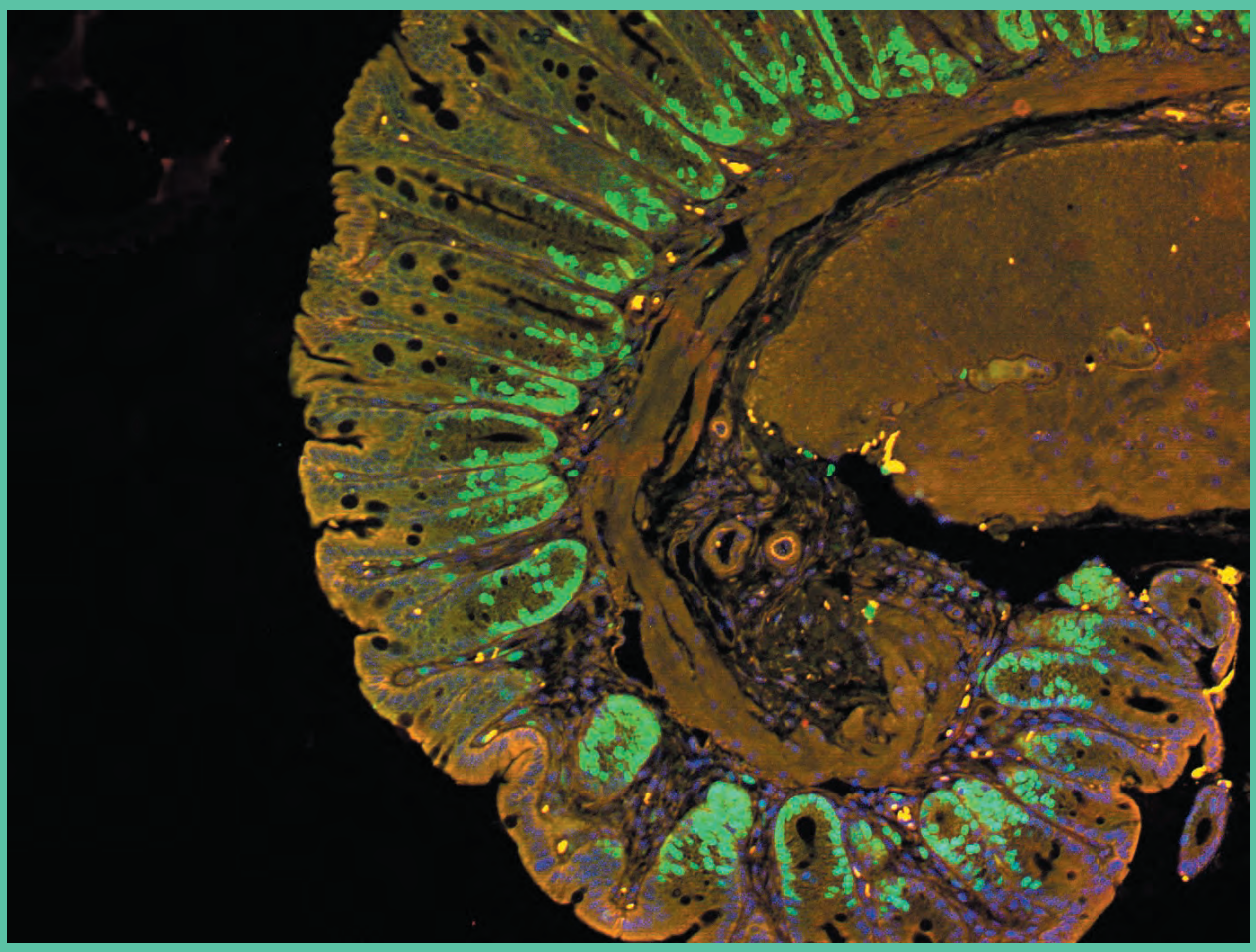

CALTECH Biology Annual Report 2009

Front Cover Illustration

Beneficial bacteria in the gut protect animals from developing intestinal inflammation. Cross sectional image of a mouse colon stained for cell proliferation with Ki-67 (green), and epithelial cell nuclei stained with DAPI (blue). Cell proliferation occurs at the base of the colonic crypts, where progenitor stem cells divide and daughter cells migrate to the surface of the epithelium. Cell proliferation is increased during intestinal disease (marked by Ki-67+ cells found near the epithelial surface), and reduced upon colonization with symbiotic bacteria. Image courtesy of Arya Khosravi and Assistant Professor Sarkis K. Mazmanian.

See abstract 226.

Back Cover Illustration

Neural crest cells in zebrafish embryos provided by Tatiana Hochgreb in the Marianne Bronner-Fraser laboratory.

Division of Biology

California Institute of Technology

Pasadena, California

Annual Report
2008 - 2009

BIOLOGY

Yolanda Duron, Annual Report Coordinator

Research Reports

Biological research summarized in this report covers the time period from June, 2008 through July, 2009. 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.

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FACILITIES

INTRODUCTION

100 Years Ago - 1909

The Bulletin of the Throop Polytechnic Institute (an earlier name for Caltech) for 1909 noted that "The school has an ideal environment. Pasadena is not only one of the most beautiful and healthful of cities, with a climate of unapproached equability and poise, but is also noted for the morality, refinement and culture of its citizenship. Saloons are prohibited by charter. Boys under age are shut out by statute from questionable places of amusement, such as pool-rooms, of which there are few."

There were only three biology courses offered in the 1908-1909 school year: Zoology, Botany, and Human Physiology and Hygiene. The Zoology course offered "the careful study of several selected animals, such as the earth-worm, cray-fish, crane-fly, star-fish, squid, toad and rabbit."

75 Years Ago - 1934

The 1934 graduates of the Biology Ph.D. program were Marston Sargent (Aspects of the physiology of the blue-green algae), Emory Ellis (The free energy of the sulfhydryldisulfide oxidation-reduction system and its physiological significance), and James Bonner (Growth substance and cell elongation). Sargent (1906-2003) left Caltech for the Scripps Institute of Oceanography, where he taught oceanography, after which he worked for the Office of Naval Research as an oceanographer. Ellis (1906 – 1993) stayed at Caltech as a postdoc until World War II; he first introduced Max Delbrück to bacteriophage, a collaboration that resulted in the classic beginning to phage molecular genetics, Ellis, E.L. and Delbrück, M. (1939) The Growth of Bacteriophage, *J. Gen. Physiol.* 22, 365-384. James Bonner (1910-1996), after a brief European postdoc, remained at Caltech as a much-honored and highly productive member of the Biology faculty, from which he retired in 1980.

50 Years Ago - 1959

Biology 1959, the Division annual report for 1958-9, quietly reported on page 127, under Honors and Awards, "G.W. Beadle received the Nobel Prize in Biology and Medicine. He also received an honorary D.Sc. degree from Oxford University." Beadle had won a share of the 1958 Nobel Prize for his work in elucidation of the nature of the gene.

The introduction to Biology 1959 stated "The activities of the Division during the year 1958-1959 are summarized in this report. The different investigations carried on during the year were concerned with such diverse matters as the molecular nature of reproduction and the transfer of learning between two halves of the brain…"

25 Years Ago - 1984

The Caltech Biology Annual Report 1984 listed three new faculty member who had joined the Division in the previous year, Scott Emr, Paul Patterson, and Mark Tanouye.

It also noted the passing of Henry Borsook on March 6, 1984; Borsook, one of the first members of the new Biology Division, was a distinguished biochemist whose work on vitamins in the 1930s and 1940s led to the establishment of the government's Recommended Daily Allowance list. He was a founder of the charity Meals for Millions, which distributed a high-protein supplement that he had developed (MPF, Multi-Purpose Food) in war-ravaged Europe: in the ten years starting in 1946, over 6.5 million pounds were distributed in 129 countries.

Assistant Professor Doris Tsao joined the Caltech Biology faculty in January 2009 to continue her research in cortical circuits for face recognition and depth perception. She is a Caltech alum having obtained undergraduate dual majors in Biology and Mathematics. She did her Ph.D. thesis with Margaret Livingstone at the Harvard Medical School where she studied cortical networks for depth perception and face recognition. She continued at Harvard for two years of postdoctoral study with Margaret Livingstone, David Hubel, and Roger Tootell. She was Head of Young Research Group at the University of Bremen before coming to Caltech. While at Harvard she pioneered the technique of using functional magnetic resonance imaging (fMRI) to target microelectrode recordings. While using this combined technique, she discovered that the inferior-temporal cortex contains several patches where the cells are selective for images of faces. She also showed that these areas are interconnected using electrical stimulation. She is now examining how nodes in this network process difference aspects of face perception and also how areas of extra-striate cortex contribute to depth perception. Her lab is using the high field magnets in the Caltech Brain Imaging Center along with a variety of other recording and imaging techniques to understand visual perception at a mechanistic level.

Assistant Professor David Prober has joined the Caltech Biology faculty to pursue his research in molecular genetics of behavior. David was born in Winnipeg, Canada, attended University of Manitoba, and worked as a research assistant for one year prior to pursuing his graduate career. His doctoral work was performed at the Fred Hutchinson Cancer Center with Dr. Bruce Edgar working on the role of oncogenes in *Drosophila melanogaster* development. Most notably, David demonstrated that the proto-oncogenes RAS, and MYC control cell growth as opposed to cell proliferation in *Drosophila*. He then joined Alex Schier's laboratory for postdoctoral research, first at the Skirball Institute at New York University, then at Harvard University. There, David initiated the study of sleep in the zebrafish, a relatively new intensively studied laboratory model organism. He convincingly demonstrated that aspects of mammalian sleep are conserved in this fish and has been exploiting the useful features of this experimental preparation to gain deeper insights into how sleep is regulated. He has carried out chemical and genetic screens to perturb sleep and wake behaviors in fish, and has identified several candidate sleep regulators. David's goal is to identify the neural circuits and genetic mechanisms that regulate sleep. His fish are thriving in the sub-basement of Church Laboratory; his lab is temporarily on the second floor of the Broad Center for the Biological Sciences while he awaits renovation of his laboratory on the second floors of the Church and Alles Buildings.

LAWRENCE L. AND AUDREY W. FERGUSON PRIZE, 2009

Athanassios Siapas Casimir Wierzynski Elliot M. Meyerowitz

Dr. Casimir Wierzynski is the winner of the Ferguson Award for the 2008-2009 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. Wierzynski performed his graduate studies in the laboratory of Professor Thanos Siapas.

His thesis work focused on understanding how the hippocampus interacts with prefrontal cortical areas. First, he showed that during sleep hippocampal-prefrontal interactions are exclusively driven by strong population bursts in the hippocampus lasting about 100 ms. Surprisingly, correlated firing is completely absent outside these bursts and in REM sleep. These observations identify the precise points in time when the hippocampus talks to prefrontal cortex during sleep, and open the door to fine tuned manipulations that can reveal the role of sleep in memory. Second, he identified a class of prefrontal cells whose firing reflects the strength of a learned association, and showed that these cells are strongly modulated by hippocampal theta oscillations. These observations provide a framework for characterizing the structure of cortical memories and their evolution throughout the course of memory consolidation.

PROFESSORIAL AWARDS, 2008 - 2009

Richard A. Andersen**, **James G. Boswell Professor of Neuroscience, presented the Mountcastle Lecture at the Johns Hopkins Medical School in 2009.

David J. Anderson has been named the ***Seymour Benzer Professor of Biology***, effective May 1. This title replaces that of Roger W. Sperry Professor of Biology. Anderson received his AB from Harvard in 1978 and his Ph.D. from the Rockefeller University in 1983. He joined Caltech's faculty as an assistant professor in 1986; becoming associate professor in 1992; professor in 1996; and Sperry Professor in 2004. He is also an investigator with the Howard Hughes Medical Institute. A professor at Caltech since 1967, Crafoord Laureate Seymour Benzer was James G. Boswell Professor of Neuroscience, Emeritus, at the time of his death in 2007.

Marianne Bronner-Fraser, Albert Billings Ruddock Professor of Biology, was honored as follows:
2009 - Fellow, American Academy of Arts and Science; Honoree: Caltech Alumni Association and Premedical Association; Keck Foundation Young Scholar's Scientific Advisory Committee; and was awarded the GSC Teaching and Mentoring Award. Is currently on the Board of Directors for the Gordon Research Conferences; National Institute of Craniofacial and Dental Research Council Member; Editor: Developmental Biology, Journal of Cell Biology, Molecular Biology of the Cell.
2008-2009 - President, Society for Developmental Biology.

David C. Chan**, **Associate Professor of Biology**, **Bren Scholar, was appointed Investigator for the Howard Hughes Medical Institute.

Michael Elowitz, Associate Professor of Biology and Applied Physics; Bren Scholar; Investigator, Howard Hughes Medical Institute, received the following awards in 2008: Presidential Early Career Award in Science and Engineering; Discover Magazine Top 20 under 40; and became an HHMI Investigator.

***Grant J. Jensen**, **Associate Professor of Biology**,* was appointed Investigator for the Howard Hughes Medical Institute.

Sarkis Mazmanian, Assistant Professor of Biology, was awarded the following: Damon Runyon Cancer Foundation Innovation Award; W.M. Keck Foundation Research Excellence Award; Excellence in Teaching Award, Associated Students of the California Institute of Technology; "Best Brains under 40" *Discover* Magazine; and Benirschke Scholar in Ulcerative Colitis Research, Crohn's and Colitis Foundation.

Elliot M. Meyerowitz**, **George W. Beadle Professor of Biology and Chair of the Division, was elected an Associate Member of the European Molecular Biology Organization in 2008.

Shinsuke Shimojo, Professor of Biology, received the "Most Creative Study" award, June 2008, by the Japanese Society of Cognitive Science; and the Nakayama Grand Prix, for "significant contributions to the science of emotion," sponsored by the Nakayama Press, Japan.

Paul W. Sternberg, Thomas Hunt Morgan Professor of Biology, was elected in 2008 to the National Academy of Sciences.

Doris Y. Tsao, Assistant Professor of Biology, received the following awards in 2009: Alfred Sloan Scholar, NARSAD Young Investigator, John Merck Scholar, Searle Scholar, and became a Klingenstein Fellow.

Other Awards

Rochelle A. Diamond, Member of the Professional Staff, has been named a fellow of the American Association for the Advancement of Science (AAAS), 2009. The association recognized Diamond for "outstanding technical contributions to the field of cytometry/cell sorting, and for distinguished professional service in development of understanding human diversity in science and engineering."

Pictures from the Jim and Ellen Strauss Symposium on Molecular Virology, held December 15, 2008

Pictures from the Biology Annual Retreat held October 24-26, 2008 at Lake Arrowhead Resort & Spa,
photos taken by Patricia Mindorff

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Summary: Our laboratory investigates the psychological and neural bases of social cognition, using a number of different approaches. Some studies focus on the psychological level, using behavioral data from healthy people to make inferences about how emotion modulates memory, attention, or conscious awareness. A second approach uses neuroimaging and electrophysiology to investigate the neural mechanisms behind emotional and social processing. A third approach studies the performances, and the brains, of special populations. At Caltech, we have been recruiting people with agenesis of the corpus callosum to investigate the functional consequences of disruption in long-range connectivity. Dr. Lynn Paul leads this work. In collaboration with Joe Piven at the University of North Carolina, we have also been studying people with autism, as well as their first-degree relatives (the parents). At the University of Iowa, we have ongoing collaborations that involve neurological populations with focal brain lesions, and that involve neurosurgical populations in whom we can record intracranially.

A major focus in the past year has been on the amygdala, and on autism. We are interested in how the amygdala modulates allocation of attention to emotional stimuli, and how it guides decisions about options whose outcomes have emotional value. One particularly active area of development is to better understand how amygdala and prefrontal cortex interact, and how they interface with other brain structures that also participate in emotional processing. Another active area of research investigates brain connectivity. We hope that a better understanding of the neural basis of social cognition will help with the diagnosis and treatment of people diagnosed with autism, agenesis of the corpus callosum, or mood disorders. We have been recruiting high-functioning adults with autism and also their parents in the past year and investigating social information processing using a wide range of approaches (behavioral, eyetracking, fMRI, and even intracranial recording from the amygdala in an individual with a diagnosis of autism).

1. Lesion mapping of intelligence

Jan Glaescher, Daniel Tranel, Hanna Damasio, Lynn Paul, David Rudrauf, Ralph Adolphs

The Wechsler Adult Intelligence Scale (WAIS) is commonly used to assess a wide range of cognitive abilities and impairments. Factor analyses reveal four underlying abilities: verbal comprehension (VCI), perceptual organization (POI), working memory (WMI), and processing speed (PSI). We used non-parametric voxel-based lesion-symptom mapping in 241 patients with focal brain damage to investigate the neural underpinnings of these factors. We found statistically significant lesion-deficit relationships in left inferior frontal cortex for VCI, in left frontal and parietal cortex for WMI, and in right parietal cortex for POI. There was no reliable single localization for PSI, which appeared to draw on a distributed set of brain regions. Statistical power maps and cross-validation analyses quantified specificity and sensitivity, and additional analyses showed that there were also effects of age and gender. Our findings provide the most comprehensive lesion maps of intelligence, provide novel criteria for their application in neuropsychological diagnosis, and suggest that the current factor structure of the WAIS may need revision in light of divergent anatomical substrates for different specific tasks comprising a single psychological factor.

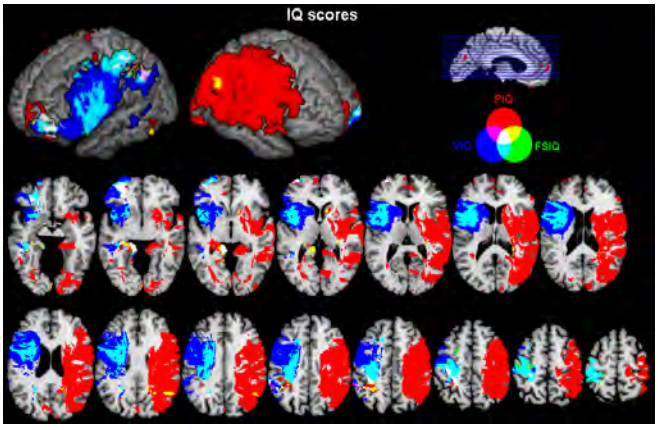

Figure 1. Voxelwise analysis for verbal, performance, and full-scale IQ. This analysis compares the IQ scores for patients with a lesion against those without a lesion at each and every voxel in the brain, thresholded at 1% False Discovery rate. In red are regions where lesions impair performance IQ (PIQ), in blue verbal IQ (VIQ) and other colors showing overlap as in the Venn diagram inset. In our paper, we carried out detailed studies for different aspects of intelligence derived from factor analyses, and

described such lesion-impairment maps for each of the different subtests of the Wechsler Adult Intelligence Scale. These studies are now being extended to look at a single common psychometric factor shared across all of the tests: the so-called "g-factor" of intelligence research. From Glaescher *et al.* (2009).

2. Intact rapid discrimination of fear in the absence of the amygdala

Naotsugu Tsuchiya, Farshad Moradi, Csilla Felsen, Ralph Adolphs

A prevalent view of the amygdala's contribution to processing facial expressions of fear is that it comes into play early and automatically, possibly through a subcortical route that mediates rapid detection of fear. We discovered that a patient with complete bilateral amygdala lesions, who is impaired in recognizing fearful faces, nonetheless showed entirely normal rapid detection of fearful faces, and normal non-conscious processing of fearful faces as well. The findings argue against an essential early-processing or non-conscious role for the amygdala, and instead favor a contribution that incorporates contextual and cognitive modulation.

Subjects were shown two faces (one emotional, one neutral) or two scenes (one threatening, one neutral) side-by-side and asked, as rapidly as possible, to push a button to indicate the side of the emotional stimulus. The amygdala patient was as fast and as accurate as controls were (Figure 2), despite the fact that her recognition and ratings of emotional stimuli are impaired.

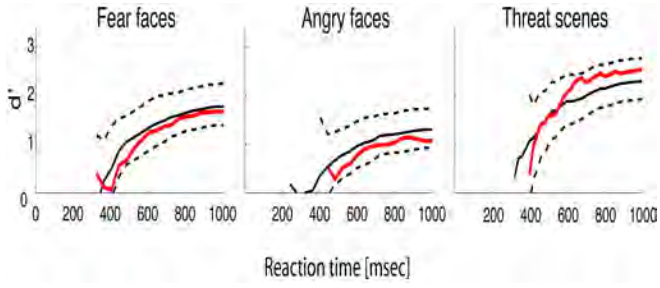

Figure 2. Amygdala patient (red) can detect fearful and angry faces as well as threat scenes as rapidly and accurately as age-matched controls (black, 95% confidence interval is dotted). The y-axis plots d', a measure of accuracy.

3. Personal space regulation by the human amygdala

Daniel Kennedy, Jan Glaescher, J. Michael Tyszka, Ralph Adolphs

The amygdala plays key roles in emotion and social behavior, yet how this translates to face-to-face interactions involving real people remains poorly understood. A series of experiments with patient SM, who has complete bilateral amygdala lesions, found that she lacks any sense of personal space. An additional fMRI study confirmed amygdala activation to close personal proximity. Our findings argue that the amygdala is required to trigger the strong emotional reactions normally following personal space violations, thus regulating interpersonal distance in humans.

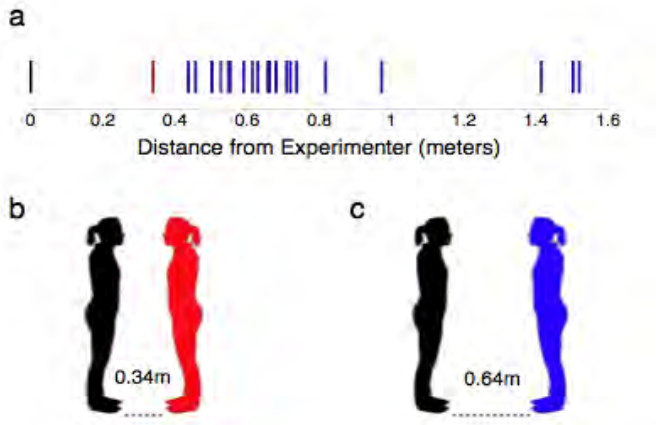

Figure 3. Lesion Study: Mean preferred distances from the experimenter. (**A**) SM's (red) preference was the closest distance to the experimenter (black), compared to age-, gender-, race-, and education-matched controls (purple, n = 5), as well as general comparison subjects (blue, n=15). (**B**) SM's mean preferred distance away from the experimenter (image drawn to scale). (**C**) Control participants' mean preferred distance away from the experimenter, excluding the largest outliers (image drawn to scale).

4. Eye movement-related EMG contamination in intracranial electrophysiology

Christopher Kovach, Nao Tsuchiya, Hiroto Kawasaki, Hiroyuki Oya, Mathew A. Howard III, Ralph Adolphs

It is widely assumed that intracranial recordings are unlikely to be affected by muscle EMG contamination. We show that this is not the case for saccade related oculomotor EMG signals. Saccade onsets are associated with a transient biphasic potential, resembling the saccadic spike potential in scalp EEG, and an increase in broad-band power affecting especially gamma and high gamma ranges. Measurements of interchannel coherence between 40 Hz and 100 Hz are especially susceptible to eye movement related contamination. To develop a precise model of the spatiotemporal contribution of oculomotor contamination, we concurrently measured eye movements and high-density intracranial EEG in five neurosurgical patients. The distribution of responses agrees with an orbital dipole source. We compare methods for eliminating and ruling out eye movements as a source of task related responses, and show that bipolar referencing and ICA filtering are effective for suppressing contamination at locations far from the orbits. After accounting for eye movement EMG, we confirm that broad-band gamma activity is a genuine feature of cortical visual responses.

5. The neuropsychological profile of autism and the broad autism phenotype

Molly Losh, Ralph Adolphs, Michele Poe, Shannon Couture, David Penn, Grace Baranek, Joseph Piven

There now exist multiple reports of a constellation of language, personality, and social-behavioral features present among relatives that mirror the symptom domains of autism, but much milder in expression. Studies of this 'broad autism phenotype' (BAP) may provide a potentially important, complementary approach for detecting the genes causing autism, by identifying more refined phenotypes which can be measured quantitatively in both affected and unaffected individuals, and which are tied to functioning in particular regions of the brain. To gain insights into neuropsychological features that index genetic liability to autism, we carried out a series of studies in high-functioning individuals with autism and parents of autistic individuals, both with and without the BAP (N=83), as well as control groups. A comprehensive battery of neuropsychological tasks tapping social cognition, executive function, and global/local processing strategies (central coherence) was used. We found that both individuals with autism as well as the parents with the BAP differed from controls on measures of social cognition, with performance in the other two domains more similar to controls. In conclusion, the findings suggest that the social cognitive domain may be an important target for linking phenotype to cognitive process to brain structure in autism, and may ultimately provide insights into genes involved in autism.

6. The inferior front-occipital fasciculus mediates recognition of facial expressions of emotion

Carissa L. Philippi, Sonya Mehta, Thomas Grabowski, Ralph Adolphs, David Rudrauf

Lesion and neuroimaging studies have implicated multiple visual and emotion-related brain regions in the recognition of emotion from facial expressions. Electrophysiological studies have demonstrated that the communication between these regions can be very rapid during this process. However, it remains unknown by what mechanism visual representations of the face can be efficiently conveyed to emotion-related brain regions that associate the face with its emotional content. Based on a recent dynamic causal modeling study, we hypothesized that long-range association fiber tracts might be critical in this process. To test this hypothesis, we used a novel approach incorporating fiber tract information into lesion mapping. We tested 103 patients with focal, stable brain lesions on their recognition of six facial expressions of emotion (happiness, sadness, fear, disgust, surprise, anger) and mapped their lesions onto a reference brain for detailed analysis. Six association fiber tracts from a probabilistic atlas (the cingulum [cingulate gyrus process] and cingulum [hippocampal process], the uncinate, inferior and superior longitudinal, and inferior fronto-occipital [IFOF] fasciculi) were registered to the same reference brain for lesion-deficit analysis. Intersections of the lesions with these tracts were used to compute probable disconnection. These tract-specific disconnection measures were then used simultaneously in a general linear model to predict emotion recognition impairments after covarying for lesion size and damage to cortical regions. Disconnection of the right IFOF significantly predicted an overall impairment in emotion recognition, as well as specific impairments for sadness, anger and fear. One subject had a pure white matter lesion mostly restricted to the IFOF. The degree of emotion recognition impairment in this subject supported the group level results. Our findings strongly implicate the right IFOF as a critical component of the large-scale neural system supporting the recognition of the facial expression of emotion.

7. Economic games quantify diminished sense of guilt in patients with damage to the prefrontal cortex

I. Krajbich, Ralph Adolphs, Daniel Tranel, N. Denburg, C. Camerer

Damage to the ventromedial prefrontal cortex (VMPFC) impairs concern for other people, as reflected in the dysfunctional real-life social behavior of patients with such damage, as well as their abnormal performances on tasks ranging from moral judgment to economic games. Despite these convergent data, we lack a formal model of how, and to what degree, VMPFC lesions affect an individual's social decision-making. Here we provide a quantification of these effects using a formal economic model of choice that incorporates terms for the disutility of unequal payoffs, with parameters that index behaviors normally evoked by guilt and envy. Six patients with focal VMPFC lesions participated in a battery of economic games that measured concern about payoffs to themselves and to others: dictator, ultimatum, and trust games. We analyzed each task individually, but also derived estimates of the guilt and envy parameters from aggregate behavior across all of the tasks. Compared to control subjects, the patients donated significantly less and were less trustworthy, and overall our model found a significant insensitivity to guilt. Despite these abnormalities, the patients had normal expectations about what other people would do, and they also did not simply generate behavior that was more noisy. Instead, the findings argue for a specific insensitivity to guilt, an abnormality that we suggest characterizes a key contribution made by the VMPFC to social behavior.

8. A connectivity map of the macaque cortex from diffusion imaging

Dirk Neumann, J. Michael Tyszka, J.H. Parvizi, Ralph Adolphs

Understanding the functions of different cortical regions requires knowledge of their inputs and outputs, yet little is known about cortical connectivity at the systems-level, in large part because such information has required labor-intensive studies using *in vivo* tracers. A recently developed alternative relies on diffusion MR imaging to

generate models of probable white-matter tracts and their orientation, but this method suffers from its indirectness. Here we validated connectivity models obtained from diffusion MRI by comparing them with published tracer studies, and then applied our method to two macaque brains to reveal global cortical connectivity. We describe the strength of connections that are already known and propose new connections never before reported. Using dimensionality reduction methods, we visualize the complete connectivity matrix of all known cortical regions and demonstrate an architecture that likely reflects functional modularity and hierarchical processing. Our results are the first complete estimate of cortical connectivity in the macaque brain, and generate new hypotheses about long-range connectivity as well as provide criteria for the application of similar methods to the study of the human brain.

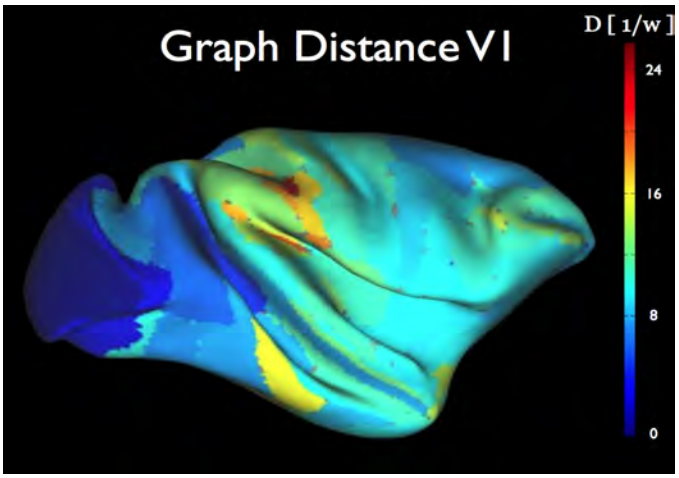

Figure 4. Inverse connection weight from V1 denoting relative connectivity distance.

9. Economic games quantify diminished sense of guilt in patients with damage to the prefrontal cortex

I. Krajbich, Ralph Adolphs, Daniel Tranel, N. Denburg, C. Camerer

Damage to the ventromedial prefrontal cortex (VMPFC) impairs concern for other people, as reflected in the dysfunctional real-life social behavior of patients with such damage, as well as their abnormal performances on tasks ranging from moral judgment to economic games. Despite these convergent data, we lack a formal model of how, and to what degree, VMPFC lesions affect an individual's social decision-making. Here we provide a quantification of these effects using a formal economic model of choice that incorporates terms for the disutility of unequal payoffs, with parameters that index behaviors normally evoked by guilt and envy. Six patients with focal VMPFC lesions participated in a battery of economic games that measured concern about payoffs to themselves and to others: dictator, ultimatum, and trust games. We analyzed each task individually, but also derived estimates of the guilt and envy parameters from aggregate behavior across all of the tasks. Compared to control subjects, the patients donated significantly less and were less trustworthy, and overall our model found a significant insensitivity to guilt. Despite these abnormalities, the patients had normal expectations about what other people would do, and they also did not simply generate behavior that was more noisy. Instead, the findings argue for a specific insensitivity to guilt, an abnormality that we suggest characterizes a key contribution made by the VMPFC to social behavior.

