was the association into complexes. We argue that these protein complexes are playing two key roles in this process: (*i*) amplifying the initial spatial cue; and (*ii*) organizing the response into distinct stages. These conclusions are supported by simulations of the model that demonstrate the robustness of this ordered sequence of cooperative protein assembly events.

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Summary: Our laboratory uses a molecular genetic approach to study basic questions in developmental biology, neurobiology and evolutionary biology: What are the molecular mechanisms of intercellular signaling? How is the fate of a cell specified in response to several intercellular signals? How do genes and neurons control complex behavior? What types of changes in development occur during evolution? How can we use the full genome sequence of closely related organisms? We primarily use the model organism *Caenorhabditis elegans* to study these questions. By understanding several aspects of this one organism in exquisite detail we hope to answer these fundamental biological questions. Our general approach is to use genetics to identify genes controlling the structure and behavior of cells and the whole organism, and to study those genes and their products, and how those genes interact to control development or behavior. In addition, we have a longstanding interest in the evolution of control mechanisms. Our research areas include: signaling by EGF-receptor and its negative regulation as a paradigm for signal transduction; vulval pattern formation and morphogenesis as a paradigm for organogenesis; sinusoidal movement including the role of G proteins Go and Gq; male mating behavior as a paradigm for the genetic control of behavior; modeling signal transduction; and bioinformatics. Our emphasis had been strongly focused on signal transduction but has more recently expanded to include transcriptional regulation.

Vulval development involves a remarkable series of intercellular signaling that coordinate the patterning of the uterine and vulval epithelia and allow them to connect precisely. Specification of the anchor cell from the ventral uterine epithelium breaks the symmetry of the gonad. The anchor cell then produces the vulval-inducing signal, LIN-3, an epidermal growth factor-like protein that acts via *C. elegans* homologs of EGF-receptor, RAS and MAP kinase. Inductive signaling is regulated at the level of ligand production as well as the responsiveness of the receiving cells. LIN-3 is produced in a highly localized and regulator manner. After the anchor cell induces the vulva, a complex program of further pattern formation, cell type specification and morphogenesis follows. The primary (1°) vulval lineage generates an E-F-F-E pattern of cell types while the 2° vulval lineage generates an A-B-C-D pattern of cell types. We now have our hands on a number of receptor proteins, transcription factors and regulated genes; we are trying to define this regulatory network to understand how organogenesis is genetically programmed. The anchor cell recognizes one of the seven vulval cell types and invades the vulval epithelium in a process akin to tumor metastasis, and we have found genes necessary for this process. Regulation by the EGF-receptor, WNT and HOM-C pathways impinge not only on vulval development but also the neuroectoblast P12 specification and male hook development. By comparing these examples with vulval development, we seek to understand the signaling specificity and signal integration.

Mating behavior, with its multiple steps, is arguably the most complex of *C. elegans* behaviors and because it is not essential for reproduction, given the presence of internally self-fertilizing hermaphrodites, is useful to elucidate how genes control behavior. We are studying several aspects of male mating behavior to understand the neuronal circuits that control the behavior and how they are genetically encoded. Most *C. elegans* behaviors rely on the heterotrimeric G proteins Go and Gq, which we have found to act antagonistically. Sinusoidal movement provides an opportunity to study interactions among G-protein signaling pathways as well as more general issues of how genes and cells program behavior. We have developed a system to observe and extract quantitative data on worm movement; in collaboration with R. Stirbl of JPL we explored using worm movement as a way of assessing environmental toxins.

We have begun to analyze the three members of the *C. elegans* TRPC family of calcium channels. We found that *trp-3* is expressed primarily in sperm and is necessary for sperm-egg interactions during fertilization.

We have begun to study transcriptional regulatory networks. One approach is comparative genomics. In collaboration with Barbara Wold's group, we have

obtained sequence from two other *Caenorhabditis* species in order to learn how to best use sequence information from multiple genomes. A second approach starts with identifying 5' ends of *C. elegans* mRNAs using a novel method. Another approach is the systematic analysis of *cis* and *trans* requirements for genes of special interest. *lin-3*, *lin-11* and *hox* genes are our initial focus.

We have continued to use *C. briggsae*, whose genome has just been sequenced, for studies of the evolution of gene function. We have isolated *C. briggsae* mutants with defects in vulval development and in dauer formation.

Our WormBase group is part of the international consortium responsible for WormBase, database of *C. elegans* biology (www.wormbase.org). Through this project, we help provide a service to the *C. elegans* and broader biological communities, as well as work on problems in bioinformatics, such as analysis of *cis*-regulatory sequences. In addition, we are developing methods for efficient retrieval and extraction of information from the literature, and provide a full-text semi-semantic queries of *C. elegans* literature (www.textpresso.org).

134. Alternative splicing in the modulation of EGF receptor signaling in ***C. elegans***

Cheryl L. Van Buskirk

A single EGF receptor ligand, LIN-3, is used for several inductive events during *C. elegans* vulval development, and it is unclear how signaling specificity is achieved. We have isolated *lin-3* cDNAs that encode alternate forms of the ligand, some of which are developmentally regulated. These isoforms differ in sequence between the EGF motif and the transmembrane domain. This juxtamembrane region contains proteolytic processing sites in the vertebrate homologs of *lin-3*, and thus the *lin-3* isoforms may possess unique cleavage properties and possibly different signaling ranges. Two variants, LIN-3A and 3B, which arise from alternate splice donor site usage, have been previously identified. LIN-3B differs from 3A by the insertion of an extra 15 residues in the juxtamembrane region. We have recently identified by RT-PCR an additional alternatively spliced, conserved exon in *lin-3* that encodes 41 amino acids. In combination with the LIN-3A/B splice choice, use of this exon results in the production of two additional isoforms, which we have called LIN-3C/D. While LIN-3A and 3B appear to be expressed at all stages, LIN-3C and 3D are detected only during early larval stages. To determine the significance of alternative splicing within *lin-3*, we are testing the ability of each isoform to rescue the vulval patterning defects of a weak *lin-3* mutant, and we are also examining the effect of heat shock-induced expression of each isoform on normal development. In addition, we will examine the expression patterns of each *lin-3* splice variant to determine which isoforms may be involved in each inductive event during normal development. We hope our studies will shed light on how alternative splicing of *lin-3* may contribute to the specificity of EGFR signaling in *C. elegans*.

135. The ***Caenorhabditis elegans fos*** family member ***Ce-fos***, promotes cell-invasive behavior

David R. Sherwood

We are interested in the cellular, genetic and molecular mechanisms that regulate the invasion of the gonadal anchor cell (AC) into the vulval epithelium of the hermaphrodite *Caenorhabditis elegans* larva. This process involves a secreted signal from the vulval cells that stimulates AC invasion, followed by the precise removal of the basement membrane separating both tissues directly bordering the AC, and concludes with the invasion of the AC between the central vulval cells. We have found that in *evl-5* mutants [Seydoux *et al*. (1993) *Dev. Biol.* 157:423-436] AC invasion is either absent or severely delayed. Visualization of the AC in mutants revealed that it extends cellular processes toward the developing vulva, and thus is still attracted to its target. However, in many cases the processes flattened at the basement membrane, suggesting an inability to cross there. We cloned *evl-5* and found that it encodes the *C. elegans* homologue of the proto-oncogene *fos* (*Ce-fos*), and appears to be the only *fos* family member in *C. elegans*. *Ce-fos* generates two transcripts utilizing distinct 5' exons, which encode a shorter protein (Ce-FOS-S), and a longer protein (Ce-FOS-L) containing an additional 136 amino acids at the N-terminus. Both proteins share the conserved DNA binding and dimerization region; however, the longer form contains a putative transactivation domain and possible leucine-zipper dimerization region. Sequencing the *evl-5* allele revealed that this mutation selectively disrupts Ce-FOS-L. Translational fusion proteins of the Cyan Fluorescent Protein (CFP) and the Yellow Fluorescent Protein (YFP) inserted into the unique 5' exons of *Ce-fos-S* and *Ce-fos-L*, respectively, showed Ce-FOS-S expression in many cells and tissues, but Ce-FOS-L primarily confined in somatic gonad cells. Furthermore, Ce-FOS-L is expressed at high levels specifically in the AC during invasion, strongly suggesting it functions there to promote invasion. To further examine the role of Ce-FOS-L in cell invasion, the expression of multiple genes within the AC in *evl-5* animals is being examined. This has led to the identification of both predicted and novel targets of Ce-FOS-L regulation in the AC, thus expanding the genes thought to regulate cell invasion and strongly implicating Ce-FOS-L as a key regulator of cell-invasive behavior.

136. Role of tissue inhibitors of matrix metalloproteases (TIMPs) and matrix metalloproteases (MMPs) in anchor cell invasion

Martha Kirouac

Cell invasion plays an important role in the formation of a normal connection between the vulva and uterine tissues in the nematode *C. elegans*. During larval development the basement membrane underneath the anchor cell is specifically broken down, and this cell sends

a process that invades between the two-vulF cells of the primary lineage of the vulva (Sherwood and Sternberg, in press). This invasion appears to be the initial step in the connection of the mesenchemally-derived uterus and the epidermally-derived vulva, which eventually will become the oviduct. One family of candidate proteins that may play a role in the breakdown of the basement membrane is the Matrix Metalloprotease Family (MMPs). This family of zinc-binding endopeptidases is capable of degrading extracellular matrix components. Specific natural inhibitors of this protein family, known as the TIMPS, play an important role in the regulation of the MMPs. A balance between the TIMPS and the MMPs activities is critical for normal physiological processes. However, there may well be a redundancy in function of the MMPs, and, therefore, teasing apart the functions of this family is tricky. By sequence similarity, we have identified six MMPs and two TIMPs in *C. elegans*. Currently, we are assessing the role of these proteins in anchor cell invasion by directed overexpression of the TIMPS in the anchor cell using a heterologous promoter, and RNAi of the MMPs alone and in combination.

137. LIN-17 frizzled, LIN-18 Ryk and Wnt ligands control the orientation of the P7.p lineage

Takao Inoue, Rashmi Deshpande[1], Russell Hill[1], Paul W. Sternberg

Many animal tissues are polarized with respect to the pattern of cell types and cell morphology, and mechanisms that control tissue orientation is an important issue in developmental biology. We are studying the mechanism by which Wnt, Frizzled and Ryk (receptor tyrosine kinase-related) signaling pathways control the orientation of the P7.p vulval lineage of *C. elegans*. The vulval precursors P5.p and P7.p execute the secondary lineage in opposite orientations. This difference in lineage orientation manifests itself at the molecular level in opposite patterns of POP-1 (TCF) localization and in the pattern of cell fates produced (assayed by *ceh-2::yfp* and *cdh-3::cfp*). In *lin-17* and *lin-18* mutants, the orientation of the P7.p is partially reversed (P5.p-like), leading to a characteristic bivulva (Biv) phenotype as well as alteration of the expression pattern of molecular markers.

We found that *lin-17* and *lin-18* function in parallel to establish the wild-type P7.p orientation. In *lin-17* or *lin-18* single mutants, we found frequent reversals in the VPC 2-cell stage (P7.p daughters), but less frequent reversals in the VPC 4-cell stage (P7.p granddaughters). In the *lin-17; lin-18* double mutant, we found high frequency of reversals in both VPC 2 and four-cell stages. As the mutual enhancement is observed with probable null alleles of *lin-17* and *lin-18*, these two genes appear to act independently of each other in parallel pathways. Based on GFP reporter fusions, *lin-17* and *lin-18* both likely function in the P7.p cell and descendants.

lin-17 encodes a Frizzled-type Wnt receptor. *Lin-18* encodes a member of the Ryk/Derailed family of tyrosine kinase-related receptors. LIN-18, Ryk and Derailed extracellular domains show homology to WIF-1, a Wnt binding protein, raising the possibility that LIN-18 functions as a Wnt receptor. To determine which Wnt proteins act as ligands, we tested various single, double and triple mutant combinations of the five *C. elegans* Wnt genes, *lin-44, egl-20, mom-2, cwn-1* and *cwn-2* using genetic mutants and RNAi feeding. We found that the *mom-2(-) lin-44(-)* combination resulted in the Biv phenotype, suggesting that these two genes encode the principal ligands for establishing the orientation of P7.p. *cwn-2* and *egl-20* had minor effects in some genetic backgrounds.

LIN-44 and MOM-2 may signal through LIN-17, LIN-18 or both. To test ligand/receptor specificities, we are testing various *wnt; receptor* mutant combinations. *lin-44* mutation strongly enhances *lin-18(-)*, but has no obvious effect on *lin-17(-)*. Conversely, *mom-2* mutation strongly enhances *lin-17(-)*, but has only a weak effect on *lin-18(-)*. These results suggest that different Wnt ligands signal through different receptors in parallel pathways both operating in P7.p.

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138. Regulation and function of ***lin-11*** during ***C. elegans*** vulval development

Bhagwati P. Gupta

We have earlier shown that the LIM homeobox gene *lin-11* plays a crucial role during vulval morphogenesis. *lin-11* is necessary for the specification of all seven vulval cell types (vulA to vulF) and in mutant animals vulval cells fail to acquire their unique identity and inappropriately fuse with each other. To understand the function of *lin-11*, we have examined its spatiotemporal expression using different *lin-11::GFP* transgenes. We find that *lin-11* is expressed in a dynamic manner in both primary and secondary lineage cells. The early expression is asymmetric (secondary cells), whereas late expression is observed in all vulval progeny.

To study the regulation of *lin-11*, we have analyzed its genomic sequences and identified a vulval-specific enhancer element. Sequence analysis of this element has revealed multiple putative binding sites for POP-1 (TCF/LEF). TCF/LEF family members are known components of the Wnt signaling pathway. LIN-17, a Frizzled receptor in *C. elegans*, is known to play a role in vulval morphogenesis. We therefore examined the role of *lin-17* in regulating *lin-11* vulval expression and find that *lin-17* is necessary for the asymmetric expression of *lin-11*. Currently we are analyzing the role of known Wnt pathway components including *pop-1* in *lin-11* vulval regulation. In addition to the *lin-17* pathway, we are also testing the role of other genes in *lin-11* vulval regulation that have been shown to play roles in epithelial morphogenesis in *C. elegans*.

139. A component of the transcriptional mediator complex inhibits RAS-dependent vulval fate specification in ***C. elegans***

Nadeem Moghal, Paul W. Sternberg

Negative regulation of receptor tyrosine kinase (RTK)/RAS signaling pathways is important for normal development and the prevention of disease in humans. We have used a genetic screen in *C. elegans* to identify genes that antagonize the activity of activated LET-23, a member of the EGFR family of RTKs. We identified two loss-of-function mutations in *dpy*-22, previously cloned as *sop-1*, that promote the ability of activated LET-23 to induce ectopic vulval fates. DPY-22 is a glutamine-rich protein, most similar to human TRAP230, a component of a transcriptional mediator complex. DPY-22 was previously shown to regulate WNT responses through inhibition of the β-catenin-like protein BAR-1. We provide evidence that DPY-22 also inhibits RAS-dependent vulval fate specification independently of BAR-1, and likely regulates the activities of multiple transcription factors during development. Furthermore, we demonstrate that although inhibition of BAR-1-dependent gene expression has been shown to require the C-terminal glutamine-rich region, this region is dispensable for inhibition of RAS-dependent cell differentiation. Thus, the glutamine-rich region contributes to specificity of this class of mediator protein.

140. Genetic analysis of the vulval mutants in ***C. briggsae***

Bhagwati P. Gupta, Shahla Gharib

To understand the evolution of developmental mechanisms, we are doing a comparative analysis of vulval patterning in *C. elegans* and *C. briggsae*. *C. briggsae* is closely related to *C. elegans* and has identical-looking vulval morphology. However, recent studies have indicated subtle differences in the underlying mechanisms of development. The recent completion of *C. briggsae* genome sequence by the *C. elegans* Sequencing Consortium is extremely valuable in identifying the conserved genes between *C. elegans* and *C. briggsae*.

To identify vulval genes in *C. briggsae*, we have taken a genetic approach and carrying out EMS screens. Our genetic screens have so far identified more than 30 vulval mutants that are vulvaless (Vul), protruding-vulva (Pvul) or multivulva (Muv). Current experiments involve mapping and phenotypic characterization. Preliminary analysis indicates that some of these have phenotypes distinct from the known *C. elegans* vulval mutants. The genetic mapping of mutants has been facilitated by the availability of makers and their linkage map. Our analysis of the Vul mutants has revealed an ortholog of *C. elegans lin-11 (ce-lin-11)*. This has been confirmed by transgene rescue and allele sequencing experiments. *lin-11* encodes a LIM homeodomain protein and functions to specify distinct vulval cell fates. We have determined the regulatory region of *C. briggsae lin-11 (cb-lin-11)* that directs gene expression in vulval cells. The temporal pattern of *cb-lin-11* expression resembles that of *ce-lin-11*, however, there are some differences. A comparison of the regulatory sequences between the two nematode species has revealed regions that are likely to be recognized by vulval-specific transcription factors. We are analyzing such elements to identify species-specific as well as conserved sequences to understand *lin-11* regulation and evolution in vulval cells.

141. Modulation of EGF receptor-mediated vulva development by the heterotrimeric G-protein Gαq and excitable cells in ***C. elegans***

Nadeem Moghal, L. Rene Garcia[1], Liakot A. Khan[2], Kouichi Iwasaki[3], Paul W. Sternberg

The extent to which excitable cells and behavior modulate animal development has not been examined in detail. Here, we demonstrate the existence of a novel pathway for promoting vulval fates in *C. elegans* that involves activation of the heterotrimeric Gαq protein, EGL-30. EGL-30 acts with muscle-expressed EGL-19 L-type voltage-gated calcium channels to promote vulva development, and acts downstream or parallel to LET-60 (RAS). This pathway is not essential for vulval induction on standard Petri plates, but can be stimulated by expression of activated EGL-30 in neurons, or by an EGL-30-dependent change in behavior that occurs in a liquid environment. Our results indicate that excitable cells and animal behavior can provide modulatory inputs into the effects of growth factor signaling on cell fates, and suggest that communication between these cell populations is important for normal development to occur under certain environmental conditions.

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142. Coordination of sensory inputs and motor control in ***C. elegans*** male mating behavior

Allyson Whittaker

We are interested in understanding how multiple sensory inputs are coordinated with motor control to generate behavior. To this aim, we are studying the neural circuits and signaling pathways that control mating behavior of the *C. elegans* male. *C. elegans* male mating behavior consists of a series of sub-steps: response to the hermaphrodite, backing along and turning near the end of the hermaphrodite, location of the vulva, insertion of spicules, and sperm transfer. Further examination of the initial steps of mating behavior, response, backing and turning, has identified additional sub-steps. Appropriate response and backing behavior requires that the male sense the presence of a potential mate, suppress foraging behavior to remain in the area of that mate, press the ventral side of the tail against the side of the hermaphrodite, and initiate backing behavior keeping the

direction of its tail constant. To turn, a male turns its tail to the side, crosses either under or over the hermaphrodite, remakes contact with the opposite side of the hermaphrodite and continues backing behavior. These behaviors likely involve chemosensation, mechanosensation and proprioception. Although these behaviors require cues from the hermaphrodite, the hermaphrodite is not likely to play an active role in mating as males will properly mate with a paralyzed hermaphrodite. Efforts to determine the hermaphrodite signals required for backing and turning behavior will be discussed.

In an attempt to dissect the neural circuits and signaling pathways controlling response, backing and turning behavior, we have performed screens for mutations that disrupt mating behavior. From one of these screens we have isolated two new alleles of *pkd-2*, *sy680* and *sy681*. We are currently focusing on one mutant line, *cod-5*. Of males homozygous for mutations in *cod-5,* 81% are disrupted in response, 62% are disrupted in turning and 44% are disrupted in vulva location. Specifically, *cod-5* mutant males take longer than normal to respond to a hermaphrodite. Although males are able to execute a turn correctly, they often fail to initiate a turn, backing off of a hermaphrodite and either swimming away or loosely making contact with the other side. Mutant males also have difficulty keeping their tails straight during backing and often briefly back along the lateral side of hermaphrodites. We are currently mapping and cloning *cod-5*. We are also mapping two mutant lines with defects in response and vulva location behaviors. In addition, we are examining the effects of disrupting known neural genes on male mating behavior.

143. ***C. elegans*** male hook development

Hui Yu

The *C. elegans* hook sensillum is a male copulatory structure that mediates vulval location behavior during mating. It includes a structural cell, a sheath cell, a socket cell, and two sensory neurons, as well as several hypodermal cells. All these cells are descended from a group of multi-potential precursor cells, "hook precursor cells" (HPCs). In hermaphrodites, six Pn.p cells, P(3-8).p, form the well-characterized vulval precursor cells (VPCs). Multiple signaling pathways are involved in VPC patterning. In males, three different Pn.p cells, the posterior P9.p, P10.p and P11.p, form HPCs, the male homolog of the hermaphrodite vulval precursor cells. To understand the details of HPC fate specification, we investigated possible roles of different signaling pathways by examining gene expression pattern and mutant phenotype. Previous studies showed that mutations in *lin-17*, a homolog of frizzle-like wnt receptor, affects HPC patterning by converting all progeny of P(9-11).p cells into a hypodermal fate (Sternberg and Horvitz, *Dev. Biol.,* 1988). We found that *lin-17::gfp* was predominantly expressed in primary P11.p cell/lineage and had a weaker expression in secondary P10.p cell/lineage. Consistent with that, *bar-1,* a β-catenin in canonical wnt pathway, was expressed in the primary P11.p cell and then was translocated into nucleus right before the first cell division. However, unlike *lin-17*, mutation in *bar-1* only had a minor effect on the hook development. It is possible that other downstream components of WNT pathway act in this patterning process. *pry-1* is a *C. elegans* homolog of Axin, a negative regulator of intracellular WNT pathway. Interestingly, *pry-1* mutant males had ectopic hooks along the ventral side of animal body. In other words, loss of inhibition of certain WNT sub-pathway allows anterior Pn.p cells to adopt posterior HPC fate.

144. Comparative analysis of ***cis***-regulatory sequences using four ***Caenorhabditis*** species

Erich M. Schwarz, John A. DeModena, Tristan De Buysscher, Eunpyo Moon, Nora Mullaney, Hiroaki Shizuya, Barbara J. Wold, Paul W. Sternberg

Comparing homologous *cis*-regulatory DNA sequences from three or more genomes has advantages over pairwise comparison of only two. *cis*-Regulatory sequences are short (6-20 bp) and tolerate substantial variation. Purely random pairing of unrelated 100-bp DNA segments is expected to yield two perfect 6 bp matches. Alignment of a third or fourth sequence should greatly lower the frequency of false-positive regions, allowing small but real *cis*-regulatory sequences to be efficiently detected. This increased resolution should also allow direct comparison between phylogenetically conserved sequences and statistically overrepresented sequences, which may yield complementary views of regulatory sequences. In the *Caenorhabditis* genus, *C. remanei* appears to be most closely related to *C. briggsae*; two other species, CB5161 and PS1010, comprise two close known and culturable *Caenorhabditis* species outside the *elegans-briggsae* group (Fitch, 2000). CB5161 is closely similar to *C. elegans*, while PS1010 is behaviorally and morphologically more divergent; these two species thus provide an evolutionarily-graded series for comparative analysis.

We have constructed fosmid libraries from CB5161 and PS1010, and begun sequencing individual fosmids for comparative analysis of genes involved in vulval or sensory neuron development. At the same time, we have devised the MUSSA software package to adapt the algorithms of Davidson and coworkers (Brown *et al*., 2002) to multiple sequence analysis. At this writing, we have ~300 kb of sequence data from both CB5161 and PS1010, comprising ~0.3% of each species' genome, with 45-60 genes in each species that have clear homologies to *C. elegans*. Examination of the *lin-11* locus in CB5161 demonstrates that a three-way comparison of this gene to *C. elegans* and *C. briggsae* clearly enhances the resolution of known and possible regulatory elements in the *lin-11* 5' regulatory region. Moreover, we find that analysis of *lin-11* with both MUSSA and the YMF/Explanators programs of Sinha and Tompa, (2003) appears to identify all known regulatory sequences in the *lin-11* 5' flank, suggesting that a two-step analysis with MUSSA to find key regions of the

genome followed by YMF to find small regulatory motifs may be highly effective in computationally mining the *C. elegans* genome for *cis*-regulatory signals. Genomic sequences from PS1010 are more difficult to analyze, but the structural organization of HOX clusters in PS1010 is identical to that in *C. elegans*, despite much reorganization of genes flanking the PS1010 clusters, which implies that subtle *cis*-regulatory sequences are likely to be detectable in this species with sufficiently powerful analysis.

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145. Genome annotation by high-throughput 5'-RNA end determination

Byung Joon Hwang, Hans-Michael Müller, Shahla Gharib, Paul W. Sternberg

The identification of the complete set of transcripts, including alternative transcripts, has proven to be technically difficult and has become a rate-limiting step in understanding genome organization. Here we describe a high-throughput technique that identifies 5'-ends of genes, which allows identification of unpredicted genes as well as alternative transcriptional control regions. The technique, TEC-RED (Trans-spliced Exon Coupled-RNA End Determination), determines 5'-ends of RNA transcripts using the *trans*-splicing reaction from splicing leader (SL) RNA to the 5'-RNA ends. It uses the common *trans*-spliced exon sequence in mRNA as a recognition site for one of type IIs and III restriction enzymes whose DNA recognition site is separated from the cleavage site. The restriction enzyme treatment extracts a piece of cDNA sequence from the 5'-end of each cDNA because its cleavage site is on the cDNA and is 14 bp apart from its recognition site on the *trans*-spliced exon. The extracted 5'-cDNA pieces ('TAG') are then concatenated for DNA sequencing, allowing each DNA sequencing reaction to determine the 5'-ends of multiple cDNAs. A pilot TEC-RED analysis on *C. elegans* mRNA containing SL1 exon identified about 1000 different 5'-RNA ends. 74% of these 'TAG' sequences overlap with known 5'-RNA ends, proving the fidelity of this method. In this analysis, 58 putative new genes and 89 new 5'-ends of the known genes were discovered. About 7% of the 'TAG' sequences indicate alternative transcriptional initiation. We are currently applying this technology to *C. elegans* and *C. briggsae* mRNA containing a *trans*-spliced SL1 or SL2 exon. This technique should be broadly applicable in nematodes. Since the *trans*-splicing reaction from endogenous splicing leader RNA has been identified in five different phyla and the reaction from exogenously expressed leader RNA has been shown in human, this technique might be applicable to genome outside the nematode phylum.

146. A Cyclin L homologue and a heat-shock transcription factor are required for heat-shock protein expression in ***C. elegans***

Yvonne M. Hajdu-Cronin, Wen J. Chen

In a screen for suppressors of activated GOA-1 (*C. elegans* $G\alpha_o$) under the control of the *hsp16-2* heat shock promoter, we identified several genetic loci that affect heat-shock induction of GOA-1. *sag-4* (V) mutants are wild type in appearance, while *sag-3* (I) and *sag-5* (III) mutants have egg-laying and fertility defects. Western analysis indicated that mutations in all three genes suppress activated GOA-1 by decreasing heat shock-induced protein expression. Although endogenous GOA-1 expression is not affected, heat-shock induction of GOA-1 decreased in the suppressor strains. We also found that mutations in *sag-3* and *sag-5*, as well as *sag-4*, were able to suppress another transgene under the control of the *hsp16-2* promoter. The suppression by *sag-4* was minimal, and suppression by the other two mutants was strong. *sag-3* and *sag-5* mutations also suppressed a transgene driven by the *hsp16-41* promoter.

We used single nucleotide polymorphisms to map *sag-3* and found that it mapped to a small region (Y53C10A) that included the *C. elegans* heat-shock transcription factor homologue (HSF). We sequenced this candidate gene in the *sag-3* mutant and found that the *sy441* mutation causes a truncation (W585stop) in HSF. The truncation eliminates the transactivation domain, an acidic, hydrophobic region at the extreme C-terminus of HSF. However, the DNA binding domain and leucine zippers remain intact in the mutant. *sag-5* has been mapped to a small interval on the left end of III, between *unc-45* and *daf-7*. The molecular nature of *sag-5* has not been determined. *sag-4* was cloned by traditional mapping and microinjection rescue experiments. We found that it encodes a cyclin homologue most similar to cyclin L, which has been shown in other species to partner with CDK11 and is involved in pre-mRNA splicing. Cyclin L in *C. elegans* contains not cyclin box in common with other cyclins, but also an RS domain characteristic of splicing factors. The three *sag-4* alleles we isolated were sequenced; all are leucine to phenylalanine mutations within the cyclin box.

147. Analysis of Dauer formation in various ***Caenorhabditis*** species

Shirley Phan[1], Takao Inoue, Hannah Kim[2]

During the normal growth of *Caenorhabditis* species, the eggs hatch into larvae, which in turn undergo a series of larval stages before reaching the adult stage. However, when surrounding conditions become adverse, worms will enter the dauer state, a developmentally-arrested third larval stage as which the worms are able to survive for long periods of time until environmental conditions are once again favorable. Two types of mutations affecting dauer formation include: Daf-c, which induces worms to form dauers even when conditions are favorable and Daf-d, which causes worms to lack the ability to form dauers under any circumstances.

To determine the molecular changes underlying the evolution of dauer formation, mutagenesis is being conducted to obtain Daf-c and Daf-d mutants from other *Caenorhabditis* species: *C. briggsae, C. remanei*, CB5161, and PS1010. A total of four Daf-d cilium structure mutants, eight other Daf-d mutants, including one daf-3 allele, and two Daf-c mutants have been obtained in *C. briggsae.* In addition, one cilium structure mutant has also been obtained in *CB5161*.

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148. Induction of ***Drosophila melanogaster*** immune response by the parasitic nematode ***Heterorhabditis bacteriophora*** and its bacterial symbiont ***Photorhabdus luminescens***

Michelle Rengarajan[*]*, Takao Inoue*

Heterorhabditis bacteriophora is a parasitic nematode capable of infecting a wide variety of insects. The infective juvenile (IJ) harbors the symbiotic bacterium, *Photorhabdus luminescens*, which is used to weaken the immune response of the host. We have found that *H. bacteriophora* is capable of infecting *Drosophila melanogaster*. This allows use of the extensive tools of *Drosophila* genetics and molecular biology to study these interactions. We have investigated the induction of the insect immune system by assaying GFP and lacZ reporters for *diptericin*, *cecropin*, and *metchnikowin*, genes known to be induced in antimicrobial immune response. By infecting fly strains with bacteria, we have determined that *P. luminescens* does not induce cecropin response, whereas *E. coli* does, suggesting that *P. luminescens* is able to either evade or suppress *Drosophila* immune response.

H. bacteriophora, *P. luminescens*, and *Drosophila* make up a potentially useful system for studying bacterial-nematode symbiosis, bacterial-insect pathogenesis, and nematode-insect parasitism. Because of the close correlation between human and *Drosophila* immune response elements, we can use *Drosophila* as a model organism to shed light on the mechanisms of immune system activation and suppression in humans. We also hope to provide information that has implications in human diseases caused by parasitic nematodes, such as lymphatic filariasis.

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149. Movement analysis system: A tool for quantifying worm behavior

Christopher J. Cronin, Jane E. Mendel, Adeline Seah, Saleem Mukhtar, Jehoshua Bruck, Robert C. Stirbl, Paul W. Sternberg

We have developed an automated movement analysis system to quantify aspects of worm behavior. Our automated worm tracking system enables us to videotape moving worms. Videos of recorded worms are analyzed by an automated worm recognition system that extracts quantitative data describing body posture and position as a function of time. An automated analysis system uses the extracted data to compute measures of several locomotion parameters. These metrics include worm centroid velocity, worm velocity along its sinusoidal path, frequency of muscle contractions (temporal), degree of dorsal-ventral body flex during muscle contractions, and the rate of movement wave propagation. Our system also derives the wavelength and amplitude (spatial) of the sinusoidal track made by worms during locomotion.

Our automated worm tracking and recognition systems are based in Visual C++ using image processing software and hardware from Matrox Imaging, and our analysis software is based in Matlab. Recent work on the tracking and recognition systems has involved porting the software to Windows 2000 and preparing for conversion to an all-digital format for image capture and archiving. Further development of the analysis software now allows us to quantify measures of worm track waveforms. Additionally, we have improved the fault tolerance of the recognition and analysis systems and improved their user interfaces for ease of use.

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Summary: In the Wold group we are interested in the composition, evolution and function of gene regulatory networks and we often use muscle development as a favored model system. We are especially interested in networks that govern how cell fates are specified and executed during development and during regeneration. This theme extends to related lineages of adult stem cells for our model system and to the way in which cells of this same lineage can become tumorgenic. Approaches to these problems increasingly use genome-wide and proteome-wide assays. To do this some of our efforts now include development of new wet-bench genomic technology and computational methods, the latter developed in an on-going partnership with Professor Eric Mjolsness of JPL/University of California, Irvine. Many of the technologies, computational tools, modeling databases, etc., are first developed and tested and vetted using yeast as the model genomic system.

A key challenge is to understand the regulatory events that drive the progression from multipotential precursor cells to determined unipotential progenitors and then to fully differentiated cells. We are currently studying these cell states and transitions using microarray gene expression analysis, global protein:DNA interaction measures, mass spec-based proteomics of multiprotein complexes, and comparative genomics. The mouse is our primary experimental animal, and the focal developmental lineage arises from paraxial mesoderm to produce muscle (also bone, skin and fat, among other derivatives). Skeletal myogenesis is governed by both positive- and negative-acting regulatory factors. The MyoD family of four closely related, positive-acting transcription factors are key. Upon transfection each can drive non-muscle recipient cells into the myogenic pathway. Given their extraordinary power to drive or redirect a cell fate decision, a central goal is to understand how the regulatory network in which they are embedded directs cell-fate selection and execution of the differentiation transition. At cellular and molecular levels, it is clear that negative regulators of skeletal myogenesis are probably just as important for regulating the outcome as are the positive regulators. The interaction between positive- and negative- acting regulators continues to be of particular interest. Multiple negative regulators of skeletal muscle are expressed in multipotential mesodermal precursors and in proliferating muscle precursors (myoblasts). It is generally believed that some of these are important for specifying and/or maintaining precursor cells in an undifferentiated state, though exactly how the system works is unknown.

To define the network more comprehensively, we have developed a major collaborative effort with the Deshaies lab here and the John Yates lab at Scripps to modify and apply MudPIT mass spectrometry, coupled with dual affinity epitope tagging, to characterize multiprotein complexes. New technology development projects are focused on large-scale measurements of protein:DNA interactions. To define the *cis*-acting regulatory elements to which these protein complexes bind, we have entered into a collaboration with Eric Greene at NIH to isolate and sequence genes from our network from ten vertebrate species each. The computational tools described below have been used to find candidate conserved regulatory elements, and these are, in turn, being subjected to functional assays via lentiviral-mediated transgenesis. These same tools are being used to analyze data from multiple species of worms related, in differing degrees, to *C. elegans*. This project is in partnership with the Sternberg lab and Hiroke Shizuya here at Caltech, and the DOE Joint Genomics Institute, where large-scale DNA sequencing is done. In this project large insert, random-shear libraries were made for two new worm species, and genes from several regulatory networks, including the myogenic one being isolated and sequenced for comparative analysis. In addition to clarifying how many and which worm genomes give us the most leverage for identifying functionally important non-coding elements in the genome, we hope to gain insights into the evolution of myogenic networks across large phylogenetic distances between vertebrates, worms and flies.

Our collaboration with Dr. Timothy Triche and colleagues at Children's Hospital is giving us a picture of how the myogenic developmental pathway relates to cells of the myogenic lineage when they run amok in cancer. Transcriptome analysis of over 100 rhabdomyosarcomas has given us several new insights into the nature of these tumors, plus identification of previously unappreciated signaling pathways that are candidates for causal contributions to tumor properties. Of particular interest was a surprising reclassification of one subgroup of tumors. These are, by histological criteria, of the alveolar class. However, by expression profiling and subsequent computational analysis, these tumors proved to be very different from classic alveolars and more similar to the other major class (embyonal). Retrospectively, we were able to relate this to the absence of a chromosomal translocation that characterizes the most alveolar tumors. This analysis also showed, surprisingly, that genes whose

expression best separates conventional alveolar and embryonal types does not correlate with their histological appearance, but rather refers to other molecular differences. We postulate that these "invisible" differences may have more powerful prognostic capacity than conventional histopathology, since the two tumor classes differ significantly in outcome, than does histopathological classification. This has implications for treatment pathways and prognosis.

An entirely new project for the lab is Tracy Teal's. She is a joint graduate student with Diane Newman in the Division of Geology and Planetary Science. The topic is environmental bacterial biofilms and the goal is to identify, visualize, and ultimately understand the multiple different metabolic cell states that comprise a biofilm at different stages of its development and under differing environmental stimuli. The degree to which principles and regulatory strategies used by metazoans during development are or are not employed by bacteria in creating biofilm structures is being probed by marking bacteria with multiple GFP derivatives driven by genes that will mark functional domains (aerobic vs. anerobic, for example).

150. Lowered oxygen culture affects skeletal muscle satellite cell yield, development and phenotype

Marie Csete, Jean Walikonis, Shuling Wang[*]

Satellite cells are normally quiescent cells present in small numbers under skeletal muscle basal lamina. Satellites re-enter the cell cycle when skeletal muscle is traumatized, and produce myoblasts and ultimately new muscle fibers. They are thought to be the major source of new skeletal muscle after trauma or degeneration. An adult muscle fiber culture model of regeneration was established in the lab by D. Cornelison (1). That work demonstrated that satellites in culture activate in a relatively synchronous fashion, with single satellites emerging from the basal lamina at about one day in culture and the first satellite division common at 48 hr, with new muscle fiber apparent at 96 hr. A characteristic expression pattern of muscle transcription factors (MyoD, myf5, myogenin, and MRF4) accompanies these morphologic patterns.

Traditional tissue culture is usually conducted at oxygen levels that are aphysiologically high, even for skeletal muscle, a generally "high"-oxygen tissue. On average, skeletal muscle tissue oxygen levels are approximately 6% O_2. Csete and Walikonis compared early satellite regeneration under physiologic oxygen levels vs. usual 20% O_2 culture conditions. Using sequential 12-hr BrdU pulses to label satellites adherent to the fibers, we found twice as many satellites proliferating at each time point (up to 60 hr) in physiologic oxygen vs. 20% O_2. Immunohistochemical labeling of satellites using immunostaining for the presence of c-Met receptors (an operational molecular marker of quiescent and recently activated muscle stem cells) confirmed the presence of twice as many satellites on fibers in low oxygen conditions at 24 and 48 hrs. In parallel studies using human myoblast cultures, we confirmed that these cells undergo enhanced proliferation under 2% O_2 conditions compared to traditional 20% O_2 cultures.

Single-cell RT-PCR was also used to assess the satellite developmental process. Individual satellite cells were assayed for the expression of the four major muscle regulatory transcription factors of the MRF family: myf5, MyoD, myogenin and MRF4. Myogenin and MyoD expression is more prominent at 24 and 48 hr under low oxygen conditions than under 20% O_2. At 24 hr, twice as many satellites in low oxygen have simultaneous expression of three MRFs. Both findings suggest that the satellites are further along in their developmental pathway under physiologic oxygen conditions. This work, together prior work with neural stem cells, raises questions about which pathways are responding to oxygen variation, and large-scale expression surveys are one obvious pathway to explore and narrow the possibilities.

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151. Characterization of pediatric sarcomas by microarray-based gene-expression studies

Sagar Damle[*]*, Joe Roden*[**]*, Ben Bornstein*[**]*, Dennis DeCoste*[**]*, Eric Mjolsness*

The central model system for our lab's developmental biological studies is paraxial mesoderm with emphasis on the myogenic pathway. In cancer biology, tumors derived from these cell lineages are the sarcomas of childhood, and those in the muscle lineage are called rhabdomyosarcomas. This work begins by probing a well defined and large set of rhabdomyosarcomas via large-scale expression analysis and ask a series of questions including: How does gene expression in the tumors relate to that seen in normal development? Can we detect groups of genes whose patterns of expression predict how aggressive a tumor will be? Can we identify patterns of genes that classify tumors into subgroups that correspond to those currently generated by pathologists? Is there a group of genes whose expression corresponds with presence of two known chromosomal translocation events that are believed to be causal for one subtype of tumor? Is there a set of genes that can help explain the bizarre observation that a small subset of these tumors are highly metastatic, yet essentially benign, while others that look histologically similar are rapidly fatal? We then seek to relate what is known about the identities of signature genes with signaling pathways, cell shape and migration, gene regulation etc., to identify candidate genes or pathways for future drug treatments.

Two histopathologically different kinds of rhabdomyosarcoma (RMS), alveolar and embryonal RMS, are associated with distinct clinical characteristics and different cytogenetic properties. Most alveolar-class RMS are characterized by a t(2:13) or t(1:13) chromosomal translocation, which results in the fusion of the DNA-

binding motifs of either PAX7 or PAX3 and the carboxy-terminal activation domain of forkhead gene. Embryonal-class RMS instead show allelic loss of regions of chromosome 11, thought to contain tumor suppressor genes. In a long-term collaboration with the Triche Laboratory and Children's Hospital LA, Affymetrix microarrays (U133A/B) were used to measure the gene-expression profiles of 31,728 unigene-derived features in 56 RMS tumors (35 ERMS 21, ARMS). Statistical analysis was performed using a two-class T-test. Despite the relatively common muscle backgrounds of these two cell types, we were able to identify several hundred differentially expressed genes (with a p-value less than 0.05). Members of this subset have been identified as having roles in specific cell-cycle regulatory and apopotosis-related pathways. Surprisingly, cannabinoid receptor 1, expressed normally in the hippocampus and responsible for phosphorylation of focal adhesion kinase (FAK), is overexpressed in ARMS elative to EMS. FAK, in turn, is upstream of several signaling pathways, including adhesion-dependent survival and cell motility, and inhibits apoptosis through pathways that inhibit degradation of AKT. FAK activation may explain the relative severity of prognosis and incidence of metastasis seen in ARMS. Alternatively, the transcription factor FoxF1 and its upstream regulator, Shh, were repressed in many ERMS, with corresponding upregulation of antagonist and growth factor, BMP4.

Other more sophisticated analyses, including both supervised and unsupervised clustering algorithms, have been applied in further characterizing the expression patterns seen in these tumors. Artificial neural networks (Bornstei; JPL), support vector machines (DeCoste; JPL) and other machine-learning algorithms are being used to identify genes whose expression differs across PAX3/PAX7 (ARMS) prognosis and metastasis boundaries in addition to the ARMS/ERMS divide. Gene subsets gathered from these lines of analysis have allowed for a finer parsing of tumor types. A two-dimensional plot of the PCA-transformed tissue space in which the genespace was restricted to these subsets (Joe Roden-JPL) has helped to provisionally reclassify translocation negative ARMS tumors on the basis of their ERMS-like gene expression patterns. The possibility that these tumors also share the more favorable ERMS prognosis and the inevitable power this will allow in the customization of cancer treatment remains to be seen, but the prospects are encouraging.

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152. Gene expression and metabolic organization in ***Shewanella oneidensis*** biofilms

T.K. Teal, B.J. Wold, D.K. Newman[*]

Bacteria have traditionally been viewed as living a primarily planktonic lifestyle. In the last decade, however, it has become more apparent that bacteria spend much of their lives as surface-attached microbial communities, i.e., biofilms. These films have considerable three-dimensional structure, meaning that the environment within different areas of the biofilm can vary markedly. These biofilms are prevalent in natural as well as man-made systems. Because of their medical and environmental importance, biofilms have recently become the subject of more intense study. We are interested in the process of biofilm formation and the metabolic organization of the biofilm. While more has become known about the biofilm lifestyle, including the existence of intercellular signaling pathways, very little is understood about the temporal and spatial metabolic states within the biofilm and how they influence further biofilm development. This collaboration between Newman and Wold labs is particularly centered on probing similarities and differences in the strategies used in forming 3-D developing biofilms, comparing and contrasting the strategies used with those used in metazoan development. It is secondarily centered on implementing genomic-scale assays, as called for in the project.

To investigate these topics we will be using biofilms comprised of *Shewanella oneidensis* strain MR-1. *S. oneidensis* is an environmentally important class of bacteria. It is a facultative anaerobe with remarkable respiratory versatility. Its genome has been sequenced, and it is relatively easy to manipulate genetically. We have started by investigating which parts of *S. oneidensis* biofilms are metabolically active. To do this we have tagged the ribosomal promoter rrnBP1 with the unstable GFP gfp(AAV) and cloned it into a plasmid that is stable in MR1. Fluorescence levels from this promoter/GFP system have been shown to be a good indicator of cell growth and replication. We are growing the biofilms in a flow cell system and imaging them using two-photon fluorescence microscopy. Using this technique we can correlate the level of fluorescence we see using microscopy with metabolic activity and can determine the regions of the biofilm in which cells are the most metabolically active, as well as explore how quickly this state can change in response to environmental perturbations. Additional constructs using promoters sensitive to other metabolic parameters are in progress.

To gain a better understanding of the process of biofilm formation, we are interested in the genes being expressed as the biofilm develops. We have created a test DNA microarray with 75 selected genes from MR-1 thought to be involved in different aspects of biofilm formation. We will assay *S. oneidensis* biofilms for gene expression over time, and perhaps selected on the basis of GFP marker gene expression. We anticipate that we will see expression of genes important in biofilm attachment and growth, as well as those involved in cell-cell signaling, or quorum sensing. Using this data we will then use 'classical genetics' techniques to determine the precise role of the identified genes. Ultimately we hope to develop a computational model of the key genetic networks involved in biofilm formation and cell-to-cell communication.

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153. Analysis of transcriptional protein complexes via MudPIT mass spectrometry

Leslie Dunipace, Johannes Graumann[*], Jae Hong Seol[**]

It is difficult to overestimate the usefulness of learning the *in vivo* protein associations that mediate a cellular process. This project focuses on protein complexes involved in regulating gene expression and is part of a larger collaborative effort between our lab, the Deshaies lab, and John Yates' lab at Scripps. The overall collaboration has the initial goal of modifying and then applying at relatively high throughput, a mass spectrometry-based method for mapping protein interactions *in vivo*. This approach couples MudPIT (MultiDimensional Protein Identification Technology) which originated in the Yates lab, with a dual affinity protein tagging / sample retrieval system constructed in the Deshaies group called HTM/HPM. Leslie has now used the tag to establish 25 yeast strains, each carrying the product of targeted recombination at a selected gene, so that the protein produced (from its normal regulatory sequences in the chromosome) carries an affinity tag at the carboxy-terminal end. This affinity tag contains two epitopes (His-9 and Myc-9) separated by a protease site. The His and Myc tags are used for sequential and rapid affinity purification. The purpose of the affinity steps is to enrich the sample for the tagged protein together with proteins associated with it, while eliminating unassociated proteins. Dual affinity is designed to eliminate the need for gel purification and "band-cutting," which has been a rate-limiting step in sample preparation in similar studies in the Deshaies lab and elsewhere in the past. The mass-spec analysis that follows identifies peptides in the sample by reference to the known genome and its predicted proteome, using a version of the Sequest software developed by Yates and colleagues. By increasing throughput, we aim to take entire groups of genes involved in a single cellular process or structure and gain a high quality mass spec map of the *in vivo* associations involved.

The 25 yeast strains were vetted for expression of the tagged protein by Western blotting against the Myc tag. Conditions for affinity retrieval were optimized and initial testing was performed by focusing on a relatively well-known, multiprotein complex (the chromatin remodeling complexes of which GCN5 is a component). Working in the Yates group, Johannes generated very encouraging and reproducible mass spec mapping results for tagged GCN5. All previously documented GCN-5-associated proteins were detected (except histone associations, which this cell preparation might not have been expected to retain), and the analysis also presented a few new reproducible candidate associations. Scale up to the remaining 24-tagged strains is in progress, as is migration and installation of the instrumentation at Caltech. The longer-term biological goals are many in our groups, but a priority for the Wold group is on transcriptional complexes, and on modifying the process it for targeting mammalian regulatory complexes and pathways.

A modest transcriptionally-oriented project in yeast will be to characterize the protein partners of all bHLH class regulators in yeast. The same tagged factors will also be used in chromatin immunoprecipitation. For studies in mammalian systems, Leslie generated a family of retroviral vectors that can be used to make transgenic mice or to mediate expression in cultured mouse or human cells. Two such vectors - one placing the dual affinity HPM tag at the amino terminus of the target protein (gene) and the other at the carboxy terminus were constructed. The lead mammalian protein for our study, the myogenic transcription factor MyoD, was introduced into both and tested for bioactivity. The carboxy-tagged MyoD was shown to have full bioactivity by the criterion of converting non-myogenic fibroblasts to myocytes following infection with the lentiviral construct. A further variation has added an IRES GFP to enable us to identify expressing cells visually, and it also showed high bioactivity. The tagged MyoD protein was shown in IP-Western analyses to bring down several expected associated proteins. We expect to begin mass spec analysis in the coming months on mammalian samples prepared from infected cells and from tissues of transgenics animals. An immediate question is whether we can detect associations that occur only on DNA (at so-called enhancesome complexes) from associations of the same proteins that do not depend on DNA (heterodimerization, for example).

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154. Developing genomic DNA as a comprehensive cohybridization standard for use in microarray gene expression measurements

Richele M. Gwirtz, Brian A. Williams

Standardization of gene expression measurements on spotted microarrays is accomplished by ratiometric quantitation. This is necessary because the mass of hybridizable material deposited on each spot is not uniform. In practice, ratiometric standardization is accomplished by simultaneous co-hybridization of the array to two samples of fluorescently-labeled nucleic acid. The general form of this ratio is that the numerator measurement contains fluorescent intensity for the experimental sample, while the denominator measurement contains fluorescent intensity for a reference sample. Subsequent comparison of multiple samples is accomplished through the common denominator RNA preparation. In any given comparison of two cell populations or experimental conditions, a number of genes will be expressed in both cell types, and will yield reliable ratiometric measurements. However, ratiometric measurements are problematic for genes that are not

expressed in the reference population (the denominator measurement). As the denominator measurements become increasingly small, the ratiometric values for the gene in question become disproportionately large and unstable.

To address the various shortcomings of RNA "standards," we are testing genomic DNA as a comprehensive microarray cohybridization standard. This standard performs three functions: 1) since genomic DNA includes all genes present in the mouse genome, it provides universal coverage of the spots on the microarray, and should avoid the problem of highly unstable denominator intensity figures in computed ratios; 2) since the vast majority of genes are represented at equimolar concentrations in genomic DNA, available sites for hybridization of labeled experimental sample will be relatively uniform and modest in intensity; and 3) genomic DNA is the same in sequence content from lab to lab and from prep to prep. RNAs are not.

For organisms with a relatively low genomic DNA complexity, such as *Arabidopsis thaliana* or *C. elegans*, or yeast, the signal expected per gene feature is expected to be robust, and we have verified that this is the case. However, for genomes of high complexity and repeat content, such as mouse and human, the signals are predictably lower. Nevertheless, after optimizing the fluorescent labeling protocol for sheared genomic DNA, we have shown that genomic DNA can be used as a reliable and reproducible "denominator" on a gene array composed of 14,000 70mer oligonucleotides. We obtain a spot coverage of greater than 95%, with median positive signals between 15 to 20 times greater than the median signal obtained from negative hybridization controls. The next step will be to move the universal DNA standard to third flour so that each experiment can deliver a direct ratio between two RNAs and universal normalization of those values to DNA. Since the concentration of each gene in DNA is known, the genomic denominator also offers the prospect of actually quantitating RNA in unknowns.

155. Defining myogenic determination and differentiation on a whole-genome scale

Brian A. Williams, Richele Gwirtz, Shuling Wang[*]

The immediate biological goal in this project is to define comprehensively the gene expression states in a model vertebrate developmental pathway. Within this goal is the aim of dissecting out responses to multiple signaling pathways that act to enhance or suppress myogenic differentiation. This pathway has three defined stable cell states linked by dynamic transitions: 1) proliferating multipotential mesodermal precursor cells that can elect the myogenic pathway or several other possibilities; 2) these multipotential precursors can be stimulated to convert to "determined" myoblasts which are proliferating unipotential muscle precursor cells; and 3) myoblasts can then be triggered to differentiate, at which point they express the genes and cellular properties of a mature muscle cell or myocyte.

The second goal is to use the data obtained to help evaluate our suite of different "clustering" algorithms to determine which ones are most useful in which ways for analyzing large-scale data of this kind (see entry from Chris Hart *et al.,* in this section). A third goal is to use the data to advance our current model of the regulatory circuitry that controls this developmental pathway. Clustered expression data is one key input in defining the circuit, which is then joined by comparative genomic DNA sequence data and protein:DNA binding studies (see entry of Tristan DeBuysscher).

Fluorescently-labeled cDNA populations representing skeletal muscle cells during several time courses of differentiation have been co-hybridized to the arrays in the presence of cDNA from each of several "reference RNAs." Biologically-relevant reference RNA sets include undifferentiated proliferating myoblasts (mono potential muscle precursor cells) and multipotential mesodermal precursor cells whose possible developmental fates include muscle cells. The comparisons of muscle differentiation courses with different reference RNA sets is designed to expose groups of genes whose expression in muscle changes relative to each of the different reference cell states. Through use of various clustering methods we have identified components of a contractile apparatus regulon. Detailed kinetics reveal that this group is subdivided into kinetic classes, and the working hypothesis is that each of these groups depends differently on input signals (calcium-dependent, FGF-dependent, insulin-dependent, etc.). These suggest, in turn, differential participation by specific transcription factors activated or repressed by each signal. The regulon was also shown to contain a number of expressed sequence tags representing previously unidentified genes in the mouse genome. To internally monitor the readout of these pathways dissected from each other, we created modified myogenic cell lines that allow us to measure the activities of individual transcription factors that we know are important in our system (MyoD family sites, MEF2 sites) to assist in attributing readout at natural complex genes. This information is then used in conjunction with computational tools to identify and categorize the *cis*-acting regulatory domains that drive genes within the regulon. Other groups of genes that belong to novel clusters have been identified and highlighted for further study, including some whose expression precedes any previously known regulator in the myogenic differentiation path way, making them candidates for hitherto unknown upstream regulatory functions.

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156. Comparative sequence analysis in vertebrate genomes: Seqcomp, FamilyRelations and MUSSA

Tristan DeBuysscher, Nora Mullaney[*]

Comparative sequence analysis in our lab is based on Seqcomp and FamilyRelations tools, developed by C. Titus Brown in Dr. Eric Davidson's lab and Tristan DeBuysscher in our lab. These tools (described in detail and available for use at http://family.caltech.edu) were

created to help identify regions of DNA that are high-quality candidates to function as *cis*-regulatory modules for nearby genes. They are based the straightforward expectation that more highly conserved domains of DNA sequence between two genomes that are at auspicious evolutionary distance from each other are good candidates for functional importance. Specifically, we expect some conserved domains located outside RNA coding regions of genes to regulate transcription by acting as promoters, enhancers, silencers, and locus control domains. Our work focuses on mammalian and, more recently, multiple nematode species with mouse and *C. elegans* as the respective reference model organisms.

The full analysis and feature set of FamilyRelations is restricted to two-way comparisons, plus a limited ability to handle three species. Our recent work focuses on the problem of many-genome comparisons, where we hope to gain further resolving power together with knowledge about genomes evolve in non-coding domains. The tool developed to view and analyze three and more genome comparisons we call MUSSA. It uses a recursive transitivity finding algorithm to analyze multiple Seqcomp files into an arbitray N-way analysis. A GUI has been developed to allow biologists to navigate the results of a N-sequence analysis using a histogram of region conservation, graphical overview of the links of highly conserved region in the manner of FR, plus tools for viewing the sequence of the conserved regions, performing simple DNA motif searches, and annotating results.

Current applications using MUSSA encompass both the myogenic regulatory pathway of interest to our lab and other genes for which we have sufficient (several tens of kilobases) orthologous sequence from multiple species. At present we are focusing on vertebrate genomes for one set of studies and, in separate but conceptually similar project, we are studying genes from four species of nematode worms in collaboration with Hiroke Shizuya and Paul Sternberg. Since many-species comparative data over >20kb regions are new, we need to answer some very basic questions: How much resolving power one can get with each additional genome being compared? How does the evolutionary distance between genomes affect resolving power? What fraction of conserved features found outside RNA coding regions contribute to transcriptional regulation of nearby genes? For example, ongoing first-pass global comparison of mouse and human genomes is revealing far more non-coding sequence similarity than most investigators expected. A basic aim in the studies for which we developed MUSSA is to learn what fraction of these conserved domains, on average, govern transcription, what fraction have some other detectable function, and what fraction may be "accidents" of evolution.

Within a region that is broadly conserved among multiple species we find, as would be expected, that divergence at some positions is species specific, while at others it is common to an order or clade. For example, one analysis in progress compares 11 species from two of the proposed placental mammial clades (III: includes rodentia, primates; IV: includes cetartiodactyla, carnivora). We find features in which divergent base pairs within a large conserved block (several tens of base pairs) are conserved within rodentia alone (or one of the other orders), conserved only within one of the two clades, or may be totally divergent for each species. Current work involves quantifying these sequence divergences and relating them to suggested evolutionary distances between the species and clades. This work is being used to refine candidate functional regions to a small set that can be experimentally tested for *cis*-regulatory functionality via lentiviral and BAC-mediated transgenesis.

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157. Identifying and testing candidate regulatory regions for genes of the myogenic regulatory circuit

Tristan DeBuysscher, Libera Berghella

Using the sequence comparisons and Family Relations tools, a dozen genes from the paraxial segmentation and myogenic regulatory circuits are being analyzed by mouse/human comparisons. Mouse BAC sequences are from sequencing performed by collaborators and the DOE Joint Genome Institute. The BACs were originally obtained from the mouse BAC library made by Hiroke Shizuya and colleagues, with screening by Mai Wang of the Mel Simon genomics group at Caltech. The conserved non-coding candidate regulatory domains are amplified by PCR and cloned into reporter vectors designed for introduction into mice by transgenesis. Because the pathways in question are active at midgestation, the transgenics can be productively assayed ~9 days after injection, giving an acceptably rapid assay compared with germline transgenesis. This project has recently been further advanced by data obtained in collaboration with Dr. Eric Green (NIH Genome Institute), which is giving us a six-genome-deep comparison of several myogenic and somitogenic pathway genes. Initial functional analysis of myogenin via multigenom sequence comparison has uncovered a highly conserved element that appears critical for expression of myogenin in adult muscle.

158. MLX – A computational framework for machine learning and data mining

Christopher Hart, Lucas Scherenbroic[*], Ben Bornstein[**], Diane Trout, Joe Roden[**], Barbara Wold, Eric Mjolsness

Genome-scale analysis, including various genome sequence features, large-scale RNA expression analysis, protein interaction maps, tissue arrays and complex collections of *in situ* images, collectively present us with the problem of large amounts of data of diverse types. The need to perform many different computational analyses on these large datasets and then to view and integrate results from multiple analyses is a common problem in bioinformatics and for end-user biologists. We have, therefore, constructed a software architecture designed to provide easy access to many machine learning algorithms and techniques and to facilitate analysis of results derived

from diverse algorithms. Using CORBA as the connection bridge, our suite of algorithms is available from several computational environments currently including mathematica, matlab, C/C++, PYTHON and Java. We have also implemented a fairly complete python application programmer interface (API), which provides a foundation for building novel analysis applications or can be used directly from the PYTHON interpreter as an interactive data analysis environment. Several of the lab's projects are now using and extending this framework.

The PYTHON API includes a powerful embeddable plotting tool, I-PLOT. I-PLOT provides for rapid construction of interactive 2D visualizations. In the case of microarray data analysis, it is critical to be able to quickly impose several data features onto a single plot. To accommodate this need, IPlot is designed to allow for the mapping of any data features onto any plot feature (i.e., color all points by cluster membership, size all data points by p-values, point coordinates determined by PCA projection of a data vector, etc.). The other major feature of IPlot is to provide interactive "clickable" linkages to any information linked to a data vector, which is accomplished using the MLX architecture.

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159. Comparison of clustering algorithms for use in large-scale gene expression analysis

C.E. Hart, Diane Trout, Sagar Damle[*], B. Bornstein[**], J. Roden[**], E. Mjolsness

DNA microarrays and other large-scale gene expression analyses generate datasets of unprecedented size and complexity. Measurements for thousands of genes across tens to hundreds of tissue types, signaling stimuli, developmental time points, genetic variants, drug doses, and the like are now routine. A pivotal step in mining large-scale gene expression data is to group genes displaying similar expression profiles and/or to group samples that are most similar to each other. A growing repertoire of clustering algorithms can be used to reveal underlying structure in other kinds of large datasets and they are rapidly being adapted and fruitfully applied to expression data. As a group, these algorithms provide the essential computational infrastructure needed to extract patterns of co-expression that are often then used, alone or in conjunction with other data, to generate hypotheses about co-regulation, biological relatedness of samples, etc. Each algorithm makes different underlying assumptions about data structure and operates by a different mechanism. These differences are expected to – and do - interact differently with data properties such as sample number, degree of sample similarity, gene number, and experimental noise. In addition, each algorithm requires the selection of distance metric and, for many, specification of the number of clusters and selection of initialization conditions. These choices also lead to differing outcomes. The end-user biologist is usually faced with a confusing array of possibilities and little guidance about relative strengths and weaknesses.

To measure and understand effects of dataset properties and algorithm properties, we constructed a framework for comparing clustering results. We then made a systematic across several major algorithms using both real and synthetic microarray data. The comparative tool framework includes a mechanism to quantitatively assess and visualize cluster overlap by generating receiver operator characteristic (ROC) curves. We also implemented, modified, normalized mutual information (NMI) and a linear assignment (LA) metrics (1) to interpret the degree of agreement and disagreement between different clusterings of the same dataset. In addition to these quantitive measures, we implemented interactive visualization tools (I-PLOT) to explore how different algorithms are organizing the data space. Thus far, we have implemented and compared: an Expectation Maximization (EM) algorithm searching for a mixture of Gaussians, K-means, phylogenetic clustering (Xclust) to which we have added a novel agglomeration step, and self organizing maps (SOMs).

Selection of the number of clusters sought is required at the outset in the majority of clustering algorithms, and we've found this choice has profound effects on the resulting clusterings that vary with the algorithm. Kmeans is exceedingly sensitive to selection of a correct cluster number (K), but correct K is almost never known to the user apriori when working with biological data. This fragility makes Kmeans, in our view, the least favored method among those tested. Determining K empirically by Monte Carlo cross-validation has been implemented for the statistically based EM algorithm, and we have found that EM itself is more robust to a range of K values than is Kmeans. Still different is XclustAgglom: When the K value is greatly over estimated, it proved remarkably robust for giving the correct overall cluster structure in many cases. However, there are notable exceptions for certain dataset structures and selections of distance metric. We have also found that several of the algorithms are quite sensitive to initialization conditions (K means is also relatively fragile in this aspect). As one might expect, most of the algorithms do well with clusters that are well separated and relatively large in size. While this is useful at some level, a feature of most real biological datasets is the existence of relatively small important clusters, and of functionally important clusters that are not greatly separated from all other data vectors. We are currently addressing these specific issues.

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160. pMesogenin1 regulates paraxial mesoderm specification and body segmentation in ***Xenopus***

Jeong Kyo Yoon[*], *Shuling Wang*[**], *Randall T. Moon*[***]

Paraxial mesoderm in vertebrates gives rise to all trunk and limb skeletal muscles, the trunk skeleton, and portions of the trunk dermis and vasculature. A defining characteristic of all vertebrates is metameric segmentation of musculoskeletal and peripheral nervous systems. This body plan arises from primary segmentation of the paraxial mesoderm into tissue blocks called somites. The bHLH class gene we have named *pMesogenin1* (isolated earlier as the E-protein heterodimerization partner from a two-hybrid screen in yeast), is specifically expressed in presomitic mesoderm in the as yet unsegmented block of paraxial mesoderm in mouse and in *Xenopus*. A striking feature of *pMesogenin1* expression is that its expression terminates abruptly in the presomitic mesoderm shortly before formation of the next somite (somites are formed continuously in a rostral to caudal gradient). Its closest relatives, MESP1 and MESP2, are expressed just after pMesogenin1 is shut down and just before the next somite forms. Thus, the bHLH subfamily of pMesogenin and MESP1/2 collectively define discrete but highly dynamic prepatterned subdomains of the paraxial mesoderm. The pattern of expression for pMesogenin1 is consistent with a functional role in specifying one or more aspects of paraxial mesoderm phenotype, and in this project I set out to test this possibility using both gain-of-function and loss-of-function experimental designs. In collaboration with Randy Moon, I found that *pMesogenin1* from either mouse or frog can efficiently drive non-mesodermal cells to assume a phenotype with molecular and cellular characteristics of early paraxial mesoderm. The assays were performed in *Xenopus* embryos or embryo explants that had been injected in early cleavage stages with RNA encoding pMesogenin1 or control RNAs of interest. Among genes induced by added *pMesogenin1* is *XWnt-8*, a signaling molecule that induces a similar repertoire of marker genes and a similar cellular phenotype. Additional target genes induced by *pMesogenin1* are *ESR4/5*, regulators known to play a significant role in segmentation of paraxial mesoderm. *pMesogenin1* differs from other known mesoderm-inducing transcription factors because it does not also activate a dorsal (future axial) mesoderm phenotype, suggesting that *pMesogenin1* is involved in specifying paraxial mesoderm. In the context of the intact frog embryo, ectopic *pMesogenin1* also actively suppressed axial mesoderm markers and disrupted normal formation of notochord. In addition, we found evidence for cross-regulatory interactions between *pMesogenin1* and T-box transcription factors, a family of genes normally expressed in a broader pattern and known to induce multiple types of mesoderm. Based on our results and results from prior studies of related bHLH genes, we propose that *pMesogenin1* and its closest known relatives, *MesP1/2* (in mouse) and *Thylacine1/2* (in *Xenopus*), comprise a bHLH subfamily devoted to formation and segmentation of paraxial mesoderm.

To further define the function of pMesogenin1 in paraxial mesoderm formation, I created a germline deletion of mouse *pMesogenin1* with the help of Shirley Pease and her colleagues in the Transgenic Mouse Facility. *pMesogenin1* homozygous knockout embryos show a complete failure of trunk and tail somite formation and segmentation of the body trunk and tail. Although several genes, including members of the famous Notch/Delta intercellular signaling apparatus, have previously been shown to partially disrupt segmentation, none have a phenotype of complete blockade, as is seen in the pMeosgenin1 animals. At the molecular level, the phenotype features dramatic loss of expression for components of the *Notch/Delta* pathway and the oscillating "somitic clock" genes that are thought to control segmentation and somitogenesis. The presumptive paraxial mesoderm also fails to execute patterning and specification steps, leading to a complete absence of all trunk paraxial mesoderm derivatives, which include skeletal muscle, vertebrae and ribs. We infer that *pMesogenin1* is an essential upstream regulator of trunk paraxial mesoderm development and segmentation in vertebrates.

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161. Isolation of target genes for pMesogenin1

Jeong Kyo Yoon[*], *Jason Chua*[**]

Functional analyses suggest that pMesogenin1 is a major regulator of paraxial mesoderm development and segmentation, based on mouse knockout and frog gain-of-function studies. We postulate that it functions at the molecular level, as do other bHLH proteins, as a sequence specific DNA-binding protein. Based on precedents from other bHLH genes that have been studied in detail, it could act as a repressor, as an activator (the most commonly reported activity for these regulators), or as either one, depending on context in a given target gene. This raises the question of what genes are direct downstream targets of pMesogenin1 regulation and which genes are indirectly regulated by it. To address these questions, we generated recombinant DNA constructs that encode chimeric proteins in which the ligand-binding domain of either glucocorticoid receptor (GR) or estrogen receptor (ER) was fused to the carboxy terminus of pMesogenin1 protein. Normally, native GR or ER function as transcription factors in the presence of their respective ligands, but they are inactive in their absence. When no hormone is bound, they are complexed with heat-shock protein (HSP). These complexes are transcriptionally inactive and are localized mainly outside the nucleus. However, upon exposure to their corresponding hormones, GR and ER are released from their HSP complexes, relocalize and function in the nucleus. Subsequently the

hormone-activated native ER and GR activate their transcriptional target genes by binding to specific DNA sequences via their DNA-binding domains. Similar to native GR or ER proteins, it has previously been shown that chimeric proteins containing the hormone-binding domains (but not the ER or GR AND binding domain) reside in the cytoplasm, and can be translocated into the nucleus upon hormone treatment. Therefore, the functional activity of the chimeric protein as a transcription regulator can be controlled in a hormone-dependent manner.

To identify target genes, the chimeric protein will be expressed in both mouse cell culture and animal cap explants culture from *Xenopus* embryo. Then, RNA will be isolated from both naïve and cells/explants treated with hormone. An important option afforded by this hormone-dependent chimeric design, is separation of direct transcriptional targets from secondary and tertiary targets. Thus, if hormone is added in the presence of a protein synthesis inhibitor, direct targets can be up or down-regulated. If hormone is added in the absence of a protein synthesis inhibitor, then primary, secondary and more indirect targets will all be affected. A microarray gene expression analysis will be applied to begin to identify genes that are either induced or repressed by the chimeric proteins in the context of appropriate cells from null animals.

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162. Expression and functional analysis of ***FP1*** during mouse development

Jeong Kyo Yoon[*], Shuling Wang[**]

Expression of *T117* RNA is first detected in the rostral floor plate of the neural tube in e8.0 mouse embryos. Expression is extended more caudally as the embryo develops. Expression of two previously known floor plate markers, *HNF-3beta* and *shh*, precedes that of *T117* in the floor plate, suggesting that *T117* may act downstream of *HNF-3beta* and *shh*. It is noteworthy, however, that unlike shh and HNF3beta RNAs, which are also expressed in other axial domains, T117 appears highly specific for the floor plate and for this reason has been named *Floorplate1 (FP1)*. The floor plate is a very interesting ventral structure in neural tube that produces important signaling molecules such as SHH and netrins function as patterning signals for of CNS neurons and for some other axial domains as well. In order to investigate the functional role of FP1 *in vivo*, both "gain-of-function" and "loss-of-function" studies are in progress. First, the FP1 bHLH coding region was engineered for ectopic expressed in the dorsal neural tube using either *Wnt-1* or *En-2* enhancer/promoters, and these have been introduced into fertilized eggs to generate transgenic embryos. The phenotypic consequences of ectopic expression, at least at the levels provided by these constructs, were unremarkable. For a loss-of-function study, the *FP1* gene was disrupted in mouse ES cells and knockout mice were generated. The initial phenotypic analysis revealed that homozygous knockouts are also largely normal in development of the floorplate and of cells known to be affected by signals from the floorplate. However, a more detailed analysis will be needed to determine whether neuronal specification or pathfinding may have been altered in more subtle ways. We isolated a presumptive ortholog for FP1 has also been isolated from *Drosophila*, based on notable similarities in the bHLH domain protein coding sequences of the two genes. Preliminary *in situ* hybridization studies indicate that it is expressed in a specific subset of glial cells.

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163. Role of a homeogene, ***msx-1*** in adult muscle regeneration

Libera Berghella

Regeneration of adult skeletal muscle after an injury or disease is understood to be mediated primarily by muscle satellite cells. In an intact muscle this population of cells is composed of rare, mononucleate cells located beneath the basal lamina of the mature fiber. In healthy adult muscle the satellite cells are mitotically quiescent. Muscle injury triggers their metabolic and mitotic activation. They emerge from G_0 mitotic arrest, proliferate and concomitantly undergo a series of developmental changes. This pathway ultimately ends, for the majority satellite cell progeny, in differentiation and fusion to form new myofibers or differentiation coupled with fusion into existing fibers. However, some cells apparently have a different fate. Instead of differentiating as muscle fibers, they return to replenish the satellite pool in order to support future regeneration.

Biochemical and molecular analysis of muscle regeneration has, in the past, been hampered by the rarity of satellite cells and the absence of reliable molecular markers for these cells, especially when they are in the quiescent state. Prior work in this lab led to a method of studying gene expression in individual satellite cells over the timecourse of an activation response (Cornelison and Wold, 1997; Cornelison *et al.*, 2000). Among the genes studied, the homeobox gene *msx-1* revealed a peculiar and interesting expression pattern, with transcripts being detectable only at extremely early timepoints (<30 minutes post-fiber isolation). This suggested that quiescent cells express *msx-1* but quickly downregulate its expression once they are activated. It is also known that forced expression of *msx-1* in cultured myoblasts inhibits differentiation and can suppress MyoD expression (Woloshin *et al.*, 1995). Based on these and other preliminary results, our working hypothesis is that *msx-1* expression marks satellite cells that are in quiescence or are returning to it, and that it may be causal in suppressing expression of MRFs in quiescent satellite cells.

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164. Expression profiling of stem-cell lines

Libera Berghella, Sagar Damle[*]*, Brian Williams, Guilio Cossu*[**]

In collaboration with the lab of Giulio Cossu at the University of Roma, we have initiated a large-scale gene expression characterization of cell lines isolated from the embryonic dorsal aorta of mice. The special interest in these cells comes from their capacity to differentiate into muscle, cardiocytes, bone, or other derivatives when grown under various culture conditions. This mimics the *in vivo* potential of dorsal aorta multipotential cells implanted into a host animal via transfusion into the blood. Such transplanted cells can subsequently be found in mature muscle fibers, and in other tissues as well. Thus, the isolation and initial characterization of this cell line supports the hypothesis that multipotent mesenchymal cells may be present embedded in vascular endothelium (De Angelis *et al*., 1999). Using cDNA microarrays made in the lab, I have begun to analyze the transcription program of these cells in comparison with more conventional myogenic cell lines that are believed to be unipotential and myogenic (C2C12, MM14), or multipotential (10T1/2), or totipotent (embryonic stem cells). Results have revealed genes expressed in common only with 10T1/2; or with 10T1/2 and ES cells, but not myoblast lines. Other genes are novel to one or both of the aorta lines, whose developmental potentials are only partially overlapping. Among genes in common with stem-like cells is CD34, a putative marker of stem cells in several other contexts. These results are being verified by independent methods, and the impact of the compound trichostatin (a global pharmacological modifier of chromatin access) are next to be tested and correlated with phenotypic impact.

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Reference
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166. A new method for assaying gene expression

John Murphy[*]*, Barbara Wold, Brian Williams, David Tirell*[*]*, Mark Davis*[*]

Gene microarrays are proving to be very powerful for large-scale gene expression analysis. However, they have significant shortcomings that we would like to bypass. They generally call for relatively large amounts of input RNA to achieve robust answers, and they are at their best with more abundantly expressed RNAs. They also have dynamic range limits that are considerably narrower than those of the RNA samples to be measured. In all their manifestations so far, they are semiquantitative, even under the best of circumstances. From an entirely different kind of methodology (multiplex single-cell RT-PCR; (Cornelison and Wold, 1997) we know that being able to simultaneously monitor multiple genes in single cells gives a different and illuminating view of the gene expression combinatoric "states" that one cannot achieve using pooled cell samples. However, the latter assays are thus far quite limited in simultaneous gene number (maximally seven simultaneous genes thus far), and they also give little information about the quantity of each RNA type in an expressing cell. It is clear that in many biological settings it would be highly desirable to get good quantitative measurements from just one or a few cells per determination for tens, hundreds, or even thousands of genes simultaneously.

To address this challenge, we are developing an approach that takes advantage of specifically-designed families of chemical tags. Oligonucleotides, each designed to hybridize to a specific RNA specie of interest, are synthesized such that each oligo carries a unique covalently-attached peptoid (not peptide) tag with a unique mass. At the end of hybridization, chemical methods are used to separate hybridized oligos from those that do not react with a complementary RNA, and then tags are cleaved from their respective oligonucleotides. In these methods, the tags, rather than the RNAs or oligos, are ultimately counted. The first method of choice for analysis is mass spectrometry. We expect that this approach will permit direct RNA quantitation over several orders of magnitude, and it has the potential to begin with very small input RNA samples. A virtue of the design is that it uses hybridization in which all components are in solution phase, and these reactions are considerably better understood and more efficient than is nucleic acid hybridization in which one member is in solid phase, as is the case for microarrays. Multiple designs have been tested for linking the oligonucleotide moiety to the peptoid tag and robust strategy has been identified. Isotopic ratioing has also been worked out to allow precise quantitation. Final proof of principle using well-defined RNA targets is in progress.

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Molecular, Cellular and Integrative Neuroscience

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Summary: This year marks the shift in our laboratory's focus to the neurobiology of social decision-making. Fronto-insular (FI) cortex (abstract 168) is strongly implicated in decisions involving risk, uncertainty, and social emotions and contains a population of large bipolar neurons which are unique to humans and African apes. This implies a potential site for recent evolutionary change in the circuitry involved in risky and emotion-laden decision-making. We have also been collaborating with the Caltech experimental economists in functional imaging studies of the brain structures involved when subjects bid at auctions and how neurologically healthy elderly subject make economic decisions (abstract 169 and abstract 170).

166. Frontal cortex fraction increases with brain size in primates but not carnivores

Eliot Bush, John M. Allman

Overall size has an enormous influence on brain structure. Some structures increase more slowly, and some more quickly than the brain as a whole. We have measured the volume of frontal gray matter in a sample of 23 primates and 13 carnivores. We find the ratio of frontal cortex to subcortical structures increases about seven fold over the primate size range. There is no size dependence in the carnivores. When the effect of size is removed in primates, frontal cortex size does not bear a strong relationship with a number of other variables, including diet, social structure, basal metabolic rate, female sexual maturity and gestation length. What causes this size dependent relationship? It has been proposed that scaling in the visual system relates to the type of neural representation held at various stages of processing. In general brain regions that must represent many different kinds of information may scale up compared to the rest of the brain. Scaling in primates may reflect the development of novel networks in the frontal cortex that are involved in planning and the management of resources and social relationships over time. These regions may increase disproportionately with brain size as a byproduct of their structure.

167. The spindle neurons of frontoinsular cortex (Area FI) are unique to humans and African apes

John M. Allman, Nicole Tetreault, Atiya Hakeem, Katarina Semendeferi[*]

Von Economo described a class of elongated bipolar cells, the spindle neurons, in layer 5 of area FI, which is located in the posterior part of orbitofrontal cortex. After close examination of the orbitofrontal and insular cortex in 25 primate species, we have found that the spindle neurons are present only in humans and African apes. We found area FI containing spindle neurons in humans, gorillas, chimpanzees and bonobos. We were not able to detect significant numbers of spindle neurons in the Asian apes (orangutans and gibbons), nor were we able find them in Old World monkeys (baboon, langur, macaque, mandrill, and mangeby, nor in New World monkeys (tamarin, owl, howler, squirrel, spider, and titi monkeys), nor in prosimians (mouse lemur, hapalemur, loris, potto, propithecus, ring tail lemur, slow loris, and tarsier). Since humans and African apes comprise a monophyletic group, the spindle neurons in FI are likely to be a derived specialization within this group that originated about ten million years ago.

We have used stereological sampling to determine the number of spindle neurons in FI in humans and African apes. The FI spindle neurons are 5 to 40 times more abundant in the adult human than in the apes. The FI spindle neurons are about 24% more numerous in the right hemisphere of both humans and apes. This asymmetry suggests a hemispheric specialization involving social emotions that evolved in apes. The spindle neurons appear to arise postnatally. A few are present in FI in 4-month old human infants. In the seven-month infant there are about 20% of the adult number and they are restricted to the lateral third of the area corresponding to area FI in adults.

Functional imaging studies indicate that area FI is activated by situations involving uncertainty, incongruity, infant cries, embarrassment, guilt, resentment, and deception. We suggest that the spindle neurons may relay to other parts of the brain a signal related to the commission or recognition of error, particular in social behavior, and may participate in the mounting of adaptive responses to errors.

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168. Neurotransmitter receptor expression in the spindle cells: A class of neurons unique to humans and apes

Karli K. Watson, Nicole A. Tetreault, Sarah P. Teegarden[*]*, John M. Allman*

The spindle cells comprise a class of large neurons that emerged recently in hominoid brain evolution. They are present in humans and apes, but not in

any other species surveyed. These neurons exist in layer 5 of the anterior cingulate (ACC) and frontoinsular (FI) cortex, the latter of which is located in posterior orbitofrontal cortex (see Allman *et al.*, 2003). We used immunocytochemical techniques to reveal the expression of various receptors on the spindle cells. The spindle neurons and their apical and basal dendrites are strongly labeled with the antibody to the dopamine D3 receptor, which implies a role in mechanisms of reward. Many spindle cells are labeled by the antibody to Trk B, the receptor for the neurotrophin, BDNF, which protects against apoptosis. BDNF also controls the expression of dopamine D3 receptors (Guillin *et al.*, 2002). Possibly related to this evidence for dopaminergic input to the spindle neurons is the recent observation that the activity of both ACC and FI increases with uncertainty during the delay period between placing a bet and receipt of reward or penalty (Critchley *et al.*, 2002). The spindle neurons are strongly labeled with antibodies to the serotonin 1B and 2B receptors. Serotonin 1B receptor agonists are known to reduce impulsive aggression in other species (de Almedia *et al.*, 2002). Thus, activation of the serotonin 1B receptors may enhance self-control in the face of the challenges of risk, negative feedback or error. The serotonin 2B receptor is rare in the brain but common in the heart and intestines (Baumgarten and Gothert, 1997).