10. The role of the amygdala in orienting attention to eyes within complex social scenes

Elina Birmingham, Moran Cerf, Ralph Adolphs

For years, the amygdala has been implicated as a brain structure dedicated to rapidly processing emotionally salient stimuli in the environment. Recently, it has been proposed that the amygdala has a more general role in orienting attention to socially salient stimuli. For instance, Adolphs *et al.* (*Nature*, 2005), found that SM, a patient with rare bilateral lesions of the amygdala, was unable to recognize fearful facial expressions because she failed to fixate the eyes of the faces. The eyes are particularly informative social stimuli, and therefore, may be ones that the amygdala is particularly biased to detect. The present study examined whether this same patient (SM) fails to fixate the eyes of people when they are presented within complex social scenes. We also investigated the role of context in which the scene occurs on SM's pattern of fixations. We presented a variety of real world social scenes under three task conditions: One asking viewers to report where people in the scene were directing their attention, a second asking viewers to describe the scene, and a third asking viewers to describe in which room the scene was taking place. Across all three conditions, SM looked less often at the eyes and more at the mouth relative to control participants (**Figure 5**). Comparing the different tasks, we found that both SM and controls looked more often at the eyes when asked to report on the social attention in the scene. These results suggest that amygdala damage may lead to a specific reduction of exploration of other people's eyes, but that task instructions can modulate this bias to some extent. The findings extend our prior work with isolated faces and corroborate the view that the amygdala helps orient attention to certain socially salient stimuli, notably and perhaps specifically, the eyes.

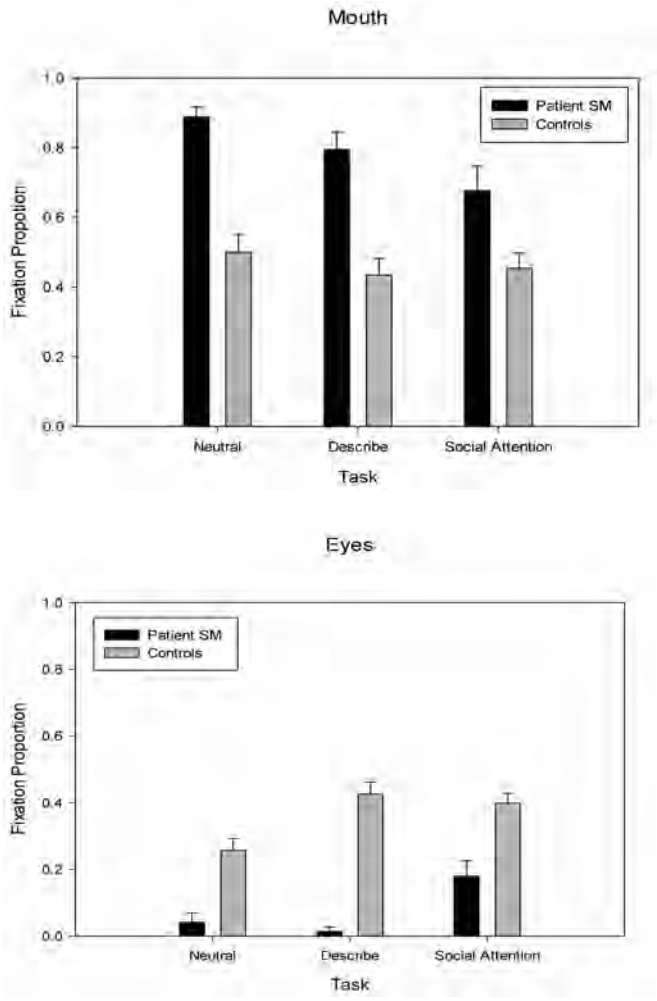

Figure 5. Proportion of fixations onto the eyes or mouth in social scenes. Shown are data from patient SM, who has bilateral amygdala lesions, and healthy controls. SM looks much less at the eyes and more at the mouth.

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Frank P. Hixon Professor of Neurobiology: John M. Allman
Graduate Students: Anna Abelin, Nicole Tetreault
Research and Laboratory Staff: Atiya Hakeem, Soyoung Park

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Summary: We are investigating the neuropathology of autism in research done in collaboration with the Wold laboratory using a new technology, RNA-Seq, to map gene expression in sites in the brain including areas FI and ACC. Our initial results have revealed strongly increased expression of genes involved in inflammation and the activation of the immune cells of the brain, the microglia, in some autistic brains. We have also found that the Von Economo neurons (VENs) strongly and rather selectively express a number of genes involved in immune functioning suggesting that the VENs may have a role in the neural modulation of immune responses in normal subjects and may play a role in neuroinflammation in autism.

Finally we are continuing our study of fiber pathways in primate brains using probabilistic tractography based on diffusion tensor magnetic resonance imaging.

11. Immune regulation and the role of Von Economo Neurons and forked cells

Nicole A. Tetreault, Atiya Y. Hakeem, Yvonne Pao, Sue Jiang, Barbara J. Wold[*]*, John M. Allman*

Von Economo Neurons (VENs) are recently evolved large bipolar neurons that were originally discovered in humans and great apes. The VENs are located in anterior cingulate cortex (ACC) and frontoinsular cortex (FI), regions of the brain associated with higher cognitive functions and monitoring intuitive responses to complex social encounters. We have several lines of evidence that the VENs and other related layer 5 neurons are involved in immune function and regulation. In this study, we immunocytochemical stained ACC and FI and found VENs and other related layer 5 neurons such as forked cells were specifically labeled by the proteins of ATF3, IL4R1alpha, IL6R, IL8R and SOCS3. Activating transcription factor 3 (ATF3) is a transcription factor implicated in nerve regeneration after injury (Seijffers *et al*., 2007) and is up-regulated in inflammation. IL6, interleukin-6, promotes the proliferation of microglia and is an indicator of inflammation (Cardenas and Bolin, 2003). Both interleukin receptors, IL4Ralpha and IL6R are involved in immune regulation and the inflammatory response (Joos *et al*., 2004), suggesting that the VENs are involved in immune and inflammatory regulation. SOCS3 (Suppressor of cytokine signaling 3) is a member of the STAT-induced STAT inhibitor (SSI) family and is a cytokine suppressor of signaling (Lebel *et al*., 2000). SOCS3 is activated by IL6, a regulator of inflammation. SOCS3 specifically labels VENs and layer 5 neurons. These results provide evidence that proteins involved in inflammation in ACC and FI are specific to VENs and their role in inflammation. IL6-mediated functioning of ACC may have a strong influence on cognitive performance as is evidenced by the following experiment by Brydon *et al*., 2008. Vaccination for typhoid increases the serum levels of IL6, which are linked to the brain concentrations of this cytokine. The Stoop test measures the reaction time of subjects when they have to discount conflicting information to perform a task, and the dorsal ACC, where the VENs are abundant, is strongly and selectively activated when subjects perform the Stroop task (Harrison *et al*., 2009a). After typhoid vaccination, the reaction time in the Stroop test is strongly and linearly related to the increased serum concentration of IL6 suggesting that the accompanying cytokine activity in the brain is influencing the VENs and other layer 5 neurons in ACC that relay information from ACC to other parts of the brain. The increased production of IL6 in these experiments is also related to the induction of negative mood changes that are closely linked to the activity of subgenual ACC, where the VENS are also abundant (Harrison *et al*., 2009b). Similarly, the induction of negative mood is related to increased activity in subgenual ACC and the increased production of inflammatory cytokines measured peripherally in the saliva (O'Connor *et al*., 2009). Thus, there are strong linkages between the activity of ACC, inflammatory cytokines whose receptors are strongly expressed in the VENs, slowed cognitive functioning and the induction of negative mood.

[*]*Professor, Division of Biology, Caltech*

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12. RNA-Seq studies of gene expression in fronto-insular (FI) cortex in autistic and control subjects reveal gene networks related to inflammation, development and synaptic function

Nicole A. Tetreault, Brian J. Williams, Andrea Hasenstaub, Atiya Y. Hakeem, Minghsun Liu, Anna C.T. Abelin, Barbara J. Wold, John M. Allman

FI is part of the neural system involved in self-awareness and social reciprocity and contains von Economo neurons (VENs) that selectively degenerate in fronto-temporal dementia (FTD) and may also be involved in autism (Seeley *et al*., *Ann. Neurol*., 2006; Allman *et al*., *TICS*, 2005). Deficits in self-awareness and social reciprocity are characteristic of both conditions. The activity of FI is reduced in autistic subjects relative to controls when they introspect their feelings (Silani *et al*., *Social Neurosci*., 2008). We have used RNA-Seq to quantify expression across the entire set of genes in FI obtained from well phenotyped autistic cases and controls. We have used an informational theoretic approach based on how informative each gene is in discriminating two types of autistic brains and control brains (normalized mutual information, NMI). The most informative genes were subjected to IPA and GO analysis. Autism-A brains have a greater number of activated microglia, key cellular participants in the inflammatory response in the brain, compared to autism-B brains, and controls. IPA based on the 225 most informative genes revealed that autism-A brains have a network of strongly upregulated genes related to immune function and inflammation. IL6 is the major hub in this network. The proteins for the IL6 receptor and several genes in the network, such as ATF3, are preferentially immuno-stained in the VENs. Previous studies have found increased amounts of IL6 protein in autistic subjects relative to controls in frontal cortex (Li *et al*., *J. Neuroimmunology*, 2009) and anterior cingulate cortex (Vargas *et al*., *Ann. Neurol*., 2005). There are many studies that implicate IL6 in social functioning (Adler, *Psychoneuroimmunology*, 2007). The VENs also preferentially express the proteins for several genes upregulated in the networks for both autism-A and B such as OLR1 (oxidized lipoprotein receptor 1), which is also implicated in inflammation and Alzheimer's disease. There is also strong immuno-staining for OLR1, IL6R and ATF3 proteins in another specialized class of layer 5 neurons, the forked cells. For the 1000 genes with the highest NMI values, we performed a GO analysis for the comparison of autism-A vs. B vs. Controls which revealed a set of significantly enriched GO terms related to stress and inflammation reflecting the network of genes centered on IL6 in autism-A. There are also large sets of significantly enriched GO terms related to nervous system development and to synaptic functioning. In summary, the VENs, forked cells and microglia are implicated in inflammatory processes in autism; also implicated in FI are gene networks involved in developmental and synaptic processes.

13. Diffusion-tensor imaging study of connectivity in the *Microcebus murinus* brain

Soyoung Park, J. Michael Tyszka, John M. Allman

We have examined the connectivity patterns in the brain of a *Microcebus murinus* - the gray mouse lemur – using diffusion-tensor imaging (DTI), an innovative magnetic resonance technique that employs the diffusion movement of water to map fiber tracts within tissues. The animal had been terminally ill with cancer and perfused with paraformaldehyde. The diffusion-tensor image, with a voxel size of 0.9 mm, was acquired using the Bruker 9.4-Tesla magnet at the Caltech Brain Imaging Center. Our main regions of interest during this study were the main olfactory bulb (MOB) and the septum pellucidum.

Our data suggest some degree of laminar organization within the MOB. As partially illustrated in the Figure 1, the regions near the outer edge of the MOB seem to project to certain regions in common (such as the accessory olfactory bulb, frontal pole, septum pellucidum, piriform cortex, olfactory tubercle, amygdala, entorhinal cortex, etc.), while the regions near the center of the bulb display another set of shared projections (reduced projection to the piriform cortex, olfactory tubercle, amygdala, and entorhinal cortex; increased projection to the septum pellucidum). This finding appears consistent with previous studies that revealed the presence of laminar cell organization in the mammalian MOB. In addition, the projection patterns in our data tend to agree with those suggested by previous rat studies (Shipley and Ennis, 1996).

Our results also indicate that the septum pellucidum can project to areas such as the hippocampus, MOB, entorhinal cortex, nucleus accumbens, and habenula. We also found intrinsic projections within the septum pellucidum (see Figure 2). The connection to the MOB suggests the septum's heavy involvement in the olfactory system. Also, the projection toward the habenula – which suppresses the activity of the dopaminergic neurons of the midbrain – implies that the septum might be involved in regulating the reward circuitry of the brain. In addition, the strong projection to the hippocampus is consistent with the evidence from previous studies, supporting the accuracy of our data (Zhou *et al*., 1999).

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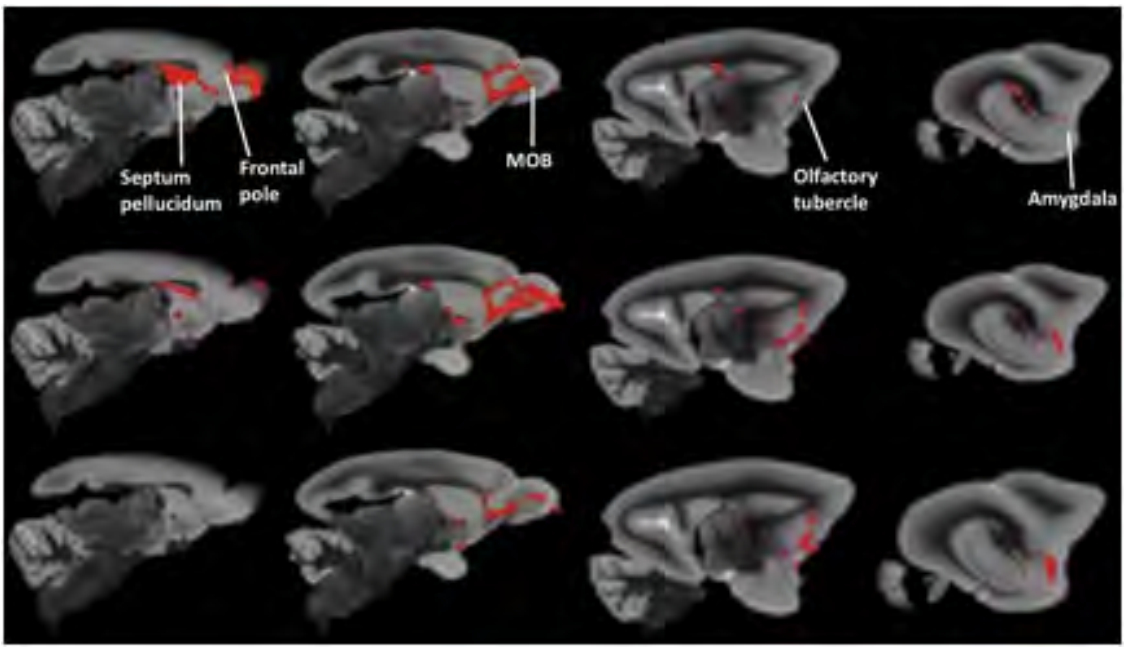

Figure 1. The parasagittal view of the mouse lemur brain with the projection data from various locations on the mediolateral axis of the MOB: the top row consists of the data from the medial region of the bulb; the middle row = near the center of the axis; the bottom row = the lateral edge of the bulb.

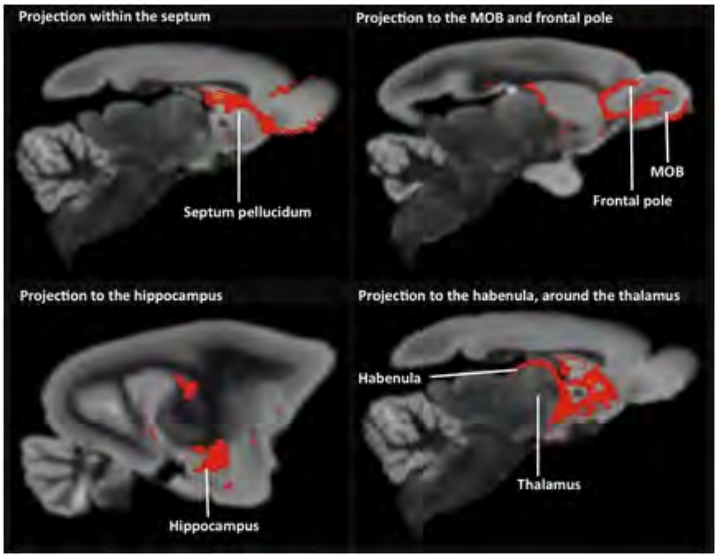

Figure 2. The projection patterns from some of the regions within the septum pellucidum of the mouse lemur brain. Upper left: The intrinsic connection within the septum itself; upper right: The projection to the MOB and the frontal pole; lower left: The projection to the hippocampus; lower right: The projection that wraps around the thalamus and connects to the habenula.

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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.

Neuroprosthetics. One project in the lab is to develop a cognitive-based neural prosthesis for paralyzed patients. This prosthetic system is 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, autonomous vehicle or a computer.

Recent attempts to develop neural prosthetics by other labs have focused on decoding intended hand trajectories from motor cortical neurons. We have concentrated on higher-level signals related to the goals of movements. Using healthy monkeys with implanted arrays of electrodes we recorded neural activity related to the intended goals of the animals and used this signal to position cursors on a computer screen without the animals emitting any behaviors. Their performance in this task improved over a period of weeks. Expected value signals related to fluid preference, or the expected magnitude or probability of reward were also decoded simultaneously with the intended goal. For neural prosthetic applications, the goal signals can be used to operate computers, robots and vehicles, while the expected value signals can be used to continuously monitor a paralyzed patient's preferences and motivation.

Movable probes. In collaboration with Joel Burdick's laboratory at Caltech, we have developed a system that can autonomously position recording electrodes to isolate and maintain optimal quality extracellular recordings. It consists of a novel motorized microdrive and control algorithm. The microdrive uses very small piezoelectric actuators that can position electrodes with micron accuracy over a 5 mm operating range. The autonomous positioning algorithm is designed to detect, align, and cluster action potentials, and then command the microdrive to move the electrodes to optimize the recorded signals. This system has been shown to autonomously isolate single unit activity in monkey cortex. In collaboration with Yu-Chong Tai's lab and the Burdick lab we are now developing an even more compact system that uses electrolysis-based actuators designed to independently move a large number of electrodes in a chronically implanted array of electrodes.

Coordinate frames. Our laboratory also examines the coordinate frames of spatial maps in cortical areas of the parietal cortex coding movement intentions. Recently, we have discovered that plans to reach are coded in the coordinates of the eye. This is 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 torques and 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 in the coordinates of the eye. This finding indicates that vision predominates in terms of spatial programming of movements in primates.

We have also been examining the coordinate frame for coordinated movements of the hand and eyes. In the dorsal premotor cortex we find a novel, "relative" coordinate frame is used for hand-eye coordination. Neurons in this cortical area encode the position of the eye to the target, the position of the hand to the target, and the relative position of the hand to the eye. A similar relative coding may be used for other tasks that involve the movements of multiple body parts such as bimanual movements.

Motion perception. 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.

Local field potentials. The cortical local field potential (LFP) is a summation signal of excitatory and inhibitory dendritic potentials that has recently become of increasing interest. We report that LFP signals in the saccade and reach regions provide information about the direction of planned movements as well as the state of the animal; e.g. baseline, planning a saccade, planning a reach, executing a saccade, or executing a reach. This new evidence provides further support for a role of the parietal cortex in movement planning. It also shows that LFPs can be used for neural prosthetics application. Since LFP recordings from implanted arrays of electrodes are more robust and do not degrade as much with time compared to single cell recordings, this application is of enormous practical importance.

fMRI in monkeys. We have successfully performed functional magnetic resonance imaging (fMRI) experiments in awake, behaving monkeys. This development 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. A 4.7 Tesla vertical magnet for monkey imaging has recently been installed in the new imaging center in the Broad building. We are using this magnet, combined with neural recordings, to examine the correlation between neural activity and fMRI signals.

14. Neural adaptation in the parietal reach region to a rotational perturbation using a closed loop brain machine interface

Paul Bailey, EunJung Hwang, Richard A. Andersen

The brain machine interface paradigm provides a novel approach to examine neural plasticity through experimenter-applied perturbations to the decoding algorithm. During brain control experiments, perturbation of the decoding model operating on neural activity creates a dissociation between the intended movement and the decoded movement. How the brain compensates for this error and regains accurate output device control allows researchers to better understand the neural basis of spatial reorganization during adaptation learning and to address the extent that selective control of neural activity is possible in a given behavioral condition.

The aim of this study was to examine how the spatial representation of a network of neurons in the reach region of the posterior parietal cortex (PRR) changes to adapt to a 180 degree rotation in the decoding algorithm. We compared the responses of neurons whose activity was used for decoding to those neurons whose activity was not used for decoding to evaluate the strategy used to compensate for the decode perturbation. Recordings were made with an eighteen channel semi-chronic microdrive (Neuralynx). During brain control experiments in primates, maximum likelihood estimation was used to decode the intended reach location in a center out task from single unit memory period firing activity in PRR. The animals were given a color cue at the beginning of each trial instructing whether the decoding model was perturbed (anti-decode task) or non-perturbed (pro-decode task). After demonstrating proficiency in the pro-decoding task, the decode algorithm was perturbed by rotating the tuning function of the decoding neural activity by 180 degrees. This anti-decode task was carried out until good performance was reestablished.

We found that the neurons used for the decoding and those which were not both changed their activity patterns similarly to offset the decoding perturbation. Thus, the predominant strategy used to regain control is highly cognitive, involving a global compensation in the activity of all neurons. With training, the transition to the new pattern required only a few trials to become proficient with the anti-decode task. This adaptation was able to generalize to new targets further indicating its cognitive nature. In summary, PRR learns new spatial contingencies using a global cognitive strategy.

15. Parietal area 5d encodes hand position and eye position in a reach-planning task

Lindsey Bremner, Richard A. Andersen

The execution of reaches to visual targets requires coordinate transformations between the neural representations of the sensory signal (in which targets are initially coded retinotopically) and the eventual motor command (in which targets are coded with respect to the effector limb). In macaques, anatomically connected circuits between the posterior parietal cortex and frontal cortex have been shown to play a role in this aspect of reach planning: neurons in the parietal reach region (PRR) represent the location of planned reach targets (T) in an eye-centered reference frame (T-E; Ref. 1), neurons in dorsal area 5 (area 5d) of parietal cortex represent the targets with respect to both the eye and the initial hand position (T-E, T-H; Ref. 2), and dorsal premotor cortex (PMd) is known to contain a complete set of vectors for relative coding of the hand, eye and target positions, including H-E (Ref. 3).

We sought to extend these findings by examining whether neurons in area 5d also code the initial position of the hand with respect to the eye (H-E) when reaches are being planned, in addition to encoding T-E and T-H. We present spike data from one rhesus macaque during a memory-guided reaching task in which the initial hand and eye positions were varied systematically. Preliminary data show a convergence of eye position and limb position effects on individual neurons, consistent with complete relative coding in area 5d. Ongoing experiments are being performed to determine whether the encoding of hand and eye position is indeed relative in area 5d.

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16. Intrinsic reward value of entertaining videos is represented in primate orbitofrontal cortex

Michael Campos, Kari Koppitch, Richard A. Andersen, Shinsuke Shimojo[*]

In the United States, adults spend over twice as much time watching television as they do eating or drinking, indicating the importance of non-appetitive rewards to modern human life. The brain's reward circuitry has been intensely studied in the past decade, but usually in the context of appetitive rewards that contribute to survival. It is unclear, however, whether the brain circuitry supporting the acquisition of appetitive rewards is the same, distinct, or overlapping with that supporting the acquisition of non-appetitive rewards. To address this issue we investigated whether neurons in the orbitofrontal cortex (OFC), a brain region largely implicated in reward processing, encoded the value of different reward categories - drops of juice and video clip presentations. Previously, we have shown that overlapping populations of neurons in the OFC were activated in a juice reward task and in a novel free-choice paradigm in which monkeys pressed buttons to watch short video clips. Since the firing activity of neurons in the primate OFC has never been correlated to the intrinsic value of visual stimuli, such as the entertainment value of video clips, we have now assessed whether the neurons that responded in the video-watching period of our experiments reflected the monkey's subjective value of watching entertaining videos, as opposed to simple visual stimulation. Here we report, based on a devaluation analysis comparing neural responses to repeated viewings of the same video sequences, that a subset of the neurons encoded the monkey's subjective value of watching videos. This parallels recent findings that some OFC neurons encode the value of drinking juices. While many individual neurons were activated in both tasks, we did not find any that encoded the subjective value of both types of rewards, suggesting that the value representations are encoded in separate OFC subpopulations. These results suggest that OFC can provide a population code that allows for the direct comparison between appetitive and non-appetitive rewards, and are consistent with our intuition that perceptual experience itself is rewarding.

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17. Adaptation of posterior parietal cortex to visually imposed perturbations of movement dynamics

Markus Hauschild, Grant H. Mulliken, G.E. Loeb, Richard A. Andersen

Posterior parietal cortex (PPC) is known to be involved in sensorimotor transformations required during planning and execution of reaching movements. Recently it has been shown that during execution of reaching movements PPC contains information about instantaneous hand position. Instantaneous hand state estimates are neither directly available from passive sensory feedback nor compatible with outgoing motor commands; they are instead assumed to be generated by internal forward models. Learning ensures that internal models are continuously updated when a new motor skill is acquired or when internal model predictions are inaccurate. In healthy subjects, proprioception and vision provide feedback signals to guide movement and update inaccurate internal models, whereas in amputees equipped with a prosthetic limb the CNS is required to rely on visual feedback only to adapt to the unknown dynamics of the artificial limb. Our study determined if forward model-related activity in PPC updates in response to an artificially imposed dynamics perturbation. We recorded from single units in PPC while a monkey was learning to control a 3D cursor in virtual reality using the movement of his right hand. Once he had learned the 1:1 mapping between hand and cursor movement, we imposed a realistic prosthetic limb dynamics model to the movement of the on-screen cursor, effectively creating a mismatch between unaltered proprioceptive and altered visual feedback. Once fully adapted, the imposed dynamics model was removed to observe re-adaptation. Initial results from ongoing

experiments suggest that PPC neural activity shifts from an initially strong instantaneous representation of the unperturbed movement toward a better representation of the perturbed cursor movement.

To study adaptation without proprioceptive inputs, the monkey was required to guide the 3D cursor using his neural signals only while not moving his arm. Cursor position was determined by an algorithm consisting of a neural decoder that translated the neural activity into cursor control commands, and a dynamics model, which constrained cursor movement. Through the brain-control phase of the experiment, we observed gradual improvement of the monkey's ability to acquire targets successfully for both the unperturbed and the dynamics-imposed cases. Behavioral performance increase was accompanied by a change of the neural ensemble's tuning properties.

Our initial results support the hypothesis that in addition to its known role in adaptation to spatial perturbations, PPC is also involved in adaptation to altered movement dynamics, thus, emphasizing its potential role in internal forward model computation.

18. Complementary multi-site LFPs and spikes for reach target location decoding in the parietal reach region

EunJung Hwang, Richard A. Andersen

Local field potentials (LFPs) in the parietal reach region (PRR) modulate the power in the beta and gamma bands depending on the impending reach target location. Therefore, the intended reach target location can be decoded from LFPs in PRR. An earlier study showed that decoding accuracy increased when the LFP signals from multiple sites were used, suggesting that LFP signals at different sites encoded independent information regarding the reach target location (Scherberger *et al*., 2005). The multi-site LFP signals used in this earlier study, however, were artificially synthesized by combining LFP signals that were sequentially recorded one site at a time. The different LFP sites in the sequential recording paradigm lack correlated noise that would have occurred during true simultaneous multi-site recording, suggesting that the observed high performance may be an unrealistic overestimate.

To address this concern, we examined actual multi-site LFPs (up to 15 channels) that were simultaneously recorded in PRR while a monkey performed center-out reaches to six different target locations. When the multi-site LFP signals were used for decoding the target location, the decoding accuracy monotonically increased with the number of simultaneously recorded LFP sites, consistent with the prediction made from the sequential data set. These results indicate that the LFP signals at different sites indeed provide a significant amount of independent information during simultaneous recordings to successfully decode intended reach locations.

Furthermore, when both spike and LFP signals were used for decoding, the accuracy was higher by 10-45% than when only spike signals were used. The enhancement by LFP signals was more critical as the decoding accuracy using spike signals decreased. Therefore, the multi-site LFP signals in PRR can provide independent information regarding the reach target location, complementing spike signals for neural prosthetic applications.

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19. fMRI dynamics in monkeys reflect spatial decisions and preferences in free choice and reward context tasks

Igor Kagan, Melanie Wilke, Richard A. Andersen

Exploring and choosing between multiple options is an important attribute of flexible behavior in primates. We investigated the neural substrates of internal preferences and external reward contingences in voluntary decision-making in three monkeys with time-resolved event-related high-field fMRI. We used two oculomotor spatial decision tasks to assess the dynamics of target representation and response selection in the distributed frontal-parietal-temporal network. In the first task, instructed single-target memory saccade trials were interleaved with choice trials, in which two *equally rewarded* targets were briefly presented and after a 10 s delay monkeys could select *either location.* The contralateral build-up of BOLD activity during the delay period reflected the monkeys' upcoming choices, and different cortical areas signaled the decision at different times. The relative ordering of BOLD time-course amplitudes during instructed and choice trials: contralateral instructed > contralateral choice > ipsilateral choice > ipsilateral instructed – demonstrated the mutually competitive interactions between sensory/attentional target representations and prospective motor plans in the two hemifields. Notably, subjects' internal behavioral spatial biases were reflected in the inter-hemispheric imbalance of left/right spatial representations: cues presented on the preferred side of visual space elicited stronger and more bilateral BOLD responses. These activation patterns are consistent with a model that postulates mutually competing interactions between two target representations/response options within each hemisphere, and between hemispheres.

Apart from internal preferences, decisions are often determined by reward expectations. Therefore, in the second task we introduced a reward magnitude context associated with target colors to study the effects of reward expectation and choice certainty in sensorimotor representations. The monkeys learned the stimulus-reward association and preferentially selected larger reward targets in the two-target choice condition. This behavioral preference was paralleled by significantly stronger cue and delay period BOLD activity in large reward trials in

several frontoparietal areas: LIP, FEF, 8B and caudate nucleus. These results support prospective evaluation of goal-directed actions. Taken together, these experiments suggest a common mechanism for integrating internal biases and external variables during oculomotor decisions.
Funding: Moore Foundation, NEI, Boswell Foundation

20. Spike phase tuning in primary visual cortex

Zoltan Nadasdy, Richard A. Andersen

We recorded single-unit activity and LFP from the V1 of two awake, behaving monkeys in a two-alternative forced-choice perceptual decision task. The monkeys were trained to hold fixation while the receptive fields of the recorded neurons were exposed to structure-from-motion stimuli. The monkeys' task involved reporting the rotation direction of a cylinder rendered by coherently moving dots while we varied the disparity and speed of the coherently moving dot field. Ambiguous and unambiguous trials were randomly interleaved. The objective of this study was to compare the spatio-temporal tuning curves between firing rates and firing phases for each neuron from the same trials. Three types of tuning curves were considered: speed, disparity, and perceptual-choice tuning. Firing-rate tuning curves were constructed by binning the 1-s spike trains with 50-ms time windows. In order to compute the firing phase, spike times were converted to phase values relative to the nearest successive peaks of the ongoing alpha and gamma oscillations. The stability of tuning curves over the stimulus presentation interval was quantified and compared between firing-rate and firing-phase tuning curves, for alpha and gamma phases separately. We found that while the disparity tuning of firing rates changes rapidly during the 1-s interval of stimulus presentation, the phase tuning in gamma band remains relatively stable. We also found that phase tuning was stronger in gamma band than in alpha band. The majority of neurons in our sample expressed more stable tuning in firing phases than in firing rates, indicating that spike phase encodes less "volatile" visual features than firing rate. Interestingly, neurons that exhibited relatively stable firing phase in disparity tuning also tended to exhibit unstable firing-rate tuning curves. These findings suggest that a population of neurons may use phase coding alternatively as opposed to in combination with firing-rate coding to encode specific stimulus features. Moreover, spike-phase differences showed higher correlation with perceptual choices than firing-rate differences during ambiguous stimulus presentations. Funding: NEI and J.G. Boswell Professorship

21. CONUS masking reveals saliency representation in reach-related areas of the posterior parietal cortex

Claudia Wilimzig[1], Markus Hauschild, Christof Koch[2], Richard A. Andersen

The parietal reach region (PRR) and Area 5 of the posterior parietal cortex (PPC) have been shown to encode reach plans. However, there have not yet been studies examining the possible effects of attention on visual responses in these parts of the PPC. We here examine whether PRR and Area 5 represent salient features in natural scenes using a newly developed masking technique, CONUS (Complementary NatUral Scene) relying on presentations of brief flashes of a natural scene (10 ms, e.g.) followed by an equally brief flash of the CONUS mask – the exact inverse of the natural scene which may simply be obtained by subtracting it from the maximum entry in each respective color channel (Wilimzig *et al*., 2008). Psychophysical results in human subjects show that this masking technique highlights salient regions of an image while it suppresses non-salient regions of the image thus, resulting in a percept that closely resembles the saliency map of a natural scene as predicted by computational algorithms of saliency (Itti and Koch, 2001 e.g.). An advantage of this paradigm is that it can produce saliency maps rather automatically and without requiring the subject to perform a behavioral-attention task.

Two monkeys (*Macaca mulattas*) passively viewed natural scenes while PRR and Area 5 spiking activity was recorded from a microelectrode array implant. We found a neural correlate of the psychophysical effect of CONUS: The activity for non-salient regions of the images decreases due to the presentation of the CONUS mask while activation for salient regions increased consistent with psychophysical results in human subjects. We show that a linear combination of the activity of 11 neurons can construct saliency maps that highly significantly correlate with predictions from computational algorithms.

The experimental phenomenon of CONUS masking and finding its neural basis provide important constraints on the current understanding of saliency processing in the brain and on computational approaches to saliency. In addition, our newly developed paradigm provides an important tool for investigating the neural basis of saliency representation for natural scenes in neurophysiological experiments.

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22. BOLD responses during pharmacologically induced hemi-neglect in the parietal cortex

Melanie Wilke, Igor Kagan, R.A. Anderson

Spatial neglect is a debilitating neuropsychological disorder occurring frequently as a consequence of parieto-temporal lesions in humans. Neglect is characterized by an impaired or lost ability to explore and perceive the space contralateral to the lesion, which cannot be explained by primary sensory or motor disorders. Visual neglect can be induced in macaque monkeys using lesions or reversible inactivations in several cortical areas, as well as subcortical nuclei. In this study, we tested the long-standing hypothesis that neglect symptoms such as spatial bias are due to an inter-hemispheric activity imbalance caused by the lesion. We reversibly inactivated portions of area LIP while a monkey performed a delayed memory saccade task and event-related BOLD activity was measured concurrently in a 4.7T scanner. The monkey was required to perform a saccade to either an instructed location ('single target condition') or was free to choose between two spatial alternatives ('two target condition'). LIP inactivation after muscimol injection did not impair memory saccade performance towards single targets, but led to an ipsilesional choice bias in the two-target condition. Specifically, the monkey showed a choice bias towards targets in the ipsilesional space (71% after inactivation as compared to 51% in control sessions). Consistent with the hemispheric imbalance model of spatial neglect, LIP inactivation modestly reduced contralateral cue/delay period BOLD activity in single target trials within the lesioned hemisphere (e.g., in the superior temporal sulcus), and slightly increased the activity in the hemisphere opposite to the lesion. However, not predicted by previous models, activity in both hemispheres was markedly increased in comparison to control sessions when the monkey chose targets in the non-preferred ('neglected') hemifield. Taken together, our results suggest that local lesions in the parietal cortex lead to widespread neural activity changes within the temporo-parietal network including reduced activity in the lesioned hemisphere and task-dependent compensatory activity enhancement in both hemispheres.

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Summary: Research in this laboratory is aimed at understanding the neurobiology of emotion. We seek to elucidate how fundamental properties common to emotional states or responses, such as arousal, are encoded in the circuitry and chemistry of the brain, and how sensory stimuli eliciting specific emotional responses, such as fear or aggression, are transformed and organized into behavior by the nervous system. Our work employs molecular genetic tools to mark, map and functionally manipulate specific circuits, to determine how identifiable populations of neurons contribute in a causal manner to behavior. These interventional studies are complemented by electrophysiology and functional imaging to measure activity in neural circuits. We use two model organisms in the laboratory: mice and the vinegar fly *Drosophila melanogaster*, with roughly equal emphasis on both.

Emotion circuits in the mouse brain

Research using the laboratory mouse *Mus musculus* focuses on understanding the neurobiology of fear, anxiety, aggression and pain, and the interrelationships between the circuitry that processes these emotions.

Our studies of fear are currently centered on the function of circuits in the amygdala, a medial temporal lobe structure that plays an important role in Pavlovian learned fear, a form of classical conditioning. We have identified genes that mark several subpopulations of neurons that form a dynamic microcircuit within the central nucleus of the amygdala. The function of this microcircuit in fear behavior is being dissected using optogenetic tools, such as channelrhodopsin, and pharmacogenetic tools, such as the ivermectin-gated glutamate sensitive chloride channel (GluCl) (Lerchener *et al*., 2007), together with acute slice electrophysiology and genetically based anatomical tracing of synaptic pathways. Computational models for this circuit are being developed in collaboration with the Elowitz laboratory, in order to understand its function at a systems level and generate testable predictions.

Similar approaches are being used to understand the role of another brain region strongly implicated in stress and anxiety, the lateral septum (LS). There are conflicting data on whether the LS promotes or inhibits anxiety, due to a lack of information about its microcircuitry. We have gained a point-of-entry into this structure by focusing on neurons expressing the type 2 corticotrophin releasing hormone receptor (Crhr2), and are using a combination of transgenic and novel viral tools to understand the functional circuitry in which these neurons participate. In the case of aggression, we are focusing on circuits within the hypothalamus, an area that has extensive connectivity with both the LS and the amygdala, through which it receives input from the olfactory system (Choi *et al*., 2005). We are using chronic *in vivo* multi-unit recording to probe the relationship between neuronal activity and aggression, as well as mating, and are also employing genetically-based functional perturbations to understand how these two related social behaviors are processed by a common structure.

Pain has both a sensory and an affective component. We are using genetically targeted methods to probe the functional roles of different subpopulations of primary sensory neurons identified by the expression of Mrgprs, a family of orphan G protein-coupled receptors that we previously characterized (Dong *et al*., 2001). We are interested in the extent to which these receptors mark neurons specialized for particular types of painful stimuli, and if so how this specificity is conveyed to the brain.

Emotion circuits in Drosophila

The pioneering work of the late Seymour Benzer proved that the powerful genetics of *Drosophila* can be used to dissect the genetic underpinnings of many types of complex behaviors. Until recently, however, it was not clear whether this model system could also be applied to understanding the neurobiology of emotion and affect. We are taking two complementary approaches to determine the extent to which this is possible, and if so what we can learn from it. One approach is to dissect the neural circuitry underlying behaviors that are analogous to defensive behaviors in higher organisms, such as avoidance (Suh *et al*., 2004, 2007), aggression (Wang *et al*., 2008) or immobility (Yorozu *et al*., 2009). The other is to model internal states or processes that are fundamental to many types of emotional responses, such as arousal, to ask for example whether arousal is unitary, or whether there are different types of behavior-specific arousal states (Lebestky *et al*., in press). In both cases, we are

developing novel behavioral assays, as well as machine vision-based approaches (Dankert *et al*., 2009) to automate the measurement of these behaviors (in collaboration with Pietro Perona, Allen E. Puckett Professor of Electrical Engineering), and are using molecular genetic-based tools to image and perturb neuronal activity in order to identify the specific circuits that mediate these behaviors.

Selected publications

Mouse behavior

Cavanaugh, D.J., Lee, H., Lo, L.-C., Shields, S.D., Zylka, M.J., Basbaum, A.I. and Anderson, D.J. (2009) Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli. *Proc. Natl. Acad. Sci. USA* **106**(27):11424-11424.

Drosophila behavior

Dankert, H., Wang, L., Hoopfer, E.D., Anderson, D.J. and Perona, P. (2009) Automated monitoring and analysis of social behavior in *Drosophila*. *Nature Meths*. **6**:297-303.

Lebestky, T.J., Chang, J.S., Dankert, H., Zelnik, L., Kim, Y., Han, K., Perona, P. and Anderson, D.J. (2009) Two different forms of arousal in *Drosophila* are independently and oppositely regulated by the dopamine D1 receptor DopR via distinct neural circuits. *Neuron*. In press.

Wang, L., Dankert, H., Perona, P. and Anderson, D.J. (2008) A common genetic target for environmental and heritable influences on aggressiveness in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **105**:5657-5663.

Yorozu, S., Wong, A., Fischer, B.J., Dankert, H., Kernan, M.J., Kamikouchi, A., Ito, K. and Anderson, D.J. (2009) Distinct sensory representations of wind and near-field sound in the *Drosophila* brain. *Nature* **458**:201-205.

23. Connectivity and function of lateral septal Crhr2$^+$ neurons

Todd Anthony

Although several brain regions have been implicated in regulating anxiety, the specific neural circuits involved remain poorly understood. The identification and analysis of these circuits is therefore a fundamental first step towards the development of improved treatments for anxiety disorders. The lateral septum (LS) is one brain region that has long been thought to be involved in controlling anxiety. However, it is unclear whether the LS is primarily anxiolytic or anxiogenic, or perhaps comprised of multiple classes of neurons each with a distinct effect on anxiety. Answering this question will require systematically manipulating defined LS neuronal populations in a precise and reproducible manner. One particular population likely to be relevant to analysis of the LS role in anxiety are neurons that express the corticotropin-releasing hormone receptor 2 (Crhr2); genetic data has demonstrated that Crhr2 modulates behavioral responses to stress and anxiety, and pharmacological studies have shown that these effects are due at least in part to Crhr2-expressing neurons within the LS. However, despite strong evidence implicating LS Crhr2$^+$ neurons in regulating anxiety, neither their synaptic connections nor exact function are known. We are addressing these questions using genetic tools to trace the connectivity and manipulate the firing properties of LS neurons that express Crhr2. By restricting analysis to neurons expressing Crhr2, these experiments are yielding highly specific results about an anatomically and genetically defined neuronal population; such information is a prerequisite in the development of targeted anxiolytic drugs.

24. Genetic dissection of the aggressive behaviors of *Drosophila* species

Kenta Asahina

Aggressive behaviors are prevalent among animals including fruit flies, a powerful genetic model for various animal behaviors. Excessive aggression in both animals and humans poses threats to our society. Little is known about the molecular machinery that dictates the neuronal circuits supporting the fly's aggressive behaviors. In order to obtain insight into such genes, we compared the frequency of aggressive behaviors among three closely related *Drosophila* species: *D. simulans*, *D. mauritiana* and *D. sechellia*. These three species share almost identical genomic structures, and all three species can generate fertile F1 hybrids with each other, allowing genetic analysis of quantitative trait loci (QTL) that may underlie any behavioral differences among the three species. The knowledge from the species comparison can be translated into their genetically versatile sister species, *D. melanogaster*, for genetic and neuronal manipulation. Such studies can reveal a molecular mechanism modulating fly aggressive behaviors in ecologically relevant manner.

Among the three species, *D. simulans* showed markedly reduced aggressive interactions compared to *D. mauritiana*, in the standard aggression chamber developed for the study for *D. melanogaster*. *D. sechellia* showed a comparative level of aggressive interactions to *D. mauritiana* only when the juice of the morinda fruit, the sole host plant of *D. sechellia*, was present in the food patch provided in the aggression chamber. I tested F1 hybrid males from a cross between female *D. simlans* and the male of *D. mauritiana*. Interestingly, the number of their aggressive interactions was as low as that of *D. simulans*. This result suggests that the genetic traits causing the difference between *D. mauritiana* and *D. simulans* are low in number. It is possible that the only one genetic trait (a single gene) causes the observed difference. In such a case, two possibilities are that 1) the less aggressive phenotype is genetically dominant, or 2) the gene promoting aggression is on the X chromosome. Further studies will aim to distinguish these alternatives and identify the relevant gene(s).

25. Neural circuitry underlying mouse fear behavior

Haijiang Cai, Wulf E. Haubensak, David J. Anderson

The amygdala plays a key role in fear conditioning; however, its cellular complexity makes it difficult to understand in detail how the underlying neural circuits regulate fear responses. Our lab has identified a genetic-marker(PKC-delta)- which labels a subpopulation of CeC/L (capsular and lateral division of central amygdaloid nucleus) neurons, which may function in circuit gating fear responses (see Wulf Haubensak's abstract). In order to understand the circuit-level mechanism of how these neurons participate in fear conditioning, we combined optogenetics and electrophysiology to map the neuronal circuits that these neurons are involved in. We demonstrated that photo-stimulation of ChR2 expressing PKC-δ neurons or their fibers in brain slices is sufficient to trigger action potential firing and post synaptic responses. Therefore, we are able to map both local and long-range (cortical) functional inputs to these CeC/L neurons, as well as outputs from the neurons. We find that the CeC/L neurons make inhibitory synapses onto projection neurons in CeM (medial part of the central amygdaloid nucleus), which can be back-labeled from the PAG (periaqueductal gray). However the local inhibition within the CeC/L population is stronger than the inhibition of projection neurons in CeM. At the same time, we are testing if the strength or dynamics of the circuits are modulated by experience or anxyolytic drugs. The structure of the neural circuits and their regulation characterized here will help us to understand better how the amygdala regulates fear responses and other emotional behaviors.

26. Reactive oxygen species modulate *Drosophila* sexual behavior

Gil B. Carvalho, Shlomo-Ben-Tabou de-Leon, Seymour Benzer, David J. Anderson

Reactive oxygen species (ROS) play an important role in numerous signaling pathways and have been implicated in aging and neurodegenerative disease. We are studying the role of ROS in modulating sexual behavior. Virgin females challenged with ROS-inducing mutations (in the mitochondrial complex II protein SdhB) or environmental agents (high ambient oxygen levels or dietary H2O2) exhibit behaviors typically triggered upon mating as a result of the transfer of the Sex Peptide (SP): high egg-laying rates, accelerated oogenesis and reduced sexual receptivity. Conversely, genetic or pharmacological treatment of oxygen-exposed or SP-expressing virgins with antioxidant agents abolishes the behavioral changes, supporting the notion that elevated ROS levels are required for the post-mating behavioral switch. Pan-neuronal expression of antioxidants is sufficient to block the effect of oxygen, suggesting that ROS act in the fly nervous system to regulate sexual behaviors. Our current efforts include the identification of genetic pathways and neuronal circuits involved in mediating this novel effect of ROS.

27. Genetic dissection of amygdala neuronal circuitry for fear and anxiety in mice

Prabhat S. Kunwar

An animal's survival depends on its capacity to identify which sensory stimuli are dangerous to its existence, and its ability to produce the appropriate responses to avoid or combat harmful stimuli. The neural circuitry that control fear, which can be learned or genetic (innate), and anxiety govern these critical judgments. In human, dysfunctions of this circuitry are thought to result in different diseases such as chronic anxiety, PTSD, depression, and autism.

The brain region, which is linked to these diseases and plays a crucial role in regulating fear and anxiety, is the amygdala, an almond shaped structure located in the medial temporal lobe of the forebrain. Precisely identifying the structure and function of the neural circuit located in the amygdala is essential for understanding how fear and anxiety occur in the normal brain, what goes wrong in the amygdala of affected people, and how appropriate treatments can be developed against fear disorders.

Among its various nuclei, the central amygdala (CeA) plays a key role in these fear behaviors. My goal is to determine the roles of different neuronal subpopulations in the CeA and to identify the neural circuits that control these behaviors and disorders. In these experiments, I plan to use molecular genetic tools of neuronal silencing and activation to modulate electrical activities of these neurons to analyze their effects on the behaviors in mice. Furthermore, I will use the genetic methods of trans-synaptic tracers to determine the anatomical and functional relationship of the CeA neuronal subpopulations in order to achieve a circuit level explanation for the behavioral phenotypes that are caused by *in vivo* functional perturbations of these subpopulations.

28. Fear control by inhibitory gating in the amygdala

Wulf Haubensak, Prabhat Kunwar, Haijiang Cai, David J. Anderson

Emotions are central to our mental self - among these fear is probably the most basic medically important emotion that can be addressed in experimentally tractable animal model systems. In general, fear is induced by stimuli that predict danger, and in turn evokes defensive behaviors necessary for survival. This is governed by: (1) integrating sensory information in the lateral amygdala, and (2) relaying this information via the central amygdala (CeA) to brainstem motor and autonomic control centers. In addition, to increase behavioral flexibility, fear and behavioral response are modulated by regulating the output from CeA. However, because of the high degree of neuronal heterogeneity in the amygdala, conventional surgical and pharmacological manipulations could not resolve which, and how, individual neuronal circuits

mediate these functions. Here, we have used novel combined viral and mouse genetic approaches to address this problem. We have used region-specific marker genes to genetically target subsets of amygdala neurons for pharmaco-genetic silencing using an ivermectin-gated chloride channel, opto-genetic activation using Channelrhodopsin, and virus-based neuroanatomical tracing. With this strategy, we have identified a neuronal population in the CeA that integrates top-down stress control from cortical areas and stress signals from within the amygdala and modulates fear by inhibiting amygdala output neurons. Interestingly, this inhibitory gate is also activated by, and partially mediates the anxiolytic effects of, benzodiazepines, anxiolytic drugs used for treatment of panic and anxiety attacks in humans. It is our hope that these findings may provide entry points for understanding neuronal circuit mechanisms of both fear and emotion in general, as well as anxiety disorders and their pharmacological treatment.

29. The role of neurosecretory cells in the modulation of *Drosophila* behavior

Timothy Tayler, Anne Hergarden

Animals exhibit countless complex and stereotyped behaviors such as aggression, courtship and the fight or flight response. These behaviors are generated and modulated by neural circuits. Other than a few simple reflex circuits, relatively little is known about how these circuits generate appropriate behaviors. *Drosophila* exhibits complex behaviors, but are anatomically less complicated and genetically more tractable than many vertebrate model systems. In addition, flies have a highly developed set of molecular tools that can be used to manipulate and analyze specific cell populations. Additionally, previous studies have demonstrated that *Drosophila* can be used to successfully identify neural correlates underlying complex behaviors such as courtship, olfactory aversion and learning and memory.

The goal of this project is to elucidate the connectivity, function, and modulation of circuits that underlie *Drosophila* behavior. Neuropeptides are an important class of signaling molecules that are involved in various aspects of animal physiology and behavior. To gain genetic control over neuropeptide-producing neurons we have identified the putative regulatory regions of 17 neuropeptide genes and have generated transgenic animals that express the GAL4 protein in the same pattern as these neuropeptides. The Gal4/UAS system is a genetic tool that enables us to express a large variety of transgenes in a spatially and temporally controlled manner. We have used fluorescent reporters to characterize these neuropeptide-Gal4 lines and have also validated the Gal4 expression patterns. We are currently using these newly generated tools to try to learn about the role of neuropeptidergic neurons in modulating behaviors. This is accomplished by using the neuropeptide-gal4 lines to express molecules that can either silence or activate neurons. We are now testing these circuit-modified animals in a wide variety of behavioral paradigms including feeding behavior, courtship, and circadian rhythm.

30. Functional dissection of the neural circuits regulating aggression in *Drosophila*

Eric D. Hoopfer, David J. Anderson

Nearly all animals exhibit some form of innate aggressive behavior that enables them to compete for food, mates and territory while defending themselves against predation. While engaging in aggression can provide a competitive advantage, it is also energetically costly and can lead to injury. Despite its social and ethological importance, we know little about the neural mechanisms that mediate aggression. An essential step in understanding this behavior is to identify the neural circuits that underlie aggressive behavior. Similar to many species, *Drosophila* exhibit aggressive behavior consisting of a distinct set of stereotyped behaviors that are innately expressed, suggesting that the development and function of the neural circuitry for aggression is genetically determined.

In order to identify neuronal populations that mediate aggression in *Drosophila* we have developed a high-throughput screen for aggressive behavior, which utilizes automated behavior tracking software[1] recently developed in the Anderson lab by Heiko Dankert, a joint postdoctoral fellow with Pietro Perona. Using the GAL4/UAS system to drive expression of *Drosophila* TRPA1, a temperature-sensitive non-selective cation channel that results in neuronal firing at a threshold of 26C[2], we will select for GAL4 lines that exhibit altered aggressive behaviors when neuronal activity is increased. In collaboration with Gerald Rubin (Janelia Farm, HHMI), we are screening a large collection of novel GAL4 lines[3] generated by his lab. These lines were generated by driving GAL4 with selected fragments of non-coding regulatory sequences from *Drosophila* genes that are expressed in the nervous system, resulting in smaller subsets of labeled neurons on average than existing GAL4 enhancer trap collections. The molecularly defined nature of this collection greatly facilitates the creation of intersectional genetic tools (i.e., GAL80 and split-GAL4 lines), which we will use to refine the expression pattern of lines uncovered by our screen to the specific neurons responsible for aggressive behavior.

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31. Monitoring and controlling neuromodulation during behaviors in fruit flies

Hidehiko Inagaki

Neuromodulators regulate the outputs of neural circuits, and allow animals to exhibit proper behaviors under various conditions. In order to understand how neuromodulators control behavior, it is critical to identify the subset of neurons modulated during different behavioral states. Despite its importance, currently there are no methods to visualize neuromodulation *in vivo*. Here I am developing such a system and applying it in the behaving fruit fly, *Drosophila melanogaster*. So far I applied the Tango assay, a system to induce reporter gene expression in response to activation of G protein coupled receptors (GPCRs) (Barnea *et al.*, 2008**)** to neuromodulator receptors of fruit flies. In both cell culture and in the brain *in vivo*, this system activated reporter gene expression in response to increases in the concentration of the appropriate neuromodulator. In future studies, I will improve this system to permit the visualization of neuromodulation during various behavioral states. Moreover, by driving neural effectors in the modulated neurons, I will determine whether the modulated neurons are necessary or sufficient for the behavioral state. As a proof of principle experiment using this approach, I am investigating neural circuits regulating stress-induced behaviors in fruit flies. In addition, I am planning to develop an anterograde transynaptic marker to analyze higher order neuronal projections.

32. Arousal and modeling emotional responses in *Drosophila*

Tim L. Lebestky, David J. Anderson

Emotional behaviors in humans convey a positive or negative response to a stimulus, and this response is typically manifest in discrete, highly conspicuous ways, such as stereotyped facial expressions and graded changes in levels of arousal. Although fruit flies (*Drosophila melanogaster)* 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 arousal, 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 aversive stimuli for high-throughput genetic screens.

One assay follows the startle effects on locomotor behaviors in response to 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. Initial results suggest that the animals show a robust, sustained increase in locomotor arousal upon receiving the stimuli, but a sustained response is not observed upon the presentation of a single stimulus. Our interpretation of the results is that the presentation of the puff stimuli raises the animals' state of acute arousal, as manifest in a sustained locomotor response. We have performed pilot genetic screens to isolate and characterize insertional mutants and potential neural circuits that mediate this startle behavior, and are currently sorting through putative mutations.

One of these mutations is an insertion in the DopR locus, which acts as a strong hypomorph and results in abnormally elevated locomotor arousal in response to air puffs. Previously it has been thought that dopamine promotes sleep-wake arousal in *Drosophila*, however our investigations have uncovered a role for DopR both in promoting sleep-wake arousal and also suppressing an endogenous acute startle response. Our data supports the hypothesis that dopamine oppositely regulates two forms of arousal, and acts via DopR within distinct neural circuits.

33. The role of TRPA1-expressing neurons in the formalin-evoked pain

Hyosang Lee, Daniel Cavanaugh, Li-Ching Lo, Shannon Shields*, Allan Basbaum*, David J. Anderson*

TRPA1 is a cation channel gated by pungent chemicals such as mustard oil. Previous studies showed that TRPA1 is expressed in a subset of $TRPV1^+$ sensory neurons in dorsal root ganglia. Consistently, we found that pharmacologic ablation of the central branches of $TRPV1^+$ sensory neurons in mice eliminates behavioral response to topical application of mustard oil, whereas conditional genetic ablation of $Mrgprd^+$ sensory neurons, which constitute a non-overlapping population of neurons dose not affect the response to mustard oil.

The formalin-evoked pain model has been used widely to test analgesics in laboratory animals. Intradermal injection of formalin into the hind paw of rodents evokes robust nocifensive responses including paw shaking and licking. The molecular mechanism of formalin-evoked pain is not yet understood. Recently, a few studies showed that formalin-evoked response is absent in TRPA1 knockout mice, suggesting that formalin exerts its effects by activating TRPA1. Interestingly, the formalin-evoked responses were tested only at a dose that is much lower than that of commonly used by other researchers.

Using $TRPV1^+$ and $Mrgprd^+$ neuron-ablated mice, we tested behavioral responses to low-dose (0.5%) and high-dose (2%) formalin. In $TRPV1^+$ neuron-ablated mice, behavioral responses to low-dose formalin were eliminated, while no deficits were observed in response to high-dose formalin. $Mrgprd^+$ neuron-ablated mice exhibited normal responses to both low-dose and high-dose formalin. These results suggest that $TRPA1^+$ neurons indeed mediate behavioral responses to low-dose formalin, but are not fully responsible for mediating responses to high-dose formalin in the formalin model.

**Department of Anatomy, University of California, San Francisco*

34. Distinct but intermixed neuronal populations mediate reproduction and aggression in a single hypothalamus nucleus

Dayu Lin, David J. Anderson

Inter male aggression and male female mating are generally considered as distinct behaviors. Nevertheless, it is increasingly recognized that these opponent behaviors may share similar neurobiological and neuroendocrine mechanisms. For example, both mating and territorial aggression are dependent upon circulating gonadal steroids. Both behaviors rely heavily on olfactory and pheromonal input. Lesions of the medial hypothalamus and medial amygdala in rodents decrease the occurrences of both mating and fighting. Taken together, these data suggest that mating and agonistic behaviors may be subserved by a common network of steroid hormone sensitive limbic areas. It is unclear how these two heavily overlapping pathways produce two opposite behavioral outcomes. One possibility is that two intermingled but distinct subpopulations of neurons mediate mating and fighting. Alternatively, the same population of neurons may mediate both mating and fighting through neuromodulation. The goal of this current study is to distinguish these two signal-processing scenarios. We first performed between-animal comparisons of the patterns of brain activation during mating and fighting, using c-fos analytic methods that permit rapid sampling across the entire brain. Our results indicate that mating and fighting indeed activate many similar hypothalamic and amygdalar regions in mice. However these areas are distinct from those activated during anti-predator defense. Next, we adapted a method to compare c-fos expression induced during the two behaviors in different regions within the same animal. Our data suggest that, at least in some commonly activated regions, two largely distinct sets of neurons are likely involved in mating and fighting; in addition, the same behavior tends to recruit a stereotyped set of neurons. Given our poor understanding of the aggression circuit in general, we decided to further investigate the functional roles of several hypothalamic regions in aggression based on our Fos results. Using reversible viral inactivation tools, we found that neurons in the ventrolateral region of the ventromedial hypothalamus and its surrounding regions are likely to be critical for the initiation of aggression. Finally, we used chronic recording in awake behaving animals to understand the physiological responses of those neurons during various episodes of aggressive and sexual behaviors.

35. Control of neural stem-to-progenitor transition by CyclinD family members

Agnes Lukaszewicz

We are interested in understanding the molecular control of neural fate specification during development. Motor neurons (MNs) are derived from a specific progenitor domain: the pMN domain of the spinal cord. While carrying out a systematic characterization of changes in gene expression within this domain, genes encoding cell cycle regulators, the CyclinDs, have been identified as potential candidates for regulating neural fate determination.

We have shown that CyclinD1 and D2 are expressed in distinct subsets of precursors, with CyclinD1 expression being spatially and temporally correlated with neurogenesis. This led us to hypothesize that CyclinD1 may regulate neurogenesis, whereas, CyclinD2 may regulate self-renewal. Loss- and gain-of-function experiments allowed us to demonstrate that CyclinD1 plays a key role in modulating neurogenesis. Furthermore, we showed that CyclinD1 re-expression is sufficient for glial-restricted progenitors to regain their neurogenic potential when transplanted into a permissive environment. Importantly, we demonstrated that CyclinD1's function as a neurogenic factor is structurally independent of its function as a cell cycle regulator. We are now interested in deciphering the molecular mechanism involved. So far, we have shown that Notch signaling can be modulated by CyclinD expression in a very specific manner: CyclinD1 is necessary for Hes6 expression (a neurogenic antagonist of Notch pathway), whereas, CyclinD2 is necessary for Hes5 expression (effector of the canonical Notch pathway). Furthermore, we have demonstrated that CyclinD1 and D2 loss-of-function phenotypes can be rescued by Hes6 and Hes5, respectively. We are currently analyzing and comparing the CyclinD1 and D2 interactome, using unbiased mass spectrometry in order to uncover new molecular partners for CyclinDs.

36. Genetic manipulation of neuronal subpopulations involved in pain and pleasure

Sophia Vrontou, David J. Anderson

Studies in our lab have identified a novel murine family of G-protein-coupled receptors (GPCRs), which are related to the proto-oncogene MAS1 and called Mas-related genes (Mrgs). Mrgs As, Bs, C and D thus far analyzed, are specifically expressed in mostly non-overlapping subpopulations of trigeminal and dorsal root ganglion (DRG) small-diameter sensory neurons, implying that they might have a potential nociceptive role. Most surprisingly it was found that the expression of MrgD is restricted to exclusively cutaneous peripheral afferents, rendering MrgD the first specific molecular marker that predicts the end-organ specificity of a subset of primary sensory neurons, and supporting the existence of a molecularly distinct subpopulation of cutaneous nociceptors. These data raise the question of: a) whether there are other molecularly distinct nociceptor subsets, innervating different targets; and b) whether the circuits they engage follow separate pathways, and if so at what point they might intersect into the brain. We are looking for molecular markers for such subsets and especially for visceral nociceptors, since visceral pain is the most common but still understudied form of pain. We will use these markers to compare the circuitry of their expressing neurons with that of nociceptor subsets innervating other targets, such as the skin and also to genetically manipulate them so as to identify their function.

Most recently, anatomical analysis of MrgB4 expressing neurons revealed that these neurons constitute a rare population of small-diameter sensory neurons, innervating exclusively the hairy skin (Liu *et al*). It was suggested that they might mark the murine analogs of the so-called C-fiber tactile (CT) afferents in humans, which respond to gentle stroking. We are interested in deciphering the connectivity of this specific subpopulation with brain regions and most importantly in identifying their function by measurements of their activity *in vivo* using gene targeting technology.

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37. Pheromonal regulation of aggressive behavior in *Drosophila melanogaster*

Liming Wang, David J. Anderson

Aggression is critical for the survival and reproduction of many animal species, though little is known about how this social behavior is regulated at the level of sensory inputs. Here we used *Drosophila melanogaster* to investigate how pheromones regulate aggression. *cis*-vaccenyl acetate (cVA), a male volatile pheromone, up-regulates the intensity of male aggression via a subset of olfactory sensory neurons (OSNs) expressing Or67d. cVA regulates the intensity of two gender-specific social behaviors, aggression and courtship, in opposite directions and through the same set of OSNs, but at different concentrations. In contrast, the gender-specificity of male social behaviors is determined by different sets of pheromones, which are likely male-specific non-volatile male-specific cuticular hydrocarbons (CHs). These data indicate that the intensity and gender-specificity of male social behaviors are regulated by distinct chemosensory systems in *Drosophila*.