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169. An fMRI study of selling strategy in second price auctions

David M. Grether[1], Charles R. Plott[1], Daniel B. Rowe[2], Martin Sereno[3], John M. Allman

Subjects participated in an auction, which contains essential, strategic elements of economic theory. The auction is simple in concept and the behavior exhibited by subjects can be compared directly to the (game) theoretical solution. After trial and error, subjects' decisions typically conform to the theoretical solution, a phenomena known in economics as equilibration. The question posed by the research concerns the mental activity that might accompany such equilibration. After sixteen auctions subjects were told the strategy that will maximize their revenues, and then sixteen more auctions were performed. We found significant activation in the frontal pole especially in Brodmann's area 10, the anterior cingulate cortex, the amygdala and the basal forebrain. There was significantly more activation in the basal forebrain and the anterior cingulate cortex during the first sixteen auctions than in the second sixteen. The activity in the amygdala shifted from the right side to the left in the first sixteen versus the second sixteen auctions.

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170. Aging and decision-making: A broad comparative study of decision behavior in neurologically healthy elderly and young individuals

Stephanie Kovalchik, Colin F. Camerer[*], David M. Grether[*], Charles R. Plott[*], John M. Allman

We investigated behavior in a series of economic decisions with two populations, one of healthy elderly individuals (with average age 82) and one of younger students (average age 20). We examined confidence, risk-aversion, the endowment effect, and the theory of mind (strategic thinking). Our findings indicate that the older adults' decision behavior is similar to that of young adults, contrary to the notion that economic decision making is impaired with age. Moreover, some of the decision behaviors suggest that the elderly individuals are less biased than the younger individuals. There is a greater prevalence of overconfident behavior in the younger population. In addition, our findings suggest that the risk behavior of older adults is more moderate and adaptive than the younger population: the older subjects are more risk seeking in a task of incomplete information but more risk-averse in a task with complete information. In addition, gender was significantly correlated to risk behavior in the older population, with men having a greater aversion to risk than women. Both populations perform similarly on the theory of mind task although there is a modest indication of a higher incidence of confused behavior in the older adults in this task. Furthermore, our results show no significant endowment effect in either population.

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Summary: Neural mechanisms for visual-motor integration, spatial perception and motion perception. While the concept of artificial intelligence has received a great deal of attention in the popular press, the actual determination of the neural basis of intelligence and behavior has proven to be a very difficult problem for neuroscientists. Our behaviors are dictated by our intentions, but we have only recently begun to understand how the brain forms intentions to act. The posterior parietal cortex is situated between the sensory and the movement regions of the cerebral cortex and serves as a bridge from sensation to action. We have found that an anatomical map of intentions exists within this area, with one part devoted to planning eye movements and another part to planning arm movements. The action plans in the arm movement area exist in a cognitive form, specifying the goal of the intended movement rather than particular signals to various muscle groups.

Recently we have begun a project attempting to develop a neural prosthesis for paralyzed patients. This prosthetic system will be designed to record the electrical activity of nerve cells in the posterior parietal cortex of paralyzed patients, interpret the patients' intentions from these neural signals using computer algorithms, and convert the "decoded" intentions into electrical control signals to operate external devices such as a robot arm or a computer to surf the internet. Currently we are testing whether intentional signals can be deciphered by recording the activity of single cells in the reach region of healthy monkeys. We have found that we can predict where an animal plans to reach in a task in which he is instructed to plan to reach in one of two directions. We provided feedback to the animal regarding this prediction, and the animal quickly learned that he could receive his juice reward at the end of each trial by simply thinking about reaching in the correct direction without actually reaching. We are currently expanding this research by recording from multiple cells using arrays of implanted electrodes. This approach allows us to increase the number of potential reach directions that can be decoded. In principle this approach could be used to record from the posterior parietal cortex of patients with paralysis, enabling them to operate external devices with their movement thoughts.

Our laboratory also examines the coordinate frames of spatial maps in cortical areas of the parietal cortex coding movement intentions. We have discovered that plans to reach are coded in the coordinates of the eye. This is a particularly interesting finding because it means the reach plan at this stage is still rather primitive, coding the plan in a visual coordinate frame rather than the fine details of the forces for making the movement. We have also discovered that when the animal plans a limb movement to a sound, this movement is still coded largely in the coordinates of the eye. This finding indicates that vision predominates in terms of spatial programming in primates.

Another major effort of our lab is to examine the neural basis of motion perception. One series of experiments is determining how optic flow signals and efference copy signals regarding eye movements are combined in order to perceive the direction of heading during self-motion. These experiments are helping us understand how we navigate as we move through the world. A second line of investigation asks how motion information is used to construct the three-dimensional shape of objects. We asked monkeys to tell us which way they perceived an ambiguous object rotating. We found an area of the brain where the neural activity changed according to what the monkey perceived, even though he was always seeing the same stimulus. In other experiments we have been examining how we rotate mental images of objects in our minds, so-called mental rotation. In the posterior parietal cortex we find that these rotations are made in a retinal coordinate frame, and not an object-based coordinate frame, and the mental image of the object rotates through this retinotopic map.

We have successfully recorded functional magnetic resonance signals following visual activation of the monkey brain. This finding is important since this type of experiment is done routinely in humans and monitors the changes in blood flow during different cognitive and motor tasks. However, a direct correlation of brain activity with blood flow cannot be achieved in humans, but can in

monkeys. Thus, the correlation of cellular recording and functional MRI activation in monkeys will provide us with a better understanding of the many experiments currently being performed in humans.

171. FMRI investigation of real and imagined visually guided movements

Kyle A. Bernheim, David J. Dubowitz, Igor Fineman, Dan Rizzuto, Richard A. Andersen

A diverse group of cortical areas are responsible for planning and executing any visually guided movement. Pathology or disease may disrupt one or more of these areas, leading to a functional deficit. To begin to understand what such a deficit may mean in terms of cortical function, we present fMRI data on the variable activation of cortex during real and imagined visually guided movements. Normal healthy subjects were instructed to fixate at a central target and make direct real or imagined pointing movements to one of eight radial targets. The targets were presented at eight degrees eccentricity in a randomized block paradigm. Instruction for the action to be performed was conveyed through a color change on the fixation point. The absence of motion artifact was verified through offline analysis. Robust, lateralized activation was observed during the real movements in contralateral premotor, motor and supplementary motor cortex, as well as along the medial bank of the intraparietal sulcus (IPS). Activation during the imagined movements was weaker, with a correlated response present along the IPS and in primary motor cortex. These data will provide a baseline for examining cortical activations in paralyzed patients while they imagine movements. These activations in patients can be used for targeting the implantation of neural prosthetics. Also we will examine whether the parietal reach region, which receives major visual input, remains relatively intact after paralysis.

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172. Moveable electrodes for autonomous cell isolation and tracking: Agorithm, experiments and hardware

Edward Branchaud, Zoran Nenadic, Daniella Meeker, Jorge Cham, Richard A. Andersen, Joel W. Burdick

Both acute and chronic multi-electrode extracellular recordings are becoming an increasingly important method for the study of populations of neurons and for the acquisition of signals needed for brain-machine interfaces. For acute recordings, human operators manually advance electrodes into tissue and position them with micron accuracy. To maintain signal quality in the presence of small tissue and electrode migrations, the electrodes are often repositioned during the course of the experiment. However, beyond a handful of electrodes, this practice is nonoptimal and perhaps intractable. To overcome these difficulties, we have developed and tested an algorithm for the autonomous isolation and tracking of neural signals. The algorithm is based on finding, by stochastic gradient ascent, the maximum of a 'cell isolation curve' - a reconstruction of a signal quality metric as a function of position. The algorithm was initially developed and tested using a simulation of the extracellular field of a small population of neurons, and then experimentally verified by autonomously isolating cells in the posterior parietal cortex of *Macaca mulatta*. Our experiments show that the algorithm autonomously isolates the best signal along a linear track. The algorithm includes detection of action potentials, alignment, clustering, and computation of a signal quality metric.

Our movable probe concept should also benefit chronic multi-electrode recordings, which are plagued by poor cell isolation and lifetimes shortened by tissue encapsulation. Chronic movable electrode systems show promise in overcoming these limitations. We are building such a chronic drive that incorporates four independently controllable electrodes (driven by miniature piezoelectric motors). This device will continually and autonomously track and isolate neural signals over long periods of time.

173. Coding of saccades to symmetrical objects

Boris Breznen, Michael Campos, Richard A. Andersen

We recorded single-unit activity in the lateral intraparietal area (LIP) of two macaque monkeys while they performed an object-based saccade task with a symmetrical object. The left or right side of an isosceles triangle was briefly cued and the triangle subsequently disappeared and reappeared after a translation and rotation. The task was to saccade to the previously cued side of the triangle. For each cell we mapped the full 2-dimensional retinotopic receptive field using a memory-guided saccade task without an object and then collected the responses in the object-based task. We found that the majority of the recorded cells were tuned for the target position in retinotopic coordinates (79% of all recorded cells). Some of the cells tuned for target position maintained the tuning in both tasks (30%). In some cells the target tuning was task specific i.e., we found cells tuned for retinotopic target position in the memory saccade task that were not tuned in the object saccade task(21%) and cells tuned for target position in the object-based saccade task that were not tuned in the memory saccade task (28%). Additionally, we found subsets of the cells that represented the orientation of the object (21% of all recorded cells), and the location of the target on the object (25%). For most of these cells the effects were modulatory i.e., the target tuned responses were modulated by the left or right position of the target on the object or by the orientation of the object on the screen. Some cells were not retinotopically tuned and yet they showed the effect of object orientation (6% of all recorded cells) or position of the target on the object (also 6%). These effects however were spatially confined and did not generalize across space. Our results suggest that LIP encodes all relevant variables necessary for solving the task but no cells

showed object-centered tuning independent of retinal location.

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174. Models of coordinate transformations for reaches toward an auditory target

Marina Brozovic, Yale E. Cohen, Richard A. Andersen

We present a theoretical study on the process of reaching toward auditory targets in the Parietal Reach Region (PRR). The experimental data show (Cohen and Andersen, 2000), that although the sound is originally coded in the head reference frame, 42% of the PRR neurons code the position of auditory targets in an eye-centered reference frame and an additional 13% code in the intermediate eye-head reference frame. Most of these neurons expressed large, complex receptive fields, which are challenging to reproduce with current theoretical models. To address the question of how these coordinate transformations are executed in the parietal cortex, we compared the spatial pattern of the observed receptive fields with the model-predicted receptive fields using two different network implementations. The first model was a three layer neural network with sigmoid transfer functions in the hidden layer and output maps representing several different reference frames. The second model was the basis function network, which uses simple linear combinations of basis set neurons to convert sound into any desired reference frame. We proposed a set of criteria for estimating how well a given model predicts the observed data. Preliminary results suggest that the three-layer network model reproduces the shape of the receptive and gain fields more accurately than the basis function model.

175. The role of area 5 somatosensory input in visuomotor transformations for reaching

Chris A. Buneo, Richard A. Andersen

We are currently exploring the role of area 5 in visuomotor transformations for reaching. Single-cell recordings were obtained from one monkey during the performance of an instructed-delay reaching task. Reaches were made from four starting positions to each of four target positions in two partially overlapping workspaces. Gaze direction was held fixed in each workspace so that reaches were made between the same starting and endpoints in eye coordinates. Starting position, target position and/or workspace effects were observed in 80% (N = 76) of the neurons during the delay period (3-factor ANOVA, $p<0.05$). The manner in which starting position and target position were encoded varied with recording depth, as did the proportion of cells with workspace effects. In the most ventrally located cells within the intraparietal sulcus, targets and starting positions were encoded separably and activity was best correlated for the same positions in eye coordinates, even though vision of the starting position was absent. Approximately 50% of these cells showed workspace effects, which manifested as a gaze modulation of an eye-centered map. In the most dorsally located cells, activity primarily reflected the difference between starting positions and target positions. Ninety percent of these cells showed workspace effects, which were largely due to differences in firing rate for movements performed at the extremes of the two workspaces. Analysis of data from the control period of the task, i.e., before target presentation, suggests these differences reflect a planning of limb trajectories in intrinsic rather than extrinsic coordinates. These results support a dual role for the somatosensory map that is known to exist in area 5. This map appears to exist to facilitate the transformation of hand position into an eye-centered coordinates, as well as to enable a transformation of early reach plans from eye-centered coordinates into an intrinsic, arm-centered frame of reference.

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176. Reward expectancy in dorsomedial frontal cortex of the macaque monkey

Michael Campos, Boris Breznen, Richard A. Andersen

We recorded neural activity from the dorsomedial frontal cortex of two macaque monkeys during the performance of memory guided and object-based saccade tasks. Target locations in both tasks were identical, and event defined intervals could be readily compared across tasks. In about 75% of the recorded neurons we observed a burst of activity during the interval following the instructed saccade in both tasks. The majority (65%) of these neurons also showed a shift in the onset time of this burst from one task to the other. The burst occurred immediately after the target-acquiring saccade in the object-based task, but with a ~250ms delay in the memory guided task. The timing of the burst corresponded to the appearance of the visual feedback that indicated to the monkey that he successfully completed the task. Furthermore, in successful trials the burst terminated with the delivery of the reward, but in error trials, in which the monkey attempted the proper saccade but was not rewarded, the burst was sustained for up to two seconds. We interpret these results to mean that the burst activity in these cells reflects an expectation of a reward, and that it persists until the reward is obtained.

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177. Representation of reward expectancy in the medial bank of the intraparietal suclus: Implications for neural prosthetics

Brian Corneil, Sam Musallam, Richard A. Andersen

Results from our laboratory have demonstrated the feasibility of using intended reach plans recorded from the medial bank of the intraparietal sulcus (mIPS) for controlling neural prosthetics. The use of the mIPS in prosthetic control provides a number of advantages, including the potential to drive learning via visual inputs in

the absence of movement. Here, we demonstrate that reach plans within the mIPS are modulated by reward expectancy, and that such modulation can be utilized to improve the decoding of intended movement plans.

Two monkeys implanted with chronic microwire arrays of 64 electrodes within mIPS performed a memory-guided reach task. The monkeys were required to reach to the remembered location of a briefly flashed cue (300 ms) after a 1.2-1.5 s delay interval. The size of the cue (small or large), conveyed either the reward magnitude (low or high) or the probability (40% or 80%), that a reward would be delivered, and the mappings of cue size to reward expectancy were interleaved. We observed robust variations in the delay-period activity of many mIPS neurons with different reward expectancies: high-reward expectancy trials were associated with both increased firing rates in all directions and an increased depth of tuning. These results demonstrate the encoding of reward expectancy within mIPS, consistent with previous reports from the lateral intraparietal area.

To examine the influence of reward expectancy on the efficiency of decoding reach plans from the mIPS, maximum likelihood estimates of intended reach directions were calculated off-line using delay-period activity segregated by expectancy. High-reward expectancies were associated with improved decoding of the intended reach location. The representation of high-level cognitive processes such as reward expectancy within the mIPS make it an advantageous target for neuro-prosthetic devices in humans who could control such cognitive processes volitionally.

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178. Real-time control of a cursor using multi-electrode arrays implanted in the medial bank of the intraparietal sulcus

Sam Musallam, Brian Corneil, Rajan Bhattacharyya, Daniella Meeker, Richard A. Andersen

A novel feature of our approach to developing a neural prosthetic for paralyzed patients is to extract cognitive control signals from the medial bank of the intraparietal sulcus (mIPS). A major sensory input to this area is visual, which is a viable source of feedback signals since it remains relatively intact after paralysis. Here we show that, using signals from the mIPS, monkeys can control the placement of a computer cursor with their thought in real time. Single and multiunit activities were simultaneously recorded from 48 out of 64 electrodes implanted chronically in the mIPS of two monkeys. During reach trials, the monkeys performed 200 delayed fixation reaches to six targets; after the monkeys acquired a central fixation point (FP), a peripheral cue appeared in one of six locations for 300 ms. Following a delay period of 1.5-1.8 seconds, the monkeys reached to the remembered cue location without breaking fixation. Feedback trials commenced in a similar fashion to reach trials. We used the delay-period activity spanning 200-1000 ms after cue offset to decode the monkey's intended reach. During this delay period, the maximum likelihood estimate of the intended reach direction was calculated, and a computer cursor appeared at the endpoint of the estimated reach. The monkeys were rewarded only when the cursor appeared in the correct position. Online performance peaked at 58% for six targets for an entire session (1000 trials), and at 77% for six targets for an average of 50 trials. Off-line decode on 200 trials resulted in a performance of 75%. These results show that cognitive signals from mIPS can be used to control external devices. Signals from other cortical areas encoding additional reach variables could be used in combination with the mIPS signals to augment prosthetics operations.

This work was supported by DARPA, HFSP, NIH, Boswell Foundation.

179. Robust unsupervised detection of action potentials using the wavelet transform

Zoran Nenadic, Joel W. Burdick

Our current understanding of the neural code relies on the assumption that information is carried in a train of action potentials. Hence, methods that can reliably separate action potentials from background noise under different scenarios [e.g., variable signal-to-noise ratios (SNR), firing rates (FR), sampling frequencies, etc.], are crucial to a proper interpretation of neural recordings. Most of the existing spike detection algorithms lack sufficient robustness, therefore periodic human intervention is required. We demonstrate how a continuous wavelet transform combined with basic signal detection and decision theory can be used for spike detection in a robust and unsupervised manner. The performance of our detector was compared against other methods, such as amplitude thresholding and power detection, and was assessed using the classical receiver operating characteristic. Cross-validation was performed on a synthesized data obtained by randomly arranging spike templates in time with the addition of neural noise whose variance is determined by SNR. The spike templates and neural noise were obtained from recordings from the posterior parietal cortex of *Macaca mulatta*. We found that our wavelet detection method (WDM), out performed other detection methods across wide range of SNR and FR. The difference in performance is particularly apparent under low SNR and FR, a situation commonly found in everyday recordings. We also found that the WDM showed more consistent behavior than other methods, which makes it more suitable for unsupervised detection. Because of its computational efficiency, the WDM can be executed almost in real-time. A simple MATLAB code together with a short tutorial is located at: http://robotics.caltech.edu/~zoran/research/detection.hmtl and its use by practicing neuroscientists is strongly encouraged.

180. Reach plan activity in macaque posterior parietal cortex during hand-eye coordination

Bijan Pesaran, Richard A. Andersen

Studies in humans have shown reaching to visual targets occurs naturally as part of a coordinated hand-eye movement. While there are few studies of hand-eye coordination in monkeys, studies of reaching with fixation have shown the posterior parietal cortex (PPC) represents reach plans in visual coordinates. To study the reach plan in PPC under more natural conditions, we trained two monkeys to perform a task on a touch-screen involving a reach search for juice rewards. Monkeys were presented with three identical reach targets one of which contained a reward. The reward target was assigned randomly each trial and they made reaches until they found it. We chronically implanted multielectrode arrays in the medial bank of the intraparietal sulcus (mIPS, 2x32 electrodes), and area 5 (A5, 1x32 electrodes), in each of two monkeys and recorded position as they performed the task. Analysis of behavior showed the monkeys looked at their hand at the end of each reach. Surprisingly, they also looked at their hand at the beginning of each reach despite intervening saccades. This could only be reliably broken by explicitly rewarding the monkey for not looking at his hand. Activity in both mIPS and A5 showed strong spatial tuning. This tuning became significant after the eye position stabilized on the hand and the hand and eye reference frames were in register. We found reach direction (1 of 8), could be most accurately decoded at reach onset (mIPS:97%, A5:84%). This declined to chance 400 ms before the reach. Our data show that, under these conditions, monkeys prefer to align the reference frame for the hand and eye before reaching. Interestingly, we find the reach plan in PPC also develops when the hand and eye are aligned. This alignment could have advantages for coordinate transformations in the neural control of reaching and suggests that during natural behavior visual-motor transformations in PPC are computed online.

181. Single-trial decoding of reach and saccade intentions in the posterior parietal cortex

Rodrigo Quian Quiroga, Larry H. Snyder, Aaron P. Batista, Richard A. Andersen

Strong evidence reported over the last years shows a crucial role of the posterior parietal cortex (PPC) in the transfer of visual inputs to motor commands. In particular, using average population responses (over several trials) it has been shown that two localized zones in the PPC, the lateral intraparietal area (LIP) and the parietal reach region (PRR) are involved in the coding of saccade and reach intentions, respectively. The question we address in this study is whether it is possible to predict (i.e., decode), on a single-trial basis both reach and saccade directions from recordings in LIP and PRR.

We analyzed single-cell recordings of neurons corresponding to the LIP and PRR areas of the macaque's PPC. Responses of these neurons were studied in a delay-reach task, in a delay-saccade task and in a dissociation task (i.e., a near-simultaneous reach and saccade in opposite directions. For doing the single-trial predictions of saccade and reach directions, we used standard classification techniques (Fisher linear discriminant), treating all cells in LIP and in PRR as simultaneously recorded. We observed that the activity in the delay period of PRR cells give an accurate prediction of reach directions but not of saccade directions. Conversely, cells in LIP give an accurate prediction of saccade directions but not of reach directions. These results stress the involvement of segregated areas of the PPC in the planning of different types of motor activity.

182. Local field potential tuning in the macaque posterior parietal cortex during arm-reaching movements

Hans Scherberger, Chris A. Buneo, Murray Jarvis, Richard A. Andersen

Single neurons in the parietal reach region (PRR) encode the target direction of upcoming arm-reaching movements and are spatially tuned to a preferred movement direction. Recently, it has been shown that the local field potential (LFP) in the lateral intraparietal area (LIP) is tuned in the gamma-band frequency range to a preferred direction similar to the spiking activity at the same location. Here we asked whether the LFPs in PRR are spatially tuned for arm movements and how reliably these LFP signals could predict upcoming arm movements.

In a delayed reach response task, the animal first had to fixate and touch a fixation point on a touch-board. Then a green cue was briefly shown in one of eight peripheral locations. The animal had to continue to look at and touch the fixation point until, after about 1sec, this point extinguished and the animal was required to touch the peripheral target while maintaining fixation at the center. Single units and the LFP were simultaneously recorded from PPR using single electrodes.

Preliminary results showed modulations of the LFP spectral power during the visual stimulation, planning, and movement period of the task. The LFP was directionally tuned similar to the simultaneously recorded single units. Using a Bayesian decoding algorithm and LFP data from 48 recorded sites, the correct movement direction (one out of eight alternatives), could be predicted from individual test trial sets (one random trial per recording site), in at least 50% of all simulations, significantly better than chance (12.5%).

These results suggest that the LFP spectrum reflects information similar to single-cell firing rates. Since the LFP is easier to record than single units, the LFP might serve as a more robust signal for the prediction of upcoming arm movements, e.g., as necessary for controlling a neural prosthesis.

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183. How does a monkey learn to avoid an obstacle during reaching?

Elizabeth Torres, Richard A. Andersen

It is not known how or where the brain computes the paths described by a hand-reaching motion. One possibility is that information about the path, whether partial or full, be encoded in parietal reach region (PRR). Alternatively, PRR might be only involved in planning the goal and not the trajectory. In order to explore this question, we have designed an experimental task that compares path generation in simple reaches vs. reaches involving an obstacle. Although in both cases the hand starts from the same point and ends at the same target, the paths it traverses in space are very different. Neuronal activity in PRR might reflect these differences. The first part of this project identifies behavioral strategies a monkey develops during learning and adaptation to the presence of an obstacle. The task consists of center-out reaching movements to six targets located on a push-button board. The positioning of the obstacles allowed for multiple solution paths in 3-dimensions. The monkey however chose planar paths confined to the region in space where the arm movement did not occlude the obstacle. Alternative solution paths would have been more efficient in the sense of path length and movement duration, but would have increased the risk of hitting the obstacle. This suggests a strategy whereby the main goal of acquiring the target initially loses priority to that of keeping the obstacle in sight. Visual information about the obstacle seems essential for solving this problem. Using the bimodal speed profiles of the obstacle-avoidance motions we identify a cluster of points near the obstacle, which tightens as adaptation progresses. Postural paths become less variable and are confined to a subspace that recruits a subset of the many available degrees of freedom. We discuss the data in the context of a simple geometric model that exploits these strategies.

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Summary: There are currently three major areas of investigation in this laboratory: the development of neural stem cells; the development of arteries and veins; and the functional neuroanatomy of emotional behaviors.

Stem cells are multipotent, self-renewing progenitor cells. In the nervous system, neural crest stem cells give rise to the PNS, while one or more classes of CNS stem cells generate the brain and spinal cord. The potential therapeutic utility of these cells makes it important to understand the molecular mechanisms that control their self-renewal and differentiation. We have prospectively isolated neural crest stem cells, and have identified both cell-extrinsic and cell-intrinsic molecules that control their differentiation and multipotency. Our experimental approaches include *in vitro* clonal analysis, *in vivo* transplantation, and loss- and gain-of-function genetic manipulations in mouse and chick embryos. Subtractive hybridization and microarray analysis are currently being employed to identify genes that distinguish stem and progenitor cells at various stages of development. The function of these genes may be tested by both gain- and loss-of-function manipulations in cultured neural crest stem cells, in the latter case using RNA interference (RNAi). Currently, we are focusing on the mechanisms that determine neurogenic potential, and its loss from committed glial progenitors, as well as molecular mechanisms of sensory neurogenesis.

Our studies of the circulatory system stem from our serendipitous discovery that arteries and veins are genetically distinct from the earliest stages of angiogenesis. Arteries express the transmembrane ligand ephrinB2, and veins express one of its receptors, EphB4. Gene-targeting studies have indicated that reciprocal ephrinB2-EphB4 signaling is essential for proper cardiovascular development, and may mediate bi-directional communication between arteries and veins. Our recent studies have included a further analysis of ephrinB2-EphB4 function in angiogenesis, using cell type-specific gene targeting; and experiments to investigate the interactions that occur between the developing nervous system and circulatory system. For example, we have recently found that sensory nerve fibers provide a template that determines the branching pattern of arteries in the developing limb skin.

A recent initiative in the lab has begun to develop and apply molecular biological tools to map and manipulate the neural circuitry underlying emotion in mice. These studies currently concern two distinct but related interoceptive states: pain and fear. In the former case, we have identified a novel family of G protein-coupled receptors (GPCRs) for neuropeptides, which are specifically expressed in restricted subsets of nociceptive sensory neurons. We are using homologous recombination in embryonic stem cells to delete these genes to study their function, as well as to study the function and connectivity of the neurons that express these receptors. In collaboration with Professor Melvin Simon's laboratory, we have identified ligands for these receptors. In collaboration with the Lester laboratory, we are analyzing the electrophysiological effects of these neuropeptides on cultured primary sensory neurons that express these receptors. These *in vitro* studies may suggest hypotheses that can be tested by behavioral experiments *in vivo*, performed in collaboration with Allan Basbaum's laboratory at UCSF. This system provides a platform from which to identify the particular nociceptive modalities mediated by these neurons, and to begin to understand the control of the perception of these noxious stimuli, by tracing their connectivity to the brain. These studies may also lead to the development of novel drugs that can be used to treat currently intractable forms of pain in humans, such as neuropathic (nerve injury-related) pain.

In a separate project, we have developed a novel experimental system to study the neural circuits that control behavioral responses to innately fearful stimuli. We have identified unimodal sensory stimuli that can elicit different fear behaviors (flight or freezing) in laboratory mice, depending on the context and/or prior experience of the animals. We are using quantitative immediate early gene expression analysis to map, with single-cell resolution, the brain regions that are active in these different behaviors. Efforts to identify genes expressed in these regions, both constitutively and in an activity-dependent manner, are also underway, using oligonucleotide microarrays and laser-capture microdissection. Identification of molecular markers for brain regions associated with emotional behaviors should permit us to determine the function of these regions, by creating transgenic animals in which the activity of

neurons in these regions can be reversibly silenced (using techniques being developed in Professor Henry Lester's laboratory), as well as to map their connectivity. These studies should better define the neural substrates of subjective emotional states, and ultimately may aid in a clearer understanding of the pathophysiological basis of affective disorders in humans, such as anxiety and depression.

In parallel with these studies in mice, we have initiated conceptually similar experiments in the fruitfly, *Drosophila melanogaster*. Our goal is to identify simple and robust innate behaviors, and then perform unbiased "anatomical" and genetic screens to map the neuronal circuits and identify the genes that control these behaviors. This dual approach will provide an opportunity to integrate molecular genetic and circuit-level approaches to understanding how genes influence behavior. The "anatomical" screen exploits the availability of "enhancer trap" lines in which the yeast transactivator protein GAL4 is expressed in specific subsets of neurons, and a conditional (temperature-sensitive) neuronal silencer gene that prevents synaptic transmission. Currently we have developed assays for an innate avoidance response triggered by a conspecific putative alarm pheromone (fly "fear"), as well as for arousal intensity and hedonic valence, two important axes underlying emotional states in humans.

184. ***In vivo*** studies of development of arterial differentiation and vascular branching

Yosuke Mukoyama

Blood vessels and peripheral nerves branch out together. Although the genetic and molecular mechanisms of vascular development have been brought together in recent years through numerous gene-targeting studies in mice, development of vascular branching pattern remains poorly understood. Recently, I have shown that the pattern of arterial branching is determined by that of the nerve in the embryonic limb skin. Moreover, the fact that nerves secrete vascular endothelial growth factor (VEGF), which is the growth factor that stimulates arterial differentiation *in vitro*, suggests that the nerves may function to induce arteries, but not veins through VEGF.

We are currently investigating VEGF involvement *in vivo* by examining conditional VEGF knockout mice that completely lack VEGF expression in peripheral nerves. The mutants show severe defects in not only vascularization in embryonic dorsal root ganglion (DRG) but also arterial differentiation in the limb skin. This latter observation is consistent with our *in vitro* studies and *in vivo* studies in zebrafish by Weinstein and colleagues, suggesting that VEGF-A is involved in arterial differentiation. However, alignment with peripheral nerves and formation of proper vascular branching appear to be maintained in the mutant.

Taken together, these data suggest that peripheral nerve promotes arterial differentiation according to its VEGF expression, whereas the nerve may control the pattern of vascular branching by low concentration of VEGF, or via other nerve-derived factors. These observations allow us to dissect the process of vascular development in the limb skin in which nerve-vessel alignment occurs.

185. Lhx6 and Lhx9 in the formation of functional connections between hypothalamic and amygdaloid nuclei that are involved in the expressions of innate behaviors

Gloria B. Choi, David J. Anderson

We are interested in innate behaviors, and more specifically, we are trying to understand how the circuitries in the brain controlling these innate behaviors form during development.

Numerous functional studies demonstrated that different nuclei in two brain regions, amygdala and hypothalamus, are involved in the expressions of either reproductive or defensive behaviors. More interestingly, there is a topographic organization of the connections between the amygdaloid and hypothalamic nuclei in such a way that the amygdaloid nuclei involved in reproductive or defensive behaviors preferentially make direct connections to the hypothalamic nuclei that are also involved in reproductive or defensive behaviors, respectively.

By doing an expression screening, we found LIM homeodomain proteins, Lhx6 and Lhx9, which occupy two adjacent nuclei in medial amygdala, the posterior dorsal (MeApd) and posterior ventral nuclei (MeApv). These nuclei are thought to be important for the expressions of reproductive and defensive behaviors, respectively. Based on the general role of LIM homeodomain proteins in specifying the axonal projection patterns of the spinal cord motor neurons, we hypothesize that these two genes may function to restrict the projections of the cells in MeApd and MeApv to only the reproductive or only the defensive nuclei in hypothalamus. In order to test this idea, we made knock in mice, in which an axonal tracer, PLAP (placental alkaline phosphatase), is inserted in place of coding sequences of Lhx6 and Lhx9. We are in the process of analyzing these mice in order to find out the hypothalamic targets of the cells expressing Lhx6 or Lhx9 in medial amygdala, and to investigate whether they are necessary for making the proper projection patterns to those targets.

LIM homeodomain proteins are, also, shown to be important for the expression of neuropeptides in various neuronal populations. We have mapped out several peptides that are co-expressed with Lhx6 or Lhx9 in medial amygdala. Using the PLAP knock-in mice, we will examine whether Lhx6 or Lhx9 are necessary for the expression of these peptides.

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186. Deregulation of dorso-ventral identity explain tripotentiality of CNS progenitors ***in vitro***

Limor Gabay

CNS stem cells are defined as self-renewing cells that can give rise to the three basic cell types of the central nervous system: astrocytes, oligodendrocytes and neurons (Taupin and Gage, 2002).

The evidence for the existence of these tripotential cells come from studies in which CNS cells were selected *in vitro* for their ability to proliferate in the presence of the mitogens FGF and EGF. It was generally assumed that this proliferation in culture does not alter the properties of the cells.

In vivo, however, there is no evidence so far that a tripotential CNS stem cell exists. In fact, recent studies of the developing embryonic mouse spinal cord show that no single cell gives rise to all three cell types. Rather, it appears that progenitors from the dorsal part of the spinal cord will generate interneurons and astrocytes, while motoneurons and oligodendrocytes are generated only in a single domain in the ventral part of the spinal cord, the motoneuron domain (Zhou and Anderson, 2002).

We decided to isolate these fate-restricted spinal cord progenitors and test whether *in vitro* they show the characteristics of CNS stem cells. We found that progenitors isolated from the dorsal part of the spinal cord and from the motoneuron domain will become tripotential *in vitro* and that this tripotentiality depends on deregulation of their dorso-ventral identity mediated by FGF.

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187. Circuit analyses of innate avoidance behavior in ***Drosophila***

Greg S. Suh, Anne Hergarden, Seymour Benzer[*]*, David J. Anderson*

We have developed a novel behavioral paradigm for an innate avoidance response in *Drosophila*. This paradigm involves avoidance of a putative "alarm" substance emitted by conspecifics subjected to stress. Responder flies were given a choice in a T-maze between a fresh tube and a conditioned tube in which a set of emitter flies was previously traumatized. Most responders choose the fresh tube; the performance index typically falls between 95 and 85 under optimal conditions. Preliminary results suggest that recognition of the "alarm" substance is likely mediated by the olfactory system. We have been trying to identify groups of neurons, downstream from the olfactory sensory neurons, essential for the avoidance behavior by carrying out an unbiased neuronal inactivation screen using the Gal4 x UAS-*Shibire*^{ts} system. Flies bearing a Gal4 enhancer trap and a UAS-*Shibire*^{ts} will be assayed in the behavior paradigm at the non-permissive temperature (30°C) and will be compared to their performance at the permissive temperature (22°C). We have isolated 13 Gal4 lines, which when bearing UAS-*shibire*^{ts} were defective in this assay at the non-permissive temperature, but retained normal olfactory responses to various odorants. To visualize the neurons functionally knocked out through the *Shibire*^{ts} system, we crossed the Gal4 lines with UAS-mCD8/GFP. Several of the lines exhibit specific expression in a defined region of the sub-esophageal ganglion (SOG). Once we have a number of candidate neuroanatomical loci mediating the avoidance behavior, we will examine the relationship between the loci by crossing the candidate Gal4 lines to UAS-WGA to trace its trans-synaptic neural pathways to identify anatomically connected loci. We have also been screening a collection of insertional mutant (EP) lines for mutant's defective in this avoidance assay.

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188. Molecular mechanism underlying genesis of autonomic neuron

Kenji Orimoto

The neural crest stem cell (NCSC) culture system including migrating NCSC (mNCSC) and sciatic NCSC (sNCSC) is characterized by: 1) clonal density culture to address the issue of multipotency of possible stem cells, and to see whether effects of a certain growth factor are instructive or selective; 2) defined instructive cues for fate determination, including agonist/antagonist to control cell fates at will; and 3) prospective isolation using cell surface markers without intervening *in vitro* culture period to minimize the possible initial alteration in cellular properties caused during the culture (sciatic NCSC).

NCSCs can differentiate into neurons, Schwann cells and myofibroblasts in response to BMP2, GGF2/Notch ligands, and BMP2/TGF-βs, respectively. Accordingly, NCSCs are postulated to possess neurogenic, gliogenic, and myofibroblastogenic capacities, as well as self-renewal capacity. When NCSCs are treated with Notch ligands for 24 hr, they completely lose their ability to differentiate into neurons even in the presence of BMP2, while they still maintain the ability to differentiate into Schwann cells and myofibroblasts under the appropriate instructive cues.

To get a better understanding of the molecular mechanism of differentiation of autonomic neurons, microarray analysis was conducted to compare gene expression profiles of Notch-activated mNCSCs and the corresponding control mNCSCs. Based on the results, genes were functionally assorted into the following categories: 1) basic cell fate; 2) chromatin remodeling; 3) cell cycle machinery; 4) signal transduction; and 5) growth factors/extracellular matrix proteins. To examine the spatio-temporal regulation of expression, *in situ* hybridization will be employed. To make sure of the functional competence of cell fate regulation, misexpression experiments including loss-of-function and gain-of-function assays will also be conducted.

The genes identified as enriched in stem cells are further analyzed to see whether they are functionally involved in Mash1 induction and neuronal differentiation

initiated by BMP2. The genes identified as enriched in non-neuronal progenitors would be downstream targets directly affected by Notch signaling cascade, as well as early markers for Schwann cell progenitors.

189. Search for genes specific for brain areas involved in innate fear responses

Walter Lerchner, Laurent vanTrigt, David J. Anderson

Fear is perhaps the most conserved emotion in our evolution. The ability to associate certain situations with fear is absolutely essential to avoid dangerous situations. However, in our complex society it is possible that the same mechanism can lead to stress and anxiety disorders. In particular, a traumatic event can lead to unspecific fear that alters the behavior in subsequent situations.

Our lab has established a behavioral procedure in which mice are exposed to an aversive ultrasound in their home environment. Naive animals respond to the sound with flight responses, i.e., active defense behavior. However, mice that were previously exposed to a series of foot-shocks respond to the ultrasound with freezing (absence of movement, i.e., passive defense response) in addition to a reduced amount of flight responses. Subsequent studies have carefully mapped differential neuronal activation in the central nervous system during and following ultra sound exposure in these two groups.

This project aims to take this effort a step further by applying molecular biology to better elucidate these circuits. Using laser capture micro-dissection (LCM) and Affimetrix oligo-nucleotide arrays, we isolated several genes with restricted expression in areas predicted to play important roles in the circuit. The promoters of some of these genes will be used to generate BAC transgenic mice in order to express axonal reporter and silencing cassettes. None of the genes show expression solely in the areas of interest. Thus, the cassettes are designed to allow spatial and temporal specificity using the promoters of two genes with overlapping expression in the area of interest.

We are currently testing the system using the *crhr2* gene (corticotrophin releasing hormone two receptor), a gene previously implicated in anxiety and fear, in combination with a gene overlapping with *crhr2* expression in the septum. These experiments should allow us to map the projections of neurons expressing the *crhr2* gene in addition to silencing the *crhr2* neurons specific in the lateral septum.

190. Olig target genes in the oligodendrocyte-astrocyte fate decision

Christian Hochstim, Qiao Zhou, David J. Anderson

This study aims to contribute to the understanding of the molecular mechanisms of cell fate determination in central nervous system development. The more specific focus will be on the glial subtype choice between oligodendrocyte and astrocyte fates. At the stage of gliogenesis, the bHLH transcription factors Olig1 and Olig2 are specifically expressed in the oligodendrocyte lineage and Olig2 is essential for oligodendrocyte generation in the spinal cord. Furthermore, lineage tracing in the Olig1,2 -/- homozygous mutant spinal cord using an *Olig2*-GFP knockin allele reveals GFP+ cells generate astrocytes, a fate transformation in the absence of Olig expression. We intend to identify Olig target genes involved in glial fate specification by screening for genes differentially expressed between Olig1,2 +/- oligodendrocyte precursors and Olig1,2 -/- putative astrocyte precursors.

We have performed a screen where GFP+ precursor cells are isolated by fluorescence-activated cell sorting (FACS) of dissociated embryonic mouse spinal cord from both Olig1,2 +/- heterozygous and Olig 1,2 -/- homozygous mutants and their mRNA expression profiles were compared using Affymetrix mouse genome cDNA microarrays (U74-A,B,C). The oligodendrocyte (+/-) and astrocyte (-/-) precursors isolated for this comparison were both taken from two independent stages of early gliogenesis, E13.0 and E14.5, and many genes were consistently differentially expressed at both stages. We wanted to look at early gliogenesis to focus on genes which, like Olig2, play a role in the specification of cell fate. Candidate genes and ESTs are being pursued further based on strong fold change and average difference change values, with a priority being given to transcription factors and molecules with known functions in the nervous system. We are currently confirming the differential expression of candidate genes by *in situ* hybridization on tissue sections of Olig1,2 +/- and Olig1,2 -/- spinal cord, as well as beginning to functionally characterize candidate genes with interesting *in situ* expression.

191. Forward and reverse genetic approaches for modeling "emotional" behavior in ***Drosophila***

Tim Lebestky

Emotional behaviors in humans convey a positive or negative response to a stimulus, and this response is manifest in discrete, highly conspicuous ways, such as stereotyped facial expressions and physiological arousal. Although *Drosophila* do not present the richness of human emotions in their behavior, they may share fundamental molecular similarities that could allow us to dissect the way that neural circuits function to provide graded responses in intensity, as measured both qualitatively and quantitatively. To this end, we are developing automated, high-resolution behavioral assays that will allow a reproducible characterization of behavioral responses to negative stimuli for high-throughput genetic screens. One such assay follows the startle effects on locomotion and escape behaviors in response to a series of air-puffs, delivered at regular intervals. We observe a reproducible escalation of locomotor activity and jump-response behaviors as a function of time and puff number. By initially screening through mutants and collections of flies that are conditionally silenced at specific circuits (Gal4-UAS system), we hope to delineate the molecular

mechanisms and neural circuits involved in the generation and modulation of such emotive responses.

Similar to mammals, *Drosophila* utilizes biogenic amines as neurotransmitters for normal neuronal function and behavior. A serotonin transporter, dSERT, with significant functional homology to the mammalian SERT family has been cloned and physiologically characterized *in vitro* (Demchyshyn *et al*., 1994); however, there is no genetic analysis of this or any other aminergic transporter *in vivo*. Given the importance of this molecular family and the successful advancement of *in vivo* RNAi techniques in *Drosophila*, we are currently developing techniques to look at gain- and loss-of-function conditions in a spatially and temporally regulated manner in adult flies. It is our hope that by tightly controlling the gene dosage and induction level of the RNAi, we may observe quantifiable phenotypic differences in behaviors that may give insights into how molecular thresholds influence the escalation or decline of distinct internal states in adult animals.

192. Identification and characterization of mrgs: A large family of neuropeptide receptors specifically expressed in nociceptive sensory neurons

Xinzhong Dong, David J. Anderson

Pain sensation is required for survival and maintenance of the integrity of an organism. However, unrelieved pain can undermine the quality of life. In mammals, pain sensation is mediated by a subset of primary sensory neurons, known as nociceptors, in the dorsal root ganglia (DRG). The nociceptors are highly diverse according to their cellular and molecular properties. To study pain sensation at the molecular level, we carried out a subtractive hybridization screen to search for genes expressed specifically in nociceptors in mice. From the screen, we were able to identify a large G protein-coupled receptor (GPCRs) subfamily comprising nearly 50 members that are homologous to MAS1, called Mas-related genes (*Mrgs*). A subset of these genes, including *MrgAs*, *MrgC11* and *MrgD*, is specifically expressed in a subpopulation of nociceptors, which has been implicated in "neuropathic" (nerve injury-induced) pain. The expression of *mrg* genes is largely non-overlapping in the subpopulation of nociceptors suggesting that there is an unexpectedly high degree of molecular diversity among them. To search for ligands and/or agonists for Mrgs, we have screened more than 100 neuropeptides against human embryonic kidney (HEK) 293 cells that stably express either *MrgA1* or *MrgC11*. By assaying increase in intracellular Ca^{2+} levels, we identified several neuropeptides that can function as agonists for the two Mrg receptors. Interestingly, most of the MrgA1- or MrgC11-specific agonists share a common RF(Y)G or RF(Y) amide motif at their C-terminus. Therefore, it is likely that the natural ligands for Mrg receptors are neuropeptides that have this common motif. In addition, we found that MrgA1 and MrgC11 show different affinities to certain agonists suggesting there is functional diversity among different Mrg receptors. Studies of *Mrgs* function could provide new insights into the functional properties of nociceptors and may eventually lead to the development of very specific therapeutic agents for alleviating pain.

193. EphrinB2 in the peripheral endothelial cells is essential for the proper embryonic vessel development

Donghun Shin, David J. Anderson

EphrinB2 conventional knockout and endothelial-specific knockout mice show the same angiogenesis defects during early embryonic development. EphrinB2 is expressed in heart endocardial cells, as well as in peripheral endothelial cells, and both mutants show heart defects, as well as peripheral angiogenesis defects in head, trunk and yolk sac. It is not clear whether the peripheral angiogenesis defects in the mutant reflect a local requirement for ephrinB2 signaling, or rather may be secondary to the heart defects.

Recently, we generated a novel line of Cre knockin mice by inserting an EGFP-Cre fusion construct in frame with the start codon of a novel gene that is expressed in peripheral arterial endothelial cells, but not in endocardial cells. Using this Cre line and a conditional ephrinB2 allele, we have deleted ephrinB2 in peripheral endothelial cells but not in heart endocardial cells. Preliminary results suggest that embryos containing this conditional ephrinB2 knockout show angiogenesis defects in the yolk sac and head at E10.5; however, the defects are less severe than in the conventional ephrinB2 mutant. This difference may reflect the fact that ephrinB2 excision mediated by the Cre line is delayed relative to the initial onset of endothelial ephrinB2 expression, and does not occur in all arterial endothelial cells.

These data suggest that the peripheral angiogenesis defects in ephrinB2 mutants may not be secondary to the heart defects, and support a local role for ephrinB2 in the angiogenic remodeling of the peripheral vasculature during embryonic development.

194. Disregulation of dorso-ventral identity of CNS precursors in mitogenic culture

Sally Lowell

The CNS is thought to be generated from cells that can self renew and generate the three major CNS cell types: astrocytes, oligodendrocytes and neurons. However, no example has been observed of a cell that generates all three-cell types *in vivo*. Rather, there appear to be two populations that follow distinct fates. One population, arising in the ventral neural tube and marked by expression of the bHLH transcription factor Olig2, generates only motor neurons and oligodendrocytes, while the complementary population generates only interneurons and astrocytes.[1,2] In contrast to their restricted fates *in vivo*, cells from both populations clonally generate all three neural cell types in mitogenic culture (see abstract by Limor Gabay). The ability of Olig2+ cells to generate astrocytes *in vitro* correlated with a down-regulation of Olig2 expression. By forcing expression of Olig genes in

culture, we were able to show that they play a causal role in suppressing astrocyte differentiation. Conversely, the ability of dorsal cells to differentiate into oligodendrocytes correlated with acquisition of Olig2 expression, consistent with the requirement for Olig genes for oligodendroctye differentiation.[1]

We investigated the mechanism by which Olig2, and thus oligodendrocyte potential, is acquired by dorsally-derived cells *in vitro*. First, we found that dorsally-derived cells were not able to express Olig2 or to differentiate into oligodendrocytes when cultured in conditions that are permissive for Olig2 expression and oligodendrocyte differentiation from ventral cells, but which lack FGF. Rather, they generate only neurons and astrocytes. This suggests that dorsal cells are not merely restricted by their local environment *in vivo* but rather differ intrinsically from ventral cells in terms of oligodendrocyte potential. We next found that dorsal cells acquire Olig2 expression and differentiate into oligodendrocytes after exposure to high concentrations of FGF. These data suggest that bFGF, a factor widely used for mitogenic expansion of neural precursors, is not neutral with respect to cell fate but rather imposes a ventral identity on spinal cord cells.

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195. Sox10 maintains multipotency of neural crest stem cells

Liching Lo, Jaesang Kim, David J. Anderson

We are interested in studying how neural crest stem cells establish and maintain their multipotency. Maintenance of the characteristics of stem cells require at least three different functions: (1) ability to inhibit overt differentiation; (2) ability to continue proliferation; and (3) ability to maintain multipotency. We used the HMG-box factor SOX10 to study neural crest stem cells (NCSCs). SOX10 is specifically expressed in neural crest cells *in vivo*, just as they delaminate from the dorsal neural tube. Expression of SOX10 is further maintained in glia but downregulated in other lineages, including neurons. *In vitro,* SOX10 is expressed in multipotent NCSCs and like *in vivo*, only maintained in glial progeny. Application of BMP2 or TGF-β to NCSCs causes the loss of SOX10 rapidly. BMP2 promotes neuronal differentiation and both BMP2 and TGF-β induces smooth muscle differentiation. Using constitutive expression of SOX10 in NCSCs, we found that SOX10 prevents the extinction of gliogenic potential by BMP2. Surprisingly, SOX10 also preserves the neuronal potential blocked by TGF-β. The ability of SOX10 to maintain glial potential correlates its ability to maintain expression of ERBB-3, a target of SOX10 and a marker for glial lineage. Constitutive expression of SOX10 also prevents cell cycle arrest induced by TGF-β. Analysis of *Dom/Dom* embryos, which are genotypically null of *Sox10,* indicated that SOX10 is required for the expression of MASH1 and PHOX2B, two transcriptional factors essential for autonomic neurogenesis. *In vitro*, constitutive expression of SOX10 also maintains MASH1 expression extinguished by TGF-β. SOX10 expression is ultimately downregulated in $MASH1^+$ and $PHOX2B^+$ neurons. Does continued expression of SOX10 interfere with neuronal differentiation? We found that forced expression of SOX10 inhibits the overt neuronal differentiation in NCSCs. In *Dom/Dom* mutants, the expression of PHOX2A, which is similar to PHOX2B but expressed one day later in autonomic neurons, is precociously and ectopically derepressed in a subset of $SOX10^+$ neural crest cells. *In vitro*, constitutive expression of SOX10 repressed the induction of PHOX2A. Forced expression of either MASH1 or PHOX2B also repressed endogenous SOX10 expression. Taken together, we found that the maintenance of neurogenic potential of SOX10 is opposed by an independent function to inhibit or delay overt neuronal differentiation. These new data suggest its function in stem cell maintenance, in addition to its known role in glial differentiation.

196. The involvement of the anterior cingulate cortex in novelty

C.J. Han,[1] David J. Anderson, C. Koch[1]*

The activation of the anterior cingulate cortex was previously shown to correlate with novelty detection. However, whether the anterior cingulate cortex is necessary to novelty detection is unclear. We set up a novelty object paradigm in mice. Mice were brought to the testing room in their home cage. A group of mice received a novel object (a corning 15 ml tube), a group received the same procedure including lifting the cage lid but not the object, and a group received nothing. We showed that the novel object readily induces the exploratory behaviors of the mice directed towards the novel object, and cage lid lifting induces general exploratory behaviors. The sum of time that the group receiving the novel object and the group receiving the lid lifting spend in exploratory behaviors are equal, but the exploratory behaviors in the group that received the novel object are mostly directly to the object. c-fos mRNA was used as a surrogate marker to detect neuronal activation by *in situ* hybridization on brains from each group. Animals from each of the three groups were sacrificed 30 minutes after the first exposure of the stimulus. We discovered that there are more c-fos positive cells in the anterior cingulate cortex of the brain that received the novel object, compared with the other two groups. To answer the question whether the anterior cingulate cortex is necessary for novelty detection, a group of mice received excitotoxic lesions of the anterior cingulate cortex and another group received sham surgery. Behavioral experiments and analyses are being conducted to determine whether the lesions to the anterior cingulate cortex cause any exploratory behavioral changes directed to the novel object.

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197. Atypical expansion in mice of the sensory neuron-specific ***Mrg*** GPCR family
Mark J. Zylka, Xinzhong Dong, Amber L. Southwell, David J. Anderson

The *Mas-related genes* (*Mrgs*) comprise a family of over 50 G protein-coupled receptors (GPCRs), many of which are expressed in specific subsets of nociceptive sensory neurons in mice. In contrast, humans contain a related but non-orthologous family of genes, called *MrgXs* or *SNSRs*, of which many fewer appear to be expressed in sensory neurons. To determine whether the diversity of murine *Mrgs* is generic to rodents or an atypical feature of mice, we characterized *MrgA, MrgB, MrgC,* and *MrgD* subfamilies in rat and gerbil. Surprisingly, while mice have ~22 *MrgA* and ~14 *MrgC* genes, rats and gerbils have just a single *MrgA* and *MrgC* gene. This murine-specific expansion likely reflects recent retrotransposon-mediated unequal cross-over events. The expression of *Mrgs* in rat sensory ganglia suggests that the extensive cellular diversity in mice can be simplified to a core subset of ~4 different genes (*MrgA, MrgB, MrgC* and *MrgD*), defining a similar number of neuronal subpopulations. Our results suggest more generally that mouse-human genomic comparisons may sometimes reveal differences that are atypical of rodents.

Neural development/stem cells

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Postdoctoral Fellows: Ted Brummel, Pankaj Kapahi, Debbie Liang, Anne Simon, W. Dan Tracey Jr., David W. Walker, Horng-Dar Wang
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Summary: Our group uses *Drosophila* as a model system in which to identify and characterize genes involved in behavior, aging, and neurodegeneration. The high degree of homology between the fly and human genomes forms the basis of a strategy for understanding the corresponding human genes.

To study the problem of aging, we use the single-gene approach to screen for mutants with enhanced longevity, and analyze the functions of the genes involved. For instance, the mutant, *methuselah,* extends the average lifespan of *Drosophila* by some 30%, and also provides increased resistance to different stresses. Another interest is the genetics of behavior, in which two paradigms are currently under investigation. One is a model of nociception that bears much resemblance to human pain, with mutants such as *painless* representing an entry into a molecular genetic analysis of this phenomenon. The second is an alarm response, in which flies subjected to vibration emit an odor that induces avoidance by other flies, which is being studied in collaboration with Professor David Anderson's group.

Exposure of flies to 100% oxygen causes early death, and we have found that an early-induced event is local disruption of mitochondrial structure in the form of internal "swirls." These also accumulate in normal aging, and their formation can be suppressed by mutations in the *methuselah* gene. Other mutants, such as *hyperswirl*, are unusually sensitive to oxygen, and display large numbers of swirls. Analysis of such mutants may provide clues to primary mechanisms of oxidation damage.

The methuselah protein is related to G protein-coupled receptors of the secretin receptor family, and has an unique N-terminal ectodomain. In collaboration with Professor Bjorkman's group, the crystal structure of the ectodomain was solved at 2.3 Å resolution. The structure represents one of only a few available three-dimensional structures of GPCRs. It reveals a folding topology likely to be conserved in Mth-related proteins, and contains a potential ligand-binding site in the form of a shallow interdomain groove with a solvent-exposed tryptophan, the only tryptophan residue in the ectodomain. Antagonists that reduce the effective activity of the receptor would be expected to mimic the defect in *methuselah,* thus possibly extending lifespan. In collaboration with Professor Richard Roberts' group, a library of peptides was generated, from which a subset was selected that show very high binding affinity to Mth protein. In collaboration with Dr. Anthony West, of the Bjorkman group, we have produced monoclonal antibodies to the protein, and expression of *mth* in cultured cells shows localization of the protein at the cell membrane. We are using such cultures, along with the small peptides as putative ligands, to test for G-protein activation in a suitable reporter system.

With Dr. Konrad Zinsmaier's group at the University of Arizona, it has been discovered that mutations in the *mth* gene reduce evoked synaptic vesicle release at the *Drosophila* larval neuromuscular junction ~50%. Pre- but not postsynaptic expression of normal Mth restores normal release in *mth* mutants. The reduction in synaptic release in the mutant in response to action potentials may be a clue to the greater resistance of *methuselah* to stress.

Caloric restriction extends lifespan in various organisms, and we are investigating that phenomenon in *Drosophila*, as well as the role of steroid hormones and bacterial flora. We have developed biomarkers to monitor the progress of aging during lifetime, and have shown that lifespan can be extended by simple feeding of a drug which alters the balance of induction and repression of different sets of genes.

198. Mitochondrial structure and function under conditions of oxidative stress and aging

David W. Walker, Seymour Benzer

A major gap in our understanding of *Drosophila* aging is the question of what happens to cells, organelles and tissues during the aging process. One hypothesis is that mitochondrial decay is the primary cause of age-related decline. Previously, we reported a striking initial pattern of mitochondrial degeneration in the flight muscle under conditions of oxygen stress and aging: the cristae within individual mitochondria become locally rearranged, in "swirl." Using a combination of confocal and fluorescent microscopy we have further studied this phenomenon.

We assayed for apoptotic cell death, via *in situ* TUNEL labeling of cryosections of flies grown under hyperoxic conditions, identified apoptosis in various tissues of flies under hyperoxia, including the retina and leg muscles, but, surprisingly, not in the flight muscle. Using immunohistochemistry, we also failed to identify cytochrome *c* release, a harbinger of apoptosis, from the flight muscle mitochondria.

In a genetic screen for mutants with increased sensitivity to hyperoxia, we have identified several that display mitochondrial defects. One of these mutants, *hyperswirl* (*hys*), is dramatically short-lived under both hyperoxia (mean survival of 1.9 ± 1.3 days) and normoxia (mean survival of 8.6 ± 3.5 days. This recessive mutant displays an overabundance of swirls, even under normoxic conditions. To assay for functional consequences of mitochondrial decay in *hyperswirl*, we compared the metabolic rate of *hyperswirl* to the parental line, $white^{1118}$ and the wild-type *Canton-S*. CO_2 production was measured in groups of 10 flies of each genotype. At seven days of age, *hyperswirl* displays ~31% decrease in mean CO_2 production. In addition, we tested the ability of *hyperswirl* mutants to fly, using a simple flight test. Control flies respond normally in this assay, whereas *hyperswirl* mutants display a severe defect in flight capacity. Molecular cloning of the *hys* gene and investigation of the gene's function and associated pathways should lead to a better understanding of mechanisms of age-related mitochondrial decay.

199. ***In vitro*** selection of peptide ligands for Methuselah, a G protein-coupled receptor associated with extended lifespan

William W. Ja, Anthony P. West, Silvia Delker, Pamela J. Bjorkman, Seymour Benzer, Richard W. Roberts

Display techniques for peptide selection serve as powerful tools for isolating novel peptides with high affinity for biological targets from large, diverse libraries of random peptides. mRNA display, a method where a peptide or protein is covalently linked to its encoding RNA sequence, is an improvement to previous selection techniques, allowing access to far greater library complexities in a more robust format. mRNA display was used here to isolate peptides that bind to the ectodomain of Methuselah (Mth), a G protein-coupled receptor previously identified from a screen for mutations that extended the average lifespan of *Drosophila* and also found to modulate stress resistance. The peptide selection method consisted of the synthesis of a large pool of RNA-peptide fusions encoding random 27-mer peptides, selection of the pool against the immobilized ectodomain of Mth, and amplification of the mRNA by RT-PCR after removal of non-binding molecules by stringent washing of the solid-support. After 8 rounds of selection, the activity of the final library (~15%) was significantly higher than that of the initial pool (~0%) and highly specific for the ectodomain of Mth. DNA sequencing of individual clones from the selected pools revealed a specific Mth-binding consensus motif of (P/R)xxWxxR. Several peptides were synthesized and their binding kinetics were determined by surface plasmon resonance. These peptides demonstrated high affinity to the Mth ectodomain with dissociation constants between 15 and 60 nM.

The synthesized peptides were also amenable to conjugation with a wide variety of useful tags, including fluorescein, rhodamine, and biotin. Peptides tagged with fluorophores were used in labeling and fluorescence-activated cell sorting of CHO cells expressing full-length Mth. These *in vivo* assays demonstrated that the selection against the ectodomain produced peptides that still recognize the full-length receptor. These peptides will be tested in a culture model for their effects in G-protein signaling.

200. Investigation of the ***methuselah*** gene family

Ted Brummel, Seymour Benzer

Mutations in the *mth* gene result in an increase in lifespan of 30%. By homology, *mth* is predicted to belong to the secretin class of G-protein coupled receptors, but has an unique N-terminal amino acid sequence. The *Drosophila* genome contains eleven *mth* like genes, for which mutants have not been reported. To determine whether any of these genes are involved in regulating lifespan and/or other phenotypes, we have generated RNAi interference constructs for them as well as for *mth*, and generation of transgenic lines is underway. This study will allow the characterization of the whole *mth* gene family.

201. Overexpression of the ***Drosophila*** orthologue of Apolipoprotein D extends lifespan and protects against acute oxidative stress

David W. Walker, Julien Muffat, Seymour Benzer

Human Apolipoprotein D (ApoD), a component of lipoproteins, especially including HDL, is a member of the family of lipocalins, small extracellular proteins present from prokaryotes to complex metazoans. ApoD is expressed in the CNS by glial cells, mainly astrocytes and oligodendrocytes, and has been implicated in numerous diseases of the nervous system. In Alzheimer's Disease, ApoD expression is increased by 60% in the hippocampus and 350% in the cerebrospinal fluid, compared with age-matched controls. Upregulation of ApoD has also been shown in amyotrophic lateral sclerosis, meningoencephalitis, and after ischemic incidents. Patients with schizophrenia and bipolar disorder also present higher brain levels of ApoD. Due to our interest in neurodegeneration and the use of *Drosophila* to model diseases, we looked in *Drosophila* for a homolog of ApoD.

A close homolog is Glial Lazarillo (Glaz) which has 40% identity and 80% similarity. Both ApoD and Glaz are similar to the ancestral lipocalins that gave rise to the family. We have isolated a mutant containing a P-element with a UAS promoter sequence inserted 5' to the *Glaz* gene. This enables the study of loss-of-function phenotypes, as well as overexpression of the gene by crossing the mutant to a line expressing GAL4 to drive the UAS promoter. We have verified, by RT-PCR, the GAL4-dependent upregulation of the *Glaz* transcript (~8 fold upregulation). Our results show that, whereas the homozygous mutant is short-lived, overexpression of the ApoD homolog results in a 35% increase in lifespan. The overexpressor is also more resistant to hyperoxia (100% O_2), to the same degree as the stress-resistant, long-lived mutant *methuselah*. Moreover, we have independently generated two new transgenic lines, each carrying the *Glaz*

cDNA. These new transgenic lines each display increased lifespan in a GAL4-dependent fashion. The protective effect of the protein may be related to the enhanced expression of its homolog in human degenerative disease.

202. Polyamines and the aging process in ***Drosophila***

Brian M. Zid, Pankaj Kapahi, Seymour Benzer

Aging can be defined as the progressive and irreversible decline in physiological functions with time. Two interventions that have been shown to extend lifespan in organisms as diverse as yeast and mice are caloric restriction (CR) and reduction of flux through the insulin-like signaling pathway. In a screen for mutants in *Drosophila* which extend lifespan, one of the long-lived lines found was DJ709, an enhancer trap containing a P-element inserted in the 5' UTR of *spermidine synthase*. Spermidine synthase is an enzyme in the polyamine biosynthetic pathway that adds an aminopropyl group to putrescine to create spermidine. Polyamines are ubiquitous polycations that are needed for cellular proliferation, differentiation and many other processes. The polyamine levels were assayed in the mutant line and spermidine levels were found to be reduced by ~30%. To investigate whether CR may work in a similar way, the polyamine levels of flies fed on different yeast concentrations were assayed, and, as in DJ709, spermidine levels were found to be lower. Mutants which affect the insulin-like signaling pathway are being analyzed for their polyamine levels and genetic interactions with DJ709.

203. Modulation of lifespan by mutation in TOR pathway genes

Pankaj Kapahi, Brian M. Zid, Daniel Koslover, Viveca Sapin, Tony Harper, Seymour Benzer

Caloric restriction, i.e., reducing calories without causing malnutrition, has been shown in many species to extend lifespan. We find that genetic reduction of flux through a nutrient responsive pathway extends lifespan in *Drosophila*. Tuberous sclerosis complex genes 1 and 2 (TSC1 and TSC2) together inhibit TOR (target of rapamycin), which mediates its downstream effects on translation via the S6 kinase. Together, they control growth and size in a pathway parallel to and interacting with the insulin-signaling pathway. Modulation of these genes in the fat and muscle tissues is sufficient to cause the lifespan extension in *Drosophila*. Examining the lifespan of flies overexpressing TSC2 under different concentrations of yeast in the diet suggests an overlap of the mechanisms of lifespan extension by the two parameters.

204. Genetic screening for multiple stress resistant EP mutants

Horng-Dar Wang, Seymour Benzer

By forward genetic screening, using both oxidative stress (paraquat) and starvation, we have found several multiple stress resistant EP mutants that are resistant to both stresses. Lifespans of the mutant lines were measured at 25°C, and seven of the eight showed significant increases in lifespan. We focused on three mutant lines that have greater lifespan extension than *methuselah*. Two have EP insertion in unknown genes and the other in a gene involved in glucose metabolism. Northern blotting and RT-PCR will be used to determine whether genes nearby to the EP element are affected. The tissues where the genes are expressed will be pinpointed by *in situ* hybridization, and transgenic flies carrying the constructs will be used to overexpress and under express the genes.

205. Characterization of a mutant that extends life by self-imposed caloric restriction

Ted Brummel, Laurent Seroude, Alisa Ching, Seymour Benzer

In the mutant *Drosophila* strain, DJ817 which has increased longevity, the gene product is expressed in sensory organs in the adult fly, as indicated by LacZ as a marker. Our analysis of this line suggests that sensory abnormalities lead the mutant to consume less food than wild-type flies, thereby constituting self-imposed caloric restriction.

The gene has three alternatively spliced messages with predicted gene products of high homology to a mosquito gene and weak homology to human and mouse proteins of unknown function. New alleles are being generated using RNA-i to confirm the involvement of the gene in the longevity and eating phenotypes.

206. Effects of bacterial flora on longevity in ***Drosophila***

Ted Brummel, Laurent Seroude, Seymour Benzer

We have found that lifelong elimination of bacteria, via either axenic culture or antibiotic treatment, results in a reduction in lifespan of approximately 30%. One possible explanation is that bacteria in the gut may affect nutrient uptake or utilization. For a mutant, which lives long as a result of a reduction in food intake, the longevity, under bacteria-free conditions, was found to be indistinguishable from that of wild-type flies.

We are currently measuring food intake in axenic and non axenic flies, as well as for strains such as *chico* and *InR,* in which insulin signaling is compromised.

207. A ***Drosophila*** model for neuronal ceroid lipofuscinosis

Atsushi Yamaguchi, Seymour Benzer

Lipofuscin is a brown-yellow, autofluorescent, electron dense substance that accumulates, with age, within the lysosomal compartment in postmitotic cells. Lipofuscin has been recognized as a universal hallmark of aging, as if the waste disposal machinery becomes less efficient. One of our goals is to clarify the significance and underlying mechanism of lipofuscinogenesis in a group of human diseases called neuronal ceroid lipofuscinoses (NCLs), the most common hereditary neurodegenerative disorders in childhood, with characteristic symptoms including progressive vision loss,

psychomotor disturbances and premature death. To date, at least eight causative genes (*CLN1-CLN8*) have been reported. The fruit fly *Drosophila melanogaster* has at least two counterpart genes, *CLN1* and *CLN3*. *CLN1* encodes lysosomal enzyme palmitoyl-protein thioesterase (PPT1), which removes fatty acid palmitate from cysteine residues in various *S*-acylated proteins *in vitro*. Palmitoylation, important in subcellular distribution of proteins, is a reversible post-transcriptional modification, unlike myristoylation and prenylation, suggesting that PPT1 is involved in not only protein degradation, but also neuronal signaling pathways.

We have applied the Gal4/UAS binary regulatory system and RNA interference (RNAi) methodology to set up a *Drosophila* CLN1 model in which a Gal4-regulated, inverted-repeat *CLN1* RNA-interference transgene disrupts PPT1 in a tissue-specific manner. Eye-specific PPT1 suppression (using a GMR-Gal4 driver) caused a severe, rough eye phenotype in which eye structure, pigment, and size were severely disrupted. This model is being used to search for genes that suppress the toxic accumulation of lipofuscin.

208. Genetic analysis of nociception

W. Dan Tracey Jr., Seymour Benzer

We are using the fruitfly *Drosophila melanogaster* as a model system to understand the molecular basis of pain. We have discovered that *Drosophila* larvae respond behaviorally to noxious stimuli in a highly reproducible and stereotypical fashion. Normal undisturbed fly larvae move through their environment with a rhythmic peristaltic motion. When touched lightly with an innocuous probe, they briefly pause motion, then resume. However, when touched with a probe heated to an unpleasant temperature (T>38^{o}C), the larvae respond by vigorously rolling in a corkscrew type motion. We are using this stereotyped behavior to perform genetic screens with the goal of identifying mutants that are insensitive or sensitized to noxious stimuli.

Thus far, we have focused on one such *Drosophila* mutant insensitive to noxious heat, which we have named *painless*. The *painless* mRNA encodes a protein of the transient receptor potential ion channel family. *painless* is required for both thermal and mechanical nociception, but not for sensing light touch. *painless* is expressed in peripheral neurons that extend multiple branched dendrites beneath the larval epidermis, similar to vertebrate pain receptors. An antibody to Painless binds to localized dendritic structures that we hypothesize are involved in nociceptive signaling. In collaboration with the Laurent lab, we found that wild-type larval nerves contained afferents of heat-sensitive neurons that initiate strong spiking above 38^{o}C, and this activity was absent in the *painless* mutant.

209. Circuit analyses of innate avoidance behavior in ***Drosophila***

Greg S. Suh, Seymour Benzer, David J. Anderson

We have developed a novel behavioral paradigm for an innate avoidance response in *Drosophila*. The avoidance of a putative "alarm" substance emitted by conspecifics subjected to stress. Responder flies are given a choice in a T-maze between a fresh tube and one in which a set of emitter flies was previously traumatized by shaking. Most responders avoid the latter tube; the performance index typically being around 90%. Preliminary results suggest that recognition of the "alarm" substance is mediated by the olfactory system.

We have been trying to identify groups of neurons in the CNS, downstream from the olfactory sensory neurons, that are essential for the avoidance behavior, by carrying out an unbiased neuronal inactivation screen using the Gal4 x UAS-*Shibire*^{ts} system. Flies bearing a Gal4 enhancer trap and a UAS-*Shibire*^{ts} were assayed in the behavior paradigm at the non-permissive temperature (30°C), compared to their performance at the permissive temperature (21°C). We have screened ~280 Gal4 enhancer trap lines and identified nine lines that are defective in this assay at non-permissive temperature, when bearing UAS-*shibire*^{ts}, but retain normal olfactory responses to various odorants.

To visualize the region in the adult brain where Gal4 is expressed in these lines, we crossed them with UAS-mCD8/GFP. Several of the lines show specific expression in a defined region in the subesophageal ganglion (SOG). We are planning to replace the Gal4 with Gal80 in each gene, to test whether that will suppress the behavioral defect when Gal80 is expressed in the same region. This experiment will show that the region in the SOG is essential for the avoidance behavior. Once we have a number of candidate neuroanatomical loci mediating the avoidance behavior, we will examine the relationship between the loci by crossing the candidate Gal4 lines to UAS-WGA to trace the transsynaptic neural pathways involved.

Publications

Simon, A.F., Shih, C., Mack, A. and Benzer, S. (2003) Steroid control of longevity in *Drosophila melanogaster*. *Science* 299:1407-1410.

Tracey, W.D., Wilson, R.I., Laurent, G. and Benzer, S. (2003) *painless*, a *Drosophila* gene essential for nociception. *Cell* 113:261-273.

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Summary: Experimental embryologists can observe single cells as they migrate and join other cells during the sculpturing of an embryo. The specificity and precision of these events is truly extraordinary. Migrating cells coalesce to create the heart. Cells originating in the neural crest migrate and continue to divide as they contribute to the shaping of teeth – and their innervation. The genetic programs responsible are so extraordinary that the upper and lower sets of teeth fit together, and even more amazingly the teeth (and indeed all other parts) of identical twins are -- well -- nearly identical.

Half a century ago Caltech's Roger Sperry showed that the growth cones of regenerating nerves have very great specificity. His results and the other examples of the specificity of migrating cells are so remarkable that even today some scientists find it hard to believe that the cell surface molecular codes are that precise. Those of us who have searched for genes and molecules with specificity adequate to account for the observed biological phenomena have made progress but have not found the answers.

We now believe that we have identified some of the genes and molecules that provide the required very high specificity on cell surfaces and thus we have begun to uncover the genetic and molecular basis of the cell surface address codes indicated by the biological phenomena mentioned above.

The new genomic databases that have become available during the past few years have allowed us to carry out research that could not be done previously in our local laboratories. Accordingly, we have made extensive use of genomic techniques and the databases to obtain the new results. We feel that we have now benefited greatly from investments made in previous decades at Caltech to develop the microchemical instruments used in the genome projects.

Recently, we have begun to examine the possibility that genetic machinery, evolutionarily related to two types of genetic switches that are known to be used in the developing immune system, are also used in other developmental systems. We have written genomic software ("Tapestry") to aid in this project. In addition, experiments have been initiated in collaboration with Scott Fraser and David Koos in the Beckman Institute Image Center to explore the role of genetic switches in development.

210. Olfactant receptors have a dual function and may play a central role in cellular dressing for many types of organogenisis

William Dreyer

Studies in our laboratory as well as many others have succeeded in identifying a large number of cell surface molecules that play a role in cellular assembly and seem to function much like the country codes, area codes and prefixes of a telephone dialing system. However, the predicted highly specific final part of the code, equivalent to the last four digits of a unique phone number, has eluded us until very recently when we uncovered a new type of candidate code molecule: the odorant receptors. These serpentine receptors (they pass through the cell membrane seven times) are represented by thousands of genes in the human and mouse genomes. There are more than 1000 members of odorant receptor related families in *C. elegans* – more than the total number of cells in that organism. Recent research has shown that the odorant receptors not only detect odorants, a relatively common type of function for a protein molecule, but also play an extremely sophisticated role in axonal targeting as axonal processes extend from the olfactory epithelium to highly specific points (glomeruli) on topological maps on the olfactory bulb. Because these receptors are capable of such a high targeting specificity they bear the hallmarks of the proposed area code molecules that we believe aid in the assembly of most tissues. It therefore seemed appropriate to ask if they might be expressed in other parts of the developing embryo (and adult) as expected for such molecular codes. A search of the genome and literature databases revealed a remarkable number of examples of these genes expressed in tissues other than the olfactory system. The widespread expression in numerous organ systems of molecules related to the olfactory receptor family obviously supports the hypothesis that such receptors perform functions other than the recognition of olfactants. Since receptors of this type play a dual role as receptors for small molecules in the olfactory epithelium and as cell surface addressing molecules that aid in the assembly of the olfactory bulb of the brain, one obvious notion is that they may also play an assembly role in other parts of the brain and embryo and are the much-sought-after key cell surface molecules used in assembling embryonic tissues.

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211. Homophilic interactions of olfactory receptors can explain otherwise perplexing experimental observations

William Dreyer

Scientists using the two-photon confocal microscopes in the Beckman Institute Biological Imaging Center (headed by Scott Fraser) have been able to observe olfactory axons as their growth cones migrate to the brain (see references below). I have been fortunate to be able to observe this work and have used it, together with studies from other labs, to draw the following conclusions: 1) Axons that express one specific receptor have a strong tendency to fasciculate (bond) to themselves to the exclusion of the 1,000 or so axons that display other receptors; 2) As a specific axon homes in on its target glomerulus, the growth cones sometimes move to the correct target after first going near a neighboring glomerulus (see below re heterophilic interactions); 3) When all olfactory axons are labeled (with olfactory marker protein) it can be seen that thick bundles of axons emerge from each adult glomerulus without obvious mixing with other fascicles.

These observations argue strongly that each of the 1,000 or so olfactory receptors is capable of recognizing itself with highly specific homophilic interactions. Since these receptors are expressed on cells of essentially all tissue types, homophilic interactions would add high specificity to cell-cell interactions throughout the organism. While the proposed homophilic interactions seem highly likely based on the experimental evidence mentioned above, heterophilic interactions with very similar receptors on neighboring cells could provide a "gradient of receptor affinities," a potentially powerful mechanism for the assembly of organisms (see Dreyer, 1998; Dreyer and Roman Dreyer, 1999).

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Summary: Research in the laboratory of Professor Christof Koch focuses on three areas: (1) biophysics of computation in single neurons; (2) understanding visual selective attention and visual consciousness at the neuronal, behavioral and computational levels; and (3) studying the role of attention, working memory and awareness in associative fear conditioning in humans and mice. For more details and all publications, see http://www.klab.caltech.edu.

Research carried out in our group as part of a program called "Biophysics of Computation" studies how the biophysics, synaptic architecture, and dendritic architecture of individual neurons subserve information processing. This research has been summarized in a textbook, *Biophysics of Computation: Information Processing in Single Neurons*, by C. Koch and published by Oxford University Press in 1999. To what extent do neuronal noise sources (thermal noise, channel noise, noise due to synaptic background firing, and so on) limit signal detection or signal reconstruction at the level of individual neurons? What are the biophysical mechanisms underlying neuronal computations? How do neurons multiply? How complex are single nerve cells? What is the code used to transmit this information? Analytical work, backed up by detailed computer simulations of nerve cells based on electrophysiological data from our experimental collaborators at the Hebrew University in Jerusalem (Idan Segev and Yossi Yarom) and elsewhere, is used to generate experimentally verifiable predictions. In collaboration with Fabrizio Gabbiani (Baylor College), we continue to pursue to understand how multiplication is implemented at the single cell level in an identified neuron in the locust's visual system. We are also analyzing the variability, reliability, and randomness of multi-unit firing activity from cells in medial temporal and frontal lobes in human patients and in monkey's visual cortex.

Understanding complex information-processing tasks, in particular the action of selective, visual attention (both saliency-driven bottom-up as well as task-dependent, top-down form) requires a firm grasp of how the problems can be solved at the "computational" level, and how the resulting algorithms can be implemented onto the known architecture of the visual cortex and associated subcortical areas. We use analytical methods, coupled with detailed computer simulations of the appropriate circuitry in the primate visual system, to study how these neuronal networks control selective visual attention and how they give rise to motion perception, object discrimination and detection (in collaboration with Tomasio Poggio at MIT and Laurent Itti at USC). The resulting algorithms are being applied to problem in image analysis and machine vision. Researchers in our laboratory study visual perception in the presence and (near)-absence of selective visual attention as well as our ability to classify and distinguish two-dimensional visual patterns using psychophysical techniques in normal subjects. We are complementing these studies using noninvasive fMRI imaging under the identical stimulus protocols. Our laboratory continues to collaborate with Itzhak Fried at UCLA in recording single units from multiple electrodes in the medial temporal cortex of awake patients during visual perception, rivalry, and imagery.

We continue to collaborate with Francis Crick (Salk) to develop a neuronally-based framework to understand how subjective feelings (e.g., as in conscious visual perception) can arise in the mammalian forebrain. In order to make experimentally verifiable progress on the mind-brain problem, it will be critical to interfere deliberately, transiently, rapidly and reversibly with groups of genetically identifiable forebrain neurons in experimental animals. In collaboration with David Anderson (Caltech), Henry Lester (Caltech) and Michael Fanselow (UCLA), we have developed an associative fear-conditioning paradigm in both mice and normal human subjects. We are investigating the role of working memory, attention and awareness in acquiring delay versus trace conditioning in both species and in identifying their

underlying neuronal representations, with a particular focus on the anterior cingulate cortex.

212 Intrinsic subthreshold noise in cultured hippocampal neurons

Kamran Diba[1], Henry Lester[2], Christof Koch

We calculate the power spectral density of current and voltage in E18 neurons cultured from the rat hippocampus by taking long (2-minute) traces and applying Welch spectral analysis. The current is measured under voltage-clamp and the voltage is measured under current-clamp. Synaptic noise is blocked by the addition of picrotoxin and DNQX. The power spectrum behaves as a lorentzian, with a cutoff due to the time constant of the cell. The overall power (and hence the variance) increases when the holding potential is increased, in a manner indicative of the activation of voltage-gated channels, as demonstrated by a model of stochastic ion channels. The model is based on a Markov-chain approximation to the channel kinetics. Then, based on the cable theory of neurons, the contribution of a distribution of ion channels is summated through the transfer impedance (or Green' s function) calculated with the NEURON simulation environment. The role of different channel types is investigated by the addition of pharmacological channel blockers, such as TTX, TEA, and 4-AP, and compared to expectations based on the model.

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213. Line source approximation predicts extra-cellular voltage for CA1 neurons recorded ***in vivo***

Carl Gold[1], Christof Koch, Darrell Henze[2], Gyorgy Buzsaki[2]

The Line Source Approximation (LSA) is a mathematical method for calculating the extracellular field from a 3-D distribution of membrane current sources. We investigate the use of the LSA combined with detailed compartmental modeling, including a model of the electrodes used, to predict the extracellular voltage waveform shape and magnitude resulting from the spiking activity of individual neurons. This provides an estimate of the maximal distance at which a neuron could be detected by an extracellular electrode. In order to tune the model we compare simultaneous intracellular and extracellular recordings of CA1 neurons recorded *in vivo* with model predictions for the same cells reconstructed and simulated. The approximate electrode position is estimated from the histologically determined track. We overcome the uncertainty regarding the values of biophysical parameters, such as the extra-cellular conductivity and the membrane Na+ conductance, by comparing the model and experimental results for numerous samples of the same class of neuron. Based upon comparisons with experimental data, we conclude that the compartmental model can accurately simulate the *in vivo* intracellular action potential and the LSA model can accurately simulate the extracellular fields of individual spiking neurons.

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214. A learning rule for local synaptic interactions between excitation and shunting inhibition

Chun-Hui Mo[1] and Christof Koch

The basic requirement for direction selectivity is a non-linear interaction between two different inputs in space-time. In some models, the interaction is thought to occur between excitation and inhibition of the shunting type within the neuron's dendritic tree. How can the required spatial specificity be obtained in an unsupervised manner? We here propose an activity-based, local learning model that can account for direction selectivity in neurons in visual cortex based on a local veto operation between excitation and inhibition. We implemented the learning rule with local calcium concentration change. Our biophysical simulations suggest that a model cell with our learning algorithm will develop direction selectivity organically after unsupervised training. The learning rule is also applicable to cells with multiple direction selective subunits on dendrites and is stable under different starting conditions.

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215. The attentional requirements of face-gender discrimination

Leila Reddy[1], Patrick Wilken, Christof Koch

The attentional cost associated with the visual discrimination of the gender of a face was investigated. Participants performed a face-gender discrimination task either alone (single-task), or concurrently (dual-task) with a known attentional demanding task (5-letter T/L discrimination). Overall performance on face-gender discrimination suffered remarkably little under the dual-task condition compared to the single-task condition. Similar results were obtained in experiments that controlled for potential training effects or the use of low-level cues in this discrimination task. Our results provide further evidence against the notion that only low-level representations can be accessed outside the focus of attention.

[1]*Computation and Neural Systems graduate student, California Institute of Technology*

216. Natural scene categorization in the near absence of attention: Further explorations

Fei Fei Li[1], Rufin VanRullen, Christof Koch, Pietro Perona[2]

Subjects are able to detect quickly animals and vehicles in previously unseen cluttered scenes presented peripherally even when their attention is distracted. They are, however, unable to discriminate rotated letters (T/L) and bisected color disks (Red/Green) in the same

conditions (Li *et al*., 2002). We explore this phenomenon further by variations of the original experiment.

The first experiment was designed to further probe the extent to which natural scene categorization is possible in the absence of attention. Subjects were instructed to respond whether there was an animal in one of the two natural images presented peripherally in random locations, while concurrently performing an attentionally demanding central task. We could measure no significant difference in performance between one and two images. This result strengthens the view that attention is not a critical resource in this task.

The second experiment was designed to verify whether T-L (or bisected color disks) discrimination was poor due to a lack of signal. The number of rotated letters (or bisected color disks) peripherally was increased to four instead of one. Even though there were more potential "features" for subjects to detect (e.g., four T junctions than just one), our subjects still failed to discriminate between T's and L's (or between Red/Green and Green/Red disks) when attention was distracted.

In a third experiment we explored the nature of the mechanisms that are critical for the T-L task. We reasoned that rotated T and L presented peripherally differ from images of animals in that they do not constitute an "object class": we are trained to recognize upright letters in the central region of the visual field. While distracting attention we presented our subject with upright T-L discrimination tasks in either the periphery or in the center of the visual field. The performance of our subjects improved significantly.

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Reference

Li, F.F., VanRullen, R., Koch, C. and Perona, P. (2002) *Proc. Natl. Acad. Sci. USA* 99(10):1073.

217. Inter-stimulus distance effects in visual search

Lavanya Reddy, Rufin VanRullen, Christof Koch

In a previous study, we showed that the attentional requirements of a task, as revealed by the dual-task paradigm, do not necessarily determine whether visual search will be parallel or serial. For example, natural scene categorization can be performed "preattentively" in a dual-task situation (i.e., a single scene containing animals can be discriminated from non-animal scenes even while attention is occupied elsewhere), and yet visual search for an animal scene among a number of non-animal scenes is a serial process. We interpreted these findings as follows: a task can be performed preattentively if there exist specific neuronal populations selective to the target and distractor categories, independent of the level of processing involved (from V1 to IT); when such selectivities exist, visual search is parallel only if the receptive fields of the relevant neurons do not significantly overlap. When receptive fields are too large, target and distractors compete within the same field and search is serial. It follows that search performance should improve if target and distractors can be separated enough to prevent them from falling into the same receptive field. We tested this prediction and found that for preattentive tasks that usually result in serial visual search (e.g., color-orientation conjunction discrimination, upright vs. inverted face discrimination), search performance improved as inter-stimulus distance was increased. For preattentive parallel tasks (color discrimination, orientation discrimination), the effect of increasing inter-stimulus distance was negligible. These results support the idea that for preattentive tasks, competition within the relevant receptive fields can affect visual search performance.

218. The neuronal correlates of change detection

Leila Reddy[1], Patrick Wilken, Rodrigo Quian Quiroga, Itzhak Fried[2], Christof Koch

At any instant, our phenomenal experience of the world is that of a rich, detailed visual environment. However, recent research shows that in fact, observers do not retain all the visual details from one view to the next and are often unable to detect dramatic changes in a scene. We are currently investigating the neuronal correlates of the detection of such changes in human patients at UCLA by recording from chronically implanted electrodes in the medial temporal lobes of epileptic patients. Specifically, our goal is to test the differences in neural responses to a scene when a change is detected compared to when the change goes undetected or even imagined. Thus far, we have recorded from a total of 91 neurons in three patients, with 15% of these units showing increased firing rates in response to a visual stimulus.

A new technique of recording neuronal data from human subjects has also been implemented. Up until recently, spikes were detected via amplitude thresholding. This method had severe limitations particularly because of the difficulty in estimating optimal thresholds for 64 channels. The new technique of continuous wide-band recordings, overcomes this limitation, and has resulted in a dramatic increase in the number of units we are able to isolate as well as in the number of spikes per unit. Specifically, a comparison done on two recording sessions in two patients shows that with the new technique we are able to isolate 43 single units (1911 spikes/unit), and 46 multi-units (6167 spikes/unit), whereas with the previous method we obtained 22 single units (982 spikes/unit), and 16 multi-units (2062 spikes/unit).

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219. Contour facilitation in a model of bottom-up attention

Robert J. Peters[1], T. Nathan Mundhenk[2], Laurent Itti[2], Christof Koch

Previously we showed that interactions among overlapping orientation-tuned units could improve a bottom-up attention model in predicting human eye movement targets. We have now extended this work to address the question of how elongated contours affect saliency in natural scenes. We used a model of contour-facilitation based on putative long-range excitatory and inhibitory interactions among orientation-tuned units in early visual cortex. Each unit tends to excite other units that are nearly collinear, and inhibit those that are nearly parallel. We tested the model on artificial images such as arrays of Gabor patches with embedded implicit contours ("snakes"), as well as natural images such as outdoor photos and overhead satellite photos. Our results agree with previous psychophysical measurements of human observers' sensitivity to implicit contours such as Gabor snakes; we found that a basic bottom-up saliency model was completely blind to such contours, while an enhanced saliency model with contour-facilitation module could consistently identify the embedded contour as the most salient element in the image. Preliminary eye tracking results suggest that observers are less sensitive to high spatial-frequency contours in natural scenes.

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220. Eccentricity-dependent changes in visual performance: An enhanced model of bottom-up attention

Asha Iyer[1], Robert Peters[2], Christof Koch

A model of bottom-up attention employed to predict human saccadic targets was previously shown to exhibit a significant discrepancy from human behavior with respect to distribution of fixations across the visual scene. The model predicts successive fixations in a uniform manner across the visual field, dependent entirely on salience of the image at that point. However, an analysis of human saccades indicates a clustering of subsequent fixations around the prior fixation point. To address the issue of spatial nonhomogeneity of the visual scene, we implemented space- and frequency-dependent filters based on psychophysical data on visual performance across the visual field (Virsu and Rovamo, 1979). Each spatial scale representation of the image is differentially attenuated as a function of eccentricity. We tested the model on artificial images such as fractals, and natural images such as outdoor scenes and overhead satellite photos. Our results show that this modified saliency model accounts for human eye movements with considerable accuracy as compared to the original model: the salience values predicted at fixated locations were roughly 2.5 times higher in the modified vs. the original model. In addition, a single exponential decay was fitted to the enhanced model. This simpler approximation shows a similar substantial increase over the performance of the original model, and a significant improvement over other approximations of eccentricity-dependent filtering in the literature (Parkhurst *et al*., 2002).

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221. Attentional selection for object recognition – A gentle way

Dirk Walther[1], Laurent Itti[2], Max Riesenhube[3], Tomaso Poggio[3], Christof Koch

We continue to develop an integrated model for the dorsal (where) and the ventral (what) pathway in the primate's visual processing system and the interaction between these two pathways. In extension of the saliency-based model for bottom-up attention by Itti, Koch, and Niebur (1998) we have developed a mechanism for extracting an estimate of the shape and size of an object or object part from low-level image properties at the attended location. We use this mechanism to modulate the activity of neurons at the V4 level in the hierarchical model for object recognition HMAX by Riesenhuber and Poggio (1999). We show that modulation of the V4 cells by as little as 20% is sufficient to sequentially recognize multiple objects in an image panel. The effect is robust over a large range of strength of modulation of the V4 cells. The objects that can be recognized in this manner by the recognition system comprise wire frame ("paperclip") objects as well as images of faces. In our future work we will explore the application of a similar attentional modulation process to machine vision for identifying multiple objects in natural scenes. We will further investigate biological and computational mechanisms of biasing the attention system for the search for particular objects in cluttered natural scenes.

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Riesenhuber, M. and Poggio, T. (1999) *Nat. Neurosci*. 2(11):1019-1025.

222. Automated video analysis for oceanographic research

Dirk Walther[1], Duane Edgington[2], Christof Koch

We develop an attentional selection system for processing video streams from remotely operated underwater vehicles (ROVs). One important application of ROVs is the possibility of obtaining quantitative video transects (QVT) with the video cameras on board the ROVs. The bottleneck for QVT is the capacity for annotation of the videos by scientists on shore. The process is tedious and time consuming. Automating parts of this process would greatly improve the usefulness of QVT. Our attention system identifies potentially interesting visual events based on salient features such as elongated edges or strong luminance contrasts in the image. Salient objects are tracked across video frames spanning multiple frames. A number of properties are extracted for shape of each object such as its apparent size in the image, its major and minor axes, and its second moments with respect to its centroid. Based on these properties, a concept for an "interesting" event is learned from training data provided by the human annotators. Frames that do not contain any "interesting" events are marked as "boring" and can be omitted from the output or re-played at an accelerated rate for the annotators. We investigated the suitability of the attention system for the task in a study with 1460 single frames from underwater video, of which 582 contained animals. In 88% of these frames the animal was marked as the most salient item in the image. In a study with five video clips of 10 seconds length each we obtained a detection rate of 93% of "interesting" events, with a false alarm rate of 9.6%. In the future we plan to develop an object classification module for the most common animals. The system will be able to process video data fully automatically for these species, and flag events that it cannot identify for human inspection.

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223. A neurobiological framework for consciousness

Francis Crick[1], Christof Koch

We summarize our present approach to the problem of consciousness, which is focused on discovering the neuronal correlates of consciousness (NCC). In the fullness of time, a complete theory of consciousness is required. However, for now, we focus on an experimentally more accessible goal, that of characterizing the NCC in mice, monkeys and humans. We describe what is meant by the term "framework." We set this out in ten working hypotheses. These are: (1) the (unconscious) homunculus; (2) zombie modes and consciousness; (3) coalitions of neurons; (4) explicit representations and essential nodes; (5) higher levels first; (6) driving and modulating connections; (7) snapshots; (8) attention and binding; (9) styles of firing; and (10) the penumbra and meaning. Most of these ideas have been suggested before, but we believe their combination is original. We also outline some possible general experimental approaches to the problem. All of this should allow this most vexing problem to be approached in an empirically verifiable manner.

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Crick, F. and Koch, C. (2003) *Nature Neurosci.* 6:119-127.

224. Selective attentional modulation of trace vs. delay fear conditioning in C57BL/6N mice

Colm M.P. O' Tuathaigh

Studies in both eyeblink and aversive conditioning in humans have reported that attentionally demanding tasks can selectively disrupt the development of both the conditioned response as well as the explicit awareness of the CS-US contingency in the trace conditioning paradigm. In the trace conditioning procedure, a decrease in the conditioned response evoked by a CS is observed following an increase in the temporal distance between it and the US, relative to delay conditioned controls where the US immediately follows the CS. The aim of our research is to model attentional modulation of trace conditioning in mice using attentional manipulations analogous to those employed in human studies. In both the delay and trace conditions, animals receive six tone CS-footshock US pairings, except that an 18 sec. trace interval is inserted between the CS and US in the trace condition. The attentionally distracting stimulus is a flashing light presentation, where both the interstimulus interval is varied throughout the conditioning session. We have found that the noncontingent presentation of a distracting stimulus during conditioning significantly attenuates learning in the trace but not the delay condition, compared to non-distracted controls, at test. It has been shown that this disruptive effect cannot be attributed to associative interference. Further studies have demonstrated that the distractor effect is not mediated by any selective anxiogenic effect evoked by the flashing light. The results indicate agreement between animal and human data with respect to attentional modulation of trace conditioning, and suggest that this paradigm may provide a putative animal model of the relationship between attention and awareness. The current goal is to identify the neural basis of this attentional distractor effect, via immediate early gene expression.

225. The involvement of the anterior cingulate cortex in novelty

Chih-Ju Han, David J. Anderson, Christof Koch

The activation of the anterior cingulate cortex was previously shown to correlate with novelty detection. However, whether the anterior cingulate cortex is necessary to novelty detection is unclear. We set up a novelty object paradigm in mice. Mice were brought to the testing room in their home cage. A group of mice received a novel object (a corning 15-ml tube), a group

received the same procedure including lifting the cage lid but not the object, and a group received nothing. We showed that the novel object readily induces the exploratory behaviors of the mouse directed towards the novel object, and cage lid lifting induces general exploratory behaviors. The sum of time that the group receiving the novel object and the group receiving the lid lifting spend in exploratory behaviors are equal, but the exploratory behaviors in the group that received the novel object are mostly directly to the object. c-fos mRNA was used as a surrogate marker to detect neuronal activation by *in situ* hybridization on brains from each group. Animals from each of the three groups were sacrificed 30 minutes after the first exposure of the stimulus. We discovered that there are more c-fos positive cells in the anterior cingulate cortex of the brain that received the novel object, compared with the other two groups. To answer the question whether the anterior cingulate cortex is necessary for novelty detection, a group of mice received excitotoxic lesions of the anterior cingulate cortex and another group received sham surgery. Behavioral experiments and analyses are being conducted to determine whether the lesions to the anterior cingulate cortex cause any exploratory behavioral changes directed to the novel object.

226. Working memory and fear conditioning

Ronald M. Carter, Constanze Hofstoetter[1], Naotsugu Tsuchiya[2], Christof Koch

Previous studies of associative learning implicate higher-level cognitive processes in some forms of classical conditioning. An ongoing debate is concerned with the extent to which attention and awareness are necessary for trace but not delay eye blink conditioning (Clark and Squire, 1998; Lovibond and Shanks, 2002). In trace conditioning, a short interval is interposed between the termination of the conditioned stimulus (CS) and the onset of the unconditioned stimulus (US). In delay conditioning, the CS and US overlap. We here investigate the extent to which human classical fear conditioning depends on working memory. Subjects had to carry out an n-back task, requiring tracking an item 1- or 2-back in a sequentially presented list of numbers, while simultaneously being tested for their ability to associate auditory cues with shocks under a variety of conditions (single-cue versus differential; delay versus trace; no task versus 0-back, 1-back and 2-back). Differential delay conditioning proved to be more resilient than differential trace conditioning but does show a reduction due to task interference similar in slope to that found in trace conditioning. Explicit knowledge of the stimulus contingency facilitates but does not guarantee trace conditioning. Only the single-cue delay protocol shows conditioning during the more difficult working memory task. Our findings suggest that the larger the cognitive demands on the system, the less likely conditioning occurs. A post-experimental questionnaire showed a positive correlation between conditioning and awareness for differential trace conditioning extinction.

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227. Computational modeling of feature inheritance

Whee Ky (Wei Ji) Ma

The proposition that reentrant interactions into the early visual system are necessary for visual awareness has lately been under close scrutiny. We examine this proposition in the context of a neuronal model which explains the phenomenology of feature inheritance.

Feature inheritance (Herzog and Koch, 2001) is a class of visual illusions in which a mask stimulus acquires a feature from a preceding brief target stimulus. We focus on the case where the target stimulus is a single tilted bar (on for 30 ms), and the mask stimulus is a grating of straight bars (on for 300 ms). In this case, only a tilted grating is perceived. If the mask is on for a short time (60 ms), target and mask are perceived superimposed.

Feature inheritance thus exhibits aspects of both backward masking and of temporal integration. Correspondingly, our model consists of two pathways: a multiplicative hypothesis-testing one and an activity-accumulating one. The multiplication pathway multiplies a "template" (a hypothesis about the world, based on early stimuli) with the current bottom-up input, in order to test the hypothesis. The template can be influenced by high-level expectations. If the match is good, the result is a bound object that is subsequently sent to perception. If it is bad, such as when the input is very brief or rapidly changing, the brain uses the output of the second pathway, which accumulates activity during the time the hypothesis-testing has not yet been completed. This yields temporal integration.

Although it is possible that the multiplicative pathway involves descending connections, no feedback in a graph-theoretical sense is necessary. Our model can explain much of the psychophysics of brief visual stimuli. In particular, we claim that the reconstruction of the contents of the perceptual gap due to the hypothesis testing is exactly Efron's "minimal perceptual moment." Furthermore, our model is consistent with the computational model of object substitution masking as presented by DiLollo *et al*., (2000).

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228. Fast Bayesian support vector machine parameter tuning with the Nystrom method

Carl Gold[1], Peter Sollich[2], Alex Holub[1]

We experiment with speeding up a Bayesian method for tuning the hyperparameters of a Support Vector Machine (SVM) classifier. The Bayesian approach gives the gradients of the evidence as averages over the posterior, which can be approximated using Hybrid Monte Carlo simulation (HMC). By using the Nystrom approximation to the SVM kernel, our method significantly reduces the dimensionality of the space to be simulated in the HMC. We show that this speeds up the running time of the HMC simulation from $O(n^2)$ (with a large prefactor) to effectively O(n), where n is the number of training samples. We conclude that the Nystrom approximation has an almost insignificant effect on the performance of the algorithm when compared to the full Bayesian method, and gives excellent performance in comparison with other approaches to hyperparameter tuning.

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229. Mismatch reduction in an on-chip image processing chip performing feature detection

Ania Mitros[1]

Feature extraction is a first step for many existing computer vision algorithms. This computation is also often one of the most time- and resource-intensive steps because the same local computation must be performed at each pixel. To head towards a real-time, small-size, energy-efficient implementation, Pesavento implemented the Tomasi-Kanade feature extraction algorithm in silicon. Although each feature detector worked splendidly, transistor mismatch killed the performance of the array. I have been re-implementing the blocks of the feature detector with floating gate transistors within each to permanently program away the mismatch. I have implemented mismatch reduction in the photoreceptor and the multiplier; both are tested and function as desired.

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230. Ecology, illusions, curiosity, cellular automata and metrics for spike train analysis

Alex Bäcker[1]

A visual illusion concerning earthshine. Described a novel visual illusion concerning the Moon –observers perceive the illuminated part as belonging to a circle of greater diameter than the dark part (Earthshine).

A robust measure for spike timing jitter. Measuring the precision of spike timing under dynamic conditions is problematic due to the difficulty of ascribing matches between spikes in the face of dropped spikes. Existing methods draw an arbitrary cutoff for the maximum allowed separation between matching spikes. We have developed a measure robust to variations in the choice of such maximum separation.

A measure for collective synchronization. Our previous work has shown neuronal synchronization is crucial to the decoding of information in neural assemblies. Yet methods to measure non-oscillatory synchronization in multineuron recordings are lacking. We propose the variance of the spike count across a neuronal assembly as a measure for collective synchronization.

Cellular automata and randomness. Proved wrong a contention of Stephen Wolfram's book, *A New Kind of Science*, about the generation of apparent randomness by simple automata with simple initial conditions by showing statistical regularities exist in the binary sequence produced by rule 30 in the central column.

A mathematical formulation for curiosity. Autonomous robots tend to cluster around what they know best after finding rewarding stimuli. We have developed a mathematical formulation for curiosity to guide exploration of robots and machine learning algorithms. Learning behavior should seek to maximize not prediction success, but *change of predictions*, venturing into spaces with poor prediction accuracy. We do this by rewarding increases in prediction confidence.

Ecological determinants of stable equilibria with multiple coexisting genotypes. Perhaps the oldest mystery of evolution and ecology is the origin of biodiversity. Different species can co-exist stably in the same niche for ages. In contrast, all intermediates between our last common ancestor with the great apes have disappeared. Additionally, all species examined exhibit a striking abundance of polymorphisms. We have put forth a theory to explain biodiversity.

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Summary: Many animals use voice for communication, but few must learn to vocalize. The known vocal learners include humans, whales, dolphins, and some bats among mammals and songbirds, parrots, and some humming birds among birds. Human babies learn some of the characteristics of speech sounds before they can speak. For example, pre-speech age babies of all races can hear the difference between L and R. Japanese babies lose this ability as they learn their native language, which does not distinguish one from the other. Similarly, young songbirds memorize the song of their father or other members of the species before they can sing. A song memory may be retained for many months in species that sing only in the spring. Birds use remembered tutor songs as templates to shape their own song. Hearing of the bird's own song or auditory feedback is essential for this process. Yasuko Funabiki used zebra finches to study memory retention in song learning. She let young birds memorize a tutor song for the first 35 days of age and then prevented them from hearing their own voice for many weeks and months. When the birds were allowed to hear their own song, they gradually reproduced the tutor song. Her work provided by far the most rigorous test for long memories in song learning.

The brain site and the nature of song memories are the subjects of Teresa Nick's projects. A group of discrete brain areas controls the production and presumably learning of song. One of these areas is called HVC, which relays signals to the major motor pathways leading to the muscles of the vocal organ. HVC neurons also respond to auditory stimuli, particularly the individual bird's own song. Teresa studied the auditory responses of HVC neurons during song development in young zebra finches. She found that HVC neurons' preference for song changed as song changed such that the "current" song was always the most effective stimulus. Her previous work indicated that HVC neurons responded to the bird's own song only during sleep or under anesthesia. According to her recent studies, HVC neurons of young finches respond best to the tutor song in the wake state and to the bird's own song in sleep. However, it is not known whether HVC generates all these response properties or receive them from its afferent sources. One of these is an area called Nif. Yuichiro Hayashi has been studying responses of Nif neurons to song. He sees gating, i.e., responses to the bird's own song in sleeping but not in waking birds. Gating was originally discovered in an area called RA, which is three stations downstream from Nif. Gating is likely to regulate auditory input to the song control system.

Our laboratory also conducts research on the brain mechanisms of sound localization in barn owls. Kazuo Funabiki studied the cellular mechanisms of coincidence detection for computation of interaural time differences (ITD). He managed to do intracellular recordings in nucleus laminaris. He found that compound postsynaptic potentials vary as a function of the waveform of the stimulus tone. We will see how the analysis of this exciting finding proceeds in Japan where he now lives. Bjorn Christianson and José Luis Peña also worked ITD processing by laminaris neurons. These cells respond to multiple ITDs that are separated by integer multiple of the period of the stimulus tone. When the stimulus is broadband noise, the response declines as the stimulus ITD departs from the best ITD. Bjorn and José analyze this phenomenon. José also analyzed the effects of binaural correlation on the intracellular response of space-specific neurons, which form a map of auditory space in the midbrain. When a broadband signal goes to one ear and its copy goes to the other ear, the binaural correlation is 1. Addition of random noise to these signals reduces the binaural correlation. The space-specific neuron does not fire any spikes in response to uncorrelated signals. However, intracellular recordings reveal subthreshold postsynaptic potentials in the neuron. This approach tells why the space-specific neuron does not respond to uncorrelated signals.

231. Long memory in song learning by zebra finches

Yasuko Funabiki

Young songbirds use memorized tutor songs as templates to shape their own songs. This process requires control of voice by auditory feedback. We prevented zebra finches from hearing their own vocalizations by exposure to loud noise after 35 days of age, before which they had been reared with song tutors from birth. When the noise stopped at 102 -200 days of age, the birds sang unstable and noisy song syllables that did not resemble the tutor syllables. The similarity to the tutor syllables steadily increased until the time of song crystallization about 30 days later. These findings show that the memory of tutor syllables survives auditory perturbations during the period when it is normally recalled and that zebra finches can use the memory well after the normal period of song development. The temporal order of syllables resembled the tutor model only in birds released from the noise before 80 days of age but not in older birds. Thus, different schedules and processes may govern the learning of syllable phonology and syntax.

232. Response bias: Neural correlates of memory in the birdsong system

Teresa Nick

The zebra finch acquires its song by first memorizing the song of an adult male tutor (sensory phase) and then matching its own vocalizations to the tutor song memory (sensorimotor phase). Vocalizations begin as slow, rambling series of begging calls and are subsequently shaped over ~40 days into a stereotyped, highly-structured, repetitive mature song which resembles the tutor song. The shaping of vocalizations by the tutor song memory must ultimately affect the song premotor circuitry, which drives singing. We have investigated auditory selectivity of the premotor song nucleus HVC (acronym used as proper name) during song acquisition using chronic multi-unit recording across the sensorimotor phase and into adulthood (45–200 days post-hatching).

HVC responds much more to the Bird's Own Song (BOS) during sleep than during waking in adult finches. We now report that the immature HVC also responds more to the BOS during sleep, even in the earliest stages of song development when the vocalization bares little resemblance to an adult zebra finch song. The response to BOS exceeds that to tutor song and several other stimuli. Moreover, responses to prior versions of BOS decline as the song matures. These data indicate that HVC response bias during sleep reflects the current vocal output of the song system. Thus, sensory and motor activities are tightly linked in this developing sensorimotor system.

During waking, the immature HVC exhibited a striking state-dependent shift in stimulus preference, preferring tutor song over BOS. Responses to tutor song exceeded those to BOS, songs of other adult male conspecific and heterospecific finches, and white noise. The waking response to the tutor song declined during the late sensorimotor phase. Collectively, these data show that the most effective waking stimulus during the template matching period of song acquisition is the tutor song from which the neural template was formed.

223. Behavioral state-dependent change of auditory responses in vocal control nucleus NIf of the zebra finch

Yuichiro Hayashi

In the songbird brain, HVC sends neural commands for song production and receives auditory input. The auditory responses in HVC are robust under anesthesia or during sleep, but suppressed during waking. However, the mechanism of this behavioral state-dependent modulation of the auditory responses is unknown. To investigate how this modulation occurs, we examined the behavioral state-change of auditory activity of NIf, a potential source of auditory input to HVc. Multiunit and single unit recordings from freely moving animals revealed that NIf neurons exhibited robust auditory responses during sleep. The responses were biased for the bird's own song, when compared with responses to the same song played in reverse or a conspecific song. On the other hand, the auditory responses were suppressed when the bird was awake. These results indicate that the auditory responses of Nif are modulated according to sleep/awake state, and the behavioral state-change of auditory activity in HVc may be relayed from NIf.

234. Coincidence detection properties in the owl's nucleus laminaris

G. Bjorn Christianson[*]*, José Luis Peña*

The response of nucleus laminaris neurons of barn owls is tuned to interaural time difference (ITD). Two copies of the sound coming from each ear are integrated in these neurons in a process that has been compared to a running cross-correlation. We recorded these neurons using a loose patch technique during sound stimulation with broadband and narrowband signals, and modeled their response to estimate the biophysical properties of these neurons. Consistent with cross-correlation, the response to ITD shows peaks and troughs whose amplitude decays as the stimulus moves away of the characteristic delay of the neurons. The decay of high frequency neurons was faster than lower frequency ones. A broader frequency tuning of high frequency neurons partially explains this observation. However, this trend persisted when narrowband signals were used, suggesting that high frequency neurons better detect phase mismatch. It is known that in the auditory system phase locking improves in high frequency neurons. However, the degree of phase locking does not affect overall peak decay in a cross-correlation model. Thus, our observation suggests that coincidence detector properties change from low to high frequency neurons. We designed a model using running cross-correlation of broadband and colored noise signals that shows how the neurons' frequency tuning width and the degree of decay are correlated. In view of the electrophysiological and morphological data available, we use this model to study the correlation between the observed decay in response and the effective time constant of coincidence detection.

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235. Response to random interaural phase in space-specific neurons

José L Peña

Two independent processing streams converge in the owl's external nucleus of the inferior colliculus (ICx). ICx neurons respond in an AND-gate manner to the combination of interaural time (ITD) and intensity (ILD) differences. Previous work has shown that, unlike lower levels of the auditory pathway, ICx neurons are silent when the sound does not contain ITD information as in uncorrelated sounds.

Intracellular recordings show that although neurons are silent, uncorrelated sound elicits large postsynaptic potentials. However, these postsynaptic potentials rarely evoke spikes. The spiking threshold prevents the generation of action potentials by sounds lacking ITD information.

The role of feedback in the synaptic mechanisms involved in the spatial tuning can also be addressed by using uncorrelated sound. The fact that these signals never elicit spikes, excludes the influence of possible feedback mechanisms in their response to sound. We observed that the tuning to ILD remained intact for uncorrelated sound. Unfavorable ILD elicited inhibitory postsynaptic potentials of similar amplitude in both correlated and uncorrelated sounds. Thus, feed forward mechanisms are mainly involved in the synthesis of these spatial receptive fields.

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Summary: We are interested in information coding in the brain and in the design principles of circuits involved in processing sensory information. We are particularly interested in understanding the role of time, synchronization and oscillations in information coding and in relating the biophysical properties of neurons and synapses to the function of the networks in which they are embedded. We therefore study the cellular, synaptic and network aspects of neural processing. We focused our research this year on the olfactory system of insects (antennal lobes and mushroom bodies, circuits analogous to the vertebrate olfactory bulbs and anterior/posterior piriform cortices), using locusts, *Drosophila* and honeybees as primary model systems. Our work combines experimental (behavioral, electro-physiological and two-photon imaging), and modeling techniques and aims at understanding functional aspects of brain circuits design and the rules of information coding used by the nervous system.

236. Cytoarchitecture of an insect olfactory system

S. Sarah Farivar

The insect mushroom body (MB) receives and processes olfactory information. The MB is a highly conserved structure, found in all but a few insect species, and has been shown to be a relevant area in learning and memory of olfactory information. The functional properties of the intrinsic cells of the MB, the Kenyon cells (KCs), has been extensively studied and their integrative properties are starting to be understood. To help decipher their role in odor processing, we are studying the morphology of these cells in an effort to map the ways in which these cells sample information from the upstream olfactory relay, the antennal lobe. Using electrophysiology and intracellular dye fills of both KCs and the projection neurons of the antennal lobe, we have begun to characterize these cells morphologically and to identify their connectivity patterns.

237. Decoding of olfactory information in the Kenyon cells of the mushroom bodies

J. Perez-Orive

In the locust olfactory system, the output of the first olfactory relay, the antennal lobe (AL) is periodic and synchronized. The Kenyon cells (KCs) of the mushroom body receive AL input via projection neurons (PNs) and are involved in olfactory learning. We examined the properties of KCs to better understand how they decode these PN inputs. We first conducted intracellular recordings of KCs while applying electrical stimulation to PNs. As more PNs are recruited by increasing the stimulation amplitude, the KC EPSPs undergo subthreshold, voltage-dependent non-linearities, well suited to facilitate responses to coincident input. Similar voltage-dependent behavior can be observed by depolarizing the KCs with intracellular current injection. KCs are very specific in their odor responses and their action potentials tend to be phase-locked with respect to the cycles of local field potential oscillations (Perez-Orive *et al*., 2002). We analyzed the relationship between the degree of phase-locking of individual KCs and their degree of odor responsiveness, and find that the KCs that respond with higher specificity present tighter phase-locking than the more promiscuous KCs (even though the average preferred phase of all KCs remains the same). Taken together, these results support the idea that KCs make use of coincidence detection and that this is related to their odor specificity.

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238. Intensity versus identity coding of odor by neuronal ensembles in the antennal lobe

Vivek Jayaraman, Mark Stopfer

We investigated the encoding and decoding of odor identity and intensity by neurons in the first and second relays of the olfactory system of the locust, the antennal lobe (AL) and the mushroom body (MB), respectively. Changes in odorant concentration led to changes in the firing patterns of individual AL projection neurons (PNs), similar to those caused by changes in odor identity. However, when we examined these time-varying response patterns across many PNs (up to 15 of which were recorded simultaneously with tetrode probes), using PCA for dimensionality reduction, the concentration-specific patterns clustered by odor identity. Using locally linear embedding (LLE) to visualize the dynamics of the

PN ensemble, we found that response trajectories for different intensities of an odor remained near one another, forming identifiable families (manifolds); trajectories corresponding to different odors formed separate manifolds. The manifolds describe intensity-invariant regions representing odors in PN coding space. We examined these representations by quantifying the variability in response patterns to repeated trials of the same stimuli and to different intensities and odors. We also compared results obtained using fixed-width or oscillatory cycle-based time bins for spike counts [Kenyon cells (KCs] in the MB integrate their PN input within single cycles); the latter approach improved classification of response patterns.

KCs had very sparse identity-specific responses with varying degrees of intensity invariance. We found that the tuning of KCs to identity and intensity and the patterning of their responses are consistent with piecewise decoding of their PN inputs over cycle length epochs.

239. Adaptive odor processing in the honeybee

Benjamin Rubin

One of the primary goals of systems neuroscience is to understand how the brain encodes and processes information about the world and uses it to produce adaptive behaviors. To achieve this goal, we have chosen to study the honeybee olfactory system, since both the anatomy and physiology of the olfactory system, as well as behaviors guided by the olfactory system, are well characterized and amenable to experimentation. In the honeybee as in other insects, odors are transduced by receptor neurons that are located in the antennae and project to the antennal lobes (ALs). In the ALs, different odors are represented by overlapping but distinct spatiotemporal activity patterns. Stimulated AL projection neurons undergo relatively slow periods of excitation and inhibition and also tend to oscillate synchronously on a faster timescale, and as a result, each odor activates a dynamic ensemble of active neurons whose membership is updated at each cycle of the 20-30 Hz oscillations (Stopfer *et al*., 1997). We are currently using tetrodes to record from ensembles of AL neurons in order to assess the population responses to a set of related odors. These recordings will be used to test whether the population responses to different odors diverge over time as they do in the zebrafish, a finding that has been hypothesized to reflect processing adapted to facilitating accurate discrimination among similar odors (Friedrich and Laurent, 2001). The recordings are also being conducted in conjunction with ongoing proboscis extension response conditioning experiments, in which honeybees are trained to form an association between and odor and a sucrose reward. While imaging experiments have shown that responses to the rewarded odor are selectively enhanced following learning (Faber *et al*., 1999), it remains unclear how the dynamic responses of populations of individually recorded AL neurons will have changed. These experiments will be used to test the hypothesis that AL responses adapt to reflect the behavioral relevance of odors; in other words, that the population responses to similarly conditioned (i.e., two rewarded) odors will converge more (or diverge less) than the responses to odors differentially conditioned (i.e., one rewarded and one unrewarded) odors.

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240. Aggregation pheromone processing in the locust antennal lobe

B. Broome

Pheromones are behaviorally relevant odor compounds used for communication and survival by many insects. Much is known about the processing of general odors in the locust antennal lobe; however, almost all of the detailed information we possess about the sensing of pheromones has been obtained in other model systems. We examined how aggregation pheromones are processed in the locust, which lacks anatomically separate pheromone responsive glomeruli. Using intracellular single unit and extracellular multi-single-unit tetrode recordings, we explored the manner in which pheromones are processed in the locust antennal lobe. We describe the various ways in which processing of these odors is similar or dissimilar to that of general odors. The response characteristics of the antennal lobe appear to be comparable for pheromones and general odors. Projection neurons are broadly tuned with respect to aggregation pheromones and respond to those pheromones similarly to general odors. In addition, pheromones stimulate synchronous and periodic firing among projection neurons in a manner similar to that observed with general odors.

241. Associative memory and olfactory representation

S. Cassenaer

An identified honeybee neuron, VUMmx1, appears to encode the reward stimulus in conditioning experiments (Hammer, 1993). VUMmx1 endogenously responds to sucrose, and activating the cell during odorant presentation subsequently gives rise to proboscis extension in response to the odorant alone. VUMmx1 projects to the antennal lobe (AL), the mushroom body (MB) and the lateral protocerebral lobe. Moreover, injection of the VUMmx1 transmitter octopamine in the AL and MB can replace sucrose as the unconditioned stimulus (Hammer and Menzel, 1998). One interpretation of these behavioral experiments is that olfactory representations in the AL and MB could be changed by newly formed associations between odors and sucrose stimuli mediated by VUMmx1.

Neurons analogous to VUMmx1 that project to the AL and to the MB have been identified in the locust as well (Braunig, 1991). We are characterizing how olfactory

representations in the locust are affected by pairing odorant presentation with direct activation of these cells or with injection of octopamine. Preliminary results show that population-level changes occur as a result of pairing odorants with octopamine injection in the AL. At this point, we are also able to identify and record from the AL- and MB-projecting cells, while carrying out extracellular tetrode recordings in the target structures.

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242. ***Drosophila*** dorsal paired medial neurons and olfactory memory

Glenn C. Turner, Rachel I. Wilson

Flies can learn to associate a particular odor (conditioned stimulus, CS), with a punishment (electric shock, unconditioned stimulus, US), or a reward (sugar water). This olfactory learning requires the activity of the dorsal paired medial (DPM) neurons, two bilaterally symmetric cells that innervate the mushroom body (MB), the memory center of the fly brain (Waddell, 2000). DPM activity is thought to modify the strength of synapses between the Kenyon cells of the MB and a population of cells termed a/b/g-lobe neurons. The resulting changes in the olfactory representations of KCs and/or a/b/g-lobe neurons presumably cause the fly to respond to the CS as it does to the US.

To determine whether DPM carries information about the CS and/or US, we are examining its responses to different sensory stimuli: odors, electric shock, and the taste of sugar water. In contrast to existing models of DPM function that suggest that DPM would represent US information (Heisenberg, 2003), we find that DPM exhibits odor-evoked activity. Whole-cell recordings from the DPM cell body indicate that it is a non-spiking neuron that responds to odors with graded potentials comprising a rapid initial depolarization and a more prolonged decay. The responses do not appear to be odor selective: DPM responds to all 14 odors tested so far, a chemically diverse panel that includes odors that are attractive or aversive to naïve flies. To determine whether learning alters DPM activity, we plan to examine its responses in flies trained to associate a particular odor with a sugar reward. We plan to investigate the representational changes that accompany learning by examining a/b/g-lobe neuron responses to rewarded and unrewarded odors. This will enable us to test whether for example a/b/g-lobe neurons become more selective for rewarded odors following learning, or whether their responses to rewarded odors become more temporally precise. The contribution of DPM to these representational changes will be assessed by genetically blocking synaptic output from DPM, and by electrophysiologically stimulating DPM.

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243. Combined two-photon laser scanning microscopy and electrophysiological recording in insect brain

Laurent Moreaux

We are currently investigating the correspondence between neuronal output (spike time series) and molecular calcium signal in *Drosophila* central olfactory neurons by combining *in vivo* intracellular electrophysiological recording and calcium imaging by two-photon laser scanning microscopy. The goal is to understand the encoding and representation of odors in the early olfactory system (antennal lobe) and more precisely to understand the mismatch between previous calcium imaging data obtained by the Axel and Misenböeck labs and the electrophysiological data obtained in the Laurent lab. The calcium imaging data are interpreted as indicating that the antennal lobe output is identical to afferent input and the antennal lobe is proposed to act as a simple relay. By contrast, physiological data indicate that antennal lobe output is distributed in space and in time, and thus profoundly transformed in this network. One simple hypothesis is that calcium imaging represents afferent synaptic input, but not spike output from the principal neurons; this could be due to the high calcium permeability of central nACh receptors and to the dominance of this mode of calcium entry over voltage-gated channels activated by action potentials. By examining the degree of correlation between calcium signal, afferent input (using fluorescent calcium indicator) and spikes from the output neurons of the antennal lobe (using whole cell recording) we should be able to elucidate this question.

244. Responses to prolonged odor presentations in the insect antennal lobe

Ofer Mazor

The antennal lobe (AL) is the first site of olfactory processing in the locust brain. It contains two main neuron types: inhibitory local neurons (LNs) and excitatory projection neurons (PNs), which form the only output. The AL responds to a 1 sec odor stimulation with a dynamic pattern of activity which includes a 20-30 Hz global oscillatory response combined with slower patterns of excitation and inhibition (100s of m sec long) in PNs. These slower dynamics are neuron- and odor-specific. Under natural conditions, insects encounter odor stimuli of varying durations, from brief encounters with turbulent odor plumes to extended periods of time on a host plant. In addition, many experimental behavioral paradigms with insects (e.g., PER conditioning), involve odor presentations many seconds in duration.

In this study, we examined the PN response to prolonged, constant odor presentations. We used silicon probes to record the simultaneous extracellular responses of 5-20 PNs to short (1 sec) and long (10 sec) "square" odor pulses. We find that the slow dynamics in PNs are

strongest in the first 2 sec after odor onset and again just after odor offset (N=246 PN-odor pairs). For the remainder of the odor presentation, each PN reaches a steady firing rate that differs from odor to odor and is typically different from its baseline rate. This result suggests that the AL response to a constant odor goes through a dynamic phase, but within a few seconds settles to a more constant response that remains fairly stable for the duration of the odor.

PNs synapse directly on to Kenyon cells (KCs), and past work from the lab (e.g., Perez-Orive *et al*., 2002), has examined the transfer of olfactory information between these two cell types during the "dynamic" portion of the response. In this study, we will look at the contribution of the two different response phases in conveying this information to the KCs. We are currently examining KC responses to prolonged odor pulses.

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245. Understanding synaptic transformation of olfactory representations in ***Drosophila*** using ***in vivo*** electrophysiology

Rachel I. Wilson, Glenn C. Turner

Drosophila is an attractive model system for studying olfaction, because in this species both primary olfactory receptor neurons (ORNs) and postsynaptic projection neurons (PNs, equivalent to mitral cells) are uniglomerular, as in the mammalian olfactory bulb, but glomerular number is reduced. Furthermore, the genetic tractability of *Drosophila* allows some spatial and temporal control of neural gene expression. We are performing whole-cell patch-clamp recordings from projection neurons and local interneurons in the *Drosophila* antennal lobe *in vivo*, using a diverse odor stimulus set. We observe TTX-sensitive action potentials in both PNs and interneurons, although spikes at PN somata appear greatly attenuated, suggesting that the spike initiation zone of these cells may be electrotonically distant. PNs are bombarded at high frequency by small, fast EPSPs, consistent with the high basal firing rate of ORNs. Even at low odor concentrations (<0.1% saturated vapor), individual PNs may respond to many odors, and many PNs are broadly tuned. PN responses can be excitatory, inhibitory, or a combination of these in consistent, odor-specific, cell-specific temporal patterns. Each interneuron recorded thus far responded to all odors (<0.1% SV), consistent with our observation that each interneuron sends neurites into every glomerulus. By filling PNs with dye, we can identify the glomerular input of each neuron. We are also performing single-sensillum extracellular recordings of spikes from GFP-expressing, identified ORNs. In agreement with other investigators, we observe that ORN responses are mainly excitatory and not temporally patterned. This implies a role for antennal lobe synapses in transforming the neural representation of olfactory stimuli. By comparing the responses of ORNs and PNs corresponding to the same glomerulus, we aim to describe this transformation more concretely, to identify the mechanistic basis for this transformation, and to understand the functional relevance of these synaptic interactions.

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246. Local connectivity within the locust antennal lobe

Ron A Jortner, Ofer Mazor*

The connectivity in a neural system, i.e., the number, pattern and strength of connections between its elements, is a fundamental property of the network architecture and underlies much of its dynamics. We present an experimental approach to studying functional connectivity in the insect antennal lobe, an intact, *in vivo* biological network, and its preliminary results.

The antennal lobe (AL), the first relay of the locust olfactory system, is a network consisting of two neuron types: excitatory projection neurons (PNs, N≈830), and inhibitory, non-spiking local interneurons (LNs, N≈300). Through its input and internal dynamics, the AL generates a synchronous spatiotemporal odor code, which is passed on to downstream networks via PN axons. It has previously been shown that LNs are necessary for mediating synchrony between PNs.

To study the connectivity underlying these synchronous dynamics, we simultaneously recorded baseline activity from single LNs using intracellular electrodes and from multiple (typically 5-15) PNs using tetrodes. We computed the average LN traces, triggered on action potentials of individual PNs. Averaging nearly always revealed excitatory postsynaptic potentials (EPSPs) in the LN, directly following a PN spike, indicative of a potentially direct synaptic connection from the PN to the LN. This finding was confirmed using dual intracellular recordings from PNs and LNs. The average amplitude of the PN-LN EPSPs (as recorded in the soma), was around 50 microvolts.

These results suggest very dense (~90%) connectivity from PNs to LNs. EPSPs could usually not be seen in raw LN traces (without averaging) due to very high background activity, in accordance with such highly convergent architecture. This dense PN to LN connectivity explains the high degree of correlation observed between LN membrane potential and odor-evoked local field potential (LFP) recorded in PN target areas. We are presently examining the connectivity between other elements of the AL network.

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247. Spatio-temporal dynamics in complex neural networks

Valentin Zhigulin

Antennal lobe is an example of complex, highly connected network of neurons that exhibits rich stimulus-dependent spatio-temporal dynamics. In general, the connection between the structure of the networks and their dynamics and function is not understood. In this project we attempt to clarify this connection by studying the dynamics of networks with different topologies. In order to concentrate our study on the influence of connectivity on the emergent network properties, we use simplified average-firing-rate description of individual neurons. All possible network structures may be divided into three major classes: uniformly random networks; small-world networks; and scale-free networks. Although the detailed connectivity of the antennal lobe network is not known yet, studies of other neural ensembles show that the majority of them have small-world structure. Hence we began by studying competitive dynamics on small-world networks. Preliminary results show that the region of parameter space in which the dynamics on such networks are deterministic is much bigger as compared to uniformly random networks, which are predominantly chaotic. Since the spatio-temporal patterns of neural activity recorder from the antennal lobe network are reproducible and, hence, deterministic, our goal is to establish the link between the structure of networks and the level of complexity and orderliness of their dynamics. Having established this link we will be able to predict topology of connections that is optimal for the type of dynamics that is observed in antennal lobe.

248. Robust and sensitive representation of odors in the dynamic competitive model of the antennal lobe

Valentin Zhigulin

Recent studies of the locust olfactory system show that it represents odors by stimulus-specific spatio-temporal patterns of neural activity. These patterns are remarkably stable from trial to trial for the same odor (robustness), but change significantly when another odor is presented (sensitivity). A new theoretical framework of "winnerless competition" (WLC), was developed recently (Rabinovich *et al*., 2001), to study possible mechanisms of such identity-temporal coding. In this project we study robustness and sensitivity of stimulus-dependent dynamics in the WLC-type model of the antennal lobe network. This model network is comprised of conductance-based neurons that are connected by nACh, $GABA_AA$ and $GABA_BB$ synapses.

We study the influence of each type of synaptic transmission and its time scales on network dynamics. Preliminary results show that the network exhibits robustness of the dynamics against noise, reproducibility of same-stimulus responses and sensitivity to small changes in the stimuli. Slow lateral inhibition ($GABA_BB$), appears to play a crucial role in the observed effects.

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Summary: We continue our work on ion channels, receptors, and transporters. We have continued to analyze several strains of knock-in mice generated in our laboratory for two ligand-gated channels, the nicotinic α4 receptor and the serotonin 5-HT3 receptor. The nicotinic receptor work is enhanced by a promising new strain, Leu9'Ala. This work has generated interesting insights into nicotine addiction, neurodegenerative disease, and epilepsy. The 5-HT3 receptor studies have generated insights into murine urologic syndrome.

Our work on selective silencing of mammalian neurons has generated a promising set of techniques and reagents based on ligand-activated chloride channels. We are now generating "proof of concept" transgenic mouse strains.

We continue our joint work with the Dougherty group, in Caltech's Chemistry Division, on aspects of ion channel structure-function. We have brought novel techniques to these studies, including mass spectrometry and fluorescence. We work on unnatural amino-acid mutagenesis, and a newly acquired instrument, the OpusXpress, speeds data collection. We have now extended unnatural amino-acid incorporation to mammalian cells.

We collaborate with both Dougherty and Doug Rees, also in the Chemistry Division, on bacterial ion channels of known atomic-scale structure. This year, we helped to accomplish total synthesis and reconstitution of a functional multipass ion channel. We also gathered data at bandwidths an order of magnitude greater than usual; but gating transitions are still too fast to measure.

Our work continues on quantitative aspects of transporter function, primarily measured with fluorescence and with knock-in mice. As an interesting side benefit of the GABA transporter knock-in mouse, we have generated and analyzed a knockout mouse for the same molecule.

The late Norman Davidson led a subgroup working on aspects of synaptic plasticity, particularly those that depend on A kinase stimulation. Members of this subgroup are now analyzing their data and preparing papers for publication.

Our group's home page has additional up-to-date information, images, and notices of positions. It's at http://www.its.caltech.edu/~lester.

249. Spatial-temporal separation of nicotine-induced seizures in knock-in mice with hypersensitive nicotinic receptors

Carlos Fonck, Bruce N. Cohen, Purnima Deshpande, Cesar Labarca

We studied nicotine-induced seizures in mice with hypersensitive nicotinic acetylcholine receptors (nAChR). These mice contain the Leu9'Ala mutation in the M2 region of the nAChR α4 subunit which increases receptor sensitivity to agonists such as acetylcholine and nicotine. Seizure studies on α4 mutated mice may be relevant to epilepsy research because all known mutations linked to autosomal dominant nocturnal frontal lobe epilepsy occur in the M2 region of the α4 or β2 subunits of nAChR. L9'A homozygous (hom) and heterozygous (het) mice, and their wild-type (WT) littermates received a single subcutaneous nicotine injection and the latency and intensity of seizures were recorded. 1 mg/kg nicotine caused rapid onset (20 sec) seizures in hom and het, but had no visible effect on WT mice. In WT mice, 10 mg/kg nicotine was necessary to elicit a seizure, which started 2-3 min following injection. Hom and het seizures (1 mg/kg) were clonic with rapid and repetitive movement of the extremities, whereas, WT seizures (10 mg/kg) were tonic-clonic and more violent. EEGs obtained from screw electrodes placed above the primary motor cortex and the visual cortex showed spike and wave activity in WT during seizures, but there were no EEG changes during hom and het seizures. A 10 mg/kg nicotine injection in hom resulted in two successive seizures: the first seizure started 20-30 sec following injection, was clonic and showed no EEG changes (similar to the 1 mg/kg seizures in mutant mice described above). The second seizure began 2-3 min after injection, was tonic-clonic and had

spike and wave shaped traces on the EEG (similar to the 10 mg/kg seizures in WT mice described above). In conclusion, seizures mediated by the mutated nAChR are initiated faster than those caused by WT receptors and, as assessed by EEG recordings, may involve the activation of a separate neuronal circuit. We are currently using various experimental approaches, such as c-fos expression, fMRI and multielectrode recordings to localize the mutant-like seizures in L9'A mice.

250. Knock-in mice carrying hypersensitive α4 nicotinic receptors: Nicotine and morphine nociception responses

*Carlos Fonck, Purnima Deshpande, Cesar Labarca, Raad Nashmi, M. Imad Damaj**

Neuronal nicotinic receptors (nAChR) are involved in a number of rodent behavioral responses including sedation, decreased nociception, hypothermia and seizures. It is not known what roles the various individual nicotinic receptor types play in the different behavioral responses. We created gain-of-function mice carrying hypersensitive α4 nAChR by introducing a Leu9'Ser mutation in the M2 region (Labarca *et at.*, 2001). In terms of agonist sensitivity, abundance and widespread brain distribution, the most important nicotinic receptor appears to be the one formed by α4 coassembled with β2 subunits. We examined the role of α4 nAChR in acute nociceptive responses by testing mice heterozygous for the L9'A mutation (hets) and their wild-type (WT) littermates in the hot plate and the tail flick apparatus (TF), following a single injection of either nicotine or morphine. It is thought that nicotine and morphine cause analgesia, through the activation of nicotinic or μ-opioid receptors, respectively, present in descending pain-modulating pathways. In the hot plate assay, nicotine increased the latency of the pain avoidance response in hets at 0.05 to 0.5 mg/kg, and in WT at 0.5 to 2 mg/kg. Hets displayed a 5.3-fold lower ED50 than WT. The specific nicotinic-binding site blocker mecamylamine (1 mg/kg) almost completely abolished nicotine effects in both WT and het. In the TF assay, hets showed no increase in response times at informative nicotine levels. Morphine (1 to 16 mg/kg), unlike nicotine, caused equal analgesia in het and WT, both in the hot plate and the TF. These data support (1) the importance of the α4 subunit in mediating nicotine analgesia in the supraspinal responses measured by the hot plate, (2) the minimal α4 modulation of the primarily spinal reflex-dominated pathway assessed by TF and (3) the independent modulation of acute nociceptive responses by α4 nAChR and morphine-sensitive receptors.

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251. Alpha4-containing neuronal nicotinic receptors modulate appetitive learning

Cesar Labarca, Seth A. Balogh, B.J. Bowers*, S.F. Logue*, J. Ernisse*, Jeanne M. Wehner**

The present study characterized the role of α4-containing neuronal nicotinic receptors (nAChRs) in learning and memory using a four-stage appetitive signaled-nosepoke task (Logue *et al.,* 1998) in 13 inbred mouse strains and in a gain-of-function α4 nicotinic receptor mutant (Labarca *et al*., 2001). In inbred mouse strains, a naturally occurring polymorphism in the α4 nAChR subunit gene encodes either an alanine or threonine (A/T) at position 529 (Stitzel *et al*., 2000). This A/T polymorphism is associated with differential receptor function and behavioral sensitivity to nicotine and ethanol in both inbred and recombinant inbred mouse strains. The first three phases of the nosepoke task consisted of training to associate an auditory cue with reinforcer availability. The last phase required that each mouse nosepoke only when the cue was presented. Inbred mouse strains with the 529alanine form of the polymorphism required a significantly greater number of days to learn to associate the auditory cue with the reward than those containing the 529threonine residue. The α4 Leu9'Ser mice are hypersensitive to acetylcholine and nicotine and have several behavioral alterations. The α4 Leu9'Ser mice showed enhanced associative learning in the signaled nosepoke task, relative to their wild-type littermates. These data suggest that nAChRs that contain the α4 subunit modulate appetitively-motivated associative learning.

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252. Nicotinic acetylcholine receptors modulate the effects of ethanol and nicotine on acoustic startle

Cesar Labarca, Jeremy C. Owens[1], Seth A. Balogh[1], Tristan D. McClure-Begley[1], Marina R. Picciotto[2], Jeanne M. Wehner[1], Allan C. Collins[1]

Recent evidence suggests that common genes influence sensitivity to both alcohol and tobacco in humans. The studies described here tested the hypothesis that α4β2-containing (abbreviated α4β2*) nAChRs are one site of overlap. This postulate was suggested by the results of a genetic mapping analysis that used recombinant inbred strains derived from Long Sleep (LS) and Short Sleep (SS) mice. An association between ethanol effects on acoustic startle and a naturally occurring polymorphism in the α4 subunit of the nAChR was found in the RI strains. This agrees with our previous finding that variability in nicotine effects on acoustic startle response and the α4 polymorphism are significantly associated in these RI strains. We tested this hypothesis further using two mouse lines carrying targeted mutations, the α4 Leu9'Ser "gain-of-function" mutant and β2 subunit null mutant mice, which do not express α4β2-type nAChRs. The α4 mutants were more sensitive to the

effects of both drugs on acoustic startle, whereas, the β2 null mutants were less sensitive to both drugs relative to wild-type controls. These results support the postulate that α4β2* nAChRs regulate the effects of both nicotine and ethanol on acoustic startle.

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253. Mice expressing a mutant form of the α4 nicotinic receptor subunit show altered GABAergic function as measured by nicotinic acetylcholine receptor-stimulated [^{3}H]-GABA release

Cesar Labarca, Michael J. Marks, Tristan D. McClure-Begley*, S.R. Grady*, Jeremy C. Owens*, Seth A. Balogh*, Jeanne M. Wehner*, Allan C. Collins**

Many behavioral effects of nicotine appear to be modulated by α4-containing nicotinic receptors (nAChRs). Leu9'Ser alleles with wild-type (WT) expression levels die neonatally, but heterozygotes with intact neo selection cassette in a nearby intron have decreased expression throughout the brain (Fonck *et al*., 2003), are viable, and were studied in these experiments. L9'S mice differ from WT in sensitivity to several behavioral effects of nicotine. For example, L9'S mice are more sensitive than WT to nicotine-induced seizures (Fonck *et al*., 2003). Many α4-containing nAChRs are expressed in GABAergic neurons. Therefore, we evaluated the effects of the L9'S mutation on nAChR function by measuring nicotinic agonist-stimulated evoked [^{3}H]-GABA release from synaptosomes prepared from several brain regions known to express α4 subunit-containing presynaptic receptors. Concentration-effect curves were constructed by stimulating [^{3}H]GABA release using acetylcholine concentrations that ranged over four log units. Acetylcholine produced a concentration-dependent release of [^{3}H]-GABA in both the WT and L9'S mice. The release profile was best fit to a two-site model in wild-type mice, but a one-site model was the best fit for the L9'S data. As expected, L9'S mice displayed an overall decrease in release; this was mostly due to a decreased low-affinity release process (stimulated by high agonist concentrations). This shift in receptor mediated GABA release is evidence for an increase in the fraction of high-affinity α4 containing receptors in the L9'S mice.

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254. Hypersensitivity to peripheral thermal nociception, decreased startle reactivity, and modulation of sensorimotor gating in knock-in mice carrying hypersensitive 5-HT3 receptors

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5-hydroxytryptamine type 3 receptors (5-HT3Rs) are the only serotonin receptors belonging to the nicotinic acetylcholine superfamily of excitatory ligand-gated ion channels. To date, 5-HT3A and 5HT3B subunits have been cloned. 5HT3A subunits can self-assemble in heterologous expression systems forming homopentameric channels while 5HT3B subunits must coassemble with 5-HT3A subunits to form functional receptors. 5-HT3 receptors have been implicated in nociception, emesis, anxiety, and alcohol abuse. In addition, 5-HT3 receptors may play a role in certain neuropsychiatric disorders such as schizophrenia and bipolar affective disorder. To gain further insight into the physiological role of 5HT3Rs we have generated a hypersensitive 5-HT3R knock-in mouse line by introducing a point mutation, V13'S, in the pore-forming M2 region of the 5-HT3A subunit via homologous recombination. When expressed in *Xenopus* oocytes, V13'S 5-HT3A subunits form receptors ~70 fold more sensitive to serotonin and become constitutively active when combined with 5-HT3B subunits. Homozygous animals exhibit reduced expression of 5-HT3A mRNA in brain and SCG as evidenced by RT-PCR. However, 5-HT induced whole-cell currents from primary cultured SCG neurons, while small, are maximally activated by 0.1 mM serotonin, indicating that hypersensitive 5-HT3 receptors are expressed on the cell surface. Homozygous mutant mice are hypersensitive to peripheral thermal nociception compared to wild-type controls, as measured by latency to respond in the hot plate assay. However, mutant mice do not significantly differ from wild-type mice in the tail flick assay, a measure of spinal nociception. To investigate the role of 5HT3Rs in anxiety and neuropsychiatric disorders, we tested these mice with the acoustic startle response (ASR) and prepulse inhibition (PPI) assays. Male homozygous mutant animals have a lower baseline ASR and a decrease in PPI compared to wild-type mice. Interestingly, female homozygous mutants show no significant difference from wild-type in ASR or PPI. Our data indicate that 5-HT3 receptors play a role in peripheral thermal nociception and, in males, can modulate startle reactivity, as well as sensorimotor gating.

255. Design and characterization of ADNFLE mutant nAChR knock-in mice

Andrew R. Tapper, Carlos Fonck, Purnima Deshpande, Cesar Labarca, Bruce N. Cohen

Autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) is an idiopathic epileptic disorder characterized by nocturnal seizures localized within the frontal lobe arising during stage 2 sleep. Mutations within the putative pore-lining M2 helix of ionotropic neuronal nicotinic acetylcholine receptor α4 (CHRNA4) and β2 (CHRNAB2) subunits have been linked to ADNFLE.

When expressed in heterologous expression systems, ADNFLE-associated mutant receptors have altered channel properties compared to wild-type, suggesting that malfunctioning nicotinic receptors are responsible for the disease phenotype. Despite the molecular identification and characterization of nAChR mutations that may underlie some cases of ADNFLE, many questions remain regarding the pathophysiology of the disease.

To test the hypothesis that a knock-in mouse carrying a human mutation for ADNFLE will display seizures like the human disease, thus providing an ADNFLE animal model, we have generated a knock-in mouse line by introducing an ADNFLE-linked point mutation, Ser10'Leu, into the M2 transmembrane region (exon 5) of the α4 nicotinic acetylcholine receptor gene using homologous recombination. Knock-in mice heterozygous for this mutation are viable and fertile. We are monitoring these animals for spontaneous seizures using chronic video and EEG analysis. Characterization of mutant mice on a behavioral, neuronal, cellular and molecular level should provide valuable insights into the pathogenic mechanism and pathophysiology of ADNFLE, as well as the role of α4β2 acetylcholine receptors in the forebrain.

256. Five ADNFLE mutations reduce Ca^{2+} dependence of the α4β2 acetylcholine response

Nivalda Rodrigues-Pinguet, Li Jia[2], Maureen Li[1], Antonio Figl[1], Alwin Klaassen[3], Anthony Truong[1], Bruce N. Cohen

Five nicotinic acetylcholine receptor (nAChR) mutations are currently linked to autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE). The similarity of their clinical symptoms suggests that a common functional anomaly of the mutations underlies ADNFLE seizures. To identify this anomaly, we constructed rat orthologs (S252F, +L264, S256L, V262L, V262M) of the human ADNFLE mutations, expressed them in *Xenopus* oocytes with the appropriate wild-type (WT) subunit (α4 or β2), and studied the Ca^{2+} dependence of their ACh responses. All the mutations significantly reduced 2 mM Ca^{2+}-induced increases in the 30 μM ACh response. Consistent with a dominant mode of inheritance, this reduction persisted in oocytes injected with a 1:1 mixture of mutant and WT cRNA. BAPTA injections showed that the reduction was not due to a decrease in the secondary activation of Ca^{2+}-activated Cl^- currents. The S256L mutation also abolished 2 mM Ba^{2+} potentiation of the ACh response. The S256L, V262L, and V262M mutations had complex effects on the ACh concentration-response relation but all three mutations shifted the concentration-response relation to the left at [ACh] $\geq$ 30 μM. Co-expression of the V262M mutation with a mutation (E180Q) that abolished Ca^{2+} potentiation resulted in 2 mM Ca^{2+} block, rather than potentiation, of the 30 μM ACh response, suggesting that the ADNFLE mutations reduce Ca^{2+} potentiation by enhancing Ca^{2+} block of the α4β2 nAChR. Ca^{2+} modulation may prevent presynaptic α4β2 nAChRs from over-stimulating glutamate release at central excitatory synapses during bouts of synchronous, repetitive activity. Reducing the Ca^{2+} dependence of the ACh response could trigger seizures by increasing α4β2-mediated glutamate release during such bouts.

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257. Selective silencing of mammalian neurons: Optimizing the strategies using chloride channels

Eric M. Slimko, Ping Li

Glutamate-gated (GluCl) chloride channels from invertebrates can be activated by ivermectin (IVM) to produce electrical silencing in mammalian neurons. To improve this GluCl/IVM strategy, we sought to mutate the *C. elegans* GluCl channels so that they become insensitive to glutamate but retain their sensitivity to IVM. Based on structure-function studies of nAChR superfamily members, we tested in oocytes 19-point mutants at 16 residues in the β subunit likely to be involved in the response to glutamate. Y182F reduces the glutamate response by greater than 6 fold, with little change to IVM responses, when coexpressed with WT GluClα. For GluCl$\alpha\beta$(Y182F), the EC_{50} and Hill coefficient for glutamate is similar to those of WT, indicating that the mutant decreases the efficacy of glutamate, but not the potency. Also, fluorescent proteins (EGFP, EYFP, ECFP; XFP) were inserted into the M3-M4 loop of the GluCl α, β and β(Y182F). We found no significant functional difference between these XFP-tagged receptors and WT receptors.

Also, organisms use synonymous codons in a highly non-random fashion. These codon usage biases sometimes frustrate attempts to express high levels of exogenous genes in hosts of widely divergent species. The *C. elegans* GluClα1 and GluClβ genes form a functional glutamate and ivermectin-gated chloride channel when expressed in *Xenopus* oocytes, but expression is weak in mammalian cells. We have constructed synthetic genes that retain the amino acid sequence of the wild-type GluCl channel proteins, but use codons that are optimal for mammalian cell expression. We have tagged the native and codon-optimized GluCl cDNAs with enhanced yellow fluorescent protein (EYFP, GluClα1 subunit) and enhanced cyan fluorescent protein (EFCP, GluClβ subunit), expressed the channels in E18 rat hippocampal neurons, and measured the relative expression levels of the two genes with fluorescence microscopy, as well as with electrophysiology.

Codon optimization provides a six- to nine-fold increase in expression, allowing the conclusions that the ivermectin-gated channel has an EC_{50} of 1.2 nM and a Hill coefficient of 1.9. We also confirm that the Y182F mutation in the codon-optimized β subunit results in a

heteromeric channel that retains the response to ivermectin while reducing the response to 100 μM glutamate by seven-fold. The engineered GluCl channel is the first codon-optimized membrane protein expressed in mammalian cells. The modified GluCl channel, without glutamate sensitivity, with a fluorescent tag, and with optimized codons, is now being used to construct transgenic mice with cell-specific expression.

258. Localization, trafficking and resonance energy transfer in α4β2 nicotinic receptor-fluorescent protein chimeras

Raad Nashmi, Mary E. Dickinson, Sheri McKinney, Mark Jareb, Cesar Labarca, Scott E. Fraser

Although the mechanisms of nicotine addiction remain unclear, altered trafficking of neuronal nicotinic receptors (nAChR) may be one contributing mechanism that modulates neuronal excitability. To study such mechanisms, we made fluorescently-tagged neuronal nicotinic receptor (nAChR) subunits. Yellow fluorescent protein (YFP) was inserted at the N-terminus or the M3-M4 intracellular loop of α4, and cyan FP (CFP) at the C-terminus or the M3-M4 loop of β2. We expressed labeled α4β2 nAChRs in HEK293T cells and cultured mesencephalic neurons, and compared their functional properties with those of unlabeled wild-type (WT) α4b2 receptors. Nearly normal ACh sensitivity and calcium permeability was noted for receptors with YFP and CFP in α4 and/or β2 M3-M4 intracellular loops; these constructs were studied further. In contrast, inserting YFP in the α4 N-terminus or CFP in the β2 C-terminus dramatically inhibited nAChR function.

The somatic and dendritic distribution of fluorescently-tagged α4 and β2 subunits was similar to that of endogenous α4-containing receptors. Co-expressing the α4-YFP and β2-CFP subunits resulted in fluorescence resonance energy transfer (FRET) between the subunits. In midbrain neurons, dendritic α4 β2 nAChRs displayed greater FRET than receptors inside the soma; and in HEK293T cells, a similar increase occurred for receptors that were translocated to the surface upon PKC stimulation. The maximal FRET efficiency between the α4 and β2 subunits was 48 ± 3% (mean ± SEM), suggesting a distance of 50 angstroms between the α4 and β2 M3-M4 intracellular loops, in rough agreement with higher-resolution structural studies. Furthermore, α4β2 nAChRs in neurons that were incubated with 1 μM nicotine for 24 hr displayed greater FRET than those in untreated neurons. Thus, fluorescently-tagged α4 and β2 nicotinic subunits provide information about α4β2 nAChR trafficking, assembly, and localization. In future experiments, we plan to examine the time-course of altered nicotinic receptor function and α4 and β2 subunit expression with chronic nicotine exposure in cultured neurons.

259. Conformational state-dependent hydrophobic photolabeling of the nicotinic acetylcholine receptor using electrophysiology-coordinated photochemistry and mass spectrometry

John F. Leite, Mona Shahgholi[1], Dennis A. Dougherty[1], Michael P. Blanton[2]

We characterized the differential accessibility of the nicotinic acetylcholine receptor α1 subunit (nAChRα1) in the open, closed and desensitized states, using electrophysiology-coordinated photolabeling by several lipophilic probes followed by mass spectrometric analysis. Voltage-clamped *Xenopus* oocytes expressing receptors were preincubated with one of the lipophilic probes and were continually exposed to acetylcholine; UV irradiation was applied during 500 msec pulses to +40 or to -140 mV (which produced closed or ~50% open receptors, respectively). In the open state, there was specific probe incorporation within the N-terminal domain at residues that align with the β8-β9 loop of the acetylcholine-binding protein. In the closed state, probe incorporation was identified at several sites of the N-terminal domain within the conserved cysteine loop (residues 128-142), the cytoplasmic loop (M3-M4), and M4. The labeling pattern in the M4 region is consistent with previous results, further defining the lipid-exposed face of this transmembrane α-helix. These results show regions within the N-terminal domain that are involved in gating-dependent conformational shifts, confirm that the cysteine loop resides at or near the protein-membrane interface, and show that segments of the M3-M4 loop are near to the lipid bilayer.

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260. Investigation of apparent mass deviations in electrospray ionization tandem mass spectrometry of a benzophenone-labeled peptide

John F. Leite, Mona Shahgholi[*], Dennis A. Dougherty[*]

In the study summarized above, using benzophenone-based topological probes to study conformational-dependent changes in mouse muscle nicotinic acetylcholine receptor (nAChR) topology, ESI-MS-MS analysis led to a consistent –2.0 Da mass deviation from expected values. In the present study we photolabeled a synthetic peptide corresponding to nAChR α1 subunit residues 130-139. MS-MS analysis of this peptide confirmed the previously observed mass deviation, associated only with fragment ions that contain the incorporated benzophenone moiety. Analysis of peak profiles for the photolabeled ions does not indicate the typical "peak fronting" that produces a mass shift when labile ions are prematurely ejected from the ion trap. Rather, H/D exchange experiments support the hypothesis that a chemical rearrangement involving phenyl migration

and ketone formation has formed an unexpected oxidized peptide, with molecular mass 2 Da less than that expected, that is isolated for collision induced dissociation in the ion trap together with the predicted precursor due to the broad ion isolation window specified.

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261. A fluorophore attached to nicotinic acetylcholine receptor βM2-domain detects high-affinity-binding and desensitization

David S. Dahan[1], Vincent A. Auyeung[2], Dennis A. Dougherty[1]

To study conformational transitions at the muscle nicotinic acetylcholine (ACh) receptor (nAChR), a rhodamine fluorophore was tethered to a cysteine side chain introduced at the β19' position in the M2 region of the nAChR expressed in *Xenopus* oocytes. This procedure led to only minor changes in receptor function. Fluorescence increases (ΔF/F) of ~10% occurred during agonist activation. The dose-response relations for ΔF agreed well with those for epibatidine-induced currents, but were shifted ~100-fold to the left of those for ACh-induced currents. Because (i) epibatidine binds more tightly to the aγ-binding site than to the αδ site and (ii) ACh binds with reverse site selectivity, these data suggest that ΔF monitors an event linked to binding at the αδ subunit interface. The data do not yet allow us to determine whether the earliest detectable fluorescent state is activation or a rapid phase of desensitization. At low [ACh] (≤ 10 μM), a phase of ΔF occurs with the same time constant as desensitization, presumably monitoring an increased population of agonist-bound receptors. Following agonist washout, ΔF returns to baseline after a noticeable lag compared with the delay of agonist-induced current, but several-fold more rapidly than the agonist-induced current recovers from desensitization, showing that one or more desensitized states have fluorescence like that of the resting channel. That conformational transitions at the αδ-binding site are not tightly coupled to channel activation, suggests that sequential rather than fully concerted transitions occur during receptor gating. Thus, time-resolved fluorescence changes provide a powerful probe of nAChR conformational changes.

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262. The role of tyrosine residues at the mouse-$5HT_{3A}$ receptor ligand-binding site investigated by unnatural amino-acid mutagenesis

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The 5-HT_3 receptor is a member of the Cys-loop family of ligand-gated ion channels and shares a high degree of homology with nicotinic acetylcholine, $GABA_A$, glycine, and GluCl receptors. Previous data show that the amino acids involved in ligand binding comprise six non-contiguous loops (A-F). Recently, tyrosine residues from binding loops C (Y234) and E (Y141, Y143 and Y153) were shown to be important for ligand binding and/or receptor gating transitions in the 5-HT_3 receptor ($5HT_3R$). To further characterize the role of these residues, we have used the *in vivo* nonsense suppression method to incorporate unnatural amino acids site-specifically into 5-HT_3Rs expressed in *Xenopus* oocytes. The results indicate that the –OH groups of Y143 and Y153 are critical for binding and/or function, whilst that of Y141 is not, as aromatic substitutions are well tolerated. At Y234, substitution with Phe leads to a ten-fold increase in the EC_{50} for 5-HT; whereas, incorporation of unnatural Phe derivatives with substituents (-F, -Br, and -CH_3) at the 4 position of the aromatic ring yield wild-type EC_{50}s, suggesting that steric bulk here enhances agonist binding. These data provide support for a homology model of the 5-HT_3R extracellular domain.

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263. Temporal control of protein phosphorylation effects with caged phosphoamino acids

E. James Petersson[*], Gabriel S. Brandt[*], Fraser Moss, Dennis A. Dougherty[*]

Phosphorylation of serine, threonine, and tyrosine residues is a ubiquitous and dynamic posttranslational protein modification that results in a wide array of alterations in protein function. In the case of ion channel proteins, these effects can result in changes in the electrophysiological properties of the channel or in the channel's trafficking. Traditionally, changes in phosphorylation have been controlled by the upregulation of kinases or the downregulation of phosphatases.

However, even in a perfectly designed experiment, these experiments are limited by the rates of the ion channel's interaction with the regulatory proteins. This prevents any precise kinetic analysis of the downstream effects of phosphorylation. We are in the process of site-specifically introducing caged, chemically phosphorylated amino acids that can be photolyzed to reveal an authentically phosphorylated amino acid. This permits one to establish a "t=0" point for any phosphorylation experiment with ms time resolution.

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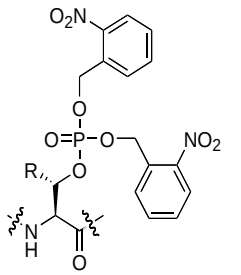
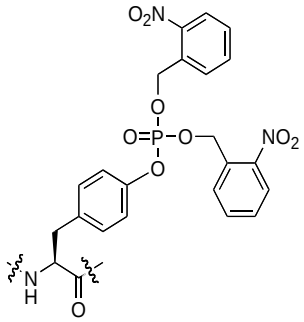


Caged pSer and pThr
Ser R = H, Thr R = Me



Caged pTyr

264. RGS9 via its DEP domain targets to D2 dopamine receptors and mice lacking RGS9 develop dyskinesia associated with dopamine pathways

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We discovered that RGS9-2, a member of the RGS family of Gα GTPase accelerating proteins, associates with the D2-dopamine receptor (D2-DR), and preferentially accelerates the termination of D2-DR signals. We established that the DEP domain of RGS9 was both necessary and sufficient for association with D2-DR. DEP domains present in other proteins, such as disheveled, associate with G protein-coupled receptors (GPCRs) in addition to D2-DR, suggesting that the DEP domain is a GPCR targeting domain. RGS9-2 is expressed specifically in the striatum, the brain region involved in the development of neuroleptic-induced tardive dyskinesia and levodopa-induced dyskinesia. We produced similar disorders in RGS9 knockout mice when inhibition of dopaminergic transmission was followed by activation of D2 dopamine receptors (D2-DR). In addition we showed that in wild-type striatal neurons RGS9-2 and D2-DR had an identical cellular distribution pattern and D2-DR abnormally inhibited glutamate-elicited currents in striatal neurons from RGS9 knockout mice. These data support a role for RGS9-2 in suppressing the side effects associated with the treatment of psychoses and Parkinson's disease.

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265. RGS9 modulates dopamine signaling in the basal ganglia

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Regulators of G-protein signaling (RGS) proteins modulate the function of heterotrimeric G proteins in part by serving as GTPase activating proteins for Gα subunits. We examined a role for RGS9-2, an RGS subtype highly enriched in striatum, in modulating dopamine D2 receptor function. Viral-mediated overexpression of RGS9-2 in rat nucleus accumbens (ventral striatum) reduced locomotor responses to cocaine (an indirect dopamine agonist) and to D2 but not to D1 receptor agonists. Conversely, mice with a null mutation in the RGS9 gene showed heightened locomotor and rewarding responses to cocaine and related psychostimulants. *In vitro* expression of RGS9-2 in *Xenopus* oocytes substantially accelerated the off-kinetics of D2 receptor-induced GIRK currents, consistent with the *in vivo* data of a net inhibitory influence of RGS9-2 on D2 receptor function. Finally, chronic exposure to cocaine increased levels of RGS9-2 in nucleus accumbens. Together, these data demonstrate a functional interaction between RGS9-2 and D2 receptor signaling and the behavioral actions of psychostimulants, and suggest that increased levels of RGS9-2 induced by psychostimulant exposure represents a compensatory adaptation that diminishes drug responsiveness.

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266. Knock-in mice with hypersensitive nicotinic α4 receptors show selective excitotoxic cell death of midbrain dopaminergic neurons

Johannes Schwarz[*], Johannes Wieacker[*], Sabine Orbl[*], Cesar Labarca, Carlos Fonck

We have previously shown that heterozygous neo-deleted or homozygous neo-intact knock-in mice with Leu9'Ser hypersensitive nicotinic α4 receptors die shortly after birth and have a severe reduction of midbrain dopaminergic neurons. However, heterozygous neo-intact mice that express only 20–30% of mutant receptors are viable and do not exhibit any gross abnormal phenotype.

Recent analyses of these heterozygous neo-intact mice show that locomotor responses to amphetamine are reduced by ~50% compared to wild-type littermates with only subtle changes in baseline behavior. Histology revealed no major cell loss except for substantia nigra pars compacta. Cell counts of tyrosine hydroxylase-immunoreactive neurons revealed a 39.1% reduction in heterozygous animals compared to wild-type littermates in young adult animals (four months). There was an age-dependent reduction of tyrosine-hydroxylase positive neurons in both wild-type and heterozygous mice (age eight months), independent of the genotype. As a control, we counted tyrosine hydroxylase-positive neurons in locus coeruleus, revealing no difference between mutant and wild-type animals. Electrophysiological recordings from dopaminergic neurons in acute midbrain slices showed an increased sensitivity of mutant dopaminergic neurons following applications of nicotine (100 μM). Spontaneous action potentials increased by 1.02 ± 0.25 Hz in mutant but did not change in wild-type neurons (0.05 ± 0.33 Hz). There was a shift in membrane potential (peak of after hyperpolarization) by 1.83 ± 0.38 mV in mutant but only 0.71 ± 0.6 mV in wild-type neurons. Thus, in heterozygous neo-intact L9'S nicotinic α4 receptor knock-in mice with limited expression of mutant receptors, there is loss of dopaminergic neurons most likely due to cholinergic excitotoxicity.

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267. GABA transporter (GAT1)-deficient mice display ataxia, tremor, reduced locomotor activity, increased body temperature fluctuation, and increased GABA receptor-mediated tonic conductance in cerebellar granule and Purkinje cells

Chi-Sung Chiu, Stephen Brickley[1], Kimmo Jensen[2,3], Amber Southwell, Sheri McKinney, Stuart Cull-Candy[1], Istvan Mody[2]

We created GABA transporter subtype I-deficient mice, mGAT1 knockout (KO), by gene targeting. The mGAT1-deficient mice reproduce and have normal muscle strength and life span, but reduced body weight (female -10%; male -20%), and motor disorders including gait abnormality, reduced time on rotarod, constant tremor at 25-32 Hz, and reduced locomotor activity in the home cage. In open-field tests, mGAT1-deficient mice display delayed exploratory activity, reduced rearing, and reduced visits to the central area, although without change in total distance traveled. Furthermore, the mGAT1-deficient mice show no difference in acoustic startle response, but a deficiency in prepulse inhibition. The open-field and prepulse inhibition results suggest that the mGAT1-deficient mice show mild anxiety or episodic nervous behavior. The knockouts also displayed higher body temperature fluctuations in 0.2-0.9 h^{-1} frequency. These behaviors are partially phenocopy effects of tiagabine, a GAT1-specific inhibitor, suggesting that they arise directly from GAT1 deficiency. Compromised levels of GABA uptake resulted in an increased $GABA_A$ receptor-mediated tonic conductance in cerebellar granule and Purkinje cells and prolonged decay of spontaneous IPSCs. The behavioral defects associated with excessive extracellular GABA in this animal illustrate the importance of GABA transporters in the regulation of correct neural function. Immunocytochemistry shows no detectable loss of GABAergic interneurons, no change in the number of $GABA_A$-, $GABA_B$-, or GAD65-immunoreactive structures, and no change in GAT3 expression pattern in hippocampus and cerebellum, suggesting little or no compensatory expression of other proteins related to GABA transmission.