38. Neural circuits responsible for *Drosophila* aggressive behavior

Kiichi Watanabe, David J. Anderson

Aggressive behavior is important for animal survival and reproduction, throughout the animal kingdom from insects to humans. With its powerful molecular and genetic tools, *Drosophila* will provide great opportunities for study of molecular and circuit-level mechanisms of aggressive behavior. Because of its complexity, the neural circuitry and molecules responsible for this behavior are still unclear. To facilitate our analysis, we have been developing a method to detect specific aggressive behaviors, in collaboration with Dr. Dankert in Perona's lab (Electrical Engineering, Caltech). This collaboration has generated a computer-based fly tracking system to detect several kinds of aggressive behaviors including "lunging" and "tussling." Using this system, I have started the following project.

The role of octopamine in aggressive behavior. Octopamine, a biogenic monoamine structurally related to noradrenaline, acts as a neuromodulator in *Drosophila*, and is implicated in aggressive behavior. Although there are some reports related to the roles of octopamine on aggressive behavior, it is still unclear which octopamine receptors, or octopamine receptor expressing neurons, are involved. In collaboration with Gerry Rubin's lab (Janelia Farm), we generated 34 driver lines expressing Gal4 in octopamine receptor positive neurons, under the control of various enhancer regions derived from the four-octopamine receptors (Oamb, Oa2, Oct_2R and Oct_3R). Using these Gal4 lines in combination with Tubulin-Gal80ts, I conditionally expressed Kir2.1 to block the activity of those neurons in adult specific manner, and analyzed the consequences of this manipulation for male-male aggressive behavior. Out of 34 lines tested, Kir2.1 expression driven by 13 Gal4 lines resulted in a suppression of aggressiveness. Using these 13 Gal4 lines, I will perform neuronal activation assays by expressing several effector genes such as dTrpA1, a warmth-activated cation channel, and NaChBac, a bacterially-derived voltage-sensitive sodium channel in the neurons that showed phenotype in silencing experiment. In this way, we hope to functionally dissect the specific neuronal circuits that control aggressive behavior.

39. Neuronal control of locomotor activity in the fruit fly

Allan M. Wong, Cynthia Hsu, Alice Robie, Michael H. Dickinson, David J. Anderson*

The fruit fly, when presented with various stimuli - be it vision, olfactory, thermal or mechanical - responds with a change in behavior. These behaviors are accomplished through a coordinated set of movements by the appendages. We are interested in how and where in the brain these different stimuli are integrated and how the signals propagated to the muscles that move the appendages. In particular we wish to understand how descending interneurons transmit signals from the brain to the thoracic ganglia: whether they carry multimodal commands from prior integrated sensory information or if they carry unimodal commands that represent discrete channels for sensory to motor action. During the past year, we pursued three different avenues to address this question.

1) We conducted a pilot behavioral screen in which we silenced subsets of neurons in the fly brain and analyzed the fly's locomotor trajectories with an automated tracking program. We found that when we silence the neurons in a line which labels primarily the fan-shaped body, the flies exhibit novel encounter behaviors where the flies collide with one another instead of the usual tendency to avoid such occurrences.

2) We also performed concurrent behavior analysis and neural activity imaging using various genetically encoded calcium indicators (GCaMP1.3, GCaMP1.6, TNXXL) in central neurons of the fly. So far, we have not been able to see correlated neural activity to behavior. We are currently trying a third generation calcium indicator with greater dynamic range and sensitivity.
3) And finally, we have been anatomically mapping and categorizing descending interneurons. We use various Gal4 lines to express photoactivatable-GFP in descending interneurons and then photo convert the pa-GFP molecules using a two-photon microscope. We see approximately 200 pairs of descending neurons in the fly brain.

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40. Distinct sensory representation of wind and near-field sound in the *Drosophila* brain

Sofia Yorozu, Allen M. Wong, Brian J. Fischer, Heiko Dankert, David J. Anderson

Behavioral responses to wind are thought to play a critical role in controlling the dispersal and population genetics of wild *Drosophila* species, as well as their navigation in flight3, but their underlying neurobiological basis is unknown. We show that *Drosophila melanogaster*, like wild-caught *Drosophila* strains 4, exhibits robust wind-induced suppression of locomotion (WISL), in response to air currents delivered at speeds normally encountered in nature. Here we identify wind-sensitive neurons in Johnston's Organ (JO), an antennal mechanosensory structure previously implicated in near-field sound detection. Using Gal4 lines targeted to different subsets of JO neurons, and a genetically-encoded calcium indicator, we show that wind and near-field sound (courtship song) activate distinct JO populations, which project to different regions of the antennal and mechanosensory motor center (AMMC) in the central brain.

Selective genetic ablation of wind-sensitive JO neurons in the antenna abolishes WISL behavior, without impairing hearing. Different neuronal subsets within the wind-sensitive population, moreover, respond to different directions of arista deflection caused by airflow and project to different regions of the AMMC, providing a rudimentary map of wind-direction in the brain. Importantly, sound- and wind-sensitive JO neurons exhibit different intrinsic response properties: the former are phasically activated by small, bi-directional, displacements of the aristae, while the latter are tonically activated by unidirectional, static deflections of larger magnitude. These different intrinsic properties are well suited to the detection of oscillatory pulses of near-field sound and laminar airflow, respectively. These data identify wind-sensitive neurons in JO, a structure that has been primarily associated with hearing, and reveal how the brain can distinguish different types of air particle movements, using a common sensory organ.

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Dankert, H., Wang, L., Hoopfer, E.D., Anderson, D.J. and Perona, P. (2009) Automated monitoring and analysis of social behavior in *Drosophila*. *Nature Meths*. **4**:297-303.

Rau, K., McIlwrath, S., Wang, H., Lawson, J., Jankowski, M., Zylka, M., Anderson, D.J. and Koerber, H.R. (2009) Mrgprd enhances excitability in specific populations of cutaneous murine polymodal nociceptors. *J. Neurosci*. In press.

Yorozu, S., Wong, A., Fischer, B.J., Dankert, H., Kernan, M.J., Kamikouchi, A., Ito, K. and Anderson, D.J. (2009) Distinct sensory representations of wind and near-field sound in the *Drosophila* brain. *Nature* **458**:201-205.

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Summary: Our group has been working on two avian neural systems since the beginning of the laboratory in 1975. One is the auditory system of the barn owl, which is known for its ability to localize prey in total darkness. We started with the discovery of neurons that responded to sounds coming from specific directions in space. These neurons form a two-dimensional map of auditory space. Each neuron responds to a specific combination of interaural time and intensity differences in sounds. We have identified every anatomical nucleus and computational process leading from the ears to the space map. We have not finished our mission, because we keep asking new questions. One of them is how neural signals disappear. For example, each auditory nerve fiber from the inner ear carries two types of information, phase and intensity of sound. As these signals reach the first set of brain stations, phase and intensity codes go to different stations. The question that we are currently addressing is how one of the codes disappears in these nuclei.

The other avian system is the song control system that was discovered in 1976 by the laboratory of Fernando Nottebohm of the Rockefeller University. It consists of several groups of neurons and connecting fibers, which are easily identifiable in brain sections. The system as it has been known is simple. Anyone familiar with the system can draw an accurate diagram of the song system. However, Gene Akutagawa has uncovered many hidden connections, making the system much more complex. This experience has taught us an important lesson, that is, we must pay attention to both structure and function.

Publications

Akutagawa, E. and Konishi, M. (2009) New connections add to the complexity of the bird's song system. Submitted.

Fischer, B. and Konishi, M. (2008) Variability reduction in interaural time difference tuning in the barn owl. *J. Neurophysiol.* **100**:708-715.

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Graduate Students: Cindy N. Chiu, Anusha Narayan*, Maria Papadopoulou, Kai Shen, Sina Tootoonian
Research and Laboratory Staff: Astrid Hoffius
* *Joint with Professor Paul Sternberg, Division of Biology, Caltech*

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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, circuit dynamics, 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 continued to focus 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 crickets as primary model systems, and of zebrafish (with focus on target regions of the olfactory bulb in the ventral telencephalon). Our work combines experimental (behavioral, electro-physiological and two-photon imaging) and modeling techniques and aims at understanding functional aspects of brain circuit design, such as the coding and learning rules used by the nervous system.

41. The consequences of STDP in the locust mushroom body

Stijn Cassenaer

Odor representations in insects undergo progressive transformations from the receptor array in the antenna, via the antennal lobe (AL), to the presumed site of odor learning, the mushroom body (MB). Broad activation of the AL by an olfactory stimulus gives rise to oscillatory population activity and diverging trajectories of projection neuron (PN) activation [1-7]. Different points along these trajectories can be thought of as representing different aspects of the odor stimulus, and cells that decode PN activity in the MB, Kenyon cells (KCs), respond sparsely at specific time-points along the PN trajectories [8]. Previous work suggests that individual oscillation cycles are meaningful units for the encoding and decoding of olfactory information by PNs and KCs, and this appears to be the case also for extrinsic neurons in the mushroom body beta-lobe (bLNs), which decode the KCs' sparse responses. We use intracellular recordings made from KCs and bLNs, to examine synaptic transmission, plasticity and odor representation in this circuit. We have found that KC-bLN synapses undergo Hebbian spike-timing dependent plasticity (STDP) on a timescale similar to the oscillatory population discharge, which is generated by the AL and propagated throughout the circuit [9]. This plasticity has a homeostatic effect on the phase of bLN firing, facilitates the synchronous flow of olfactory information, and maintains the segregation between oscillation cycles. We have found an additional component that contributes to this segregation, namely lateral inhibition among bLNs. We construct a simple network model to evaluate the consequences of the interaction between STDP and the competition among bLNs due to this phase-locked inhibition. Considered within the context of the circuit in which the KC-bLN network is embedded, these results suggest a mechanism for learning different aspects of an odor stimulus, after formatting as a function of oscillation cycle in the AL.

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42. Active sampling in locust olfaction

Stephen J. Huston

I am studying how the processing of sensory information changes when an animal actively investigates its environment. Much is known about how the locust nervous system encodes olfactory information. This knowledge comes from experiments on locusts with restrained antennae (the main olfactory organs). However, during normal function locusts move their antennae - possibly to actively sample odors. I have characterized how these antennal movements change in the presence of an odor and are currently engaged in experiments to determine how antennal movements affect the odor responses of olfactory neurons.

I developed equipment to automatically record the direction of walking and track the 3D antennal movements

of a tethered locust while it is presented with spatially localized odors. I have found that, when presented with an odor, locusts change the location (**Figure 1a**) and frequency (**Figure 1b**) of antennal movements. This behavioral strategy leads to an increase in the frequency at which the locusts are able to sample odors.

In collaboration with Stijn Cassenaer I have started recording neural activity from the locust olfactory system while the locust is free to move its antennae (**Figure 1c**). Can we predict the neural responses to olfactory stimuli from previous experiments on restrained locusts, or does neural processing in the olfactory system change as the locust moves its antennae to investigate the environment? Hopefully this work will help us to understand how behavioral and neural coding strategies interact.

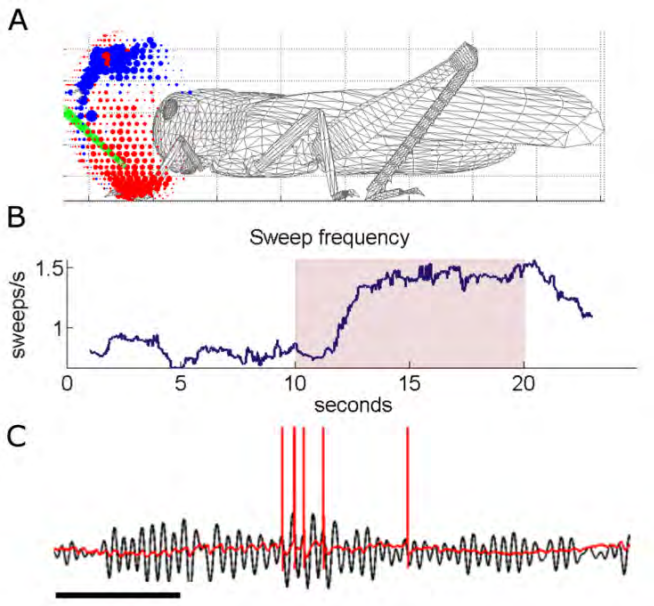

Figure 1. (a) Difference between the distribution of antennal tip positions before (blue) and during (red) odor presentation. Wheat grass odor was added to an air-stream originating on the locust's left, represented by the green tube. **(b)** Change in median antennal sweeping frequency with the presentation of wheat grass odor (red box). **(c)** Neural activity recorded during free antennal movements. An intracellular recording from an olfactory system projection neuron is shown in red overlaid with local field potentials (black). Scale bar = 500 ms.

43. Sensory coding and perception in *Drosophila*

Mala Murthy

Over the past year, I have investigated 1) the logic of neuronal connectivity in the *Drosophila* mushroom body, a higher order brain center required for complex processes such as learning and memory (1), and 2) the neuronal basis for species-specific preferences to courtship songs in *Drosophila.*

[1] The mushroom body is required for olfactory learning in flies (2-4). In adults, the principal neurons of the mushroom body, the Kenyon cells (KCs), form a large-cell population usually divided into three classes (α/β, α'/ β', and γ) based on anatomical criteria. Both neuronal populations from which their olfactory input derives, the antennal lobe projection neurons (PNs) and the presynaptic partners of the PNs, the olfactory sensory neurons, can be individually identified by their glomerular projections, by the expression of specific genes, and by their responses to odors (5-10). I have investigated whether or not Kenyon cells are similarly individually identifiable across flies – this issue is important because it relates to the specification of sensory network connectivity in general: at what level (if at all) does the order that exists in early sensory circuits break down, such that connections and cellular properties become specific to each individual animal?

To record from potential KC functional replicates across flies, I used *in vivo* physiology methods for *Drosophila* developed in the Laurent lab and a GAL4 line with restricted expression in a small subset of ~23 α/β KCs (11). If KCs are identifiable neurons, with recordings from one labeled KC each in 27 flies, at least 13 replicates (non-singletons) should occur in our dataset with p>0.99. Odor response profiles (both spiking and subthreshold activity) across recordings from GAL4-labeled KCs provided no evidence for obvious repeats. Further, across-animal responses were as diverse within the genetically-labeled subset as across all KCs in a larger sample. However, identifying functional repeats, supposing that they exist, required some knowledge of expected inter-individual variability. For this reason, I recorded from the presynaptic partners of the KCs, the PNs, under the same stimulus conditions. In collaboration with Dr. Ila Fiete (Caltech Broad Fellow), we used these data as inputs to a KC model: different KC types were generated by summ

ation of odor responses from PNs of different glomerular types, and different KC individual responses of one type were generated based on the measured inter-fly variability across responses from PNs of a single glomerular type. In the model, regardless of the convergence factor between PNs and KCs, KC responses could easily be clustered by type and across individuals. We applied clustering thresholds derived from this model to the experimental KC data, and in several thousand runs of the simulations, the thresholds identified an average of 14 non-singletons among the model KCs, but fewer than three among the real data. Our data and analyses collectively indicate that measured PN response variability combined with stereotyped PN-to-KC connectivity across individuals cannot account for the variability of KC responses we observe.

Our result, that one-to-one connections between PNs and KCs may vary substantially from animal to animal, is surprising given the genetic, anatomical and functional stereotypy observed with most *Drosophila* neurons studied to date, and in particular, with the olfactory inputs to the mushroom body. However, the lack of precise circuit specification in the mushroom body may result from two features of this structure: (i) Mushroom

bodies are required for olfactory learning and recall; thus, inputs to KCs may be plastic; and (ii) KCs are very numerous (~2,500 in *Drosophila*); this makes the precise specification of pairwise PN-to-KC connectivity a significant mechanistic challenge. Further, functional stereotypy across animals is not a prerequisite for memory formation. In a system in which associative memories are stored as patterns of synapses between KCs and their targets, those memories could be defined without *a priori* specification. However, these target neurons may need to respond to odors in more stereotyped ways than KCs, in order to generate reproducible learned behaviors. It will thus, be of great interest to determine if and how identifiably reemerges downstream of the Kenyon cells.

[2] During courtship, virgin females display preferences for males of their own species; following courtship and persisting for several hours to days, mated females reject conspecific males, suppressing courtship behavior in favor of egg-laying. While the importance of auditory cues in mating in *Drosophila* has been well documented (12-15), how courtship songs are encoded within the auditory pathway and how the perception of song leads to different behaviors depending on the song (conspecific or heterospecific) and the state of the female (mated or unmated) is unknown. Songs are produced by courting males via wing vibrations. Males of each species (there are >1700 *Drosophila* species) sing a unique courtship song, consisting of low frequency pulses with species-specific pulse carrier frequencies and inter-pulse intervals (16-18). I have chosen to take a comparative approach to understanding the neural coding of courtship song by presenting auditory stimuli from and performing electrophysiological recordings in several of these species.

In *Drosophila*, sounds, like odors, are received by the antenna where they are transformed into neuronal signals in stretch receptor neurons, which connect with second-order neurons in the brain (19, 20). Our preliminary studies have involved: a) Analyzing sound traces of male courtship singing, in order to build a behaviorally-relevant stimulus set; and b) Conducting electrophysiological recordings from primary and genetically-labeled secondary neurons in response to both synthetic and recorded courtship song. These studies suggest a basis for species-specific auditory tuning at the level of the antenna. However, antennal tuning curves are broad, indicating that song preference is mostly encoded downstream. Future characterization of the system will involve more detailed song analysis, continued patch recordings, genetic silencing and activation of neuronal subsets, and cross-species comparisons, in order to decipher how flies distinguish between conspecific and heterospecific courtship songs, and how this distinction leads to reproducible song-elicited attraction or avoidance behaviors.

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44. Dynamic coding in an olfactory system

Ingmar Riedel-Kruse

I wish to understand how the architecture of neuronal networks determines its dynamics ("firing patterns"), and how such dynamics constitute computational and coding tasks. This is a central question in systems neurobiology. Specifically, I study how the sense of smell is processed in insects in a brain structure called the antenna lobe. In this lobe the neurons have been shown to exhibit synchronized, oscillatory firing patterns. I use multi-unit recording techniques ("tetrodes"), to measure these firing patterns of a large number of neurons at the same time. In order to increase the number of simultaneously recorded neurons, I collaborated with Sotiris Masmanidis and Jiangang Du to develop and test new tetrodes (Du, Riedel-Kruse *et al.*, *J. Neurophysiol.* 2009). These new tetrodes enabled us to record from significantly more neurons simultaneously, and to locate

neurons in 3D. The analysis of my data has revealed so far – among others - that these firing patterns become more reliably over the time course an odor is encountered, furthermore, that the phase of spikes relative to the oscillatory field potential is strongly correlated to the firing reliability of these spikes, which suggests an error correction mechanism for downstream neurons. In collaboration with Ila Fiete, Ila Varma and Joshua Milstein, we use information theoretical approaches to quantify the information content in these firing patterns. I hope that my results will lead to new conceptual insight into olfactory processing and, more generally, into the role of the widely observed oscillatory activity in other neuronal networks.

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45. Decoding of multiplexed odor representations by mitral cell downstream target neurons

Cindy N. Chiu[*]

Previous work in the Laurent lab has demonstrated that the zebrafish olfactory system is a useful model for understanding how brains can perform complex computational tasks such as stimulus categorization and identification. Odors can evoke synchronous action potentials among stimulus-specific subsets of mitral cells (MCs), the principal output neurons of the olfactory bulb (OB). MCs can also respond dynamically to odors without synchrony. Previously, our lab has shown that patterns of synchronous MC spikes evoked by related stimuli converge whereas patterns of non-synchronous MC spikes diverge during an odor response (Friedrich *et al*., 2004). These observations led to the proposal that two types of complementary information about stimulus quality can be carried within a single OB output stream (i.e., the population of spiking MCs): information about odor category is conveyed by converging patterns of synchronous spikes while information about odor identity is conveyed by diverging patterns of non-synchronous spikes.

We aim to address how brain areas downstream of the olfactory bulb decode MC activity and what types of computations they might perform in turn. Anatomical evidence suggests that there are several downstream targets including areas in the zebrafish telencephalon. We predict that these brain regions use different decoding strategies such as using different levels of temporal sensitivity to integrate synaptic input to extract information about stimulus quality.

We are studying the morphological, biophysical, and odor response properties of MC target neurons to characterize their role in processing of olfactory information, with particular interest in their sensitivity to synchronous input. We have performed MC axon stimulation experiments coupled with simultaneous whole-cell patch clamp recordings in the ventral nucleus of the ventral telencephalon (Vv) to demonstrate the existence of both direct and indirect synaptic connectivity between these brain areas. Preliminary whole-cell patch clamp recordings suggest that Vv neurons do not exhibit intrinsic resonance at relevant frequencies nor have we observed subthreshold odor-evoked oscillations, but further investigation is necessary to characterize the temporal sensitivity of Vv neurons.

[*]*Cindy Chiu is funded by the Gordon and Betty Moore Foundation*

46. Transfer at a thermosensory synapse in *C. elegans*

Anusha Narayan[*]

C. elegans is an attractive model organism for neural circuit analysis. In order to characterize the functional dynamics of the circuits that control behavior, it is necessary to understand the synaptic transformations that take place. Thermotaxis is an established behavior in *C. elegans* [1, 2]. We attempt to characterize the transfer function at a prominent synapse within the thermotactic circuit. AFD is the primary thermosensory neuron [3], and AIY is its principal post-synaptic partner.

We drive expression of Channelrhodopsin-2, a light-activated cation channel [4], solely in AFD using a cell-specific promoter, and use whole cell patch clamp recording techniques [5] to measure the light-evoked synaptic response at AIY.

We are able to reliably activate the presynaptic cell AFD with blue light, evoking depolarization of up to 40 mV and inward currents of up to 15 pA, with responses lasting the duration of the stimulus.

The postsynaptic response at the AIY cell body is small - less than 5 mV, with inward currents of less than 1 pA, and appears to be graded and tonic, lasting the duration of the stimulus. Our results indicate that this synapse has low gain, and transmits information from AFD to AIY with short latencies and high fidelity. It will be interesting to see how AIY uses this information and integrates it with other incoming streams, and to examine processing further downstream in the thermosensory circuit.

[*]*Joint with Paul Sternberg, Professor, Division of Biology, Caltech*

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47. Feedback inhibition in the mushroom body and gain control

Maria Papadopoulou

The giant GABAergic neuron (GGN) is a single, paired, non-spiking neuron that arborizes extensively in the mushroom body (MB) (Leitch and Laurent, 1996), where it overlaps with the dendrites and the axons of Kenyon cells (KCs). KCs are the intrinsic neurons of the MB and are thought to be required for learning and memory (Heisenberg, 2000). We are interested in understanding the function of GGN in olfactory processing: in particular, its pattern of arborization makes it an attractive candidate for controlling or modulating KC responses to odors, with potential implications for learning and recall. Physiological recordings of KCs in locust show that these neurons respond sparsely to odors, by contrast with their excitatory input from the antennal lobe (projection neurons or PNs) (Perez-Orive *et al*., 2002). Inhibition appears to be critical to control KC response threshold, probability and duration during odor stimulation (Perez-Orive *et al*., 2002). We show that there exists a feedback loop whereby KCs provide excitatory input to GGN, which, via its GABAergic output contributes to the control of KC excitability. Using electrophysiological techniques, we are studying the properties and modes of action of GGN in locust. Our data suggest that this neuron acts to control the gain of PN-to-KC information transfer and normalize KC-population output, making it independent of input strength. Manipulating GGN activity during odor stimulation suggests a global action of GGN, as measured both at the KC input and output level. We further examine the ability of GGN to control the gain by directly monitoring the activity of β-lobe neurons, downstream from the KCs, during such manipulations. Specifically, enhancing or diminishing GGN activity dramatically affects the output of the β-lobe neurons and provides a framework in which to consider the importance of GGN's gain control effects.

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48. Sparse and invariant representations of odor mixtures in the mushroom body

Kai Shen

Principal neurons (PNs) of the locust antennal lobe (AL) exhibit odor-specific dynamic responses. And because not all PNs express the same patterns at the same time, the state of the AL network is dynamic, carried by an assembly of neurons that evolves in a stimulus-specific manner over time. These neuronal responses can be described geometrically as stimulus-specific trajectories reflecting the state of the AL network. How do these trajectories change with changes in the stimulus? Are there general rules to how these trajectories evolve? We addressed this question by first probing the PN network with small changes to the stimulus that varied progressively along a continuum. By varying the ratio of concentrations of two components within a binary mixture, we effectively morphed one unique odor to another. We observed a progressive rather than abrupt transformation from one odor-specific trajectory to another; thus, the PN network optimizes its encoding space to fully complement the stimulus parameter space.

To observe transformations across a more broad range of stimulus space, we systematically increased the complexity of the mixture by adding single odorants in a stepwise manner from 2 to 8 (e.g., AB, ABC, ABCD, ABCDWXYZ) and presenting different combinations that overlap by varying amounts (e.g., ABCD, ABCX, BCWX, BDWX, DWYZ, WXYZ). To test the linearity of mixture representation, we estimated the ensemble PN response vectors to odor mixtures from the mixture components using a simple linear model. We then examined the degree of deviation between these estimated response vectors and the experimentally-observed response vectors. We find that for binary mixtures, there is good approximation between the estimated and experimental response vectors, but this approximation degrades rapidly when more odorants are added to the mixture. As expected, the PN ensemble output (as represented by stimulus-specific trajectories) due to single odor components (e.g., A), are very different to that of multi-component mixtures (4-, 5-, 8-, e.g., ABCDW).

Interestingly, when we recorded from the Kenyon cells (KCs), the downstream targets of the PNs in the mushroom body, we find many KCs that are invariant across odor mixtures. For example, many KCs respond invariantly to mixtures containing A, for e.g., to A, AB, ABC, ABCD, ABCDW, etc., but to no other single components, like B, C, D. How is it possible that any downstream decoder can do this? We gain some intuition by analyzing the many PN ensemble subspaces that each downstream KC sees. We suspect that there exist many such subspaces within the AL, where the neural representations of odor components and more complicated mixtures overlap. In addition,

and in collaboration with Anusha Narayan and Sina Tootoonian, we are building a simple feed-forward model of the PN-KC network, with minimal assumptions and nonlinearities. By comparing the statistics of the output of this model (model KCs) to our real experimental KCs, we hope to elucidate the essential mechanisms that give rise to such rich computations.

49. Computational models of locust olfaction, and analysis of *Drosophila* courtship song

Sina Tootoonian

The projection neurons (PNs) of the locust antennal lobe project to the lateral horn interneurons (LHIs), and to the Kenyon cells (KCs) of the mushroom body, a structure implicated in learning. The KCs fire much more sparsely than the PNs and can show odor and odor-and-concentration specificity. A computational model of the PN-LHI-KC interaction has been developed in the lab over the last few years to help determine the aspects of the network that produce the observed KC behavior. The original model was found to be incapable of producing realistic KC behavior. In collaboration with Kai Shen, Anusha Narayan, and Maria Papadopoulou, I have added nonlinearities to this model that mimic excitatory post-synaptic potential (EPSP) 'sharpening' caused by KC voltage-sensitive sodium channels, as well as the global feedback provided by the giant GABA-ergic neuron (GGN). We have found that the addition of these features makes the model dynamics more realistic, but still does not produce realistic KCs. We are currently investigating connectivity bias to address this discrepancy.

The males of many *Drosophila* species will 'sing' to females during courtship by extending and vibrating their wings. These species-specific 'fly songs' modulate the success of courtship. I have been working with Mala Murthy, a post-doctoral scholar in the lab, on the analysis and characterization of fly songs that she has recorded from males of several *Drosophila* species. The main goals of the analysis are to allow quantitative comparisons of songs across species, and the synthesis of realistic fly song for playback to females whose auditory neurons are being recorded from. I have written software that uses simple wavelet techniques to automatically extract fly song from the audio recordings, and we are presently in the early stages of song analysis.

Publications

Du, J., Riedel-Kruse, I.H., Nawroth, J.C., Roukes, M.L., Laurent, G. and Masmanidis, S.C. (2009) High-resolution three-dimensional extracellular recording of neuronal activity with microfabricated electrode arrays. *J. Neurophysiol.* **101**(3):1671-1678. (doi:10.1152/jn.90992.2008)

Geffen, M.N., Broome, B.M., Laurent, G. and Meister, M. (2009) Neural encoding of rapidly fluctuating odors. *Neuron* **61**(4):570-586. (doi:10.1016/j.neuron.2009.01.021)

Moreaux, L. and Laurent, G. (2008) A simple method to reconstruct firing rates from dendritic calcium signals. *Front. Neurosci*. **2**(2):176-185. [PMCID:PMC2622756]

Murthy, M., Fiete, I. and Laurent, G. (2008) Testing odor response stereotypy in the *Drosophila* mushroom body. *Neuron* **59**(6):1009-1023. (doi:10.1016/ j.neuron. 2008.07. 040).

Rabinovich, M., Huerta, R. and Laurent, G. (2008) Transient dynamics for neural processing. *Science* **321**:(5885)48-50. (doi: 10.1126/science. 1155564).

Turner, G.C., Bazhenov, M. and Laurent, G. (2008) Olfactory representations by *Drosophila* mushroom body neurons. *J. Neurophysiol*. **99**(2):734-746. (doi:10.1152/jn. 01283.2007).

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Summary: Our lab studies ion channels that respond to the neurotransmitters acetylcholine, serotonin, GABA, glycine, and (among invertebrates) glutamate. These are termed "Cys-loop receptors." At the most fundamental level, with Professor Dennis Dougherty's group in Caltech's Division of Chemistry and Chemical Engineering and Professor Sarah Lummis of Cambridge University, we apply new types of chemistry to understand how Cys-loop receptors transduce the binding of agonists into the opening of the channels.

We are describing the neural events that occur when an animal is chronically exposed to nicotine. We hypothesize that this set of responses underlies the pathophysiology of nicotine addiction, the world's largest preventable cause of death. We also hypothesize that the same processes underlie the benefits of two inadvertent therapeutic effects of smoking: the inverse correlation between a person's history of smoking and his/her susceptibility to Parkinson's disease, and the preventive effect of nicotine in autosomal dominant nocturnal frontal-lobe epilepsy. We are studying these complex neural processes at several appropriate levels: the genes, the receptor proteins, the effects on neurons, the organization of neurons in circuits, the resulting behavior of animals, and even neural events in humans.

Several lab members are describing the molecular/biophysical aspects of SePhaChARNS, for "selective pharmacological chaperoning of acetylcholine receptor number and stoichiometry." We hypothesize that SePhaChARNS is a thermodynamically driven process leading to the classical observation that chronic exposure to nicotine causes "upregulation" of nicotinic receptors. If the hypothesis is proven, SePhaChARNS is the molecular mechanism that shapes an animal's response to chronic exposure to nicotine. We are now producing subcellular movies depicting the first 24 hours of nicotine addiction—thought to be the most crucial stage in the process, especially for adolescents. These images display the spread of newly chaperoned, fluorescent receptors as they travel from the endoplasmic reticulum to the cell membrane.

Other lab members are generating mice with genetically modified nicotinic receptors—gain of function, not knockouts. Some mice have a hypersensitive subunit; in such mice, responses to nicotine represent selective excitation of receptors containing that subunit. Other mice have a fluorescent subunit, so that we can quantify and localize upregulation of receptors containing that subunit.

The human end of the scale is represented by our collaboration with Professor Johannes Schwarz of the University of Leipzig. Deep brain stimulation for Parkinson's disease often requires an initial session of microelectrode-based single unit recording, in order to localize the stimulating electrodes. We're analyzing the effects of nicotine in these single-unit recordings.