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268. GABA transporter-1 (GAT1) deficient mice: Differential tonic activation of $GABA_A$ ***versus*** $GABA_B$ receptors in the hippocampus

Chi-Sung Chiu, Irina Sokolova, Kimmo Jensen[*], Istvan Mody[*]

Following its release from interneurons in the central nervous system (CNS), the major inhibitory neurotransmitter GABA is taken up by GABA transporters (GATs). The predominant neuronal GABA transporter GAT1 is localized in GABAergic axons and nerve terminals, where it is thought to influence GABAergic synaptic transmission, but the details of this regulation are unclear. To address this issue, we have generated a strain of GAT1-deficient mice. We observed a large increase in a tonic postsynaptic hippocampal $GABA_A$ receptor-mediated conductance. There was little or no change in the waveform or amplitude of spontaneous IPSCs or miniature IPSCs. In contrast, the frequency of quantal GABA release was one-third of WT, although the densities of $GABA_A$-receptors, $GABA_B$-receptors, GAD65 and VGAT1 were unaltered. The GAT1-deficient mice lacked a presynaptic $GABA_B$-receptor tone, present in WT mice, which reduces the frequency of spontaneous IPSCs. We conclude that GAT1 deficiency leads to enhanced extracellular GABA levels resulting in an overactivation of $GABA_A$-receptors responsible for a postsynaptic tonic conductance. Chronically elevated GABA levels also downregulate phasic GABA release and reduce presynaptic signaling via $GABA_B$ receptors, thus causing an enhanced tonic and a diminished phasic inhibition.

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269. Resting tremor of GABA transporter type I (GAT1)-deficient mice is modulated by benzodiazepine treatment

Amber L Southwell, Chi-Sung Chiu

Tremor, or the involuntary contraction of opposing muscles in a rhythmic or oscillatory way, is the most common movement disorder in humans. To

investigate the role of GABA in tremorgenesis, our laboratory has studied a GAT1-deficient mouse. This mouse exhibits several abnormal phenotypic characteristics, including a 25-32 Hz resting tremor. The anticonvulsant Tiagabine, a specific inhibitor of GAT1, induces tremor in human patients, indicating that the tremor of the GAT1-deficient mouse is the result of increased extracellular GABA concentrations rather than developmental alterations. Power spectrum analysis of mechanical transducer data gathered from wild-type mice, GAT1-deficient heterozygous and GAT1-deficient homozygous mutants show a peak at 25-32 Hz that occurs only in the homozygous mutant representing the pathological tremor. Flunitrazepam treatment (10 and 15 mg/kg IP) dose dependently increases the amplitude and decreases the frequency of the pathological tremor peak, while having very little effect on the spectrum of wild-type mice. This indicates that the tremor of the GAT1-deficient mouse results from over stimulation of benzodiazepine-sensitive $GABA_A$ receptors

270. Activity of ***E. coli*** mechanosensitive channels recorded at bandwidths of up to 300 kHz

George Shapovalov

We have modified a patch-clamp setup to record and analyze at low noise and high temporal resolution recordings from bacterial ion channels. We modified the headstage to house a specially created Teflon pipette holder following Parzefall, Wilhelm *et al.* (1998) and used quartz pipettes in order to decrease stray capacitance. The chamber was shielded by placing it inside a specially prepared aluminum shed that formed a Faraday cage. An inner layer of shielding was created from aluminum foil near chamber and pipette assembly. The Axoclamp 200B amplifier was modified to bypass the internal 10 kHz filter, allowing faster than 100 kHz signals to be recorded. We used an external 8-pole Bessel unit to provide anti-alias filtering. The signal was digitized at 1 MHz with a modified Digidata 1322A by Axon.

Bacterial MscL and MscS channels have an unusually large conductance, which renders them suitable for achieving higher signal-to-noise ratio. For example, the MscL channel produces single channel currents of 300 pA at 100 mV applied across the membrane. Combination of these properties of the channels and modifications that we did to instrumentation allowed us to achieve an acquisition bandwidth of 300 kHz with a corresponding temporal resolution of 2-3 μs. We registered closed channel rms. noise of 15 pA. At such resolution *E. coli* MscL channels demonstrated rich gating kinetics, visiting many subconductive states. The *E. coli* MscS channel has smaller total conductance of 1 nS, however, only one conductive state has been reported. This renders the MscS channel a more favorable candidate for studying properties of state transitions.

Elementary transition events were collected via the template search routine provided by Clampfit 9. Selected fragments were further aligned with a sub-microsecond resolution to minimize deviation of central parts of individual fragments from average. Mean 10-90 rise time of observed transitions corresponds to the resolution limit set by the instrumentation (2-3 μs).

Reference

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271. Synthesis and reconstitution of functional mechanosensitive channels

Daniel Clayton, George Shapovalov, Joshua Maurer[1], Dennis A. Dougherty[1], Gerd Kochendoerfer[2]

Our objective was to achieve robust synthetic access to functional MscL protein. We have succeeded in producing multi-mg quantities of biotin-labeled full-length MscL from both *M. tuberculosis* and *E. coli.* Biotin was incorporated as an affinity tag for Alexafluor-488-labeled streptavidin to facilitate analysis of the reconstitution of MscL into lipid vesicles. We then proceeded to incorporate both polypeptides into multilamellar vesicles. Bright-field and fluorescence images of *E. coli*-MscL incorporated into azolectin vesicles showed intense fluorescence in the lipid bilayer regions of the vesicles only after MscL reconstitution, indicating preferential incorporation of the synthetic channel protein into the lipid bilayer. Single- and multi-channel conductance recordings were then obtained on inside-out patches from vesicle preparations similar to those used in fluorescence experiments. Synthetic MscL exhibited comparable activity to recombinant wild-type protein, as indicated by a single-channel conductance of ~2.5 nS, and similar gating characteristics under suction [average midpoints for the probability of opening curves are -1.0 (SD 0.16, n=6) for synthetic and -1.3 PSI (SD 0.25, n=10] for recombinant *E. coli*-MscL]. This reconstitution procedure was successfully applied to full-length Tb-MscL protein, with comparable results for reconstitution, and wild-type channel behavior was observed.

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272. Gain-of-function mutation of 5-HT3 receptor leads to obstructive uropathy in mice: ***In vivo*** and ***in-vitro*** pharmacology

Annindya Bhattacharya[1], Quan-Ming Zhu[1], Hong Dang, Gary Cain[1], Nora Rozengurt[2], Debra A. Cockayne[1], Anthony P.D.W. Ford[1]

Gene knock-in mice carrying a gain-of-function (V to S) mutation in the 13' position of the a subunit of the 5-HT3 receptor (5-HT3*) exhibit premature death due to obstructive uropathy. To characterize this urological pathology, we measured bladder activity in homozygous 5-HT3* mice and investigated responses to pharmacological manipulation. Histopathology of lower urinary tract tissues from 5-HT3* mice revealed mucosal and smooth muscle hyperplasia of urinary bladder and prostate with secondary inflammation and bacterial

infection. Cystometric evaluation of 14 week-old 5-HT3* mice revealed highly distended bladders with impaired voiding. In contrast to the micturition contractions seen in wild-type controls, 5-HT3* mice exhibited urinary dribbling and frequent non-micturition bladder contractions (NMBC). NMBC in 5-HT3* mice were attenuated by prazosin (0.3 mg/kg, sc), and bladder contractions appeared to be less responsive to carbachol (ivc) with contractions seen only at 10-4 M, compared to the potent contractions seen in wild-type controls at 10-6 – 10-4 M. *In vitro* analysis on isolated bladder strips from these animals demonstrated that carbachol (10-5 M) and KCl (67 mM) produced significantly reduced contractions. In contrast, isolated bladder strips from naïve eight week-old 5-HT3* mice contracted to carbachol and KCl with same maximal response as that of wild-type controls, but failed to exhibit bladder contractions to ATP (10-4 M) and neurogenic electrical field stimulation. Therefore, 5-HT3* mice exhibit histopathological and cystometric changes indicative of bladder outlet obstruction. Compared to wild-type controls these mice show bladder and prostate hyperplasia, bladder distension, urine dribbling and frequent NMBCs. Bladder tissue from 5-HT3* mice fail to contract to nerve stimulation and ATP, and show a progressive loss of carbachol and KCl-induced maximal contraction. This is the first study showing that disturbances of 5-HT3 receptor function lead to genitourinary disease.

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273. Site-specific incorporation of unnatural amino acids into receptors expressed in mammalian cells

Sarah L. Monahan, Dennis A. Dougherty**

We describe an approach to achieve unnatural amino acid incorporation into channels and receptors expressed in mammalian cells. We show that microelectroporation provides a general method to deliver DNA, mRNA, and tRNA simultaneously. In both CHO cells and cultured neurons, microelectroporation efficiently delivers an *in vitro* transcribed, serine amber suppressor tRNA, leading to nonsense suppression in a mutant EGFP gene. In CHO cells both natural and unnatural amino acids chemically appended to a suppressor tRNA are site-specifically incorporated into the nicotinic acetylcholine receptor (nAChR). Electrophysiology confirms the expected functional consequences of the unnatural residue. The microelectroporation strategy described here is more general, less tedious, and less damaging to mammalian neuronal and non-neuronal cells than previous approaches to nonsense suppression in small cells, and provides the first example of unnatural amino acid incorporation in mammalian cells using chemically aminoacylated tRNA.

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274. Different binding orientations for the same agonist at homologous receptors: A lock and key or a simple wedge?

Tingwei Mu, Dennis A. Dougherty**

We studied two homologous binding sites for the neurotransmitter serotonin (5-HT) the 5-HT_3 receptor and the MOD-1 receptor of *C. elegans*. Key aromatic residues of the agonist-binding site are conserved in the two. We saw MOD-1 as presenting an opportunity to quantify a cation-π interaction between serotonin and a Tyr, allowing a direct comparison with the serotonin•••Trp interaction in 5-HT_3. Therefore we used the *in vivo* nonsense suppression methodology for unnatural amino acid incorporation to substitute Tyr 180 of MOD-1 with several Tyr analogues, including fluorinated residues of the sort that were so informative in studying the tryptophan interaction. These studies led to no clear conclusions. However, another canonical aromatic residue of the agonist-binding site is Tyr 198 of the nAChR, aligning with Tyr 234 of 5-HT_3 and Tyr 192 of AChBP. This Tyr is conserved in essentially all members of the family, but it is Trp 226 in MOD-1. Given our earlier successes with studies of fluorinated Trp derivatives, we applied the same protocol to Trp 226 of MOD-1. The same trend seen for Trp 183 of 5-HT_3 is seen for Trp 226 of MOD-1. Both serotonin lines have steeper slopes than the analogous ACh line (as expected based on electrostatic arguments), and the two serotonin lines have identical slopes. Such agreement means that the primary ammonium of serotonin makes a strong cation-π interaction with a Trp in both systems, but the two homologous receptors use a different tryptophan to make the cation-π interaction to serotonin. Our results show that, instead of settling for the tyrosine, the agonist reorients in the binding site to contact a nearby tryptophan and thus, maximize the cation-π interaction.

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275. Modulation of postsynaptic proteins is essential for forskolin-induced potentiation of synaptic transmission

Irina V. Sokolova, Norman Davidson

Activation of protein kinase A (PKA) by forskolin induces enhancement of glutamatergic synaptic transmission in the CA1 area of the hippocampus. Classical quantal analysis of synaptic transmission suggests that PKA-induced potentiation arise via increased presynaptic release probability. To investigate whether postsynaptic events also contribute to the forskolin-induced potentiation of excitatory synapses in culture, we studied dissociated hippocampal cultured neurons using either perforated patch or whole-cell configuration (PPC or WCC, correspondingly). Paired recordings using PPC revealed that 15 min forskolin perfusion leads to the short-term potentiation (STP) of evoked EPSCs (eEPSCs) in all six cell pairs tested [234±10%(SE)]. STP was followed by the long-term potentiation (LTP) of eEPSCs in five of six cell pairs tested (143±7% (SE)). LTP lasted for ≥80 min after forskolin washout. When a postsynaptic neuron was

voltage-clamped by WCC, however, forskolin-induced STP was significantly attenuated (152±8%(SE), $p<0.01$) and LTP was disrupted in all six-cell pairs tested. In experiments using PPC, 15 min forskolin perfusion also induced an increase of mEPSC frequencies. The frequency of mEPSCs was maximal after 15 min of forskolin perfusion (445±106% (SD) of baseline level, five cells) and gradually declined reaching the baseline level 45 min after the start of forskolin washout (three cells). The time-course of mEPSC frequencies in WCC experiments resembled that in the PPC experiments; however, in WCC the maximal upregulation of mEPSC frequency 15 min after the start of forskolin perfusion was significantly attenuated (275±18% (SD) of baseline, three cells, $p<0.05$). Overall, these results demonstrate that modulation of postsynaptic proteins by PKA, and diffusible postsynaptic factors, are essential for the forskolin-induced potentiation of synaptic transmission.

276. Requirement of a critical period of GABAergic receptor blockade for induction of a cAMP-mediated long-term depression at CA3-CA1 synapses

Tzu-ping Yu, Norman Davidson

Previous reports show that bath application of the adenosine 3':5'-cyclic monophosphate (cAMP) analog, Sp-cAMPS, induces a protein kinase A (PKA)-dependent and protein synthesis-dependent long-term potentiation (LTP) at hippocampal CA3-CA1 synapses. Recently we reported a novel form of long-term depression (LTD) induced by concurrent application of Sp-cAMPS and picrotoxin, a γ-aminobutyric acid type A ($GABA_A$) receptor antagonist. In the present study, we further investigate the mechanisms underlying such cAMP-mediated LTD. Synaptically connected CA3 and CA1 cells of hippocampal slice cultures were impaled by sharp electrodes. Excitatory postsynaptic potentials recorded from a CA1 pyramidal cell were evoked by single action potentials in a CA3 cell. Picrotoxin was applied to slices at various time points after Sp-cAMPS was perfused. We found that Sp-cAMPS-induced potentiation could be converted to depression when picrotoxin was applied within 30 min after perfusion of Sp-cAMPS. Picrotoxin applied 1 h after perfusion of Sp-cAMPS had no effect on Sp-cAMPS-induced synaptic potentiation. Once LTP was induced by Sp-cAMPS and expressed for 1 h, the subsequent application of Sp-cAMPS and picrotoxin produced no new changes in synaptic strength. Also, once LTD was induced and expressed for 1 h, subsequent Sp-cAMPS produced no new changes in synaptic strength. These findings suggest that a synapse is committed irreversibly to cAMP-mediated LTP or LTD during a critical period, and that later signals cannot interconvert these two fates.

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Summary: Much of the research in this laboratory involves the study of interactions between the nervous and immune systems. Using knockout (KO) mice and over-expression *in vivo* with adeno- and lentiviral vectors, we are exploring the role of neuropoietic cytokines in PNS and CNS injury and repair, epilepsy, Alzheimer's disease, pain and inflammation. In the same context of neuroimmune interactions, we are investigating a mouse model of mental illness based on the known risk factor of maternal influenza infection. We are also investigating potential therapies for Huntington's disease (HD), using intracellular expression of antibodies, and manipulating NFκB activity. An additional project involves the study of the mechanism of how stress affects melanoma tumor progression.

Cytokines are diffusible, intercellular messengers that were originally studied in the immune system. Our group contributed to the discovery of a new family that we have termed the neuropoietic cytokines, because of their action in both the nervous and hematopoietic/immune systems. We have demonstrated that one of these cytokines, leukemia inhibitory factor (LIF), can coordinate the neuronal, glial and immune reactions to injury. Using both delivery of LIF *in vivo* and examination of the consequences of knocking out the LIF gene in mice, we find that this cytokine has a powerful regulatory effect on the inflammatory cascade, both within and outside the nervous system, and it can regulate neurogenesis. We are exploring the implications of these findings in the study of animal models of nerve and spinal cord injury and regeneration, epilepsy and inflammatory pain. We find, for instance, that LIF is a critical regulator of astrocyte activation following stroke or seizure, and that this cytokine also regulates inflammatory cell infiltration, neuronal death, gene expression, as well as the production of new neurons from stem cells following injury. These results highlight this cytokine as an important therapeutic target. We are also examining the role of LIF in a transgenic mouse model of Alzheimer's disease.

Cytokine involvement in a new model for mental illness is also being investigated. This mouse model is based on findings that maternal viral infection can increase the likelihood of schizophrenia or autism in the offspring. We are using behavioral, neuropathological and brain imaging methods to investigate the effects of maternal influenza infection on fetal brain development and altered behavior in adult offspring.

We are utilizing intracellular antibody expression to block the toxicity of mutant huntingtin (Htt), the protein that causes HD. We have produced single-chain antibodies (scFvs) that bind to various domains of Htt, and these can either exacerbate or alleviate Htt toxicity in cultured cells, acute brain slices, and in a *Drosophila* HD model. Work has begun on delivering these scFvs in mouse models of HD. We have also implicated the NFκB signaling pathway in the pathogenesis of HD, and identified several steps in this signaling cascade as potential therapeutic targets.

277. Effects of LIF on adult neural stem cells and neurogenesis: Can LIF stimulate adult brain repair?
Sylvian Bauer, Bradley Kerr

In the adult mammalian brain, new neurons are generated from neural stem/progenitor cells residing in particular areas and these neurons are integrated locally in the olfactory bulb and the hippocampal dentate gyrus under normal conditions. After injury, however, this ongoing neurogenesis does not lead to significant neuronal replacement in affected regions, unless exogenous growth factors are supplied. We have shown that the cytokine leukemia inhibitory factor (LIF) is necessary for the lesion-induced replacement of neuronal precursors that regenerate olfactory sensory neurons in the adult mouse. Preliminary results also indicate that delivery of LIF in the normal adult mouse brain enhances bromodeoxyuridine (BrdU) incorporation. We are now determining the nature of these newly generated cell types. LIF is being delivered in the ventricular system as a recombinant protein (using osmotic mini-pumps) or via viral vectors (adenovirus or lentivirus). The new cells are then characterized with immunohistochemistry and confocal microscopy. We are also investigating the possibility that LIF could stimulate neuronal replacement in a mouse model of Alzheimer's disease that displays neuronal death.

278. The role of leukemia inhibitory factor (LIF) in the regulation of the inflammatory cascade following spinal cord injury
Bradley J. Kerr

The inflammatory response to injury in the central nervous system is significantly slower than that seen after a similar insult to a peripheral nerve. In the latter case, there is a rapid recruitment of macrophages to the site of injury, which clear away damaged myelin distal to the

injury, a process known as Wallerian degeneration. This process is critical for the successful regeneration of damaged peripheral axons. However, Wallerian degeneration after injury to the CNS proceeds at a much slower rate (weeks to months) compared to the PNS (7-14 days). The delayed onset of this process has been postulated to contribute to regenerative failure in the injured CNS, as known regeneration inhibitory factors released from damaged myelin are not adequately removed. Therefore, treatments that can stimulate a more rapid immune cell response in the CNS can be beneficial in promoting successful regeneration of damaged fibers. Since leukemia inhibitory factor (LIF) is a critical mediator of the inflammatory response in cortex and peripheral nerve (Sugiura *et al*., 2000), we are studying how LIF regulates the inflammatory cascade after spinal cord injury. Results from over-expression of LIF using a recombinant adenoviral construct are compared with the responses of LIF knockout mice following spinal injury. Results thus far indicate that LIF regulates reactive gliosis, inflammatory cell infiltration, and the expression of pro-inflammatory cytokines and chemokines after injury. These studies are also aimed at determining whether manipulation of LIF levels can alter the onset of Wallerian degeneration, promote axonal regeneration, and improve functional outcome after spinal cord injury.

279. The role of leukemia inhibitory factor in the regulation of the response to seizure

Kristina H. Holmberg

Our group previously found that the cytokine leukemia inhibitory factor (LIF) is induced in astrocytes in response to seizure. We are now using adult LIF knockout (KO) mice to study the functional role of LIF in post-seizure sequelae in the hippocampus. Twenty-four hours after seizure, immunocytochemical staining (ICC) for glial fibrillary acidic protein is upregulated in the wild-type (WT) but not in the KO. This indicates that LIF is an autocrine regulator of astrocyte activation following seizure, as it is following physical injury to the cortex (Sugiura *et al*., 2000). LIF also regulates the expression of several neuropeptides in response to seizure. While seizure reduces calretinin ICC in the supragranular layer in WT mice, we see little change in the LIF KO. In contrast, staining for neuropeptide Y (NPY) in mossy fibers is much higher in the LIF KO following seizure than in the WT. This is of particular interest because NPY is known to have anti-convulsant effects. Other peptides such as vasoactive intestinal polypeptide and cholecystokinin do not show any differences between WT and KO mice, normally or after seizure. Overall, these results show that LIF can control neuropeptide phenotype in the brain *in vivo*, as it is known to do in the PNS.

280. A viral model for mental illness based on a known risk factor

Limin Shi, Nora Tu[1], Paul H. Patterson

There is considerable evidence that exposure of pregnant women to viral infection during a critical period can increase the incidence of schizophrenia and possibly autism in their offspring. We previously reported that influenza respiratory infection of pregnant BALB/c mice can cause highly abnormal behavioral responses in their offspring, tested as adults. We have carried out further histological analysis on the brains of these offspring and find morphologic changes in the hippocampus, a structure that consistently displays pathology in schizophrenia, and in the cerebellum, a structure that consistently displays pathology in autism. The advantage of the mouse model is the opportunity to investigate when and how these changes occur.

To study whether the effects of the virus are due to direct infection of the fetus, we have used RT-PCR to search for viral RNA. While the maternal lung is strongly positive, the fetal brain and the placenta are negative for viral RNA, adding to our previous evidence that the maternal anti-viral response is causing the changes in the fetal brain.

[1]*Caltech undergraduate student*

281. Do cytokines mediate the effects of maternal viral infection on fetal brain development?

Stephen Smith, Jennifer Li[1], Limin Shi, Paul H. Patterson

We have found that influenza respiratory infection of pregnant mice induces behavioral abnormalities in their offspring that are consistent with behaviors seen in schizophrenia and autism. We also have evidence that these changes in fetal brain development are due to the maternal anti-viral response rather than to direct viral infection of the fetus. We are now testing the hypothesis that certain cytokines produced by the mother are responsible for altering fetal brain development. The approach is to: (i) block cytokine function in the infected pregnant mouse by injecting anti-cytokine antibodies; and (ii) attempt to mimic the effects of infection by injecting non-infected pregnant mice with particular cytokines. The adult offspring are then tested behaviorally and their brains examined histologically.

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282. The use of recombinant antibodies to dissect the neuropathology of Huntington's disease and as potential therapeutics

Ali Khoshnan, Jan Ko, Paul H. Patterson

Huntington's disease (HD) is caused by expansion of a polyglutamine stretch in the N-terminus of the protein, huntingtin (Htt). Expression of mutant Htt results in selective death of neurons in the striatum and the cortex. Large nuclear aggregates and amyloid-like complexes containing Htt are observed in post-mortem HD tissue. We generated eight monoclonal antibodies (mAbs), which recognize the expanded polyglutamine (polyQ) domain,

the polyP domains, or the C-terminus of exon-1 of Htt (HDx-1). We have cloned the complementary DNAs of the antigen binding regions of several of these mAbs and have assembled recombinant single-chain antibodies (scFvs). Intracellular expression of the MW7 scFv targeted to the polyP domains of Htt reduces cell death caused by expression of mutant Htt in cultured cells, in acute brain slice cultures, and in a *Drosophila* HD model. In contrast, expression of two scFvs recognizing the polyQ domain exacerbates mutant Htt toxicity. Studies are in progress to explore the therapeutic potential of MW7 and how it achieves its effects. Anti-polyQ scFvs are being used as reagents to understand the molecular mechanism of aggregation induced by expanded polyQ.

283. Mutant huntingtin activates NF-κB through interaction with IKKγ

Ali Khoshnan, Jan Ko

Transcriptional dysregulation by mutant huntingtin (Htt) protein has been implicated in the pathogenesis of Huntington's disease (HD). We find that mutant Htt protein activates the NF-κB pathway. Mutant Htt physically associates with IKKγ, a regulatory component of the IκB kinase complex (IKK). In cultured cells, this interaction results in the activation of IKK, leading to the phosphorylation and degradation of the inhibitory protein IκBα. These findings have *in vivo* relevance, as striatal extracts from HD transgenic mice have higher levels of IKK than extracts from control mice, and activated NF-κB is found in the nucleus of striatal and cortical neurons in HD mice. Binding to IKKγ is mediated by the expanded polyglutamine domain in mutant Htt, and is augmented by the proline-rich motifs of Htt. Over-expression of IKKγ promotes mutant Htt aggregation and nuclear localization. Conversely, a N-terminally truncated form of IKKγ, which interferes with IKK activity, blocks Htt-induced NF-κB activation and reduces the toxicity of mutant Htt in cell culture and in an acute brain slice model of HD. Toxicity is also inhibited by expression of a mutant F-box deleted E-3 ubiquitin ligase, ΔF-βTRCP, which specifically blocks degradation of IκB inhibitory proteins. Thus, aberrant interaction of mutant Htt with IKKγ, and subsequent NF-κB activation, may be important for HD pathology. Studies are in progress to understand the interplay between mutant Htt protein, the IKK complex and signaling pathways that are influenced by these interactions.

284. The effect of stress on the immune system and melanoma progression

Jennifer Montgomery

We are investigating the relationship between stress, the immune system and melanoma tumor progression. We have found that a paradigm of alternating rotational stress, restraint stress and cold-water swim stress raises serum levels of the stress hormone corticosterone while avoiding habituation. In these mice, the corticosterone binding globulin does not change with stress indicating that the elevated corticosterone is free to activate the hypothalamic-pituitary-adrenal axis. Additionally, we are assaying changes in cytokine levels caused by stress. Specifically, we are looking at IL-1β, TNF-α, IL-6, and IFN-γ, as they have been implicated in both the stress response as well as in melanoma growth in culture. We are also investigating the mRNA levels of these cytokines by RT-PCR in the hypothalamus, spleen and liver.

We are about to begin the same stress protocol using a new mouse model of melanoma that is based on the genetics of human tumors. In the transgenic mouse model, cutaneous melanoma occurs spontaneously at a predictable time point, allowing study of the disease *in situ* as it naturally progresses. We will be monitoring the timing of tumor onset as well as disease progression in mice that undergo behavioral stress and comparing them to control mice that remain unstressed.

Publications

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Summary: Synapses, the points of contact and communication between neurons, can vary in their size, strength and number. These differences in synapses and their ability to change throughout the lifetime of the animal contributes to our ability to learn and remember. We are interested in how synapses are modified at the cellular and molecular level. We are also interested in how neuronal circuits change when synapses change their properties. We conduct all of our studies in the hippocampus, a structure known to be important for memory in both humans and animals. We use molecular biology, electrophysiology and imaging to address the questions detailed below.

A major focus of the lab concerns the cell biological mechanisms that govern modifications at individual synaptic sites. In particular, we are interested in the idea that dendritic protein synthesis and degradation may contribute to synaptic plasticity. We are also interested in mRNA and protein trafficking during synaptic plasticity.

We are also examining the role of the cadherins family of cell adhesion molecules in synaptic plasticity. Several labs have shown that cadherins are localized to synapses in the hippocampus. Earlier, we demonstrated that function-blocking cadherin antibodies or peptides can prevent long-term potentiation, without interfering with basal synaptic transmission. We hypothesize that cadherin bonds may be sensitive to local fluxes in extracellular calcium imposed by action potential activity. We are now examining the molecular mechanisms by which cadherins influence synaptic strength and the involvement of cadherins in the formation and maintenance of synapses, using fluorescence resonance energy transfer and endocytosis assays.

285. Pumilio is a potential regulator of local protein synthesis in hippocampal neurons
Chiranjib Dasgupta, Changan Jiang

Pumilio is the founding member of the Puf-family of translational repressors. In *Drosophila* oocytes, it represses the translation of *hunchback* mRNA by binding to a *cis*-RNA element, the nanos response element (NRE), and recruiting two other co-repressors, Nanos and Brain-tumor. Recent studies showed that *Drosophila pumilio* mutants have defects in long-term memory, suggesting that it functions in synaptic plasticity. To study the function of *pumilio* in mammalian synaptic plasticity, we have cloned a rat *pumilio* homologue, *pum2*. Immuno-staining of hippocampal slices and cultured hippocampal neurons revealed high levels of Pumilio protein in the cell body and proximal dendrites, suggesting a possible function in the transport of dendritic mRNA and/or the regulation of local protein synthesis. To address whether pumilio regulates protein synthesis in neurons, we have developed GFP-based translational reporters, which either contain or lack an NRE in the 3'-UTR. Overexpression of Pum2 significantly reduced the expression of the NRE-containing reporter without affecting translation of the control (NRE-) reporter. These data suggest that Pum2 can repress the translation of NRE containing mRNAs in neurons. Currently, we are using these reporters to examine whether *pumilio* plays a role in synaptic plasticity and whether its function as translational repressor can be regulated by synaptic activity.

286. Dendritic protein synthesis and electrical stimulation in hippocampal neurons
Melinda Owens

In neurons, new proteins are synthesized not only in the cell body but also in the dendrites. Dendritic protein synthesis provides a logical mechanism for achieving and maintaining specificity during synaptic enhancement. However, the connection between dendritic protein synthesis and specific patterns of neural activity has yet to be firmly established. Using field stimulation of cultured hippocampal neurons, I attempt to characterize the relationship between dendritic protein synthesis and patterns of electrical stimulation associated with hippocampal long-term potentiation (LTP), a well-characterized model for synaptic plasticity associated with learning and memory.

First, I established that my field stimulation chamber evoked activity. Trains of 1 Hz, 10 Hz, and 100 Hz caused neurons to extensively release the dye FM 4-64,

indicating exocytosis and vesicle release. Then, I used a diffusion-limited green fluorescent protein (GFP)-based protein synthesis reporter to follow dendritic protein synthesis after electrical stimulation. Cultured hippocampal neurons were infected with a virus containing the reporter and stimulated with different electrical patterns associated with LTP induction. Different stimulation protocols for inducing LTP make use of different molecular mechanisms and might have different roles for dendritic protein synthesis, so the frequency and duration of the stimulus were varied as well. Then, changes in the expression level and distribution of the GFP reporter were analyzed over a period of several hours.

In the time-lapse experiments, it appears that a high frequency stimulation protocol consisting of four 1s trains of 100 Hz depresses protein synthesis relative to control neurons not receiving stimulation, while neurons stimulated with low frequency stimulation, 400s at 1 Hz, appear to keep pace with controls. However, there seems to be no difference in protein synthesis levels between the proximal and distal dendrite. Presently, I am performing a theta-burst protocol (ten bursts of four pulses at 100 Hz, with 200 ms between pulses). This work, combined with future studies in electrically stimulating hippocampal slices, will further elucidate the relationship between LTP and protein synthesis in dendrites.

287. The regulation of N-cadherin turnover at the synapse

Chin-Yin Tai

Synaptic plasticity likely requires the dynamic growth of synaptic contacts, which are modulated by cadherins and other cell adhesion molecules. We are studying one such molecule, N-cadherin, a member of the classic cadherin family. Little is known about the turnover of N-cadherin at the synapse and the mechanism that underlies its exo- and endocytosis. To begin to address this issue, we have conducted antibody-live labeling of N-cadherin in cultured hippocampal neurons. After acid-stripping, dendrites are decorated with N-cadherin antibody, suggesting an active internalization of N-cadherin in these regions. We are now examining whether synaptic activity alters this pattern of internalization. A recent study demonstrated that the ubiquitination of E-cadherin plays a role in regulating its endocytosis in epithelial cells. To examine whether ubiquitination plays a role in neuronal cadherin trafficking, we have immunoprecipitated cadherin from whole-brain lysates and conducted Western blot analysis with anti-ubiquitin antibodies. We have observed the presence of several higher molecular weight cadherin species suggesting neuronal cadherins are also ubiquitinated. We are currently examining whether ubiquitination contributes to N-cadherin internalization and degradation.

288. Miniature synaptic events regulate local protein synthesis in dendrites of hippocampal neurons

Michael Sutton, Nick Wall

Although it is now well established that neuronal dendrites are capable of mRNA translation, the signals that regulate dendritic protein synthesis are still largely unknown. Here, we demonstrate that, in the context of chronic activity blockade, miniature synaptic events (mEPSPs), potently regulate protein synthesis in the dendrites of cultured hippocampal neurons. Using a GFP-based protein synthesis reporter (a Sindbis viral vector in which the coding sequence for a destabilized, myristoylated GFP is flanked by the 5' and 3' UTR of α-CAMKII), in combination with time-lapse confocal microscopy, we examined dendritic protein synthesis from 9-12 hrs after chronic activity blockade (1 μM TTX). When the impact of mEPSPs are chronically blocked by the glutamate receptor antagonists CNQX and APV (TTX+Block), an increase in reporter synthesis in both proximal and distal dendrites is observed (relative to controls). When mEPSPs are permitted (TTX alone), however, a marked decrease in protein synthesis is observed in both of these compartments. Acute application of CNQX and APV (+TTX), 9 hrs after pre-incubation in TTX alone produces a rapid increase in reporter synthesis in both proximal and distal dendrites, suggesting that in the absence of evoked synaptic transmission, mEPSPs tonically inhibit dendritic protein synthesis. Similarly, blocking spontaneous vesicle release with botulinium toxin A (BoNT/A; 100 nM), when cells were pre-treated chronically (7 hrs), with TTX alone also stimulated dendritic translation, providing parallel evidence that mEPSPs tonically inhibit dendritic protein synthesis. Finally, the acute effects of BoNT/A were occluded by chronic mini blockade (TTX+Block), suggesting that the increase in dendritic protein synthesis observed in each case derives from blocking mEPSPs. These results reveal a previously unknown role for miniature synaptic events in regulating dendritic protein synthesis in neurons. The spatial resolution and mechanisms of translational regulation by mEPSPs are currently being investigated.

289. A lentiviral vector-based reporter system for studying local protein synthesis in hippocampal neurons

Changan Jiang

Local protein synthesis in neuronal dendrites plays an important role in the induction of long-lasting forms of synaptic plasticity, but the mechanisms by which neural activity triggers translation are not well understood. To study the regulation of local protein synthesis, we have developed a lentiviral vector-based system for expressing protein synthesis reporters in neurons at physiological levels. In this system, a PEST sequence is first fused with the open reading frame of a superbright yellow fluorescence protein, Venus, to reduce its half-life to 3-4 hours. Next, a myristoylation signal is attached to the

destabilized Venus to target the protein to the membrane. To mimic the dendritic synthesis of CaMKIIα protein, we have flanked the $_{myr}$dVenus with the 5' and 3' untranslated regions (UTRs), of CaMKIIα mRNA, the *cis*-elements required for its translation regulation and mRNA targeting. Expression of this reporter by a lentiviral vector in cultured hippocampal neurons results in bright fluorescence in the dendrites. Experiments are currently underway to determine whether synaptic activity can induce the dendritic synthesis of the reporter and the signaling pathways involved in translation regulation.

290. A potential role for ubiquitin-mediated degradation in AMPA receptor recycling and synaptic plasticity

Gentry Patrick[*], Baris Bingol[*], Gerald Reis[1], Holli Weld

The availability of proteins likely plays an important role in synaptic function and plasticity. Protein synthesis has been clearly shown to play a role in synaptic plasticity; however, little is known about the potential role of ubiquitin-mediated protein degradation. Using immunostaining techniques, we show that ubiquitin (ub) is present in the soma, dendrites, and spines of cultured hippocampal neurons. Treatment with AMPA changes the pattern of ub staining. We therefore asked whether ubiquitin-mediated protein degradation plays a role in the AMPA-stimulated internalization of GluRs. In agreement with published work, short-term treatment with AMPA induced an average 8-10 fold increase in internalized GluRs in cultured hippocampal neurons. In contrast, brief (5-15 min) pretreatment with the proteasome inhibitor MG132 dramatically reduced the amount of GluR1 and two internalization induced by AMPA or NMDA. MG132 did not affect the GluR1 or two surface receptor population prior to AMPA treatment. The block of AMPA-stimulated GluR internalization is not due to a dimunition of the free ub pool during MG132 incubation. The expression of a chain elongation defective ub mutant (Ub K48R) also severely diminished AMPA-induced internalization. The requirement for proteasome activity and polyubiquitination in GluR internalization suggest an important role for ub-mediated degradation in GluR trafficking. We are currently pursuing the identity of the degraded protein(s) involved in GluR trafficking. One obvious target is the GluR itself. Although both GluR1 and GluR2 are polyubiquitinated, GluR mutants, in which cytoplasmic ubiquitination is prevented, still exhibit MG132-sensitive AMPA-induced internalization. These results suggest that the degradation of a protein other than GluR is required for GluR internalization.

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291. Visualization of cadherin-cadherin association in neurons

Eric Mosser

Classic cadherins, in particular N- and E-cadherins, are expressed and localized at synaptic sites in the brain and involved in synaptic plasticity. Cadherins exhibit Ca^{2+}-dependent homophilic interactions across cell junctions: the removal of Ca^{2+} from the extracellular solution results in a loss of adhesion. Thus, it is possible that changes in extracellular Ca^{2+} associated with synaptic activity may alter cadherin-cadherin interactions. We are attempting to visualize cadherin-cadherin associations at cell-cell junctions with the eventual goal of monitoring hippocampal synapses during synaptic activity. This will enable us to determine if synaptic activity and plasticity affects cadherin dynamics and synaptic structures. We have expressed multiple E-cadherin constructs with HA or myc epitope tags and several recombinant single-chain antibodies (scFvs), specific to the myc and HA epitopes. These scFvs are either green fluorescent protein (GFP), variant fusions (yellow or cyan), or have been engineered such that they will allow specific fluorescent labeling using maleimide derivatives of fluorescent dyes. Fluorescence Resonance Energy Transfer (FRET), between appropriate fluorophore pairs will be used to visualize the homophilic interactions of cadherins forming junctions between adjacent cells. Viral vectors will be used to express these constructs in neurons. A cadherin containing one GFP variant (FRET donor), will be expressed in presynaptic neurons while cadherin containing the other GFP variant (FRET acceptor), will be expressed in postsynaptic neurons. FRET will then be used to detect the cadherin interactions between pre- and postsynaptic cells in differing conditions. In particular, we will examine the effects of synaptic activity and varying extracellular calcium concentrations on cadherin-cadherin dynamics.

292. Local protein synthesis stimulated by D1/D5 dopamine receptor agonists

W. Bryan Smith, Shelley R. Starck[1], Richard W. Roberts[2]

It is well established that *de novo* protein synthesis is required for long-term memory storage. Furthermore, the activity-dependent regulation of local protein synthesis (LPS) in the dendritic compartment is believed to be essential for the persistence of late-phase LTP. While it has been demonstrated that isolated dendrites of mature hippocampal neurons are able to synthesize proteins, the synaptic mechanisms governing this local translation have yet to be determined. Previous studies have implicated the D1/D5 class of dopamine receptors in a wide range of behavioral and cellular processes: the receptors are critically involved in spatial learning, and have also been shown to enhance glutamatergic transmission in the hippocampus. In order to test the hypothesis that D1/D5 receptors regulate LPS, we have examined the effects of two D1/D5-selective agonists - SKF-38393 and Dihydrexidine (Dhx) - on

protein synthesis in cultured hippocampal neurons. Between-dish comparisons as well as single-cell time-lapse imaging experiments show that bath application of the D1/D5 agonists stimulates synthesis of a GFP reporter molecule possessing the untranslated regulatory regions of CamKIIα. On average, the increases in GFP signal seen with the dopamine agonists are greater than those seen with glutamate receptor activation via NMDA or with bath incubation of bicuculline.

Given the maturation time of the GFP fluorophore, which may be as long as 30 minutes, one cannot definitively rule out the cell body as a potential source of dendritic GFP signal in these experiments. To address this issue, we are using a novel protein synthesis reporter based on puromycin, an aminoacyl-tRNA analog. In order to visualize translation in living cells, puromycin has been covalently attached to fluorescein, thus generating fluorescein-dC-puromycin (F2P). The free amino group on puromycin reacts with the carboxyl end of proteins as they are being synthesized in the ribosome, thereby labeling newly synthesized proteins with fluorescein. Focal application of a solution containing F2P and Dhx to a small region on a dendrite (10-20 μm), results in substantial fluorescein incorporation. Importantly, the fluorescein signal is almost completely absent when cells are pre-treated with anisomycin, a potent translation inhibitor. Our results clearly demonstrate the ability of dopamine receptor agonists to activate protein synthesis machinery in the dendrites of hippocampal neurons. Future experiments will investigate the necessity of this pathway for LPS induction and learning behavior *in vivo*.

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293. A method to locally control protein synthesis

Michael Goard, Girish Aakalu[1], Olesya Fedoryak[2], Carlo Quinonez, Jamii St. Julien[3], Stephen J. Poteet[2], Tim Dore[4]

The targeting of mRNA and localized synthesis of proteins to specific cellular domains enables cells to carry out asymmetric functions. In neurons, for example, the local synthesis of proteins may allow specific regions of dendrites to show long-lasting protein synthesis-dependent plasticity, such as long-term potentiation. In the case of LTP, the relative contributions of somatic vs. dendritic protein synthesis have been difficult to address because existing techniques do not permit selective inhibition in either cellular compartment. In order to inhibit protein synthesis in specific cellular domains (e.g., dendrites vs. cell bodies), we have developed a photo-releasable anisomycin compound. The cage consists of a 6-bromo-7-hydroxycoumarin-4-ylmethyl group, which can be removed through exposure to UV light. Therefore, the area of protein synthesis inhibition can be restricted to a small region defined by the limits of UV light exposure, or, potentially, multiphoton excitation if a pulsed IR laser is the light source. We have tested the compound's effectiveness using *in vitro* protein translation systems, cultured CHO cells, and cultured neurons; and are currently testing the compound in hippocampal slice preparations. These tests indicate that the uncaged compound can effectively inhibit protein synthesis in a spatially restricted manner but does not affect protein synthesis in its caged form. This ability to "locally" inhibit protein synthesis will enable experiments in brain slices and cultured cells where one can specifically inhibit protein synthesis in the cell body or synaptic layers. These experiments will elucidate the roles of somatic and/or dendritic protein synthesis during plasticity with both spatial and temporal precision.

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294. 'Memory' in the maintained discharge patterns of neurons in the human hippocampal-amygdala complex

Joydeep Bhattacharya, Jessica Edwards, Adam Mamelak, M.D.

The spontaneous *in vivo* activity of single neurons is less frequently studied than stimulus-induced activity. Classically, the spontaneous firing pattern of a neuron, which is often irregular, is considered to be neuronal noise that can be modeled as a stochastic point process (a renewal process). A renewal process is essentially memory-less; successive intervals in the inter-spike-interval (ISI) sequence are independent and identically distributed. An alternative view is that spontaneous activity exhibits long-range correlations in its ISI structure. We used detrended fluctuation analysis (DFA) and multiscale entropy (MSE) to analyze the ISI sequences of 22 neurons located in either the hippocampus or the amygdala of human subjects implanted with intracranial hybrid depth electrodes. Activity was recorded in the absence of any explicit cognitive task while the subjects were awake. Both the DFA and MSE techniques indicate the spontaneous firing patterns of most neurons are not well described by a renewal process; rather they show long-range power-law correlations, representing ongoing memory effects in the ISI sequence, which arise from an underlying non-renewing process. Such long-range correlations were not observed when the analysis was conducted on shuffled data sets that preserve the mean firing rate (and histogram), of the original data set. The presence of long-range correlation is a characteristic of a fractal-like dynamics, representing *memory* in the firing pattern. Such memory may be used to optimize information transfer and storage at synapses.

295. ***In vivo*** human electrophysiology: Investigations of learning and memory

Jessica Edwards

Questions of how humans learn and remember information about the world around us have long fascinated scientists and philosophers. The majority of information about human learning and memory has come

from psychological testing – methodologies that provide significant observational results, but little explanatory power. Electrophysiological work in animals has been able to elucidate many of the mechanisms and structures involved in non-human learning, but cannot simply be extrapolated to human behavior. Human electrophysiology provides a unique means by which data from diverse fields can be integrated to describe a more complete system for human learning and memory. We have the rare opportunity to record neuronal activity at the single-unit level from alert, behaving humans. Patients suffering from medically intractable epilepsy are resistant to drug therapies that are traditionally used for seizure control. When the epileptogenic focus is well-localized, removal of the small portion of offending tissue can cure a patient of epilepsy in over 85% of cases. To localize the area for surgery, patients are implanted with up to twenty electrodes, including several microwire electrodes (40 micron, Pt/Ir wires) in the hippocampus and amygdala. Because electrodes are being placed for clinical diagnostic purposes, an opportunity exists to simultaneously record individual neuronal activity. The duration of the medical procedures allows us to monitor the neuronal activity of cells in the hippocampus and amygdala for up to one week. While we record neuronal activity, the patients complete a series of psychological tasks designed to elicit rapid learning and declarative memory. Patient performance is evaluated using both recall and recognition tasks to evaluate different forms of memory retrieval. We also have developed a virtual Water Maze, a video game simulation of the Morris Water Maze task, to test object-cued place memory. Preliminary analysis of these data have shown marked differences between neuronal responsiveness to correctly and incorrectly recognized items. We hope to extend these analyses to examine the correlation between local field potentials (EEG) and single-unit activity.

296. Relevance of the temporoammonic pathway in hippocampus memory system

Armando Miguel Remondes

Medical and experimental evidence has progressively linked the hippocampal formation with the acquisition and maintenance of contextual memories in the mammalian brain. Early anatomical studies described most of the connections between the whole brain and the hippocampus, but the functional relevance of the different connections and their interplay remains largely unknown. The entorhinal cortex (EC) provides most of the anatomical input to the hippocampus and is thereby believed to carry the cortical information, which is eventually processed in order for memories to be formed. The entorhinal input to the hippocampus comprises two projections. One of them connects EC layer II neurons with the dentate gyrus and initiates what is known as the tri-synaptic circuit: EC II neurons send axons to make synapses onto the DG granular cells, these send their axons to the hippocampus CA3 field, and these send their axons, Schaffer collaterals, to the CA1 pyramidal cells. The CA1 pyramidal cells produce the major output from the hippocampus back to the EC, and this structure conveys its output to the rest of the cortex. The least studied connection between the hippocampus and the cortex connects EC layer III cells directly with the CA1 region of the hippocampus. This connection has earlier been named the temporoammonic pathway (TA). After having studied extensively the physiological properties of the TA pathway and described its interesting modulatory powers over the CA1 information processing, we are currently assessing its relevance in the memory formation by studying the behavioral consequences of TA lesions. Using two hippocampus-dependent behavioral tasks, the Morris Water maze and the Transitive Inference test, we hope to ascertain the necessity of this direct cortical projection for the performance of known hippocampus-dependent behaviors.

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297. Development of multimodal spatial integration and orienting behavior in humans

Christine Chee-Ruiter, Patricia A. Neil, Joydeep Bhattacharya, Alice Lin, Elinor Lin, Ben Matthews, Susan Dao

The spatial location of objects and events is often specified by concurrent auditory and visual inputs. Adults of many species, including humans, take advantage of such multimodal redundancy in spatial localization. Previous studies have shown that adults respond more quickly and reliably to multimodal compared to unimodal stimuli localization cues. The current study investigated for the first time the development of audio-visual integration in spatial localization in infants, 1-10 months of age. Infants were presented with a series of unimodal or spatially and temporally coincident bimodal lights and sounds, +/-25 and +/-45 degrees from center, and their head and eye orienting responses were measured from digital video records. Results showed that infants older than four months responded significantly faster to bimodal stimuli versus visual or auditory only stimuli; younger infants responded to all stimuli, either bimodal or unimodal, uniformly. This is consistent with neurophysiological findings from multimodal sites in the superior colliculus of infant monkeys that multimodal enhancement of responsiveness is not present at birth but that it emerges during the first months of life. Additionally, age-dependent effects of position and modality on response latency are supportive of multiple developmental stages preceding the onset of adult-type bimodal spatial localization responses. Other ongoing studies expand on these findings using spatially and temporally discordant stimuli and comparing these results with those previously obtained, as well as investigating the development of attention in audio-visual localization using the Gap/Overlap and Inhibition-of-Return paradigms. Additionally we are using EEG/ERP analysis with both infants and adults for the same localization paradigm, to study the neural basis for these behaviors.

298. A moving visual stimulus progressively drags the perceived timing of a sound

Bhavin R. Sheth, Shinsuke Shimojo

Here, we report a new cross-modal effect. When observers had to time the occurrence of a brief sound (S) relative to a moving visual stimulus, the S appeared to be dragged later and later in time over successive motion sweeps, despite the fact that its timing relative to the motion was the same on each sweep. Over the course of the trial, the perceived drag would abruptly and randomly reset to a value close to the initial one, and then start all over again on the succeeding sweeps. Method: A bright circular disk (100.4 cd/m^2, 1 deg. dia., 10 deg. eccentricity below fixation) drifted 21 deg. from left to right (or right to left, depending on the trial) over 465 ms on a dark background. This motion repeated 15 times (sweeps) on each trial. A 2.2 ms long S (65 dBa SPL) occurred at the same instant relative to the visual motion on all sweeps. After all sweeps were concluded on the trial, observers (n=5) had to indicate where the disk was in space when the Ss occurred. Os made two reports with a mouse: The locations of the disks coincident with the first and the last S. Results: Congruent with informal observations, the perceived disk location coincident with the final S was further along in the motion than that of the first S (difference = 3.84 deg. +/- 1.56 deg., $p<.001$ for n=3, $p<.05$ for n=1). We asked whether multiple Ss or multiple motion sweeps contributed to the sound drag. In one experiment (2 sounds), we played the S only in the second and last motion sweeps. In another (2 sweeps), we showed the moving stimulus only for the second and last Ss. The sound drag in the motion direction was not as large in either experiment compared to the original (2 sounds: n = 3, difference = -2.27 deg.; 2 sweeps: n = 2, difference = 1.81deg). Thus, both the sound and the motion contributed to the sound drag. Other factors and the relationship of the effect with the flash-lag effect (Nijhawan, 1994) and the flash-drag effect (Whitney and Cavanagh, 2000) will be discussed.

299. Gaze, decision processes and cortical activation: A joint eye-tracking/EEG study

Claudiu Simion, Joydeep Bhattacharya, Shinsuke Shimojo

Using eye tracking, we reported (VSS02) a gaze bias towards choice in several 2AFC tasks involving human faces and abstract shapes. The gaze bias translated in a progressive increase in the likelihood that the future choice is inspected, culminating with a significant (>80%)

bias prior to decision; this bias, termed the "gaze cascade effect" was incorporated in a model where orienting behavior (gaze) plays an active role in preferential decisions.

We investigated the neural basis of such decision processes by simultaneous recordings of multivariate EEG signals and eye-tracking, while subjects inspected pairs of faces or abstract shapes. Four separate conditions were considered, where subjects had to decide: 1) which face was more attractive; 2) which face was less attractive; 3) which face was rounder; and 4) which shape was more attractive. A passive-viewing condition was included, where subjects inspected faces with for future recognition. Wavelet-based time-frequency analysis revealed the dynamics of middle and high frequency band (14-70 Hz) activity. Significant increases from pre-stimulus intervals were found in the gamma frequency band (>30 Hz) in prefrontal regions; this effect was mostly pronounced in the last second before decision. Stronger gamma band neural responses were found in the face attractiveness task, compared to the other 2AFC tasks, whereas minimal increase during the passive condition was noted. Moreover, left hemispheric lateralization was found in the last 400 ms before decision in the face attractiveness task. Contrarily, activity in the beta and gamma frequency bands decreased during the same time period during the "rounder" and "less attractive" tasks.

This suggests that the "more attractive" task is functionally distinct from the other two tasks, maybe because it naturally links orienting and liking, while the others involve top-down suppression and control.

300. Gaze manipulation biases preference decisions

Claudiu Simion, Shinsuke Shimojo

We continue our investigation of the relationship between preference judgments and orienting behavior. We reported (VSS'01) a gaze bias towards choice in preference tasks (gaze cascade effect), when stimuli were either faces, or abstract, unfamiliar shapes. We proposed a model in which orienting behavior (active gaze) interacts with cognitive assessment of stimuli in a positive feedback loop leading to the decision.

If our model is correct, biasing observers' gaze should influences preference. To assess the validity of this claim, we showed human faces, whose baseline attractiveness was matched, on a computer screen, while subjects were instructed to actively follow the display with their eyes. Faces were presented side by side alternatively, so that only one was present on the screen at any given time. We biased the fixation duration by always presenting one face for a longer time (900 vs. 300 ms). This sequence was repeated 2, 6 or 12 times before subjects were allowed to respond which face appeared more attractive. The results show a clear bias towards the longer presented face when the number of repetitions was larger than two (59% for 6 repetitions, 59.2% for 12 repetitions, both $p<0.01$), consistent with our model.

To exclude the possibility that the preference bias was solely due to mere exposure, we performed a control experiment in which the presentation sequence was identical, but both images were presented in the middle of the screen, at the point of fixation. Retinotopic exposure sequence was identical, yet this experiment did not reveal any preference for the longer exposed face. Moreover, to show that the effect is not due to general perceptual fluency, we repeated the original experiment asking subjects to report which face was rounder, instead of more attractive. No bias towards the longer shown face was found.

We conclude that orienting behavior, in the form of active gaze shift, is critical in deciding preference, directly influencing it.

301. Color-spreading is selective for shape and configuration, and operates along perceptual surfaces

Daw-An Wu, Ryota Kanai, Shinsuke Shimojo

It is commonly held that color filling-in phenomena stop at luminance edges (e.g., Troxler Fading). We have reported a counter-example where edges facilitate the spreading of color. Subjects fixating on a broad, smooth color gradient containing a pattern of dark gaps perceived the central color to spread and replace the peripheral colors, 'jumping' over the gaps.

We now report that color spreads only to regions consistent with the pattern present near fixation, and multiple colors can spread at the same time. We also investigate perceptual surface segregation as a mechanism.

Configuration: Subjects fixate on a dark field containing an array of color patches. Each patch consists of a "square" surrounded by a "frame." Squares vary from red to green in a graded fashion: they are red near fixation, green in the periphery. Frames are all green (so peripheral patches are homogeneous). Subjects perceive the color configuration present at the fovea to spread, until all patches appear as sharply delineated red squares surrounded by green frames.

Multiple color gradients: Color-spreading persists when both squares and frames vary in color. It is fastest when they are given opposite color gradients (center = red squares and green frames, periphery = green squares and red frames). Often, the extreme periphery quickly takes on the foveal pattern, even as areas of intermediate eccentricity retain their actual color (homogeneous yellow) for a time. This suggests that color-shape misbinding can facilitate the process of color-spreading.

Surfaces: In stimuli with only one color gradient, colinearity of patches doesn't affect spreading. However, for the above multi-colored stimuli, color spreading is slower when patches are not colinear. When squares and frames are arrayed as separate objects, color spreading is again independent of colinearity; the percept is of red squares and green frames lying on separate surfaces. We propose that elements (colors, shapes, dark gaps) on the same perceptual surface can interfere with each other, but this is alleviated by perceptual segregation onto different surfaces.

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302. Color-spreading selective for visual surfaces in transparent motion

Ryota Kanai, Daw-An Wu, Shinsuke Shimojo

Filling-in phenomena have been assumed to occur retinotopically based on luminance edge signals (e.g., Cornsweet Illusion, Troxler Fading, etc.). In contrast to this view, we have previously reported that a prolonged fixation at a color gradient interrupted by sharp luminance edges, results in a color-spreading beyond the luminance edges (Shimojo, Wu, and Kanai, 2002, *Perception* 31, supplement).

One way to account for this illusion is that color-spreading occurs along perceptual surfaces, rather than retinotopically. Here, we tested this idea using transparent motion in which two surfaces are moving in opposite directions, each having a different color gradient from center to periphery.

In a typical stimulus, the color of dots gradually changed from red to green as the eccentricity increased on one surface, and on the other surface, the color of dots gradually shifted from green to red. Thus, at an intermediate eccentricity, there arises a yellow area where the colors of two surfaces are close to each other. The width of this area was varied by changing the steepness of the gradient and the effect of occlusion on this area was tested.

Our findings include: 1) the color spreading occurred selectively for each surface, supporting the surface-based account; and 2) a shallow gradient did not necessarily result in an immediate color-spreading, as opposed to the classical edge-based filling-in phenomena.

Although the final percept of the color for each surface was perceptually similar between the shallow and the steep gradients, two separate effects seem to be involved. First, the color information from the fovea dominates over that of the periphery and spreads in this direction, as in our previous report. Second, when the peripheral colors consist of the colors same as in the fovea, but combined with the opposite motion direction, these colors are actively used to facilitate a percept of homogeneously colored surface via a motion-color misbinding process.

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303. Color spreading beyond luminance edges and space

Shinsuke Shimojo, Daw-An Wu, Ryota Kanai

When there is a color/luminance gradient from the fovea to the periphery, a prolonged fixation leads to color spreading in this direction. We report a variation in which color spreads beyond spatial gaps.

An array of small colored squares with luminance contours was displayed on a black background. When the color/luminance inside the squares had a global color/luminance gradient from the fovea to the periphery, a prolonged fixation led to vigorous color spreading in this direction. After 10 - 20 s of gazing, nearly the entire array appeared to be homogeneous in terms of the inside color, while the perceptual distinction between background and patches remained obvious. (Artist Julian Stanczak published a series of works with this type of configuration and effect.)

The effect is similar to color spreading or filling-in reported in the literature, but distinctive in that the spreading occurs beyond luminance edges and background space.

Contribution of factors such as luminance, gap, presence and collinearity of contours is discussed.

We argue that, while the effect is seemingly related to occlusion, early sensory mechanisms, such as edge detection and adaptation, play important roles. The effect has implications on bottom - up processes towards a cortical representation of multiple visual surfaces.

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304. Disappearance of target after sustained adaptation induced by high contrast

Farshad Morad[1], Shinsuke Shimojo

Filling-in can be induced by high-contrast edge adaptation (Shimojo and Kamitani VSS '01), or after prolonged adaptation to a peripheral low-contrast object (Troxler, 1904). Adaptation to sustained low-contrast vs. adaptation to transient high-contrast suggests synergy between contrast and edge adaptation, but the possible interactions are not well understood. We observed that presenting a low-contrast edge for 5-10 seconds and then flashing a high-contrast edge over it could elicit the perceptual disappearance of a subsequent low-contrast edge at the same location. Neither adaptation to the low-contrast edge nor flashing the high-contrast edge alone had any significant effect. We investigated this effect using Gabor signals (2 cpd, 5 deg eccent., sd=1, mean lum. 50cd/m2, background 50cd/m2). Target (contrast=4%) followed either a) a sustained (8 sec) low (4%) contrast stationary or drifting Gabor signal (adaptation only), b) a brief (20ms) high (~100%) contrast Gabor signal (flash only), or c) adaptation followed by flash (combined condition). A random-dot mask followed the target after 1 second. The task was to identify whether the target was present or not. Subjects (n=5) failed in less than 3% of the

trials in adaptation only or flash only conditions, but more than 30% in the combined condition ($p<.0001$). For combined condition trials, failure of detection was more pronounced after adaptation to a drifting Gabor than a stationary one ($p<.05$). There was no significant difference between same or opposite contrast polarity (phase insensitivity). In other experiments we found: a) suppression is selective for orientation; and b) disappearance could be transferred to other locations. Results suggest: 1) contrast gain adjustment to transient change is processed separately from adaptation to sustained stimuli; 2) the two mechanisms interact non-linearly.

305. Metaphor of 'high' and 'low' in pitch revisited: Visual motion illusion induced by auditory pitch

Fumiko Maeda[1], Ryota Kanai[2], Shinsuke Shimojo

Why are pitches described as being 'high' or 'low'? Are these terms merely metaphors? Studies demonstrate interactions between pitch, space and other variables within and across modality. While several theories have been proposed, the nature of the interactions including the neural mechanisms involved remains unclear. Here we demonstrate a novel cross-modal illusion, one that does not appear to be predicted by current theory. A pure tone with changing pitch (0.3 – 2.0 kHz) was presented while observers viewed counter-phase gratings. The direction of motion was perceived in accord with the pitch change, i.e., when the tone changed from high to low pitch, downward motion was perceived, and vice versa. Control data indicate that this is a perceptual effect unexplained by eye movements, response bias, or linguistic semantic top-down influences. Furthermore, it is unlikely that the phenomenon is a low-level sensory effect or could be explained by the intrinsic spatial attributes of sound. These results may provide insight into the neural mechanisms underlying cross-modal interactions. In addition, they may have implications for understanding the possible perceptual origin of the metaphoric use of 'high' and 'low' in pitch.

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306. Visual cortex as a site of cross-modal integration

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It has been shown that visual perception can be strongly affected by auditory stimuli. It is unclear, however, what the brain circuitry subserving these interactions may be. We used the sound-induced illusory flash effect (a single flash accompanied by two auditory beeps is perceived as two flashes) as a tool to investigate this question. This illusion is much stronger in the periphery than fovea. In a previous study using event-related brain potentials, we had investigated whether this illusion is due to modulation of activity in the visual pathway or a higher perceptual area, such as associative cortex. We compared the visual evoked potentials (VEPs) in the presence and absence of sound. Activity was modulated significantly already prior to 200 ms poststimulus in the illusion trials (periphery) but not in trials where no illusion occurred (fovea). In addition, the VEP associated with the illusory second flash was qualitatively very similar to the VEP associated with a physical second flash, suggesting that similar representations underlie the percept of the illusory and a physical flash. These results suggested that the observed modulation of activity by sound occurs within the visual cortex. In the present study we tried to localize the brain regions involved in the perception of the illusory flash more directly and more accurately using event-related fMRI. We collected the functional images of three participants in the following conditions. Unimodal conditions V_p and V_f consisted of visual stimulation: a small disk flashed once in the periphery or fovea, respectively. Bimodal conditions AV_p and AV_f consisted of auditory-visual stimulation: combination of two beeps with visual stimuli V_p and V_f, respectively. In another unimodal condition, V_p^2, a physical double flash was presented in the periphery. Trials were randomized. Contrasting the (illusion) condition AV_p against V_p resulted in activity in Brodmann's areas 17, 18, and 19. Contrasting (no-illusion) condition AV_f versus V_f, however, did not show any activity in the occipital lobe ruling out the possible role of attention in the aforementioned enhanced visual activity. Considering that the visual stimulus was identical in AV_p and V_p, the enhanced activity of early visual areas in AV_p can only be attributed to the perception of the illusory flash caused by sound. Similar brain areas were indicated when contrasting V_p^2 against V_p. The common brain areas involved in these two contrasts confirm our previous ERP results suggesting similar mechanism underlying the percept of a physical and an illusory flash. These results altogether indicate that the activity in the early visual cortical areas is modulated by sound.

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307. Perceiving the present: Foundations, review, and a general theory of illusions of projected size, projected speed, luminance contrast and distance

Mark A. Changizi, Romi Nijhawan, Shinsuke Shimojo

"Perceiving-the-present" denotes the theory that natural selection has favored a visual system capable of correcting for the roughly 100 msec latency between the time light hits the retina and the time of the consequent perception. In particular, perceiving-the-present posits that, for an ecologically appropriate stimulus occurring at

time t, the visual system generates at time t + 100 msec a perception of the scene most probably present at time t + 100 msec; that is, the visual system attempts to perceive the present, not the recent past. Although this may seem to be only a small departure from the orthodox perceiving-the-recent-past view, it is, in fact, a significant departure, for it is able to accommodate many classes of otherwise inexplicable illusions. In this paper we first discuss the foundations of perceiving-the-present, contrasting it to the orthodox theoretical framework within visual perception, and then briefly review existing evidence for perceiving-the-present, including the class of flash-lag effects and the geometrical illusions. We then demonstrate how perceiving-the-present can unify over two dozen classes of illusion dealing with the effects of: (1) projected size; (2) projected speed; (3) luminance contrast; (4) distance; (5) eccentricity; (6) converging lines; and (7) optic flow on perceived (A) projected size; (B) projected speed; (C) luminance contrast; and (D) distance.

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Summary: Our research focuses on the study of information processing across networks of neurons, with emphasis on the neuronal mechanisms that underlie learning and memory formation. Many lines of evidence suggest that the process of memory formation occurs by gradual integration of recently-learned information into distributed cortical networks through the interactions between cortical and hippocampal circuits. However, the direct experimental investigation of these interactions has been difficult since, until recently, simultaneous chronic recordings from large numbers of well-isolated single neurons were not technically feasible. These experiments became possible with the advent of the technique of chronic multi-area tetrode recordings in freely-behaving rodents. Tetrode recordings provide a powerful technology for studying networks of neurons because they allow (a) reliable isolation of single neuron activity, (b) significant yield, enabling the simultaneous monitoring of large numbers of neurons, and (c) long-term stability of individual neuron recordings over many days, a property critical for studying the neuronal basis of learning.

Using this technique we monitor the simultaneous activity of large numbers of cortical and hippocampal cells during the acquisition and performance of memory tasks, as well as during the sleep periods preceding and following experience. Tracking neuronal activity across different behavioral and brain states is important as memory formation is believed to occur over several stages, with the *encoding* of mnemonic information in hippocampal networks occurring during active exploratory behavior, and the gradual *consolidation* of memories in neocortical sites occurring under the influence of hippocampal activity during off-line periods, such as sleep. Consistent with this idea, brain activity during these different brain states is drastically different, with each state marked by a characteristic combination of network oscillations.

Our main working hypothesis is that these network oscillations play a key role in structuring cortico-hippocampal communication, and that entrainment of neuronal subpopulations through phase-locking to these oscillations is an important mechanism of memory encoding and consolidation. This hypothesis is based on two main results: (1) We have shown that during *awake behavior* prefrontal cortical neurons fire at specific phases of the hippocampal theta rhythm even in the absence of any cortical theta rhythmicity, demonstrating the existence of intricate temporal coordination across cortical and hippocampal circuits; (2) We have shown that during *slow wave sleep* cortico-hippocampal interactions are not uniform across time, but are structured around discrete identifiable episodes characterized by the co-occurrence of hippocampal fast (200 Hz) ripple oscillations and cortical slower (7-15 Hz) spindle oscillations.

Our current work focuses on the quantitative characterization of the fundamental spatial and temporal structure of cortical and hippocampal phase-locking to network oscillations, and the modulation of these properties by learning. Furthermore, we investigate the cellular and molecular basis of network interactions by analyzing the effects of pharmacological and genetic manipulations on the organization of ensemble neuronal activity. Our experimental work is complemented by theoretical studies of network models and the development of tools for the analysis of multi-neuronal data.

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Summary: Our overall focus is on the molecular mechanisms of axon guidance and synaptogenesis in *Drosophila*. Our approach combines genetics, molecular biology, biochemistry, and cell biology. We are especially interested in cell-surface and signal-transduction proteins that function in growth cones, presynaptic terminals, and their postsynaptic partners.

Receptor tyrosine phosphatases and axon guidance: Genetics of receptor tyrosine phosphatases. In the 1990s, we showed that four receptor-linked protein tyrosine phosphatases (RPTPs}, are selectively expressed on CNS axons and growth cones in the *Drosophila* embryo, and that these RPTPs regulate motor and CNS axon guidance during embryonic development. RPTPs are likely to directly couple cell recognition *via* their extracellular domains to control of tyrosine phosphorylation *via* their cytoplasmic enzymatic domains. The extracellular regions of the fly RPTPs all contain immunoglobulin-like (Ig) and/or fibronectin type III (FN3) domains, which are usually involved in recognition of cell-surface or extracellular matrix ligands. Their cytoplasmic regions contain either one or two PTP enzymatic domains. The fly genome encodes six RPTPs.

We have performed a detailed characterization of the genetic interactions among five of the six RPTPs. We find that each growth cone guidance decision in the neuromuscular system has a requirement for a unique subset of RPTPs. In some cases, the RPTPs work together, so that defects are only observed when two or more are removed. In other cases, however, phenotypes produced by removal of one RPTP are suppressed when a second RPTP is also absent. Our results provide evidence for three types of relationships among the RPTPs: partial redundancy; collaboration; and competition [Sun *et al*., (2001) *Mol. Cell. Neurosci.* 17:274-291], (Ratnaparkhi abstract). We have also recently obtained mutants lacking expression of the sixth, and apparently final, RPTP, DPTP4E, and are investigating their phenotypes.

Searching for RPTP substrates. It is difficult to identify PTP substrates biochemically because PTPs usually do not display strong specificity *in vitro*. One current approach is to perform yeast two-hybrid screens with 'substrate-trap' versions of DPTP10D, DPTP69D, and DPTP99A. We introduced a constitutively activated chicken Src tyrosine kinase into the yeast together with the PTP 'bait' constructs, in the hope that it would phosphorylate relevant substrate fusion proteins made from cDNA library plasmids. The screen should also recover non-substrate interacting proteins that may form complexes with the RPTPs *in vivo*. We have identified several classes of clones whose interactions with the substrate-trap RPTPs are dependent on coexpression of the tryosine kinase, suggesting that they may be substrates. We are currently analyzing these further to determine which ones are likely to be of interest for the future (Bugga abstract).

Searching for RPTP ligands, and development of a new method for identification of ***Drosophila*** cell-surface proteins. We have conducted several mammalian COS cell expression screens to attempt to identify ligands and/or coreceptors for the fly neural RPTPs, so far without success. This has been a major problem in the RPTP field; indeed, *in vivo* ligands for RPTPs have not been identified in any system. In order to understand how RPTPs regulate axon guidance, it is essential to know when and where they engage ligands, and how ligand binding affects enzymatic activity and/or localization.

Our current approach to identifying ligands is based on our observation (Aloisia Schmid, unpublished; Zinn abstract), that fusion proteins in which the extracellular domains of RPTPs are joined to human placental alkaline phosphatase (AP) can be used to stain live *Drosophila* embryos. Each of four fusion proteins (DLAR-AP, DPTP69D-AP, DPTP10D-AP, DPTP99A-AP) binds in a specific manner to the embryonic CNS. Most of the observed staining is on CNS axons. We are now screening a 'deficiency (Df) kit' of ~200 fly lines, each of which lacks a specific region of the genome, by staining homozygous Df embryos from each line with each of the fusion proteins. This method should identify the genomic regions encoding each of the RPTP ligands (Fox abstract). We have already identified Dfs that contain genes required for DLAR-AP staining, and have tentatively defined the gene encoding the putative DLAR ligand.

We are also developing a novel method for expressing the entire repertoire of fly cell-surface proteins on the surfaces of transfected mammalian cells. Cells expressing this collection of cell-surface proteins can be screened using RPTP-AP fusion proteins in order to identify and characterize ligands. We will also generate monoclonal antibodies (mAbs) to these cell-surface proteins, and we have developed an approach which should allow us to directly clone the genes encoding the proteins recognized by these mAbs without any biochemical purification or screening of expression libraries. These genes and mAbs should be of interest in understanding axon guidance, as well as other processes that occur during fly development.

Genes controlling axon guidance and synaptogenesis in the larval neuromuscular system. The 32 motor neurons in each hemisegment of a *Drosophila* embryo innervate 30 muscle fibers, and each motor axon extends along a stereotyped route and always targets the same fiber. Motor growth cones reach their muscle targets during late embryogenesis and then gradually mature into presynaptic terminals. These synapses continue to expand and change as the larva grows, because their strengths must be matched to the sizes of the muscle fibers they drive. The pattern of Type I neuromuscular junction (NMJ) synapses in the third instar larva is simple and highly stereotyped, with boutons restricted to specific locations on each muscle fiber.

We devised and executed a gain-of-function (GOF) screen of live larvae to find genes involved in axon guidance and synaptogenesis in this system [Kraut *et al*. (2001) *Curr. Biol.* 11:417-430.] Our screen identified 41 'known genes' (those with published mutant alleles), and 35 'new genes' for which high-level neuronal expression produces axonal/synaptic phenotypes. We assembled published phenotypic data on the 'known genes,' and examined larval neuromuscular loss-of-function LOF phenotypes for some of them ourselves. These results showed that at least 3/4 of the 'known genes' are important for nervous system development or function in wild-type flies. An analysis of homology relationships displayed by the 'known gene' and 'new gene' sets suggests that most of the 'new genes' will also have neural loss-of-function LOF phenotypes. The products encoded by the 76 genes identified in our screen include kinases, protein and lipid phosphatases, GTPases, guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), ATPases, cell-surface receptors, RNA-binding proteins, transcriptional regulators, and a variety of other molecules likely to be involved in protein trafficking, modification, and degradation.

We have initiated further study of a number of genes identified in the screen that are defined by existing mutations. *pumilio* (*pum*) encodes an RNA-binding protein that shuts down translation of specific mRNAs by binding to their 3' untranslated regions. We found that Pum protein is expressed in a small subset of CNS neurons, and *pum* LOF mutations alter motor axon guidance in the embryo. Pum protein is also localized to NMJs in third instar larvae, and is primarily postsynaptic. *pum* LOF larvae have NMJs that do not grow in a normal manner. Postsynaptic Pum regulates expression of a translation initiation factor, eIF-4E, which is localized to post-synaptic aggregates; thus, in *pum* mutant larvae we observe a dramatic increase in the number and size of eIF-4E aggregates. Pum protein binds directly to the 3'UTR of eIF-4E mRNA (Menon abstract). We are also investigating aggregation of Pum protein (Salazar abstract).

Robo2 is a cell-surface protein that mediates growth-cone repulsion from sources of its ligand Slit. We find that Robo2 overexpression alters the migration of sensory neuron cell bodies in the peripheral nervous system (PNS), and LOF mutations in Robo family genes also affect PNS cell migration (manuscript submitted for publication; (Kraut abstract 313).

'New' genes identified by the screen, and by an earlier GOF embryonic screen conducted by Qi Sun, a former graduate student, include a number of molecules involved in protein trafficking in neurons. We have investigated two of these thus far. *Spastin* encodes an AAA-ATPase that is the ortholog of human *spastin*, a gene mutated in the disease autosomal dominant spastic hemiplegia. AAA-ATPases are a class of proteins that form multimeric complexes that regulate a variety of protein trafficking events within the cell, including vesicle sorting, protein degradation, and microtubule dynamics. There are about 30 such proteins encoded in the fly genome. We made LOF mutations in the *spastin* gene, and found that these produce synaptic defects. Mutant adults cannot fly or jump (Sherwood abstract).

Beached/Blue cheese is a huge protein that is closely related to the human protein whose loss causes Chediak-Higashi syndrome, a lethal disease affecting lysosomes and related organelles (Kraut abstract 315). We are undertaking a systematic study of the 'new genes' by making transgenic RNAi lines, so that we can knock out expression of each gene in a defined set of cells (Jeon abstract).

308. Analysis of genetic interactions among receptor tyrosine phosphatase genes

Anuradha Ratnaparkhi

The neural receptor tyrosine phosphatases (RPTPs) encoded by the *Ptp* 69D, *Ptp*99A and *Lar* genes are required for motor axon guidance during *Drosophila* embryonic development. In *Lar* mutants, the ISNb motor axons often fail to enter their target ventrolateral muscle (VLM) field. The failure to innervate these muscles results in an ISNb 'parallel bypass' phenotype. The bypass phenotype produced by *Lar* mutations is suppressed when *Ptp99A* is also mutant. This result is consistent with a model in which the decision by ISNb axons to enter the VLM field is controlled through negative regulation of DPTP99A signaling by DLAR. Such regulation might be direct (through protein-protein interactions between DLAR and DPTP99A), or indirect (through their downstream signaling pathways). To further examine these models, we overexpressed DPTP99A in the embryonic CNS using an EP line (from Ernst Hafen's group), carrying a insertion 5' to the *Ptp*99A gene. If either the direct or indirect model is correct, one would predict that overexpression of DPTP99A in a wild-type background should be able to produce ISNb parallel bypass. (We had tried to perform this experiment earlier using a variety of DPTP99A cDNA constructs, but these apparently did not express at a high enough level to produce bypass.) With the EP-DPTP99A line, however, we find that overexpression of DPTP9A in postmitotic neurons produces ISNb bypass phenotypes identical to those seen in *Lar* mutants. We are currently performing co-overexpression experiments to see if driving expression of wild-type DLAR together with

DPTP99A can suppress the bypass phenotype. If this is observed, it would indicate that the activities of the two RPTPs can be titrated against each other, consistent with direct or indirect regulation of DPTP99A signaling by DLAR.

Cross-regulation of RPTP signaling is also observed for DLAR and DPTP52F. Here removal of DLAR suppresses a *Ptp52F* CNS phenotype, suggesting that DPTP52F is a negative regulator of DLAR signaling. We are performing experiments to look at this and other genetic interactions with *Ptp52F*.

309. Identification of putative RPTP ***in vivo*** substrates

Lakshmi Bugga

To understand fully the function and regulation of *Drosophila* RPTPs, it will be necessary to identify the physiological substrates of the individual RPTPs. To isolate RPTPs in a complex with their target substrates, we are using yeast two-hybrid system. We are looking for proteins that interact with *Drosophila* neuronal RPTPs-10D, 69D, 99A and 52F. To attempt to achieve stable binding of the RPTPs to a tyrosine phosphorylated substrate, we have used 'substrate-trap' mutants of the RPTPs, which can bind to substrates but do not catalyze dephosphorylation, instead remaining bound to substrate in a stable complex.

We constructed plasmids encoding GAL4 DBD/RPTP bait proteins and introduced them into yeast together with fly cDNA libraries encoding GAL4AD-cDNA fusion proteins (from Steve Elledge, Baylor). We also introduced a plasmid containing a constitutively active form of chicken c-Src, driven by a constitutive yeast promoter. The bait (with or without src), and prey (cDNA), hybrid proteins are transformed into separate yeast mating strains (A and alpha), that contain three reporter genes-adenine, histidine, and lacZ, and, also an auxotrophic marker. Positive interactions are detected by selecting on plates lacking the auxotrophic marker and screening for reporter expression.

Our screen with all four DPTPs resulted in several positive clones - about 15 genes that interact with either of the four DPTPs. We eliminated some of these genes that are either bait independent or not specific to the interacting RPTP. We identified seven genes that interact specifically with a given DPTP; four of these genes as potential substrates based on Src dependence. Of the seven genes four are known genes-Tartan (a cell adhesion molecule expressed in embryonic CNS and PNS), cysteine string protein (a chaperone protein expressed in larval neuro-muscular junction and adult brain), Xmas-1 (an RNA binding protein that is involved in spermatogenesis, oogenesis and embryogenesis), and BEST:LD07122 (a DNA binding protein). Of the three unknown genes, one is rich in proline residues and also has proline motifs that are known to bind to SH3 domains. RNA *in situs* with this gene showed expression in the embryonic CNS. We are currently testing these interactions *in vitro* by transient transfection experiments with *Drosophila* cell line-S2 cells and finding out the expression of rest of the unknown genes by RNA *in situs*, and looking for double-mutant phenotypes with DPTP and tartan mutant flies.

310. Screening for ligands of DPTP10D, DPTP99A, and DPTP69D

Nicki Fox

The structural similarity of receptor protein tyrosine phosphatases (RPTPs), to receptor tyrosine kinases (RTKs), has led to the hypothesis that ligands might also modulate receptor protein tyrosine phosphatase (RPTP), activity, as they do with RTKs. To date, most RPTPs are "orphan receptors," as their physiologically relevant ligands are unknown. The identification of ligands for RPTPs is crucial for better understanding of the exact mechanisms by which RPTP signaling affects the growth cone.

A screening method is being undertaken that utilizes recombinant proteins consisting of the extracellular domain of either RPTP10D or RPTP99A (the portion of the RPTP that would be expected to interact with a ligand), fused to a molecule of human placental alkaline phosphatase (PLAP). The fusion proteins are incubated with live *Drosophila* embryo 'filets' (flat dissections in which the CNS is flanked by muscle and epidermis), and then replaced with fixative. The fixed embryo filets are then incubated with an anti-PLAP secondary antibody and finally a fluorescent probe-conjugated tertiary antibody. This procedure results in a staining pattern that reveals where DPTP-binding proteins are expressed. This procedure will be carried out on wild-type embryos and embryos containing chromosomal deficiencies (Dfs). A Df 'kit' of about 200 Df lines (Bloomington Stock Center), which together encompass about 80% of the genes, will be screened for Dfs that cause aberrant DPTP-PLAP staining. A Df that removes specific DPTP-PLAP staining would presumably contain a gene encoding the protein to which the DPTP extracellular domain is binding. Overlapping Dfs can then be screened to narrow down the genes that might encode a ligand or binding partner of DPTP10D or DPTP99A.

311. The translational repressor ***pumilio*** regulates local translation and synaptic growth at the larval NMJ

Kaushiki P. Menon, Subhabrata Sanyal[1], Robin P. Wharton[2], Mani Ramaswami[1], Kai Zinn

Pumilio (Pum) is a translational repressor protein involved in early development in *Drosophila*. Repression of *hunchback* mRNA translation by maternal Pum is required for embryonic patterning. We have now characterized the functions of zygotic *pumilio* in the nervous system. Our results show that *pum* is required for synaptic growth in the larval neuromuscular system. Pum protein localizes to the postsynaptic side of the NMJ synapse, and it is also expressed in neuronal cell bodies. We find that presynaptic (neuronal), and postsynaptic (muscle), Pum have distinct roles in synaptic development and function. In *pum* mutants, there are fewer and larger boutons at larval NMJs. This phenotype is rescued by

low-level neuronal expression of Pum. Overexpression of Pum in neurons produces a reverse phenotype, in which NMJs have boutons that are much smaller and more numerous than in wild type.

Postsynaptic Pum represses local translation of mRNA encoding the translation initiation factor eIF-4E. In *pum* mutants, eIF-4E accumulates in large aggregates around the synapse. eIF-4E accumulation is reversed by low-level expression of Pum in muscles. Pum protein binds directly to the 3' UTR of eIF-4E mRNA, suggesting that this mRNA is a direct Pum target.

The GluRIIA receptor is also overexpressed at the NMJ in *pum* mutants, especially at 1s boutons. We are currently examining whether this overexpression is a consequence of derepression of translation of synaptic eIF-4E mRNA. We are also investigating how presynaptic and postsynaptic Pum regulate the electrophysiological properties of larval NMJs.

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312. Protein aggregation and synaptic function

Anna Salazar

Protein aggregation has been implicated in numerous human diseases, including prion-based encephalopathies. Prions are capable of catalyzing their own propagation in a process whereby the protein serves as a seed or template in which the WT protein becomes folded into the abnormal structure and aggregates. This process has been observed in various organisms, including yeast, in which a system has been developed to better understand prion-like behavior. In yeast, the [psi+] phenotype is caused by the aggregation of Sup35p, a translational termination factor, causing it to become inactivated. Because the aggregation of other proteins enhances the aggregation of Sup35p, this system can be used as an assay for proteins that display prion-like behavior. In response to a talk by Kandel, which implicated the existence of prion-like proteins at synapses, the *C. elegans* and *Drosophila* databases were analyzed to search for RNA binding proteins with Q/N rich domains. One of the genes obtained in this search, *pumilio*, encodes a protein that was obtained through an axon guidance screen at the larval neuromuscular junction by Kaushiki Menon in the lab (Menon abstract). Two Q/N rich domains of this protein, the full-length protein, as well as the *C. elegans* homolog are being cloned into yeast expression vectors in order to probe their ability to exhibit prion-like behavior in a well-established yeast assay.

313. Roundabout 2 controls segment-specific migration and orientation of sensory organs

Rachel Kraut

Roundabout (Robo) cell-surface receptors and their secreted ligand Slit regulate axon guidance and cell migration in a variety of systems. We are studying the role of Slit-Robo signaling in regulating the segment-specific positioning and orientation of *Drosophila* chordotonal (cho) sensory organs. Cho organs in the abdomen are located laterally and have dorsal dendrites, while their thoracic counterparts are located dorsally and have ventral dendrites. Robo2 overexpression in cho neurons converts the abdominal organs to a thoracic position and morphology by blocking their ventral migration and altering their orientation. *slit* (or *robo robo2*), loss-of-function mutations produce a reverse transformation in which thoracic cho organs migrate ventrally and take on the appearance of abdominal organs. These phenotypes suggest that Slit-Robo signaling is normally stronger in the thoracic cho organs, and that this increased signaling blocks their ventral migration. There are no obvious segmental differences in expression of Robos in cho neurons that could account for this. However, thoracic visceral mesoderm (VM) adjacent to non-migrating thoracic Chos expresses Robo2, and misexpression of Robo2 in more posterior abdominal VM, adjacent to abdominal Chos, produces abdominal-to-thoracic cho organ transformations. Finally, a homeotic mutation that converts abdominal cho organs to a thoracic morphology causes derepression of Robo2 expression in abdominal VM. The results are consistent with a novel signaling model (the 'Slit sandwich'), in which Robo2 on the thoracic VM binds to Slit and presents it to Robos on cho neurons. Our findings also suggest that the specification of cho organ position in wild-type embryos involves repression of *robo2* in the abdominal VM by Hox protein complexes.

314. ***Drosophila*** spastin is required at the larval NMJ and for adult motor function

Nina Tang Sherwood, Qi Sun, Mingshan Xue[1], Bing Zhang[1], Kai Zinn

We identified the *Drosophila* ortholog of the human *Spastin* gene through a neuronal overexpression (EP) screen for novel molecules that affect axon guidance in the embryo. Mutations in human *Spastin* (also called SPG4), underlie the most common form of autosomal dominant hereditary spastic paraplegia (AD-HSP). This disease is characterized by progressive spasticity and weakness of the lower limbs due to degeneration within the descending corticospinal tracts.

Spastin encodes a member of the large and functionally diverse AAA ATPase family of proteins, and our data indicate that Spastin protein is localized to the cytoplasm. Members of the AAA family function as multimers that catalyze the assembly or disassembly of protein complexes. Spastin is related in sequence to the microtubule-severing AAA ATPase katanin-60, and overexpression of human Spastin in tissue culture cells causes disassembly of the microtubule network. These results suggested that Spastin may also regulate microtubule polymerization. In support of this model, we have found that the microtubule network disappears when *Drosophila spastin* is overexpressed in muscles.

Overexpression of Spastin in neuronal precursors and neurons generates a dramatic phenotype in which the CNS is compressed onto the midline. To determine the

spastin loss-of-function phenotype, we generated several mutant lines by imprecise excision of our EP insertion. These excisions delete the N-terminal portion or all of the Spastin coding sequence. In *spastin* null excision mutants, there is a 30% decrease in excitatory junction current amplitude at third-instar larval NMJs. Correspondingly, immunocytochemistry reveals that synaptic bouton number increases by ~70%, while average bouton size is concomitantly reduced. Similar but less severe changes in synaptic physiology and morphology are observed with the partial deletion lines, which by RT-PCR still appear to express truncated forms of *spastin* mRNA and are thus, most likely hypomorphs.

Consistent with these defects at the larval NMJ, adult flies mutant for *Spastin* display compromised motor behavior. Both the null and partial deletion lines are homozygous viable, but adult flies homozygous for the complete deletion are found infrequently due to late-pupal lethality. In those flies that do eclose successfully, walking and climbing ability are greatly reduced, and neither flying nor jumping are observed. Furthermore, flies homozygous for the partial deletion are also slow-moving, and those that can fly do so poorly. Together with the physiological and morphological phenotypes observed at the larval NMJ, these data demonstrate a requirement for Spastin in *Drosophila* movement, and provide a potential model system for study of human AD-HSP.

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315. Analysis of ***blue cheese/beached***, a vesicle trafficking protein required for synaptogenesis

Rachel Kraut, Ashley Wright, Kim Finley[1]

We are establishing fluorescent transgenic markers in *Drosophila* that will allow us to visualize the movement of endocytic, cytoskeletal, and membrane lipid compartments in neurons during axon pathfinding in live embryos and in primary neuronal cultures. These will enable us to analyze the dynamic and coordinated behavior of these components of the growth cone in embryos and cells that are either deficient for, or that overexpress genes involved in pathfinding that were discovered in our overexpression screen. We will primarily focus on using these tools to identify the functions of a novel gene we called *beached*, which belongs to a family of BEACH-domain containing proteins. The BEACH domain has been implicated in sphingolipid breakdown and occurs in proteins that have a role in endolysosomal trafficking. Beached also contains a FYVE domain, which localizes proteins to endosomal membranes. Beached protein localizes to vesicles in a subset of CNS neurons and sensory neurons.

Mutations in *beached* (known as *blue cheese*), were recently described by Dr. Finley as causing progressive neurodegeneration in adult flies, and the human homolog of *beached* maps very close to a locus that has been implicated in late-onset familial Alzheimer's disease. Therefore, in uncovering Beached's role in pathways that regulate pathfinding, we will also shed light on mechanisms that underlie the neurodegenerative phenomena that are observed in *beached* mutants.

A second branch of the project will involve the establishment of primary embryonic CNS cultures derived from transgenic animals carrying the above fluorescent markers. This will achieve two purposes: the first will be to visualize at higher resolution the dynamic processes in axons and growth cones during pathfinding using the markers detailed in the proposal; the second will be to develop an assay for assessing the effects on pathfinding and endocytic activity of sphingolipid signaling molecules (S-1-P, sphingosine, and ceramide, that may be produced by Beached activity.

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316. Identification of genes involved in axon guidance and synaptogenesis by transgenic RNAi

Mili Jeon

An overexpression/misexpression screen for genes that are involved in axon guidance and synaptogenesis in the larval neuromuscular system identified 35 'new' genes that had not been previously characterized using genetics (Kraut *et al*., 2001). These genes encode a variety of proteins, including cell-surface receptors, RNA-binding proteins, transcriptional regulators, and proteins involved in trafficking, modification and degradation. To further analyze these genes, we wished to develop a faster way to study their loss-of-function (LOF) phenotypes than making individual P-element excision mutants. We also needed to be able to block gene expression in specific tissues at specific times, because many of the genes identified in the gain-of-function (GOF) screen are likely to have multiple roles during development. Accordingly, we selected a subset of the 35 genes for further analysis using transgenic RNAi methods to inhibit their expression. We picked genes that: 1) encoded interesting molecules; 2) produced strong GOF phenotypes; and 3) showed expression in the wild-type nervous system by *in situ* hybridization. We generated constructs containing UAS elements linked to invertedrepeat sequences of these genes and generated transgenic flies with them. Transgenic flies were crossed to different driver lines (Elav-GAL4 for neuronal expression, 24B-GAL4 for muscle expression, and OK107-GAL4 for mushroom body expression). We are currently examining embryos, larvae, and adults from these crosses to look for defects in axon guidance and synaptogenesis.

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Structural, Molecular and Cell Biology

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Summary: In the past year, the analysis of the role of human mitochondrial DNA (mtDNA) mutations in aging has made significant progress. We had previously shown that a C150T transition in the mtDNA main control region occurred in homoplasmic or near-homoplasmic form at a strikingly higher frequency in leukocyte mtDNA from centenarians (~17%) than in leukocyte mtDNA from younger subjects of the same Italian population (~3%). We had also obtained evidence that, in immortalized lymphocytes from centenarians, the C150T mutation was consistently associated with the disappearance of the normal origin of mtDNA heavy strand replication at position 151 and the appearance of a functional novel origin at position 149, and that somatic events contributed to the accumulation of the mutation in centenarians. To gather information about the role of inheritance in the occurrence of the C150T transition, leukocyte mtDNA from a large number of monozygotic and dizygotic twins 27 to 95 years old from the same Italian population was analyzed. Surprisingly, the homoplasmic or near-homoplasmic C150T mutation was found in high proportion of the monozygotic twins (30%) and of the dizygotic twins (22%). Eight of the ten pairs of monozygotic twins investigated had identical frequency of the mutation in the two members of each pair, strongly pointing to the inheritance of the mutation. The centenarians are a group of people who have survived the aging-related decline in critical functions and increasing risks of diseases, while the twins have survived the 5-fold to 10-fold higher rate of perinatal mortality and the increased post-natal morbidity characteristic of twin gestations as compared to singleton gestations. Therefore, it is a plausible hypothesis that the observed marked increase in frequency of the C150T transition in the centenarian and twin "survivors" results from a selection process favoring the individuals who have inherited it from their mother and those who have accumulated it during life.

The tissue specificity of the C150T mutation and its remodeling effect on the origin of mtDNA heavy-strand replication at position 151 have strongly pointed to the role of a specific protein(s). A search for such protein has led to identification of a 37 kDa protein which specifically binds to a sequence encompassing the C150T mutation. This protein is presently being investigated in detail.

In another area of our research program, concerning the nuclear control of mitochondrial organization, an important finding has been the identification of two isoforms of the mitochondrial fusion mediator mitofusin 2 (Mnf2), which are characterized by different electrophoretic mobility. Particularly significant has been the observation that the relative abundance in different human cell lines of the slower migrating isoform appears to correlate with a filamentous/network mitochondrial morphology. Another significant finding has been that the two Mnf2 isoforms do not differ in the reading fame of the encoding gene, an observation which strongly points to a secondary modification(s) underlying their electrophoretic mobility difference.

A strong interest in our laboratory continues to be centered around the crucial role played by mitochondria in the apoptotic process in mammalian cells. Thus, a considerable amount of experimental work is being carried out in two main lines of research in this area. The first line is directed towards elucidating the mechanism underlying the hypersensitivity of the outer mitochondrial membrane to digitonin that was previously shown in our laboratory to characterize an early phase of the mitochondrial response to two apoptotic stimuli, i.e., Fas receptor-ligand interaction and staurosporine. Particular attention is being given to the possible role of lipid peroxidation or of changes in cholesterol abundance and/or distribution. The second line of research aims at identifying the basis for the hypersensitivity to apoptosis exhibited by cells carrying a mutation in the ND4 subunit of the respiratory chain NADH dehydrogenase (Complex I), which affects the activity of this enzyme. The main hypothesis which is being tested here is that the inefficient reduction of ubiquinone due to the Complex I ND4 subunit mutation causes an increased superoxide production. Furthermore, the capacity of the *S. cerevisiae* single-subunit mitochondrial NADH dehydrogenase, transfected and overexpressed in the mutant cells, to compensate for the endogenous Complex I defect and to restore normal sensitivity to apoptosis is being investigated.

In a different line of research, the early observation in our laboratory of the existence of two overlapping transcription units operating on the mtDNA heavy strand, one specific for the mtDNA segment coding for the two mitochondrial rRNA species and the $tRNA^{Phe}$ and $tRNA^{Val}$, and the other one covering the whole heavy strand and transcribing the 12 protein-coding genes and the remaining tRNA genes encoded in this strand, has been unequivocally confirmed *in vivo*.

317. Investigation of proteins interacting with sites of human mtDNA main control region specifically targeted by aging-dependent accumulations of mutations

Jaehyoung Cho

The D-loop region of human mitochondrial DNA (mtDNA) harbors very critical control sequences such as replication origins and transcription promoters. Most interestingly, this control region is targeted by mutations which accumulate in aging-dependent manner (1). To investigate the molecular basis of this aging-dependent mutagenesis and of the associated selection mechanism(s), we have planned to identify possible protein-DNA interactions in this region. In the initial phase of the mtDNA replication process, a triple-stranded mtDNA displacement loop (D-loop) is formed. The stability of the single-stranded region of D-loop may have an important role in the regulation of the mtDNA copy number. Thus, we have searched for possible protein-DNA interactions of both single-stranded DNAs (heavy and light strands) of this region. In the case of DLP4, a mtDNA segment harboring major replication origins, only the light strand showed binding activity for several proteins in DNA mobility shift assay. We purified these proteins by single-stranded DNA affinity chromatography, and showed them to include species of 70, 55, 37, 28 and 15 kDa molecular weight. Among them, the 37 kDa protein exhibited specific binding to a secondary origin of heavy-strand replication (OH2). This OH2 region is the site with a very interesting aging-dependent mutation (C150T transition).

Previous work in this laboratory showed that, in leukocytes from subjects of an Italian population, a homoplasmic C150T transition occurs in approximately 17% of 52 subjects 99-106 years old, but, in contrast, in only 3.4% of 117 younger individuals. Interestingly, the 37 kDa protein shows a stronger affinity to the mutant mtDNA sequence than to wild-type sequence. Since 5'-end analysis of nascent heavy strands consistently revealed a new replication origin at position 149, substituting for that at 151, only in C150T mutation-carrying mtDNA samples from fibroblasts or immortalized lymphocytes (2), the 37 kDa protein may have some important role in the selection of a remodeled replication origin. In order to elucidate the molecular nature of the DLP4 light-strand DNA binding proteins, we plan to purify them by DNA affinity chromatography and sequence them by nanoelectron-spray tandem mass spectrophotometry with the final goal of cloning the related genes.

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318. Analysis of the functional effects of the aging-dependent T414G mutation in the human mitochondrial DNA main control region

Jennifer Fish

The tissue-specific, aging-dependent T to G transversion at position 414 in the mtDNA main control region from human fibroblasts has previously been described in work from our laboratory (1). This mutation lies within the promoter for the RNA primer of mtDNA H-strand synthesis. A unique feature of mitochondria is the premature abortion of a high proportion of nascent H-strands, which thus forms the displacement loop (D-loop). These aborted nascent H-strands constitute the so-called 7S DNA (2). The function of 7S DNA is still unknown. However, it has been shown that the distribution and relative amounts of 7S DNA molecules starting from various origins and of the "true" replicating molecules starting from the same origins are identical (3,4). Therefore, given the location of the 414 mutation, we hypothesized that it might affect the rate of 7S DNA synthesis, and possibly, the rate of total mtDNA replication.

In order to investigate the effects of the T414G mutation on mtDNA replication, we are using a primer extension technique which allows us to identify the nascent H-strands starting at the various origins within the D-loop and aborting at the 3' end of this loop or extending beyond it. This technique is being applied to transformant cell lines carrying mitochondria that contain mtDNA either 100% mutant or 100% wild type for the 414 mutation.

If it turns out that the 414 mutation provides a replicative advantage to mtDNA molecules that carry it, it would be important to establish if it is selective. That is, does the 414 mutation preferentially occur in and/or is selected for in mtDNA molecules that function better or worse than average? In order to make a general assessment of the function of mitochondria with and without the mutation, we plan to measure the respiratory capacity and the efficiency of oxidative phosphorylation of several different pairs of transformed cells sharing the same nuclear background, which carry 100% or 0% of mutant mtDNA.

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319. Two mitofusin Mfn2 isoforms differentially expressed in various human cell lines and tissues correlate with distinct mitochondrial organization

Petr Hájek, Ansgar Santel[1], Margaret Fuller[1], Giuseppe Attardi

Mitochondria are dynamic structures that can rapidly fuse or divide. In man, mitochondrial fusion is mediated by mitofusins Mfn1 and Mfn2. In the present work, a relationship between the mitochondrial organization, as determined by staining with tetramethylrhodamine methyl ester, and the endogenous level of the fusion mediator Mfn2 protein was established in various human cell lines. The highest level of Mfn2 protein was found in HeLa CCL2 cells, that have long-filament mitochondrial morphology, medium levels, in A549 and HepG2 cells, that have punctate to short-filament mitochondrial morphology, and the lowest level, in 143B cells, that have mostly punctate mitochondrial morphology. Two protein species of different electrophoretic mobilities immunoreactive with anti-Mfn2 antibodies were observed on Western blots of mitochondrial lysates from HeLa CCL2, A549, HepG2 and HeLa S3 (suspension) cultures. A high proportion of the Mfn2 form with the slower electrophoretic mobility (s-Mfn2) was detected in mitochondrial lysates from HeLa CCL2 (~92%) and A549 (~88%) cells, whereas a low proportion of the s-Mfn2 was observed in mitochondrial lysates from HepG2 (~10%) and HeLa S3(~7%) cells. Only the faster migrating form of Mfn2 (f-Mfn2) was detected in the mitochondrial lysate from 143B cells. These findings revealed a tendency to an association of the filamentous mitochondrial morphology with the expression of the slower migrating s-Mfn2 form.

Proteins with electrophoretic mobilities corresponding to those of one or the other or both of the two forms of Mfn2 and immunoreactive with anti-Mfn2 antibodies were also detected in total cell extracts from human heart, liver, skeletal muscle, kidney and testis. These findings strengthened considerably the *in vivo* significance of these isoforms. In order to obtain the entire cDNA sequence from the Mfn2 mRNA(s), the RACE protocol was applied to synthesize and amplify 5'- and 3'-end-containing cDNA subfragments from poly(A)$^+$ RNA of 143B, HeLa CCL2 and HeLa S3 cells. For this purpose, divergent Mfn2-specific primers were used which were derived from the internal sequence encoding the antigenic peptide utilized to generate the anti-Mfn2 antibodies. Analysis of individual clones of the RACE products revealed sequences derived from the same ORF, indicating the presence in 143B, HeLa CCL2 and HeLa S3 cells of only one type of Mfn2 mRNA, destined presumably to be post-translationally modified. This conclusion was supported by the observation that the electrophoretic mobility of Mfn2 synthesized *in vitro* from the cDNA obtained from a human myeloid cell line was similar to that of f-Mfn2, and clearly different from the mobility of the slower migrating s-Mfn2 form. Appropriate experiments excluded that the different electrophoretic mobilities of the two Mfn2 isoforms can be attributed to differences in their content of disulfide bonds or to differential phosphorylation.

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320. New cellular model for a mitochondrial disease

Miguel Martín-Hernández

Previous work in our laboratory had addressed the question of the pathogenetic mechanisms of different mitochondrial DNA (mtDNA) mutations involved in mitochondrial diseases. Important advances were made, showing that mtDNA point mutations can cause several mitochondrial dysfunctions wich play a key role in the cellular pathological phenotype (1,2). However, further work needs to be done in order to improve the knowledge of the relationship between the mutant mtDNA content and the threshold for the pathological phenotype in these diseases, as well as the role of the nuclear background in these events.

For this purpose, a ρ^0 (mtDNA-less) cell line derived from the SH-SY5Y neuroblastoma cell line has been constructed to be used for mitochondria-mediated transformation in order to develop new cellular models of mtDNA-linked diseases affecting the brain. These neuroblastoma cells, as well as the ρ^0 derivative cell line, have the ability to differentiate, after an appropiate chemical stimulus, into neuronal cells, giving us a useful tool to mimic the conditions *in vivo*.

We have successfully constructed mitochondria-mediated ρ^0 cell transformants using as mitochondrial donors, cell lines carrying the mtDNA tRNA$^{Leu(UUR)}$ A3243G point mutation associated with the MELAS encephalomyopathy syndrome (constructed in our laboratory from patient donors), or cell lines carrying the wild-type mitochondrial tRNA$^{Leu(UUR)}$ gene (constructed in our laboratory from unaffected individuals). Both types of transformant cell lines are able to become differentiated *in vitro* after retinoic acid treatment.

With this new cellular model now available in our laboratory, we can characterize more reliably the pathogenetic mechanism of the MELAS mutation. In particular, we want to address the question of the relationship between mtDNA mutation content and phenotype threshold for the different mitochondrial disfunctions related to the MELAS syndrome, as well as the question of the role of the nuclear background in the manifestation of the biochemical phenotype, by taking advantage of the differentiated transformants as the model nearest to the *in vivo* situation.

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321. Definitive characterization of the two initiation sites of the human mitochondrial DNA heavy-strand transcription

Miguel Martín-Hernández[*], Jaehyoung Cho[*]

Early investigations in our laboratory were directed towards analyzing in detail human mitochondrial DNA (mtDNA) transcription. *In vivo* and *in vitro* DNA transcription experiments showed that the initiation sites for mtDNA transcription in mammalian cells are located in the gene-free region close to the origins of heavy (H)-strand synthesis. There is a single initiation site for the light (L)-strand transcription, from which a giant polycistronic transcript originates, which extends over the whole L-strand. As to the H-strand transcription, it takes place in two different events. One involves the synthesis of the two rRNAs and two tRNAs ($tRNA^{Phe}$ and $tRNA^{Val}$) in the form of a transcript wich terminates at the 16S rRNA/$tRNA^{Leu(UUR)}$ boundary, and is processed to the mature species. A second transcript is also extended almost over the entire length of the H-strand, and is destined to be processed, probably co-transcriptionally, to the mature species, resulting in the synthesis of all other H-strand-encoded tRNAs and mRNAs

Two initiation sites for H-strand transcription were previously identified in our laboratory. The upstream site, H1, controls the rDNA transcription, and the downstream site, located near the 5' end of the 12S RNA, H2, the synthesis of the whole H-strand polycistronic transcript (1,2,3). The aim of this project is provide a definitve characterization of the two initiation sites for the H-strand transcription, and the understanding of the regulation of the two transcription events

In recent experiments, we have used two different experimental aproaches for the fine 5'-end mapping of the H-strand primary transcripts: i) highly-specific labeling (by the GTP-guanylyltransferase capping reaction) of the *in vivo* primary transcripts, followed by S1 protection assays and ii) primer extension by reverse transcriptase of the *in vivo* synthesized H-strand transcripts. The two aproaches have identified at the nucleotide level these two initiation sites, H1 and H2, so that we have now unambigous tests of these two *in vivo* initiation events

A significant advance in our understanding of the mechanism and regulation of the human mtDNA transcription was the identification and characterization of the protein factor mTERF (mitochondrial termination factor), which is involved in termination of transcription of the mitochondrial rDNA. mTERF protects in DNase I footprinting assays the DNA sequence at the 5' end of the $tRNA^{Leu(UUR)}$ immediatelly adjacent and downstream to the 3' end of the *in vivo* 16S rRNA gene product (4).

Our goal, closely related to the previous aim, is to investigate the possible role of mTERF in the regulation of the two H-strand transcription initiation events. Very recent preliminary results have been obtained in our laboratory, which points to an interaction of the mTERF with the sequence containing the H-strand initiation site(s).

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322. Aging-related accumulation of tissue-specific point mutation in the human mtDNA control region for replication

Jin Zhang, Jordi Asin-Cayuela[1], Jennifer Fish

A decline of mitochondrial oxidative phosphorylation with aging in some mammalian tissues has been linked to the accumulation of somatic mtDNA rearrangements. These findings have led to the idea that progressive damage to mtDNA during life may contribute to aging processes. However, the involvement of point mutations in this mtDNA damage has remained very controversial. In the past several years, a considerable part of our research efforts has been aimed at determining the occurrence and the possible role of mtDNA point mutations in human aging. An important breakthrough has been provided by the discovery of a tissue-specific, aging-dependent large accumulation of point mutations in the human mtDNA main control region at critical sites for replication. The T414G transversion, within the promoter for the RNA primer of heavy (H)-strand mtDNA synthesis, was observed in a generally high proportion (up to 50%) of mtDNA molecules in skin fibroblast cultures from 8 of 14 normal individuals above 65 years of age, but was absent in fibroblast cultures from 13 younger individuals. The age distribution and the results of two longitudinal studies indicated clearly that the T414G mutation was not inherited. A search for possible aging-dependent point mutations in the mtDNA main control region of skeletal muscle revealed, surprisingly, the absence of the T414G mutation and the presence of two mutations that had not been observed in fibroblast mtDNA, in particular, a A189G transition, very close to the main origin of H-strand synthesis (position 191), and a T408A transversion, again within the H-strand synthesis RNA primer promoter. The A189G mutation was found in 11-64% of the mtDNA and the T408A transversion in 2-16% of the mtDNA in the muscle from the majority of 27 normal individuals 53 to 92 years old, while being absent or marginally present in the muscle from 19 individuals younger than 34 years. Both the fibroblast T414G mtDNA mutation and the muscle A189G and T408A mutations showed a striking tissue specificity, being absent in heart, liver, lymph nodes, leukocytes and spleen. In a more recent large-scale screening of the mtDNA control region in leukocytes from subjects of an Italian population, another breakthrough was provided by the discovery that a

homoplasmic C150T transition near an origin of H-strand synthesis occurs in ~16.4% of 55 subjects 99-106 years old, but, in contrast, in only 3.3% of 123 younger individuals (p = 0.0037). Evidence was obtained for the contribution of somatic events, under probable nuclear genetic control, to the striking selective accumulation of the mutation in centenarians. Support for a somatic contribution to the latter phenomenon also came from the observation in a different system, i.e., in five human fibroblast longitudinal studies, of an aging-related somatic expansion of the C150T mutation, up to homoplasmy.

In another study, among leukocyte mtDNA samples from 36 monozygotic and 18 dizygotic twins, 27 to 95 years old, 28% (p < 0.0001) and 22% (p = 0.011), respectively, of the individuals involved exhibited the homoplasmic C150T mutation. As to the possible functional relevance of the C150T mutation, another surprising finding was that this mutation was consistently associated, in fibroblasts and in immortalized lymphocytes, with the disappearance of the normal origin of replication at position 151 and the appearance of a functional novel origin at position 149. Considering the aging-related health risks that the centenarians have survived and the developmental risks of twin gestations, the association of the C150T mutation with centenarians and twins suggests that selection for a remodeled replication origin, inherited or somatically acquired, may provide a survival advantage. The accumulation of tissue-specific point mutations identified in fibroblasts and skeletal muscle of old subjects is conceivably also a part of an aging-related remodeling of the control of mtDNA replication. Further work is needed, however, to establish the functional relevance of this remodeling.

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323. Early changes in the mitochondrial outer membrane during apoptosis

Katia Altomare, Anne Chomyn

Mitochondria play a central role in apoptosis in mammalian cells. In fact several signalling molecules, that act as effectors, are released from mitochondria during the apoptotic process. Cytochrome *c* is one of these.

It has been shown that treatment of 143B.TK$^-$ cells with staurosporine, an apoptosis inducer, causes an early decrease in endogenous respiration rate. This decrease precedes the release of cytochrome *c* from mitochondria, and we have previously obtained evidence that it is caused by an alteration in the permeability of the outer membrane (1). This is caused, presumably, by closure of the voltage-dependent anion channel, which prevents the uptake of oxidizable substrates into mitochondria.

Digitonin permeabilization after staurosporine treatment of 143B.TK$^-$ causes a dramatic decrease in respiration rate due to disruption of the outer membrane. In fact cytochrome *c* addition completely restores respiration (1). Most significantly, Bcl-2 overexpression prevents the staurosporine-induced hypersensitization of the outer membrane (1).

We are investigating the basis of hypersensitivity to digitonin that occurs when cells are induced to undergo apoptosis. Our hypothesis is that reactive oxygen species produced in mitochondria during apoptosis induce lipid peroxidation. Membranes containing peroxidized lipids are more susceptible to disruption by detergents; therefore, peroxidation of lipids could explain the digitonin effect which we observe. Another possibility can be that as digitonin binds specifically to cholesterol, the abundance or distribution of cholesterol in the outer mitochondrial membrane undergoes some change during apoptosis.

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324. Increased sensitivity to apoptosis in complex I mutants

Catherine Lin, Anne Chomyn

We observed several years ago that human cell lines carrying a pathogenic mtDNA mutation in the ND4 gene underwent apoptosis induced by staurosporine much more rapidly than did a related cell line having the same nuclear background, but wild-type mtDNA (unpublished). The ND4 gene encodes an essential subunit of the 43-subunit complex I, the respiratory chain NADH dehydrogenase. The mutation mentioned above is the most common cause of Leber's heritary optic neuropathy (LHON), an inherited disease that manifests itself as a sudden loss of vision.

The mutation compromises complex I activity, but does not abolish it. Hofhaus and colleagues (1996) showed that complex I-dependent respiration is decreased by about 50% in cells carrying the mutation. However, when they measured NADH:ubiquinone oxidase activity

spectrophotometrically by following the conversion of NADH to NAD^+, they observed that the activity was normal. This apparent contradiction suggested the following interpretation: electrons transferred by the defective complex I from NADH did not always stably reduce ubiquinone, and therefore, did not always travel down the respiratory chain to oxygen. If this hypothesis were correct, then free radicals, in particular superoxide, would be expected to be generated from the inefficient reduction of ubiquinone.

We are testing the hypothesis that the increased sensitivity to apoptosis of cells carrying the ND4 LHON mutation is due to increased superoxide production in the cells. As part of our project we are overexpressing NDI1, the single-subunit yeast mitochondrial NADH dehydrogenase, in the mutant cells in order to determine whether the exogenous enzyme will restore in them normal sensitivity to apoptosis. NDI1 gene expression has been shown to restore respiratory activity in a human mutant cell line that was completely deficient in complex I activity (Bai *et al*., 2001).

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325. Point mutations in the human mitochondrial DNA D-loop region and aging

Nicola Raule

Point mutations in human mitochondrial DNA (mtDNA) can be responsible for severe biochemical alterations in the cell often resulting in major diseases (1).

A considerable amount of effort has been spent to study the effects of several point mutations in the coding regions of mtDNA. On the contrary, very little is known about the effects of point mutations in the main non-coding region (D-loop region), although, because of its regulatory function, one would expect that they could lead to major alterations. Recently, a paper from our laboratory has shown a strikingly increased frequency of a C150T mutation in the mtDNA D-loop region from leukocytes of centenarians and twins and from fibroblasts of aged individuals (2). Previous papers from our laboratory had identified a novel aging-dependent T414G transversion in the main mtDNA non-coding region of human fibroblasts (3) and two different aging-dependent mutations in human skeletal muscles, i.e., an A189G transition and a T408A transversion (4). These papers have raised the question of the effects of these mutations on mtDNA.

Because of their close proximity to origins of mtDNA heavy strand replication, we have hypothesized that the C150T and A189G mutations could interfere with the mechanism of replication. To address this question, we decided to construct cybrid cell lines derived from the fusion of ρ^0 (mtDNA-less cells) and different cells carrying the specific mutation of interest. The ρ^0 206 cell line is a derivative of a $143B.TK^-$ cell line, which has been completely depleted of mtDNA (5). The best aspect of this technique is that it eliminates most of the interference of different nuclear backgrounds, since all the transformants have almost the same ρ^0 206 nuclear background.

The attempts to obtain the fusion between $\rho^0$206 cells and fibroblasts carrying the C150T mutation have been successful. Further analysis is starting to show some interesting differences between cells carrying the mutation and those carrying wild-type mtDNA.

As to the muscle mutations, we utilized the standard fusion protocol starting from a fibroblast sample carrying the A189G mutation. This attempt gave a positive result. To obtain some transformants carrying the T408A muscle mtDNA mutation, we plan to utilize muscle precursor cells (satellite cells) that can be isolated from the muscle of aged subject. These cells can be grown attached to a plate, thus allowing the standard fusion protocol.

The analysis of the transformants will aim at investigating any possible effects of the mutation on mtDNA replication, as well as at testing their respiration capacity and several biochemical parameters related to the efficiency of oxidative phosphorylation.

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Summary: Our laboratory currently explores multiple areas: the control of NF-κB activation, the role of NF-κB plays in neurons, the function of the *abl* gene, use of interfering RNAs to inhibit HIV growth, reprogramming of the specificity of T lymphocytes, and other questions.

The largest program in the laboratory involves study of the NF-κB transcription factor. Our major tool is a collection of mice in which we have knocked out one or more of the genes encoding proteins that are part of the NF-κB complex or control its activity. Using these mice and cells derived from them, we are studying the role of the individual proteins in controlling specific genes in various cell types. A major program involves using fibroblasts of defined genotype. We are also studying macrophage functions. At the level of transcriptional regulation, questions we are investigating include whether the various NF-κB-related proteins have different functions and whether the sequences of the individual sites that bind NF-κB have regulatory relevance

NF-κB is found in high concentrations in neurons and, being present at synapses, is ideally positioned to serve a role as a mediator of synaptic activity. We are examining this possibility using the knockouts and direct study of neuronal and synaptosomal preparations. We are also examining knockouts for behavioral defects.

There are many pathways for activation of NF-κB. We used a novel screen to find new components of the signaling pathways and discovered that CARD 11 is such a protein. We have connected it to the T cell receptor-mediated signaling pathway using interfering RNA as a probe. It contains CARD, coiled-coil, PDZ, SH3, and GUK domains. We are also using cellular genetic approaches to find new signaling components.

NF-κB is important in oncogenesis and we are investigating the roles of particular forms of NF-κB as well as the relationship of oncogenesis to senescence.

We are also trying to understand why different genes bind NF-κB using binding sites of differing sequence. We found that in mouse and humans, there is conservation of the particular sites, indicating that the individual nucleotides have important roles in the binding process. We have developed a system for studying this question in the context of endogenous gene sequences using retrovirus-mediated transfer of large segments of DNA. With this system, we can demonstrate the specificity of different sites and are now examining the consequences of altering the sequences.

We have used RNAi and lentivirus-mediated transgenesis in many studies in the laboratory. We combined these techniques to show that we could implant an RNAi directed against the receptor for HIV in T cells and protect the cells against HIV infection. We are now trying to increase the efficacy of the process with an eye to developing the system for therapeutic purposes.

This laboratory has had a long-standing interest in the role of the *abl* gene in oncogenesis and in normal cellular physiology. We have shown that the closely related *arg* gene overlaps in its functions with *abl*, which had confused understanding of *abl*. The double knockout is an embryonic lethal. *C. elegans* has only one *abl* gene, facilitating genetic studies. Our present results suggest that *abl* acts through an interaction with EPH receptors and we are investigating that possibility in detail in mammalian cells. We have also started investigating the role of the Wnt pathway in the nervous system.

The immune system can evolve in an individual a wide range of specificities but there are times when it would be advantageous to impart a particular specificity to a fraction of a person's immune cell repertoire. We have used retrovirus-mediated transgenesis of bone marrow stem cells in mice to try to create this situation. We have been successful with both CD4 and CD8 cells, creating mice that have up to 20% of their T cells carrying one specificity. We are investigating the value of this system in fighting cancer using mouse tumors with a defined antigenic marker.

A further interest of our laboratory is the role of a group of large protein kinases in intracellular signaling processes. One is ATR—we have found that a knockout of the gene encoding this protein leads to very early embryonic lethality. We are investigating ATR in more detail through a conditional allele.

In another program, we are trying to understand how the AID protein is regulated. This is a critical component of the somatic mutation and class switching properties of lymphocytes.

326. Analysis of TNF and Fas receptor signaling to activation of gene expression

Mark Boldin, David Baltimore

Members of the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor superfamily play a crucial role in activation, proliferation, survival and death of cells in the immune system. The two prototype death receptors of the family are type I TNF receptor (TNFR1) and Fas/APO-1. The biological functions triggered by these two receptors are characterized by a remarkable duality – infliction of tissue damage and cell death goes hand in hand with activation of tissue repair and expansion. The physiological reason for this duality

lies in the ability of these receptors to trigger two kinds of intracellular signaling programs: a proteolytic cell death cascade and a number of kinase cascades leading to activation of gene expression, some of which may protect cells against cytotoxicity. The major transcription factors activated in response to TNF and Fas ligand are NF-κB and AP-1 proteins.

Despite significant recent progress in understanding the molecular mechanisms of TNFR1 and Fas receptor signaling towards activation of gene expression, there is still a great deal of confusion and argument with regard to the exact molecular scheme of events. The main cause for much of the disagreement in the field is the use of dominant-negative mutants and overexpression studies in order to elucidate the role of specific players in the pathway. Our rationale is that such approaches should be complemented by genetic studies. Genetic approaches often provide more clear-cut answers to the question of which molecules play an essential role in a given signal transduction pathway. Therefore, in order to identify molecules that play a crucial role in relaying the signals from the TNFR1 and Fas receptors towards NF-κB and AP-1 activation, we are performing genetic screens similar to that developed by George Stark and his colleagues for the analysis of interferon-receptor signaling (1). The method is based on creation and subsequent genetic complementation of mutant mammalian cell lines that are defective in cytokine-induced activation of a signal transduction pathway leading to transcriptional activation.

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327. Essential and dispensable roles of ATR in cell cycle arrest and genome maintenance

Eric J. Brown, David Baltimore

A cre/lox conditional mouse line was generated to evaluate the role of ATR in checkpoint responses to ionizing radiation (IR) and stalled DNA replication. We demonstrate that after IR treatment, ATR and ATM each contribute to early delay in M phase entry but that ATR regulates a majority of the late phase (2-9 hours post-IR). Double-deletion of *ATR* and *ATM* eliminates nearly all IR-induced delay, indicating that ATR and ATM cooperate in the IR-induced G2/M phase checkpoint. In contrast to the IR-induced checkpoint, checkpoint delay in response to stalled DNA replication is intact in *ATR* knockout cells and *ATR/ATM* and *ATR/p53* double knockout cells. The DNA replication checkpoint remains intact in *ATR* knockout cells even though the checkpoint-stimulated inhibitory phosphorylation of Cdc2 on T14/Y15 and activating phosphorylation of the Chk1 kinase no longer occur. Thus, incomplete DNA replication in mammalian cells can prevent M phase entry independently of ATR and inhibitory phosphorylation of Cdc2. When DNA replication inhibitors are removed, ATR knockout cells proceed to mitosis but do so with chromosome breaks, indicating that ATR provides a key genome maintenance function in S phase.

328. AID as an oncogene

Rafael Casellas

During an immune response, naïve B cells often generate germinal centers in secondary lymphoid organs. In this specialized environment, B lymphocytes proliferate extensively and their antibody molecules undergo two dramatic genetic changes: their affinity for the antigen is increased by somatic hypermutation and their constant region (Cμ) gene is replaced for downstream isotypes (Cγ, Cε, Cα) by class switch recombination.

The molecular mechanisms that generate switching and hypermutation are mostly unknown however, both reactions are abrogated in mice and humans deficient in AID, a B-cell restricted enzyme capable of catalyzing deamination of C to U on single-stranded DNA. This genetic alteration is believed to result in DNA double-stranded breaks that not only give rise to hypermutation and recombination, but also may lead to chromosomal translocations and B cell malignancy. This hypothesis is based on the observation that transgenic animals expressing AID all rapidly succumb to tumors showing translocations. In addition, most human lymphomas express AID and carry translocations to the heavy- or light-chain loci, suggesting that abnormal recombination might juxtapose oncogenes to the immunoglobulin loci. Furthermore, enforced AID expression in human fibroblasts leads to overall hypermutation and genomic instability. Therefore AID deregulation *in vivo* may lead to lymphomagenesis in humans.

To fully understand how AID is regulated under physiological conditions and in tumors, we are currently characterizing the AID promoter and transcriptional regulatory sequences by means of comparative genomics, chromatin immunoprecipitation assays, and transgenic animals expressing AID-GFP bacterial artificial chromosomes.

329. Developing mobile group II introns for mammalian gene targeting

Huatao Guo, David Baltimore

Mobile group II introns can be designed for efficient gene targeting in bacteria. These introns are not only ribozymes, but also transposable elements. Intron transposition is mediated by the intron-encoded DNA endonuclease, which is a ribonucleoprotein (RNP) particle, containing both the intron RNA and the intron-encoded protein. The DNA target site for intron transposition is long (~31 nucleotides), therefore highly specific. The DNA target site is primarily recognized by the intron RNA through base pairing, allowing the intron to be flexibly retargeted to virtually any genes predictably.

To develop the LI.LtrB intron for efficient mammalian gene targeting, we decided to directly express the LI.LtrB RNP particles in mammalian cells. We have now achieved efficient expression of the intron-encoded protein in mammalian cells. Intron RNA can also be

generated in mammalian cells. In addition, we have observed protein-dependent intron RNA splicing *in vivo*. Preliminary results also suggest that RNP particles generated in mammalian cells can mediate intron insertion into plasmid DNA target sites. We are now determining if the LI.LtrB intron-encoded DNA endonuclease expressed in mammalian cells is able to cleave chromosomal DNA target sites. If mobile group II introns can be developed to integrate into chromosomal DNA target sites efficiently, they could have broad applications in gene targeting and gene therapy.

330. Signal transduction in the nervous system

Wange Lu, David Baltimore

Intercellular communication plays essential roles in the development and function of the nervous system. One of our major interests is the function and mechanism of signal transduction of Abl/Arg family protein kinase. In *Drosophila, abl* acts within developing neurons to regulate fasciculation and axonal outgrowth. In mammals abl gene family consists of Abl and Arg. $Arg^{-/-}$ mice physically develop normally, but exhibit many subtle behavioral abnormalities. *Abl* and Arg double knockout mice are embryonic lethal due to a severe neurulation defect. The signaling mechanism of Abl/Arg remains unclear in either system.

To identify the potential signal transduction pathway, we have knocked out the *Abl* gene in *C. elegans*. RNAi-mediated inhibition of Abl synthesis leads to defects similar to Vab-1 and Vab-2, whose mammalian homologues are Eph receptor tyrosine kinases and their ligands respectively. We then moved to mammalian cells to study a possible Eph/Abl/Arg connection because of the availability of various Abl/Arg-deficient cell lines.

Eph receptor tyrosine kinases belong to the biggest family of receptor tyrosine kinases. They play essential roles in neuron migration, axon guidance and synaptic plasticity. Arg and Eph were found to interact with each other at the overexpression level in HEK cells. Deletion analysis showed that the C-terminus of Arg, which is unique for Arg, is required for binding Eph. This is consistent with the fact that Arg binds to Eph strongly. Similar study has shown that the SAM domain in Eph plays essential roles in binding Arg.

Comparison of Eph activity in wild type and Abl/Arg double knockout cell lines further demonstrate that Abl/Arg are required for Eph activation. Furthermore, Arg alone can rescue the Eph activity in the Abl/Arg double knockout cell lines, being consistent with that Arg interacts and phosphorylates Eph specifically. In primary hippocampal/cortical neurons, Eph activity is significantly reduced in the Arg knockout mice, comparing with that in the wild-type neurons. Since Eph receptors have been shown to interact with NMDA receptor and involved in both excitatory synapse and spine formation. We have looked at the roles of Arg in these areas, especially with the knowledge that Arg knockout mice have some subtle behavior defects. NMDA receptor phosphorylation in both wild type and knockout mice are at the same level. We are currently looking at both synapse and spine formation in Arg knockout and wild-type mice.

Another focus of our interest is to study the function of the Wnt pathway in the nervous system. We have utilized transgenic RNAi technology to knockdown the expression of components in the Wnt pathway. The phenotypes of these transgenic mice are being investigated.

331. Function of neuronal NF-κB

Mollie K. Meffert, David Baltimore

Long-lasting alterations of neuronal properties, such as those involved in plasticity and apoptosis, depend upon changes in gene expression. We have explored the neuronal function of a potent transcription factor, Nuclear Factor kappa B (NF-κB), which is critical to host defense and is found in almost all cells. NF-κB is the prototype of a family of dimeric transcription factors made from monomers containing Rel regions that bind DNA, interact with each other, and also bind the IκB inhibitors. We find that basal synaptic input in mature mouse hippocampal neurons activates the NF-κB transcription factor and induces κB DNA-binding activity consisting of p50:p65 and p50:p50 dimers (measured by EMSA). Experiments using synaptosomes (isolated nerve terminals) indicate that both p50 and p65, as well as IκBα and IκBβ, are found at synapses. While both p50:50 and p50:p65 dimers occur in intact neurons, synapses contain only p65:p50. We have confirmed this interesting finding using a p65 knockout mouse made in our laboratory. While intact neurons from this p65 null mouse contain p50:p50 dimers, no NF-κB or IκB can be detected at synapses.

Using intracellular Ca^{2+} buffers, we have found that neuronal NF-κB activation and the induction of κB-dependent transcription requires only local submembranous Ca^{2+}-elevation and that this Ca^{2+}-dependent pathway involves the Ca^{2+}/calmodulin-dependent kinase, CaMKII. Inducible NF-κB resides in the cytoplasm complexed to IκB; cytoplasmic storage enables it to receive upstream signals more directly than transcription factors localized solely to the nucleus and also could allow it to transport signals from distant synaptic stimuli to the nucleus. We constructed a GFP-p65 fusion gene to explore NF-κB localization and translocation in neurons. Our GFP-p65 fusion protein localizes to both the cell body and processes of neurons and undergoes nuclear translocation following stimulation by glutamate or depolarization. The intracellular localization of NF-κB and its sensitivity to submembranous Ca^{2+} elevation make it ideally situated to provide an additional level of synapse-to-nucleus transcriptional control. The ability of NF-κB to regulate transcription in response to locally-restricted Ca^{2+} fluxes and CaMKII activation could permit it to sum multiple, even subthreshold, synaptic inputs and might form the basis of a unique activity-dependent function in neurons. Further experiments will be performed to explore this speculation.

Ca^{2+}-regulated gene transcription is essential to diverse physiological processes including the adaptive plasticity associated with learning. We have utilized $TNFR^{-/-}$, $p65^{-/-}$ mice to address the possible functions of NF-κB in adaptive learning. Mice lacking p65 exhibited a selective learning deficit in a version of the radial arm maze requiring the use of spatial information, while learning in a cued-version of the maze did not differ from p65-wild-type sibling controls. These observations suggest that long-term changes to adult neuronal function caused by synaptic stimulation can be regulated by NF-κB nuclear translocation and gene activation.

332. CARD11 mediates factor-specific activation of NF-κB by the T-cell receptor complex

Joel L. Pomerantz, Elissa M. Denny, David Baltimore

NF-κB is a transcription factor that functions as a pleiotropic regulator of genes involved in inflammation, the development and function of the immune system, and in antiapoptotic responses. NF-κB is commonly composed of two subunits, p50 and p65, which are held inactive in the cytoplasm by the binding of an inhibitor molecule, IκB. A variety of extracellular signals activate NF-κB by inducing the targeted destruction of IκB, thereby liberating NF-κB to translocate to the nucleus, bind to specific target sites, and activate a program of gene expression. Signals induce IκB degradation by activating the catalytic activity of a kinase complex (IKK complex) which phosphorylates IκB, targeting IκB for ubiquitination and destruction by the 26S proteasome.

Mice deficient in NF-κB subunits, or in molecules that signal to NF-κB, have revealed that the proper regulation of NF-κB activity is critical for normal innate and adaptive immune responses. In the adaptive immune response, NF-κB is a critical target of antigen receptor signaling in B and T cells. In T cells, the activation of NF-κB by T-cell receptor (TCR) triggering, in concert with costimulatory signals (CD28), is required for T-cell activation and proliferation. In addition, the properties of mice transgenic for nondegradable forms of IκB have suggested a role for NF-κB in TCR-mediated thymocyte selection and in pre-TCR survival signals.

The mechanisms by which TCR signaling activates the IKK complex are not well understood. Recently we have developed an expression cloning strategy for the isolation of components of signaling pathways that activate NF-κB. In our strategy, a cDNA expression library is assayed in pools for the ability to activate an NF-κB-responsive reporter when cotransfected into tissue culture cells. Positive pools are tested for specificity using a reporter containing mutated NF-κB binding sites. In addition, positive pools are tested in the presence of a kinase-dead IKKβ subunit to confirm that they activate NF-κB through the activation of the IKK complex. Clones responsible for a particular pool's activity are purified by sib selection.

Using this strategy, we screened a mouse thymus expression library and cloned CARD11, a signaling adaptor molecule containing CARD, coiled-coil, PDZ, SH3, and GUK domains. Using dominant-negative and RNA-interference approaches, we demonstrated that CARD11 mediates the activation of NF-κB by TCR signaling. Importantly, we showed that the role of CARD11 is pathway-specific and factor-specific. CARD11 did not mediate NF-κB activation by TNFα or dsRNA, and CARD11 did not mediate the activation of the NFAT and AP-1 factors by TCR signaling. CARD11 functions upstream of the IKK complex and functionally cooperates with Bcl10, a signaling molecule overexpressed in mucosa-associated lymphoid tissue (MALT) lymphomas. Currently we are investigating how CARD11 functions mechanistically to transmit signals between the TCR and IKK complexes.

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333. Lentivirus-mediated gene silencing by RNA interference in mammalian cells

Xiao-Feng Qin, Vincent Auyeung, David Baltimore

RNA interference (RNAi) is an evolutionarily conserved mechanism of posttranscriptional gene silencing through target specific degradation of messenger RNAs. In mammalian cells, RNAi can be selectively triggered by 21 to 25 nt short RNA duplexes (small interfering RNA, or siRNA) without activation of the IFN response pathway. To harness its potential as a general tool for gene knockdown, we have recently developed a lentivirus-based system to deliver hairpin form of siRNAs expressed from a pol III promoter cassette. In collaboration with Irvin Chen's lab at UCLA, we first explored the efficacy of this siRNA delivery system as a therapeutic means of intracellular immunization for the treatment of HIV/AIDS disease. We designed a number of siRNA constructs directed to CCR5, the key co-receptor for primary HIV-1 infection in humans. With high-titer siRNA vector stocks, greater than 40% of human peripheral blood T lymphocytes (PBLs) were transduced. In these productively transduced PBLs, the expression of a potent CCR5-siRNA resulted in up to 10-fold stable inhibition of CCR5 expression on the cell surface, whereas levels of the closely related co-receptor CXCR4 was not affected. Virus challenge experiments showed that knockdown of CCR5 expression by the anti-CCR siRNA vector provided a substantial protection for the lymphocytes from CCR5-tropic HIV-1 infection, dropping infected cells by 3- to 7-fold. Future studies will aim to incorporate multiple siRNA units targeting to both the host gene (CCR5) and the viral factors (LTRs, gag/pol, ect) into a single delivery vector to achieve optimal antiviral protection.

In a second line of research, we investigated the feasibility in generating knockdown transgenic animals

using the lenti siRNA vector in combination of a single-cell embryo injection technology developed earlier in the laboratory. With anti-lacZ reporter gene as our model, we found the lentivector encoded siRNA transgene was successfully expressed in various tissues and cell types (including the liver, kidney and brain) resulting in potent inhibition (up to 50 fold) of the target gene at both mRNA and protein levels. More importantly, this level of gene silencing can be achieved by a single copy of the siRNA transgene, and the knockdown effect can be stably transmitted to offspring in the Mendelian fashion. Taken together, our studies have demonstrated the broad utility of the lentiviral siRNA vector for both functional genomics and therapeutic applications.

Publication

Qin, X.-F. An, D.S., Chen, I. and Baltimore, D. (2003) Inhibiting HIV-1 infection in human peripheral blood leukocytes by lentiviral vector mediated delivery of siRNA against CCR5. *Proc. Natl. Acad. Sci. USA* 100:183-188.

334. The role of NF-κB in cell growth and transformation

Jeff Wiezorek

The NF-κB transcription factor is composed of protein homo- and heterodimers of five gene products (p65, p50, p52, c-Rel, and RelB). Diverse stimuli act through the IκB kinases (IKKs) to promote the degradation of IκB and allow NF-κB translocation to the nucleus. NF-κB activation has been implicated in several biological processes including inflammation, immunoregulation, apoptosis, and cell proliferation. Although the transforming ability of the v-REL oncoprotein was established many years ago, recent evidence suggests other human NF-κB family members may be important in oncogenesis. NF-κB DNA-binding activity is constitutively increased in many lymphoid and epithelial tumors. The RAS, BCR-ABL, and HER2 oncogenes and transforming viruses can activate NF-κB. Furthermore, several genes thought to be essential to the cancer phenotype- those controlling angiogenesis, invasion, proliferation, and metastasis-contain κB-binding sites. However, direct genetic evidence demonstrating the role of NF-κB in transformation and cancer progression is lacking.

Our lab has generated strains of knockout mice in which one or more of the NF-κB family members are deleted. 3T3 and primary fibroblasts of a specific genotype generated from these mice are powerful tools for dissecting NF-κB signaling pathways. These cells and mice are being used to study different aspects of cellular senescence, transformation, and cancer progression.

The direct role of the NF-κB proteins in transformation and oncogene-induced senescence is being investigated in primary mouse embryonic fibroblasts and immortalized fibroblasts. *In vitro* assays have identified differing requirements for NF-κB family members in transformation. RelA and c-Rel are most important, whereas p50 and p52 may inhibit transformation. As this signaling pathway may be important in some breast cancers, the contribution of NF-κB to the cancer phenotype is being evaluated *in vivo*. I have identified RelB as necessary for alveolar proliferation during pregnancy in a knockout model. This signaling pathway and its role in mammary transformation is under investigation. Additionally, by crossing the MMTV-cNeu transgene into NF-κB knockout strains, the contribution of constitutive NF-κB activity to tumor initiation, hypoxic survival, invasion/metastasis, and angiogenesis is being directly studied.

335. Imparting desired specificities to T cell repertoire

Lili Yang, David Baltimore

Previously, we have developed an alternative to transgenesis for producing antigen-specific T cells *in vivo*. In this system, clonal naïve T cells with defined antigen specificity are generated by retrovirus-mediated expression of TCR cDNAs in RAG1-deficient murine hematopoietic precursor cells. These T cells can be stimulated to proliferate and produce cytokines by exposure to antigen *in vitro* and they become activated and expand *in vivo* following immunization. IL-2-deficient T cells generated by this technique show decreased proliferation and cytokine production, both of which can be rescued by exogenous addition of this growth factor. Thus, retrovirus-mediated expression of TCR cDNAs in hematopoietic precursor cells permits the rapid and efficient analysis of the life history of antigen-specific T cells in different genetic backgrounds, and may allow for the long-term production of antigen-specific T cells with different functional properties for prophylactic and therapeutic purposes.

We further explored the therapeutic potential for the system. As a primary requirement we need to prove that we could apply the system to generate antigen-specific T cells from wt hematopoietic stem cells. In this case the endogenous TCR rearrangement is a potential hurdle. Our results show that retrovirus-mediated expression of antigen-specific TCR in wt hematopoietic stem cells allows the generation of clonal naïve T cells with defined antigen-specificity, which maintain a stable pool size in the periphery. These T cells show normal response to antigen stimulation *in vitro*. And upon immunization, the T cells show clonal expansion *in vivo*. Furthermore, we proved that both CD4+ helper T cells and CD8+ cytotoxic T cells could be generated using this system. Thus, we have established a system that can impart desired specificities to the T cell repertoire. This system has potential to provide therapeutic opportunities for curing cancer, AIDS and other infectious diseases.

336. Specificity of transcriptional activation in the NF-κB/Rel protein family

Thomas Leung, David Baltimore

The study of mammalian gene transcription is often complicated by the fact that multiple members of a transcription factor family recognize the same regulatory sequence. While several models have been proposed, little is known about how genes recruit specific members of transcription factor families for activation. The Nuclear Factor kappa B (NF-κB)/Rel transcription factor family is an evolutionarily conserved gene regulation system involved in the coordination of an organism's response to infection, stress, and injury. Many different stimuli activate NF-κB, but the transcriptional response to each stimulus is unique. Within the NF-κB/Rel protein family, four members are involved in gene activation: p50, p52, p65, and cRel. They homo- or heterodimerize with one another to bind DNA.

Our lab has developed a genetic system to elucidate whether specific NF-κB/Rel family members are required for gene activation in a physiological manner. Single and multiple knockouts for each member of the NF-κB/Rel family have been created, and 3T3 fibroblast cell lines have been derived. Our recent genetic analysis with this comprehensive panel of knockout cell lines shows that NF-κB-dependent genes have specific NF-κB/Rel family member requirements for activation by TNFα. This finding suggests that the variety of NF-κB/Rel dimer isoforms allows for stimulus-specific gene expression programs.

How is the requirement for specific NF-κB/Rel family members generated? We are investigating that issue by a novel methodology using viral-mediated implantation of genes to allow ready access to regulatory sequences. Combining this technology with knockout cells and chromatin immunoprecipitation, we can identify what promoter element requirements are necessary for NF-κB-dependent gene activation and begin to elucidate potential mechanisms generating transcriptional specificity. As a first step to understanding the promoter code, we are addressing how this requirement is established by studying the sequence of the κB site in the DNA.

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Summary: My laboratory is interested in the structure and function of molecules involved in cell surface recognition, particularly those mediating recognition in the immune system (Figure 1). We use a combined approach of x-ray crystallography to determine structures, molecular biological techniques to produce proteins for crystallization and to modify them, and biochemistry to study the properties of the proteins we make. Much of our work has focused upon homologs of class I MHC proteins, which function in many ways that are distinct from their immunological role in peptide presentation to T cells. MHC homologs being investigated in our laboratory include the neonatal Fc receptor (FcRn), which transports immunoglobulin G across epithelial cells; HFE, which helps to regulate iron metabolism; Zn-α_2-glycoprotein (ZAG), which is involved in regulation of lipid catabolism, and viral MHC homologs, which help viruses evade the host immune system.

Transfer of maternal IgG molecules to the fetus or infant is a mechanism by which mammalian neonates acquire humoral immunity to antigens encountered by the mother. The protein responsible for the transfer of IgG is the MHC class I-related receptor FcRn. MHC class I molecules have no reported function as immunoglobulin receptors; instead they bind and present short peptides to T cells as part of immune surveillance to detect intracellular pathogens. We solved the crystal structures of rat FcRn both alone and complexed with Fc. Our crystallographic and biochemical studies suggest that FcRn dimerizes in response to IgG binding, which may serve as a component of a signal to initiate internalization of FcRn/IgG complexes. We also hypothesize that formation of an oligomeric ribbon of FcRn dimers on adjacent membranes bridged by IgG molecules (Figure 2) is required for FcRn function. We are now interested in understanding the roles of the FcRn dimer and the oligomeric ribbon in IgG transport. We are in the process of characterizing oligomeric ribbon formation *in vitro* using biophysical assays, and have extended these studies to real-time confocal imaging of cells undergoing FcRn-mediated transcytosis of IgG. We are also doing structure/function studies of two other Fc receptors that are not MHC homologs: gE/gI, a viral Fc receptor for IgG, and FcαR, a host receptor for IgA.

HFE is a recently discovered class I MHC homolog that is involved in the regulation of iron metabolism, an unexpected function for an MHC-related protein. HFE was discovered when its gene was found to be mutated in patients with the iron overload disease hereditary hemochromatosis. HFE has been linked to iron metabolism with the demonstration that it binds to transferrin receptor, the receptor by which cells acquire iron-loaded transferrin. We solved crystal structures of HFE alone and HFE bound to transferrin receptor. The interaction of HFE with transferrin receptor is a fascinating system to study because we can use crystal structures to answer biochemical, functional, and evolutionary questions that address how binding of HFE interferes with transferrin binding, if conformational changes in the receptor are involved in the binding of either transferrin or HFE, which part of the MHC-like HFE structure binds transferrin receptor, and how the HFE interaction with the receptor compares with interactions of ligands with MHC and MHC-like (e.g., FcRn) proteins. We are expanding our studies to include cell biological investigations of HFE and transferrin receptor intracellular trafficking in transfected cell lines using confocal microscopy and other imaging techniques.

We also study Zn-α_2-glycoprotein (ZAG), a soluble MHC class I homolog present in low concentrations in most bodily fluids. ZAG was isolated from blood more than 30 years ago, but it's been a molecule in search of a function for a long time. Recently researchers at Aston University in the U.K. discovered that ZAG is involved in cachexia, a wasting syndrome that can affect people with terminal illnesses. ZAG is responsible for the fat-depletion component of cachexia, since it stimulates lipid breakdown in adipocytes and reduces fat stores in laboratory animals. We have purified ZAG from serum and completed its crystal structure, which revealed an as yet unidentified non-peptide compound in the ZAG counterpart of the MHC peptide binding site. We are using a combination of structural studies, computational analysis, and ligand binding experiments to analyze the

function and potential roles of ZAG in lipid catabolism under normal and pathological conditions.

We are also interested in other MHC homologs, including proteins encoded by viruses. Both human and murine cytomegalovirus (HCMV, MCMV) express relatives of MHC class I heavy chains, probably as part of the viral defense mechanism against the mammalian immune system. Our biochemical studies show that the HCMV homolog associates with endogenous peptides resembling those that bind to class I MHC molecules. Our current efforts focus upon defining the structure and function of these homologs in order to understand why viruses make them and how they interfere with the host immune system.

Our structural work on class I MHC homologs has elucidated new and unexpected recognition properties of the MHC fold. For FcRn and HFE, the structural and biochemical studies have revealed a similar fold and some common properties, including the assumption that both receptors "lie down" parallel to the membrane when binding ligand, and a sharp pH-dependent affinity transition near neutral pH. In the case of FcRn, we have elucidated the structural basis of its pH-dependent interaction with IgG and will now focus upon cell biological studies of intracellular trafficking, for which the pH dependent interaction is critical. The pH-dependence of the HFE-TfR interaction suggested to us that intracellular trafficking studies of HFE would be interesting, so much of our future efforts on both the FcRn and HFE systems will center around probing their function in a cellular context using imaging techniques. Our functional studies of ZAG are at an earlier stage, since a receptor for ZAG has not been identified and the mechanism by which ZAG promotes lipid degradation is unknown. We are at an even earlier stage in our studies of the viral MHC homologs, in which our primary goal will be to solve crystal structures of a viral homolog alone and complexed with its cellular receptor.

Figure 1

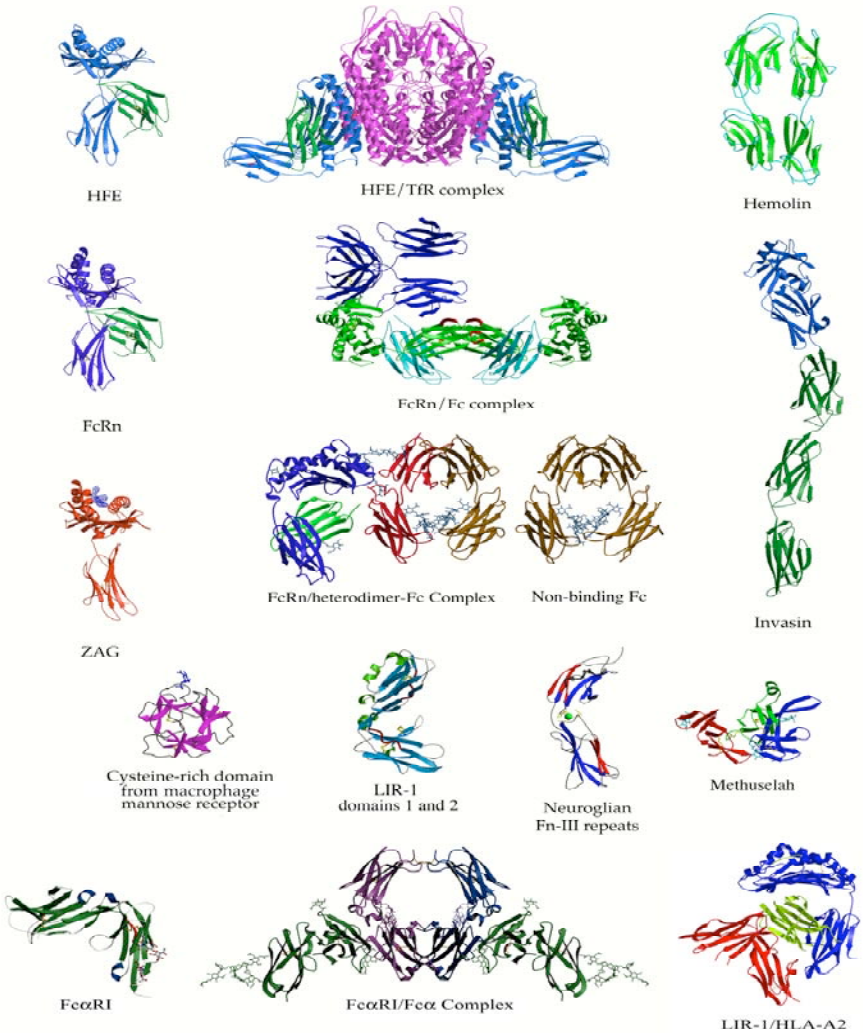
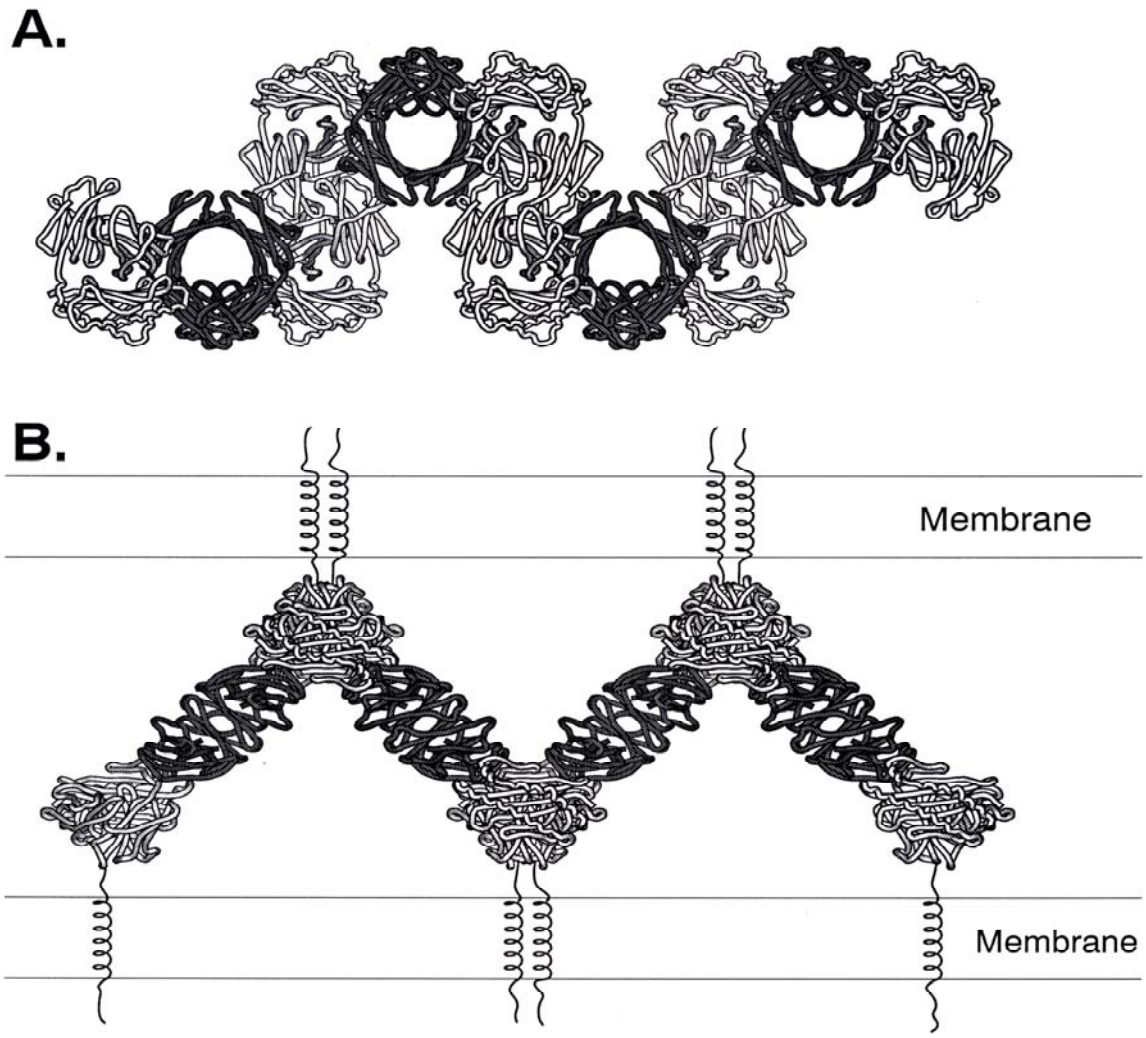


Figure 2



337. Characterization of FcRn-mediated transport pathways via confocal microscopy

Devin B. Tesar, Noreen Tiangco

Movement of specific protein molecules across epithelial cell barriers by their cognate receptors is achieved via a multivesicular transport pathway known as transcytosis. Discrete steps in the procession of a receptor-bound ligand through the transcytotic network are characterized by association with distinct subpopulations of endosomal compartments. These subpopulations of endosomes can be identified by confocal microscopy using fluorescent markers (such as transferrin) or antibodies against such markers (such as anti-Rab antibodies). We are currently working to decipher the transcytotic itinerary of FcRn, with or without its IgG ligand, by colocalizing FcRn and IgG with different endosomal markers. To achieve this, we have transfected polarized MDCK cells with expression vectors encoding either full-length rat FcRn or FcRn-GFP (a chimeric molecule in which green fluorescent protein is recombinantly fused to the C-terminal cytoplasmic tail of FcRn). We have demonstrated that both cell lines are immunoreactive towards monoclonal antibodies against rat FcRn, demonstrating that FcRn trafficking can be followed by monitoring GFP fluorescence in FcRn-GFP cells. We have also demonstrated that these cells are competent to bind and internalize fluorescently labeled rat IgG (Cy5-IgG). Endosomal populations that are positive for both FcRn-GFP and Cy5-IgG fluorescence have been identified by confocal microscopy in FcRn-GFP cells that were incubated with 500 nM Cy5-IgG at pH 6.0, proving that trafficking movements associated with FcRn-mediated transport of IgG can be monitored over time. Antibodies against specific endosomal markers can be viewed using specific antibodies conjugated to a red dye (such as RITC or Cy3). This allows for three-color confocal analysis to determine the intracellular localization of FcRn, it ligand, and marker proteins for particular compartments at different stages during the transport process. These data can then be compared to the transport pathways of more well-characterized receptors such as the polymeric Ig receptor (pIgR) to evaluate which steps might be unique to, or particularly important during FcRn-mediated transport of IgG.

338. Assay for ligand-induced dimerization of FcRn

*Devin B. Tesar, Mary Dickinson**

The 6.5 Å crystal structure of FcRn complexed with Fc (1) demonstrates the formation of an oligomeric array of FcRn dimers bridged by Fc ligands (Figure 2). This "oligomeric ribbon" may have functional implications in living cells. First, transport vesicles containing the oligomeric ribbon would have their opposing membrane faces brought into close (~200 Å) proximity of one another. In addition, ribbon formation would induce an ordered linear arrangement of FcRn cytoplasmic tails. The cytosolic trafficking machinery may use one or both of these features as a means of distinguishing between vesicles that contain FcRn-IgG complexes and those that contain FcRn alone or unbound IgG. Oligomeric ribbon formation would require the simultaneous occurrence of two phenomena: 1) FcRn dimerization; and 2) bridging of FcRn molecules on opposing membrane faces by Fc ligands. To test for dimerization of FcRn, either in the presence or absence of ligand, we have devised an *in vivo* assay using fluorescence resonance energy transfer (FRET). We have created chimeric FcRn molecules that either enhanced cyan fluorescent protein (ECFP) or enhanced yellow fluorescent protein (EYFP) fused that C-terminus of the cytoplasmic tail. In collaboration with Mary Dickinson and Scott Fraser at the Caltech Beckman Imaging Center, we are using laser scanning confocal microscopy in conjunction with a Zeiss LSM Meta system, capable of deconvoluting the CFP and YFP spectra, to detect energy transfer from FcRn-CFP to FcRn-YFP in stably transfected Madin Darby canine kidney (MDCK) cells, both in the presence and absence of IgG. These results will be compared to FRET measurements of engineered FcRnCFP/YFP molecules containing putative dimer-disrupting mutations. We are also testing for FRET in HLA-DR, an MHC class II molecule and an obligate heterodimer as a positive control, and, as a negative control, between FcRn-CFP and HFE-YFP. HFE is another MHC class I homolog that does not bind to IgG or interact with FcRn.

**Scott Fraser Laboratory, Caltech*

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339. Three-dimensional EM studies of IgG transport by FcRn

Yongning He

FcRn is a cell receptor responsible for passive acquisition of humoral immunity in the vertebrate newborn. It also regulates the IgG half-life in serum and IgG transport in different tissues. The physiological roles of FcRn rely on its pH-dependent binding interaction with IgG and its transport activity after ligand binding. Based on the known biochemical and biophysical data, it has been suggested that FcRn:IgG complexes inside transcytotic vesicles form an ordered oligomeric structure in which FcRn molecules on the adjacent vesicular membrane are bridged by IgG molecules (Figure 2). The formation of this oligomeric structure may enable vesicles containing receptor:ligand complexes to be distinguished from the vesicles containing free FcRn or IgG, thereby allowing only those vesicles containing FcRn:IgG complexes to be recognized by the cytoplasmic vesicular trafficking machinery. CryoEM tomography of IgG bridged membrane-bound FcRn incorporated into synthetic liposomes will provide three-dimensional information of the bridging structure formed by FcRn molecules on adjacent membranes bound to IgG. Structural details of FcRn:IgG transcytotic vesicles will improve our understanding of the pathway and mechanism of IgG transport by FcRn, which can then serve as a general model for intracellular vesicular transport of receptor: ligand complexes. In addition, increased understanding of FcRn function will aid in efforts to take advantage of FcRn's function as the protection receptor that increases the lifetime of IgG in the blood, thereby facilitating current therapeutic strategies to use monoclonal antibodies to combat cancer and other diseases.

340. 3D EM tomography of intracellular vesicles in FcRn-expressing MDCK cells

Pamela J. Bjorkman, Devin B. Tesar, Noreen E. Tiangco, Mary Morphew, Eileen O'Toole*, J. Richard McIntosh**

The neonatal Fc receptor (FcRn) transports IgG across epithelial cell barriers to provide maternal antibodies to offspring and serves as a protection receptor for IgG by rescuing endocytosed IgG from lysosomal degradation. In both its transport and protection roles, FcRn binds to IgG at acidic pH inside endosomes and releases IgG at the pH (7.4) of blood. We solved crystal structures of rat FcRn alone and complexed with Fc. The FcRn/Fc structure revealed an oligomeric ribbon of FcRn dimers bridged by Fc molecules (Figure 2), which we propose forms inside an acidic transport vesicle. Oligomeric ribbon formation inside a vesicle is predicted to create a local region in which adjacent membranes are spaced ~200 Å apart. The ~0.2 μm resolution of a fluorescence confocal microscope is not sufficient to ascertain directly whether or not oligomeric ribbon formation is occurring inside transport vesicles. In order to compare the dimensions of transport vesicles predicted to contain the oligomeric ribbon with those in which ribbon formation should be disrupted, we need a technique that permits a higher resolution analysis. In collaboration with Dr. J. Richard McIntosh and the Boulder Laboratory for 3D Electron Microscopy of Cells, we are using EM tomography to analyze FcRn-expressing cells transcytosing IgG. This technique allows us to derive 3D images of transport vesicles within FcRn-expressing MDCK cells to a resolution of ~6 nm, which is sufficient for measuring the distance between adjacent membranes. We are using monofunctional nanogold-labeled Fc fragments internalized after incubation of live cells at pH 6

to facilitate identification of compartments containing FcRn-Fc complexes.

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341. Cloning of the chicken yolk sac IgY receptor

Anthony P. West, Jr.

The passive transfer of immunity in the form of immunoglobulins from mother to young occurs in many vertebrate species. In mammals, this occurs *in utero* (e.g., for humans) or after birth through milk (e.g., for rodents). In both of these cases, immunoglobulin G (IgG) is transported across a cellular barrier via transcytosis by the neonatal Fc receptor (FcRn), a major histocompatibility complex (MHC)-related protein that binds IgG at the acidic pH of intracellular endosomes (pH $\leq$ 6.5) and releases IgG at the pH of the bloodstream (pH 7.4). In birds and some reptiles, the counterpart of IgG, immunoglobulin Y (IgY) is first packaged in the yolk compartment of eggs, then transported across the yolk sac membrane into the embryonic bloodstream. A yolk sac membrane IgY receptor has been characterized and shown to have pH-dependent, FcY-specific binding similar to that observed for mammalian FcRn. To gain insight into the evolutionary origins of FcRn, which has been identified thus far only in mammals, and class I MHC molecules, which are found in various non-mammalian vertebrates including birds, we purified, cloned, and characterized the chicken yolk sac membrane IgY receptor (FcRY). FcRY is a 1459 amino acid protein that is unrelated to mammalian FcRn. Instead, FcRY appears to be the avian homolog of the mammalian secretory phospholipase A_2 (PLA_2) receptor.

342. Structural studies of the polymeric Ig receptor

Agnes Hamburger

The human polymeric immunoglobulin receptor, pIgR, belongs to the Ig superfamily and is expressed on the basolateral surface of secretory epithelial cells. pIgR binds dimeric IgA (dIgA) and pentameric IgM (pIgM) and transports them to the apical surface of the cell, where the complexes are cleaved and deposited into secretions such as milk or mucus. Infrequently, uncomplexed pIgR that has reached the apical surface is reverse transcytosed back to the basolateral surface. In addition to its intended purpose in mucosal immunity, pIgR is also used by a strain of *Streptococcus pneumoniae* to gain entry into epithelial cells by taking advantage of the reverse transcytosis. This interaction is mediated by a choline-binding protein (CbpA) on the surface of *S. pneumonia*.

pIgR is a glycosylated type I transmembrane protein containing five extracellular domains that most closely resemble V-like Ig domains. We have expressed different domains of pIgR using baculovirus-infected insect cells and crystallization attempts are underway. We are also recombinantly expressing pIgR's binding partners, the Fc portion of dIgA (dFcα) and the pIgR binding domain of CbpA. Future efforts will be directed at performing binding experiments and co-crystallization attempts.

343. Biophysical and structural characterization of an FcαRI:IgA1 complex

Andrew B. Herr, Edward R. Ballister[*#], *Darcy A. Ballister*[**]

FcαRI, a receptor specific for the Fc region of IgA (Fcα), is responsible for IgA-mediated immune responses. We have characterized the interaction between FcαRI and Fcα by a number of biophysical techniques (Herr *et al*., 2003a). We showed that two FcαRI molecules bind to a single Fcα homodimer by analytical ultracentrifugation. Surface plasmon resonance studies confirm the 2:1 stoichiometry of binding, with similar affinities at each site. The binding affinity is pH-dependent, in a manner consistent with protonation of a histidine residue in the binding site. A thermodynamic analysis indicates that the histidine does not participate in a salt bridge in the complex; indeed, less than 7% of the free energy of binding was contributed by electrostatic interactions.

We have also solved the crystal structures of human FcαRI alone and its complex with Fcα (Herr *et al*., 2003b). FcαRI has two immunoglobulin-like domains oriented approximately 90° apart. Fcα resembles IgG and IgE Fcs but has differently located interchain disulfide bonds and external rather than interdomain N-linked carbohydrates. Unlike 1:1 FcγRIII:IgG and FcεRI:IgE complexes, two FcαRI molecules bind each Fcα dimer, one at each Cα2-Cα3 junction. The FcαRI binding site on IgA1 overlaps the reported pIgR binding site, which may explain why secretory IgA cannot initiate phagocytosis or bind to Fcα RI-expressing cells in the absence of an integrin co-receptor.

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(b) Herr, A.B., Ballister, E.R. and Bjorkman, P.J. (2003) Insights into IgA-mediated immune responses from the crystal structures of human FcαRI and its complex with IgA1-Fc. *Nature* 423:614-620.