We are also engineering Cys-loop receptors in order to manipulate neurons. Now we are optimizing suitably mutated *C. elegans* GluCl receptor channels to have strong responses to the minuscule concentrations of the anthelmintic ivermectin and its derivatives. In collaboration with Professor David Anderson's group, we have introduced these channels into mouse neurons. When the animal is injected with ivermectin, the neurons can be selectively silenced—and eventually, we hope, selectively activated. There are important applications for both research and therapy.

Several of our projects lead naturally to drug discovery procedures. We have a drug discovery collaboration with Michael Marks and his group at the University of Colorado, Boulder; and with Targacept, Inc.

We also have interests in new techniques at the intersection of biophysics, single-molecule imaging, chemistry, mouse genetics, and neuroscience. We're

delighted to host visitors in our lab on the third floor of the Kerckhoff Laboratory.

50. A hydrogen bond in the complementary subunit of α4β2 that is important for agonist binding

Angela Blum[*]*, Henry A. Lester, Dennis A. Dougherty*[*]

Nicotinic acetylcholine receptors (nAChRs), pentameric ligand-gated ion channels that bind nicotine, acetylcholine and structurally related agonists, are implicated in smoking addiction and many neurological disorders, including Alzheimer's disease, Parkinson's disease and schizophrenia. Previous studies in our lab have shown that nicotine and acetylcholine bind to the neuronal receptor α4β2 though a cation-π interaction and (for nicotine) a hydrogen bond with W154 of the receptor's ligand binding "aromatic box." We now report a third interaction, a hydrogen bond with L119 of the complementary subunit, which is important for agonist binding. Mutant cycle analyses with the unnatural residue α-hydroxyleucine (Lah) and agonist analogs reveal that the backbone NH of b2L119 makes a hydrogen bond to the carbonyl and pyridine N of acetylcholine and nicotine, respectively. This interaction is present in both receptor stochiometries, $(a4)_2(b2)_3$ and $(a4)_3(b2)_2$. Epibatidine binding was also shown to be sensitive to mutations at b2L119. Taken together, these binding interactions will offer insight into the design of new therapeutic agents that selectively target these receptors.

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51. The effect of nicotine and nicotinic receptors (nAChR) on α-synuclein oligomerization and ER stress

Hajer Brahem[*]*, Rahul Srinivasan, Henry A. Lester*

α-synuclein (aSyn) is a widely expressed neuronal protein, the misfolding, oligomerization and fibrillization of which is implicated in Parkinson's disease (PD). In this study, a bimolecular complementation assay (BiFC) was used for the direct visualization of aSyn oligomer formation in transiently transfected mouse neuroblastoma (N2a) cells. Tagging aSyn to non-fluorescent GFP fragments, GFP (1-155) on its N-terminal end and GFP (156-238) on its C-terminal end generated two constructs: aSyn-Nter-GFP and aSyn-Cter-GFP, such that GFP fluorescence is detected when aSyn oligomerization occurs. The intensity of green fluorescence can then be used to quantify aSyn oligomerization. Co-transfection of N2a cells with aSyn-Nter-GFP, aSyn-Cter-GFP and the α4 and β2 subunits of the mouse neuronal nicotinic receptor (nAChR) reduced aSyn oligomerization. Similar results were obtained following co-transfection with α4-β4, GAT1 (GABA transporter) and GluClα (Glutamate gated chloride channels). Co-transfection of N2a cells with aSyn-Nter-GFP, aSyn-Cter-GFP and an empty control pcDNA3.1 backbone vector did not alter aSyn-GFP expression thus, ruling out non-specific transfection-dependent effects on oligomerization. The effects of GAT1 and GluClα on aSyn oligomerization indicate a possible role for promoter competition of transfected plasmids. Alternatively, the increased expression of proteins within the endoplasmic reticulum (ER) of transfected cells could result in the initiation of an ER stress response and a consequent reduction in aSyn oligomerization. Future studies will focus on the possible relationship of ER stress and aSyn oligomerization.

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52. Epileptiform activity and nicotine-induced tonic seizures in an ADNFLE mouse

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Autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) is a rare inherited partial epilepsy that is linked to point mutations in the α4 and β2 nicotinic subunits. ADNFLE patients present brief nocturnal seizures with symptoms such as tonic extension of the neck, trunk, and/or forelimbs; tonic finger splaying; and forelimb clonus. Dr. Jian Xu at the Salk Institute constructed a knock-in mouse line bearing one of the ADNFLE mutations (β2:V287L). To determine whether nicotine induced ADNFLE-like seizures in these mice, we injected mutant mice and wild-type (WT) littermates with 2-mg/Kg nicotine (i. p.) and videotaped their behavior. The β2:V287L mutation significantly ($p < 0.01$) increased the incidence of nicotine-induced tonic seizure symptoms in the mutant mice. In homozygous mutant mice, nicotine elicited a diagonal retrograde tail in 12 of 12, tonic forelimb extension in 11 of 12, forelimb digit splaying in 10 of 12, and forelimb clonus in 5 of 12 mice tested. In contrast in WT littermates, nicotine only elicited the first three symptoms in one of 13 mice tested and failed to elicit forelimb clonus in any of the 13 WTs tested. The incidence of nicotine-induced tonic seizure symptoms in 12 heterozygous mice was intermediate between the WT and homozygotes. We recorded chronic electroencephalographic (EEG) activity from a separate group of four homozygous mice. One of the group displayed spontaneous sharp-wave epileptiform activity and most showed signs of generalized EEG suppression. Thus, the β2:V287L mutation dramatically increases the incidence of nicotine-induced tonic seizure symptoms, and appears to generate spontaneous baseline and epileptiform EEG abnormalities, in knock-in mice. We are continuing to monitor WT and mutant mice for signs of abnormal EEG activity.

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53. Probing the binding interaction of cytisine to the low affinity α4β2 nAChR

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The α4β2 nicotinic acetylcholine receptor (nAChR) is a pentameric neuronal ligand-gated ion channel (LGIC) which exists in two stoichiometries, $(\alpha4)_2(\beta2)_3$ called high affinity and $(\alpha4)_3(\beta2)_2$ termed low affinity. It has previously been shown in our laboratory that nicotine binding to the high affinity α4β2 nAChR is mediated through a cation-π interaction at a tryptophan residue (αW149) in loop B of the extracellular domain, as well as a hydrogen bond to the backbone carbonyl at threonine 150 (αT150). Cytisine is a partial agonist at the α4β2 nAChR and selectively activates $(\alpha4)_3(\beta2)_2$ over $(\alpha4)_2(\beta2)_3$. This compound has been used as a smoking cessation drug and is the parent compound for Varenicline (CHANTIX). We plan to determine the binding mechanism of cytisine to $(\alpha4)_3(\beta2)_2$ nAChR. Using unnatural amino acid mutagenesis and nonsense-suppression, a series of fluorinated unnatural amino acids will be systematically introduced into the aromatic residues of the binding box of the $(\alpha4)_3(\beta2)_2$ receptor to probe the existence of a cation-π interaction. The hydrogen bond interaction to the backbone carbonyl will be examined by substituting αT150 for threonine α-hydroxy acid (Tah), thus weakening the carbonyl as a hydrogen bond acceptor. These experiments will further our understanding of cytisine binding to $(\alpha4)_3(\beta2)_2$ nAChR.

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54. Photochemical proteolysis of an unstructured linker of the $GABA_AR$ extracellular domain prevents GABA but not pentobarbital activation

Crystal Dilworth, Henry A. Lester*

Nicotine binds and activates nicotinic acetylcholine (ACh) receptors (nAChRs) in the peripheral and central nervous system. The nAChRs belong to the Cys-loop family of ionotropic receptors, which share a pentameric architecture arranged around a central ion permeable pore. Many distinct subunit combinations can form receptors; and these combinations have distinct pharmacology in two areas: responses to acute applications of nicotinic drugs, and to chronic or repeated applications. This project studies receptors containing the α5 subunit (α5* nAChRs) because genome-wide association and candidate gene studies have identified polymorphisms in the α5 gene that are linked to an increased risk for nicotine addiction, alcohol addiction, and lung cancer.

Using high-resolution fluorescent microscopy techniques including Förster resonance energy transfer (FRET) and total internal reflection fluorescence (TIRF) we are able to visualize the nAChR's life cycle from assembly to degradation, within living cells. Here we propose to use these methods to study the assembly of α4β2α5* nAChRs, to determine the subcellular localization of α5* nAChRs, and to measure the pharmacological differences between α4β2* and α4β2α5* receptors. This study will therefore, determine the molecular and subcellular processes that may underlie the response to chronic or repeated nicotine exposure of cells containing α5* receptors. Several health-related phenomena involve nAChRs: nicotine is the major addictive component of tobacco; there is an inverse correlation between smoking and Parkinson's disease; and patients with autosomal dominant nocturnal frontal lobe epilepsy who smoke have fewer seizures.

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55. Critical role of α4 subunits in α6* nicotinic ACh receptor function in regulating striatal dopamine release and locomotor behaviors in mice

Ryan M. Drenan, Sharon R. Grady, Michael J. Marks*, Andrew Steele, Cheng Xiao, Julie M. Miwa, Henry A. Lester*

Nicotinic acetylcholine receptors (nAChRs) containing α6 subunits with a sensitizing L9'S mutation ($\alpha6^{L9'S}$*) can be selectively activated by low concentrations of nicotinic agonists. Functional α6* nAChRs are largely restricted to dopaminergic (DA) neurons and striatal DA terminals, allowing *in vivo* administration of low doses of nicotinic agonists into $\alpha6^{L9'S}$ transgenic mice to selectively activate the mesostriatal and mesolimbic DA system. Functional $\alpha6^{L9'S}$* nAChRs contain β2 subunits but may be expressed with or without α4 subunits. To test whether α4 subunits are required for functional α6β2* nAChRs *in vivo*, we crossed $\alpha6^{L9'S}$ mice with α4 KO mice to construct α4KO/$\alpha6^{L9'S}$ mice. Electrochemical measurements of striatal dopamine levels showed that both single and multiple (4p, 100 Hz) stimulations evoked dopamine release transients that were greater in magnitude and duration in $\alpha6^{L9'S}$ mice versus α4KO/$\alpha6^{L9'S}$ or WT mice. There was no difference in dopamine transporter numbers in any genotype as measured by [^{125}I]-RTI-121 binding. These data suggest that the prolonged dopamine release in $\alpha6^{L9'S}$ mice may be due to activation of α4$\alpha6^{L9'S}$β2* nAChRs and a subsequent augmented release of DA and not a defect in dopamine reuptake. Consistent with previous findings that *in vivo* activation of $\alpha6^{L9'S}$* nAChRs causes hyperlocomotion via selective stimulation of the mesostriatal dopamine system, we found that in home cage behaviors, $\alpha6^{L9'S}$ mice, but not α4KO/$\alpha6^{L9'S}$, α4 KO, or WT mice, showed hyperactivity, including increased travel distance and more frequent walking and jumping. Neither the $\alpha6^{L9'S}$ mutation or the α4KO/α $6^{L9'S}$ mutation changed [^{3}H]-SCH23390 binding for dopamine D_1 receptors or [^{3}H]-raclopride binding for dopamine D_2 receptors, suggesting the hyperactivity in $\alpha6^{L9'S}$ mice may result from enhanced dopamine release due to selective activation of

$\alpha4\alpha6^{L9'S}\beta2$* nAChRs by ambient ACh. Thus, these data show that α4 subunits play a critical role in α6* nAChR function *in vitro* and *in vivo.*

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56. Interpreting the biphasic dose-response curve of a mutant nAChR

Shawnalea J. Frazier[*], Kristin Rule Gleitsman[*], Henry A. Lester

Muscle nicotinic acetylcholine receptors are ligand-gated ion channels allosterically regulated by neurotransmitter binding events. Attempts at understanding the mechanism of allosteric transduction between the binding site and putative channel gate some 60 Å away have led to the categorization of particular extracellular domain residues with specific functional roles in either ligand binding or channel gating events. One such residue, γW55/δW57, which participates in formation of the 'aromatic binding box', i.e., the site at which agonist binds, has been reported to elicit greater effects on the channel gating equilibrium than agonist affinity when conventionally mutated. A backbone mutation at this site was accomplished by incorporation of the unnatural amino acid α-hydroxy tryptophan (Wah), which forms an ester rather than an amide peptide bond. The resulting channel exhibited anomalous biphasic dose-response behavior when this mutation was combined with another known gating mutation in the channel pore, βL9'S. TIRF imaging studies and stochastic simulations of this αβ9'γW55WahδW57Wah receptor confirm that the biphasic response is produced by a population of receptors with a single, rather than mixed, stoichiometry. Single-channel recordings obtained at a variety of concentrations contain two main types of channel behavior, one with a high probability of channel opening ($P_o$high) and one with a low probability of channel opening ($P_o$low). Both types show their own agonist-induced, concentration-dependent increase in channel opening events. These two types contribute to the two components of the macroscopic dose-response curve.

It has since been determined that the biphasic dose-response curve observed for αβ9'γW55WahδW57Wah is a mixture of two functionally different receptor populations with the same subunit stoichiometry, where the first $P_o$high component activated by lower agonist doses reflects the concentration-dependent activation of αβ9'γW55WahδW57Wah and the second $P_o$low component reflects the concentration-dependent activation of αβ9'γW55QδW57Q, that is, a similar mutant receptor but with a glutamine (Q) mutation rather than Wah. This undesired mutant receptor is the result of 76mer-tRNA (the limiting constituent of unnatural amino acid incorporation by nonsense suppression via microinjection of RNA) being endogenously aminoacylated with the natural amino acid glutamine. This 76mer-tRNA may be present in unknown quantities either as an unreacted side product from *in vitro* ligation of 76mer-tRNA-Wah, or as a natural end product following *in vivo* incorporation of Wah during translation, thereby accumulating with increasing with post-injection time. For both macroscopic and single-channel studies, a post-injection incubation of 48 - 72 hrs was typically used to give a current response suitable for analysis. Thus, the biphasic dose-response curve observed for αβ9'γW55WahδW57Wah was identified as an artifact of the nonsense suppression method due to extended post-injection incubation periods.

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57. Long-range coupling in an allosteric receptor revealed by mutant cycle analysis

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The functional coupling of residues that are far apart in space is the quintessential property of allosteric proteins. We studied an exemplar nicotinic acetylcholine Cys-loop; the gating of an intrinsic ion channel is allosterically regulated by the binding of small molecule neurotransmitters 50-60 Å from the channel gate. Although structurally these receptors have two distinct domains, some residues in the extracellular ligand-binding domain must be involved in communicating the binding event to the channel gate. These gating pathway residues are quite important, but their identification and characterization can be challenging. The present work introduces a simple strategy, derived from mutant cycle analysis, for identifying gating pathway residues using macroscopic measurements alone. This method employs a well-characterized reporter mutation (βL9'S) known to impact gating, in conjunction with additional mutations of residues in the ligand-binding domain hypothesized or previously found to be functionally significant. We then show that this method, elucidating long-range functional coupling in allosteric receptors (ELFCAR), can be applied to a wide variety of reporter mutations to identify both previously characterized and novel mutations that impact the gating pathway. We support our interpretation of macroscopic data with single-channel studies. ELFCAR should be broadly applicable to determining functional roles of residues in allosteric receptors.

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58. Correlations of affinity and potency of compounds for α4β2* and α6β2*-nAChR subtypes show differing relationships

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A library of compounds was assessed for affinity and potency at α4β2* and α6β2*-nAChRs, generating sufficient data to correlate a number of parameters. For these studies, affinity was assessed at α4β2*-nAChRs by inhibition of [^{125}I]-epibatidine (epi) binding to mouse cortex membranes, a region where high-affinity epi binding is restricted to α4β2*. Affinity for α6β2*-nAChRs was assessed by two methods, inhibition of [^{125}I]-alpha-conotoxin MII (CtxMII) binding to mixed membranes from striatum, olfactory tubercle, and superior colliculus from mouse brain, regions with relatively high expression of this subtype, and by inhibition of [^{125}I]-epi binding to membranes of the same regions from α4 null mutant mice which eliminates all high affinity binding of epi except that of the α6β2*subtypes. Function of these two subtypes was measured by $^{86}Rb^{+}$ efflux from mouse thalamic synaptosomes, and [^{3}H]-dopamine release from striatal synaptosomes. The $^{86}Rb^{+}$ efflux assay measures exclusively α4β2*-nAChRs while the [^{3}H]-dopamine release assay with the use of CtxMII assesses both α4β2* and the α6β2*-nAChR function. The two independent measures of α6β2* binding were compared by correlation analysis. The results indicated that these methods give equivalent binding Ki values (r^2=0.91, slope=0.86, for 17 compounds). The two methods for assessing α4β2*function were also compared and the data indicate the EC50 values assessed by these two methods are the same (r^2=0.89, slope=0.96, for 13 compounds). In a correlation of fifteen agonists for binding affinity (Ki) vs. potency for function (EC50), these parameters have the expected relationship between binding Ki and functional EC50 of about 100-fold difference (r^2=0.82, slope=1.06, for 15 compounds). However, the α6β2*-nAChR appears to have a distinctly different relationship with binding affinity displaced less than 10-fold from EC50 values (r^2=0.87, slope=0.97, for 15 compounds). These data may imply that α6β2*-nAChRs have a different relationship between ground state and desensitized state from the α4β2*-subtypes.

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59. Photochemical proteolysis of an unstructured linker of the $GABA_AR$ extracellular domain prevents GABA but not pentobarbital activation

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In addition to activation by their cognate neurotransmitter, γ-aminobutyric acid (GABA), the γ-aminobutyric type A receptor ($GABA_AR$) is subject to allosteric modulation by a variety of compounds, including benzodiazepines. The structural elements associated with allosteric modulation by this important class of pharmaceuticals is not well understood. The α subunit of these inhibitory cys-loop ligand gated ion channels is involved in both benzodiazepine and GABA binding events, suggesting a role for this subunit in allosteric modulation. Herein, we use the nonsense suppression methodology to incorporate a photoactivated unnatural amino acid to photochemically cleave the backbone of the α subunit of the $\alpha_1\beta_2$ $GABA_AR$ between loop A, which contains the putative benzodiazepine binding site and loop E, which is involved in GABA binding. Proteolytic cleavage impairs GABA but not pentobarbital activation, strongly suggesting the unstructured linker between loops A and E is critical to the GABA activation pathway.

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60. Optimizing ivermectin-activated anion channels for neuronal silencing

Sindhu Kadambi, Henry A. Lester

To block neuronal firing in selected areas of the brain, we developed a silencing strategy that utilizes a glutamate-activated chloride channel (GluCl) cloned from *C. elegans* to inhibit neuronal firing. GluCl receptors are pentameric Cys-loop receptors formed by α and β subunits around a central pore. We made a Tyr182Phe mutation in the GluCl β subunit to eliminate the normal glutamate response of the channels, codon-optimized both subunits for enhanced mammalian expression, and labeled them with fluorescent proteins for visualization. The mutant channels respond exclusively to the anti-parasitic drug ivermectin (IVM). However, the nonspecific effects of IVM *in vivo* limit the usefulness of this approach. To minimize these nonspecific effects, we decided to increase the IVM sensitivity of the mutant GluCl channels and their single-channel conductance by making additional mutations in the M2 region, a domain that has been shown to be important in modulating agonist sensitivity of other Cys-loop receptors. These mutations should reduce the IVM dose required to silence neuronal firing *in vivo*. The effects of the mutations were tested by expressing the mutant channels in *Xenopus laevis* oocytes, and measuring their IVM response electrophysiologically. Mutating the leucine residue at the 9' position in the M2 domain to an alanine or valine significantly increased IVM sensitivity. The L9'A mutant constructs were, however, constitutively active, whereas, the L9'V mutant constructs produced only a two-fold increase in sensitivity to IVM (compared to

WT). We are testing additional mutations at the 9' position, as well as at other residues within the M2 domain to see if we can increase IVM sensitivity more than twofold without significantly increasing the probability of spontaneous channel openings. Mutations are also being introduced within the large cytoplasmic loop of the receptor (the HA-stretch) to increase single channel conductance. Mutant constructs that successfully increase IVM sensitivity and minimize spontaneous openings will be eventually expressed in cultured neurons to test their ability to inhibit neuronal firing. If successful, these modifications will reduce the IVM dose required to activate GluCl *in vivo* and enhance the usefulness of this silencing strategy.

61. Structure-function studies on the 5-HT_3 receptor ligand-binding site reveal polar residues critical for receptor activation and identify an intersubunit salt bridge

Kiowa S. Bower[*], *Henry A. Lester, Dennis A. Dougherty*[*]

The 5-hydroxytryptophan$_3$ (5-HT_3) receptor is a member of the Cys-loop family of neurotransmitter-gated ion channels (LGIC). This family of receptors also includes the nicotinic acetylcholine (nACh), $GABA_A$, and glycine receptors, and is involved in fast synaptic transmission. The receptors function as a pentameric arrangement of subunits where each subunit is composed of a large extracellular domain and four transmembrane α-helices (REFS). Each of the five 5-HT_3 binding sites is located at an interface between the extracellular domains of the pentamer (REFS). Sequence similarity between the 5-HT_3 receptor and the acetylcholine binding protein (AChBP), as well as mutational studies (REFS), have enabled the construction of homology models that have provided approximate representations of the 5-HT_3 ligand-binding site (REFS). Current models show that the 5-HT_3 ligand-binding site is most likely formed by three loops (A-C) in the principle subunit and three loops (D-F) in the complementary subunit (REFS) (Figure 1b). However, as homology models are inherently speculative, experimental validation of predictions based on these models are invaluable to the elucidation of the actual binding interactions between 5-HT and its receptor. Previous studies have established an important role for aromatic residues that are conserved across the Cys-loop family, including a cation-π interaction between the positive charge of 5-HT and the side chain of Trp183 on loop B of the principle subunit.

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62. Molecular interaction between nicotinic acetylcholine receptors and the modulator protein lynx1

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Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels expressed throughout the brain and at neuromuscular junctions. These receptors are homo- or hetero-pentameric with homologous subunits arranged around a central ion pore. The lynx family of proteins have been shown to physically associate with nAChRs and are expressed in brain areas heavily involved in nicotinic function. Lynx modulators are thought to support proper nAChR function *in vivo*. *In vitro*, lynx has been shown to associate with the neuronal α4β2 and α7 nAChR subtypes and the muscle-type nAChR. The interaction with lynx affects receptor function at the molecular level, including acetylcholine sensitivity and desensitization kinetics.

Molecular-scale interactions between the lynx proteins and the nAChR are the target of our investigation. Specifically, we seek to identify the sites of interaction between nAChR and lynx1, the first protein discovered of the family. Lynx1 is a small protein containing 72 amino acids with a C-terminal glycophosphoinositide-linked (GPI-linked) sequence. Lynx's cysteine-rich motif is characteristic of the class of elapid snake venom neurotoxins such as α-bungarotoxin (αBtx) and cobratoxin, which are known competitive antagonists to specific nAChR subtypes. The GPI-linked motif would topologically allow the lynx proteins to bind in a similar fashion to αBtx at the intersubunit interface on nAChR. However, the antagonistic effect, as seen with the toxins, has not been demonstrated with lynx binding. This raises the possibility that lynx binds to the non-agonist interfaces of the receptor that are allosterically important to gating. Interestingly, our preliminary electrophysiology results in *Xenopus* oocytes support this view: co-injection with lynx1 primarily affects $(\alpha4)_3(\beta2)_2$ but not $(\alpha4)_2(\beta2)_3$ stoicheometry of the α4β2 nAChR although their agonist binding interfaces are identical. With photobleaching experiments on total internal reflection (TIRF) microscopy, we aim to determine whether or not lynx1 binds to nAChR at the agonist binding interfaces (as αBtx does) and to identify the number of lynx1 binding sites per receptor. Once the broad regions of the binding site are located, we hope to focus further onto specific binding residues using site-directed mutagenesis.

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63. Investigating the upregulation of the α6 nAChR subunit in response to chronic nicotine using a modified bacterial artificial chromosome (BAC)

Elisha Mackey, Henry A. Lester

As receptors containing α6 nicotinic acetylcholine receptor (nAChR) subunits have the highest affinity to nicotine yet measured, α6 could play an important role in understanding nicotine addiction. To investigate upregulation of the α6 nAChR subunit in response to chronic nicotine, we will visualize receptors containing α6 in a mouse line expressing fluorescently-labeled α6 and observe changes in expression in response to nicotine. This approach is similar to previously published efforts using a knock-in mouse line containing a YFP-labeled α4 nAChR subunit and a mouse line with a CFP-labeled α4 nAChR subunit.

In our investigation, instead of developing a fluorescently-labeled α6 knock-in mouse, we have taken advantage of the speed with which transgenic mice can be developed by using a modified bacterial artificial chromosome (BAC) to develop a BAC transgenic mouse line. A GFP sequence was inserted into the M3-M4 loop on exon 5 of Chrna6 (α6 nAChR) located on a 156kb BAC using recombination in a two-step selection and counter selection technique. The BAC includes significant 5' and 3' flanking regions for faithful expression of α6. The modified BAC was then randomly introduced into the mouse genome via pronuclear injection of fertilized mouse eggs.

Those mice that express the transgene at levels close to wild type will then be crossed to an appropriate α6 knock-out line such that the only α6 present contains a fluorescent tag. The resulting mice will be tested for appropriate receptor function by testing levels of ACh and nicotine-induced Ca^{2+} influx, nicotinic receptor binding, and nicotine-induced dopamine release. The mice will then be exposed to chronic nicotine or chronic saline for 10 days using osmotic mini-pumps, thereafter, the brains sectioned and examined for changes in levels of α6 receptor subunit expression. These results will provide insight into the relationship between specific changes in number and distribution of nicotinic receptors and nicotine thus, helping to understand the role of α6 and addiction.

64. Evaluating interfacial residues of the NMDA receptor with unnatural amino acids: A novel probe of the clamshell

Kathryn A. McMenimen[*], Samuel J. Ettinger[*], Henry A. Lester, Dennis A. Dougherty[*]

Ionotropic glutamate receptors (iGluRs) are ligand-gated ion channels that play a pivotal role in learning and memory in the mammalian central nervous system. The N-methyl-D-aspartate (NMDA) receptor is activated by the binding of glutamate and glycine to clamshell-like domains in the NR2 and NR1 subunits, respectively. Ligand binding rotates the D2 lobe of the clamshell toward the D1 lobe, and this closure promotes channel gating. Studies of partial agonists in the related AMPA receptor establish that submaximal activation results from partial clamshell closure. However, structural studies of NR1 have not revealed a similar mechanism for partial agonist activation. Using unnatural amino acid mutagenesis we convert an NR2-conserved tyrosine to homotyrosine and an NR1 glutamine to homoglutamine, residues designed to disrupt clamshell closure by expanding the side chain without altering its functionality. Homotyrosine incorporation indeed produces larger functional changes for full agonists relative to partial agonists in NR2, while homoglutamine produces a much smaller change for full agonists relative to partial agonists. These data show that a clamshell closure mechanism, previously shown for AMPA receptors, likely also applies to NMDA receptors, but to different degrees in the NR1 and NR2 subunits. This study extends the unnatural amino acid methodology to serve as a functional probe of the clamshell mechanism in iGluR ligand binding.

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65. Molecular mechanisms underlying cholinergic regulation in the brain

Julie M. Miwa, Henry A. Lester

Nicotinic acetylcholine receptors (nAChRs) of the cholinergic system affect a wide array of biological processes including learning and memory, attention, and addiction. Because of the ability of the cholinergic system to effect global changes in excitability, the regulation of its receptor system is quite critical. Several mechanisms exist to regulate this process; we are investigating the effect that alterations in receptor sensitivity.

This is accomplished by studying gain-of-function mutations in nAChRs, and through alterations in the regulator elements of the nACh receptor complex. *Lynx1*, the founding member of a family of mammalian prototoxins, binds nAChRs and modulates their function *in vitro* by altering agonist sensitivity and desensitization kinetics. *In vivo*, lynx balances cholinergic tone to influence complex brain processes, such as learning and memory enhancements observed in lynx1 null mutant mice.

In order to explore the mechanism by which lynx achieves its effects on nAChRs, we are utilizing several fluorescence microscopic techniques to label nAChRs in living cells and track these receptors during assembly and export to the cell surface. In mammalian cells, we have discovered unique static and dynamic features when the receptor is co-transfected with lynx1, indicating that lynx alters nAChR trafficking in the cell and can influence receptor expression and stoichiometry on the cell surface. These experiments will be extended to evaluation of mutant mice lacking the lynx1 gene, in order to compare receptor trafficking against those of neurons from wild-type mice. These studies will be helpful in explaining the global modulatory effects of the lynx1 gene in normal and disease processes, and can contribute to our understanding

of disease states when normal nAChR regulation is disrupted.

66. Subcellularly resolved FRET explains GABA transporter function in N2a cells by detecting oligomerization states and plasma membrane anchoring

Fraser J. Moss, P.I. Imoukhuede[1], Kimberly Scott, Jia Hu[2], Joanna L. Jankowsky[3], Michael W. Quick[2], Henry A. Lester

The γ-aminobutyric acid transporter mGAT1 was expressed in neuroblastoma 2a cells. Nineteen fluorescent mGAT1 designs incorporating fluorescent proteins were functionally characterized by [^{3}H]GABA uptake in an expression system that responded to several experimental variables, including the mutations and pharmacological manipulation of the cytoskeleton. Oligomerization and subsequent trafficking of mGAT1 were studied in several subcellular regions using localized fluorescence, donor recovery after acceptor photobleaching (DRAP), and pixel-by-pixel analysis of normalized Förster resonance energy transfer (NFRET) images. Nine constructs were functionally indistinguishable from wild-type mGAT1 and provided information about normal mGAT1 assembly and trafficking. The remainder had compromised [^{3}H]GABA uptake and presented oligomerization and trafficking deficits that helped determine regions of sequence involved in these processes. DRAP FRET detected mGAT1 oligomerization, but richer information was obtained from histograms derived from pixel-by-pixel analyses of the NFRET signal. These were fit to either two or three Gaussian components. Two of the components, present for all mGAT1 constructs that oligomerized, may represent dimers and high-order oligomers (probably tetramers), respectively. Only wild-type functioning constructs displayed three Gaussians; the additional component apparently had the highest mean FRET intensity. Directional radius analysis determined that the pixels with the greatest magnitude NFRET from wild-type functioning constructs were localized at the cell periphery. At the periphery, the highest-NFRET component represented ~30% of all pixels, similar to the percentage of mGAT1 from the acutely recycling pool resident in the plasma membrane in the basal state. Blocking the mGAT1 PDZ interacting domain or disrupting the actin cytoskeleton abolished the third, highest NFRET component from the total signal of wild-type functioning transporters. Thus, pixel-by-pixel NFRET analysis resolved two distinct forms of GAT1 oligomer, distinguished plasma membrane resident from nearby subsurface transporter complexes, and revealed sequences governing PDZ-mediated interactions of the transporter with the cytoskeleton and/or the exocyst.

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67. Coassembly of fluorescently tagged α7 and β2 nicotinic acetylcholine receptor subunits in mammalian cell line detected by FRET

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Mammalian nicotinic acetylcholine receptors (nAChRs) generally assemble as heterooligomers. A notable exception is the α7 subunit, which assembles as homopentamers in mammalian heterologous expression systems. Some reports also suggest that α7 forms hetero-oligomers in mammalian cells and in both non-mammalian and mammalian neurons. Thus, a mammalian heterologous expression system stably expressing both α7 and β2 subunits would be useful to both confirm α7β2 coassembly and to study its behavior in mammals. The present study demonstrated that mouse α7 and β2 subunits, each fused to a different fluorescent protein, can be stably co-expressed for over 20 passages in a mammalian cell line, SH-EP1, which is nAChR native null. Moreover, this work shows for the first time that fluorescence resonance energy transfer (FRET) occurred between the fluorescent proteins on the differentially labeled subunits in intracellular compartments. Additionally, preliminary results of trafficking and electrophysiological studies suggest that co-expression of the β2 subunit affects surface trafficking of α7. This cell line will prove useful in studying the assembly, interaction with chaperone proteins, trafficking and function of α7β2 heterooligomers.