344. Structural studies of a herpes simplex virus immunoglobulin G receptor

Elizabeth R. Sprague, W. Lance Martin[#], *Robin Deis*[*], *Elizabeth E. Leyton*[**]

The herpes simplex virus (HSV) immunoglobulin G (IgG) receptor is a heterodimer composed of the gE and gI proteins that is found on the surface of the HSV virion or HSV-infected cells. The gE/gI heterodimer binds the Fc portion of IgG, and this interaction has been implicated in several mechanisms of immune evasion, including inhibition of virus neutralization, interference with

complement- and cell-mediated lysis pathways, and antibody-bipolar bridging. The gE protein has also been implicated in the cell-to-cell transmission of HSV. In order to facilitate understanding how the gE/gI heterodimer binds the Fc portion of IgG and how this recognition event affects the ability of the host immune system to eradicate the virus, we have undertaken biochemical and crystallographic studies of the gE protein, gE/gI heterodimer, and the gE/gI-Fc complex. Using baculovirus-infected insect cells, we have produced sufficient quantities of various pieces of the gE and gI proteins, both as separate proteins and as heterodimeric complexes. The Fc portion of human IgG1 is expressed in CHO cells. We have determined the stoichiometry of the gE/gI-Fc complex and further defined the interaction interface by constructing recombinant Fc molecules where residues in the putative gE/gI-binding site have been mutated on one or both of the subunits. We used an analytical ultracentrifugation sedimentation velocity assay to compare the binding of the gE/gI heterodimer to wild type (two copies of the wild-type subunit), "heterodimeric" (one mutant and one wild-type subunit) and "nonbinding" (two copies of the mutant subunit) Fc molecules with several gE/gI:Fc molar ratios. Our data show that the nonbinding Fc does not bind gE/gI, the heterodimeric Fc binds one molecule of gE/gI, and the wild-type Fc binds two molecules of gE/gI. Thus, we conclude that one gE/gI heterodimer binds to each half of the Fc molecule and at least some of the residues that were mutated contribute significantly to the binding. This data complements our previous data demonstrating that gE/gI shows strongly pH-dependent ligand binding properties, analogous to FcRn, but in the opposite direction (gE/gI binds tightly to IgG at neutral or basic pH, but not at acidic pH). Additionally, we have continued our attempts at improving the resolution of the gE/gI-Fc crystals by varying the protein fragments used in crystallization as well as applying alternative techniques in manipulating the crystals.

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345. Crystal structure of HLA-A2 bound to LIR-1, a host and viral MHC receptor

Benjamin E. Willcox[*], Leonard M. Thomas

LIR-1, an inhibitory receptor expressed on monocytes, dendritic cells, and lymphocytes, binds to a broad range of classical and non-classical class I MHC molecules and to the human cytomegalovirus class I MHC homolog UL18. We have solved the 3.4 Å crystal structure of a complex between the LIR-1 D1D2 domains and the classical class I MHC molecule HLA-A2. LIR-1 binds HLA-A2 using a mode distinct from other class I MHC-binding immunoreceptors, including killer inhibitory receptors (KIRs), T-cell receptors, and CD8, by engaging a surface on the primarily conserved β2-microglobulin and α3 domains of HLA-A2, thereby explaining the broad recognition of class I MHC molecules and UL18. The LIR-1 binding site comprises residues at the interdomain hinge, analogous to the class I MHC-binding surface on the evolutionarily-related KIRs, and additionally, a patch at the D1 tip. The LIR-1 residues involved are largely unchanged in some other LIRs, suggesting a similar class I MHC recognition mode.

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346. Structural and functional studies of MHC class 1 homologs in HCMV

Zhiru (Jenny) Yang

HCMV is widespread among all human populations and can be life threatening for immunocompromised individuals, such as cancer, transplant, and AIDS patients. HCMV achieves its lifelong persistence in host cells by adopting multiple mechanisms to subvert the primed immune system, including down-regulation of host class I MHC molecules.[1] HCMV encodes two MHC class I homologs, UL18 and UL142, which may be components of a strategy to avoid immune detection of class I-MHC negative cells. UL18 is heavily glycosylated (13 potential N-linked glycosylation sites) and able to bind host-derived β2-microglobulin (the class I MHC light chain) and endogenous peptides. The host cell receptor for UL18 is LIR-1, which is most abundant in B cells, monocytes, macrophages, and dendritic cell.[2] The UL18/LIR-1 interaction is likely to contribute to the latency and persistence of HCMV as well as the viral evasion of host NK cell surveillance.[3] In addition to UL18, LIR-1 also binds a broad range of host MHC class I molecules, but with an affinity that is over 1000 times reduced compared to its affinity for UL18.[4] The structures of LIR-1 and a LIR-1/HLA-A2 complex have recently been solved in our lab.[5,6] Currently, we are working on the co-crystallization and structure determination of UL18 with LIR-1. We are also generating deglycosylated mutants of UL18 to use in crystallization trials. These studies will be extended to include a newly identified HCMV class I MHC homolog, UL142, which is present in clinical HCMV strains such as Toledo.[7] UL142 shares about 20% amino acid sequence identity with MHC class I molecules. It may play a role in the virulence of the wild-type HCMV. Immunological, biochemical and structural studies are underway to characterize this protein. Altogether, our work on UL18 and UL142 will expand our basic understanding of the MHC class I homologs and the virus-host interaction. As suggested by their potential relevance to the virus-host interplay, UL18 and UL142 could also serve as a lead for drug designs for HCMV.

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347. Expression of Zn-α_2-glycoprotein: Investigating the structural and functional importance of ligand binding within the groove

Anthony P. West, Jr., Silvia L. Delker

Zn-α_2-glycoprotein (ZAG) is a 41 kDa soluble protein that is present in most bodily fluids. Recent studies by Michael Tisdale and his colleagues at Aston University have suggested that ZAG plays a role in the extreme weight loss (cachexia) that occurs in some cancer patients.[1] Since 30-50% of cancer mortality is a direct result of this wasting syndrome, developing therapies to reduce the systemic strain associated with wasting is an important goal. We previously determined the 2.8 Å crystal structure of ZAG isolated from human serum demonstrating the structural similarity between ZAG and MHC I molecules.[2] Unlike MHC I molecules, ZAG appears to bind a small non-peptidic ligand within its groove. The importance of this ligand in ZAG's structure and function is unclear. As part of an on-going effort to identify the ZAG ligand, we are attempting to generate crystals that diffract to higher resolution. We expressed ZAG in Chinese hamster ovary cells and in baculovirus-infected insect cells with point mutations removing two neighboring glycosylation sites (N89K, N92T). These forms of ZAG crystallize in a different space group from serum ZAG. Both forms diffract to ~2.0 Å and also show a density in the groove similar to the aforementioned crystal structure. We are currently in the process of trying to resolve the identity of the non-peptidic ligand based on the higher resolution crystallographic data.

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348. Biochemical and structural studies of Ferroportin1

Adrian E. Rice, Douglas C. Rees[*]

Iron is both a boon and a bane to life on earth. All organisms require it as a cofactor in several essential functions, yet an overabundance of this mineral can wreak cellular havoc by creating highly reactive OH· radicals. Higher organisms have developed specialized network of molecules designed to monitor and maintain iron homeostasis. When these networks are genetically compromised, diseases such as anemia and iron overload result. Our current study focuses on ferroportin1, an integral membrane protein implicated in the transport of iron across the basolateral membrane of mature enterocytes into the blood. Ferroportin1 is highly conserved through mammals and has a predicted mass of ~65 kD. Since its discovery, ferroportin1 has been directly implicated in an autosomal dominant form of the iron overload disease hemochromatosis. Genetic studies have shown that at least four different point mutations in the coding region of the human ferroportin1 gene lead to this disease. Structure prediction software predicts that ferroportin1 has between eight and eleven transmembrane helices. Little is currently known about the biochemistry of ferroportin1. We are interested in characterizing its iron export function from a biochemical and structural standpoint and in identifying any functional interactions with other biological molecules. Current plans in our laboratory aim at obtaining purified recombinant ferroportin1 for these studies.

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349. Mutageneic mapping of transferrin receptor reveals differential binding footprints for iron-loaded and iron-free transferrin

Anthony M. Giannetti

Transferrin Receptor 1 (TfR) is responsible for the primary iron uptake pathway in most mammalian cells. Cell surface TfR binds to circulating iron-loaded transferrin (Fe-Tf) and transports it to acidic recycling endosomes where the acidic pH causes iron to dissociate from transferrin (Tf) in a TfR-assisted process. The iron-free form of Tf (apo-Tf) remains bound to TfR and is recycled back to the cell surface, where the complex dissociates upon exposure to the slightly basic pH of the blood. The binding of TfR to another ligand, the Hereditary Hemochromatosis protein HFE, is thought to be critical for HFE's role in regulating iron homeostasis. A previous mutagenesis study revealed that Fe-Tf and HFE compete for the receptor by binding to an overlapping site on the TfR helical domain. We have completed a study of the affinities of a more extensive set of human TfR mutants (30 in all) for binding to HFE and Fe-Tf at pH 7.4 and to apo-Tf at pH 6.3, and derived binding footprints for Fe-Tf and apo-Tf on TfR. Although most TfR mutations exert similar effects on binding to Fe-Tf and apo-Tf, some TfR residues exert differential effects, suggesting differences in the contact points between TfR and the two forms of Tf that could be caused by pH-dependent conformational changes in TfR, Tf, or both. Using these results, we have proposed a structure-based model to explain the mechanism of TfR's activity in assisting iron release from Fe-Tf. The results from the mutagenesis have been used to create a heterodimeric TfR that binds HFE and Fe-Tf each on only side of the obligate TfR dimer. This protein has been used to study the stoichiometry of ligand binding to TfR and investigate preliminary reports of cooperativity in ligand binding by TfR.

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350. Structural studies of prostate-specific membrane antigen

Mindy. I. Davis, Leonard Thomas, Adam Paiz[*]

Prostate-specific membrane antigen (PSMA) is a 84 kDa type II transmembrane protein that is expressed predominantly on the surfaces of prostate epithelial cells. PSMA shares ~30% amino acid sequence identity with transferrin receptor (TfR), which binds iron-loaded transferrin as part of the iron uptake pathway in most cells. TfR includes a protease-like domain that lacks the key zinc-binding residues seen in aminopeptidases and does not bind Zn to form an active peptidase. In contrast, PSMA does not bind transferrin (as determined by a surface plasmon resonance-based assay) and is an active zinc peptidease. An isoform of PSMA (glutamate carboxypeptidase II) found in the brain cleaves the abundant neuropeptide N-acteyl-aspartyl-glutamate releasing the neurotransmitter glutamate. PSMA also hydrolyzes folate, releasing the terminal glutamate. Prostascint®, an antibody to the intracellular region of PSMA, is currently used for imaging the extent of prostate cancer. Many researchers are studying PSMA for development of prostate cancer imaging agents to the extracellular region of PSMA (sPSMA) and for immunotherapeutics and prodrugs (inactive until cleaved *in vivo* by PSMA) for prostate cancer. Although PSMA is a promising target for the development of therapeutics and diagnostics for the treatment of prostate cancer, its oligomeric state and structure are currently unknown. Our goal is to determine the oligomeric state and x-ray crystal structure sPSMA. We have expressed (~10 mg/L) sPSMA in a baculovirus/insect cell system and purified it using standard methods. sPSMA elutes from the size exclusion chromotography column slighter after the TfR dimer despite its higher dimer molecular weight. We will investigate the oligomerization state of sPSMA using analytical ultracentrifugation to determine whether it is a compact dimer or an elongated monomer. We have obtained crystals of PSMA and determined a molecular replacement solution for the sPSMA structure, which indicates that sPSMA forms a dimer resembling the TfR homodimer. Current efforts are directed towards obtaining crystals that diffract to higher resolution and crystals of sPSMA-inhibitor complexes. In addition to the prostate cancer applications, the structure of sPSMA will provide an interesting evolutionary comparison to the structure of TfR.

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351. Biophysical and structural studies of huntingtin exon 1 and anti-polyglutamine scFvs

Kathryn E. Huey-Tubman, Pingwei Li

Huntington's disease is an inherited neurodegenerative disorder associated with a mutated form of huntingtin (Htt). Htt, a large protein (~350 kDa) of unknown function, contains an unstable polyglutamine [poly(Gln)] tract. It has been proposed that when the poly(Gln) tract exceeds a "pathogenic threshold" of 36 glutamines, a conformational transition occurs, causing disease. We have shown that soluble huntingtin exon 1 fusion proteins with 16 (normal) to 46 (pathogenic) glutamines are extended structures with random coil characteristics, with no evidence of a global conformational change. We have shown that the Fab fragment of an anti-poly(Gln) antibody, MW1 (1), specifically binds both normal and expanded poly(Gln) tracts in soluble huntingtin exon 1. Sedimentation equilibrium analytical ultracentrifugation and surface plasmon resonance studies of these interactions show that the stoichiometry and affinity of the Fab increase with poly(Gln) length. These results support a "linear lattice" model in which the number of binding epitopes increases with the length of the poly(Gln) tract (2).

Previous therapeutic efforts have targeted the proposed pathogenic conformation of poly(Gln) with small molecules such as single-chain antibody variable region fragments (scFv). Our results suggest that a pathogenic conformation does not exist, and that such monovalent molecules are unlikely to distinguish between normal and expanded poly(Gln) tracts. The linear lattice model provides a new structural framework for drug design. The covalent linkage of monovalent compounds having micromolar affinities can produce multivalent compounds in which their binding affinities is nanomolar or higher. These multivalent compounds will bind with high avidity and specificity to expanded, rather than normal poly(Gln) tracts. To test this, we have produced divalent and trivalent scFvs from a monovalent scFv generated from the MW1 hybridoma (3). We will compare the binding affinities of the original monovalent scFv versus the divalent and trivalent scFvs to huntingtin exon 1 fusion proteins containing normal and pathogenic poly(Gln) tracts. Ultimately, we want to solve the crystal structures of poly(Gln) containing polypeptides complexed with the monovalent and multivalent scFvs.

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352. Structural and biophysical studies of transferrin receptor 2

*Mindy I Davis, Caroline A. Enns**

Transferrin receptor 2 (TfR2) is a type II membrane glycoprotein that is expressed predominantly on the surface of hepatocytes. The function of TfR2 is unknown although there are similarities to transferrin receptor 1 (TfR1), including 45% amino acid identity in the human extracellular regions. Previous surface plasmon resonance (SPR) results demonstrated that TfR1 binds both iron-loaded Tf (Fe-Tf) and the hemochromatosis protein HFE at the pH of the cell surface (pH=7.4).[1] The complex is endocytosed into acidic endosomes, where iron is released from Tf to be stored in the cytoplasm in the iron storage protein ferritin. The iron-free version of Tf (apo-Tf) recycles back to the cell surface along with TfR, where apo-Tf is released upon encountering the slightly basic pH of blood. Like TfR1, TfR2 binds Fe-Tf at pH 7.4, but unlike TfR1, TfR2 does not bind HFE at basic pH.[2] The ability to bind apo-Tf to TfR2 at pH 6 has not been tested. Mutations in TfR2 or HFE leads to hereditary hemochromatosis, a disease of iron overload, and thus understanding the role of TfR2 in iron homeostasis is important. Our goal is to extend the previous TfR2 SPR binding assays to investigate the potential for apo-Tf binding and to determine the x-ray crystal structure of TfR2 and TfR2 bound to Tf. Unlike TfR1, TfR2 loses binding activity rapidly and tends to aggregate, so a large focus of the past year has been upon obtaining stable protein. Full-length and truncated forms of the extracellular region of human and mouse TfR2 were expressed in a baculovirus/insect cell expression system at pH 7 using media containing Fe-Tf. These proteins all lost activity rapidly as determined by SPR. We are currently developing additional constructs that we hope will be stable throughout the SPR and crystallization experiments. These results may lead to an understanding of the different roles of TfR1 and TfR2 in iron homeostasis and how mutations in TfR2 lead to hereditary hemochromatosis.

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353. Cloning and expression of human TLR5 and its ligand, flagellin from ***Salmonella typhimurium***

Pingwei Li

Toll-like receptors (TLRs) are a family of immunoreceptors mediating the recognition of bacterial lipids, sugars, proteins and nucleic acids, and initiating inflammatory responses. The engagement of TLRs with their ligands induces the expression of chemokines such as TNFα. TLR5 of the toll-like receptor family recognizes flagellin, a major structural component of bacterial flagella. In order to understand the molecular mechanism of the recognition between TLR5 and flagellin, we have generated several different clones of the extracellular domain of human TLR5 and expressed the protein in both bacteria and insect cells. One of the ligands of human TLR5, flagellin from *Salmonella typhimurium*, was also cloned and over-expressed in bacteria. We will try to crystallize TLR5 and its complex with falgellin and determine the crystal structures of these proteins.

354. In search of the ligand of Methuselah, a ***Drosophila*** GPCR associated with extended lifespan

Anthony P. West, Jr., Silvia L. Delker, Bill Ja[1], Seymour Benzer[2]

The *Drosophila* mutant *methuselah* was identified from a screen for single gene mutations that extended average life-span. The *methuselah* mutant has a 35% increase in average life-span and increased resistance to several forms of stress. The protein affected by this mutation appears to be a G protein-coupled receptor (GPCR), though the upstream and downstream pathways that Methuselah (Mth) connects are unknown. Mth has a large extracellular N-terminal domain, which may be the ligand-binding domain of the receptor by analogy to related GPCRs, whose isolated extracellular domains function in ligand binding. We have determined the 2.3 Å crystal structure of the Mth ectodomain. The structure did not closely resemble any other protein and thus did not suggest what the natural ligand might be. Nevertheless, the ectodomain structure reveals a potential ligand-binding site, in a shallow groove between two flexible domains.

Using the Roberts' lab *in vitro* peptide selection technique (based on RNA-protein fusions) we have screened a large random library for Mth-binding peptides. We prepared a biotinylated form of the Mth ectodomain, which was used in the selection protocol. After eight rounds of selection, a group of strongly binding fusions was sequenced. Although most of the peptides were unrelated (with respect to the selection process), the majority contained a common pattern: (P/R)xxWxxR.

Surface plasmon resonance-based assays of the interaction of these peptides with the Mth ectodomain shows that they bind with affinities between 30 nM and 1 mM. We are attempting to co-crystallize these peptides with the Mth ectodomain to possibly reveal the ligand binding site. Previous studies have shown that peptides selected in a similar manner tend to bind at 'hot spots' for ligand binding. We have obtained small crystals of the Mth-peptide complex, and we are now optimizing the crystallization conditions.

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Professor Emeritus: Charles J. Brokaw

Summary: Motor enzymes —- dyneins, kinesins, and myosins — convert energy from ATP dephosphorylation into most of the movements performed by eukaryotic cells. We think that myosin and kinesin are reasonably well understood, although new experimental results from time to time surprise us. On the other hand, we have very little knowledge or understanding of the functioning of the axonemal dyneins that power the movements of flagella and cilia; these molecular complexes are a major challenge for the future. My current work uses computer simulation methods to explore ideas about motor enzyme function in situations ranging from experimental studies on individual motors to an intact flagellum containing tens of thousands of dyneins. Most of the simulation programs, as Macintosh applications, are available at www.cco.caltech.edu/~brokawc/software.html

355. Swimming with three-dimensional flagellar bending waves

Charles J. Brokaw

Computer simulation methods have been developed for study of bending wave generation by a model flagellum that can bend in three dimensions. Helical bending wave generation appears to be the default result obtained with the simplest possible method for controlling activity of the axonemal dyneins. At the time of last year's report, the method used for computing the moments resulting from external viscous resistance of the fluid medium was not sufficiently accurate. This problem has now been solved by an improved method for calculating the velocity, relative to the base, of a point on the flagellum from knowledge of the rates of bending (in three dimensions) at joints between the base and the point of interest (see: www.cco.caltech.edu/~brokawc/Suppl3D/YYWcomb.pdf).

Using these methods, swimming velocities can be computed for cells propelled by helical bending waves. These velocities can be compared directly with swimming velocities computed with the same model, except for control constraints that cause the generation of planar bending waves. For large cells (similar to the situation with bacteria propelled by helical flagella), helical waves are effective propellers. For small cells (such as most simple spermatozoa), helical bending waves are much less effective propellers than planar bending waves, because rotating the flagellum and cell body is much easier than pushing it through the fluid. This is not a new conclusion, but earlier work (such as work at Caltech by Alan Chwang and T. Y.-T. Wu) compared helical and planar waves with fixed wavelength and amplitude. In the new computations, the internal dynein motors are held constant, and the flagellum chooses its preferred bending wave parameters. This may be a much more biologically relevant comparison. Sperm flagella that produce planar bending waves must overcome the simple tendency for an axoneme to produce helical bending waves. The mechanism that has evolved to allow them to generate planar bending waves is not known, although there is considerable evidence that it may involve regulation of dynein activity by components associated with the central pair of microtubules found in almost all flagella that produce planar bending waves.

356. Self-organization in models for flagellar bending

Charles J. Brokaw

Large arrays of cilia, found on ciliated epithelia and the surfaces of protozoons such as *Paramecium*, beat with patterns known as metachronal waves. In one direction along the surface, the beat cycles of individual cilia are synchronous. Perpendicular to this direction, the beat cycles show a regular progression of phases, which produces a traveling wave. Both experimental and modeling studies indicate that metachronal waves are "self-organized." Each individual cilium is an independent oscillator, but its oscillation is modified by the movement of the fluid by neighboring cilia, and metachronal waves are the result.

Self-organization has also been discovered by computer simulations of models of flagella that can bend in three dimensions. In these models, each of the nine outer microtubular doublets is controlled by the curvature of that doublet, and each doublet is identical. In this case a pattern referred to as "doublet metachronism" appears, in which regions of dynein activation propagate along each doublet, with a phase shift of approximately $2\pi/9$ radians between each adjacent pair of doublets. Although self-organization appears to occur easily in these models, we cannot say whether the doublet metachronism that presumably occurs in real flagella that propagate helical bending waves is the result of a similar type of self-organization.

In the most successful models of flagellar movement, in which dynein activity is turned on and off by curvature, oscillation is a global property of the model. There are problems with these models, not the least of which is the fact that although these curvature-controlled models have been studied for more than 30 years, there is still no direct evidence for control of dynein activity by curvature. In contrast, there is experimental evidence, beginning 30 years ago, that oscillatory sliding between outer doublets is a local capability. In a typical flagellum, connections between doublets at the basal end ensure that sliding must produce some bending. So the question arises, can a distributed oscillatory sliding capability be self-organized to produce propagated bending waves? Investigations of this question with two-dimensional computer models of flagellar bending gave disappointing results. Even worse results are now obtained with the three-dimensional computer models. However, these computations provide a clue, indicating that the problem lies within the model used for local oscillatory sliding. A newer model for local oscillatory sliding is now available that may be more appropriate for finding out whether self-organization of oscillatory sliding is a reasonable mechanism for flagellar bending wave generation.

Publication

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Summary: A hallmark of cancer cells, in addition to uncontrolled proliferation, is genomic instability, which appears in the form of chromosome loss or gain, gross chromosomal rearrangements, deletions, or amplifications. The mechanisms that suppress such instability are of the utmost interest in understanding the pathogenesis of cancer. Our lab studies the components of the DNA replication apparatus that promote genomic stability, primarily using yeast genetics and biochemistry.

Several years ago, Rajiv Dua in the laboratory discovered that DNA polymerase ε, one of four essential DNA polymerases in yeast, had not one, but two essential functions. Deletion of the polymerase domain left the cells viable because another polymerase activity could substitute. Conversely, deletion of the remaining, non-catalytic half of the protein was lethal. Shaune Edwards in the laboratory carried out a two-hybrid screen for proteins that interact with the enigmatic C-terminal region of pol ε in order to discover its function. She found that pol ε interacts with Trf5, a protein involved in establishing cohesion of sister chromatids during passage of the replication fork. She has gone on to develop evidence that the essential function of the C terminus of pol ε is to aid in establishing efficient sister chromatid cohesion during S phase. Another postdoctoral fellow in the laboratory, Caroline Li, has characterized the Trf5 protein. She has shown that it encodes a previously unknown poly(A) polymerase and that it stimulates the activity of pol ε dramatically. Future studies are aimed at defining the mechanism by which these two proteins regulate interaction of the replisome with the cohesin complex, the glue that holds the chromosomes together, and how failure of cohesion leads to genomic instability.

At least seven human diseases characterized by cancer predisposition and/or premature aging are correlated with defects in genes encoding DNA helicases. The yeast genome contains 134 open reading frames with helicase motifs, only eight of which have been characterized. Martin Budd in our laboratory identified the first eukaryotic helicase essential for DNA replication, Dna2. He showed by interaction studies that it was a component of the machine that is required for accurate processing of Okazaki fragments during lagging strand DNA replication. Enzymatic studies to elucidate the sequential action of the DNA polymerase, helicase, and nuclease required for this processing form an ongoing mechanistic biochemistry project in the laboratory.

Stimulated by various reports in the literature implicating Dna2 in telomere biogenesis and structure, Wonchae Choe made the interesting observation that the bulk of Dna2 is localized to telomeres and that this localization is dynamic. During G1 and G2 phases of the cell cycle, Dna2 is at telomeres. During S phase Dna2 is present on the replicating chromatin. Current studies are aimed at defining the genes that regulate the localization. In addition to defects in replication, *dna2* mutants are also very sensitive to agents that induce double-strand breaks (DSBs). Osamu Imamura has shown that Dna2 is mobilized from telomeres in response to the induction of double-strand breaks. He is carrying out experiments to test the model that Dna2 delocalization from telomeres is part of the signaling system that induces the DNA damage and S-phase checkpoints, as has also been suggested for yKU, a protein involved in non-homologous end joining and in stabilizing telomeres.

One model of cellular aging suggests that accumulation of DNA damage leads to replicative senescence. Most endogenous damage occurs during S phase and leads to replication fork stress. At least three human diseases of premature aging or cancer predisposition, Werner, Bloom, and Rothmund-Thompson, are caused by defects in helicases similar to Dna2. Martin Budd and Laura Hoopes found that *dna2* mutants have a significantly reduced lifespan. Microarray analysis by Isabelle Lesur shows that the *dna2* mutants' age by the same pathway as wild-type cells; they just age faster. Interestingly, the human Bloom gene, (though not human Werner or Rothmund-Thompson) complements the replication defect of *dna2* mutants. Future work will take advantage of the yeast system to further delineate the role of BLM protein in mammalian cells. The work of Tao Wei in the lab suggests that instability of repetitive DNA, such as the ribosomal locus and telomeric DNA, is a major cause of genomic instability in the aging *dna2* mutants.

357. Aging cells show a global stress and DNA damage response

Isabelle Lesur

One of the characteristics of the *dna2-1* mutant is that it is aging prematurely. The median replicative lifespan of most *S. cerevisiae* wild-type cells is about 25 generations; the maximum is about 40 generations (Jazwinski, 1993). It has recently been shown that several DNA replication mutants show drastically reduced replicative lifespan (Hoopes *et al.*, 2002). This led to the suggestion that replication mutants represent an exaggerated case of spontaneous replication errors that occur in wild-type cells in every generation, and that cessation of cell division in both mutant and wild type is the consequence, in part, of chromosome damage occurring during DNA replication. Such a model would be consistent with the observed instability of the rDNA

repeats in old cells (Sinclair, 2002). One of these DNA replication mutants is *dna2-1*. Its median lifespan is eight generations, and maximum about 15 generations, and this mutant ages in the absence of ERCs (Hoopes *et al.*, 2002).

Our current study was designed to further define the causes of aging in wild-type yeast and of the premature aging of the *dna2-1* strain by further defining the pathologies of old cells, both wild type and mutant. Our initial study has been to determine the genome-wide transcriptional response to progression through the lifespan using microarray analysis. In order to obtain sufficient quantities of sufficiently old cells for reproducible production of RNA and cDNAs for microarray hybridizations, we developed a method for isolating old cells based on size selection. Our results suggest that a shift toward energy storage is associated with aging. Also an extensive subset of the environmental stress response genes (ESR) were activated in the old cells, as well as genes involved in DNA repair. The response shows striking similarity to the telomerase delete response, a gene expression pattern observed in cells senescing due to deletion of the catalytic subunit of telomerase.

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358. Biochemical characterization of Pol σand it's interaction with Pol ε

Caroline Li, Peter Snow[*]*, Judith L. Campbell*

Our group has previously reported genetic and physical interaction of *Saccharomyces cerevisiae* Pol2 (the catalytic subunit of the essential holoenzyme DNA polymerase ε) with either Trf4 or Trf5. The DNA polymerase activity of Pol ε was dramatically stimulated in the presence of Trf4 or Trf5. Trf4 and Trf5 are part of the Pol σ gene family that is essential for the viability of *S. cerevisiae*. Trf4 and Pol2 have been reported to have DNA polymerase activity, to be involved in DNA damage repair, and to play a role in sister chromatid cohesion.

S. cerevisiae Trf4 and Trf5 were expressed and purified from insect cells. Biochemical assays revealed that Trf4 and Trf5 have limited DNA polymerase activity and poly(A) polymerase activity. The DNA polymerase activity is likely a contaminant of protein purification because when a putative active site mutant of Trf5 was made, the DNA polymerase activity remained the same as that of the wild-type protein. However, the poly(A) polymerase activity was significantly reduced in the putative active site mutant of Trf5. We are currently producing a Trf4 putative active site mutant to check for the loss of poly(A) polymerase activity. Poly(A) polymerase was observed in two proteins from *S. pombe*, Cid1 and Cid13, that are homologous to *S. cerevisiae* Pol σ. The activity is thought to be involved in gene regulation of proteins involved in the S-M checkpoint. Cid1 and Cid13 are cytoplasmic proteins, whereas Trf4 and Trf5 are found in the nucleus, so their roles may be distinct. The poly(A) polymerase activity observed was characterized and found to be maximal in the presence of manganese instead of magnesium. It is possible that the activity seen in the presence of manganese is not physiological. We are currently pursuing the mechanism by which Trf4 and Trf5 stimulate Pol ε.

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359. Trf4 and Tfr5 are nuclear proteins that associate preferentially with origins of replication in S-phase cells

Shaune Edwards, Judith L. Campbell

Our group has previously reported genetic and physical interaction of *Saccharomyces cerevisiae* Pol2 (the catalytic subunit of the essential holoenzyme DNA polymerase ε) with both Trf4 or Trf5, two closely related and functionally redundant proteins required for DNA replication and sister chromatid cohesion. The association led to the discovery that that pol ε is also involved in sister chromatid cohesion. In *S. pombe*, Cid1 and Cid13, two proteins homologous to *S. cerevisiae* Trf4 and Trf5, were recently shown to be cytoplasmic poly(A) polymerases. The activity is thought to be involved in enhancing the stability of RNAs encoding proteins involved in the S-M checkpoint. Since Caroline Li in our group has found that Trf4 and Trf5 do catalyze a poly(A) polymerase reaction, albeit inefficiently, we were interested in to what extent Trf4 and Trf5 are similar to and different from the *S. pombe* homologs.

Unlike the *S. pombe* Cid1 and Cid3 proteins, we find that both Trf4 and Trf5 are nuclear proteins, though we cannot rule out some small fraction of Trf4 and Trf5 being localized to the cytoplasm. Furthermore, consistent with a role in DNA replication and in cohesion, we showed by chromatin immunoprecipitation assays that Trf4, and Trf5 colocalize in S phase, along with Pol2, the catalytic subunit of DNA polymerase ε, to origins of DNA replication. This work establishes that the Cid1 and Cid13 proteins in *S. pombe* are probably unrelated in function to Trf4 and Trf5. Recently, a third homolog of Trf4 and Trf5 in *S. pombe*, has been shown to have an exclusively nuclear localization, and may represent the functional Trf-homolog.

360. Overproduction of the replication initiation protein Cdc6 inhibits the metaphase to anaphase transition

Susanna Boronat, Judith L. Campbell

The replication protein Cdc6p is synthesized at the end of mitosis, binds to the chromosomally-bound ORC, and in turn enables the subsequent binding of Mcm proteins at the replication origins during G1 to form pre-replicative complexes (preRCs). During late G1 phase, preRCs are activated by Clb5,6/Cdc28 and Dbf4p/Cdc7p protein kinases leading to the onset of S phase. At this stage, the preRCs are lost from origins and cannot reassemble until passage through the next mitosis, since the Clb/Cdc28 kinases block preRC formation. The levels

of Cdc6p fluctuate during cell cycle: Cdc6p is synthesized at the end of mitosis, and is subject to degradation at the onset of S phase.

Cdc6p contains six potential target sites for the Cdc28 protein kinase, three at the N-terminus and three at the C-terminus. The mutation of all these sites, a subset of them, or just one site at C-terminus (site 6), into non-phosphorylatable sites results in the stabilization of Cdc6p.

We and others have reported previously that Cdc6p ectopic expression and especially the expression of phosphorylation site mutants, produces a delay in mitosis, specifically at the metaphase to anaphase transition. We had proposed that this delay could be explained by two different, although not mutually exclusive, explanations. One possible explanation is that Cdc6p ectopic expression triggers or mimics a checkpoint that arrests cells at the metaphase to anaphase transition. We showed that no checkpoint mutants that we have tested could suppress the mitotic delay, assayed by monitoring the levels of the Pds1 protein. This result strongly suggested that Cdc6p ectopic expression is not triggering the DNA replication or the spindle checkpoint.

The second explanation would involve a direct inhibition of the APC (Anaphase Promoting Complex)/Cdc20, the complex involved in the metaphase to anaphase transition, as opposed to the alternative form of the APC, APC/Cdh1, involved in the exit of mitosis. The activation of APC/Cdc20 requires active Cdc28 kinase. Therefore, inhibition of the APC/Cdc20 could be due either to inhibition of the Cdc28 kinase or to activation of the phosphatase PP2A, known to dephosphorylate the APC, or to inhibition of both the APC and PP2A.

We have shown that Cdc6p inhibits Cdc28 kinase activity *in vitro*. However, when we analyzed the Cdc28 kinase activity, using either histone H1 or the B-subunit of the polymerase α as substrates, we did not detect a significant inhibition of Cdc28 activity. Also, some of our phosphorylation site mutants did not bind to Cdc28 and were still capable to produce a mitotic delay. These results strongly suggested that there had to be mechanism other than the direct inhibition of Cdc28p by binding to the Cdc6p.

Therefore, we investigated if activation of the PP2A phosphatase was responsible for our observations and we found that this was the case. We found that mutant yeast strains without Cdc55, one of the regulatory subunits of PP2A, did not show mitotic delay when Cdc6 phosphorylation site mutants were expressed. We showed that APC/Cdc20 function, as measured by Pds1 degradation, was restored. We also found that *cdc55*Δ restored the viability of *cdc16* strains overexpressing Cdc6 phosphorylation site mutants. We analyzed the relationship between Cdc6 and PP2A in more detail and we found that Cdc6 co-immunoprecipitated with Cdc55 and that interaction is through Cdc6 C-terminus. We also found that Cdc6 was able to interact with Cdc16, one of the APC core subunits, known to be phosphorylated to activate APC/Cdc20. The simplest and most obvious interpretation of our results was that Cdc6 interacts with PP2A through the Cdc55 regulatory subunit and this interaction results in the recruitment of PP2A to the APC, which is then dephosphorylated and deactivated.

361. Mechanism of and model for Cdc6 inhibition of mitosis

Sue Boronat, Chris Raub[*]*, Judith L. Campbell*

We are interested in understanding the mechanism of inhibition of mitosis by overproduction of Cdc6, but most importantly the biological significance of these interactions. Since the levels of Cdc6 were thought to be very low at the metaphase to anaphase transition in normal cycling cells, the fact that our results were obtained by overproduction of Cdc6 raised the question of the role, if any, of inhibition of APC/Cdc20 by Cdc6 in normal cells. We think that the answer lies in the fact that the metaphase to anaphase transition is an important node in the cell cycle upon which many regulatory systems converge.

We have proposed a model in which Cdc6 that is synthesized after anaphase, and which is present at very high levels, also serves in a normal cell cycle to fully inhibit APC/Cdc20 before Cdc20 degradation in late mitosis. In other words, we propose that the constitutive overproduction used in our experiments to date reflects a physiologically significant cell cycle specific, transient, high level of Cdc6. *CDC6* transcription peaks simultaneously with *CDC20* and protein synthesis occurs shortly after. Cdc6 may then interact with PP2A through the regulatory subunit Cdc55, recruit PP2A to the APC/Cdc20 and inactivate it by dephosphorylation of the core subunits. At this point, the levels of Cdc6 protein may not suffice to inhibit Clb/Cdc28, but we cannot rule out this possibility. Such inhibition would provide an additional means to inactivate APC/Cdc20, however. Later in telophase, it is possible that Cdc6 levels are high enough to inhibit Clb/Cdc28, in conjunction with Sic1, and, therefore, contribute to the activation of the APC/Cdh1 and exit of mitosis, as reported by others. Although dephosphorylation of the APC core subunits is not essential for APC/Cdh1 interaction, the fact that Cdh1 binds to an unphosphorylated APC core makes it likely that Cdc6-associated PP2A dephosphorylates the APC/Cdc20, contributing to the switch from Cdc20 to Cdh1.

We are currently working on the verification of this model. We have carried out Western blots of myc-Cdc6 expressed from its own promotor as a function of the cell cycle. The peak of myc-Cdc6 correlates with timing of Pds1 degradation, that is, it is early in mitosis and not late or in G1. Also, the levels of Cdc6 protein peak when the levels of Clb2 are still very high, an additional sign that cells are still in early mitosis. However, due to those high levels of Clb2, most probably Cdc6 is not forming pre-RCs. The profile of Cdc6 expression is, therefore, in agreement with our model.

We are also studying the effect of lack of Cdc6 during mitosis, since we expect a faster degradation of the APC/Cdc20 substrates like Pds1 and Clb5, because in the

absence of Cdc6, the APC/Cdc20 would not be inactivated. Our preliminary results support our hypothesis. In a biochemical approach, we are trying to prove that endogenous levels of Cdc6 bind to Cdc55 in anaphase and that this interaction affects APC/Cdc20 activity.

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362. Interaction between ***Saccharomyces cerevisiae*** DNA polymerases epsilon and sigma

Clara C. Reis, Judith L. Campbell

Polymerase epsilon (pol ε) is an essential DNA polymerase required for DNA replication, repair, and the S/M checkpoint. Recently it was discovered that the essential but non-catalytic C-terminal domain of yeast DNA pol ε interacts both physically and genetically with pol σ, encoded by *Saccharomyces cerevisiae TRF4* and *TRF5* genes.

Mutants of both polymerases have been shown to have defects in sister chromatid cohesion. The overall goal of our studies is to explore further this interaction with respect to checkpoint response, sister chromatid cohesion, and chromosome dynamics during S phase.

If the essential functions of pol σ require interaction with pol ε, then one would expect pol σ and pol ε to be present at similar levels in the cell. The relative amounts of endogenous Pol2 subunit of pol ε, Trf4, and Trf5 were therefore studied in asynchronous cells, revealing that Trf4 was present in about 2.5-fold excess over Trf5, which was present at levels approximately equivalent to Pol2. We will now evaluate Dpb2 subunit of pol ε levels. We have also shown that the levels of the Pol2, Trf4 and Trf5 proteins are constant throughout cell cycle progression.

One possible role for this interaction is to participate in the S-phase checkpoint. Therefore, the genetic interaction between *pol2* and *trf4* and *trf5* with respect to the checkpoint response, which leads to increase in the levels of *RNR1* (ribonucleotide reductase subunit) transcription, was investigated. We found that overexpression of the *RNR1* partially suppresses the ts phenotype of *trf5Δpol2-12* and the cs phenotype *trf4Δpol2-12*, suggesting that the interaction may be part of the checkpoint signaling pathway.

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363. Analysis of mutations in potential Mec1 phosphorylation sites

Martin Budd, Judith L. Campbell

Dna2 protein is involved in DNA replication and DNA repair. A proteomic screen has identified an interaction of Dna2 and Ddc2 protein. Ddc2 protein interacts with Mec1 protein, a protein kinase that is the initiating kinase for the DNA replication and DNA damage checkpoint. DNA2 protein is phosphorylated by Mec1 kinase, and is not phosphorylated by Mec1 kinase with defects in the catalytic domain. Since Mec1 regulates both the checkpoint response and the DNA repair response, the interaction with Dna2 may provide a pathway for Mec1 regulation of the DNA repair response. To identify the residues that are phosphorylated, we have initiated a collaboration with Rudi Aebersold at the Institute for Systems Analysis in Seattle. Mec1 phosphorylates SQ and TQ residues. In order to determine which possible sites might be significant, we have compared the sequence of *Saccharomyces cerevisiae* Dna2 with the protein from four other related *Saccharomyces* species to identify potential sites for Mec1 regulation. All these sites were mutated to alanine and strains containing the mutations were tested for temperature-sensitive growth, sensitivity to MMS (methyl methane sulfonate), and sensitivity to x-rays. One strain has defective growth at 37ºC, and is sensitive MMS. The mutations are being combined to test for an interaction among the mutations.

364. Identification of mutations synthetically lethal with ***dna2-1*** and ***dna2-2*** mutants

Martin Budd, Judith L. Campbell, Amy Tong[1], Charles Boone[1]

A set of deletion mutations in *S. cerevisiae* has been constructed for all known genes (about 6200). About 5100 of these mutations are nonessential. These nonessential genes can be used for a synthetic lethal screen with a strain with a mutation in an essential gene. We have collaborated with the laboratory of Charles Boone at the University of Toronto to do a synthetic lethal screen with *dna2-1* and *dna2-2* mutants. We had previously identified *rad27, sgs1, srs2, rrm3, rad52, est1* mutations as being synthetically lethal or sick when combined with *dna2* mutant strains. Rad27p is a nuclease that is the main player in Okazaki fragment processing. Sgs1p, Srs2p, and Rrm3p are DNA helicases involved in DNA replication, DNA repair, and the DNA checkpoint response. Sgs1p is homologous to the helicases defective in Werner Syndrome patients and Bloom Syndrome patients. Est1 protein is a subunit of telomerase the protein required to synthesize the chromosome ends. The screen has been done with *dna2-1* strains and has identified additional genes involved in DNA repair. In addition genes involved in the DNA replication checkpoint were identified. *dna2-1* is not synthetically lethal with *mec1* mutants, thus the identification of DNA replication checkpoint genes is surprising. Genes involved in DNA repair, transcriptional silencing, chromatin assembly, and the osmotic stress response are also as synthetically with dna2-1 mutants.

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365. Conserved genes associated with controlled magnetite mineralization in bacteria

Cody Z. Nash[1], L. Elizabeth Bertani, Joseph L. Kirschvink[1]

The biologically controlled mineralization of magnetite (BCMM) within lipid-bilayer membranes (magnetosomes), which is the biophysical basis of magnetotactic behavior in some bacteria, appears to be under strict genetic control. In an effort to identify genes involved in this process we compared the publicly available genomes of two magnetotactic bacteria, *Magnetospirillum magnetotacticum* MS-1 and *Magnetococcus* sp. MC-1 to each other and to the NCBI protein database. Our comparison focused on finding proteins conserved, particularly between these magnetotactic bacteria. We define a conserved gene in MS-1 as a gene (i.e., gene A) which best matches some gene from MC-1, gene B, where gene B also best matches a gene from MS-1, although not necessarily gene A. Conserved genes were likewise defined for MC-1. In MS-1 and MC-1 we found 122 and 104 conserved genes respectively, showing that the two species use similar systems for BCMM. These genes appear in multiple clusters, arranged according to function suggesting that BCMM is ancestral to the α-proteobacteria instead of having been transferred laterally among the class. The conserved genes include most of the genes known to localize to the magnetosome and they also include a significant number of genes involved in functions consistent with models of magnetotaxis, including glycoprotein synthesis, redox reactions, inorganic ion transport, and signal transduction. We find support for the hypothesis that BCMM is a metabolic strategy, as well as a navigational one.

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366. Evidence that yeast ***SGS1***, ***DNA2***, ***RRM3, SRS2***, and ***FOB1*** interact to maintain rDNA stability

Tao Weitao, Martin Budd, Laura L. Mays Hoopes, Judith L. Campbell*

We have proposed that faulty processing of arrested replication forks leads to increases in recombination and chromosome instability in *Saccharomyces cerevisiae* and contributes to the shortened lifespan of *dna2* mutants. Now we use the ribosomal DNA locus, which is a good model for all stages of DNA replication, to test this hypothesis. We show directly that DNA replication pausing at the ribosomal DNA replication fork barrier (RFB) is accompanied by the occurrence of double-strand breaks near the RFB. Both pausing and breakage are elevated in the early-aging, hypomorphic *dna2-2* helicase mutant. Deletion of *FOB1* suppresses the elevated pausing and DSB formation, and represses initiation at rDNA ARSs. The *dna2-2* mutation is synthetically lethal with *rrm3* or *srs2,* encoding DNA helicases involved in rDNA replication/recombination. Our current work also shows that mutations inactivating yeast RecQ helicase Sgs1 cause the replication fork to stall and DSBs at rDNA RFB, but either the mutation in *FOB1* or an increase in *SIR2* gene dosage suppresses the fork stalling. In conclusion, the replication-associated defects in the rDNA are symbolic of similar events occurring either stochastically throughout the genome or at other regions where replication forks move slowly or stall, such as telomeres, centromeres, or replication slow zones. The data support a notion of concordant action of Sgs1, Dna2, Sir2, Rrm3 and Srs2 in pathways processing stalled replication forks and DSBs, such as those occurring at the Fob1-dependent RFB.

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Summary: Diverse biological systems -- including viruses, cells, and organelles -- are enclosed within lipid membranes that serve to compartmentalize the systems and distinguish their contents from the environment. Under special circumstances, however, these membrane-bound systems undergo membrane fusion, in which the lipids of two compartments fuse and the internal contents ultimately mix. In general, these fusion events are multi-step processes, involving recognition of two membrane surfaces, membrane apposition, and finally, lipid and content mixing. Such membrane fusion events are central to many fundamental cellular processes, including entry of enveloped viruses into cells during infection, entry of sperm into an egg during fertilization, and fusion of organelles during protein trafficking and organelle biogenesis. Using cell biological, biophysical, and genetic approaches, our lab is studying the mechanisms through which these membrane fusion events occur.

Regulation of mitochondrial dynamics: Our major research focus is on the regulation of mitochondrial dynamics. Mitochondria are dynamic organelles that undergo cycles of homotypic fusion and fission. Such membrane fusion and fission events play important roles in controlling organelle number, subcellular distribution, morphology, and ATP production. In some cells, fusion of numerous mitochondria into a well-organized reticulum is thought to enable transmission of mitochondrial membrane potential, thereby facilitating ATP generation to active regions of the cell. In some cases, mitochondrial fusion is developmentally regulated; for example, during insect spermatogenesis, the mitochondria of immature spermatids aggregate and fuse into giant mitochondria. Such regulated mitochondrial fusion is likely required in order to accommodate the changing metabolic state of the cell. In flies and budding yeast, developmentally regulated fusion of mitochondria is necessary for mitochondrial function and is controlled by the mitochondrial GTPase, Fzo. In addition, genetic studies in mammalian and yeast cells have identified a second GTPase, Drp1/Dnm1, that plays a central role in fission of mitochondria.

Although the importance of mitochondrial fusion in lower eukaryotes is well documented, its role during mammalian development remains unclear. Accordingly, we have placed a major emphasis on addressing this issue through the analysis of mice deficient in mitofusins Mfn1 and Mfn2, homologs of Fzo (Hsiuchen Chen). This work has definitively shown that mitochondrial fusion in mice requires mitofusins and has revealed an essential role of mitochondrial fusion during mouse development, particularly in formation of the placenta. In addition, our analysis leads to a model in which fusion protects mitochondrial function by enabling cooperation between mitochondria. Finally, these studies may also lead to mouse models of human mitochondrial diseases, a diverse group of diseases characterized by defective mitochondrial function.

Genetic studies have uncovered Fzo/mitofusin as a central molecule in the fusion of mitochondria, but its mechanism of action is unknown. It may act as a truly fusogenic molecule, analogous to viral envelope proteins that directly mediate membrane fusion. Alternatively, it may act as a regulatory protein, directing assembly of a fusion machinery in a GTP-dependent manner. We are currently performing extensive structure-function analyses of Fzo to address these issues (Scott Detmer, Erik Griffin, Takumi Koshiba). We have also begun attempts to genetically and biochemically characterize proteins that interact with Fzo in order to identify other proteins involved in mitochondrial fusion.

Mammalian Drp1 (dynamin-related protein) is essential in mediating mitochondrial membrane fission. This GTPase is structurally related to dynamin, a well-studied protein involved in fission of the plasma membrane during endocytosis. We have begun biochemical studies on Drp1 with the goal of understanding its mechanism of action (Tobias Rosen).

HIV Entry: In a second line of research, we are continuing our investigations of membrane fusion by viral envelope proteins. A key step in the life cycle of enveloped viruses is fusion of viral and host cell membranes. A virally-encoded glycoprotein, gp160, is responsible for mediating the entry process of HIV-1, the etiological agent of Acquired Immunodeficiency Syndrome (AIDS). The envelope precursor, gp160, is cleaved to form the subunits gp120 and gp41. gp120 directs target cell recognition, while gp41 mediates the merging of viral and cellular membranes. gp41 is composed of several distinct domains including a hydrophobic fusion peptide, two coiled-coil domains (termed N- and C-terminal helices), a membrane-spanning region, and a cytoplasmic tail.

Our current model for HIV membrane fusion invokes a series of conformational changes in the gp120/gp41 complex. Interaction between gp120 and cellular receptors liberates the gp41 fusion peptide from its native conformation and allows its insertion into the host cell membrane. A transient species termed the prehairpin intermediate is created, in which the N-terminal helices form a trimeric coiled-coil but do not interact with the C-terminal region. Subsequently, a hairpin structure is generated, in which the C-terminal helices pack in an anti-parallel manner around the trimeric N core to form a

fusion-active six-helix bundle. This N-C interaction brings the viral fusion peptide, inserted into the host-cell membrane, and the transmembrane segment, associated with the viral membrane, into close proximity. In a process that remains poorly defined, fusion of the closely apposed viral and cellular membranes follows. A mechanistic understanding of HIV membrane fusion may lead to new strategies to inhibit HIV entry into human cells.

To test this model of HIV entry, we are determining the mechanism through which peptide inhibitors prevent gp41-mediated membrane fusion. Some of these peptide inhibitors bind to fusion intermediates of gp41, and our analysis has revealed sequential steps in the fusion pathway. We are also characterizing a series of gp41 mutants that fail to fuse in order to better understand how formation of the six-helix structure leads to membrane fusion (T. Suntoke).

367. Developmental and cellular biology of mitochondrial fusion in mammals

Hsiuchen Chen

Mice contain two Fzo homologs, termed mitofusin 1 and mitofusin 2 (Mfn1 and Mfn2), that are highly similar to yeast and fly Fzo. We have generated mice deficient in each mitofusin, and have shown that both are required for normal development. In each case, homozygous mutant mice die in mid-gestation. Mfn2-deficient mice show a marked reduction in the trophoblast giant cell layer of the placenta, leading to placenta insufficiency and slight developmental delay of the embryo proper. Mfn1-deficient embryos, in contrast, are severely runted and deformed. Since the embryonic lethality associated with Mfn-deficiency precludes analysis of mitochondrial fusion in adult tissues, we have also generated conditional knockout mice for both mitofusins.

Cells from either Mfn1-deficient or Mfn2-deficient mice contain fragmented mitochondria, a morphology consistent with loss of mitochondrial fusion. Indeed, cell fusion assays demonstrate a severe reduction in mitochondrial fusion when either mitofusin is absent. In addition, mutant cells display a loss of membrane potential in some mitochondria. Mitochondrial fusion, therefore, is essential for full mitochondrial function, probably because it enables cooperation between mitochondria.

To further explore the effects of mitofusins on mitochondrial morphology and function, we have used RNAi directed against Mfn1 in Mfn2-deficient cells to generate mitofusin-null cells. Other genes known to be involved in mitochondrial fusion and fission, including Opa1 and Drp1, have also been targeted by RNAi. We are examining how knockdown of different combinations of these genes disrupt mitochondrial dynamics and function. To assay mitochondrial function, we are using oxygen electrode measurements to determine respiration rates. In addition, we are performing cell growth experiments to assay the effects of disrupted mitochondrial fusion on overall cell health. Preliminary results indicate that mitochondrial fusion is indeed required for optimal mitochondrial, and thus, cellular function.

368. Structure-function analysis of murine Mfn1 and Mfn2

Scott Detmer

An understanding of the mechanism of mitochondrial fusion will likely yield novel insights into membrane biology, because mitochondrial fusion is a unique process that fuses four lipid bilayers (the outer and inner membranes of both fusing mitochondria). Mfn1 and Mfn2 are mitochondrial outer membrane proteins required for this fusion; however, their mechanism of action in the fusion process is unknown. We have identified point mutations in the GTPase and heptad repeat domains of Mfn which lead to loss-of-function, indicating that each of these domains is critical for function.

To gain insight into mechanism, we are studying the inter- and intramolecular interactions of the Mfn proteins. Using a differential epitope tagging strategy and co-immunoprecipitation, we find that Mfn1 and Mfn2 interact both homo- and heterotypically. Thus, three distinct Mfn oligomers can form: Mfn1 and Mfn2 homotypic complexes and Mfn1-Mfn2 heterotypic complexes. The significance of these distinct complexes is being investigated. Mfn1 homotypic interactions appear to be occurring through multiple domain interactions, because non-overlapping fragments of Mfn1 interact both with themselves and with each other. In addition, we find that a region of Mfn1 and the GTPase domain interact raising the possibility that this region may be acting as an intramolecular GTPase activator or effector. Further *in vivo* and *in vitro* studies will characterize this interaction as well as determine its functional consequences.

Genetic and biochemical analysis in yeast have identified Fzo1-interacting proteins that are required for mitochondrial fusion. Using immunoprecipitation techniques, we are trying to identify binding partners of Mfn. We have generated cell lines that lack endogenous Mfn1 or Mfn2 and that express epitope-tagged Mfn1 or Mfn2, respectively. These tagged proteins will be immunoprecipitated and analyzed by SDS-PAGE; promising interacting bands will be identified by mass spectroscopy and protein sequencing.

369. Inter- and intramolecular Fzo1p interactions

Erik Griffin

To understand the mechanism of yeast Fzo1p action, it is necessary to identify its critical domains. Our structure/function analysis has defined several critical regions in Fzo1p required for mitochondrial fusion. The loss-of-function point mutants resulting from this analysis are currently being characterized in more detail using both *in vitro* and cell-based assays for GTPase activity, oligomerization, and mitochondrial fusion. Additionally, allelic complementation between null point mutants has allowed us to determine which regions are required on a single molecule, and which can be located on separate molecules.

To understand the inter- and intramolecular interactions between Fzo1p molecules, we are testing the effect of mutations and truncations in co-immunoprecipitation assays. Interestingly, non-overlapping N- and C-terminal fragments of Fzo1p interact with each other and restore mitochondrial fusion. This interaction may define a critical step in Fzo1p function because it is sensitive to several point mutations. We are currently further defining this interaction in heterologous expression systems and *in vitro*. These approaches should allow us to generate a model for Fzo1p action in mitochondrial fusion.

370. Protein dissection study of murine Fzo-homologs, mitofusins Mfn1 and Mfn2

Takumi Koshiba

Like other Fzo-homologs, murine Mfn1 and Mfn2 contain several interesting sequence motifs. These include the GTPase domain, which we have shown is critical for function, and two heptad hydrophobic repeat regions (HR1 and HR2), which are similar to the sequences found in viral envelope proteins and SNARE proteins involved in membrane fusion.

As a first step towards understanding the mechanism of Mfn1 and Mfn2 action, it is necessary to identify critical functional domains. Therefore, we designed expression plasmids to produce three major regions (GTPase, HR1 and HR2) of Mfn1 and Mfn2 as recombinant histidine-tagged proteins in bacteria host cells. Recombinant proteins have been overexpressed and refolded. Currently, we are characterizing these recombinant proteins through biochemical, biophysical, and cell biological strategies.

371. Biochemical and structural analysis of assembly by Drp1

Tobias Rosen

Dynamin-related protein (Drp1) is a large (~80 kD) GTPase involved in the regulated scission of mitochondria in mammalian cells. Drp1 has significant sequence homology to dynamin, a well-characterized molecule involved with endocytic membrane cleavage events, and therefore Drp1 has been proposed to utilize a similar mechanochemical mechanism of action. This protein concentrates to areas of mitochondrial membranes which subsequently become sites of membrane fission. Inhibition of Drp1 function leads to abnormally long and interconnected mitochondria.

I am interested in understanding how the various domains of Drp1 interact to promote membrane scission. By conducting a series of limited proteolysis experiments, I have identified a number of interesting domains which are similar to those that have been identified in dynamin. In particular, I have identified a region analogous to dynamin's GTPase effector domain (GED), which has been functionally implicated in both assembly and GTPase regulation. This region is predicted to be primarily coiled coil, and may be a key moiety in integrating this molecule's transition from a soluble to an assembled state. I have expressed several forms of this GED region in *E. coli* and found them to be helical by circular dichroism. I am now attempting crystallization trials to determine an atomic level structure. In addition, I am investigating the *in vivo* role of the GED by characterizing a dominant negative allele of Drp1 with deletions in the GED domain.

372. Analysis of HIV-1 gp41 mutants

Tara Suntoke

To better understand the mechanism of gp41-mediated fusion, we are examining the role of gp41's six-helix hairpin structure. The fusion-active six-helix bundle is stabilized predominantly by hydrophobic interactions between N and C helices. Using structural data for the fusion-active core, we have created a series of mutations that are designed to reduce or prevent N-C association and thus, six-helix bundle formation. A range of hydrophobic substitutions at five different positions is being used to test the hypothesis that hairpin formation directly correlates to fusion activity. All gp41 mutants show wild-type levels of expression and precursor processing in mammalian cells, suggesting that the native structure of Env remains unaffected by the mutation. Cell-cell fusion assays reveal that mutants with bulky hydrophobic substitutions are more capable of mediating fusion than those with small aliphatic substitutions. In order to compare the stability of the six-helix bundle structures formed by each mutant, we performed a biophysical analysis of bacterially purified six-helix peptides. The helical content and thermal stability are quantitated by circular dichroism. In general, stability of the six-helix core correlates with hydrophobic bulk of the substitution: glycine and alanine mutants are less stable than phenylalanine substitutions at the same position. Those mutants with a more stable six-helix core structure are the same ones that exhibit greater fusogenic potential. Thus, evidence indicates that formation of the six-helix bundle structure directly correlates with gp41 fusion activity. These fusion defective mutants will be further characterized *in vivo* in transfected mammalian cells. To examine their progress through the fusion pathway, immunoprecipitation experiments with gp41 conformation-specific antibodies and peptide probes will be performed. Using fluorescent dyes to label target cell membranes and cytosol, fusion kinetics of the mutants will also be analyzed.

Publications

Chen, H., Detmer. S.A., Ewald, A.J., Griffin, E.E., Fraser, S.E. and Chan, D.C. (2003) Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. *J. Cell Biol.* 160:189-200.

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Summary: Accurate control over the growth and division of cells is crucial for the development of complex, multicellular organisms. Consequently, aberrations in cell cycle control can have profound consequences; the inability to restrain cell division, for example, contributes to the genesis of cancer. We have been studying cell cycle control in the baker's yeast *Saccharomyces cerevisiae*, because this organism offers many advantages. Yeast provides a unique opportunity to apply both genetic and biochemical approaches to resolve a multicomponent system. A large catalog of yeast genes required for cell cycle progression and regulation has been assembled from genetic studies. Many of the cell cycle genes from yeast have homologs that perform identical tasks in human cells. This striking conservation of function allows for comparative studies of the cell division cycle in yeast and mammalian cells.

A key challenge in the study of cell cycle regulation is to understand how cells transit from one phase of the cell cycle to another. Cell cycle transitions are dramatic events in the life of a cell; at the transition from G2 phase to mitosis, for example, the cytoplasm and nucleus are extensively remodeled to promote the coordinated execution of chromosome segregation. Recent results suggest that cell cycle transitions also serve as regulatory watersheds for 'checkpoint' signaling pathways that maintain the temporal organization of cell cycle events and ensure the fidelity of chromosome replication and segregation. Thus, a molecular description of the mechanism and regulation of cell cycle transitions will greatly illuminate our understanding of how cells grow and divide, and how these processes are regulated. It is currently thought that transitions from one phase of the cell cycle to another are coupled to fluctuations in the activity of a family of cyclin-dependent protein kinases (CDKs). These kinases represent a special family of kinases that are activated by regulatory proteins known as cyclins. Cyclins bind to the catalytic kinase subunit and trigger a battery of posttranslational modifications that culminate in the activation of the kinase. Eventually, the kinase activity is extinguished by proteolysis of the stimulatory cyclin subunit.

Over the past few years, we have focused our effort on two cell cycle transitions: the entry into S phase and the exit from mitosis. Surprisingly, both of these transitions are triggered by the ubiquitin-mediated destruction of key cell cycle regulatory proteins. Ubiquitin is a small protein that can be ligated posttranslationally to substrate proteins. Upon attachment of ubiquitin, the substrate protein is rapidly destroyed by a multisubunit protease complex known as the proteasome. The realization that ubiquitination plays such a critical role in cell cycle control has led my lab to focus increasingly on understanding how selective proteolysis via the ubiquitin pathway is employed to regulate a variety of processes inside cells, including cell proliferation, signal transduction, and transcription.

Below, I summarize in more detail the four major areas of investigation in the lab, and provide thumbnail descriptions of all current projects.

Mechanism and regulation of protein degradation by the SCF pathway. Our prior investigations into the biochemical mechanism by which budding yeast cells transit from the G1 phase of the cell cycle to the S phase led to the discovery of the SCF ubiquitin ligases. SCF ubiquitin ligases are comprised of four subunits: Skp1, the Cullin family member Cul1 (or Cdc53), an F-box protein, and the RING-H2 domain protein Hrt1 (also known as Rbx1 or Roc1). The F-box protein serves as a receptor that docks substrates to the catalytic core of SCF, which is comprised of the cullin and RING-H2 subunits. We have proposed that the catalytic core of SCF recruits the E2 (ubiquitin-conjugating), enzyme Cdc34, and switches on the ability of Cdc34 to transfer ubiquitin to substrate that is docked to the F-box subunit. Skp1 serves as a tether that links together the substrate-binding and catalytic modules. Interestingly, there are ~20 F-box proteins encoded in the yeast genome, and many more in metazoan genomes. At least some of these F-box proteins can assemble with the cullin/RING-H2 catalytic core to form SCF complexes, suggesting that eukaryotic cells harbor many distinct SCF ubiquitin ligases, each with a unique substrate specificity. The prototype for the SCF family - SCF^{Cdc4} (where Cdc4 refers to the identity of the F-box subunit) - enables the transition from G1 phase to S phase in budding yeast by promoting the ubiquitination and degradation of the S-phase cyclin-dependent kinase inhibitor Sic1 (see Figure 1). Five ongoing projects in the lab build upon our discovery of SCF^{Cdc4}. Rati Verma is using a purified system comprised of SCF^{Cdc4}, 26S proteosome, and

Sic1/S-phase CDK to investigate the mechanism by which ubiquitinated Sic1 is recognized by the proteosome and selectively degraded to yield active S-phase CDK. Matt Petroski is investigating the biochemical mechanism by which SCF^{Cdc4} selects sites of ubiquitination in its substrates, and how it activates the ubiquitin conjugating enzyme Cdc34 to transfer ubiquitin to substrate. Rusty Lipford is investigating how SCF^{Cdc4} regulates transcription via ubiquitin-dependent degradation of transcriptional regulatory proteins. Kathleen Sakamoto's project addresses the hypothesis that tethering a protein to the F-box subunit of SCF is sufficient to activate its ubiquitination and eventual destruction. A small molecule-based approach to achieve this goal might lead to the development of a novel class of therapeutics. Finally, Nazli Ghaboosi is attempting to isolate conditional lethal temperature-sensitive mutants of the ubiquitin-activating enzyme Uba1. These mutants will enable us to address how ubiquitin metabolism modulates the recruitment of SCF^{Cdc4} and a host of other proteins to the 26S proteosome.

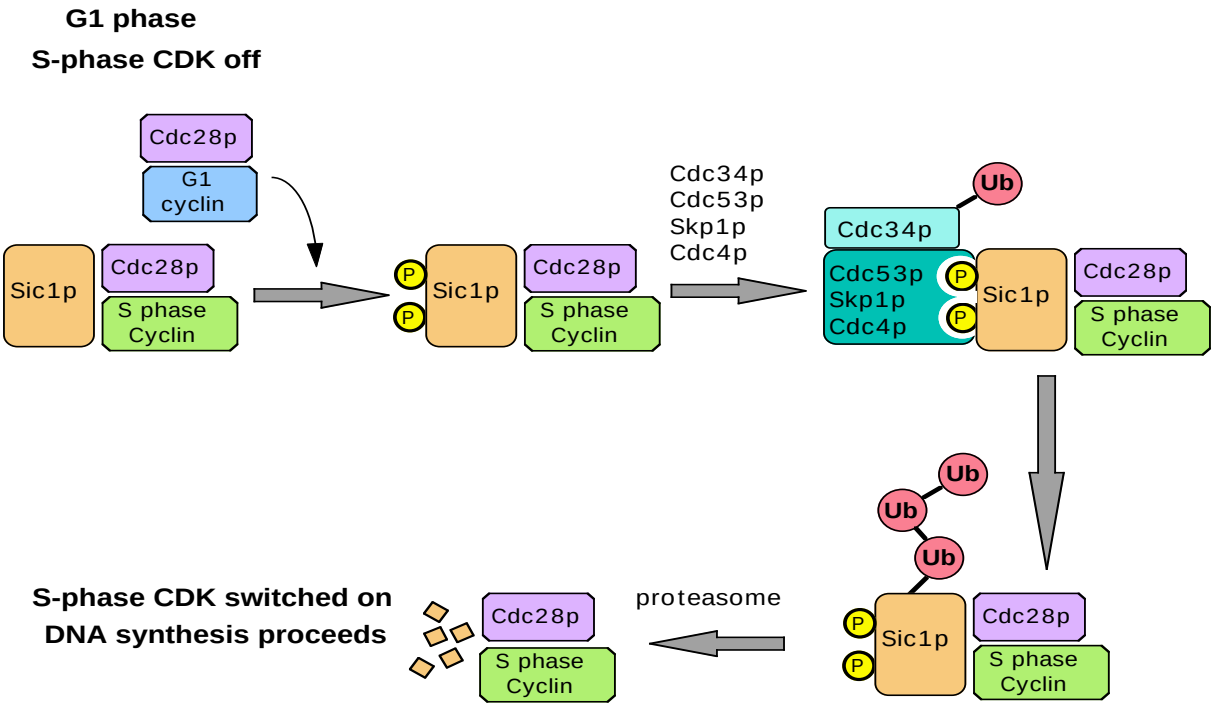

Figure 1: A molecular model for the G1/S transition in budding yeast.

Mechanism of action, substrates, and regulation of the COP9 signalosome. During the course of our studies on SCF ubiquitin ligases in animal cells, Svetlana Lyapina (now departed), discovered that SCF co-purifies with the COP9 signalosome. The COP9 signalosome (CSN) is an eight-protein assemblage that is evolutionarily related to the lid subcomplex of the 26S proteosome. CSN was originally discovered by Xing-Wang Deng and colleagues as a regulator of photomorphogenetic development in plants, and has since been implicated in a wide range of cellular and developmental processes. However, essentially nothing was known about the mechanism of action, biochemical targets, and regulation of this fascinating protein complex. In the past year, Greg Cope has shown that a novel metalloprotease-active site located in the Csn5 subunit of CSN promotes cleavage of the ubiquitin-like protein Nedd8 from the Cul1 subunit of SCF. Interestingly, this active site, which we have dubbed, "JAMM" (JAb1/Mpn domain Metalloenzyme), is found in six other human proteins, including the Rpn11 subunit of the proteasome, where it serves as an essential ubiquitin isopeptidase that cleaves ubiquitin from proteins as they are being degraded by the proteasome (Verma *et al.*, 2002). This finding suggests that the diverse biological activities of CSN may all relate to its ability to cleave Nedd8 from Nedd8-conjugated proteins. This discovery has opened up new opportunities to investigate how CSN works, and what its role is in cell physiology. Greg now seeks to build upon his discovery by constructing point-mutated animal cells that are specifically defective in the Nedd8 isopeptidase activity of CSN. To test more directly whether the JAMM motif actually specifies an enzyme active site, Xavier Ambroggio has crystallized a JAMM protein from an archaebacterium to analyze its three-dimensional structure. Finally, Gabriela Alexandru is examining the biology of an interesting human protein that contains a JAMM domain, and has been implicated in signaling within the TGF-β pathway.

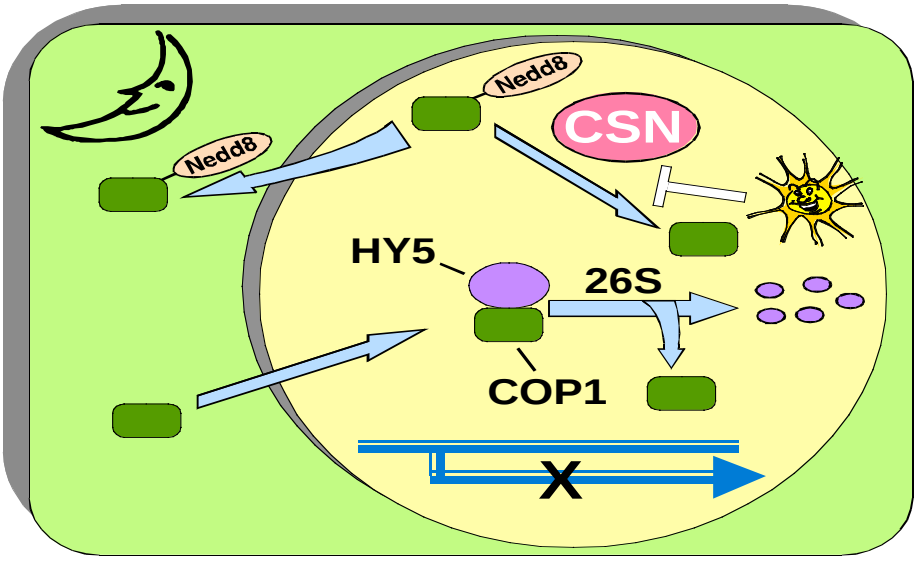

Figure 2: A model for how CSN controls photomorphogenesis, and how this may relate to the Nedd8-deconjugating activity of CSN. In the dark, CSN activates COP1, which in turn mediates turnover of the transcription factor HY5. In sunlight, the stimulatory action of CSN on COP1 is abrogated, rendering COP1 inactive and thereby allowing the accumulation of HY5. HY5, in turn, activates transcription of light-induced photomorphogenic genes. We postulate that one of the components of this circuit (e.g., COP1, HY5, or affiliated polypeptides) is regulated by reversible modification with Nedd8. In the example drawn, Nedd8 modification of COP1 would promote its export from the nucleus, which is exactly what is seen in CSN-deficient cells.

Proteomics. Budding yeast - with its formidable arsenal of genetic, molecular genetic, biochemical, and cell biological techniques - is an ideal system in which to develop and test new approaches in proteomics. Currently, there are two proteomic-type projects in the lab. Johannes Graumann is using a novel mass spectrometry technique developed by John Yates at Scripps and known as "MudPIT" (for multidimensional protein identification technology) to identify proteins that interact with a suite of yeast proteins involved in control of mitosis. MudPIT enables the sequencing of complex protein mixtures without the need for prior separation by SDS-PAGE, and so can identify proteins that are present at low stoichiometry, stain poorly, or are otherwise difficult to detect by conventional approaches. Thibault Mayor's project is to use MudPIT to identify the proteins that are covalently modified with ubiquitin in budding yeast. We hope to apply this method to the mapping of substrate networks for ubiquitin conjugating and deconjugating enzymes.

Functions of the RENT complex in cell cycle control and nucleolar biogenesis. Several years ago, Wenying Shou and Jae Hong Seol discovered the RENT complex, and characterization of this complex led to the model shown in Figure 3. RENT is comprised of the nucleolar anchor protein Net1, the cell cycle regulatory protein phosphatase Cdc14 and the chromatin silencing protein Sir2. Cdc14 is required for the exit from mitosis, which it promotes by dephosphorylating (and thereby activating), proteins that mediate the inactivation of cyclin/CDK activity at the end of mitosis. Throughout the cell cycle, Cdc14 is confined to the nucleolus through its interaction with Net1. At the end of mitosis, the successful completion of anaphase activates a signaling pathway referred to as the mitotic exit network (MEN), which disengages Cdc14 from Net1. The emancipated Cdc14 goes on to inactivate cyclin/CDK and thereby trigger the exit from mitosis. This hypothesis for how the exit from mitosis is controlled in budding yeast was dubbed 'RENT control' by Shou *et al.*, 1999. Intriguingly, in addition to its role in cell cycle control, Net1 also mediates silencing of RNA polymerase II transcription in the nucleolus, activation of transcription by RNA polymerase I, and retention of a broad set of resident proteins within the nucleolus. The molecular mechanisms underlying the cell cycle-regulated disassembly of RENT and the role of RENT in organizing nucleolar chromatin remain largely unknown.

Over the past year, Ramzi Azzam has sought to identify and characterize molecules involved in the regulated disassembly of RENT at the end of mitosis. Angie Mah has characterized the substrate specificity of protein kinase Dbf2, which is the last known component of the MEN that lies upstream of RENT. Angie hopes to use this information to understand how Dbf2 promotes the dissociation of RENT. Dane Mohl seeks to understand how nuclear transport may contribute to regulation of the RENT complex and exit from mitosis.

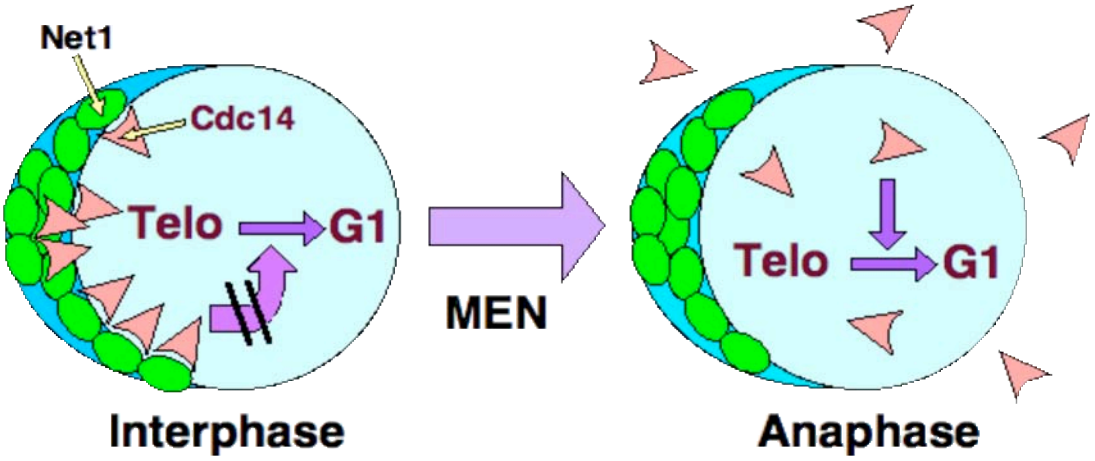

Figure 3: The mitotic exit network (MEN), triggers release of Cdc14 from Net1 during late anaphase. During interphase (left side), Cdc14 is sequestered in the nucleolus by virtue of its interaction with Net1. Upon activation of the MEN, Cdc14 is released from Net1, and diffuses throughout the nucleus and cytoplasm (right side). Free Cdc14 triggers the telophase → G1 transition in part by activating APC-dependent ubiquitination and degradation of mitotic cyclin.

373. Specificity and biological function of JAMM domain proteins

Gabriela M. Alexandru

The JAMM motif (Jab1/MPN domain metalloenzyme), was initially identified in the Jab1/CSN5 subunit of the signalosome and found to be essential for its deneddylase activity (Cope *et al*., 2002). The same motif is present in the Rpn11 subunit of the proteasome, where it specifies a similar isopeptidase activity directed against ubiquitin conjugates (Verma *et al*., 2002). Different JAMM domain proteins are present in organisms ranging from archaea to humans. Besides CSN5 and Rpn11, the family of human JAMM domain proteins comprises at least six other members, whose biological function is either unknown or poorly understood. Among these, AMSH has been previously characterized as a positive regulator of TGF-beta signaling. However, very little is known about the mechanisms underlying AMSH function at molecular level. I plan to identify potential binding partners for AMSH by MuDPIT analysis of AMSH-immunoprecipitates from human tissue culture cells, using rabbit antibodies sensitive enough to detect endogenous amounts of AMSH. This will reveal whether AMSH, like CSN5 and Rpn11, is also part of a big multi-component complex and it might also give us an insight as to what its targets could be. The final goal would be to link AMSH biochemical activity to its biological function in mammalian cells.

374. Structural analysis of the proteasome and JAMM domain proteins

Xavier Ambroggio

The proteasome is a large protein complex composed of four distinct layers: the alpha and beta subunits which form the 20S core, and the lid and AAA ATPase base that form the 19S regulatory particle. The hydrolytic activity of the 20S core has been well characterized through structural and biochemical analysis, yet little is known about the mechanism by which proteasome substrates are recognized and translocated through the 20S core. These later activities have been associated with the 19S regulatory particle. The AAA ATPase base is homologous to other complexes, such as NSF, which have been implicated in the unfolding of proteins. The lid of the proteasome is an eight subunit hetero-oligomeric complex homologous to the COP9 signalosome (CSN) and the eukaryotic initiation factor 3 (eIF3). Through a battery of bioformatic, biochemical, and structural studies we found that a particular subunit of the proteasome lid (Rpn11) and signalosome (Csn5) has a metalloprotease domain (JAMM domain) responsible for the removal of ubiquitin or ubiquitin-like proteins from proteasome or CSN substrates, respectively. These studies suggest that the proteasome lid is involved in substrate recognition. In order to understand these bits of data in the larger context of the proteasome, we are working on obtaining a crystal structure of the proteasome lid. In addition, through structural studies we seek to understand the function of JAMM domain proteins and their evolutionary relationships.

375. The role of the mitotic exit network and Net1 phosphorylation in the M/G1 transition

Ramzi Azzam, Angie Mah

Exit from mitosis is an essential step in the progression of cells through the cell cycle. In late mitosis, inactivation of the mitotic cyclin/Cdk complexes causes mitotic spindle disassembly, chromosomal condensation, and return of cells to G1 phase. In budding yeast, mitotic exit requires a network of seven genetically interacting proteins that include three protein kinases (Cdc15, Cdc5, Dbf2), a protein phosphatase (Cdc14), a GTPase (Tem1), a GTP/GDP exchange protein (Lte1), and a protein kinase regulator (Mob1). This group is collectively known as the mitotic exit network (MEN). Cdc14 is sequestered in the nucleolus, tethered to Net1 and Sir2 in a complex known

as the regulator of nucleolar silencing and telophase (RENT), throughout interphase. Upon activation of the MEN, Cdc14 is released from Net1, diffuses throughout the cell, and initiates cyclin proteolysis and the telophase → G1 transition by dephosphorylating and thereby switching on the APC activator Hct1/Cdh1. Recently, Cdc14 was demonstrated to be transiently released from the nucleolus into the nucleus, and this early release depends on a protein network collectively termed the "FEAR" pathway named for (cdc fourteen early release). The MEN are now thought to maintain this FEAR-mediated release.

We are trying to deduce how the release of Cdc14 from RENT complex is accomplished. We undertook the mapping of *in vivo* phosphorylation sites on Net1 as a possible mechanism of release. Mutation of all 13 phosphorylated Ser and Thr residues to alanine and subsequent partial mutants confirmed that the Net1 phospho-site mutants were defective in the FEAR-mediated release and genetically interact with MEN components. Phospho-specific antibodies made against phosphorylated residues on Net1 demonstrated that Net1 phosphorylation is cell-cycle regulated. Phosphorylation was dependent on the mitotic Cdc28/Clb1-2 Cdk activity both *in vitro* and *in viv*o. Furthermore, the phospho-epitope formation was dependent on the FEAR network and did not occur in a subset of FEAR mutants. Thus, our data indicate that the role of Net1 phosphorylation is to bring about the FEAR-mediated release of Cdc14. Further experiments are in progress to determine the epistatic relationship of FEAR and Clb/Cdk with regards to Net1 phosphorylation.

376. Nedd8 protein modification and the COP9 signalosome

Gregory Cope

The COP9 signalosome (CSN), is a multi-subunit complex conserved from human to fission yeast *S. pombe*. This complex has been attributed to play a role in multiple processes, including photomorphogenesis in plants, development in *Drosophila*, and cell cycle control in *S. pombe*. Given that all eight subunits of the CSN are highly homologous to the proteasome lid, a role in proteolysis has been proposed for the CSN.

We recently found that the CSN is associated with multiple cullin proteins (termed Cul1-5), in mammalian and yeast cells [Lyapina *et al.* (2001) *Science* 292:1382-1385]. Cullins are members of a class of E3 ubiquitin ligases that target specific protein substrates for ubiquitin-dependent proteolysis. There are multiple ways in which cullins are regulated, one of which is through the covalent modification with the ubiquitin-like protein Nedd8. This modification increases Cul1 ubiquitin ligase activity toward substrates *in vitro* and is essential in the fission yeast *S. pombe*. We found that CSN promotes the cleavage of the ubiquitin-like molecule Nedd8 from *S. pombe* Cul1 *in vitro* and *in vivo* and this activity requires a putative metallo-enzyme motif in Jab1/Csn5, which we term JAMM (Jab1/MPN domain associated metalloenzyme). Through genetic and biochemical analysis, we have found that JAMM is essential for Cul1 deneddylation and acts positively on Cul1 activity *in vivo* [Cope *et al.* (2002) *Science* 298:608-611]. Moreover, a point mutant in the JAMM motif of Csn5 is unable to rescue lethality of a Csn5 null *Drosophila*, suggesting that deneddylation underlies at least some of the previously characterized functions of CSN.

In an effort to more thoroughly understand the function of CSN deneddylating activity in mammalian cells, we are undertaking an approach to remove Csn5 from human culture cells. Using recently developed siRNA techniques, it is hoped that the loss of Csn5 (and thus, deneddylation) *in vivo* will provide us with a useful way to study the numerous questions regarding SCF in human cells. For example, how does loss of deneddylation effect SCF substrate abundance, SCF composition, and SCF localization? In addition, establishing a cell line that has lost deneddylation would help to identify other proteins that are regulated by the covalent modification with the Nedd8 protein.

377. Role of ubiquitin-activating enzyme in ubiquitin-dependent degradation

Nazli Ghaboosi

Ubiquitin-dependent proteolysis by the multi-subunit 26S proteasome is the major non-lysosomal pathway for protein degradation in eukaryotic cells. The pathway begins with the activation of ubiquitin by the E1 ubiquitin-activating enzyme. The ubiquitin moiety is transferred to one of several E2 ubiquitin-conjugating enzymes and is subsequently attached to the substrate, sometimes with the aid of an E3 ubiquitin ligase. The multi-ubiquitinated substrate is then targeted for degradation by the 26S proteasome through a poorly understood mechanism. It was originally thought that all substrates for the 26S proteasome must be ubiquitinated for proper targeting. However, there is convincing evidence that some proteins are targeted to the proteasome without ubiquitin modification. These ubiquitin-independent substrates call into question the necessity of ubiquitination in proteasomal targeting, as well as the mechanism of substrate-proteasome interactions.

We would like to characterize the role of ubiquitination in substrate targeting to the proteasome by identification of ubiquitin-independent substrates and ubiquitin-independent, proteasome-interacting proteins. While the list of known E2s, E3s, and substrates is steadily growing, there is only one E1 enzyme in all somatic eukaryotic cell types. At the apex of this intricate network, E1 offers a unique perspective from which to address the many unanswered questions regarding proteasomal targeting.

We have created and characterized several temperature-sensitive mutants of the essential yeast E1 gene, *UBA1*. We have demonstrated that yeast E1 mutants are defective in the ubiquitination and degradation of a variety of known substrates, in effect conditionally disrupting the entire downstream ubiquitination pathway.

This system is being employed to determine the ubiquitin dependence of proteasome-interacting proteins by comparing mass spectrometric profiles of affinity-purified 26S proteasomes from wild-type and *uba1* mutant cells. In addition, E1-independent substrates are being identified using quantitative proteomics to screen for short-lived proteins that are not stabilized in *uba1* mutant cells.

378. Medium-scale interaction analysis in a non-specialized environment

Johannes Graumann

The question of how to determine interacting proteins for any given bait protein in the yeast *S. cerevisiae* has recently been approached from several directions. The techniques applied are genome-wide two-hybrid screens, protein chip analysis, and affinity purification combined with polyacrylamide gel separation and mass spectrometry analysis. All these techniques share major drawbacks. In the case of the two hybrid and protein chip approaches, genomic libraries of tagged proteins have to be established and maintained and interaction networks have to be pieced together from binary interaction information. In the case of gel separation-based mass spectrometry approaches, cutting individual protein bands from stained gels leads to a sample number explosion.

An alternative approach, which has not been tested for large-scale data sets so far and avoids these problems was developed by Link *et al.* (1999) *Nat. Biotechnol.* 17(7):676-682, purified protein mixtures are digested in solution and the resulting complex peptide mixture is separated by multidimensional capillary chromatography connected in-line to an ion trap mass spectrometer. Analyzed peptide fragmentation spectra are computationally identified and assigned to their protein of origin.

Analyzing interaction partners of a set of 20 dually-tagged yeast proteins involved in mitosis, we have recently shown that this approach - termed MudPIT for multidimensional protein identification technology - is mature enough to enable analysis of large complexes and moderately-sized interaction networks in a biological laboratory (manuscript in preparation). The analysis of moderate-scale interaction networks, so far confined to an industrial or semi-industrial context and highly specialized analytical chemistry laboratories, is ready to be a tool for biologists - with enormous potential. Current work aims at the extension of the approach to search for substrates of ubiquitin ligase enzymes.

379. Regulation of transcription by ubiquitin-mediated proteolysis

Rusty Lipford

Previous work established a link between ubiquitin-mediated proteolysis and transcriptional activation by Gcn4, an activator of amino acid biosynthetic genes. Our current analysis has led to the counterintuitive conclusion that proteolysis of Gcn4 contributes to its activity. Stable Gcn4 mutants that are not ubiquitinated are defective for transcription, despite increased association with target promoters and normal recruitment of RNA polymerase. Similarly, mutations in ubiquitin and in the ubiquitin ligase SCF stabilize Gcn4, yet compromise its activity. Finally, mutations in the 26S proteasome or in proteasome-targeting factors, or treatment with proteasome inhibitors increase Gcn4 abundance and ubiquitination, but decrease its ability to activate transcription. We propose that ubiquitin-mediated proteolysis is required to complete a step in the transcription cycle, e.g., promoter release or promoter recycling. Our present efforts are focused on determining the molecular details of the role of proteolysis in transcription. In addition, we are assessing the generality of this phenomenon, and have discovered that inhibition of the 26S proteasome also attenuates the transcriptional activity of Gal4.

380. The mitotic exit network in ***S. cerevisiae***

Angie Mah, Ramzi Azzam

Exit from mitosis is ultimately triggered by the loss of cyclin-dependent kinase (Cdk) activity by degradation of mitotic cyclins and accumulation of Cdk inhibitors. Cdc14 plays a critical role in triggering this event as it dephosphorylates, thereby activating the inhibitors of mitotic Cdk. Cdc14 activity is modulated by a regulatory group of proteins, the mitotic exit network (MEN). The mitotic exit network consists of the group of genes TEM1, LTE1, CDC15, DBF2, DBF20, MOB1 and CDC5. Mutations in these genes have revealed similar phenotypes of arrest in late anaphase with separated chromosomes, an elongated spindle, and elevated Cdc28/Clb2 kinase activity. Genetic interactions with each other have also been shown, with Cdc14 as the endpoint of this pathway. Tem1 is a ras-like GTPase, which is likely to be a target of Lte1, a guanine nucleotide exchange factor. Tem1 is thought to then activate Cdc15, a protein kinase. We have demonstrated Cdc15 directly activates Dbf2, another protein kinase, but only when Dbf2 is bound to Mob1.

However, the substrate for the Dbf2-Mob1 kinase complex remains elusive. In collaboration with Michael Yaffe's group, we have determined the optimal phosphorylation motif for Dbf2. By defining a consensus phosphorylation sequence, we hope to characterize Dbf2 substrate(s), which ultimately links the MEN pathway to its effector, Cdc14. To narrow the list of candidate substrates that contains this sequence, we are working with Micheal Snyder's group. Their lab has produced protein chips imprinted with the yeast proteome. By using these chips, we can detect proteins that are phosphorylated by the Dbf2-Mob1 kinase complex. Further analysis of these putative substrates will allow us to determine the downstream target(s) of the Dbf2-Mob1 kinase complex, which in turn leads to Cdc14 activation and therefore mitotic exit.

381. Identification of ubiquitin-ligase (E3) substrates

Thibault Mayor

The presence of conserved motifs on ubiquitin-ligases such as HECT or RING domains has led to the identification of a vast array of E3's. However, very little is known about their substrate specificity. Our goal is to establish a global approach allowing the identification of ubiquitin-ligase substrates. The current study is focused on purification of ubiquitin conjugates using both polyubiquitin-binding proteins and hexahistidine affinity tags on ubiquitin. We have established an efficient purification method that should now allow us to analyze the composition of ubiquitin conjugates in cells by mass spectrometry. Ultimately, we will try to perform quantitative mass spectrometry on samples from cells that either express or do not express a particular ubiquitin ligase. In this way, we hope to identify specific substrates for different known ubiquitin ligases.

382. Regulation of mitotic exit

Dane Mohl

Faithful segregation of chromosomes requires tight control over cytokinesis and exit from mitosis. In budding yeast, mitotic exit involves the proteolytic removal of B-type cyclins (Clb), and concomitant accumulation of Sic1, a Clb/Cdk inhibitor. The mitotic exit network (MEN) links cell cycle progression to the completion of DNA segregation by regulating the release of a phosphatase, Cdc14, from the RENT complex, where it is bound to and inhibited by Net1.

Activation of Cdc14 appears to be regulated through its release from RENT and subsequent relocalization to the cytoplasm. Two pathways regulate these changes, FEAR and MEN. The FEAR pathway drives dissociation of Cdc14 from RENT by a mechanism that involves the phosphorylation of Net1, and directly affects the ability of Cdc14 to bind the Net1 complex.

MEN-dependent partitioning of Cdc14 from the nucleus to the cytoplasm may facilitate sustained release from the nucleolar RENT complex. We believe that the nuclear/cytoplasmic localization of Cdc14 is regulated by phosphorylation of a C-terminal NLS in Cdc14. Our work has shown that the same residues of the C-NLS that confer cell cycle-dependent nuclear import to GFP *in vivo*, can be phosphorylated by the MEN pathway kinase, Dbf2/Mob1, *in vitro*. In order to further define the mechanisms that regulate Cdc14 localization, we are using live cell fluorescence microscopy, MUDPIT technologies (multi-dimensional mass spectrometric protein identification tools), phospho-peptide mapping, as well as standard genetic and biochemical tools to investigate how the mitotic exit network controls the interaction of Cdc14 with both the RENT complex and the nuclear import/export machinery.

383. Regulation of Sic1 stability through the SCF^{Cdc4}/Cdc34 pathway

Matthew D. Petroski

In general, E3s (ubiquitin ligases) serve as a bridge between the substrate to be ubiquitinated and the ubiquitin-conjugation machinery. Two major classes of E3s have been identified, based on the presence of either a HECT domain or a RING motif. Our studies have largely focused on the multiprotein RING E3 SCF. SCF consists of Skp1, a cullin protein, an F-box protein, and the RING protein Hrt1 (also known as Rbx1 or Roc1). This complex has been identified in all eukaryotic systems and has been implicated in the regulation of diverse processes such as cell cycle progression, transcription, signal transduction, and inflammation response.

Our studies have concentrated on the mechanism of ubiquitination of the yeast cyclin-dependent kinase inhibitor Sic1 by the activity of SCF^{Cdc4} and its E2 Cdc34. Recognition of Sic1 by SCF^{Cdc4} requires phosphorylation on at least six of its eight phosphorylation sites, due to the low affinity of these sites for the phosphate-binding pocket of the F-box protein Cdc4. As Cdc4 can only accommodate a single phosphate molecule, perhaps multiple low-affinity binding sites "kinetically trap" Sic1 to optimally position lysine residues for ubiquitination. We have shown that the six lysines important in the turnover of Sic1 are closely juxtaposed near the most critical phosphorylation sites and that a single multiubiquitin chain attached to one of these lysines is the minimal signal required for destabilization. Our current analyses focus on elucidating a relationship between a chosen site of ubiquitin attachment and the phosphate recognized by SCF^{Cdc4}.

384. Targeting proteins for proteolysis by ubiquitin ligases in cancer therapy

Kathleen M. Sakamoto[*]

A central goal of the pharmaceutical and biotechnology industries is to identify small, stable, cell-permeable molecules that inhibit the activity of cellular proteins that contribute to cancer. Despite the importance of identifying new protein inhibitors for use as therapeutics, there is no simple, straightforward strategy for doing so that works for all target proteins. We propose a general method for developing inhibitors that exploits the unique characteristics of the ubiquitin-dependent proteolytic system of eukaryotic cells. The ultimate goal of my project is to identify a cell-permeable molecule that binds to the substrate-docking site of a ubiquitin ligase. By covalently linking this molecule to other compounds that bind specific cellular proteins, we intend to develop a novel class of drugs or ProTacs (Proteolysis Targeting Chimeric Pharmaceuticals), that can trigger the destruction of any protein in eukaryotic cells for which there exists a small, cell-permeable ligand. The specific aims of my project are to develop an experimental system to prove that this novel concept for drug design is experimentally feasible. At one end, ProTac contains a peptide that binds with high affinity to the substrate-docking domain of the

ubiquitin ligase b-TRCP. We then chemically linked the peptide to the fungal metabolite ovalicin, which binds covalently and specifically to the cellular enzyme methionine aminopeptidase-2 (MetAP-2). We have demonstrated as "proof of principle" that the resulting peptide-ovalicin ProTac chimera tethers MetAP-2 to b-TRCP, and targets MetAP-2 for ubiquitination and degradation. To determine whether ProTacs could recruit a different substrate to the SCFb-TRCP ubiquitin ligase for ubiquitination through non-covalent interactions, we tested the estrogen receptor (ER). Ligand binding to both the ER and androgen receptor (AR) have been shown to enhance growth of breast and prostate cancer cells, respectively. A ProTac was synthesized containing the IκBα phosphopeptide linked to the ligand, estradiol. The estradiol-IκBα phosphopeptide ProTac was able to recruit the ER to the SCFb-TRCP, resulting in ubiquitination and degradation of ER. We are currently testing the ability of microinjected and cell-permeable ProTacs to target the estrogen receptor and the androgen receptor in cancer cells. Our goal is to demonstrate that ProTacs can be used to target cancer-promoting proteins for ubiquitination and degradation in hopes of developing novel approaches to treat breast and prostate cancer.

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385. Determining the requirements for proteolysis by purified 26S proteasomes

Rati Verma

Labile substrates of the 26S proteasome are earmarked for proteolysis by the covalent attachment of a multiubiquitin chain on acceptor lysines. Substantial biochemical evidence has been garnered demonstrating that UBA- and UIM-domain containing proteins can bind multiubiquitinated proteins. However, since binding is just the first step in a multistep 26S degradation reaction, and binding can occur in the absence of degradation, the exact role played by the above proteins in proteolysis has remained ill-defined and is currently the subject of intense debate with both positive and negative roles being postulated. We have utilized our defined degradation assay to investigate the relative contributions of the putative receptor proteins. 26S proteasomes were isolated from wild-type and mutant budding yeast cells lacking the UBA and UIM-domain containing 26S proteins (Rad23 and Rpn10, respectively). The composition and gross assembly of 26S was unimpaired in these mutants. Functionally, *rad23*Δ 26S were only partially defective for degradation, whereas there was an absolute requirement for the UIM-containing proteasomal subunit Rpn10 for degradation and deubiquitination. This preferred requirement for Rpn10 was also mirrored *in vivo*. Full complementation of the *in vitro* defect by recombinant Rpn10 allowed us to demonstrate that the requirement for Rpn10 function was bipartite. The multiUb binding contribution of the UIM was redundant with that of the UBA. In addition, the N-terminal VWA was required for full *functionality* of the UBA-domain protein *in vitro*. The significance of the VWA domain was underscored by the observation that it could suppress the temperature sensitivity, canavanine sensitivity, and protein turnover defect of the *rpn10*Δ*rad23*Δ double mutant. These studies lead us to conclude that UBA- and UIM-domain proteins are required for proteolysis both *in vitro* and *in vivo*, and that their individual contributions to proteolysis were masked in prior studies due to redundancy of the receptors *in vivo*, and lack of functional assays from mutant preparations *in vitro*.

386. Chemical biology approach identifies ubiquitin chain recognition as a potential target for therapeutic intervention in proteasome function

Rati Verma

Recent approval of Velcade (PS-341) by the U.S. FDA for treatment of relapsed multiple myeloma establishes the 26S proteasome as an important new target in oncology. PS-341 is a peptide boronate that acts by forming a transition state intermediate with the chymotryptic active site of the 20S proteasome. To address whether there might be other ways to inhibit the ubiquitin-proteasome pathway, a search was initiated by Randy King's laboratory (ICCB, Harvard Medical School), that involved screening a large collection (>100,000) of organic compounds for those that inhibited the degradation of a cyclin B-luciferase fusion protein in frog extract. This screen netted 28 compounds, some of which inhibited specifically the turnover of cyclin B-luciferase, and others that inhibited both cyclin B and β-catenin turnover. Two of the latter compounds, #59 and #92, were investigated in more depth by us to determine if they inhibit the 26S proteasome. Both compounds ablated turnover of ubiquitinated Sic1 by yeast 26S proteasome in a completely reconstituted system. In dose response analyses, #59 and #92 yielded 50% inhibition at ~1 μM and ~0.5 μM, respectively. Analysis of proteasome sub-reactions revealed that both compounds blocked binding of ubiquitinated Sic1 to 26S proteasome. Moreover, both compounds blocked binding of ubiquitinated Sic1 to recombinant ubiquitin chain receptors Rad23 and Rpn10 at concentrations similar to those that blocked degradation. Consistent with these observations, #92 did not block ubiquitin-independent degradation of ornithine decarboxylase by the 26S proteasome. Most remarkably, native gel-shift experiments indicated that #92 bound to K48-linked tetraubiquitin chains, but not K63-linked chains. This result is supported by NMR analysis by David Fushman's laboratory (University of Maryland, College Park, MD), which revealed that #92 bound at the ubiquitin-ubiquitin interface that is unique to the K48-linked chain. Main chain atoms in residues at the di-ubiquitin interface that are involved in binding to the CUE domain showed a strong chemical shift upon binding #92. Together with our biochemical data, the NMR analysis indicates that #92 competes directly with ubiquitin chain

receptors for binding to the ubiquitin chain, and thereby blocks targeting of ubiquitinated polypeptides to the 26S proteasome. This remarkable inhibitor of a key protein-protein interaction that lies at the heart of ubiquitin-dependent proteolysis suggests a clear strategy for the development of a novel class of 26S proteasome inhibitors

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Summary: In eukaryotic cells, the cyclin-dependent kinases (Cdks) control the progression of the cell cycle by regulating the accurate replication of the genome during S-phase and the faithful segregation of the chromosomes at mitosis (M-phase). The entry into these phases of the cell cycle is controlled by Cdks called S-phase promoting factor (SPF) and M-phase promoting factor (MPF). The action of these Cdks must be controlled both temporally and spatially in a very stringent manner. This strict regulation is imparted by a number of checkpoint mechanisms. For example, cells containing unreplicated DNA cannot enter mitosis due to the mobilization of the replication checkpoint. The Dunphy laboratory is engaged in the elucidation of the molecular mechanisms underlying the regulation of SPF and MPF during the cell cycle. Most of these experiments are conducted with *Xenopus* egg extracts, a system in which the entire cell cycle can be reconstituted *in vitro*.

The first member of the cyclin-dependent protein kinase family described is M-phase promoting factor (MPF), which contains the Cdc2 protein kinase and a regulatory subunit known as cyclin B. Since the identification of the molecular components of MPF, there has been rapid and extensive progress in unraveling the biochemistry of mitotic initiation. It is now well established that MPF acts by phosphorylating a myriad of structural and regulatory proteins that are involved directly in mitotic processes such as nuclear membrane disintegration, chromosome condensation, and mitotic spindle assembly. An ongoing challenge to the cell cycle field is the elucidation of how these phosphorylation reactions regulate the structural and functional properties of the various targets of MPF.

We have been most interested in how the cyclin-dependent protein kinases are regulated during the cell cycle. The principal focus of our laboratory has been on the regulatory mechanisms that govern the activation of MPF at the G2/M transition. Some immediate and long-term issues that we are tackling include:

1. What controls the timing of MPF activation so that it occurs at a defined interval following the completion of DNA replication?
2. How do various checkpoint or feedback controls influence the Cdc2/cyclin B complex?
3. What are the molecular differences between the simple biphasic cell cycle found in early embryonic cells and the more complex cell cycles that arise later in development?

More recently, we have been able to study at the molecular level some of the key events leading to the initiation of DNA replication at the G1/S transition. These events involve a cooperative interaction between the Origin Recognition Complex (ORC), the Cdc6 protein, and members of the Mcm family. These studies may ultimately help us understand how S-phase and M-phase are integrated with one another.

In principle, the regulation of cyclin-dependent kinases such as MPF could occur at any of several levels, including synthesis of the cyclin protein, association between the Cdc2 and cyclin proteins, or posttranslational modification of the Cdc2/cyclin complex. The posttranslational regulation of the Cdc2/cyclin complex is particularly important, even in early embryonic cells which manifest the simplest cell cycle programs. In recent years, many of the elaborate details of this Cdc2 modification process have been defined. For example, the binding of cyclin results in three phosphorylations of Cdc2: one at threonine 161 that is required for Cdc2 activity, and two dominantly inhibitory phosphorylations at threonine 14 and tyrosine 15. A variety of genetic and biochemical experiments have established that the inhibitory tyrosine phosphorylation of Cdc2 is an especially important mechanism of cell cycle regulation. As described in greater detail below, there is now strong evidence that the decision to enter mitosis involves considerably more than the tyrosine dephosphorylation of Cdc2. However, a thorough understanding of the kinase/phosphatase network that controls the phosphotyrosine content of Cdc2 will provide a firm foundation for understanding other facets of mitotic regulation.

Our laboratory has made substantial contributions to understanding the molecular mechanisms controlling the activation of the Cdc2 protein. For our studies, we utilize cell-free extracts from *Xenopus* eggs. Due to pioneering work in a number of the laboratories, it is now possible to re-create essentially all of the events of the cell cycle in these extracts. Consequently, it is feasible to study the molecular mechanisms of Cdc2 regulation in intricate detail with this experimental system. To facilitate these studies, we make extensive use of recombinant DNA technology to overproduce cell cycle proteins in either bacteria or baculovirus-infected insect cells. Moreover, in conjunction with our biochemical studies, we are taking advantage of the fission yeast system to exploit genetic approaches to identify novel *Xenopus* regulators of the cell cycle.

387. Coordination of S-phase with mitosis

Soo-Mi Kim, Stephanie Yanow, Juan Ramirez-Lugo

In eukaryotic cells, biochemical pathways have evolved to ensure that initiation of a late cell cycle event is dependent upon an early event. Particularly, entry into mitosis normally depends on successful completion of DNA replication. Perturbation of DNA synthesis by DNA replication inhibitors or DNA damage arrests the cell cycle at a point termed the mitotic entry checkpoint. Mutants that disrupt the checkpoint control have been isolated from various organisms, suggesting that checkpoint control is due to an active mechanism rather than an intrinsic feature of mitosis itself. By using the *Xenopus* egg extract system, it has been shown that ongoing chromosome replication can induce the mitotic checkpoint, while inhibition of DNA replication by DNA polymerase inhibitors enhances this effect. Furthermore, DNA synthesis on artificial single-stranded DNA templates in S-phase extract is sufficient to delay mitosis, indicating that the DNA replication itself generates a signal triggering the arrest at S/M transition. Based on these observations, it is generally believed that the S-M checkpoint mechanism can sense a particular structure of DNA replication, e.g., protein complex assembled at the replication fork or the single-stranded DNA, and transmit a signal to the downstream cell cycle machinery. We are currently trying to employ a few different approaches to isolate the key players in the checkpoint pathway and to characterize their function in *Xenopus* egg extracts.

388. Regulation of ***Xenopus*** Plx1, a protein kinase that phosphorylates and activates the Cdc25 protein phosphatase

Hae Yong Yoo

The Cdc25 protein is a dual-specificity phosphatase that dephosphorylates both threonine-14 and tyrosine-15 on Cdc2 and activates the kinase activity of Cdc2 at the onset of mitosis. The Cdc25 protein has a highly conserved C-terminal catalytic domain and less conserved N-terminal regulatory domain rich in Ser-Pro and Thr-Pro motifs. The activity of the Cdc25 protein is regulated by phosphorylation. During mitosis, the N-terminal regulatory domain becomes highly phosphorylated on serine and threonine residues and the catalytic activity increases 5-10 fold. Many studies have indicated that both the Cdc2/cyclin B complex, as well as an additional unidentified kinase phosphorylate Cdc25 at mitosis.

We set out to purify the unknown kinase responsible for activating Cdc25 from *Xenopus* extracts. This attempt was aided greatly by our finding that a major Cdc25-specific kinase distinct from Cdc2/cyclin B associates with the N-terminal domain of Cdc25. Using a *Xenopus* Cdc25 affinity column and several conventional chromatographic methods, we purified a Cdc25-specific kinase activity approximately 2500-fold. We isolated several micrograms of this protein (p67) and sequenced four of its tryptic peptides. PCR primers designed for two of these peptides were used to amplify a segment of the cDNA encoding p67. The PCR fragment was used to isolate a full-length 2.4 kb cDNA that contains all four tryptic peptide sequences. The amino acid sequence indicates that it is a typical Ser-Thr kinase with its catalytic domain in the N-terminal half of the polypeptide. It is clearly a member of the Polo family of protein kinases. Accordingly, we have named it Plx1, for Polo-like kinase from *Xenopus*. Recombinant six-histidine-tagged Plx1 was then produced in Sf9 insect cells and purified on nickel agarose. This kinase phosphorylates and activates Cdc25 *in vitro*. We are now studying the regulation of this enzyme, which may be a key regulator of mitosis in all eukaryotic cells.

389. The ***Xenopus*** Chk1 protein kinase mediates a caffeine-sensitive pathway of checkpoint control in cell-free extracts

Akiko Kumagai, Seong-Yun Jeong

The entry into mitosis is controlled by regulatory proteins that ensure the proper segregation of replicated chromosomes to daughter cells. The integrity of chromosomal DNA is under constant surveillance during the cell cycle. In eukaryotic cells, when chromosomes become damaged or cannot be replicated completely, mitosis is prevented by checkpoint mechanisms until two intact copies of the genome can be produced. A key target of these checkpoint pathways is the Cdc2-cyclin B complex, also known as maturation or M-phase promoting factor (MPF). The Cdc2-cyclin B complex, once activated at the G2/M transition, phosphorylates a myriad of proteins that carry out the various processes of mitosis such as nuclear disassembly and chromosome segregation.

In the presence of damaged or unreplicated DNA, Cdc2-cyclin B is kept inactive due to inhibitory phosphorylation of the Tyr-15 and Thr-14 residues of Cdc2. These phosphorylations are carried out collectively by the kinases Wee1 and Myt1. At the onset of mitosis, the phosphatase Cdc25C removes these inhibitory phosphate groups and thereby activates Cdc2-cyclin B. The activity of Cdc25C is strictly regulated, being low during interphase and high at mitosis. In recent studies in humans and *Xenopus*, it has been shown that Cdc25 is negatively regulated during interphase by the binding of 14-3-3 proteins. The inactive form of Cdc25 found prior to mitosis is phosphorylated on Ser-216 and Ser-287 in humans and *Xenopus*, respectively. This phosphorylation, which occurs in a consensus 14-3-3 binding site, mediates the association between Cdc25 and 14-3-3 proteins. This phosphorylation-dependent interaction appears to suppress the activation and/or action of Cdc25. Mutants of Cdc25 that cannot be phosphorylated at this residue and thus, cannot bind 14-3-3 proteins override the unreplicated/damaged DNA checkpoint(s), suggesting that Cdc25 is a target of checkpoint regulation.

We have analyzed the role of the protein kinase Chk1 in checkpoint control by using cell-free extracts from *Xenopus* eggs. Recombinant *Xenopus* Chk1 (Xchk1) phosphorylates the mitotic inducer Cdc25 *in vitro* on

multiple sites including Ser-287. The Xchk1-catalyzed phosphorylation of Cdc25 on Ser-287 is sufficient to confer the binding of 14-3-3 proteins. Egg extracts from which Xchk1 has been removed by immunodepletion are strongly but not totally compromised in their ability to undergo a cell cycle delay in response to the presence of unreplicated DNA. Cdc25 in Xchk1-depleted extracts remains bound to 14-3-3 due to the action of a distinct Ser-287-specific kinase in addition to Xchk1. Xchk1 is highly phosphorylated in the presence of unreplicated or damaged DNA, and this phosphorylation is abolished by caffeine, an agent which attenuates checkpoint control. The checkpoint response to unreplicated DNA in this system involves both caffeine-sensitive and caffeine-insensitive steps. Our results indicate that caffeine disrupts the checkpoint pathway containing Xchk1.

390. Positive regulation of Wee1 by Chk1 and 14-3-3 proteins

Joon Lee

Wee1 inactivates the Cdc2-cyclin B complex during interphase by phosphorylating Cdc2 on Tyr-15. The activity of Wee1 is highly regulated during the cell cycle. In frog egg extracts, it has been established previously that *Xenopus* Wee1 (Xwee1) is present in a hypophosphorylated, active form during interphase and undergoes down-regulation by extensive phosphorylation at M-phase. We report that Xwee1 is also regulated by association with 14-3-3 proteins. Binding of 14-3-3 to Xwee1 occurs during interphase, but not M-phase, and requires phosphorylation of Xwee1 on Ser-549. A mutant of Xwee1 (S549A) that cannot bind 14-3-3 is substantially less active than wild-type Xwee1 in its ability to phosphorylate Cdc2. This mutation also affects the intranuclear distribution of Xwee1. In cell-free kinase assays, Xchk1 phosphorylates Xwee1 on Ser-549. The results of experiments in which Xwee1, Xchk1, or both were immunodepleted from *Xenopus* egg extracts suggested that these two enzymes are involved in a common pathway in the DNA replication checkpoint response. Replacement of endogenous Xwee1 with recombinant Xwee1-S549A in egg extracts attenuated the cell cycle delay induced by addition of excess recombinant Xchk1. Taken together, these results suggest that Xchk1 and 14-3-3 proteins act together as positive regulators of Xwee1.

391. Respective roles of Xchk1 and Xcds1 in checkpoint pathways

Zijian Guo, Wenhui Li, Jianghai Wang

In eukaryotic cells, biochemical pathways have evolved to ensure that initiation of a late cell cycle event is dependent upon an early event. Particularly, entry into mitosis normally depends on successful completion of DNA replication and of DNA repair. Perturbation of DNA synthesis or the presence of irreparable DNA damage arrests the cell cycle at a point termed the mitotic entry checkpoint. Mutants that disrupt the checkpoint control have been isolated from various organisms, indicating that the checkpoint control is due to an active mechanism involving kinase cascade signaling.

We have isolated one of the checkpoint kinases, *Xenopus* Cds1 (Xcds1), by using database analysis, polymerase chain reaction (PCR) amplification, and library screening. Xcds1 is phosphorylated and activated by the presence of some simple DNA molecules with double-stranded ends in cell-free *Xenopus* egg extracts. Xcds1 is not affected by aphidicolin, an agent that induces DNA replication blocks. In contrast, another checkpoint kinase *Xenopus* Chk1 (Xchk1), responds to DNA replication blocks, but not the presence of double-stranded DNA ends. Both Xcds1 and Xchk1 phosphorylate Cdc25 within a 14-3-3 binding site, which is required for a normal checkpoint response. Immunodepletion of Xcds1 (and/or Xchk1) from egg extracts does not attenuate the cell cycle delay induced by double-stranded DNA ends, suggesting that there are redundant mechanisms in this system that prevent mitosis in the presence of this type of DNA damage. Our findings indicate that in *Xenopus*, and perhaps other vertebrates, there are two pathways that respond to different kinds of signals from the DNA. Xcds1 and possibly another kinase(s) are activated by double-stranded DNA ends. In contrast, Xchk1 is unaffected by double-stranded DNA ends, but responds efficiently to replication blocks and UV damage, which also may act at least in part by perturbing replication.

392. Requirement for Atr in phosphorylation of Chk1 and cell cycle regulation in response to DNA replication blocks and UV-damaged DNA in ***Xenopus*** egg extracts

Zijian Guo

The checkpoint kinase Xchk1 becomes phosphorylated in *Xenopus* egg extracts in response to DNA replication blocks or UV-damaged DNA. Xchk1 is also required for the cell cycle delay that is induced by unreplicated or UV-damaged DNA. In this report, we have removed the *Xenopus* homolog of ATR (Xatr) from egg extracts by immunodepletion. In Xatr-depleted extracts, the checkpoint-associated phosphorylation of Xchk is abolished, and the cell cycle delay induced by replication blocks is strongly compromised. Xatr from egg extracts phosphorylated recombinant Xchk1 *in vitro*, but not a mutant form of Xchk1 (Xchk1-4AQ) containing nonphosphorylatable residues in its four conserved SQ/TQ motifs. Recombinant human ATR, but not a kinase-inactive mutant, phosphorylated the same sites in Xchk1. Furthermore, the Xchk1-4AQ mutant was found to be defective in mediating a checkpoint response in egg extracts. These findings suggest that Xchk1 is a functionally important target of Xatr during a checkpoint response to unreplicated or UV-damaged DNA.

393. Claspin, a novel protein required for the activation of Chk1 during a DNA replication checkpoint response in ***Xenopus*** egg extracts

Akiko Kumagai, Joon Lee, Daniel A. Gold

We have identified Claspin, a novel protein that binds to *Xenopus* Chk1 (Xchk1). Binding of Claspin to Xchk1 is highly elevated in the presence of DNA templates that trigger a checkpoint arrest of the cell cycle in *Xenopus* egg extracts. Xchk1 becomes phosphorylated during a checkpoint response, and we demonstrate directly that this phosphorylation results in the activation of Xchk1. Immunodepletion of Claspin from egg extracts abolishes both the phosphorylation and activation of Xchk1. Furthermore, Claspin-depleted extracts are unable to arrest the cell cycle in response to DNA replication blocks. Taken together, these findings indicate that Claspin is an essential upstream regulator of Xchk1. We are currently analyzing various facets of the structure and function of Claspin.

394. Repeated phosphopeptide motifs in Claspin mediate the regulated binding of Chk1

Akiko Kumagai

In vertebrates, the checkpoint-regulatory kinase Chk1 mediates cell cycle arrest in response to DNA replication blocks and UV-damaged DNA. The activation of Chk1 depends on both the upstream regulatory kinase ATR and Claspin. Claspin is a large acidic protein that becomes phosphorylated and binds to Chk1 in the presence of checkpoint-inducing DNA templates in *Xenopus* egg extracts. Through deletion analysis, we have identified a 57 amino acid region of Claspin that is both necessary and sufficient for binding to *Xenopus* Chk1. This Chk1-binding domain (CKBD) contains two highly conserved repeats of approximately ten amino acids. A serine residue in each repeat (Ser-864 and Ser-895) undergoes phosphorylation during a checkpoint response. A mutant of Claspin containing non-phosphorylatable amino acids at positions 864 and 895 cannot bind to Chk1 and is unable to mediate its activation. These experiments indicate that two phosphopeptide motifs in Claspin are essential for checkpoint signaling.

395. Claspin, a Chk1-regulatory protein, monitors DNA replication on chromatin independently of RPA, ATR, and Rad17

Joon Lee, Akiko Kumagai, William G. Dunphy

Claspin is required for the ATR-dependent activation of Chk1 in *Xenopus* egg extracts containing incompletely replicated DNA. We show here that Claspin associates with chromatin in a regulated manner during S-phase. Binding of Claspin to chromatin depends on the pre-replication complex (pre-RC) and Cdc45 but not on replication protein A (RPA). These dependencies suggest that binding of Claspin occurs around the time of initial DNA unwinding at replication origins. By contrast, both ATR and Rad17 require RPA for association with DNA. Claspin, ATR, and Rad17 all bind to chromatin independently. These findings suggest that Claspin plays a role in monitoring DNA replication during S-phase. Claspin, ATR, and Rad17 may collaborate in checkpoint regulation by detecting different aspects of a DNA replication fork.

396. ***Xenopus*** Drf1, a regulator of Cdc7, accumulates on chromatin in a checkpoint-regulated manner during an S-phase arrest

Stephanie K. Yanow, Daniel A. Gold, Hae Yong Yoo, William G. Dunphy

We have cloned a *Xenopus* Dbf4-related factor named Drf1 and characterized this protein by using *Xenopus* egg extracts. Drf1 forms an active complex with the kinase Cdc7. However, most of the Cdc7 in egg extracts is not associated with Drf1, which raises the possibility that some or all of the remaining Cdc7 is bound to another Dbf4-related protein. Immunodepletion of Drf1 does not prevent DNA replication in egg extracts. Consistent with this observation, Cdc45 can still associate with chromatin in Drf1-depleted extracts, albeit at significantly reduced levels. Nonetheless, Drf1 displays highly-regulated binding to replicating chromatin. Treatment of egg extracts with aphidicolin results in a substantial accumulation of Drf1 on chromatin. This accumulation is blocked by addition of caffeine and by immunodepletion of either ATR or Claspin. These observations suggest that the increased binding of Drf1 to aphidicolin-treated chromatin is an active process that is mediated by a caffeine-sensitive checkpoint pathway containing ATR and Claspin. Abrogation of this pathway also leads to a large increase in the binding of Cdc45 to chromatin. This increase is substantially reduced in the absence of Drf1, which suggests that regulation of Drf1 might be involved in the suppression of Cdc45 loading during replication arrest. We also provide evidence that elimination of this checkpoint causes resumed initiation of DNA replication in both *Xenopus* tissue culture cells and egg extracts. Taken together, these observations argue that Drf1 is regulated by an intra-S-phase checkpoint mechanism that downregulates the loading of Cdc45 onto chromatin containing DNA replication blocks.

397. Phosphorylated Claspin interacts with a phosphate-binding site in the kinase domain of Chk1 during ATR-mediated activation

Seong-Yun Jeong, Akiko Kumagai, Joon Lee, William G. Dunphy

Claspin is essential for the ATR-dependent activation of Chk1 in *Xenopus* egg extracts containing incompletely replicated or UV-damaged DNA. The activated form of Claspin contains two repeated phosphopeptide motifs that mediate its binding to Chk1. We show that these phosphopeptide motifs bind to Chk1 by means of its N-terminal kinase domain. The binding site on Chk1 involves a positively-charged cluster of amino acids that contains lysine 54, arginine 129, threonine 153, and arginine 162. Mutagenesis of these residues strongly compromises the ability of Chk1 to interact with Claspin. These amino acids lie within

regions of Chk1 that are involved in various aspects of its catalytic function. The predicted position on Chk1 of the phosphate group from Claspin corresponds to the location of activation-loop phosphorylation in various kinases. In addition, we have obtained evidence that the C-terminal regulatory domain of Chk1, which does not form a stable complex with Claspin under our assay conditions, nonetheless has some role in Claspin-dependent activation. Overall, these results indicate that Claspin docks with a phosphate-binding site in the catalytic domain of Chk1 during activation by ATR. Phosphorylated Claspin may mimic an activating phosphorylation of Chk1 during this process.

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Summary: If we could simply look inside a cell and see its molecular components in all their complexes and conformations, cell biology would be all but revealed. We are developing cryoelectron microscopy–based technologies to do this for at least the largest macromolecular complexes, hoping to show both how individual proteins work together as large "machines," and how those machines are organized into "assembly lines" within living cells. Our projects range from imaging individual macromolecules to complex biochemical reactions to organelles and even to intact cells. We use two basic data collection strategies. The first, called "tomography," involves imaging a single unique object (such as a cell) from multiple directions and then merging those projections into a three-dimensional reconstruction. The second, called "single particle analysis," involves imaging a large number of identical copies of a target object (such as a purifiable protein complex), and again merging the images to produce a three-dimensional reconstruction. Both techniques are in their infancy, but together hold the promise of completing what I like to call the "structural biology continuum" in a step-wise fashion by showing first how individual proteins (visible by X-ray crystallography and NMR spectroscopy) come together to form complexes (visible by single particle analysis), and how those complexes are organized within living cells (visible by electron tomography).

Both single particle analysis and tomography begin by spreading the sample in a thin film (~300 nm) across an electron microscope grid and then plunging it into liquid ethane, which causes the water to form vitreous, rather than crystalline, ice, and preserves the sample in a native state without any unnatural fixatives, resins, or stains. For tomography, the sample is imaged from a series of angles by incrementally tilting a goniometer through ~140 degrees. For single particle analysis, each copy of the sample ("particle") structure freezes with a random orientation with respect to the plane of the grid so that tilting is not necessary. Instead, images of hundreds of thousands of individual particles are recorded and then aligned and averaged in three-dimensions computationally. The target resolution for single particle analysis is .4-.8 nm, where the secondary structure of a particle can be clearly resolved and fitting atomic models of the components is unambiguous. The target resolution for tomography is 2-4 nm, where the identity, location, and orientation of individual macromolecules can be seen in their cellular contexts.

This first year of the lab has been invested into establishing a state-of-the-art cryoelectron microscopy laboratory and starting a number of projects covering both technology development and its application to an initial sampling of key biological problems.

398. Electron tomography of the major model cells from the "minimal" cell to yeast

Greg Henderson, Gavin Murphy, Achilleas Frangakis, Surelys Galano, William Tivol, Prabha Dias, Grant Jensen

Within just the past few years it has become possible to visualize the structures of small, whole cells in their native, hydrated state at resolutions sufficient to identify some of the largest macromolecular complexes by cryoelectron tomography. We are preparing to image all the major model cells from a "minimal" cell to yeast by this technique to compare and contrast the ultrastructure of cells throughout a range of complexities and provide the information about their molecular organization needed for future computational modeling. As a "minimal" cell we have cultured *Mycoplasma genitalium*, which has only ~500 genes and is simultaneously the smallest known free-living organism and the most comprehensively characterized in terms of its structural genomics. As a "minimal" but more genetically modifiable cell we are imaging *Mesoplasma florum*, which again has only ~600 genes and is extraordinarily small. The next most complicated, historical "model" cells are *Caulobacter crescentus* and *E. coli,* which are both being cultured and imaged in the lab. *Caulobacter* represents a simple example of asymmetric cell division and is the target of an ambitious program to comprehensively elucidate its genetic regulatory circuitry en route to whole cell modeling. *E. coli* is of course the most widely studied cell of all. We have identified the smallest and simplest known eukaryotic cell, *Ostreococcus tauri*, which like *E. coli* and *Caulobacter* has only ~3000 genes and is less than a micron in diameter, but which represents a large step up the complexity ladder and has a fully enclosed nucleus, multiple linear chromosomes, a tiny Golgi apparatus, mitochondrion, and chloroplast. This will likely be the first eukaryotic cell to be computationally modeled. Finally at the most complex end of the spectrum we have begun to image a small yeast mutant which we hope will place in structural context the vast body of research on this classic model cell. In each case the goal is to determine the native, three-dimensional structures of the cells and search them computationally for consistent features such as cytoskeletal elements and patterns of protein localization.

399. Tomography of HIV at various stages of maturation

Jordan Benjamin, Bill Tivol, Grant Jensen

HIV is perhaps the most extensively studied "organism" in terms of structural biology. It has three layers, including an outer lipid bilayer envelope, a protein shell of "matrix" subunits, and an inner protein "capsid" shell housing the genome. The structures of nearly every component of HIV have been determined in isolation, some in many different conformations and with various inhibitors, but it is still unclear how individual subunits come together to form the intact virion. Furthermore, the virus goes through multiple stages of maturation wherein the structures of these shells change in poorly understood ways. We are working to determine the three-dimensional structure of HIV at various stages of maturation by cryoelectron tomography. The structures that we obtain will allow us to confirm or refute various models that have been proposed about the organization of the protein shells and the changes underlying maturation.

400. Single particle analysis of the proteasome

Cristina Iancu, Grant Jensen

The proteasome is a large (~800 kDa) protein complex that proteolyses ubiquitinated substrates. The structure of its 20S core is known by X-ray crystallography, but the structures of the multi-component regulatory "base" and "lid" are unknown. We are imaging the 20S core alone as part of an attempt to understand and expand the resolution limitations of the technique. For this the core particle is ideal in that it is commercially available in highly purified form, its structure is known, and it is large and rigid. We are preparing to record on the order of 10 million particle images and average them to determine a near-atomic resolution (4-8 Angstrom) structure.

In a complementary study we are also imaging full 26S proteasome to determine the structure of the base and lid components.

401. Multi-slice image simulations

Bernard Heymann, Grant Jensen

As part of our technology development efforts, we are writing custom software to simulate electron microscope images of frozen-hydrated protein complexes. Electron microscope images contain a number of imperfections, including the loss of information at certain spatial frequencies described by the contrast transfer function, spatial distortion, and beam-tilt induced coma. When thousands of images of individual proteins are averaged, these imperfections must be computationally corrected as well as possible before the images are mutually aligned and averaged. We have many ideas for new and improved algorithms for these tasks that must be rigorously tested through application to simulated images. Further, we wish to explore how well experimental images can be aligned by quantitatively comparing experimental images to simulated images in a neighborhood of projection directions.

There are several methods available to simulate electron microscope images of varying accuracy. We have chosen to implement a "multi-slice" approach, wherein the phase changes in the incident beam are tracked as a function of depth through the specimen. In addition, we are explicitly treating surrounding water molecules and the various effects of spherical aberration, defocus, astigmatism, and shot noise in the detectors.

402. Reducing charging effects at helium temperatures

Bill Tivol, Tim Barnes, Grant Jensen

When a high-voltage electron beam passes through a vitrified biological sample, covalent bonds are destroyed, secondary electrons escape, and the structure is gradually destroyed. Past experience has shown that cooling the sample to liquid nitrogen (~90K) or even liquid helium temperatures (~10K) seems to slow this radiation damage, perhaps by constraining molecular fragments and reactive intermediates. Unfortunately, such cooling also dramatically reduces the conductivity of the underlying carbon support film and exacerbates the problem of having temporary unmatched positive charges that cause the sample to move and distort.

We are testing whether thin films of titanium on our samples can alleviate this charging problem at liquid helium temperatures (~10K). Among the metals that have high conductivity near absolute zero, titanium is relatively light (thus producing less background signal) and attractive for this purpose.

403. Extrapolation of data into the "missing wedge"

Bill Tivol, Bernard Heymann, Grant Jensen

In electron tomography, the sample is prepared as a thin sheet of material and imaged throughout a range of tilt angles. This range is limited, however, to approximately +/- 70 degrees because the path length through the sample becomes exponentially large as the tilt angle approaches 90 degrees. Following the projection theorem, the Fourier transform of each projection is a central slice of the object's three-dimensional transform. Thus if the projection angles are limited, there arises a "wedge" of missing data in the three-dimensional transform of the object that gives rise to an anisotropic resolution degradation in the reconstruction. Nevertheless there are two physical laws that constrain the scattering phenomena: unitarity, which means that the sum of the scattering in all directions must be equal to the incident beam; and analyticity, which means that the scattering amplitudes and all their derivatives must be continuous. We are trying to use these constraints to extrapolate data into the missing wedge and improve the quality of our tomographic reconstructions. Initially the ideas and possible algorithms will be tested with simulated data.

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Summary: The focus of the lab has been the coupling of theoretical, computational, and experimental approaches for the study of structural biology. In particular, we have placed a major emphasis on developing quantitative methods for protein design with the goal of developing a fully systematic design strategy that we call "protein design automation." Our design approach has been captured in a suite of software programs called ORBIT (Optimization of Rotamers By Iterative Techniques) and has been applied to a variety of problems ranging from protein fold stabilization to enzyme design.

404. Evaluating a force field via fixed composition sequence design calculations

Oscar Alvizo[1], Stephen L. Mayo

A finely-tuned force field is essential to protein design and protein folding. Proper tuning is problematic, however, due to difficulties in determining the appropriate scale factors for the force field parameters and in calculating the energy of proteins in the denatured state. ORBIT's force field was improved through iterative rounds of protein design calculations and experimental measurement of predicted sequence stabilities. In order to keep the energy of the denatured state constant, design calculations were performed under fixed amino acid composition restraints. Energy discrepancies between computational predictions and experimental results could then be attributed to flaws in the force field's ability to predict folded state sequence energies. The accuracy of the force field was quantified in three ways: by comparing the amino acids of the predicted sequences to the wild type, by determining the sequence bias required to recover the wild-type sequence, and by comparing the experimental stabilities to the predicted sequence energies. The high native stability of the designed protein (the β1 domain of protein G) and the reduced sequence space resulting from the fixed composition restriction make it highly unlikely that predicted sequences that are vastly different from wild type will be more stable. Therefore, if the predicted sequences are significantly different from wild type, then the force field needs improvement.

Calculations started at a wild-type sequence bias of 0.0 kcal/mol, which was increased by increments of 1.0 or 0.5 kcal/mol until the wild-type sequence was recovered. With standard parameters, a large bias of 6.0 kcal/mol was required to recover the wild-type sequence, highlighting the inaccuracies in the force field. Tuning decreased this to 2.5 kcal/mol. It was concluded that a solvent exclusion-based solvation model is more accurate than a surface area-based one. Disallowing formation of hydrogen bonds at the surface and use of a larger rotamer library also improved accuracy. These improvements to the force field increased the predictive power of the unbiased calculation by a factor of 5, 4.1, and 1.2 at core, boundary, and surface positions, respectively.

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405. Antibody design

Eun-jung Choi, Stephen L. Mayo

Antibodies are prominent targets of many different protein engineering efforts. This is primarily due to their wide use in the laboratory, as well as in medicine. The fact that recombinant antibodies and their fragments now represent over 30% of all biological proteins undergoing clinical trials illustrates the enormous medical application potential of antibody research. Currently available antibodies have many limitations including large size, immunogenicity, poor stability, lack of diversity, and insufficient specificity. These problems have prompted researchers to adopt several different strategies to engineer improved antibodies. We are applying our protein design algorithms to computationally design antibodies with specific modifications to overcome these limitations. The multidomain characteristic of antibodies encouraged us to work with single-chain Fv antibodies (scFv), which consist of a single variable heavy-chain and variable light-chain domains connected by a linker. This is the smallest fragment that retains the binding specificity and affinity of the whole antibody. We are in the process of selecting a scFv that can serve as a scaffold for functional antibody designs. Our lab has already shown that computational methods can be used to build functionality into a neutral protein scaffold. Antibodies have also been shown to be capable of catalyzing reactions based on their binding affinity to transition state analogs. We intend to build on these results and use our computational methods to design catalytic function into antibodies.

406. Determining the crystal structure of a fully redesigned α-helical protein

Geoffrey Hom[1], Stephen L. Mayo

Computational protein design is used to determine an amino-acid sequence that will adopt a target structure. Previously, we had designed a novel sequence to adopt a 51-amino-acid, alpha-helical fragment of the engrailed homeodomain (EH). The design was riskier than previous ones of EH, because no knowledge of the wild-

type sequence (except for one glycine) was included in any step of the design process; i.e., EH was fully redesigned (EH-FR). The design appears to have been successful: EH-FR has a thermal denaturation temperature >99°C (versus 49°C for the wild type) and unfolds cooperatively in guanidinium chloride. 1D ^{1}H-NMR spectra indicate EH-FR is well-folded and α-helical.

It is unknown how closely EH-FR adopts the wild-type EH structure. To that end, we are determining EH-FR's crystal structure. Two distinct crystallization conditions have been found, and one yielded crystals diffracting to $\leq$2 Å. However, we were not successful in using molecular replacement to determine the phases, and so we are currently using heavy-metal derivatives to obtain experimental phases.

EH-FR's crystal structure will invariably differ at least slightly from the target structure. Once the crystal structure has been solved, it may be interesting to compare sequences designed to adopt the EH structure with sequences designed to adopt the EH-FR structure.

The EH-FR sequence was selected computationally from ~10^{45} possibilities, well beyond the limits of physical selection methods. Nevertheless, our search algorithms may be insufficient for the full redesign of large or even average-sized proteins. Accordingly, we are also attempting to improve the suite of algorithms used in protein design.

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407. Protein-protein recognition

Possu Huang[1], Stephen L. Mayo

Biologically functional proteins carry out their actions by interacting with other components in the cell, and protein-protein association serves a very important role. Proteins can bind directly to their targets to carry out a function or they can bind specifically to themselves forming higher-order structures to perform their duties. We are interested in learning how proteins can utilize their surface residues to interact with other proteins. We are also curious about the influence protein backbone geometry has on complex formation. A special fast fourier transform (FFT)-based docking algorithm was developed to study dimer formation. We can reproduce certain wild-type dimer geometries based on a monomer backbone and we can also generate *de novo* dimers based on protein surface complementarities. The protein design algorithm developed in the Mayo group is being used in conjunction with the FFT docking algorithm to redesign the binding interface. We intend to capture the physics and chemistry in the protein-protein recognition process by simulating the process on computers. Redesigned protein dimers are being synthesized and tested. Their binding specificities and affinities are being assessed by NMR and analytical ultracentrifugation methods.

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408. Computational design of a novel enzyme for catalysis of a pericyclic reaction

Kirsten S. Lassila[1], Stephen L. Mayo

We are working towards the goal of fully automated enzyme design. In particular, we hope to apply computational protein design methods to the task of creating a completely novel catalyst for the Claisen rearrangement of chorismate to prephenate. Naturally catalyzed by the chorismate mutases, this reaction offers many desirable features as an early test of enzyme design methods. The reaction is a first-order sigmatropic rearrangement of a single substrate and has neither intermediate steps nor involvement of catalytic groups such as general acids or bases. The reaction has been extensively studied in many contexts—as a rare enzyme-catalyzed pericyclic process, as an essential step in the biosynthesis of aromatic compounds, and as a rare example of a reaction that occurs through identical mechanisms enzymatically and in solution. Our method of enzyme design involves identifying amino acid sequences likely to bind to an *ab initio* transition state structure of the chorismate-prephenate rearrangement. As a part of this process, we are testing new methods that allow translation and rotation of the transition state structure within a binding cavity while simultaneously optimizing protein side chain identity and conformation. These methods will be generally applicable to the design of ligand binding sites and enzyme active sites. The ability to computationally design new reaction catalysts can be expected to have important implications for organic synthesis, bioremediation, and biotechnology.

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409. Computational design of a novel aldolase

Jessica Mao[1], Stephen L. Mayo

Designed enzymes are attractive industrially for their efficiency, substrate specificity, and stereoselectivity. To date, there are few enzymes used in organic synthesis. The aldol condensation is one of the most important and utilized carbon-carbon bond-forming reactions in synthetic chemistry. It is the reaction between two aldehyde/ketone groups, yielding a β-hydroxy-aldehyde/ketone that upon dehydration by acid or base affords an enone. While natural aldolases are efficient, they are very limited in their substrate range. Novel aldolases that catalyze reactions between desired substrates would be a powerful synthetic tool. They would expand the limited repertoire of designed enzymes and further our understanding of their structure/function relationship. We are adopting the "compute and build" design cycle that combines theory, computation, and experiment to rationally design a novel aldolase. Our initial design will catalyze the reaction between acetone and benzaldehyde via the enamine mechanism that natural class I aldolases utilize. The backbone structure of triosephosphate isomerase (TIM) serves as the scaffold for building a novel protein catalyst. We chose the $(\alpha/\beta)_8$-barrel fold because of its prevalence; this fold accounts for ~10% of all known proteins and all

but one are enzymes. The ubiquity of the fold and its ability to catalyze a wide variety of reactions make it an interesting system to study. Potential catalytic sites are being identified using the ORBIT protein design software, modeling a high-energy state in the reaction mechanism as a series of rotamers. The validity of this approach was indicated by identification of the natural catalytic site of catalytic antibody 33F12, which catalyzes the target reaction. After the potential catalytic sites are identified, we apply positive design to increase the affinity of the protein for the substrate. This can be done by designing in noncovalent interactions that stabilize the high-energy state. Next, we will use negative design to improve the specificity of the designed enzyme. By modifying the active site, binding of molecules other than the desired substrate can be made unfavorable. Experimentation will follow to confirm and optimize the design.

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410. ***In silico*** prediction of metallo-β-lactamases with improved catalytic efficiency

Peter Oelschlaeger, Stephen L. Mayo

Metallo-β-lactamases (MBLs) have recently raised major concern due to their ability to hydrolyze a broad spectrum of β-lactam antibiotics and thus, confer resistance to bacteria. In contrast to serine-β-lactamases, which use a serine-dependent mechanism to cleave the amide bond of the β-lactam ring, MBLs use zinc ions to activate a hydroxide for the nucleophilic attack. Two MBLs, IMP-6 and IMP-1, differ by a single residue, but have significantly different substrate spectra, and it is assumed that IMP-1 evolved from IMP-6. Molecular dynamics (MD) simulations of IMP-6 and IMP-1 in complex with near-transition-state intermediates of different β-lactam substrates were performed using a cationic dummy atom approach to represent the zinc ions (1). Stability scores for these complexes, obtained from multiple simulations with different starting conditions and at different temperatures, correlated well with experimental data (k_{cat}/K_M's) and provided insights into the molecular basis of substrate specificity (2).

Motivated by the observed correlation for wild-type enzymes, we selected point mutants of IMP-6 and submitted them to MD simulations in complex with ceftazidime, a broad-spectrum cephalosporin. Mutants with high stability scores (suggesting high catalytic efficiency and improved antibiotic resistance) were selected for experimental validation: they were expressed in *E. coli*, purified, and characterized biochemically. Catalytic efficiencies (k_{cat}/K_M's) toward ceftazidime and six additional β-lactam antibiotics were determined. Preliminary results with three well-expressed and well-folded mutants indicate that the predictions are correct. This *in silico* selection method led to mutants that have not been described previously but that represent a potential clinical threat because they can easily evolve from and are superior to IMP-6. In addition, the new mutants improved our understanding of how enzymes, i.e., metalloenzymes, can increase catalytic efficiency toward given substrates in the course of evolution: mutations close to the active site stabilized the near-transition-state intermediates by decreasing the flexibility of zinc-coordinating amino acids.

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411. Protein design on gp41 and the inhibition of HIV viral fusion

Joseph J. Plecs, Stephen L. Mayo

We are using protein-design techniques to increase the effectiveness of antiviral peptides in binding the HIV protein gp41. The gp41 protein facilitates the fusion of the viral membrane with a human cell membrane, an essential step in the life cycle of HIV. The active conformation of gp41 is known to include a six-helix bundle, an insight that has led to the development of successful antiviral strategies. Peptides derived from gp41 can interfere with viral fusion by forming nonproductive complexes with the native protein, replacing one of the six helices in the active conformation. At least one such peptide has been shown to be a useful therapeutic agent. Because the structure of the six-helix bundle has already been determined, we can use structure-based design to optimize the interactions of a peptide molecule with the viral complex. We have employed both fixed-backbone and flexible-backbone methods to design new peptides based on the gp41 sequence, and several of our designed peptides exhibit tighter helical-bundle formation than the wild-type protein sequences. Such improvements could result in peptides that are more effective fusion inhibitors. For instance, improved gp41-derived peptides could inhibit viral fusion at lower doses, remain associated with the viral complex for a longer time, or be included in an array of anti-gp41 peptides so that the virus cannot easily become resistant to them all. In order to better understand the mechanisms of stabilized helical-bundle formation, we are pursuing the structures of our redesigned helical bundles by X-ray crystallography. To date, we have obtained diffraction-quality crystals of one mutant that appear to incorporate a heavy metal and be suitable for multiple anomalous dispersion experiments.

412. Total computational design using approximate sequence optimization algorithms

Premal S. Shah[1], Geoffrey Hom[1], Scott A. Ross, Stephen L. Mayo

Sequence optimization in protein design can be performed using both exact and approximate algorithms. Exact algorithms are often preferred because the solution obtained for a target backbone is guaranteed to be the global minimum energy conformation (GMEC), while this is not the case for approximate algorithms. However, large designs often cause exact algorithms to stall, failing to produce a solution. In such cases, approximate algorithms

may be employed; these include Monte Carlo (MC) methods (1), genetic algorithms (2), and self-consistent mean field techniques (3). Two recently-developed approximate algorithms, Vegas (4) and FASTER (5), may also be used; these have been shown to provide better solutions than previous approximate algorithms.

Due to the enormous computational complexity, exact algorithms stalled and failed to provide a solution for the total sequence design (TSD) of the 51-residue engrailed homedomain (ENH). Therefore, we used three approximate algorithms, MC, Vegas, and FASTER, to obtain low-energy solutions for this design. The sequences obtained using Vegas and FASTER (ENH_VF) were both 40-fold mutants of ENH and were identical. The sequence obtained from MC (ENH_MC) was also a 40-fold mutant of the wild-type sequence (an 8-fold mutant of ENH_VF) but had a higher simulation energy than ENH_VF.

We constructed proteins composed of the ENH_VF and ENH_MC sequences and found that they had identical unfolding free energies (DG_{unfold} = 4.2 kcal mol^{-1}) as monitored by chemical denaturation; they were more stable than wild-type ENH by over three kcal mol^{-1}. Circular dichroism wavelength scans of the two variants were identical and showed both to be folded at 99ºC. 1D ^{1}H NMR spectra displayed sharp, narrow line widths that were well-dispersed, indicating well-folded proteins. The solution structure of ENH_VF was solved and comparison with the simulation-predicted structure reveals a common topology, indicating high fold specificity. Based on the thermodynamic and spectral similarity of ENH_VF and ENH-GKH, we concluded that ENH-GKH also shares the high specificity observed with ENH_VF.

Our design of ENH is only the second structurally-verified TSD performed to date. Our results also show that use of approximate sequence optimization algorithms in protein design when exact algorithms fail can lead to proteins that have significantly higher stabilities than the wild-type protein.

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413. Exploring the origins of binding specificity through the computational redesign of calmodulin

Julia M. Shifman, Stephen L. Mayo

Computational protein design was used to improve the binding specificity of calmodulin (CaM), a ubiquitous second messenger protein that has evolved to bind tightly to a variety of targets, and as such exhibits low binding specificity. Residues in or near the CaM binding interface were redesigned for improved interactions with one of its targets, smooth muscle myosin light-chain kinase (smMLCK); CaM interactions with alternative targets were not directly considered in the optimization. The predicted CaM sequences were constructed and tested for binding to a set of eight targets including smMLCK. The initial redesign of CaM boundary and surface residues in the binding interface yielded a CaM variant with suboptimal affinity to smMLCK. In order to improve these results, additional calculations were performed to explore the effects of altering a number of optimization parameters. The best CaM variant was obtained using a dielectric constant of 4.0, a large rotamer library, and a modified energy function that emphasizes intermolecular interactions. When tested for binding to the set of selected targets, this CaM variant exhibited up to a 155-fold increase in binding specificity. The increase in binding specificity was not due to improved binding to smMLCK, but to decreased binding to the alternative targets. This finding is consistent with the fact that the sequence of wild-type CaM is nearly optimal for interactions with numerous targets.

414. Implementing new models for protein electrostatics

Christina Vizcarra[1], Stephen L. Mayo

There are many possible models describing the interactions between the complex charge distribution in a protein and a highly polarizable solvent like water. Until now, only very simple methods have been used to include electrostatic solvation energy in protein design. We are implementing the Surface Area/Generalized Born method, which allows us to more accurately describe the energy of a protein's charge distribution in the context of its molecular geometry and surrounding solvent. Such improvements in the electrostatic energy component in protein design will have a significant impact in the areas of enzyme design and molecular recognition, for which polar and charged amino acids are key.

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415. Improving protein design calculations

Eric Zollars[1], Stephen L. Mayo

My projects since entering the Mayo lab have involved the development of the computational protein design package. One of the major assumptions that allows protein design to occur is the assignment of amino acid side chains to discrete conformations known as rotamers. Without this discretization, the complexity of computational protein design would be astronomical.

However, rotamer usage also leads to error in side-chain placement and consequently, errors in the predicted sequence and/or energy of the designed protein. An optimal rotamer library would contain a minimal number of rotamers that would still yield reasonably accurate results. The current rotamer library in use in the Mayo lab is derived from a published statistical survey of the conformations of side chains observed in protein crystal structures. Research by other labs since the published survey has shown inherent problems with this approach. Two new approaches were put forward: a Bayesian analysis of the structural database to compensate for areas of conformational space that were inadequate to produce statistical significance, and a study that included energy considerations in developing the conformations of the discrete rotamers. We developed these new libraries for use in our protein design algorithms, used them to design variants of a test protein, and confirmed the theoretical results experimentally by building the variants in the lab and characterizing them thermodynamically.

A burgeoning area of research in protein design is the development and implementation of better models to approximate the electrostatic interactions that occur in proteins in solution. We are in the process of validating the efficacy of a variety of these models for use in protein design calculations. Additionally, we are working on methods to improve the accuracy of the force field used in these calculations. By analyzing a large database of protein structures that have been characterized thermodynamically, it will be possible to determine the relative importance of the different energy terms in our force field. In the foreseeable future, this information should also allow a more accurate description of the electrostatic terms to be developed.

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Summary: We are interested in two groups of animal viruses, the alphaviruses and the flaviviruses. These viruses contain an RNA genome of about 11,000 nucleotides and are enveloped, having a lipid envelope that surrounds an icosahedral nucleocapsid. Both alphaviruses and flaviviruses are vectored by mosquitoes and infect both mosquitoes and a wide range of vertebrates. We wish to understand the replication of alphaviruses and flaviviruses at a molecular level and to study the evolution of these two virus groups and more broadly the evolution of all RNA viruses.

The flaviviruses contain about 70 members, most of which are important human pathogens causing hundreds of millions of cases of human disease annually. Diseases caused by the flaviviruses include yellow fever, dengue fever, and encephalitis. West Nile virus, which first appeared in the Americas four years ago in New York state and then spread rapidly over most of the United States, is a member of this family. The genome RNA of about 10,700 nucleotides is the only viral message RNA and is translated into a long polyprotein that is cleaved to give both structural proteins required for virus assembly and nonstructural proteins required for RNA replication.

The alphaviruses contain about 28 members and many cause febrile illness in humans that in some cases may progress to encephalitis or polyarthritis. The genome organization of alphaviruses differs from that of flaviviruses in that the genome of 11,700 nucleotides is translated into a long nonstructural polyprotein, whereas the structural proteins are translated as a distinct polyprotein from a subgenomic RNA. Remarkedly, however, the structures of the virions in the two groups have been found recently to be related. One of the two glycoproteins present on the exterior of the virions, called E in the case of flaviviruses and E1 in alphaviruses, are structurally identical. There are other similarities in the structures of the virions in the two groups, including the formation of heterodimers between E or E1 and the second external glycoprotein, which function in equivalent fashions. It seems clear that the virus structures, although they differ in important ways, are derived from a common ancestral source. We have suggested that this common structure may be important in the life of these viruses as arboviruses, capable of infecting both hematophagous arthropods and vertebrates.

We have started an ambitious collaboration with structural biologists at Purdue University to determine the structures of alphaviruses and flaviviruses to high resolution, and to determine the structures of all of the proteins encoded by the viruses to atomic resolution. We have now determined the structure of dengue virus to 9.5 Å and the structure of immature dengue virus that contains uncleaved prM to 14 Å. This resolution is high enough that the glycoproteins can be positioned with confidence in the virus particle, that carbohydrate side chains of the glycoproteins can be seen, and even the membrane spanning domains that anchor the glycoproteins can be identified and traced. We are also continuing a program to obtain atomic structures of a number of virus-encoded proteins, and to construct and test mutants of the viruses in order to understand the details of virus replication and assembly.

416. Structure of mature dengue virus particles

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As described last year (1), we have prepared highly purified dengue virions for structure determination by cryo-electron microscopy and subsequent image reconstruction. The structure has now been refined to a resolution of 9.5Å, which allows many additional features of the virion, such as the membrane spanning domains of the glycoproteins and the position of carbohydrate chains attached to the glycoproteins, to be visualized. Thus, carbohydrate chains attached to asparagines 67 and 153 of E can be seen, and it is interesting that a previous report based on biochemical data (2) found that only Asn-67 was glycosylated in dengue 2 virions produced in mosquito cells, as were our preparations of dengue 2. The reason for the differences in these results in not known, but our present results clearly demonstrate the power of high-resolution cryo-EM studies.

In the mature virion, which is 500 Å in diameter and has a fairly smooth surface, E is present in homodimers that associate side-to-side to form a herringbone pattern. Thus, the E dimers are not all present in quasi-equivalent positions, but can interact with other dimers in two different ways so as to result in two different environments for the dimers. The proteins of most regular viruses occupy quasi-equivalent positions, and the flaviviruses deviate from this rule. In the dimers, the fusion peptide is protected by being covered by the E partner in the homodimer.

The alpha-helical regions of the E molecules previously predicted to serve as stems that attach the ectodomain of the protein to the membrane-spanning anchors (3), as well as some of the N-terminal region of the M proteins, are buried in the outer leaflet of the viral membrane. Thus, these regions are membrane-associated rather than serving as true stems. As is the case for the

immature particles, the anchor regions of E and M each forms antiparallel E:E and M:M transmembrane alpha-helices, leaving their C-termini on the exterior of the viral membrane. The visualization of the transmembrane domains of E and prM/M is one of only a few determinations of the disposition of transmembrane proteins *in situ*.

The high-resolution structure does not show any direct interaction between the nucleocapsid core and envelope proteins. In contrast, in most enveloped viruses interactions between internal components of the virus (inside the lipid bilayer) and the external glycoproteins are known to occur. Thus, it is unclear how the nucleocapsid acquires an envelope during virus assembly.

The organization of the glycoprotein lattice in the prM-containing flavivirus particle is quite different from that in the mature virion. Further, the sizes of the immature and mature particle are very different (600 Å versus 500 Å). Thus, conversion of the immature to the mature particle requires a gross rearrangement of the E protein, during which prM-E heterodimers are converted to E-E homodimers and the particle condenses to 80% of its former size. It is predicted that fusion of the infecting virion with a cellular membrane during virus entry requires another gross rearrangement in which E homodimers reassemble into E homotrimers accompanied by an expansion of the particle by 10-20%.

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417. Structure of immature flavivirus particles

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Flaviviruses produce two surface glycoproteins that are used in the assembly of virus particles, called prM and E. These form a heterodimer when first produced. At some point in the assembly of virions, prM is cleaved by a cellular protease called furin to produce M. E then rearranges to form homodimers in the mature virion. It is possible to produce immature virus particles that contain uncleaved prM by growing the virus in the presence of ammonium chloride. This lysosomotrophic agent causes the pH of endosomal vesicles through which virus is transported to rise, which in turn prevents cleavage of prM by furin. It is not known if these immature particles are true intermediates in virus assembly, but reports in the literature have shown that it is possible to convert noninfectious, prM-containing particles into infectious virions by treatment with furin (1).

We have produced prM-containing dengue virus particles in this way. The structure of these particles has been determined to 14 Å resolution by cryo-electron microscopy and subsequent image reconstruction techniques. The particle is 600 Å in diameter and has 60 icosahedrally-organized, trimeric spikes on its surface. The structure of E of the related flavivirus tick-borne encephalitis virus has been determined to atomic resolution by conventional X-ray crystallography (2), and this structure has been fitted into the electron density of the prM-containing particle. Electron density not accounted for by E can then be assigned to prM and to the so-called stem-loop region of E, as well as to the membrane spanning domains of the glycoproteins. In this way it has been shown that each spike consists of three prM:E heterodimers. The three heterodimers in a spike do not occupy quasiequivalent positions, however, unlike the case for the structural proteins in most regular viruses. The pre-peptide components of the prM proteins of any one spike cover the fusion peptides at the distal ends of the E glycoproteins, preventing these hydrophobic fusion peptides from aggregating or interacting prematurely with cellular membranes, a step that occurs during entry of virus into host cells. This arrangement is similar to that of the E2 glycoproteins covering the fusion peptides of the E1 glycoproteins in the fusion competent, trimeric spikes of alphaviruses (3).

Each heterodimer on the surface of the prM-containing particle is associated with two transmembrane densities, one corresponding to the E and the other to the prM glycoprotein. Each transmembrane density represents two alpha helices arranged in an anti-parallel coiled-coil derived from the C-terminal region of E or M, leaving the carboxy terminus of each protein on the exterior of the virus, consistent with the predicted membrane spanning domains of the unprocessed polyprotein. The organization of the glycoprotein lattice in the prM-containing flavivirus particle is quite different from that in the mature virion, as described in the previous abstract. These results and those in the previous abstract represent one of the first visualizations of the membrane-spanning domains of glycoproteins *in situ*.

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418. Production and ***in vitro*** reactivation of immature yellow fever virus particles containing prM

Pritsana Chomchan, Jeroen Corver[1]

Recently, a cryoelectron microscopic reconstruction of immature, prM-containing particles of yellow fever virus (YFV) was determined as part of a collaboration between our group and the Structural Biology group at Purdue University. The structure of the immature particle of YFV is very similar to that of immature dengue (DEN) virions (see abstract 417). Whether prM-containing particles can be reactivated to mature infectious virions by furin, which cleaves at Arg-X-Arg-Arg or Arg-X-Lys-Arg, is still a question. In YFV virus, the primary furin cleavage site to generate the M protein is believed to be between amino acids 89 and 90 in the amino acid sequence ^{83}Arg-Ser-Arg-Arg-Ser-Arg-Arg-Ala^{90} but there is a second potential site in this sequence between amino acids 86 and 87. We have mutated the furin cleavage site in the full-length cDNA clone of YFV to Arg-Ser-Arg-Gly-Ser-Arg-Arg-Ala. RNA was transcribed *in vitro* and used to transfect BHK cells. RNA replication was detected by immunofluorescence, but infectious virions were not released into the medium. This is in agreement with a published report that immature particles of tick-borne encephalitis virus, a related flavivirus, are not infective. We attempted to confirm the production of immature YFV particles by precipitation of virus particles with polyethylene glycol followed by immunoprecipitation with mouse antiserum against YF E protein, but were unable to detect YFV E protein by western blot analysis due to the low efficiency of transfection. Furthermore, after 24 hr, some virus revertants arose that were able to produce progeny virus. To avoid problems with reversion, we are constructing another mutant YFV with a killed-furin cleavage site by deleting amino acids 87 and 88 of prM and changing ^{88}Arg-Arg^{89} to Ser-Ala.

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419. Construction of a full-length cDNA clone of dengue 2 virus

Pritsana Chomchan, Bong-Suk Kim[+]*, Edith Lenches, Michael Suh*

We have begun a collaborative project to construct a full-length clone of dengue 2 virus, using our laboratory strain, the S1 vaccine strain of the PR159 isolate. We have obtained the full-length clone of dengue 2 in a pBAC vector which was assembled by Professor Richard Kuhn's group at Purdue University. However, using SP6 RNA polymerase to transcribe RNA we obtained multiple transcription products due to an extra SP6 promoter present at the 3' end of the full-length sequence. Moreover, these RNA transcripts were not infectious for either C6/36 cells (mosquito) or BHK cells (vertebrate) due to errors introduced during PCR amplification. In addition there is some extra sequence derived from yellow fever virus at the 3' end. To get away from the restrictions inherent in pBAC clones, the full-length dengue 2 sequence (with the extra SP6 promoter and YF sequences) was subcloned by the Purdue group into the pACNAR plasmid, a moderate copy plasmid. We have removed the extra SP6 promoter and 3' yellow fever sequences from the 3' end of the dengue 2 sequence in the pACNAR plasmid and now can generate a single transcript using SP6 polymerase, but the transcript is still not infectious.

A full-length cDNA clone of dengue 4 virus has been described which was produced using low temperature (17°-25°C) and a low concentration of selective antibiotic (25 μg/ml ampicillin) for growing bacterial colonies after DNA transformation (1). New cDNA was generated by reverse transcription of dengue 2 genomic RNA and cloned into the pCR cloning vector; transformed bacteria were grown at 25°C on medium containing only 25 μg/ml of ampicillin. Although only a few plasmids contained dengue cDNA due to toxic sequences within the flavivirus sequence, we are using these cDNAs to replace sequences in the noninfectious pACNAR clone, hoping to restore infectivity.

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420. Purification and assay of the Sindbis virus nsP2 protease

Maria Farkas

In collaboration with the Structural Biology group at Purdue University, we cloned the protease domain of Sindbis nsP2 into a pET bacterial expression plasmid. Large quantities of the protein were purified and set up for crystallization under various combinations of temperature, buffer, salt and pH conditions at Purdue University. While we are still waiting for crystals to form, we developed a protease assay to determine whether the purified protein is functional under the various crystallization conditions.

Previously in our lab, full-length, wild-type Sindbis virus was cloned into a high-copy bacterial plasmid, and then the active site of the nsP2 protease was mutated to inactivate the protease. From this clone RNA was transcribed *in vitro* by SP6 RNA polymerase; and translated *in vitro* in a rabbit reticulocyte lysate (Promega). The translation product under optimal conditions is the polyprotein of approximately 280 kDa composed of nsP1, nsP2, nsP3, and nsP4 which served as a substrate for the purified nsP2 protease. We have optimized the protease assay, using various temperatures, incubation times, concentrations of enzyme and substrate, and buffers. After incubation at 30°C for 1 hour the protease cleaved the polyprotein in *trans* into the four nonstructural proteins, which were identified by SDS-PAGE. A time-course study showed that the protease cut the nsP1-nsp2 bond most rapidly, and cleavage of that bond was complete

within ten minutes. Incubations longer than 1.5 hours had no effect on the enzyme kinetics under our conditions.

We found that some of the crystallization buffers rendered the protease inactive. In addition, storage conditions affected the activity of the protease. Active purified protease preparations were stored at both 4˚C and -80˚C and periodically assayed. Preparations kept at -80˚C, and subsequently thawed and frozen many times were not stable and lost activity faster than preparations kept at 4˚C.

As a positive control, wild-type Sindbis viral RNA was translated and labeled under the same conditions. The products are the four nonstructural proteins, which are cleaved by the active nsP2 protease. These products could be identified by immunoprecipitation using polyclonal antibodies to each of the nonstructural proteins.

421. Expression of pr and prM of yellow fever virus and dengue virus

Pritsana Chomchan, Michael Suh, Maria Farkas

The flavivirus genome encodes three structural proteins: C, the capsid protein; prM, a precursor of the membrane protein M; and E, the envelope protein. Recently, capsid proteins from dengue virus (DEN) and from yellow fever virus (YF) have been purified and structure of the proteins determined by NMR techniques. Comparable structural data for flavivirus prM or pr (the portion cleaved from prM by furin during maturation) have not yet been reported. We are attempting to express and purify pr and a truncated form of prM, in which the membrane anchor domains were removed to improve protein solubility, from both YF and DEN. PCR products encoding the entire sequence of pr (amino acids 115-205 in the DEN polyprotein comprising amino acids 1-91 in pr; amino acids 121-209 in the YF polyprotein comprising amino acids 1-89 in pr) were cloned into pET28a (+) and pET30a (+) vectors. Similarly, PCR products encoding prM lacking the membrane fusion region (amino acids 115-243 in the DEN polyprotein comprising amino acids 1-129 of DEN prM; amino acids 121-247 in the YF polyprotein comprising amino acids 1-129 of YF prM) were cloned into the same vectors. Plasmid DNAs from several clones whose sequence had been confirmed were used to transform either IPTG- or salt-inducible *E. coli*. To date, no expressed protein of the correct size (10-13 kDa) has been detected by SDS-PAGE after induction and it is possible that the *E. coli* used degrades small proteins. Therefore, we are trying to construct a new expression plasmid using the pET41a (+) vector in which pr or truncated prM will be produced as a GST protein fusion.

422. Purification of mutant yellow fever virus for cryoelectron microscopy

Maria Farkas

The three-domain envelope protein of flaviviruses has been crystallized as a dimer (1). Dimers of E protein have also been fitted into the cryoelectron density envelope of dengue virus and there has been interest in determining how important dimer formation by E protein is for the overall configuration (and perhaps the stability) of flaviviruses. Two years ago, Ellen Westerhout generated a number of mutations in the dimer interface of the yellow fever virus E protein. These mutations (E537K, S539P, T596A, V604E, an insertion of 3 Ala after 439, and a deletion of amino acids TG598-599) were placed into a transient expression construct, but have now been introduced into the full-length yellow fever virus clone. Mutants S539P and the 3 Ala inserted after 439 were not infectious, but the other mutants grew to titers comparable with that produced by wild-type yellow fever virus. We have optimized the infection conditions in BHK-21 cells, prepared large amounts of the different mutants for further purification, and are currently trying new protocols for concentration and purification of the mutant virions. It is possible that one or more of these mutants can be purified to sufficient homogeneity for cryoelectron microscopic reconstruction.

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423. Mutagenesis of the BC-loop of Sindbis E1 glycoprotein

Pritsana Chomchan, Kayla Smith

Glycoprotein E1 of alphaviruses is required for virus assembly and membrane fusion when the released virus infects a new cell. The structure of the alphavirus glycoprotein E1 has been shown to be similar to that of the flavivirus glycoprotein E (1). Both alphavirus E1 and flavivirus E contain sequences characteristic of fusion peptides at one end of an extended domain. It has been suggested that the loop with the fusion peptide interacts with adjacent loops of the E/E1 protein to form a classical "beta-barrel" configuration. In Sindbis virus E1, the loop containing the fusion peptide is stabilized by two disulfide bonds. Previously, we generated single mutants at each of the cysteine residues (amino acids 63 and 94 from the N-terminus of E1), changing each to both alanine and serine. BHK cells were transfected with RNA and monitored for viral replication by immunofluorescence. Infection by all of these mutants failed to spread to neighboring cells, presumably due to disruption of the fusion competent configuration of E1. However, revertants quickly arose within 24 hr post-transfection, since all mutations had changed only a single nucleotide within each cysteine codon. When new mutants with the cysteines at amino acids 62, 63 and 94 from the N-terminus of E1 changed to both alanine and serine using two nucleotide changes within each cysteine codon were

made, none of the mutants spread from cell to cell. From these results we conclude that the two disulfide bonds examined are required for cell to cell movement of Sindbis virus. The finding that revertants arise so quickly appears to indicate that the presence of the mutant E1 within the infected cell does not interfere with the assembly of infectious virus once an RNA encoding revertant E1 has arisen within that cell.