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68. Pharmacological chaperoning of nicotinic receptors begins in the endoplasmic reticulum. High-resolution imaging

Rigo Pantoja, Rahul Srinivasan, Sindhuja Kadambi, Elisha D.W. Mackey, Shelly Tzlil, Fraser J. Moss, Henry A. Lester*

Nicotine addiction is the world's leading preventable cause of mortality. Smokers also have a much lower incidence of Parkinson's disease. Previous experiments show that ligand interactions with α4- and β2-nicotinic receptor subunits are necessary and sufficient for nicotine addiction. A plausible cellular/molecular mechanism for some responses to nicotine exposure is selective pharmacological chaperoning of acetylcholine receptor number and stoichiometry (SePhaChARNS). To investigate SePhaChARNS in a neuron-like environment, we used single- and few-molecule resolution fluorescence microscopy to monitor localization and trafficking of α4GFP β2 and α4GFP β4 receptors expressed in mouse neuroblastoma (N2a) cells. As in previous investigations on native neurons and heterologous expression systems, we find large pools of endoplasmic reticulum (ER) localized α4GFP β2 receptors. Strikingly, cells expressing α4GFPβ4 display plasma membrane (PM) localized receptors. Pharmacological chaperoning was investigated by incubating N2a cells expressing α4GFP β2 receptors in nicotine or the partial agonist cytisine. Furthermore, we simultaneously imaged α4GFP β2 receptors and an

mCherry-tagged ER exit site (ERES) marker to monitor the ER exit of α4GFP β2 receptors and the associated changes in ERES. Nicotine induced an increase of ER localized α4GFP β2 receptors, and ERES activity did not change markedly. In contrast, cytisine treatment increased the number of ERES fusion events, which may be an ERES adaptive response to increased cargo load. Consistent with an increase in cargo load we observed increased α4GFP β2 receptor PM localization. Data from cytisine and nicotine treatments directly indicate that pharmacological chaperoning is initiated in the ER. Nicotine and cytisine induce assembly of differential α4 β2 nicotinic receptor stoichiometries (see accompanying abstract by Srinivasan *et al*.), which leads to differential receptor localization, trafficking and ERES response. Thus, high-resolution imaging of SePhaChARNS is providing data required to understand, and manipulate nicotinic receptors with drugs.

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69. α4* nAChR modulation of glutamate release from the medial perforant path

Rachel Penton, Henry A. Lester

Despite extensive research, the physiological mechanisms underlying nicotine addiction are still largely unknown. Nicotine may be unique compared to other psychostimulants as it has the capability of interacting with nicotinic acetylcholine receptors (nAChRs) in many areas of the brain outside of the classical reward system and is involved in the release of other neurotransmitters in addition to stimulating dopamine release. One pathway in which this may be important is the medial perforant path (MPP) projection from medial entorhinal cortex (MEC) layer II stellate cells to dentate gyrus (DG) which may be involved in the cognitive effects associated with chronic nicotine exposure. It has previously been shown that the α4* nAChRs are upregulated on MPP axons following chronic nicotine treatment and activation of these upregulated receptors by nicotine decreased the threshold for long-term potentiation induction, presumably by increasing glutamate release (Nashmi *et al*., 2007). These current studies will determine how α4* nAChRs located on MPP axons modulate glutamate release and how upregulation of these receptors alters this modulation.

The α4* nAChRs may be expressed in multiple locations (presynaptically, preterminally, and/or axonally) along the MPP axons and will likely impact glutamate release differently depending on their location. Initial experiments conducted in acute hippocampal slices prepared from chronic saline- and nicotine-treated mice will include whole-cell recordings of the postsynaptic target neurons, the DG granule cells. These recordings will be used to determine whether activation of α4* nAChRs alters miniature, spontaneous, or evoked excitatory postsynaptic currents (EPSCs) indicating the likely location of the receptors as presynaptic, preterminal, or axonal, respectively. Additionally, the timecourse of the acute nicotinic effects will be important for determining if α4* nAChRs located on MPP axons are differentially sensitive to desensitization after upregulation. We predict that the results will indicate preterminal and/or axonal localization of the receptors; therefore, additional experiments will include direct axonal bleb recordings to observe nAChR-mediated depolarization and currents. Recordings of the MEC layer II stellate cells will also be performed to determine if the entire pathway is hypersensitive to nicotine after chronic treatment. These studies will give insight to possible mechanisms underlying the cognitive effects of chronic nicotine.

70. Investigating the roles of residues in the vicinity of the α4β2 nAChR aromatic binding site

Nyssa Puskar[*], Henry A. Lester, Dennis A. Dougherty[*]

The neuronal α4β2 nicotinic acetylcholine receptor (nAChR) is strongly associated with nicotine addiction and belongs to the larger family of highly homologous nAChRs that are involved in various aspects of brain function. Research in our lab uses a combination of unnatural amino acid mutagenesis and electrophysiology to develop a chemical-scale understanding of the neuroreceptors and ion channels involved in brain and central nervous system function. We recently reported that the high affinity for nicotine at the α4β2 nAChR is a result of a strong cation-π interaction and backbone hydrogen bond to a specific aromatic amino acid of the receptor, tryptophan 154. Interestingly, both of these interactions are either absent or extremely attenuated in the homologous receptor found at the neuromuscular junction, which explains why smokers do not become paralyzed when inhaling nicotine. Currently, we are continuing chemical-scale investigations of the α4 β2 nAChR to determine the molecular origins of nAChR subtype specificity. Specifically, we are probing the manner in which residues positioned in the immediate vicinity of the binding site play a role in either facilitating or destabilizing the previously identified interactions (i.e., cation-π interaction and hydrogen bond).

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71. Oscillatory firing of single neurons in the human subthalamic nucleus: a cross-hemisphere synchrony analysis

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The human subthalamic nucleus (STN) plays a crucial role in Parkinson's disease. Bilateral deep brain stimulation (DBS) in the STN is an effective way to suppress Parkinsonian tremors. In patients with tremors, a substantial fraction of spontaneously active STN neurons fire rhythmically. Within a single STN, many neurons fire synchronously. The circuit mechanisms that cause this strong synchrony are unclear. In particular it is unknown whether this phenomenon is restricted to the STN of hemisphere or is more widespread. To elucidate this mechanism, we investigated whether neurons in one STN are also firing in synchrony with neurons in the contralateral STN.

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72. Single-channel recording of nicotinic receptors with pore mutations

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To activate the nicotinic acetylcholine receptor, agonist must first bind in the extracellular domain and then induce conformational change(s) leading to activation, which includes movement in the channel pore that allows for conductance. At the whole-cell level, a variety of functional effects are observed when mutations are made in the pore of nicotinic acetylcholine receptors. These include changes in agonist potency and extent and rate of desensitization.

To determine more specifically how mutations in the channel pore impact receptor function, we are performing single-channel recording of nicotinic acetylcholine receptors with no agonist and varying concentrations of acetylcholine. Thus far, our analysis of long apparent openings of receptors with one or more pore mutation has revealed surprisingly frequent brief closures that may be inconsistent with models that suggest these mutations significantly hinder the channels ability to close quickly. These single-channel experiments aim to produce a model that will explain the effects of one or more pore mutations to a single receptor. Such a model will then be validated against the systematic functional effects on agonist potency (EC_{50}) that have been previously reported.

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73. Single-channel recording of neuronal nicotinic acetylcholine receptors

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The neuronal nicotinic acetylcholine receptors, including the $\alpha4\ \beta2$ heteromer and $\alpha7$ homomer, are pentameric ligand gated ion channels. These receptors are opened when two molecules of an agonist, endogenously acetylcholine, bind to the receptor at binding sites located in the extracellular domain. Agonist binding causes a conformational change in the channel pore that produces a change from the nonconducting to conducting form. Previous whole-cell studies have shown that the $\alpha4\ \beta2$ receptor can form in two distinct subunit combinations: three α subunits and two β subunits (A3B2) and two α subunits and three β subunits (A2B3). Nicotine has been shown to have a higher potency for the receptor with the A2B3 stoichiometry (high sensitivity) than at the A3B2 receptor (low sensitivity). However, nicotine is a partial agonist - with efficacy lower than acetylcholine - at both stoichiometries.

Previous single-channel recording of $\alpha4\ \beta2$ channels have produced conflicting conclusions regarding which of the two stoichiometries has a larger single-channel conductance, or if there is even a difference in single-channel conductance between the A2B3 and A3B2 stoichiometries. We seek to use both whole-cell and single-channel recording to determine the functional differences between the two stoichiometries, which can be controlled by modulating the ratio of α to β mRNA. For both $\alpha4\ \beta2$ receptors, as well as the $\alpha7$ receptor, experiments at varying concentrations of several different agonists will then be performed in order to develop a model to explain the differences in efficacy of various agonists for these receptors.

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74. Nicotine normalizes intracellular subunit stoichiometry of nicotinic receptors carrying mutations linked to autosomal dominant nocturnal frontal lobe epilepsy

Cagdas D. Son, Fraser J. Moss, Bruce N. Cohen, Henry A. Lester

Autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) is linked with high penetrance to several distinct nicotinic receptor (nAChR) mutations. We studied $(\alpha4)_3(\beta2)_2$ vs. $(\alpha4)_2(\beta2)_3$-subunit stoichiometry for five channel-lining M2 domain mutations: S247F, S252L, 776ins3 in $\alpha4$, and V287L, V287M in $\beta2$. $\alpha4$ and $\beta2$ subunits were constructed with all possible combinations of mutant and wild-type (WT) M2 regions, of cyan and yellow fluorescent protein (CFP, YFP), and of fluorescent and non-fluorescent M3-M4 loops. Sixteen fluorescent subunit combinations were expressed in N2a cells. Förster resonance energy transfer (FRET) was analyzed by donor recovery after acceptor photobleaching and by pixel-by-pixel sensitized emission, with confirmation by

fluorescence intensity ratios. Because FRET efficiency is much greater from adjacent than for non-adjacent subunits and the α4 and β2 subunits occupy specific positions in nAChR pentamers, observed FRET efficiencies from $(\alpha4)_3(\beta2)_2$ carrying fluorescent α4 subunits were significantly higher than for $(\alpha4)_2(\beta2)_3$; the converse was found for fluorescent β2 subunits. All tested ADNFLE mutants produced 10-20% increments in the percentage of intracellular $(\alpha4)_3(\beta2)_2$ receptors compared to wild-type (WT) subunits. In contrast, 24-48 h nicotine (1 μM) exposure increased the proportion of $(\alpha4)_2(\beta2)_3$ in WT receptors and also returned subunit stoichiometry to WT levels for α4S248F and β2V287L nAChRs. These observations may be relevant to the decreased seizure frequency in ADNFLE patients who use tobacco products or nicotine patches. Fluorescence-based investigations of nAChR subunit stoichiometry may provide efficient drug discovery methods for nicotine addiction or for other disorders that result from dysregulated nAChRs.

75. Pharmacological chaperoning of nicotinic receptors begins in the endoplasmic reticulum: Compartments and stoichiometries

Rahul Srinivasan, Rigo Pantoja, Sindhuja Kadambi, Elisha D.W. Mackey, Shelly Tzlil[*], Fraser J. Moss, Henry A. Lester

Pentameric α4β2 neuronal nicotinic acetylcholine receptors (nAChRs) assemble in two possible stoichiometries, $(\alpha4)_2(\beta2)_3$ or $(\alpha4)_3(\beta2)_2$. The proportion of the total receptor population represented by each stoichiometry in a cell is influenced by the local environment. Selective pharmacological chaperoning of nicotinic acetylcholine receptor (nAChR) number and stoichiometry (SePhaChARNS) is an important aspect of nicotine addiction and can explain the inadvertent therapeutic effects of smoking in Parkinson's disease. We employed fluorescent protein (FP)-tagged nicotinic acetylcholine receptor (nAChR) subunits to study the effects of nicotine and cytisine on: (1) intracellular receptor stoichiometry using pixel-by-pixel Förster Resonance Energy Transfer (FRET); and (2) trafficking of assembled nAChRs to the plasma membrane (PM) by total internal reflection fluorescence microscopy (TIRFM). Neuroblastoma (N2a) cells were transiently co-transfected with α4mCherry and β2GFP nAChR subunits. Nicotine (1 μM for 4 h) incubation increased the assembly of the $(\alpha4mCherry)_2(\beta2GFP)_3$ nAChR stoichiometry. Subcellular stoichiometry analysis revealed that nicotine-induced preferential $(\alpha4mCherry)_2(\beta2GFP)_3$ receptor assembly in the endoplasmic reticulum (ER). TIRFM showed that nicotine exposure restricted localization of the newly assembled $(\alpha4GFP)_2(\beta2)_3$ receptors to the ER. Conversely, cytisine treatment (1 μM for 4 h) of α4mCherry and β2GFP transfected N2a cells resulted in preferential assembly of the $(\alpha4mCherry)_3(\beta2GFP)_2$ stoichiometry in the ER and an increase in surface trafficking of assembled nAChRs relative to non-treated controls. To study the influence of β2 on α4 β2 nAChR trafficking, N2a cells were transiently co-transfected with either α4GFP β2 or α4GFP β4 and imaged 48 h post-transfection by TIRFM. mCherry with a lyn kinase membrane localization signal was included in transfections to visualize the PM. The α4GFP β2 receptors trafficked to the PM in ~10% of the cells while ~90 % of cells displayed α4GFPβ4 at the PM. Together, these data reveal a rate-limiting role for β2 subunits in ligand-induced α4 β2 nAChR trafficking and stoichiometry-based differences in subcellular receptor localization.

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76. Probing the binding interactions between the GluR2 AMPA receptor subunit and several agonists and partial agonists

Margaret W. Thompson[*], Kathryn A. McMenimen[*], Henry A. Lester, Dennis A. Dougherty[*]

Ionotropic glutamate receptors (iGluRs) are ligand-gated ion channels that mediate synaptic plasticity in the mammalian nervous system. As a result, these receptors are essential for learning and memory and have been implicated in related processes such as addiction and Alzheimer's disease. The α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor is activated by the endogenous neurotransmitter glutamate, as well as several non-native partial agonists. Upon ligand binding, the two lobes of the AMPA binding domain rotate towards one another like the closing of a clamshell with the partial agonists closing the clamshell less than the full agonist. Using nonsense suppression, the steric and electrostatic properties of several residues in the binding site of the GluR2 AMPA receptor subunit have been varied in order to understand the differences among their interactions with glutamate and the less efficacious willardiines. Preliminary results indicate that a negatively charged residue at position 705 is required for receptor activation.

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77. Probing the role of the cation-π interaction in the binding sites of GPCRs using unnatural amino acids

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We describe a general application of the nonsense suppression methodology for unnatural amino acid incorporation to probe drug-receptor interactions in functional G protein-coupled receptors (GPCRs), evaluating the binding sites of both the M2 muscarinic acetylcholine receptor and the D2 dopamine receptor. Receptors were expressed in *Xenopus* oocytes, and activation of a G protein-coupled, inward-rectifying K(+)

channel (GIRK) provided, after optimization of conditions, a quantitative readout of receptor function. A number of aromatic amino acids thought to be near the agonist-binding site were evaluated. Incorporation of a series of fluorinated tryptophan derivatives at W6.48 of the D2 receptor establishes a cation-π interaction between the agonist dopamine and W6.48, suggesting a reorientation of W6.48 on agonist binding, consistent with proposed "rotamer switch" models. Interestingly, no comparable cation-π interaction was found at the aligning residue in the M2 receptor.

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78. A hydrogen bond in the complementary subunit of α4β2 that is important for agonist binding

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Nicotinic acetylcholine receptors (nAChRs), pentameric ligand-gated ion channels that bind nicotine, acetylcholine and structurally related agonists, are implicated in smoking addiction and many neurological disorders, including Alzheimer's disease, Parkinson's disease and schizophrenia. Previous studies in our lab have shown that nicotine and acetylcholine bind to the neuronal receptor α4β2 though a cation-π interaction and (for nicotine) a hydrogen bond with W154 of the receptor's ligand binding "aromatic box." We now report a third interaction, a hydrogen bond with L119 of the complementary subunit, which is important for agonist binding. Mutant cycle analyses with the unnatural residue α-hydroxyleucine (Lah) and agonist analogs reveal that the backbone NH of b2L119 makes a hydrogen bond to the carbonyl and pyridine N of acetylcholine and nicotine, respectively. This interaction is present in both receptor stochiometries, $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$. Epibatidine binding was also shown to be sensitive to mutations at b2L119. Taken together, these binding interactions will offer insight into the design of new therapeutic agents that selectively target these receptors.

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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 mice and over-expression *in vivo* with viral vectors, we are exploring the role of the neuropoietic cytokine leukemia inhibitor factor (LIF) in regulating neural stem cell proliferation and fate in the adult brain. In the context of neuroimmune interactions during fetal brain development, we are investigating a mouse model of mental illness based on the known risk factor of maternal influenza infection. Huntington's disease (HD) is another focus, where we are investigating potential therapies using intracellular expression of antibodies (intrabodies) and also manipulating NFκB activity.

Cytokines are diffusible, intercellular messengers that were originally studied in the immune system. Our group contributed to the discovery of a family that we have termed the neuropoietic cytokines, because of their action in both the nervous and hematopoietic/immune systems. We demonstrated that one of these cytokines, 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. Moreover, LIF can regulate neurogenesis and gliogenesis. LIF is a critical regulator of astrocyte and microglial activation following stroke, seizure or trauma, and this cytokine also regulates inflammatory cell infiltration, neuronal and oligodendrocyte death, gene expression, as well as adult neural stem cell renewal. These results highlight LIF as an important therapeutic target. We are currently examining the role of LIF in a chemical model of multiple sclerosis, where exogenous LIF can increase oligodendrocyte number and stimulate remyelination.

Cytokine involvement in a model for mental illness is also being investigated. This mouse model is based on epidemiological findings that maternal infection can increase the likelihood of schizophrenia or autism in the offspring. We are using behavioral, neuropathological, molecular, and brain imaging methods to investigate the effects of activating the maternal immune system on fetal brain development and how this leads to altered behavior in young and adult offspring. Recent results indicate that the cytokine IL-6, acting on both the placenta and fetal brain, is key in mediating the effects of maternal immune activation on the fate of the 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 intrabodies that bind to various domains of Htt, and these can either exacerbate or alleviate Htt toxicity in cultured cells, acute brain slices, and in *Drosophila* HD models. Current work is evaluating the efficacy of viral delivery of intrabodies in a variety of mouse models of HD. One of the intrabodies displays striking efficacy in ameliorating the motor and cognitive deficits, as well as the neuropathology caused by mutant Htt in these models. 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

79. Identifying the sites of interleukin-6 action following maternal immune activation

Elaine Hsiao, Paul H. Patterson

Maternal infection increases the risk for schizophrenia and autism in the offspring. In rodents, maternal influenza infection or maternal immune activation (MIA) with the double-stranded RNA, poly(I:C), causes behavioral, histological and transcriptional changes in adult offspring that are consistent with those seen in schizophrenia and autism. This indicates that MIA, rather than a specific pathogen, is responsible for the increased risk of mental illness in the offspring of mothers with infections during pregnancy. In investigating the possibility that cytokines may mediate the effects of MIA, it was determined that the cytokine interleukin-6 (IL-6) is essential for the manifestation of a variety of abnormalities in the adult offspring of poly(I:C)-treated mothers. Therefore, localizing the sites of IL-6 action may illuminate the anatomical and molecular pathways through which MIA alters fetal brain development. Our data indicate that IL-6 acts on both the placenta and the fetal brain. Downstream IL-6 responses indicative of IL-6 activity, such as SOCS3 induction, are observed in the fetal brain and placenta following maternal poly(I:C) injection. Moreover, IL-6 signaling pathways are activated in the placenta and fetal brain shortly after maternal poly(I:C) injection, as measured by increased phosphorylation of STAT1, STAT3 and ERK1/2. Effects in the placenta are localized to the spongiotrophoblast and labyrinth layers, indicating that MIA is transduced in the

fetal compartment. Maternal poly(I:C) also increases IL-6 mRNA expression itself in the fetal brain and placenta, raising the possibility of a positive feedback loop. That these effects of poly(I:C) are due to IL-6 induction is indicated by an experiment in which an anti-IL-6 blocking antibody is co-injected with poly(I:C), which abrogates the observed increases in IL-6 and its downstream sequelae. These results support a key role for IL-6 in the placenta and fetal brain in shaping neural development and behavior in the MIA offspring.

80. Interaction between genes and environment in a mouse model of mental illness

Catherine Bregere, Paul H. Patterson

Various environmental and genetic factors are suggested to be involved in the etiology of schizophrenia and autism. Infections during pregnancy represent an environmental risk factor for the development of these disorders in the offspring, and this can be modeled in mice. Indeed, progeny of pregnant mice infected with the flu virus, or injected with poly(I:C), a viral mimic, display neuropathology and behavioral impairments reminiscent of autism and schizophrenia. As candidate genes for these disorders are identified, and genetically-modified mice modeling these mutations are generated, it is possible to test whether genes and environment interact synergistically in the pathogenesis of mental illness. Thus, our hypothesis is that neuropathology and behavioral impairments will be exacerbated in mutant offspring of mothers given infections or maternal immune activation (MIA). To test this, we are using a mutant of disrupted in schizophrenia-1 (DISC-1), which is implicated in schizophrenia and other severe mental illnesses, as well as a mutant of urokinase-type plasminogen activator receptor (uPAR), which is relevant for autism. Preliminary data do not reveal an exacerbation of behavioral deficits in DISC-1 progeny born to mothers treated with poly(I:C), as measured by prepulse inhibition and latent inhibition. However, additional experiments involving a larger number of mice will be conducted to validate this observation. MIA-exposed uPAR progeny are currently being generated.

81. Imaging hallucinations in mice

Natalia Malkova, Paul H. Patterson

Hallucinations are defined as normal activation of the visual or auditory system in the absence of appropriate sensory input. A corollary is that such activity should be enhanced by drugs that are known to induce hallucinations in normal people and that exacerbate this symptom in schizophrenic subjects. Activation of 5-HT2A receptors (5-HT2AR) is responsible for the psychomimetic properties of hallucinogens in humans. 5-HT2A receptor agonists such as 2,5-dimethoxy-4-iodoamphetamine (DOI) and lysergic acid diethylamide (LSD) stimulate head twitches in mice, which are not seen in 5-HT2AR null mutant mice. We find that DOI induces this stereotyped behavior in mice in a dose-dependent manner. At the molecular level, this drug activates expression of the immediate early genes *egr-1* and *c-fos* but not *egr-2* or *period-1* in auditory, visual and somatosensory cortices. In addition, 15 min exposure to DOI leads to c-jun phosphorylation in the somatosensory cortex and amygdala. Thus, in the absence of external acoustic and visual input, the hallucinogen DOI activates surrogate markers of neuronal activity in the sensory cortex and amygdala, which perform a primary role in the processing and memory of emotional reactions.

The second stage of the project involves our mouse model (Shi *et al*., 2003, 2005) that is based on the epidemiological finding that maternal infection increases the risk of schizophrenia in the offspring. We find that, compared to controls, the offspring of mothers whose immune systems were activated at mid-gestation show increased stereotypical behavioral responses (head-twitching, grooming and rearing) to DOI. The finding that these offspring are more sensitive to the hallucinogen raises the question of whether they also experience spontaneous, hallucination-like, neuronal activity.

82. Information processing in the hippocampus of the offspring of immune-activated mothers

Hiroshi Ito, Stephen Smith, Elaine Hsiao

Maternal immune activation by injection of the dsRNA, poly(I:C), in pregnant mice causes the offspring to display a series of behavioral abnormalities that are consistent with those seen in schizophrenia and autism. As abnormalities in the hippocampal network are one of the most consistent observations in the brains of schizophrenia patients, we examined the synaptic properties in the hippocampus of this mouse model *in vivo* and in acute brain slices. To assess hippocampal network function *in vivo*, we use expression of the immediate early gene, c-Fos, as a surrogate measure of neuronal activity. Compared to controls, the offspring of poly(I:C)-treated mothers display a distinct c-Fos expression pattern in area CA1 following novel object exposure. Because dopamine (DA) has a selective influence on object, rather than spatial, information processing in the hippocampus, we examined the effect of this neurotransmitter on temporoammonic-CA1 synaptic transmission in slices. Compared to controls, the offspring of poly(I:C)-treated mothers display a significantly increased sensitivity to DA in temporoammonic-CA1 synapses. There is also a reduced frequency and increased amplitude of miniature excitatory postsynaptic currents (mEPSCs) in CA1 pyramidal neurons. We do not find a significant difference in paired-pulse facilitation or LTP at Schaffer collateral-CA1 synapses. These various findings suggest that this animal model has altered object information processing. Indeed, the offspring of poly(I:C)-treated mothers display enhanced novelty discrimination in a novel object recognition paradigm. Thus, our analysis of object and spatial information processing at both synaptic and behavioral levels reveals a largely selective abnormality in object information processing in this mouse model of maternal immune activation.

83. The effect of maternal immune activation on the ultrasonic vocalizations of the neonatal offspring

Natalia Malkova, Paul H. Patterson

We are investigating the neurobehavioral development of mouse pups born to mothers whose immune systems were activated at mid-gestation. A behavioral assay that can be used very early in development involves ultrasonic vocalizations, which are important for mother–infant social interaction. We find that injection of double stranded RNA (poly(I:C)), which evokes an inflammatory response in the mother similar to that induced by influenza virus, alters the behavior of the offspring. Compared to controls, 10 day old C57BL/6J male pups born to mothers given poly(I:C) have lower rates of ultrasonic calling when separated from their nest. In addition, these pups produce shorter calls of higher frequency modulation. No difference is found in the physical abilities and general health between the control and experimental groups. Thus, the alteration in vocalizations of pups born to mothers with an activated immune system is not due to a delay in physical development. The absence of detectable differences in maternal responsiveness towards the pups also suggests that maternal care is normal and that the deficit in pup distress calls is the result of a reduced sensitivity to isolation or bonding with the parent.

84. Characterization of huntingtin fibrils by electron paramagnetic resonance

Charles W. Bugg, Paul H. Patterson, Ralf Langen[*]

Huntington's disease is a progressive, incurable neurodegenerative disease caused by an expansion in the polyglutamine tract in the protein Htt (huntingtin). Neurons in affected brain regions exhibit inclusion bodies that include Htt fibrils. Amyloid-like fibrils of several polyglutamine proteins, including Htt, exhibit a cross-β-pattern in X-ray diffraction studies. Much of the previous work has focused on polyglutamine peptides, and the exact structure of Htt fibrils is still unknown, although several structures have been proposed. We are characterizing Htt fibrils made *in vitro* using site-directed spin labeling, coupled with electron paramagnetic resonance (EPR). Mutant Htt exon 1 is purified and labeled with a paramagnetic reagent (MTSL) at various specific cysteines that have previously been introduced by site-directed mutagenesis. EPR spectra are collected before and after making fibrils. Protein EPR can give information about the environment around the paramagnetic reagent. The basic EPR spectrum gives information about side-chain mobility. Underlabeling experiments can reveal intermolecular distance constraints. Information about side chain exposure and secondary structure can be gleaned from power saturation EPR experiments using paramagnetic reagents that collide with MTSL. Using the data from these experiments, we will construct a model of huntingtin fibrillary structure.

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85. IKKα and IKKβ regulation of DNA damage-induced cleavage of Huntingtin

Ali Khoshnan, Jan Ko

Proteolysis of huntingtin (Htt) plays a key role in the pathogenesis of Huntington's disease (HD). N-terminal fragments of mutant Htt have the propensity to form oligomers and amyloid structures that are neurotoxic. However, the environmental cues and signaling pathways that regulate Htt proteolysis are poorly understood. We have discovered that treatment of neurons with the DNA damaging agent etoposide or γ-irradiation promotes cleavage of endogenous wild-type and mutant Htt, generating N-terminal fragments of 80-90 kDa. One prominent pathway induced by DNA damage and implicated in HD pathogenesis is the IκB kinase (IKK) complex. The complex has two kinases, IKKα and IKKβ, and a regulatory subunit IKKγ. HD patients have elevated levels of cytokines that are induced by IKK and our previous work showed that HD mice have persistent IKKβ/NF-κB activity in the CNS. Inhibition of IKKβ blocks the neurotoxicity of mutant Htt in an acute brain slice culture model of HD. We find that DNA damage-induced Htt cleavage requires IKKβ and is suppressed by IKKα. Elevated levels of IKKα, or inhibition of IKKβ expression by a specific small hairpin RNA (shRNA), or its activity by sodium salicylate, prevents Htt proteolysis and increases neuronal resistance to DNA damage. Moreover, IKKβ phosphorylates the anti-apoptotic protein Bcl-xL, a modification known to reduce Bcl-xL levels and activate caspases that can cleave Htt. Similar to silencing of IKKβ, increasing the level of Bcl-xL in neurons prevents etoposide-induced caspase activation and Htt proteolysis. These results indicate that DNA damage triggers cleavage of Htt, and identify IKKβ as a prominent regulator. Moreover, IKKβ-dependent reduction of Bcl-xL is important in this process. We are testing the hypothesis that reduction of IKKβ expression can ameliorate HD pathology. To this end, we have deleted its expression in the CNS and are crossing these knockout mice with an HD mouse model.

86. Regulation of MeCP2-mediated gene expression by IKKα

Ali Khoshnan, Paul H. Patterson

Homeostasis of methyl-CpG binding protein 2 (MeCP2) is critical for neuronal function and development. Mutations in the coding region of MeCP2 are responsible for most cases of Rett syndrome. The levels of MeCP2 are also reduced in the brains of a subset of idiopathic autism patients. MeCP2 is expressed at high levels in mature neurons and it is suggested to play a role in dendritic growth and synaptic plasticity. MeCP2 is known to bind to methylated CpG islands in the promoters of many genes, where it recruits a repressor complex to inhibit gene expression. However, recent studies indicate that MeCP2 is also a transcriptional activator. MeCP2 may influence the expression of up to 2,200 genes, including BDNF and CREB. The context and

environmental signals that regulate the transcriptional activity of MeCP2 are poorly understood. Phosphorylation of MeCP2 at several serine residues is important for its biological function. Activity-induced phosphorylation of MeCP2 at Ser 421 by calcium/calmodulin-dependent protein kinase II (CaMK-II) in neurons induces gene expression from several promoters including the exon-IV BDNF, enhances dendritic branching, and regulates complex behaviors like seizures and circadian rhythm in animal models. Ser 80 phosphorylation on the other hand, is essential for association of MecP2 with chromatin. It is notable that mass spectrometric analysis of MeCP2 isolated from brain reveals the presence of several phosphorylated sites, some which have not been characterized. Thus, phosphorylation-mediated regulation of MeCP2 function remains an exciting area of investigation. We have discovered that IκB kinase alpha (IKKα) regulates MeCP2 function and expression. IKKα binds and phosphorylates MeCP2 *in vitro*. Furthermore, IKKα promotes neurogenesis and neurite outgrowth, which coincides with the accumulation of MeCP2 and the induced expression of several gene products including BDNF. We are investigating whether IKKα-MeCP2 interaction is a molecular switch that controls the expression of MeCP2-regulated genes in differentiating neurons.