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424. RNA replication and viral assembly of chimeras between yellow fever virus and dengue 2 virus

Pritsana Chomchan, Edith Lenches, Lindsay Hufton[*]

In order to study the interactions of the structural proteins of flaviviruses with one another and with the replication machinery, we have constructed several chimeric flavivirus cDNA clones. We have previously shown that full-length yellow fever virus (YF) containing prM and E from dengue 2 virus (DEN) replicated, as well as wild-type YF. But after transfection with a plasmid containing full-length YF with all three structural proteins (capsid, prM, and E) from DEN, there was no spreading of infection to neighboring cells, and no detectable virus was released into the medium. Cyclization of flavivirus viral RNA due to complementary sequences in the 5' end-of-capsid and in the 3' non-translated region (NTR) is required for viral RNA replication. Although the core 8 nucleotides of the YF and DEN sequences required for cyclization and replication are identical, our results show that YFV RNA with the DEN capsid sequence does not replicate. In order to test RNA sequences required for replication, the 5' cyclization sequence in DEN capsid in the YF/DEN(C-prM-E) chimera was changed to that of YF (nt 125-144 of DEN replaced by nt 147-165 of YF). After transfection of BHK by this mutant chimera, RNA replication could be detected by immunofluorescence. RNA replication was also restored in a mutant chimera in which part of the 3' NTR of YF was changed to that of DEN (nt 10748-10766 of YF to nt 10598-10616 of DEN). However, no virion assembly was detected in either case and the RNA transfection efficiency was quite low.

It has been shown by NMR spectroscopy that YFV and DEN virus capsid proteins contain three and four domains of alpha-helical structure, respectively (1). The three alpha-helical domains of YFV are similar to domains 2, 3, and 4 of DEN. We propose that these alpha-helical domains play an important role in virus assembly and are constructing chimeric capsid proteins between YFV and DEN by exchanging these helical domains to determine how these affect assembly of flavivirions.

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425. Glycosylation mutant of yellow fever virus

Edith Lenches, Michael Suh, Maria Farkas

A number of laboratories, including our own, have had difficulties in generating purified flavivirus preparations of sufficient homogeneity for cryoelectron microscopy reconstructions. We have produced such a preparation of dengue virus (see abstract 416) but despite repeated attempts, have been unsuccessful with yellow fever virus. Unlike other flaviviruses, yellow fever virus contains an envelope protein which is lacking glycosylation, and we reasoned that inserting a glycosylation site into the YF E protein, at the location of the conserved glycosylation site in other flaviviruses, might improve virus stability and permit cryoEM reconstruction. The "glycosylation mutant" of yellow fever virus was constructed by making a change at amino acid 153 of E (amino acid 436 of the polyprotein) from Asn to Thr to create the glycosylation site N-W-T-T from the wild-type sequence N-W-N-T. The glycosylation mutant grew better than wild-type yellow fever virus, producing yields of up to $1x10^8$ pfu/ml. To verify that the site on the E protein of this mutant virus was used, parallel cultures of BHK cells infected with either mutant or wild-type viruses were labeled from 24 to 48 hr post-infection with ^{14}C glucosamine (a glycosylation precursor) or ^{35}S methionine. At 48 hr post infection the infected cell monolayers were lysed, immunoprecipitated with antiserum against yellow fever virus E protein, and the precipitated proteins were analyzed by SDS-PAGE. Comparison of the patterns for 14C and ^{35}S indicated that only half of the intracellular YF E protein was glycosylated. We are currently examining the degree of glycosylation of E protein incorporated into virions released into the medium. In addition we are preparing large amounts of the glycosylation mutant for further study.

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Smits Professor of Cell Biology: Alexander Varshavsky
Postdoctoral Scholars: Christopher Brower, Cheol-Sang Hwang, Rong-Gui (Cory) Hu, Jun Sheng, Zanxian Xia, Zhenming (Jack) Xu, Jianmin Zhou
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Summary: Our main subject is the ubiquitin (Ub) system. In the 1980's, my colleagues and I produced the first evidence that Ub-protein conjugation is required for protein degradation in living cells and for cell viability, discovered the first physiological functions of Ub-dependent proteolysis (in the cell cycle, DNA repair, protein synthesis, transcriptional regulation, and stress responses), deciphered the first degradation signals in short-lived proteins, and identified critical mechanistic attributes of the Ub system, particularly the poly-Ub chain and subunit selectivity of protein degradation. Our work continues to focus on Ub-dependent circuits.

The effect of an intracellular protein on the rest of the cell depends on the protein's concentration. The latter is determined by the rate of synthesis and/or import of the protein in relation to the rates of its degradation, inactivation by other means, or export from the compartment. The *in vivo* half-lives of intracellular proteins range from a few seconds to many days. Over the last decade, a vast number of biological circuits were shown to contain either constitutively or conditionally short-lived regulators. In addition, damaged or otherwise abnormal intracellular proteins tend to be recognized as such and selectively degraded, largely by the same Ub-proteasome system that mediates the controlled proteolysis of short-lived regulatory proteins. The metabolic instability of a regulator provides a way to generate its spatial gradients and allows for rapid adjustments of its concentration (or subunit composition) through changes in the rate of its synthesis or degradation.

Ubiquitin (Ub) is a 76-residue protein that exists in cells either free or conjugated to many other proteins. Degradation of intracellular proteins by the Ub-proteasome system regulates a multitude of processes: cell growth, division, differentiation, signal transduction, responses to stress, and thus, a broad range of metacellular, organismal processes as well, from embryonic development to the immunity and functions of the nervous system. Ub-dependent proteolysis involves the "marking" of a substrate through covalent conjugation of Ub to a substrate's internal Lys residue. Ub conjugation is mediated by the E1-E2-E3 enzymatic cascade. E1, the ATP-dependent Ub-activating enzyme, forms a thioester bond between the C-terminal Gly of Ub and a specific Cys residue of E1. In the second step, activated Ub is transesterified to a Cys residue of a Ub-conjugating (E2) enzyme. Thereafter a complex of E2 and another enzyme, E3, conjugates Ub to a Lys residue of a substrate. The functions of E3 include the recognition of a substrate's degradation signal (degron). The numerous proteolytic pathways of the Ub system have in common their dependence on Ub conjugation and the 26S proteasome (which processively degrades Ub-protein conjugates), and differ largely through their utilization of distinct E2-E3 complexes. Specific E3s recognize (bind to) specific degrons of their substrates. The diversity of E3s and E2s underlies the enormous range of substrates that are recognized and destroyed by the Ub system, in ways that are regulated both temporally and spatially.

One pathway of the Ub system is the N-end rule pathway (Fig. 1). The N-end rule, which relates the *in vivo* half-life of a protein to the identity of its N-terminal residue, was discovered by this laboratory in 1986, in experiments that explored the fate of a fusion between Ub and a reporter protein such as *E. coli* β -galactosidase (βgal) in the yeast (fungus) *S. cerevisiae*. In eukaryotes, Ub-X-βgal is cleaved, cotranslationally or nearly so, by deubiquitylating enzymes (DUBs) at the Ub-βgal junction. This cleavage takes place regardless of the identity of the residue X, proline being the single exception. By allowing a bypass of the normal N-terminal processing of a newly formed protein, this result yielded an *in vivo* method (the Ub fusion technique) for generating different residues at the N-termini of otherwise identical proteins, a technical advance that led to the finding of the N-end rule. The *in vivo* half-lives of resulting X-βgal proteins were shown to range from ~2 min (e.g., Arg-βgal or Leu-βgal) to longer than 20 hr (e.g., Met-βgal or Gly-βgal), depending on the identity of their N-terminal residue (Fig. 1). The N-end rule pathway is present in all organisms examined, from mammals and plants to fungi and prokaryotes. The N-end rule-based degradation signal, called the N-degron, consists of a destabilizing N-terminal residue and an internal lysine (or lysines) of a substrate, the Lys residue being the site of formation of a substrate-linked poly-Ub chain. The ubiquitylated substrate is degraded by the 26S proteasome.

Functional and mechanistic studies of the N-end rule pathway in yeast and mammals are a major theme of our current work.

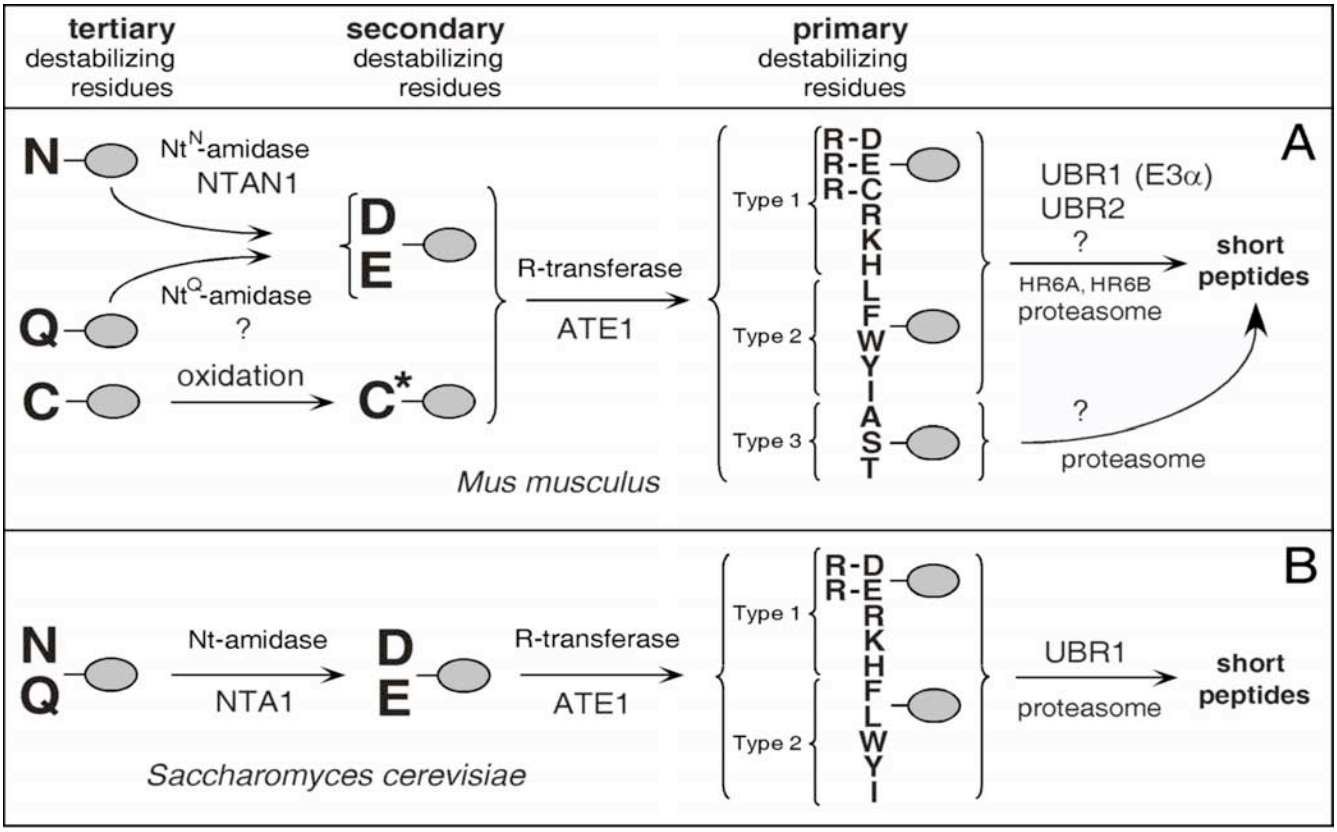

Fig. 1. The N-end rule pathway in mammals (A) and yeast (B).

426. An essential role of N-terminal arginylation in cardiovascular development

Yong Tae Kwon[1], Anna Kashina, Rong-Gui Hu, Jee Young An[1], Jai Wha Seo[1], Ilia Davydov[2], Alexander Varshavsky

The N-end rule has a hierarchic structure. Specifically, N-terminal Asn and Gln are tertiary destabilizing residues in that they function through their deamidation, by N-terminal amidohydrolases, to yield the secondary destabilizing residues Asp and Glu, whose activity requires their conjugation, by *ATE1*-encoded Arg-tRNA-protein transferases (R-transferases), to Arg, one of the primary destabilizing residues. The latter are recognized by the Ub ligases (E3 enzymes) of the N-end rule pathway. In mammals, the set of destabilizing residues that function through their arginylation includes not only Asp and Glu but also Cys, which is a stabilizing (non-arginylated) residue in the yeast *S. cerevisiae*. In vertebrates, the set of secondary destabilizing residues contains not only Asp and Glu but also Cys, the latter being a stabilizing residue in the yeast N-end rule.

The two characterized species of mammalian Arg-tRNA R-transferases (R-transferases), ATE1-1 and ATE1-2, are produced through alternative splicing of *ATE1* pre-mRNA (Kwon *et al*., 1999). The ratio of *ATE1-1* to *ATE1-2* mRNA varies greatly among the mouse tissues: it is ~0.1 in the skeletal muscle, ~0.25 in the spleen, ~3.3 in the liver and brain, and ~10 in the testis, suggesting that the two R-transferases are functionally distinct. However, the substrate specificities of ATE1-1 and ATE1-2 are similar to that of the *ATE1*-encoded R-transferase of *S. cerevisiae*, in that they can arginylate N-terminal Asp and Glu, but cannot arginylate N-terminal Cys (Kwon *et al*., 1999). This raises the question of how N-terminal Cys is arginylated in mammalian cells. To address this issue and the physiological functions of arginylation, we constructed *ATE (-/-)* mouse strains (Kwon *et al*., 2002). It was found that *ATE1(-/-)* cells are incapable of arginylating all three of the secondary destabilizing N-terminal residues, Asp, Glu and Cys, raising the possibility that N-terminal Cys may undergo a chemical modification that converts it into a substrate of ATE1. Through biochemical and mass spectrometric analyses, we showed that N-terminal Cys, in contrast to N-terminal Asp and Glu, is oxidized prior to its arginylation by R-transferase, suggesting that the arginylation branch of the N-end rule pathway functions as an oxygen sensor. *ATE1(-/-)* embryos die *in utero* around day E15, primarily from hemorrhages. *ATE1(-/-)* embryos exhibit both defective cardiogenesis and defective angiogenic remodeling of the early vascular plexus. Thus, *ATE1* is required for cardiovascular development, a new set of functions of the N-end rule pathway.

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427. Mechanistic and functional analyses of N-terminal arginylation

Christopher Brower, Cory Hu, Jun Sheng, Jack Xu, Jianmin Zhou, Alexander Varshavsky

Several functional and mechanistic studies of the N-terminal arginylation in mammals and yeast are under way, following the advances described in Abstract 426 (Kwon *et al.*, 2002). These studies include:

(i) Dissection of the substrate specificity of *ATE1*-encoded R-transferases in *S. cerevisiae* and the mouse, including analyses of the mechanism of selective oxidation of N-terminal Cys (see Abstract 426) (*Cory Hu*). One of our aims is to understand physiological significance of the differences in the activity of R-transferases toward N-terminal Cys versus its oxidized derivatives such as a cysteic acid residue. Recent work (*Cory Hu*) has shown that R-transferases, at alkaline pH, are capable of arginylating not only N-terminal $CysO_3$ (cysteic acid) but unmodified N-terminal Cys as well. However, the latter reaction proceeds very slowly at physiological pH, in contrast to efficient arginylation of N-terminal $CysO_3$ under the same conditions.

(ii) Construction and functional analyses of mouse strains (and cells derived from them) in which the expression of *ATE1*-encoded Arg-tRNA-protein transferases R-transferases is selectively and conditionally abolished in specific cell lineages during embryogenesis, or postnatally (*Christopher Brower*). This set of projects should make possible, among other things, a functional dissection of N-terminal arginylation in specific organ systems and cell types of adult mice. (A nonconditional *ATE1(-/-)* genotype is embryonic lethal (Abstract 426).

(iii) Construction and functional analysis of knock-in mouse strains that contain a doxycycline-inducible allele of *ATE1*, and thus, can overproduce R-transferases, in a controllable manner, in specific cell types during embryogenesis, or postnatally (*Jianmin Zhou, Christopher Brower*).

(iv) Analysis of chromosome stability and regulation of apoptosis in mouse *ATE1(-/-)* cells (*Jianmin Zhou, Jun Sheng, Cory Hu, and Christopher Brower*). This set of projects stems from the discovery of the function of the *S. cerevisiae* N-end rule pathway in the maintenance of chromosome stability (Rao *et al.*, 2001), and from the conjecture that an analogous function in mammalian cells involves the arginylation (*ATE1*-dependent) branch of the N-end rule pathway. Recent work (*Jianmin Zhou*) indicated that N-terminal arginylation is essential for the *in vivo* degradation of the separase-produced fragment of SCC1, a subunit of mouse cohesin. *ATE1(-/-)* embryonic fibroblasts (EFs) are strongly different from +/+ EFs in their resistance to a variety of cytotoxic drags (*Jianmin Zhou, Cory Hu*). The molecular mechanisms of these phenotypes remain to be understood. *ATE1(-/-)* EFs are phenotypically unstable as established cell lines, in that independently produced *ATE1(-/-)* cell lines tend to differ in the sets of proteins they overproduce, relative to +/+ EFs (*Jun Sheng*).

(v) Identification of *ATE1*-dependent circuits (i.e., the circuits which involve N-terminal arginylation) through the identification of mouse genes whose expression is significantly altered during embryonic development in *ATE1(-/-)* embryos, using microarray techniques, differential display and analogous methods with mRNA preparations from *ATE1(-/-)* and congenic +/+ embryos (*Jun Sheng*).

(vi) Identification of physiological substrates of R-transferases through the testing of putative substrates of caspases and calpains that bear secondary or tertiary destabilizing N-terminal residues (*Jianmin Zhou and Christopher Brower*). *In vivo* and cell-free approaches to discovery of physiological substrates of R-transferases (*Jack Xu* and *Cory Hu*).

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428. Female lethality and apoptosis of spermatocytes in mice lacking the UBR2 ubiquitin ligase of the N-end rule pathway

Yong Tae Kwon[1], Zanxian Xia, Jee Young An[1], Takafumi Tasaki[1], Ilia Davydov[2], Jai Wha Seo[1], Jun Sheng, Youming Xie[3], Alexander Varshavsky

The absence of gross defects in *UBR1(-/-)* mice (Kwon *et al.*, 2001) suggested that the E3 function of the mouse N-end rule pathway is mediated by at least two functionally overlapping genes, one of which is *UBR1*. The previously identified mouse gene, termed *UBR2*, encodes a 200 kD protein highly similar to UBR1 (Kwon *et al.,* 1998). *UBR2(-/-)* mouse strains were recently constructed and characterized, revealing a sex-specific phenotype: male infertility and female lethality (Kwon *et al.*, 2003). The relative frequency of *UBR2(-/-)* males produced from heterozygous (+/- × +/-) matings was similar to that of +/+ males. Adult *UBR2(-/-)* males were outwardly normal, but exhibited severe defects in spermatogenesis and were sterile. Until 4 weeks after birth, the size of their testes was close to that of congenic +/+ mice. However, during the 5th and 6th weeks, the mass of *UBR2(-/-)* testes decreased by ~2-fold. The degeneration of testes in *UBR2(-/-)* mice was caused by massive apoptosis of spermatocytes and their progeny, round spermatids. The number of apoptotic germ line cells

in the mutant testes dramatically increased five weeks after birth. The first detectable abnormality of germ cell differentiation in the *UBR2(-/-)* testes was a much lower number of early meiotic spermatocytes at week 2. At week 3, when meiotic divisions of spermatocytes produce round spermatids, ~2,800 round spermatids per 100 seminiferous tubules were observed in the +/+ testes, but virtually no round spermatids could be detected in the mutant testes. This and related data suggested that defective meiosis in *UBR2(-/-)* male mice was the primary cause of their sterility. Despite the meiotic defect, a small number of cells that appeared to be round spermatids could be detected in the *UBR2(-/-)* testes after week 4. However, most of these cells did not continue to differentiate, and died through apoptosis, together with meiotic spermatocytes, after week 6. As a result, the testes of two months-old *UBR2(-/-)* males were severely shrunk and vacuolized; they nearly completely lacked spermatids and spermatozoa. In contrast, the spermatogonia, Sertoli cells, and Leydig cells remained apparently intact in the *UBR2(-/-)* testes. Thus, the absence of UBR2 (but not of its close homolog UBR1) leads to a severe defect in spermatogenesis that stems primarily, if not exclusively, from a meiotic defect. Further analysis of this phenotype showed that *UBR2(-/-)* spermatocytes are arrested largely at the pachytene stage of meiosis, and fail to form the synaptonemal complex that holds together the homologous chromosomes and is essential for meiotic recombination. GST-pulldown binding assays with purified N-end rule substrates and extracts from *S. cerevisiae* overexpressing mouse UBR2 have shown that UBR2 can recognize N-degrons *in vitro*. Thus, mouse UBR2 functions *in vivo* as an E3 component of the N-end rule pathway that functionally overlaps with the previously known E3 of this pathway, UBR1 (E3α) (Kwon *et al*., 2003).

Yet another feature of the *UBR2 (-/-)* phenotype was extremely low frequency of the *UBR2(-/-)* female progeny from heterozygous (+/- × +/-) matings, in contrast to the normal (mendelian) frequency of *UBR2(-/-)* males. Strikingly, the rare *UBR2(-/-)* females that were born were fertile and apparently normal otherwise. This phenotype is being analyzed. It might be caused by perturbed X-chromosome inactivation in early *UBR2(-/-)* female embryos.

To investigate the functional interaction of UBR1 and its close homolog UBR2, we used [*UBR1(+/-)UBR2(+/-)*] mice to produce [*UBR1(-/-)UBR2(-/-)*] double-mutant mouse strains. Disruption of both *UBR1* and *UBR2* was lethal: none of the [*UBR1(-/-)UBR2(-/-)*] embryos survived beyond day E12 (Y.T. Kwon and J.W. Seo, unpublished data). Whereas most of the double-mutant male embryos died at ~E9.5, most of their female counterparts died significantly earlier. Remarkably, *UBR1(-/-)UBR2(-/-)* embryonic fibroblasts did contain the N-end rule pathway, albeit of significantly lower activity, indicating the presence of at least one other (third) E3 of this pathway.

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429. Construction and analysis of mouse strains lacking the UBR3 ubiquitin ligase

Takafumi Tasaki[1], Yong Tae Kwon[1], Alexander Varshavsky

Kwon *et al*. (1998) identified two distinct mouse (and human) genes, termed *UBR2* and *UBR3*, which encode proteins that are similar to mouse UBR1 (E3α), the previously characterized E3 of the N-end rule pathway. In contrast to the highly similar mouse UBR1 and UBR2 proteins (47% identity and 68% similarity), the mouse UBR3 protein, while clearly a member of the UBR family, is less similar to UBR1 (25% identity and 51% similarity) and UBR2 (25% identity and 48% similarity). In addition, mouse UBR3 lacks some of the residues in its N-terminal region that have been shown to be essential for the function of yeast UBR1, and are also present in the mouse (and human) UBR1 and UBR2 proteins. We mapped and partially sequenced the mouse *UBR3* gene, and more recently constructed *UBR3(-/-)* mouse strains. *UBR3(-/-)* mice are viable, and are being characterized.

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Kwon, Y.T., Reiss, Y., Fried, V.A., Hershko, A., Yoon, J.K., Gonda, D.K., Sangan, K., Copeland, N.C., Jenkins, N.A. and Varshavsky, A. (1998) *Proc. Natl. Acad. Sci. USA* 95:7898-7903.

430. Dissection of c-MOS degron

Jun Sheng, Akiko Kumagai, William G. Dunphy, Alexander Varshavsky

c-MOS (called MOS below) was originally identified as a cellular homolog of the v-MOS oncoprotein of Moloney murine sarcoma virus. MOS is a serine/threonine protein kinase that is normally expressed largely, if not exclusively, in oocytes and spermatocytes. MOS functions as a regulator of oocyte maturation. A fully-grown (stage-VI) *Xenopus laevis* oocyte is arrested in the G2 phase of the first meiotic cell cycle. Oocyte

maturation, initiated by progesterone, involves the completion of meiosis I, followed by meiosis II and the arrest at metaphase of meiosis II. At this stage, the mature oocyte, called an egg, is ready for fertilization. MOS phosphorylates, and thereby activates, the kinase MEK1. In the MAP cascade's terminology, MOS is a MAP kinase kinase kinase (MAPKKK). The synthesis of MOS begins shortly after progesterone stimulation, and ceases near the end of oocyte maturation. The concentration of MOS is controlled not only through its synthesis but also through its conditional degradation by the Ub system.

Studies by Sagata and colleagues (Nishizawa *et al.*, 1992, 1993) have shown that wild-type MOS, bearing the N-terminal Pro residue, is short-lived upon its expression in oocytes, whereas the otherwise identical MOS derivative bearing N-terminal Gly is long-lived. These and related findings led Nishizawa *et al*. (1992, 1993) to propose the "second-codon rule," in which the N-terminal Pro residue of MOS targets MOS for degradation. In the N-end rule terminology, the above conjecture meant that the MOS protein contains an N-degron whose N-terminal Pro is recognized by a specific Ub ligase. One difficulty with this model was that N-terminal Pro, in the context of previously characterized N-end rule reporters, was clearly a stabilizing residue.

To address this problem, we analyzed the MOS degron in greater detail, found it to be a portable degron, and demonstrated that, contrary to the model above, the N-terminal Pro residue of c-MOS is entirely dispensable for c-MOS degradation if Ser-2 (encoded Ser-3) of c-MOS is replaced by a small nonphosphorylatable residue such as Gly (Sheng *et al.*, 2002). The dependence of c-MOS degradation on N-terminal Pro was shown to be caused by a Pro-mediated down-regulation of the net phosphorylation of Ser-2, a modification that halts c-MOS degradation in oocytes. Thus, the N-terminal Pro residue of c-MOS is not a recognition determinant for a ubiquitin ligase, in agreement with the earlier evidence that Pro is a stabilizing residue in the N-end rule.

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431. The UFD4 ubiquitin ligase lacking the proteasome-binding region catalyzes ubiquitylation but is impaired in proteolysis

Youming Xie[1], *Alexander Varshavsky*

We have previously shown that *S. cerevisiae* UBR1 and UFD4, the E3 enzymes of, respectively, the N-end rule pathway and the UFD (Ub/fusion/degradation) pathway, directly interact with specific subunits of the 26S proteasome (Xie and Varshavsky, 2000). These and other recent results suggested a route for the substrate's delivery to the proteasome distinct from the previously explored proteasome binding to a substrate-linked poly-Ub chain. The GST (glutathione S-transferase) pulldown assay was used to test nine subunits of the 19S particle for binding to UFD4, identifying RPT6 as the UFD4-binding subunit in that set (Xie and Varshavsky, 2000). More recently, we advanced this analysis for the UFD4 Ub ligase, and showed that it interacts with RPT4 and RPT6, two of the ~17 subunits of the 19S particle (Xie and Varshavsky, 2002). The 201-residue N-terminal region of UFD4 was found to be essential for its binding to RPT4 and RPT6. UFD4$^{\Delta N}$, which lacks this N-terminal region, is apparently wild type in ubiquitylating UFD substrates *in vivo*, but is impaired in conferring short half-lives on these substrates. We suggest that interaction of a targeted substrate with the 26S proteasome involves contacts of specific proteasomal subunits with the E3 of an E3-substrate complex, with the substrate's multi-Ub chain, and with the substrate itself. This broadly-spaced, multi-site binding may serve to slow down substrate's dissociation from the proteasome and to facilitate the unfolding of substrate upon ATP-dependent movements of the chaperone subunits of the 19S particle (Xie and Varshavsky, 2002).

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References

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Xie, Y. and Varshavsky, A. (2002) *Nature Cell Biol.* 4:1003-1007.

432. Pairs of dipeptides synergistically activate the binding of substrate by ubiquitin ligase through dissociation of its autoinhibitory domain

Fangyong Du[1], *Federico Navarro-Garcia*[2], *Zanxian Xia, Takafumi Tasaki*[3], *Alexander Varshavsky*

Two substrate-binding sites of UBR1, the E3 of the N-end rule pathway in *S. cerevisiae*, recognize basic (type 1) and bulky hydrophobic (type 2) N-terminal residues of proteins or short peptides. A third substrate-binding site of UBR1 targets CUP9, a transcriptional repressor of the peptide transporter PTR2, through an internal (non-N-terminal) degron of CUP9 (Byrd *et al.*, 1998; Turner *et al.*, 2000). Previous work (Turner *et al.*, 2000) demonstrated that dipeptides with destabilizing N-terminal residues allosterically activate UBR1, leading to accelerated *in vivo* degradation of CUP9, a strong decrease of CUP9 concentration, and the induction of PTR2 expression. Through this positive feedback, *S. cerevisiae* can sense the presence of extracellular peptides, and react by accelerating their uptake. In the present work (Du *et al.*, 2002), we found that dipeptides with destabilizing N-terminal residues cause dissociation of the C-terminal autoinhibitory domain of UBR1 from its N-terminal region that contains all three substrate-binding sites. This dissociation, which allows the interaction between UBR1 and CUP9, is strongly increased only if both type 1 and type 2 binding sites of UBR1 are occupied by dipeptides. An aspect of autoinhibition characteristic of

yeast UBR1 was also observed with mammalian (mouse) UBR1 (E3α). Autoinhibition has not been reported previously for Ub ligases. UBR1, UBR2 and UBR3, the mouse homologs of *S. cerevisiae* UBR1, contain the conserved (and similarly arranged) UBHC, RING-H2, and UBLC domains similar to those of yeast UBR1. In addition, mouse UBR1 and UBR2 contain binding sites for the type 1 and type 2 destabilizing N-terminal residues. Moreover, the functional properties of these sites, such as the ability of type 1 dipeptides to stimulate the degradation of type 2 N-end rule substrates, are also similar to those of yeast UBR1. Finally, an aspect of autoinhibition characteristic of yeast UBR1 was also observed with mouse UBR1. Thus, it is highly likely that metazoan E3s of the UBR family are also controlled through autoinhibition, similarly to yeast UBR1. The *S. cerevisiae* UBR2 E3 and mouse UBR3 E3, whose functions remain unknown, are not a part of the N-end rule pathways in the respective organisms (H. Rao, T. Tasaki, Y.T. Kwon and A. Varshavsky, unpublished data). Thus, the (postulated) autoinhibition of yeast UBR2 and mouse UBR3 may be regulated by naturally occurring small compounds distinct from dipeptides. Identifying these compounds would provide a clue to still unknown physiological roles of the UBR-family Ub ligases that function outside the N-end rule pathway. The discovery of autoinhibition in the Ub ligases of the UBR family (Du *et al.*, 2002) indicates that this regulatory mechanism may control the activity of other Ub ligases as well. Our findings also suggest that natural compounds (either small molecules or proteins) may regulate the activity of diverse Ub-dependent pathways through the suppression or induction of autoinhibition in Ub ligases.

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Du, F., Navarro-Garcia, F. Xia, Z., Tasaki, T. and Varshavsky, A. (2002) *Proc. Natl. Acad. Sci. USA* 99:14110-14115.

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433. Quantitative analyses of interactions between components of the N-end rule pathway and their substrates or effectors

Zanxian Xia, Alexander Varshavsky

Detailed understanding of the N-end rule pathway requires, among other things, the knowledge of equilibrium binding constants for the reversible interactions between UBR1 and its effectors or substrates. (With some interactions, it would be desirable to know the corresponding rate constants as well.) We are carrying out these measurements using the fluorescence polarization (FP) technique, with purified *S. cerevisiae* UBR1 (or its fragments) and a variety of UBR1 ligands, including peptides with destabilizing N-terminal residues. These analyses will be expanded to include other physiological ligands of UBR1 such as the RAD6 Ub-conjugating enzyme, and CUP9, a homeodomain repressor recognized through its C-terminal degron by a distinct (third) substrate-binding site of UBR1.

434. Phosphorylation of UBR1: Its regulation and functions

Cheol-Sang Hwang, Zanxian Xia, Alexander Varshavsky

S. cerevisiae UBR1, the E3 of the yeast N-end rule pathway, is phosphorylated *in vivo*, but the role(s) of this UBR1 modification in the multiple functions of the N-end rule pathway is unknown. Phosphorylation sites on UBR1 and the kinases/phosphatases involved remain to be identified as well. We are using biochemical and genetic approaches to understand, in functional and mechanistic detail, this modification of UBR1.

Publications

Du, F., Navarro-Garcia, F. and Varshavsky, A. (2002) Pairs of dipeptides synergistically activate the binding of substrate by ubiquitin ligase through dissociation of its autoinhibitory domain. *Proc. Natl. Acad. Sci. USA* 99:14110-14115.

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Facilities

Biopolymer Synthesis Center
Gene Expression Center
Flow Cytometry and Cell Sorting Facility
Genetically Altered Mouse Production Facility
Monoclonal Antibody Facility
Nucleic Acid and Protein Sequence Analysis Computing Facility
Protein Expression Center
The Protein Microanalytical Laboratory

Biopolymer Synthesis Center
Supervisor: Suzanna J. Horvath
Staff: Andrej A. Ausing, John P. Bleech, Ryan A. Ellingson, John Racs

The Biopolymer Synthesis Center has continued to provide high quality chemical synthesis, purification, and analysis of a wide range of biopolymers at the request of the scientific community at Caltech. We also provide strong technical support to individual research projects through the introduction and the use of the latest technology in both chemistry and biology. Today's emerging new techniques challenge us with their sophistication and complexity, as well as their rapid space of evolution. For a growing number of scientists we have made complex experiments possible by advance design, synthesis, and purification of unique molecules. The intellectual interaction between our staff and the research scientist was essential to the success of each project.

The DNA/RNA Synthesis Laboratory

The total number of DNA molecules synthesized in FY'02 is 10,194 for 42 Caltech faculty members and seven outside collaborators. This represents 309,446 base additions.

The total number of RNA molecules synthesized in FY'02 was 74 for five Caltech faculty members. This represents 1,507 base additions.

Our primary instruments are two ABI Model:3948 synthesizers. Each instrument is able to synthesize 48 purified deoxyoligonucleotides up to 91 bases in length every 36 hours. The synthesis scale is 0.05 μM.

The three four-column DNA/RNA synthesizers (ABI Model:394-08) operate with full capacity all the time. These synthesizers can be set up as 0.04, 0.2, or 1.0 μM scale synthesis. They are flexible for the use of a variety of modified chemistries, including RNA synthesis. A catalog of DNA reagents for chemical modifications is available in our laboratory. With the expert assistance of our DNA synthesis staff, Caltech scientists can choose from this catalog and design "special oligos" for synthesis. These special oligos are now widely used at Caltech in a large variety of experiments.

Requests for chemically-modified oligonucleotides have been increasing dramatically and continuously and they are more challenging than ever before.

The Peptide Synthesis Laboratory

There are four automated peptide synthesizers in our Peptide Synthesis Laboratory. Our most advanced solid-phase automated synthesizers are the two ABI Model:433A. They are set up for the application of Fmoc chemistry with optimized chemical cycles. These cycles are automatically monitored by a conductivity cell. Using Fmoc chemistry, we can selectively introduce phosphorylated sites on any Ser, Thr, or Tyr residues in the peptide sequence. There are two automated peptide synthesizers, ABI Model:430A, set up for the application of t-Boc chemistry. We have synthesized large numbers of short peptides, peptide-protein conjugates to generate heterogeneous antisera or monoclonal antibodies with desired specificity for a large variety of applications. Each peptide made in our laboratory includes CE, HPLC, and MS analysis. MS analysis is usually done at the Protein/Peptide Microanalytical Laboratory (PPMAL).

The number of peptide molecules synthesized so far in FY'03 has remained high, with a large increase in their complexity and purity requirements.

In FY'02 we synthesized 106 peptides for 18 Caltech faculty members and nine outside collaborators. This represents a total of 2,298 regular and special amino acid couplings.

A series of technically difficult peptide synthesis were carried out successfully in our laboratory that contributed to the substantial progress made in the laboratories of the following professors: David Baltimore (Biology); William Dunphy (Biology); Harry Gray (Chemistry); Steve Mayo (Biology); Alexander Varshavsky (Biology); Peter Dervan (Chemistry); and David Tirrel (Chemistry).

We have synthesized a large number of extremely difficult, very insoluble hydrophobic peptides for Dr. Boris Minev and Professor Maurizio Zanetti at the UCSD Cancer Center.

For Professor David Eisenberg's request, we continued to make a series of peptides with exceptional high purity for his crystallography studies. He is at the UCLA-DOE Laboratory of Structural Biology, Molecular Medicine.

For the sixth year in a row, in collaboration with Professor Michael Grunstein at the Molecular Biology Institute, UCLA, we have been synthesizing a large number of histone-peptides that involves the combined application of both t-Boc and Fmoc synthesis. We have found a way to introduce selective acetylation of one or more Lys side chains in a given peptide sequence.

Flow Cytometry and Cell Sorting Facility

Supervising Faculty Member: Ellen Rothenberg
Facility Manager: Rochelle Diamond
Operators/Technical Specialists: Rochelle Diamond, Patrick Koen

The Caltech Flow Cytometry Cell Sorting Facility has been serving the Caltech community and outside clients for over 19 years. This multi-user facility provides flow cytometric technology on a fee-for-service basis. Just about any cell or cell-like particle can be analyzed and purified by sorting from a heterogeneous population based on its scatter and fluorescent properties. The information obtained is not only qualitative, but also can be quantitative, and the properties are correlated on a single-cell basis to reveal statistical population characteristics for all cells in the sample. For this reason, flow technology is a useful tool to study many aspects of cell biology including cell cycle analysis; defining and isolating cell populations in immunology, developmental biology, and neurobiology: isolation of transfectants exhibiting desired properties; real-time measurements of physiological responses in cells; quantitation of cell death; and isolation of clones for high-throughput genomics. The particular applications provided by the Caltech Facility have been driven by the needs of the Caltech user community.

The Flow Cytometry & Cell Sorting Facility is equipped with a FACS Vantage SE research-grade flow cytometer/cell sorter capable of sorting 3,000 events/second based on multiparameter analysis of cell size, density, and intensities of up to five colors of fluorescence. In addition, the facility owns a Coulter Epics Elite, an older machine that is only optimized for three-color analysis. Nevertheless, it still provides superior optical resolution for cell cycle work and for low-level fluorescence detection. The staff, rather than the user, perform all experiments on these cell sorters due to the complexity of the instrumentation. In addition, a non-sorting four-color analyzer (FACSCalibur) and a free-standing work station are available to researchers for self-service analysis around the clock, provided that the investigators demonstrate competence with the analyzer or take training provided by the facility.

The advent of fluorescent proteins engineered as reporter gene products to monitor specific promoter activities has greatly increased the demand on the facility for sorting large quantities of purified cells with specific marker expression patterns. Many new fluorochromes have been brought on the market, and commercially-available antibodies conjugated to them have pushed the envelope on the number of fluorescent parameters that can be used to dissect heterogeneous populations of cells. Novel uses of flow technology with these reagents continue to be developed. These new developments, and the demand for purification of increasingly rare cell populations, have made it necessary to upgrade our instrumentation.

In the spring of 2003, Caltech faculty obtained a multiuser instrumentation grant from NIH to purchase a new cell sorter that will greatly enhance the speed and capabilities of the Facility. The newest state-of-the-art instruments offer tenfold higher sorting speeds than our existing, 5-year-old sorter, with over eight colors available for analysis and all-channel digital compensation that gives far more accurate separation of signals from the different fluorochromes. The instrument that we will be able to purchase will also make it possible for the first time for fluorescent resonance energy transfer (FRET) analyses using Cyan Fluorescent Protein and Yellow Fluorescent Protein to be conducted on the sorter.

This past year the facility has serviced 16 groups in the Biology, Chemical Engineering, and Chemistry Divisions representing 50 researchers. The facility has also trained 11 new FacsCalibur analyzer users.

For further information about the Flow Cytometry/Cell Sorting Facility or to schedule an appointment or discuss your research application, please consult the Facility web site: http://www.its.caltech.edu/~cellsort.

Gene Expression Center
Director: José Luis Riechmann
Staff: Brandon King, Vijaya Rao, Kayla Smith

Support: The work described in the following research report has been supported by:
Beckman Institute
L.K. Whittier Foundation
Moore Discovery Grant Fund
Muriel and Millard Jacobs Fund for Genomics and Genetic Technology
Sun Microsystems

The goal of the Center for Gene Expression, in the Division of Biology, is to provide a complete suite of cutting edge genomic research tools to all interested Caltech scientists, with an initial emphasis on large-scale gene expression profiling. The Center performs gene expression analyses using DNA microarray technology, and is equipped with the necessary experimental and bioinformatics infrastructure that is needed to generate, store, and analyze large-scale datasets from a variety of microarray technological platforms.

The variety of commercially available platforms and reagents differs considerably among different organisms. For mouse and human, these reagents include Affymetrix GeneChips, commercial oligonucleotide microarrays printed on glass slides (from Agilent, Amersham, and MWG), and whole genome oligonucleotide sets for in-house printing (from QIAGEN). On the other hand, the *Arabidopsis* or *C. elegans* genomes are only fully represented on Affymetrix GeneChips and in oligonucleotide sets from QIAGEN. Reagent availability and technical and cost considerations determine our choice of microarray platform for each particular project.

For *Arabidopsis* (work performed together with Professor E.M. Meyerowitz's group), we use a whole genome 70 mer oligonucleotide set, that we print on glass slides (either in-house prepared poly-lysine slides, or commercial slides, such as UltraGaps) using our MicroGridII microarrayer. In this manner, we generate in a cost effective way hundreds of microarray slides. In the past, before this oligonucleotide set was available, we also produced PCR-product microarrays based on in-house prepared, subtracted cDNA libraries. For mouse (work performed together with Professor E. Rothenberg's group), we have used both Affymetrix GeneChips and Amersham microarrays. Available equipment in the laboratory for experiments with the different platforms includes a QIAGEN 3000 liquid handling robot, a MicroGridII arrayer (BioRobotics), a GenePix 4000A scanner (Axon Instruments), a BioAnalyzer (Agilent Technologies), and the necessary instruments to use Affymetrix GeneChips (Fluidics Station 400, Hybridization Oven 640, and GeneArray 2500 scanner).

We have also assembled the basic bioinformatics infrastructure that is needed for microarray data storage and analysis, combining both commercial and in-house written software. The Center for Gene Expression uses Resolver (from Rosetta BioSoftware) as its primary gene expression data analysis system. Resolver is a robust, enterprise-scale, gene expression system that combines a high capacity, MAGE-compliant database and advanced analysis software in a high-performance server framework. The system is accessible through client stations using a web-based interface. Resolver has been developed with a plug-in framework architecture, which allows for customization and extension, and integration with third-party products. We are extending the system with links to other public and proprietary databases, and we also plan to integrate into Caltech's customized Resolver additional analysis tools that have been developed by the groups of Barbara Wold and Eric Mjolsness. In addition, we are contributing to an academic, multi-site effort to develop a public gene expression database, GeneX2. Our interest in that project is that a commercial database might not be nimble enough to accommodate all of our needs, which can change over time as new analysis methods are developed. We also have at our disposal additional microarray software tools and analysis packages, both public and commercial. The hardware infrastructure of the Gene Expression Center currently includes a Sun Fire V880 server (from Sun Microsystems), that we use for the Resolver database (Oracle) and analysis system.

Publications

Broun, P., Pointdexter, P., Osborne, E., Jiang, C.-Z. and Riechmann, J.L. WIN1, a transcriptional activator of epidermal wax accumulation in *Arabidopsis*. Submitted.

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Wellmer, F., Riechmann, J.L., Ferreira, M.A. and Meyerowitz, E.M. Identification of floral organ specific transcripts by gene expression profiling of *Arabidopsis* floral homeotic mutants. Submitted.

Genetically Altered Mouse Production Facility
Director: Shirley Pease F.I.A.T.
Mouse Facility Manager: Bruce Kennedy, M.S., RLATG
Mouse Facility Supervisor: Jenny Arvisu, ALAT
Embryonic Stem Cell Culture: Jue Jade Wang, M.S.
Cryopreservation and Microinjection: Juan Silva, B.S., LAT
Staff: Jennifer Alex, AA, RALAT; Armando Amaya; Lilia Anonuevo, LAT; Cirila Arteaga; Donaldo Campos; Hernan Granados; Carlos Hernandez; Joon Kang; Jorge Mata, ALAT; Jose Mata; Gustavo Munoz, B.A.; John Papsys, BS; Lorena Sandoval; Shannon Witherow, A.A.; Carolina Young, B.S.

The transgenic technique for gene addition (Gordon *et al*., 1980) and targeted gene modification (Zijlstra *et al*., 1989) have been established at Caltech since 1984 and 1993, respectively. Gene addition in the mammalian system is accomplished by injecting DNA into the pronucleus of a fertilized egg. Targeted disruption of specific genes, however, requires the manipulation of pluripotent embryonic stem (ES) cells *in vitro* and their subsequent return to the embryonic environment for incorporation into the developing embryo. The resulting chimeric mouse born is useful for two purposes: 1) it is comprised of tissue from two sources, the host embryo and the manipulated stem cells. More importantly, 2) it can be mated, so as to produce descendants that are entirely transgenic, resulting from the ES cell contribution to the germline of the chimeric mouse. More recently, the Facility together with the Baltimore lab, participated in the development of a new method for the introduction of DNA into early-stage embryos (Lois, C. *et al*., 2002). This method makes use of non-recombinant lentivirus as a vector for the introduction of DNA into one-cell embryos. The method has proven to be highly efficient and promises to be useful for studies in mice and rats, where large numbers of constructs need to be tested. This new methodology also makes feasible the generation of transgenic animals in species that were hitherto impractical to work with, due to the very low numbers of embryos available for use.

The newly refurbished, pathogen-free barrier-operated mouse facility was opened and re-stocked with pathogen-free strains in February 1995. In addition to 86 transgenic, knockout and knockin strains, we also maintain colonies of inbred and outbred animals, which are used to support the development of new lines, by investigators at Caltech. We also have many mouse models on both an inbred and an outbred background, plus intercrosses between two or three different but related mouse models. In total, we maintain 153 separate strains of mouse. Some of these strains are immune deficient and require specialized care, to protect them from bacteria commonly present, (and non-pathogenic), in immune competent animals. In immune deficient animals, these hitherto harmless organisms can cause a problem. This may interfere with the well being of the animal and the extraction of reliable experimental results. Facility staff provides a complete service to investigators. All colony management operations are carried out by staff at the request of investigators. The work of the staff and manager of the facility continues to reflect Caltech's commitment to good laboratory animal practice and our adherence to NIH guidelines. In 2001/2002, the Facility participated in the campus effort to become accredited by the International Association for the Accreditation of Laboratory Animal Care.

The facility, in collaboration with Anderson, Baltimore, Lester, Simon, Wold and Varshavsky laboratories, has generated multiple transgenic, knockout and knockin mouse strains. Presently, twelve principal investigators and their post-doctoral fellows or graduate students use the facility.

New transgenic lines have continually been produced and in all, 64 new transgenic lines for gene addition and 48 new knockouts have been produced since February 1995. Since the lentiviral vector method was established, 55 transient or established mouse models have been generated by this means, together with one Tg rat model. Facility staff has performed all embryo manipulation involved in the production of these new lines. Microinjection equipment has been set up within the barrier facility, which operates on restricted access as part of the "barrier" itself. A room outside the facility has been allocated by the Division to be used primarily for teaching grad students, technicians and postdocs the techniques involved in transgenic mouse production. This room has been operating since July, 1996. Investigators have the option of using this room to perform their own microinjection of embryos, rather than using the full technical service available from the Genetically Altered Mouse Facility.

In tissue culture and the use of embryonic stem cells, the Facility participated in the derivation of new ES cell lines derived from genetically altered mice (see Simon laboratory Annual Report, 2001). Goals for the coming year for this part of the Facility include the acquisition and use of hybrid ES cell lines for their reported vigor, and also, the establishment of the tetraploid embryo complementation technique for the generation of animals wholly of ES cell origin.

In the generation of mouse models involving the use of embryonic stem cells, we continue to pre-screen candidate clones for injection by the preparation of chromosome spreads. In establishing the chromosome count of each clone prior to injection, we find we are reliably able to predict which clones will generate good chimeras that will transmit through the germline. In effect, we have been able to increase efficiency by 100% in the production of germline transmitting chimeras.

In cryopreservation, 50 strains are either stored or partially stored. For each strain, between 200 and 500 embryos at 8-cell stage have been preserved in liquid nitrogen. There are currently over 16,000 embryos frozen in total. We shall continue to preserve embryos from every single mouse strain the Core maintains. The

advantages of such a resource are many. Unique and valuable mouse strains that are currently not in use may be stored economically. In the event that genetic drift should affect any strain, over time, then the option to return to the original documented genetic material is available. Lastly, in the event of a microbiological or genetic contamination occurring within the mouse facility, we have the resources to set up clean and genetically reliable mouse stocks in an alternative location.

In addition to producing a total of 167 new animal models since February 1995, we have re-derived 48 pre-existing genetically altered strains of mice. This practice is being continued by the Office of Laboratory Animal Resources.

The production of pathogen-free animals enables Caltech to exchange valuable genetically-altered mouse models with other academic groups around the world. As more and more mouse models become available and the exchange of animals more frequent, it is essential for Caltech to be in a position to deal effectively with animal lines generated in the facility and in other laboratories. To this end, the Division has funded the refurbishment of two quarantine animal holding areas. This now enables us to safely contain and "clean up" other mouse models coming in from other institutions without putting our own colony at risk.

Listed below are the names of the twelve principal investigators and their postdoctoral fellows or graduate students who are presently using the transgenic facility.

Pepe Alberola-Ila
Susannah Barbee, Christie Beel, Harry Green, Gabriela Hernandez-Hoyos, Micheline Laurent, Eric Tse

David Anderson
Gloria Choi, Xinzhong Dong, Limor Gabay, C.J. Han, Christian Hochstim, Walter Lerchner, Li Ching Lo, Sally Lowell, Raymond Mongeau, Yosuke Mukoyama, Donghun Shin, Qiao Zhou, Mark Zylka

David Baltimore
Eric Brown, Rafael Casellas, Jolene Chang, Alexander Hoffman, Wange Lu, Mollie Meffert, Lili Yang, Xiao-Feng Qin

Scott Fraser
Mary Dickinson, Angelique Louie, Carol Readhead, Seth Ruffins, Chris Waters

Mary Kennedy
Jenia Khoroseva, Irene Knuesel, Pasquale Manzerra, Leslie Schenker

Henry Lester
Purnima Deshpande, Carlos Fonck, Joanna Jankowsky, Steven Kwoh, Cesar Labarca, Sacha Malin, Sheri McKinney, Raad Nashmi, Johannes Schwarz, Irina Sokolova, Amber Southwell, Andrew Tapper

Paul Patterson
Sylvian Bauer, Kristina Holmberg, Bradley Kerr, Ali Koshnan.

Ellen Rothenberg
Alexandra Arias, Angela Weiss, Mary Yui

Erin Schuman
Changan Jiang

Melvin Simon
Pam Eversole-Cire, Lingjie Gu, Jong-Ik Hwang, Valeria Mancino, Kum Joo Shin

Alexander Varshavsky
Cory Hu, Jun Sheng

Barbara Wold
Jennifer Ambroggio, Libera Berghella, Brian Williams

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Zijlstra, M., Li, E., Sajjadi, F., Subramani, K.S. and Jaenisch, R. (1989) *Nature* (London) 342:425-638.

Publication

Lois, C., Hong, E.J., Pease, S., Brown, E.J. and Baltimore, D. (2002) Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* 295:868-872.

Monoclonal Antibody Facility

Supervisor: Paul H. Patterson
Director: Susan Ker-hwa Ou
Staff: Shi-Ying Kou

The Monoclonal Antibody Facility provides assistance to researchers wishing to generate monoclonal antibodies (Mabs), polyclonal ascites *Abs* (Immunizing the mice with antigen until the serum titer is high enough then induce the mice with sarcoma cells to obtain high titer polyclonal ascites. This method can provide 10-18 ml polyclonal ascites per mouse by using small amount of antigen), ascites fluid or other tissue culture services. In addition to these service functions, the Facility also conducts research on the development of novel immunological techniques.

In its service capacity, the Facility produced Mabs for the following groups during the past year. Wange Lu from Dr. Baltimore's laboratory obtained polyclonal ascites against peptide sequence from RYK (receptor involved in neuron axon guidance). Qiao Zhou from Dr. Anderson's laboratory obtained polyclonal ascites against peptide sequence of chick Olig 2 (novel basic-helix-lop-helix transcription factor). Dr. Strauss' laboratory obtained polyclonal ascites against Sindbis virus and yellow fever virus E protein. Dr. Witte from UCLA obtained hamster Mabs against G2A protein C terminal. G2A is a G protein–coupled receptor, which is involved in chronic inflammation and autoimmunity, Dr. Witte also obtained mouse Mabs against G2A N terminal and C terminals. Dr. Kaufman from UCLA obtained Mabs against peptide sequence from rare protein in mouse brain. Dr. Rees obtained Mab against MSC1- mechanalsensitive channel protein from *E. coli*.

We are currently working with the following groups. The Patterson lab tries to generate Mabs against TAU (a family of microtubule-associated proteins, which regulate the stability and organization of microtubules in neuronal cells. The Parker lab tries to generate polyclonal ascites against peptide sequence from viral proteins V5-KLH and V5-OVA. The Rajasekaran lab (UCLA) tries to generate Mabs against human embryonic kidney cell. The Baltimore lab tries to generate polyclonal ascites against RYK GST-fusion protein (receptor involved in neuron axon guidance). The Baltimore lab also tries to generate polyclonal ascites against human B94 protein induced by cytokine. The Rees lab tries to generate Mabs against membrane protein, which is a Vit B12 transporter. The Ou lab (USC) tries to generate Mabs against HCV F protein. The Benzer lab tries to generate Mabs against peptide sequences from Painless protein from *Drosophila*. The Zinn Lab tries to generate Mabs against bleached-*Drosophila* protein involved in axonogenesis.

Publications

Bennett, M.J., Huey-Tubman, K.E., Herr, A.B., West, A.P., Ross, S.A. and Bjorkman, P.J. (2002) A linear lattice model for polyglutamine in CAG-expansion diseases. *Proc. Natl. Acad. Sci. USA* 99:11634-11639.

Khoshnan, A., Ko, J. and Patterson, P.H. (2002) Effects of intracellular expression of anti-huntingtin antibodies of various specificities on mutant huntingtin aggregation and toxicity. *Proc. Natl. Acad. Sci. USA* 99:1002-1007.

Lo, L., Dormand, E., Greenwood, A. and Anderson, D.J. (2002) Comparison of the generic neuronal differentiation and neuron subtype specification functions of mammalian *achaete-scute* and *atonal* homologs in cultured neural progenitor cells. *Development* 129:1553-1557.

O'Nuallain, B., Ou, S., Ramsey, H., Ko, J., Patterson, P., Berthelier, V., Macy S. and Wetzel, R. Conformational monoclonal antibodies against polyglutamine aggregates. Manuscript in preparation.

Zhou, Q. and Anderson, D.J. (2002) The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification. *Cell* 109:61-73.

Nucleic Acid and Protein Sequence Analysis Computing Facility

Supervisor: Stephen L. Mayo

Staff: David R. Mathog, Manager, Sequence Analysis Facility

The Sequence Analysis Facility (SAF) provides software, computers, and support for the analysis of nucleic acid and protein sequences. Currently the SAF hardware consists of a Sun Netra running Solaris (mendel.bio.caltech.edu), a small Beowulf cluster of 20 Athlon XP nodes, a file server, a 32 page per-minute, duplexing laser printer, and a color laser printer. The Biology Division's slidemaker, and the PCs that comprise the "structure analysis facility" are also located in our facility. The SAF has over 250 registered users distributed among 50 research groups.

Most common programs for sequence analysis are available on Mendel. These include the GCG and EMBOSS Packages, PRIMER3, Phred, Phrap, Cross_Match, Phylip, and HMMER. Many of these may be accessed through the W2H or Pise web interfaces. Users may supply their own sequences or use those in the locally maintained databases (Genbank, PIR, SwissProt, and others). Other programs, custom written programs, or special databases are available on request. The PCs boot either Linux or Windows XP and support hardware stereo in either mode. Under linux the programs O, Molscript, XtalView, CCP4, and Delphi are available. Under Windows XP Swiss PDB Viewer, O, POVray, and various drawing and animation programs may be used. The searchable documentation for these programs is available on the SAF web server (http://saf.bio.caltech.edu/). The lecture notes and homework from the introductory course "Fundamentals of Sequence Analysis" are also available on the SAF web server. BLAST jobs submitted through the SAF web interface run on the SAF Beowulf cluster (in parallel) faster than they do at the NCBI server.

Nucleic acid sequences from the DNA sequencing facility are distributed through the SAF web and FTP servers and may be analyzed on our servers. We also distribute site-licensed software such as DNASTAR, Gene Construction Kit, and X-Win.

Protein Expression Center

Supervisor: Pamela Bjorkman
Director: Peter M. Snow
Staff: Cynthia Jones, Inderjit Nangiana

The Protein Expression Center was established to meet the needs of the Caltech community in all areas relevant to protein expression. The services provided range from the generation of recombinant DNA constructs to expression of recombinant proteins to purification of the expressed proteins.

The tissue culture portion of the facility, which is central to our operation, is equipped with an inverted phase contrast microscope designed for examination of cells, two laminar flow hoods suitable for sterile manipulations, two incubators capable of supporting the growth of mammalian cell lines, and two refrigerated incubators housing eight spinner plates which are used for the growth of insect cells. The center is currently capable of small to medium scale (100 ml to 2 liter scale) production of recombinant proteins in the baculovirus system, which is the most widely used eukaryotic system for the production of recombinant proteins. We have also explored the use of a second eukaryotic expression system (the methylotropic yeast *Pichia pastorius*), which has the potential to generate large amounts of recombinant proteins. We are currently able to generate at least 10 liters/week of infected cells in the baculovirus system using a combination of spinner flask and shaker flask configurations. A 15-liter bioreactor which is suitable for larger-scale production of proteins expressed in baculovirus as well as other eukaryotic systems (such as mammalian cells) is also available. A second 15 liter fermenter gives us the capability to grow both bacteria and yeast in volumes up to 10 liters. In addition, smaller quantities of both bacteria and yeast (50 ml-2 liters) may be grown in our shaking incubator. We are also capable of growing mammalian cell lines on a more limited basis (25 ml-500 ml). This year the Center acquired a Cell Pharm, a hollow-fiber bioreactor designed for high-level expression of secreted proteins in mammalian cells which gives the Center additional versatility with respect to the variety of expression systems we are capable of exploring and utilizing.

The Expression Center is equipped to perform most common techniques in molecular biology (including PCR, construction and analysis of recombinant DNA vectors, and molecular cloning of DNA molecules). In addition, we are capable of protein concentration, protein purification (FPLC), and analysis using standard biochemical techniques (for example most types of electrophoresis).

This year, the Center has expressed more than 80 different proteins in varying quantities (requiring the generation of more than 250 liters of baculovirus-infected insect cells for a number of investigators both at Caltech and outside of the Institute. During this period, we have made 75 new recombinant viruses, with the recombinant DNA used for the generation of 30 of these viruses being constructed at the Center. In addition, 36 of the expressed proteins were concentrated and at least partially purified by Expression Center personnel for the investigators.

The proteins that have been expressed are diverse in their biochemical and functional characteristics, ranging from nuclear proteins involved in DNA replication to cell surface proteins mediating cell-cell interactions. Similarly, the purposes to which the expressed or purified proteins have been put to use are also varied. Some have been used as immunogens for the generation of antibodies, while some have been used as bait in attempts to characterize putative ligands. In addition, a number of the proteins have been utilized in crystallization trials in attempts to determine their three dimensional structure.

The Center has provided services for the following investigators at Caltech: David Anderson, Pamela Bjorkman, Jacqueline Barton, Judith Campbell, Ray Deshaies, Mary Kennedy, Jay Nadeau (JPL), Douglas Rees, Richard Roberts, Paul Sternberg, Alexander Varshavsky, Linda Hsieh-Wilson, and Kai Zinn.

We have also provided services to Carmelo Bernebeu at the Centro de Investigaciones Biologicas, Madrid, Spain; A.C. Bianco at Harvard Medical School; Anthony Cooper at the University of Chicago; Joyce Fingeroth at Harvard Medical School; Balazs Gereben and Imre Kacskovics at the Institute of Experimental Medicine; Hungarian Academy of Sciences, Budapest, Hungary; Hiromi Kubagawa at the University of Alabama; Chih-Pin Liu at City of Hope Hospital; Anne Mason at the University of Vermont College of Medicine; Minnie McMillan at USC School of Medicine; Malini Raghavan at The University of Michigan Medical School; Scott Rajski at the University of Wisconsin School of Pharmacy; Michael Stallcup at USC Medical School; Thilo Stehle at Massachusetts General Hospital; Cox Terhorst at Harvard Medical School; Eric Turner at the University of California, San Diego; Cynthia Wolberger at Johns Hopkins University School of Medicine; Galina Yahubyan, at the University of Utah; and Olga Zak at the Albert Einstein College of Medicine.

Publications

Dua, R., Levy, D.L., Li, C.M., Snow, P.M. and Campbell, J.L. (2002) *In vivo* reconstitution of *Saccharomyces cerevisiae* DNA polymerase epsilon in insect cells. Purification and characterization. *J. Biol. Chem.* 277(10):7889-7896.

Edwards, S., Li, C., Levy, D.L., Brown, J., Snow, P.M. and Campbell, J.L (2003) *Saccharomyces cerevisiae* DNA polymerase ε and polymerase *s* interact physically and functionally, suggesting a role for pol ε in sister chromatid cohesion. *Mol. Cell. Biol.* In press.

Louie, K.A., Dadgari, J.M., DeBoer, B.M., Weisbuch, H., Snow, P.M., Cheevers, W.P., Douvas, A. and McMillan, M. (2002) Caprine arthritis-encephalitis virus (CAEV)-infected goats can generate human immunodeficiency virus (HIV)-gp120 cross-reactive antibodies. *J. Virol.* In press.

Protein Micro Analytical Laboratory
Director: Gary M. Hathaway
Senior Research Assistant and MPS: Jie Zhou
Associate Biologist: Felicia Rusnak
Faculty Advisors: William G. Dunphy, Kai Zinn
Website: http://www.its.Caltech.edu/~ppmal, or
http://www.caltech.edu/subpages/analyt.html, or
http://www.its.caltech.edu/~bi/bicatalog.html#ppmal

The Protein Micro Analytical Laboratory (PPMAL) facility's main function is to sequence proteins and peptides and perform mass spectrometry on these and other biopolymers including polynucleotides. The laboratory employs well-documented procedures and develops new methods for analyzing biopolymers for a broad user base at Caltech. Cost recovery is by charge-back with supplemental support from the Beckman Institute.

PPMAL is located in Room 204 with offices in Rooms 232 and 215 of the Beckman Institute, 626-395-6388/2769, FAX: 626-449-3414

Activity Centers

Biopolymer Mass Spectrometry and Proteomics

- Protein identification from in-gel digests by mass mapping and cLC/MS/MS
- Tandem mass spectrometry of biomolecules
- Identification of post-translational and chemical modifications
- MALDI-TOF Post source decay and prompt fragmentation analysis
- Database searching for protein identification

Edman Sequencing

- Subpicomole, N-terminal sequence analysis

High Performance Liquid Chromatography

- Submillimeter and capillary HPLC for analysis and purification

Specialty Analyses

- *De novo* sequence analysis of MS/MS derived data
- Identification by "mass tagging" procedures
- Chemical modification of proteins and peptides for mass spectrometry. Including reduction, alkylation, acetylation, and O-methylisourea conversion of lysine residues, chemical targeted identification (CTID) of phospho- and glyco-peptides for mass and chemical sequence analysis
- Consultation on sample preparation and error analysis including dissemination of information including the maintenance of our web site

SPECIFICS

Mass Spectrometry of Biopolymers: The facility is currently operating three mass spectrometers: a triple quadrupole, a research level MALDI time-of-flight, and a quadrupole/time-of-flight (Qstar-pulsar) mass spectrometer. The liquid chromatography (cLC/MS) interface for the Qstar uses laminar flow, static mini-gradients (1). Reverse phase capillary columns are self-packed and are an integral part of the electrospray nozzle (1). With this system, fragmentation spectra can be collected using less than 10 femtomoles of peptide (2).

Protein/Peptide Chemical Sequence Analysis by Edman Degradation: A dual reaction column, capillary microsequencer with subpicomole sensitivity is used. The instrument extends the use of the technology for combined chemical and MS sequencing projects (2). Our modified chemistry is used to improve performance (3).

Chemical Modification of Proteins and Peptides: Chemical conversion of peptides, particularly those with post-translational modifications, is carried out. The facility also publishes its original research efforts in this area such as our new CTID technology (4).

Database Search and Analysis: The laboratory maintains access to database search via the web. MASCOT is used for identifying proteins using information obtained from mass and chemical sequencing experiments.

Collaborations and Accomplishments: During the course of the year the lab interacted with 15 laboratories of the Division of Biology. Additional faculty were supported in Chemistry, Environmental Engineering and Applied Mathematics, and by analyses performed for the biopolymer synthesis (89 mass analyses) and protein expression facilities (56 proteins identified).

The facility published a new method for detecting and analyzing large glycosylated or phosphorylated peptides and proteins (4) and presented three posters. The director presented two posters, the first at the ASMS "Mass Spectrometry in Glycobiology and Glycochemistry," Asilomar (October, 2002), and a second at the ABRF "Translating Biology Using Proteomics and Functional Genomics," Denver (February 2003). At the latter meeting our oral presentation received best in show recognition. Amersham Biosciences presented the award that consisted of a cash prize, certificate of recognition, and commemorative plaque. Dr. Zhou presented a poster entitled: "A Method for the Identification of Post-translationally Modified Peptides by Chemical Targeting," at the ASMS in Montreal, (2003).

Future expectations and directions: Our laboratory continues to develop new and unique procedures, which permit high throughput analysis while maintaining high sensitivity and accuracy. Results are reflected in short turnaround times with reduced costs, which are competitive with similar facilities at other premier institutions. While the lab's main focus of enhanced efficiency has enabled it to complete more than 25,000 analyses, it remains committed to improvement through its research effort.

Publications

(1) Zhou, J., Rusnak, F., Colonius, T. and Hathaway, G.M. (2000) Quasi-linear gradients for capillary liquid chromatography with mass and tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* 14:432-438.

(2) Hathaway, G.M. (2000) Direct gradient-elution of peptides for capillary LC/MS/MS application to the ABRF-00SEQ protein sequencing test peptide. In: "Vydac Advances," summer issue.

(3) Rusnak, F. and Hathaway, G. (2001) Application of a coupled reducing system to Edman sequencing. *J. Biomol. Tech.* 12:40-43.

(4) Rusnak, F., Zhou, J. and Hathaway, G.M. (2002) Identification of phosphorylated and glycosylated sites in peptides by chemically targeted, proteolytic cleavage. *J. Biomol. Tech*. 13:228-237.

Graduates

Doctor of Philosophy - 2003

Yên Kim Buì – (Biology)
B.A., Pomona College, 1996
M.S., California Institute of Technology, 1999
Thesis: Genetic Analysis of LET-23 Mediated IP_3 Signaling in *Caenorhabditis elegans*.

Bruce Seymour Burkemper – (Biology)
B.A., University of Vermont, 1989
Thesis: Biochemical Characterization of *Drosophila* Receptor Tyrosine Phosphatases.

Grace C. Chang – (Computation and Neural Systems)
B.S., Stanford University, 1992
M.S., Stanford University, 1994
Thesis: Neural Representation of Surface Ordering in Visual Areas V1, V2 and MT.

Deepshikha Datta – (Biochemistry and Molecular Biophysics)
B.Sc., University of Delhi, 1996
Thesis: Protein-Ligand Interactions: Docking, Design and Conformation Change.

Andrew Josef Ewald - (Biochemistry and Molecular Biophysics)
B.S., Haverford College, 1997
Thesis: The Molecular Control of Cell Movements during Early Vertebrae Development.

Martha Kirouac – Biology
B.S., Union College, 1996
M.S., California Institute of Technology, 2000
Thesis: *cis*-Regulatory Control of Three Cell Fate-Specific Genes in Vulval Organogenesis of *C. elegans* and *C. briggsae*.

Chunhui Mo - (Biochemistry and Molecular Biophysics)
B.S., Peking University, 1994
Thesis: Synaptic Learning Rules for Local Synaptic Interactions: Theory and Application to Direction Selectivity.

Cindy María Quezada - (Biochemistry and Molecular Biophysics)
B.S., University of California, Davis, 1995
Thesis: Histidine Phosphorylation in Bacterial Chemotaxis.

Carlo Joseph Quiñónez – (Biology)
B.S., San Diego State University, 1997
M.S., California Institute of Technology, 1999
Thesis: Theory and Design of Relaxometric Probes.

Christopher Ashby Voigt - (Biochemistry and Molecular Biophysics)
B.S.E., University of Michigan, 1998
Thesis: Computationally Optimizing the Directed Evolution of Proteins.

Qiao Zhou – (Biology)
B.S., Qingdao Ocean University, 1993
M.S., Boston University, 1997
Thesis: Glial Cell Development in the Vertebrate Central Nervous System.

2003 – Master of Biology

Deepshikha Datta – (Biochemistry and Molecular Biophysics)

Andrew Gabriel-Antonio Medina-Marino – (Biology)

2003 - Bachelor of Science, Biology

Owen Peter Aftreth – (Biology)
Sangeeta Bardhan* – (Biology)
Audrey Beth Carstensen – (Biology)
James Zachary Chadick* - (Biology and Chemistry)
Shay Chinn – (Biology)
Lusine Danakian* – (Biology)
China An Hanson – (Biology)
Daniel Huibo Kim – (Biology)
Hannah Kyungjoo Kim – (Biology)
Stephanie Ann Kovalchik* - (Biology and Literature)
Miguel Edmundo Lemus – (Biology)
Alexander Peter Lin – (Biology)
Arjun Venkat Narayanan – (Biology)
Timothy Van Pfeiffer* – (Biology)
Rey Natividad Ramirez – (Biology)
Colin Witter Rudel – (Biology)
Anthony James Michael Salter* – (Biology)
Isaac See* - (Mathematics and Biology)
Saken Sherkhanov* – (Biology)
Elizabeth Lee Stameshkin – (Biology)
Sarah Lynn Teegarden* – (Biology)
Christina Lee Telles - (Biology and Literature)
Sonia Crago Timberlake – (Biology)
Kevin Yee-Bien Tse – (Biology)
Nora Na Tu* – (Biology)

*Graduated with honors in accordance with a vote of the faculty

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Entrepreneurial Program Fellowship
Evelyn Sharp Fellowship
John and Ellamae Fehrer Endowed Biomedical Discovery Fund
Ferguson Fellowship
Ferguson Fund for Biology
Lawrence L. and Audrey W. Ferguson Prize
Fletcher Jones Foundation
Fling Charitable Trust
Frank P. Hixon Fund
French Foundation Fellowship
French Foundation for Alzheimer Research

Gates Grubstake Fund
German Government Fellowship
William T. Gimbel Discovery Fund in Neuroscience
Ginger and Ted Jenkins
Gordon E. and Betty I. Moore Foundation
Gordon Ross Fellowship
Gordon Ross Medical Foundation
Gosney Fellowship Fund
Grubstake Presidents Fund

William D. Hacker Trust
Lawrence A. Hanson, Jr. Professorship of Biology
Helen Hay Whitney Fellowship
Helen Hay Whitney Foundation
Hereditary Disease Foundation
Hicks Fund for Alzheimer Research
Hixon Professorship of Psychobiology
Dr. Norman Horowitz
Howard Hughes Medical Institute
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Human Frontiers in Science Program (HFSP)
Huntington Hospital Research Institute

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Jet Propulsion Lab
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Josephine V. Dumke Fund
Joyce Charitable Fund
Joyce Fund for Alzheimer's Disease Research

Keck Discovery Fund
W.M. Keck Foundation for Discovery in Basic Medical Research
Steven and Michele Kirsch Foundation
The Susan G. Komen Breast Cancer Foundation
Kroc Foundation

L.A. Hanson Foundation
Leukemia and Lymphoma Society
Life Science Research Foundation
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McKnight Endowment Fund for Neuroscience
McKnight Foundation
McKnight Neuroscience of Brain Disorders Award
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Moore Foundation
Muriel and Millard Jacobs Fund for Genomics and Genetics Technology

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National Imagery and Mapping Agency
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National Institute of Arthritis and Musculoskeletal and Skin Disease
National Institute of Child Health and Human Development
National Institute of Dental and Craniofacial Research
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National Institute on Deafness and Other Communication Disorders
National Institute on Drug Abuse
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National Multiple Sclerosis Society
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Norman Chandler Professorship in Cell Biology
Norman Davidson Lectureship
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Rosalind W. Alcott Scholarship Fund
Anna L. Rosen Professorship
Benjamin Rosen Family Foundation
William E. Ross Memorial Student Fund
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Damon Runyon Fellowship

Sandia National Laboratories
Warren and Katherine Schlinger Foundation
Edwin H. Schneider Fund
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Sun Microsystems, Inc.
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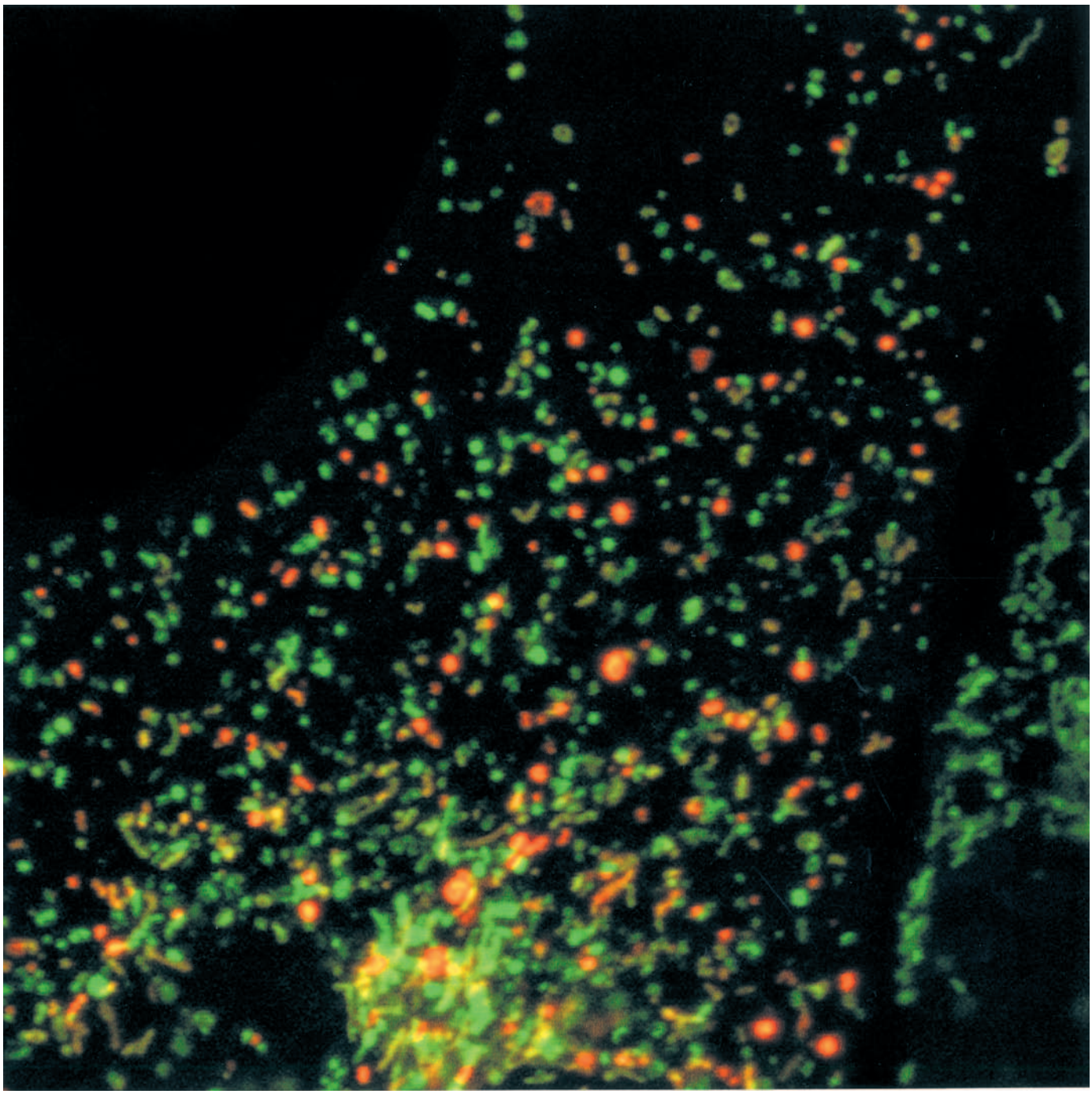
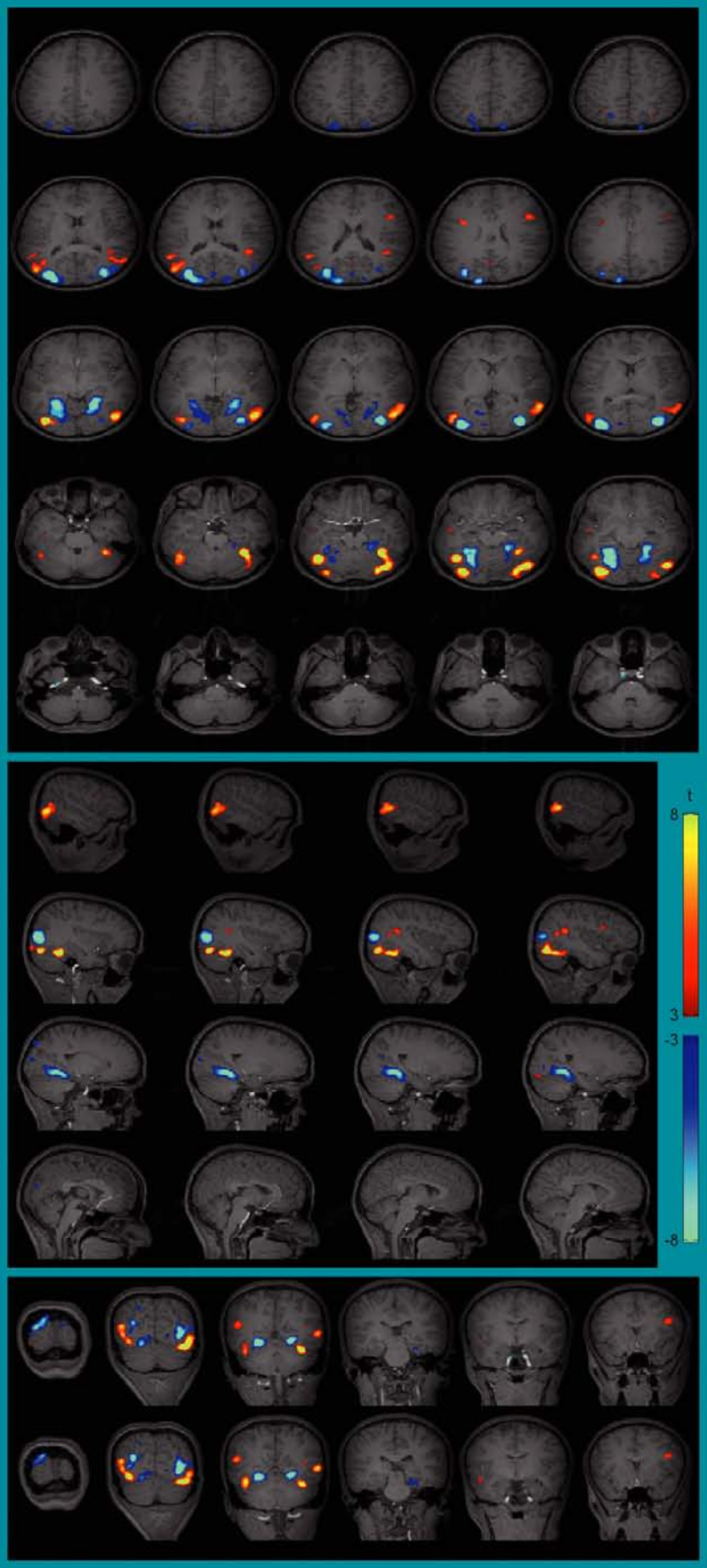
Index of Names

Index of Names



Assistant Professor of Biology, Bren Scholar, David C. Chan

Disruption of mitofusin 1 (Mfn1) in mouse cells results in a severe reduction in mitochondrial fusion. When Mfn1 mutant cells containing GFP (green) or dsRed (red) labeled mitochondria are fused by polythylene glycol (PEG), the mitochondria in fused cells fail to fuse and therefore remain distinctly green or red.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-3
-8