87. IKKα promotes the expression of brain-enriched miRNAs in human neurons

Ali Khoshnan, Paul H. Patterson

MicroRNAs (miRNAs) are ~22 nucleotide noncoding RNAs that play important roles in the posttranscriptional regulation of gene expression. miRNAs usually bind to the 3-UTR of mRNA and block translation, regulating many cellular functions including oncogenesis, neurodegeneration and neural stem cell differentiation. Several miRNAs including mir-7, mir-9, mir-124 and mir-132, are enriched in the CNS and regulate neurogenesis and dendritic growth. The underlying mechanism of miRNA expression in differentiating neurons is not well characterized. We are exploring the function of the IκB kinase complex (IKK) in neurons and how its activation by environmental stimuli can influence neuronal development and survival. We find that expression of an extra copy of IKKα in a human stem cell line, isolated from the midbrain of an eight weeks-old embryo, accelerates neuronal differentiation, promotes neurite sprouting and enhances the expression of brain-derived neurotrophic factor (BDNF). Microarray studies reveal that IKKα-induced neurogenesis overlaps with accumulation of brain-enriched miRNAs. Expression of some of the IKKα-induced miRNAs has been validated by qRT-PCR. It is interesting that IKKα promotes degradation of NRSE (neuron restrictive silencer element), which is known to inhibit the expression of certain miRNAs implicated in neurogenesis. Future studies will focus on understanding the mechanism of IKKα-induced miRNAs expression and its interaction with NRSE in human neurons. We are also examining whether IKKα influences the fate of neuronal stem cell differentiation. It is notable that expression of brain-enriched miRNAs is repressed in differentiating neurons treated with interleukin-6 (IL-6). In rodents, maternal exposure to elevated IL-6, as during infection, induces abnormal neurogenesis in the fetus and produces behavioral abnormalities resembling those in autism. We are characterizing the molecular dynamics of IL-6 in neural stem cell differentiation and how it may influence IKKα signaling to regulate miRNA expression and thus neurogenesis.

88. Exogenous LIF stimulates oligodendrocyte progenitor cell proliferation and remyelination

Benjamin E. Deverman, Sylvian Bauer, Paul H. Patterson

We have found that delivery of leukemia inhibitory factor (LIF) to the adult brain promotes neural stem cell self-renewal and stimulates oligodendrocyte progenitor cell (OPC) proliferation. Based on these data, we hypothesized that if exogenous LIF could enhance the OPC response in the context of chronic demyelination it may, in turn, promote the generation of new oligodendrocytes (OLs) and aid remyelination. To test this, we first feed mice a diet containing cuprizone, which induces demyelination in the corpus callosum (CC) and hippocampus, and then inject the mice with either a LIF- or lacZ-expressing adenovirus (Ad-LIF or Ad-lacZ) in the lateral ventricle. Using a combination of BrdU and retroviral labeling, we find that LIF significantly enhances OPC proliferation in the CC and hippocampus. These OPCs mature into OLs that contribute to remyelination. In the CA3 region of the hippocampus, spontaneous generation of new OLs and remyelination is minimal after a 12-week course of cuprizone, allowing us to assess the effects of LIF administration. After six weeks of Ad-LIF treatment, the number of OLs is restored to near normal levels, while in control mice the number of OLs fails to recover. Furthermore, compared to controls, remyelination in CA3 is significantly greater in Ad-LIF-treated mice, and immunohistochemical analysis suggests that nodal architecture is restored on a subset of these myelinated axons. In the converse experiment, we find that the generation of OLs is delayed in LIF KO mice as compared to WT mice following cuprizone treatment, confirming the importance of endogenous LIF during remyelination. Finally, we are breeding several lines of genetically-modified mice that will allow us to inducibly knockout a LIF receptor subunit in OPCs or more mature OLs. Using these mice, we will determine (i) whether LIF stimulates the proliferation of OPCs directly or through factors produced by intermediate cell types; and (ii) whether LIF also promotes OPC differentiation into mature OLs.

89. Intrabody gene therapy for Huntington's disease mice

Amber L. Southwell, Jan Ko, Paul H. Patterson

Huntington's disease (HD) is a neurodegenerative disease resulting from the expansion of a glutamine repeat in the huntingtin (Htt) protein. Transmission of the gene for the mutant Htt protein (mHtt) is autosomal dominant, allowing for pre-symptomatic diagnosis and potential treatment. Despite the simple genetic nature of HD, current therapies are directed at managing symptoms such as chorea and psychiatric disturbances. In an effort to develop a therapy directed at disease prevention we have investigated anti-Htt intrabodies that bind the regions flanking the glutamine expansion. We have previously shown that V_L12.3, an intrabody recognizing the N-terminus of Htt, and Happ1, an intrabody recognizing the proline-rich domain of Htt, reduce mHtt-induced toxicity and aggregation in cell culture and brain slice models of HD. Although V_L12.3 is more potent than Happ1, V_L12.3 also increases the level of nuclear Htt. Happ1 has no effect on Htt localization and also increases the turnover of mHtt. Due to the apparently different mechanisms of action of these two intrabodies, we have delivered both to the brains of HD model mice using a chimeric AAV2/1 viral vector with a modified CBA promoter. V_L12.3 treatment, while beneficial in a lentiviral model of HD, has no effect on the YAC128 HD model and actually increases severity of phenotype and mortality in the R6/2 HD model. In contrast, Happ1 treatment confers significant beneficial effects in assays of motor and cognitive deficits, as well as in the neuropathology found in the lentiviral, R6/2, N171-82Q, YAC128 and BACHD mouse models of HD. These results indicate that increasing the turnover of mHtt using AAV-Happ1 gene therapy represents a highly specific and effective treatment in diverse models of HD.

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90. Genetic and neural circuits that regulate sleep

A person can reject food, abstain from sex and control their thirst, but cannot keep from falling asleep. What is the genetic and neural basis of this most insistent bodily need? Why is it essential in nearly all multicellular animals, and how is it regulated?

Answers to these questions remain elusive despite years of study, and we are taking a new approach using zebrafish. Zebrafish are amenable to high-throughput genetic and small molecule screens that are powerful approaches to identify genetic pathways that regulate sleep. The optical transparency of zebrafish larvae makes them an ideal system to monitor and manipulate neural circuits that regulate sleep, and zebrafish and mammalian brains are structurally similar. Zebrafish are therefore uniquely suited to unravel the mysteries of sleep.

We are interested in two general questions: What are the genetic circuits that regulate sleep and what are the neural circuits that regulate sleep?

Genetic Regulation of Sleep: To test whether similar genetic pathways regulate zebrafish and mammalian sleep, we are studying Hypocretin/Orexin, whose loss causes the mammalian sleep disorder narcolepsy. We found that overexpression of zebrafish Hypocretin impairs the initiation and maintenance of sleep, consolidates wakefulness and induces a state of hyperarousal, as it does in mammals. We are now performing screens to identify novel genes and small molecules that regulate sleep, and are characterizing several candidate sleep regulators.

Neural Regulation of Sleep: Hypocretin is expressed in ~20 hypothalamic neurons in zebrafish larvae compared to 3,000 neurons in mice and 70,000 neurons in humans, making it much simpler to characterize the zebrafish Hypocretin circuit in detail. We found that zebrafish Hypocretin neurons project to wake-promoting centers of the brain and are active during periods of consolidated wakefulness, as in mammals.

We are now taking advantage of the optical transparency of zebrafish larvae, the relative simplicity of zebrafish neural circuits and recently developed optical tools to study the development and function of Hypocretin neurons, as well as other hypothalamic neural circuits that may regulate sleep. We are characterizing these neural circuits down to the level of individual neurons, testing the effects of activating and inhibiting specific neurons on behavior, and monitoring the activity of genetically-defined neurons in freely behaving larvae.

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Summary: Synapses, the points of contact and communication between neurons, can vary in their size, strength and number. The ability of synapses to change throughout the lifetime of the animal contributes to the 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 develop microfluidic platforms to optimize the stimulation and visualization of cell biological processes at synapses.

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.

A unique endeavor in the lab involves the recording of single neuron activities in the medial temporal lobe of human epilepsy patients. In these studies, we are able to correlate single neuron responses with behavioral experience and performance. These studies should elucidate some fundamental mechanisms of brain coding and representation. In addition, we conduct experiments in hippocampal slices to examine how activity and neuromodulators influence synaptic transmission and plasticity.

91. Regulation of mRNA stability in the hippocampus

Iván J. Cajigas, Erin M. Schuman

It is clear that *de novo* protein synthesis plays an important role in synaptic transmission and plasticity. A substantial amount of work has demonstrated that mRNA translation in the hippocampus is spatially controlled and that dendritic protein synthesis is required for different forms of long-term synaptic plasticity. Despite many recent observations that emphasize the importance of local protein synthesis in memory formation, little is known about the determinants that aid dendritic mRNA translation. In this work, we are testing the hypothesis that neuronal activity regulates mRNA stability in order to facilitate local protein synthesis. Using confocal microscopy and immunofluorescence experiments, we have gathered evidence showing that factors involved in modulating the half-life of mRNAs are present in dendrites of hippocampal neurons. In addition, we have data suggesting that application of the Brain-Derived Neurotrophic Factor (BDNF) to neurons triggers the relocalization of a subset of neuronal RNA binding proteins to synapses. In order to obtain additional insights on the role of mRNA stabilization in the hippocampus, we are currently developing techniques to identify and track the localization of RNAs in neurons. Specifically, we are taking advantage of the [3 + 2] copper-catalyzed cycloaddition (click chemistry) to label newly synthesized RNAs. Briefly, neurons are incubated with a uridine analog that contains an alkyne group. This modified uridine is incorporated into the RNA and after fixing the cells the alkyne group is reacted with an azide tag conjugated to Alexa 488. Using this technique, we have been able to show the formation of neuronal RNA granules in cultured hippocampal neurons. Our long-term goal is to visualize and identify the RNAs that are synthesized in response to application of different neuromodulators. Altogether, these results suggest that mRNA stability plays and important role in the hippocampus and that it can contribute to synaptic plasticity and memory processing.

92. Investigating synapse-to-nucleus-to-synapse signaling using a novel microfluidic perfusion chamber

Anne M. Taylor, Erin M. Schuman

Investigating signaling between distal synapses and the nucleus and how this leads to the accumulation of new proteins required at synapses during learning and memory has been particularly challenging to study due to the highly polarized nature of CNS neurons and the extreme density of synapses. We developed a novel microfluidic chamber to selectively manipulate discrete synaptic regions and visualize changes at the cell body and locally at synapses. Compartmentalized microfluidic chambers were used to direct the formation of synapses in isolated parallel arrays connecting two separate neuron populations. Using these platforms, we can manipulate pre- and post-synaptic populations of neurons, both genetically and pharmacologically. We added a microfluidic perfusion channel to perfuse neurotransmitters, drugs, and other soluble factors to distinct synaptic regions with spatial resolution as low as 10 μm and with good temporal resolution. We use this device to show that the metabotropic receptor agonist, DHPG, locally applied to synaptic regions leads to increases in ARC transcription and also increases in local ARC mRNA accumulation within the perfused dendrites, demonstrating that this microfluidic perfusion chamber is uniquely suited to investigate synapse-to-nucleus-to-synapse signaling.

93. Identifying and visualizing the hippocampal dopaminergic subproteome using BONCAT and FUNCAT

Jennifer J.L. Hodas, Daniela C. Dieterich[1], David A. Tirrell[2], Erin M. Schuman

Both synaptic and behavioral plasticity require new protein synthesis. Dopamine is a critical neuromodulator, and abnormalities in dopaminergic regulation underlie disorders like Parkinson's disease, Alzheimer's disease, and schizophrenia—diseases that impair the ability to form and retrieve memories. The stimulation of D1/D5 dopaminergic receptors in the hippocampus is thought to be critical for protein synthesis-dependent long-term potentiation (LTP), a process important for long-term synaptic plasticity and memory. Despite considerable effort, the proteins synthesized upon activation of dopaminergic pathways, the dopaminergic subproteome, are still largely unknown.

Using bioorthogonal noncanonical amino acid tagging (BONCAT), we are able to specifically identify components of the hippocampal dopaminergic proteome in an unbiased, non-toxic manner. Moreover, we utilize a related technique, fluorescent noncanonical amino acid tagging (FUNCAT), to visualize the effect of D1/D5 dopaminergic receptor stimulation in regulating global and local protein synthesis in the hippocampus. Both techniques employ a methionine surrogate, azidohomoalanine (AHA), which is conjugated to an azide-bearing tag via [3+2] copper-catalyzed click chemistry. Upon stimulation with a D1/D5 dopamine receptor-specific agonist, there are significantly increased levels of FUNCAT signal present in dendrites when compared to unstimulated dendrites. Since our interests also extend to dendritic protein synthesis, we have combined the use of FUNCAT with a Transwell culture system and have observed protein synthesis in both the somatic and dendritic compartments. Therefore, we demonstrate the application of BONCAT and FUNCAT to probe one of the protein synthesis-dependent functions in the hippocampus.

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94. Calcium-dependent dynamics of cadherin interactions at synapses and cell-cell junctions

S.A. Kim, C.-Y. Tai, L.-P. Mok, E.A. Mosser[*], Erin M. Schuman

Cadherins, a family of homophilic adhesion proteins, play an important role in the calcium-dependent dynamics of structural and synaptic plasticity along with cell-cell contact formation and remodeling. A unique feature of cadherin-cadherin interactions is a strong dependence on extracellular Ca^{2+}—which serves to rigidify the molecular structure of cadherins and promote trans-junctional interactions. Fluctuations in extracellular Ca^{2+} could thus, in principle, modify the dynamics of cadherin homophilic interactions across junctions, but this remains unexplored. We have developed a genetically encoded Förster Resonance Energy Transfer (FRET) reporter system that permits the direct visualization and quantification of spatiotemporal dynamics of N-cadherin interactions across intercellular junctions in live cells. We found that upon rapid chelation of extracellular Ca^{2+} N-cadherin exhibited a sudden, but partial, loss of homophilic interactions. A cadherin mutant that lacks adhesive activity (W2A) exhibited a more substantial loss of homophilic interactions, suggesting two types of cadherin interactions—one that is rapidly modulated by changes in extracellular Ca^{2+} and another with relatively stable adhesive activity that is Ca^{2+}-independent. These data indicate a previously unrecognized sensitivity of cadherin molecules to fluctuations in extracellular Ca^{2+}. To transfer our FRET reporter system into neurons, N-cadherin constructs are expressed using viral gene delivery such that a FRET donor (cerulean-cadherin) is expressed in presynaptic neurons while a FRET acceptor (venus-cadherin) is expressed in postsynaptic neurons. FRET will then be used to detect cadherin interactions between pre- and postsynaptic cells under various conditions. In particular, we will examine the effects of synaptic activity and varying extracellular calcium concentrations on cadherin-cadherin dynamics. These experiments will test our hypothesis that cadherins act as extracellular calcium detection system to coordinate synaptic plasticity across the synapse.

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95. Visualization of dynamic 80S ribosome formation by inter-subunit FRET in living cells

Young J. Yoon, Elisabeth Wang, Michael Zobel[*], *Erin M. Schuman*

The ribosome is a large molecular machine that is responsible for synthesis of proteins in all organisms. During translation, the large and small subunits of the ribosome assemble to catalyze peptide-chain elongation. Observations of assembled ribosomes or polysomes have long been the hallmark of active protein synthesis within cells. In addition, characterization of changes in ribosome profiles has been a focus of cell biology for many years. To monitor ribosome assembly and dynamics we used the structural information available for the eukaryotic 80S ribosome to optimize the position of fluorescent protein labels within the small and large subunit ribosomal proteins (RP). In this system, the assembly of the 80S ribosome would result in an increase in the proximity of fluorescently-labeled RPs. Taking advantage of this feature, we labeled a pair of RPs and employed inter-subunit Förster resonance energy transfer (FRET) by acceptor photobleaching to assess 80S formation in heterologous cells. As proof of principle, the FRET assay was able to demonstrate 80S formation. Moreover, we show that our fluorescently-labeled RPs incorporate into ribosomal subunits and localize to proper subcellular compartments. Applications for visualizing dynamic 80S ribosome formation by inter-subunit FRET in living cells can be extended to a morphologically polarized cell, such as a neuron. Local protein synthesis in dendrites and synapses of neurons is important for certain types of synaptic plasticity. Furthermore, electron microscopy (EM) observations indicate that the number of polysomes increased in synaptic spines after stimulation. Our ability to monitor local changes in 80S ribosome formation over time will allow us to address many of the outstanding questions regarding ribosome dynamics during synaptic plasticity.

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96. Is telencephalon the mirror to the zebrafish mind?

Mark Aizenberg, Erin M. Schuman

There is ample evidence that various cognitive functions, including learning and memory, depend on coordinated activity of large networks of neurons, sometimes in distant brain areas. However, the understanding of brain's activity is challenged by enormous complexity of neural circuitry and restricted ability to access activity of large neuronal populations. Zebrafish is emerging as animal model particularly amenable to bypass those problems, as zebrafish juveniles possess a rich behavioral repertoire and a relatively simple brain. Importantly, larvae are transparent, which makes possible visual monitoring of neural activity. In this study, we employed *in vivo* confocal calcium imaging of the telencephalon of 5-8 days-old larvae to study how sensory inputs of different modalities converge to form higher order representations. We demonstrated that light and touch evoke activity of different subsets of the cells in telencephalon with a little overlap. Also, light stimuli with different spatiotemporal pattern evoke activity of different sub-populations of the cells. Additionally, we combined confocal imaging with high-speed video recording of the fish tail. Using this technique we found that ultra-slow network oscillations in the telencephalon are correlated with locomotor activity of the fish. Finally, we developed a classical conditioning paradigm in the restrained zebrafish, which allowed us to monitor Ca^{2+} activity in the brain during learning. Using a moving light spot as the conditioned stimulus (CS) and a mild touch as unconditioned stimulus (US) we demonstrated that larval zebrafish alter its behavioral response to the CS following learning. This work provides a rare opportunity to study how dynamic patterns of activity in neural networks in higher brain areas contribute to animal learning and memory.

97. Fluorescent non-canonical amino acid tagging in larval zebrafish

Flora I. Hinz, Daniela C. Dieterich[1], *Jennifer J.L. Hodas, David A. Tirrell*[2], *Erin M. Schuman*

Recently, the introduction of small bio-orthogonal functional groups, such as azides and ketones, into proteins, glycans and lipids by using their own biosynthetic machinery has been developed as a way to tag these molecules in cells. Bio-orthogonal Non-Canonical Amino Acid Tagging (BONCAT) and Fluorescent Non-Canonical Amino Acid Tagging (FUNCAT) are two such methods. BONCAT and FUNCAT utilize non-canonical amino acids such as Azidohomoalanine (AHA), which can be used as a surrogate for methionine by methionyl tRNA synthetase (MRS), to introduce new functional groups, in this case an azide group, into newly synthesized proteins in cells. These azide groups can be used to tag proteins with either a biotin-alkyne tag or a TexasRed-alkyne tag, by selective Cu(I)-catalyzed azide-alkyne ligation. Biotin tagged proteins can then be separated from the preexisting proteome by affinity purification and identified by tandem mass spectrometry, while the TexasRed-alkyne tag enables visualization of tagged proteins. Azides and alkynes are essentially absent from vertebrate cells, which makes this azide-alkyne ligation ('click chemistry') very selective. FUNCAT has been successfully used in dissociated hippocampal culture, organotypic hippocampal slices and acute hippocampal slices. However, the present study using larval zebrafish is the first *in vivo* demonstration of FUNCAT. We have shown that newly synthesized proteins can be specifically fluorescently labeled in intact larval zebrafish nervous system after a four-hour incubation with AHA.

The BONCAT / FUNCAT techniques have the advantage that they can also be genetically restricted to different cell groups within a population. Instead of AHA as a non-canonical surrogate for methionine, Azidonorleucine (ANL), a different azide bearing non-canonical amino acid can be used for the BONCAT /

FUNCAT reaction. However, its' azide bearing side-chain is too bulky to fit into the binding pocket of wild-type MRS and can therefore, not be charged onto Met-tRNA in wild-type cells. The Tirrell group, using a high-throughput screening approach, have identified three specific mutations in MRS, which enable charging of the non-canonical amino acid ANL onto mutated Met-tRNA in *E. coli*. This enables incorporation of ANL into proteins only in cells which express this mutated MRS. As an extension to this study, we have introduce these same mutations into the *D. rerio* MRS to enable ANL charging onto Met-tRNA and thereby allow incorporation of this non-canonical amino acid into newly synthesized proteins. We have transfected a GFP fusion construct into COS7 cells to demonstrate that the *D. rerio* mutant MRS is capable of charging ANL onto Met-tRNA. This approach may enable localization of the FUNCAT and BONCAT techniques to specific cell populations in *D. rerio in vivo*. In future, such methods could possibly be paired with a conditioning paradigm to elucidate what neurons and neuronal circuits underlie the formation of a specific memory in larval zebrafish.

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98. Functional division of hippocampal area CA1 via modulatory gating of entorhinal cortical inputs

Hiroshi Ito, Erin M. Schuman

Many studies have indicated that the hippocampus is important for establishing associative memories that involve an animal's environment, space or context. The hippocampus indeed receives two streams of information, spatial and nonspatial, via major afferent inputs from the medial entorhinal cortex (MEC) and the lateral entorhinal cortex (LEC). The MEC and LEC projections in the temporoammonic (TA) pathway are topographically organized along the transverse-axis of area CA1. The potential for functional segregation of area CA1, however, remains relatively unexplored. Here, we demonstrated differential novelty-induced c-Fos expression along the transverse-axis of area CA1 corresponding to the topographic projections of MEC and LEC inputs. In hippocampal slices, we found distinct presynaptic properties between LEC and MEC terminals, and application of either DA or NE produced a largely selective influence on one set of inputs (LEC) and differentially modulated frequency-dependent signal transmission and synaptic plasticity. Finally, we demonstrated that differential c-Fos expression along the transverse-axis of area CA1 was largely abolished by an antagonist of neuromodulatory receptors, clozepine. Our results suggest that differential neuromodulator-mediated control in topographic TA projections allows for the hippocampus to independently control spatial and nonspatial information processing, which may provide a unique role of the TA pathway in hippocampal function.

99. Characterization of the brain proteasome and its interacting proteins

Hwan-Ching Tai, Henrike Besche[*], Alfred L. Goldberg[*], Erin M. Schuman

We are interested in studying protein degradation in the neuron because it plays important roles in modifying synaptic components and contributes to synaptic plasticity. The majority of intracellular proteins in the cell are degraded by the ubiquitin-proteasome system, but the molecular mechanism of proteasome-mediated proteolysis at the synapse is not well understood. Therefore, we biochemically isolated synaptosomes from rat cortices and applied a newly developed method[1] to isolate its proteasome content. For comparison, we also purified cytosolic proteasomes from the cortex. By mass spectrometric analysis, we found many factors associated with the proteasome complex. Proteasome interacting factors are likely to function in substrate handling and proteasome regulation. There are differences in the complement of proteasome-interacting proteins between the cytosolic and synaptic compartments of the neuron, and between the brain and other tissues. For instance, ECM29, a 26S proteasome stabilization factor, is only found in cytosolic proteasomes. Synapse-only factors include TAX1BP1, a ubiquitin binding protein, and SNAP-25, a synaptic vesicle protein

We also observed that proteasome levels and its interactors can be dynamically regulated by neuronal activity. Following transient NMDA stimulation, neuronal 26S proteasomes dissociate into 20S core particles and 19S regulatory particles, and the latter gets degraded. Two E3 ligases associated with proteasomes also become degraded, UBE3A/E6-AP and HUWE1/ARF-BP1. Interestingly, both E3s have been linked to human genetic disorders associated with mental retardation. But little is understood about their roles in synaptic plasticity and learning. Our study reveals that neuronal proteasome regulation is much more complex than previously assumed. Further work is required to elucidate how it is involved in facilitating proteomic changes and maintaining proteome homeostasis under different physiological conditions.

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100. Spike-field coherence correlates of memory encoding in the human medial temporal lobe

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The medial temporal lobe (MTL) is required for the acquisition of episodic memories. At the level of single neurons, however, it is unclear what sequence of events leads to the storage of a new memory. In particular, it is unclear whether mechanisms that induce synaptic plasticity *in vitro* and *in vivo* are utilized at the level of populations of neurons to encode a new memory. We simultaneously recorded single-unit activity and the local field potential (LFP) from the amygdala and hippocampus from the MTL of epileptic patients, who had electrodes implanted for the purpose of localizing seizures. Patients viewed a sequence of unique images. In a later recognition

memory test, patients indicated whether they had seen the image before or not on a 6-point confidence scale. Patients had good memory and a good sense of confidence. Here, we focus on neural activity during learning. We observed that a substantial fraction of single units (20-30%) fired action potentials that were phase-locked to the theta band of the LFP. Further, the spectrum of the spike-triggered LFP showed a prominent peak in the 3-8Hz range. Can differential phase locking explain why LFP power differences are a good indicator of memory formation? The spike-field coherence (SFC) computed for single units showed significant peaks in the SFC for theta and lower frequencies. The amplitude of the SFC was modulated by memory strength - subsequently, remembered stimuli exhibited a larger SFC. An increased SFC could be observed even for units that do not respond to the stimulus by changing their firing rate. The increased SFC cannot be attributed to increased LFP power or changes in firing rate. This indicates that tight coordination between stimulus-evoked neuronal firing and the activity of the local population can predict successful memory formation.

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Summary: While we continue to examine the dynamic/adaptive nature of human visual perception – including its crossmodal, representational, sensory-motor, developmental, emotional, and neurophysiological aspects - we put more emphasis on the ERATO (Exploratory Research for Advanced Technology) Shimojo "Implicit Brain Functions" project (supported by JST, Japan Science and Technology Corporation, officially started in October '04), with its emphasis on implicit cognitive processes, emotional decision making, and their neural correlates. Vigorous collaborations are conducted among our psychophysics laboratory here and the ERATO Japan site located at NTT Communication Science Laboratories, Atsugi, Kanagawa, Japan, as well as Harvard MGH, Boston University, Gordon College London, National Academy of Science Austria, and Decode Inc., Germany. Besides, we just started new collaborative efforts on "social brain," under the Caltech-Tamagawa gCOE (grand Center of Excellence) program (supported by MECSST, Ministry of Education, Culture, Sports, Science and Technology, Japan, started in Sept '08).

Using a variety of methods including eye tracking, high-density EEG, fMRI and MEG, we examine how exactly peripheral sensory stimuli, neural activity in the sensory cortex, and the mental experience of perception are related to each other. As for objectives of the ERATO project, we aim to understand implicit, as opposed to explicit or conscious, somatic and neural processes that lead to, and thus, predict conscious emotional decision such as preference. As a more overarching general theme including both the perceptual psychophysics and the ERATO studies of emotional decision making, we are in particular interested in the intriguing interactions between *predictive* processes (prior to and thus, predicting the mental event or behavior) and *postdictive* processes (posterior) to understand conscious experience of perception, sensory-motor learning, memory, and emotional decision modulated by internal and external factors. The new gCOE program put additional scientific emphases on social, emotional, and reward-related neural circuits. Toward these ends, we employ interpersonal EEG recording, TMS/tDCS stimulation, and fMRI-base neural feedback method.

In extensions of our core interest in visual preference decision making, we collaborated with neuroeconomists on campus to investigate neural correlates of choice decision in more realistic real-world situations (such as purchasing snacks).

(1) We continue our work applying TMS (Transcranial Magnetic Stimulation) to the visual cortex of alert normal subjects, to reveal neural mechanisms underlying conscious visual perceptual experience. In our earlier finding of TMS-triggered "visual replay" effect, we had demonstrated that when a simple visual stimulus is presented followed by a dual-pulse TMS (which typically yields an illusory light field in the contralateral visual field, called "phosphene," the stimulus "reappears" perceptually, mainly within the area of phosphene. In a subsequent study, we found that: (a) natural scenes often generate a vivid perceptual replay; (b) the phenomenology of replay varies across observers, from a photopic to filling-in-like to contours alone; and (c) the replay often goes beyond the spatial range of phosphene (e.g., bilateral, symmetrical percepts). The results revealed new aspects of visual cortical traces and dynamics that lead to conscious perception. Later, we also found that after repeated paired presentation of the visual transient stimulus and the dual-pulse TMS (say ten trials or more), the TMS alone can trigger the replay without visual stimulation ("entrainment effect"). This paired association effect was sensitive to the temporal delay between the visual stimulus and TMS in the pairing phase. Also after various attempts, we finally found firm evidence that the replay is *not* just a "phenomenological illusion" but rather executing real effects on visual information encoding. To prove this, we used a variation of forward masking paradigm in which the visual mask itself has no power of suppressing the target, but the "replay" of the mask did.

(2) Emotional decision making has been the central focus of our ERATO project (JST.ERATO Shimojo Implicit Brain Function Project). We have investigated behavioral and neural correlates of such preference decision in both the human and the monkey. In particular, we addressed the issue of memory-preference relationship, in order to resolve the seeming conflict

between the Familiarity and the Novelty principles in the literature. Surprisingly, we originally found a segregation between these two principles across object categories - the Familiarity dominates across trials in face preference, whereas the Novelty dominates in natural scene preference. Our subsequent studies suggested that similarity/variance within category and task matters to the preference. Most recently, we made a few significant contributions: (a) In hybrid stimuli in which a central face was surrounded by a natural scene background, <a familiar(old) face + a novel(new) scene> combination was rated as most attractive on average, as predicted by the previous results. However, this was only in the case when the subject was asked to evaluate attractiveness of the overall image. When asked to evaluate the face only neglecting the background, surprising non-linear interactions occurred. Some interactions between implicit (ground) and explicit (figure) are suggested; (b) Initial gaze was recorded and correlated with old/new of the stimulus and the final preference decision. In the case of natural scenes, initial gaze tended to go to the novel stimulus, and to be correlated with the final choice. In the case of faces, however, the results were less straightforward in which the initial gaze pattern was dominated by Novelty whereas later gaze pattern was dominated more by Familiarity from previous experience; (c) We came up with a descriptive model and a review, in which preference is not inconsistent with the classical concept of "utility" and (d) We performed an fMRI study to identify neural correlates of Familiarity and Novelty preference in each object category. As a result, distinct brain regions were found to be associated with familiarity preference for FC (e.g., DLPFC, vACC) and novelty preference for NS (e.g., FG). We also noticed that choice history modulated the brain activity. These results suggest that there exist separate neural mechanisms involved in familiarity and novelty preference and their recruitment depends on the object category.

(3) To see if seeing itself is rewarding even in the monkey, we recorded the OFC (Orbito-frontal cortex) neurons' activity via multiple-electrodes, while the monkey was engaged in either the classical reward task (i.e., visual discrimination for juice reward) or just watching videos without juice reward. We found three types of reward-related neurons, those who responded only to the juice, those responded only to the videos, and those responded to both. Recently, we also found neurons that are specifically responding immediately after spontaneously switching from one kind of drink reward to the other ("switch neurons").

(4) Animal and human behavior can be modified by a procedure called Instrumental conditioning. We aimed to extend this traditional approach in order to directly shape neural activity instead of overt behavior. Using real-time fMRI feedback, we were previously successful in conditioning hand- and foot-movement related areas in the human motor cortex separately. More recently, we were also successful in modifying the OFC activity, albeit transiently. We further demonstrated that when the OFC activity is elevated by the feedback, attractiveness of face was evaluated higher.

(5) We earlier reported an intriguing auditory-visual effect called the "double flash" illusion (*Nature*, '09). In order to examine the degree to which this cross-modal effect is immune to top-down control and motivation, we manipulated feedback and monetary reward for "correct" answer (in the physical sense). The results overall demonstrated a surprising degree of robustness of the illusion, indicating earlier and bottom-up nature of it.

(6) We have been investigating how the probability and the reward structures affect arm-reaching tasks. The results suggest that as the difference in value between the targets increases, subjects' biases also increase. Moreover, changes in biases across trials are better predicted by recent experience, rather than global experience. This suggests that people learn value structure through recent experience, and this knowledge is used to guide reach planning.

(7) We reported a temporal "blind spots" in the human visual system, employing a gap detection paradigm with a long-lasting visual stimulus. The "blindness" in the gap detection turned out to be sensitive to the timing of the gap with regard to the onset of the visual stimulus.

Although not published or reported yet, we are conducting a project that has its focus on interactions between two brains and bodies. For this we came up with original psychophysical tasks in which implicit synchrony of bodily movements between the two players affect the performance *against* instructions/tasks. We have already obtained some intriguing behavioral findings, and currently collecting high-density EEG data from the players' brains simultaneously.

101. A functional explanation for the effects of visual exposure on preference

Mark Changizi[*†], *Shinsuke Shimojo*

Visual exposure to an object can modulate an observer's degree of preference for it, initially enhancing preference (a "familiarity preference" regime), and eventually lowering it again (a "novelty preference" regime). Here we investigate whether there may be a functional advantage to modulating preference in this way. We put forth the simple hypothesis that degree of preference for an object of type X is the brain's estimate of the expected value of acting to obtain X. In light of this view of what preferences fundamentally represent, we are able to explain the "exposure effect" and many of the connected phenomena.

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102. Linking memory to preference: an fMRI study of familiarity and novelty principles

Junghyun Park, Hackjin Kim[*], Shinsuke Shimojo

Preference decision is largely based on past experience, but exactly how remains elusive. Indeed, there are evidences for the two apparently incompatible principles, familiarity and novelty. Recently we showed (VSS'07) a clear segregation of the two principles across object categories: familiarity preference gradually increased in faces (FC) as a function of exposure, while novelty preference was dominant in natural scenes (NS).

There have been considerable studies on the neural basis of either memory or preference decision making, but few attempts were made to link them. In the current study, we looked for neural correlates of familiarity and novelty principles for preference, to see if there exists a unitary mechanism, which responds differentially depending on the object category, or two separate mechanisms specialized for each. While scanned with fMRI, 17 subjects judged relative preference between a pair of images (FC or NS) presented sequentially. Within a block of 20 trials, one image was repeatedly presented and paired with a new image. Thus, throughout a block, comparisons were always between familiar (F) and novel (N) images (except for the 1st trial) with increasing number of exposure of F toward the end of the block.

FCs and NSs were tested in separate sessions. Behaviorally, the opposite effects of repetition for different object categories were replicated: repetition of FC and NS resulted in more and less preference, respectively. When F was simply contrasted with N without considering subjects' choice, there was a significant overlap between activated areas in FC and NS conditions, suggesting that the same memory system may be involved in the representation of familiarity and novelty for images of different categories. To examine how experience (familiarity/novelty) interacts with preference decision, we grouped 20 trials in each block into four levels of repetition and searched for brain areas significantly activated by the interaction between F-N and choice behavior in four levels of repetition. Distinct brain regions were found to be associated with familiarity preference for FC (e.g., DLPFC, vACC) and novelty preference for NS (e.g., FG). We also analyzed the data in terms of choice history: switch (SW) or No-switch (NS). When F was chosen in a trial, grater activation was found if F was also chosen in the preceding trial (NS) than when the previous choice was N (SW). In contrast, when N was the current choice, greater activation was observed if the previous choice was F (SW) rather than N (NS). All these findings suggest that there exist separate neural mechanisms involved in familiarity and novelty preference and their recruitment depends on the object category.

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103. Temporal blind spots in the human visual system

Yasuki Noguchi, Shinsuke Shimojo

Images presented at a specific region of the visual field cannot be perceived monocularly, due to the lack of photoreceptors on the retina (blind spot). Here we report that our visual system has a similar kind of local weakness also in the temporal domain. Subjects were presented with a long-lasting visual stimulus (a bright ring on the black background) and judged whether or not it had a brief disappearance (gap) embedded in a random timing after the stimulus onset. Although the duration of the gap was always constant, its detection rate transiently decreased when the gap was introduced at several specific timings after the onset. This temporally limited impairment in detecting the gap was not observed when subjects reported an increase in luminance (not disappearance) of the same stimulus. These results altogether indicate that our visual system has several 'insensitive' periods after the stimulus onset that could selectively work for the decreasing brightness. Although this insensitivity to the decrements would be normally advantageous to maintain the constant representations of the long-lasting stimuli while resisting a rapid-adapting characteristic of neural activity, it also makes us blind to the physical decrement of the luminance if the decrement is synchronized with those insensitive periods.

104. Learning probability and reward through experience: Differences in behavior and neural correlates of value resulting from expertise

Erick J. Schlicht[1], Alice Lin[2], Antonio A. Rangel[3], Colin Camerer[3], Shinsuke Shimojo, K. Nakayama[1]

Throughout our everyday routine we must make actions in the face of uncertainty. From a decision theoretic standpoint, optimal actions are those that maximize the value associated with the task. However, in order for humans to act optimally, it necessitates the brain has an accurate representation of both the reward and probability associated with each outcome. Previous research investigating how humans use value structure to perform reaching movements has exclusively focused on asymptotic performance, ignoring how this structure is learned. Therefore, the first portion of this talk will outline work investigating how target value is learned to use for reach planning. The results show that as the difference in value between the targets increases, subjects' biases also increase. Moreover, changes in biases across trials are better predicted by recent experience, rather than global experience. This suggests that people learn value structure through recent experience, and this knowledge is used to guide reach planning. The second portion of this talk will explore differences in brain activity due to expertise when computing value in gambling tasks. We compared brain activity of poker experts and novices when making gambling decisions in both poker and lottery tasks. Although both tasks are statistically identical, we found

bilateral activation in the Insula that is correlated with value to be greater in the poker task for poker experts, but in poker novices, bilateral Inslua activation is greater for the lottery task. These results suggest that expertise influences how the brain codes and uses value information in decision theoretic tasks. Overall, this work demonstrates the importance of experience in how our brain uses value to guide actions and decisions.

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[Lecture, ERATO Meeting, Shuzenji, Japan, July.]

105. Transcranial magnetic stimulation (TMS) consolidates and retrieves a percept from short term memory

Hsin-I Liao[1,2], Daw-An Wu[3], Hsiu-Yu Yeh[2], Shinsuke Shimojo[1†]

Transcranial magnetic stimulation (TMS) to visual cortex dynamically interacts with retinal input and thus, alters conscious perceptual experience. When a visual stimulus is presented and followed by a dual-pulse TMS, an "instant replay" of the visual stimulus is observed (Halelamien, *et al*., VSS '07; Wu *et al*., VSS '01, '02, '04). While following this finding, we found that repeatedly pairing the same visual stimulus with TMS can lead to a state where the replay percept can be retrieved by TMS alone without any visual stimulus presentation.

The experiment consisted of two phases, the training phase and the test phase. In the training phase, a geometric pattern (a disk or a line) was presented for 100 ms, followed by a dual-pulse TMS (50 ms inter-pulse interval) with 300 ms delay. After repeated trials (10 trials or more), TMS alone was delivered without any visual stimulus presentation (the test phase). The replay percept was often retrieved in the area of TMS-triggered phosphene. Among 18 subjects we have tested, five reported no replay percept either in the training phase or in the test phase; another four reported a replay percept only in the training phase; the remaining nine reported a replay percept both in the training phase AND in the test phase. Increasing the delay between visual stimulus and TMS during the training phase substantially weakened the vividness of the replay percept, with complete abolition at 3 sec. in most subjects. In such cases, no replay was seen in the test phase, either.

That the replay precept can be entrained and then retrieved by TMS alone not only provides evidence for the existence of a neural representation for the "replay," but also implies a Hebbian-like associative learning mechanism that can be a basis of conscious perceptual experience.

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[Poster presented at ASSC (Association for the Scientific Study of Consciousness), 12th Annual Meeting, Taipei, Taiwan.]

106. Retrieval of visual percept by paired association of a visual stimulus and transcranial magnetic stimulation (TMS): Effect of TMS delay

Hsin-I Liao[1,2], Shinsuke Shimojo[1†]

TMS to visual cortex dynamically interacts with retinal input, altering conscious perception. When a brief visual stimulus is followed by a dual-pulse TMS, an "instant replay" of the percept could be observed. After repeating the pairs, the replay percept could be retrieved even by TMS alone without accompanying visual stimulus (Liao *et al*., ASSC '08). We manipulated the delay between the visual stimulus and TMS to further investigate how the paired association leads to the TMS-triggered perceptual retrieval. When the delay increased to 3 sec., the replay was no longer observed, nor the retrieval when TMS alone was delivered. It implies that pairing the visual stimulus and TMS without inducing the replay is not sufficient to cause the retrieval. The most vivid replay and the retrieval were both observed with delay of 300 ms. It may be expected from "cortical coincidence," considering the neural conductance delay from retina plus cortical processing time required for conscious perception. In line with it, we also found a positive correlation between the strength of replay and that of retrieval later. The replay percept may be directly consolidated and then retrieved. Alternatively, there may be a common mechanism underlying the replay and the retrieval.

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107. Gaze orienting, and novelty vs. familiarity preference

Hsin-I Liao[1], Junghyun Park, Eiko Shimojo[2†], Junko Ishizaki[3], Su-Ling Yeh[1], Shinsuke Shimojo[2†]

We have shown a segregation of memory effect on preference choice between object categories: familiarity preference for faces and novelty preference for natural scenes (Shimojo *et al*., '07, '08; Liao and Shimojo, '08 VSS). It has also been shown that in preference choice, the gaze is biased towards the picture that will be chosen, called "gaze cascade effect" (Shimojo *et al.*, '03 *Nat. Neurosci*.). Considering that novelty elicits orienting reflex (van Olst, '71), we aim to see whether gaze orienting is directed to novel pictures initially, and whether the initial gaze bias interacts with the later gaze cascade effect between object categories and choices (familiar or novel stimulus). Eye movements were recorded while the subject performed a two-alternative force-choice (2AFC) preference task between an old and a new pictures. The

picture that was pre-rated as median attractive was used as the old picture and paired with a new picture in each trial. The results showed that as the trial repeated, the old face was chosen more frequently whereas the new natural scenes were more preferred, replicating our previous findings. Eye movement data showed a general initial gaze bias towards novel pictures, especially for natural scenes, but later on, the gaze patterns differed. For faces, the gaze bias reversed towards familiar pictures to overcome the initial novelty bias in most trials. For natural scenes, on the other hand, the gaze remained on the new picture to bias further towards the final choice. These results suggest that initial gaze bias towards novel pictures contributes to the preference for novelty, but not directly to the preference for familiarity. Indeed, the initial gaze towards novel stimulus is more pronounced in the trials with a final novelty preference for natural scenes, but not for faces.

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108. Visual replay effect: Objective evidence from a masking paradigm

Harish Vasudevan, Neil Halelamien, Shinsuke Shimojo

Transcranial magnetic stimulation (TMS) is a non-invasive method employing a magnetic coil to generate an electrical signal in the human brain. When applied directly to the visual cortex, TMS induces either scotomas (blind spots) or phosphenes (flashes of light) depending on coil type and stimulation conditions. Interestingly, if TMS is applied at a particular timing after a visual stimulus, an "instant replay" of the original stimulus is perceived, typically within the phosphene area (Halelamien, *et al.*, VSS 2007). This replay effect potentially offers unique insight into the formation and storage of mental representations. To obtain objective evidence of replay's perception and determine if replay shares the same early perceptual circuit as retinally triggered stimuli, we use a masking paradigm to examine replay's spatial and temporal specificity. Subjects were presented a physical mask followed by a letter, which they were asked to report. In the TMS condition, a double pulse of TMS was administered after mask presentation to facilitate "replay" of the mask, obscuring letter detection. The timing was carefully selected such that there was no direct forward masking by the physical mask but a potential masking effect by the replayed mask. Three conditions were tested with and without TMS: 1) no physical mask presented; 2) physical mask and letter in different positions; and 3) physical mask and letter in the same position. We found that the replay effect was most pronounced when the delay between mask and TMS pulse was 150-250 ms. Further, our data shows statistically significant performance degradation with the addition of TMS only when the physical mask and letter are presented in the same visual location, suggesting a replay-induced masking effect. These results provide evidence for the ability of TMS induced replay to interact with the same perceptual circuits as retinal input in a spatiotopically-organized manner.

109. Integration of attractiveness across object categories and figure/ground

Eiko Shimojo[†], Junghyun Park, Shinsuke Shimojo*

When a 2AFC preference task is performed within the same object category, face (FC) or natural scene (NS), a distinctive segregation occurs – a familiar FC is chosen more than a novel FC across trials, whereas a novel NS tends to be chosen more than a familiar NS (Shimojo *et al.*, VSS'07, '08). How would the preference choice change if a FC is presented with a surrounding NS (just like a typical commercial advertisement)? Thus, we combined a central FC [old (o) or new (n)] and a surrounding NS (o or n) as an integrated single stimulus, for which subjects were asked to rate attractiveness in a 7-point scale. We selected 21 pictures, excluding too high- and too low-rated, from a pre-rated set in each category and used the 11th as a repeating (o) stimulus. There were two experiments with different instructions: 1) Rate the attractiveness of the entire picture; or 2) Rate the central face only, neglecting the surrounding. The results of Exp 1 clearly betrayed the prediction by simple summation of attractiveness across objects where the FCo-NSn combination would have the highest slope of accumulated attractiveness. Instead, the results were: FCn-NSo > FCo-NSn > FCo-NSo = FCn-NSn in the order of slope of the rating X trial number plot. The results of Exp 2 were m0ore consistent with the simple summation model: FCo-NSn = FCn-NSn > FCo-NSo > FCn-NSo. The 1) results suggest that attractiveness is integrated in a non-additive way across object categories and figure/ground. The 2) results can be accounted for by two factors; Familiarity preference in FC at an explicit level, and novelty preference in NS at an implicit level. These results together indicate a high degree of nonlinearity and implicit processing in memory-based attractiveness integration.

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Associate Professor of Computation and Neural Systems, Bren Scholar: Athanassios G. Siapas
Postdoctoral Scholars: Evgueniy Lubenov, Laurent Moreaux, Casimir Wierzynski
Graduate Students: Ming Gu, Andreas Hoenselaar

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Summary: Our research focuses on the study of learning and memory formation in freely behaving animals at the level of networks of neurons. Previous research has shown that the hippocampus is critical for the formation of long-term declarative memories, and that this hippocampal involvement is time-limited. The current predominant conjecture is that memories are gradually established across distributed neocortical 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. 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.

Our research efforts focus on analyzing the structure of cortico-hippocampal interactions in the different brain states and on characterizing how this structure is modulated by behavior; how it evolves throughout the learning process; and what it reflects about the intrinsic organization of memory processing at the level of networks of neurons.

Our experimental work is complemented by theoretical studies of network models and the development of tools for the analysis of multi-neuronal data.

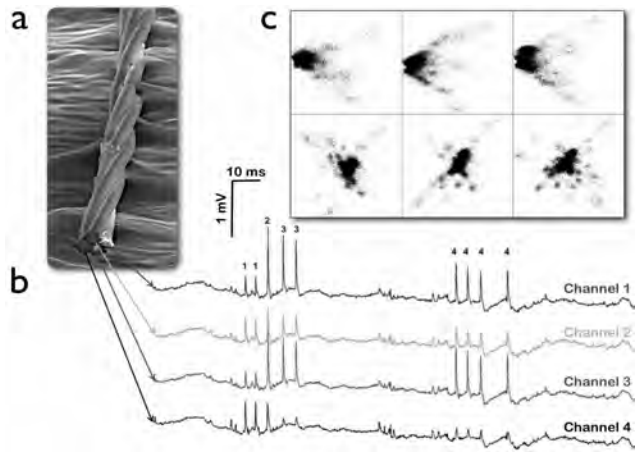

Figure 1: *Hippocampal Tetrode Recordings.* (a) Scanning EM image of a tetrode, which consists of four microwires twisted together to form a single recording probe. (b) Because of the spatial separation between the wire tips and the signal sources, each action potential is recorded simultaneously on all four wires, but with different amplitudes. This enables isolating the activity of multiple neurons in the vicinity of the tetrode tip, through a process analogous to triangulation. (c) The six 2-D projections of the Hadamard transformation of the 4-D space of amplitudes. Individual cells correspond to clusters in this space.

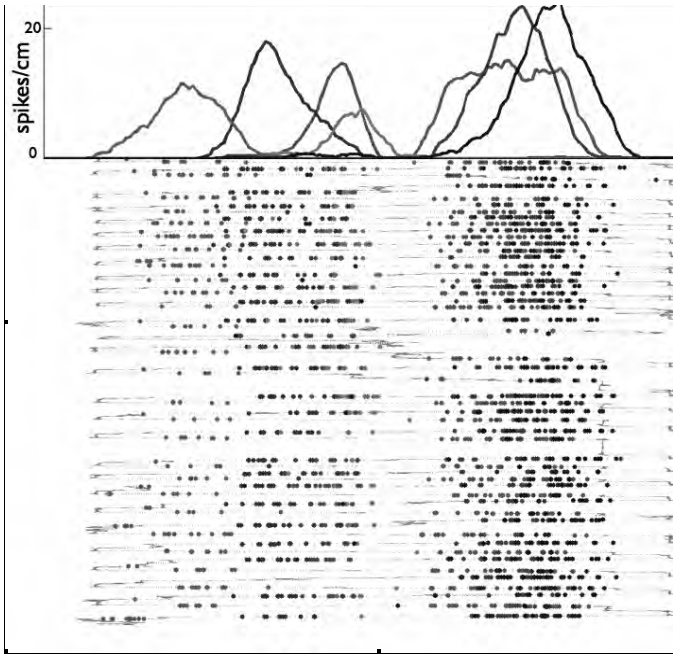

Figure 2: *Place fields.* The bottom panel shows that position of a rat in gray as a function of time (total time 28 minutes). The rat runs on a linear track back and forth and the positions and times of the spikes of seven simultaneously recorded place cells are shown in different colors. The top panel displays histograms of all spikes of each cell as a function of position along the track. Each of these cells ("place cells") fires in a specific location on the track ("place field"). The histograms are binned at 1cm and smoothed with a Gaussian with σ =10 cm.

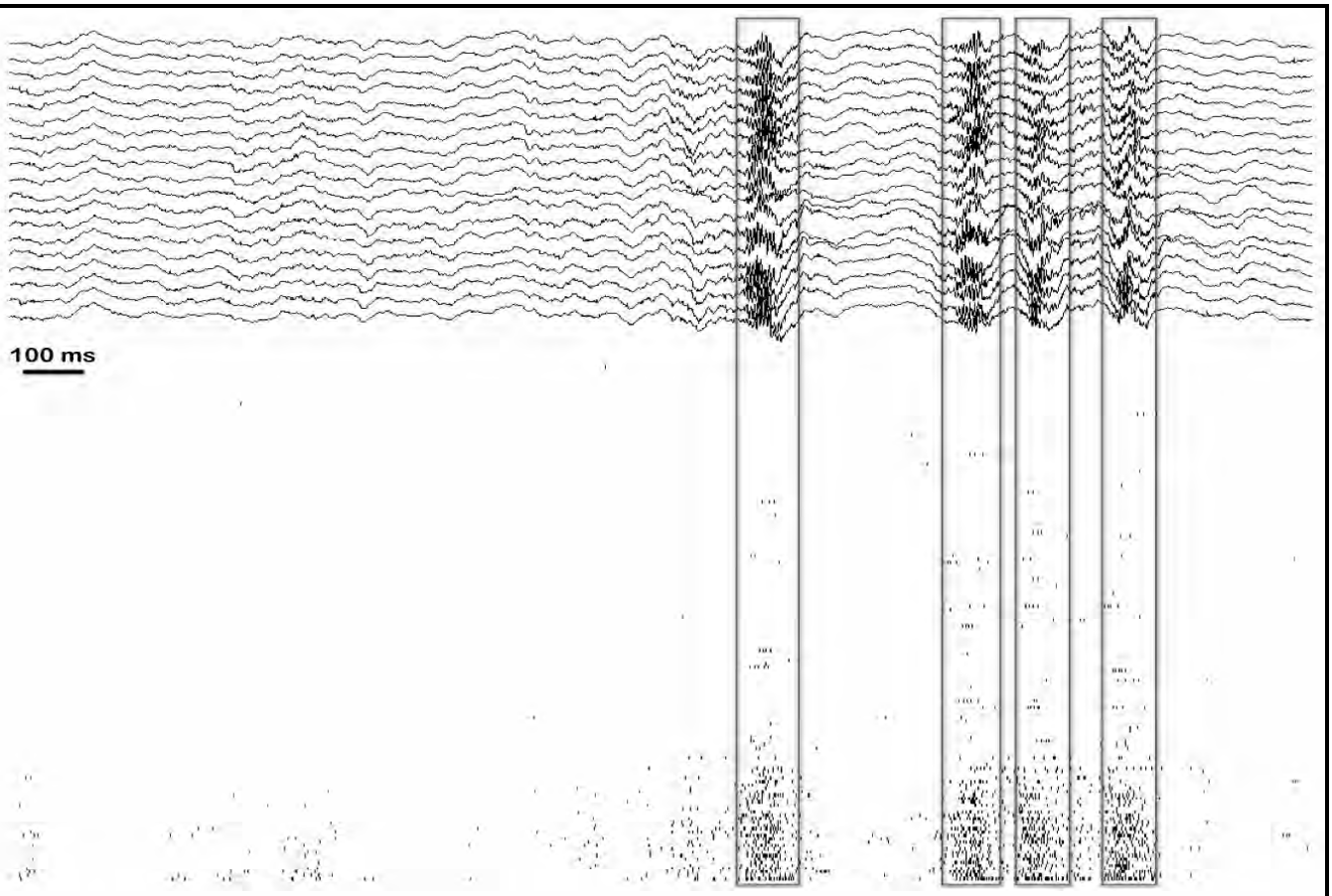

Figure 3: *Hippocampal synchronous bursts during slow-wave sleep.* Local field potential (LFP) recordings from several tetrodes in CA1 (top), together with rasters of 154 simultaneously recorded CA1 cells during slow-wave sleep. Rasters are ordered in terms of firing rates. A block of high firing interneurons is at the bottom of the figure. Sharp-wave bursts (SPWs) are marked by the red rectangles. Notice that CA1 cells tend to fire synchronously around SPWs, and that high-frequency oscillations (~200 Hz ripples) are observed in the LFPs together with sharp-wave bursts.

110. Hippocampal theta oscillations are traveling waves

Evgueniy Lubenov, Thanos Siapas

Theta oscillations clock hippocampal activity during awake behaviour and rapid eye movement (REM) sleep. These oscillations are prominent in the local field potential, and they also reflect the subthreshold membrane potential and strongly modulate the spiking of hippocampal neurons. The prevailing view has been that theta oscillations are synchronized throughout the hippocampus, despite the lack of conclusive experimental evidence. In contrast, here we have shown that in freely behaving rats, theta oscillations in area CA1 are travelling waves that propagate roughly along the septotemporal axis of the hippocampus. Furthermore, we found that spiking in the CA1 pyramidal cell layer is modulated in a consistent travelling wave pattern. Our results demonstrate that theta oscillations pattern hippocampal activity not only in time, but also across anatomical space. The presence of travelling waves indicates that the instantaneous output of the hippocampus is topographically organized and represents a segment, rather than a point, of physical space.

Reference

Lubenov E.V. and Siapas, A.G. (2009) *Nature* **459**:534-539.

111. Decoupling through synchrony in recurrent networks

Evgueniy Lubenov, Thanos Siapas

Synchronization is a key property of many biological, physical, and engineering systems. The level of synchronization in distributed systems is often controlled by the strength of the interactions between individual elements. In brain circuits the connection strengths between neurons are constantly modified under the influence of spike-timing-dependent plasticity rules (STDP). However, the consequences of these local rules for the global dynamics of brain networks are not fully understood. We have shown that when recurrent networks exhibit population bursts STDP rules exert a strong

Figure 4: *Hippocampal activity during REM sleep.* The same neurons and LFP traces as in **Figure 3** during a REM episode (2 minutes earlier than in **Figure 3**). Notice the scale change from **Figure 3**. Theta oscillations (4-10 Hz) are clearly visible in the LFPs, and interneurons fire rhythmically phase-locked to the theta oscillations. Pyramidal cells are rhythmically activated over multiple theta cycles, similar to the activation during a pass through a place field (for example the segment marked by the red rectangle).

decoupling force that desynchronizes activity. Conversely, when activity is random, the same plasticity rules can have a coupling and synchronizing influence. The presence of these opposing forces promotes the self-organization of spontaneously active recurrent networks to a state at the border between randomness and synchrony. These results may have implications for the transfer of information in cortico-hippocampal networks during memory formation, and for understanding the therapeutic effects of deep brain stimulation for Parkinson's disease and epilepsy.

Reference

Lubenov E.V. and Siapas A.G. (2008) *Neuron* **58**:118-131.

112. Mechanisms and functional consequences of synchronous hippocampal bursts during slow-wave sleep

Evgueniy Lubenov, Casimir Wierzynski, Ming Gu, Thanos Siapas

Hippocampal activity during slow-wave sleep is characterized by the presence of highly synchronous bursts (sharp-wave bursts, **Figure 3**). Within each of these bursts about 40,000 CA1 cells (~10%) fire within a window of less than 100 ms. These massive population events are believed to be very effective in driving hippocampal postsynaptic targets and engaging plasticity mechanisms. We study the patterns of neuronal firing during these bursts, and analyze how these patterns evolve throughout sleep. These experimental efforts are complemented with the development of computational models of the mechanisms underlying the generation of synchronous bursts within recurrent networks.

113. Hippocampal activity patterns during REM sleep

Evgueniy Lubenov, Ming Gu, Casimir Wierzynski, Thanos Siapas

REM sleep activity looks remarkably similar to the awake activity, hence the name, *paradoxical sleep*. As illustrated in **Figure 4**, hippocampal LFPs are characterized by theta oscillations, regular 4-10 Hz oscillations that also characterize hippocampal activity during the awake behavior. Hippocampal cells fire phase-locked to the theta rhythm both in awake behavior and REM sleep, but the preferred phases in these two brain states need not be the same [1]. The timing of spikes of hippocampal neurons with respect to the theta rhythm has strong effects on synaptic plasticity [2,3] hence quantifying changes in phase-locking may provide important insights into the functional role of sleep in

memory formation. We study the evolution of hippocampal activity across multiple sleep-wake cycles. In addition, we develop tools for analyzing and identifying network activity motifs in REM sleep and study their experience specificity.

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114. Prefrontal-hippocampal interactions in associative learning

Casimir Wierzynski, Thanos Siapas

Eyeblink conditioning is a form of associative learning that has been shown to engage the hippocampus across a wide range of species and parameters [1]. Moreover, in its trace form, where the conditioned and unconditioned stimuli do not overlap in time, eyeblink conditioning has been shown to require an intact hippocampus for successful acquisition [2]. This hippocampal dependence falls off with time, implying that the long-term locus of the CS-US association is extra-hippocampal. Furthermore, lesions to the medial prefrontal cortex in rats have been shown to disrupt the long-term recall of the eyeblink response, but not its acquisition [3]. Using simultaneous chronic recordings from the hippocampus and medial prefrontal cortex, we are characterizing the relationships between the activity patterns in these brain areas during the acquisition of the CS-US association. These experiments enable us to study how the CS-US association is represented across prefrontal and hippocampal circuits, and how this representation evolves with learning.

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115. Prefrontal-hippocampal interactions during slow-wave sleep

Casimir Wierzynski, Evgueniy Lubenov, Ming Gu, Thanos Siapas

Cortico-hippocampal interactions during sleep are believed to reorganize neural circuits in support of memory consolidation. However, spike-timing relationships across cortico-hippocampal networks - key determinants of synaptic changes - are poorly understood. We have shown that cells in prefrontal cortex fire consistently within 100 ms after hippocampal cells in naturally sleeping animals. This provides evidence at the single cell-pair level for highly consistent directional interactions between these areas within the window of plasticity. Moreover, these interactions are state dependent: they are driven by hippocampal sharp-wave/ripple (SWR) bursts in slow-wave sleep (SWS) and are sharply reduced during REM sleep. Finally, prefrontal responses are nonlinear: as the strength of hippocampal bursts rises, short-latency prefrontal responses are augmented by increased spindle band activity and a secondary peak approximately 100 ms later. These findings suggest that SWR events are atomic units of hippocampal-prefrontal communication during SWS and that the coupling between these areas is highly attenuated during REM sleep.

Reference

Wierzynski C.M., Lubenov E.V., Gu M. and Siapas A.G. (2009) *Neuron* **61**:587-596.

116. Comparison of CA3 and CA1 place fields

Ming Gu, Thanos Siapas

Pyramidal cells ("place cells"), in the hippocampal subfields CA3 and CA1 fire in specific regions of space ("place fields" - **Figure 2**). These subfields have very different intrinsic architecture: CA3 is characterized by extensive recurrent collaterals that are not prominent in CA1. Does the different architecture imply different place field properties in CA3 and CA1? We characterize the precise temporal structure of firing, and the relationships of place field firing across these subfields. We, furthermore, examine how place field representations evolve and mature when the animal is exposed to novel environments.

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Lubenov, E.V. and Siapas, A.G. (2008) Decoupling through synchrony in neuronal circuits with propagation delays. *Neuron* **58**(1):118-131.
Lubenov, E.V. and Siapas, A.G. (2009) Hippocampal theta oscillations are travelling waves. *Nature* **459**(7246):534-539.
Wierzynski, C.M., Lubenov, E.V., Gu, M. and Siapas, A.G. (2009) State-dependent spike-timing relationships between hippocampal and prefrontal circuits during sleep. *Neuron* **61**(4):587-596.
Zhang, T., Zhang, L., Liang, Y., Siapas, A.G., Zhou, F.M. and Dani, J.A. (2009) Dopamine signaling differences in the nucleus accumbens and dorsal striatum exploited by nicotine. *J. Neurosci.* **29**(13):4035-4043.

Assistant Professor of Biology: Doris Tsao
Postdoctoral Scholars: Piercesare Grimaldi, Sebastian Moeller
Graduate Student: Shay Ohayon

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Summary: Research in our lab aims to uncover the mechanisms for visual perception in primates, specifically, how objects are identified, and how 3D shape and location are computed. We use a combination of fMRI, electrophysiology, and anatomy in monkeys, as well as mathematical modeling. In order to elucidate the principles of information flow underlying object recognition, we are investigating a specialized system of fMRI-identified face-selective regions in the temporal and frontal lobes of the macaque brain. A major goal is to understand the functional specialization of each of these face patches. To this end, we are interrogating the face system from four directions:

1. *Representations*: What are the selectivity and invariance properties of cells in each face patch?
2. *Behavioral Role*: What is the effect of inactivating specific face patches on various face-related behaviors?
3. *Connectivity:* What is the wiring diagram of the face patches?
4. *Transformations*: What are the key functional differences between input and output cells within each face patch?

We believe that understanding in detail the processing in the face patches will not only yield insights into the general mechanisms underlying object recognition, but will clarify basic organizational principles of primate neocortex.

In the coming years, we plan to follow a similar approach to understanding the neural mechanisms underlying spatial perception.

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Postdoctoral Scholars: Bader Al-Anzi, Hyung-Kook Lee, Ed Silverman
Graduate Student: Ashley Palani Wright
Staff: Elena Armand, Amy Cording, Mili Jeon, Kaushiki Menon, Violana Nesterova, Yelizaveta Nesterova, Anna Salazar

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Summary of Zinn group research:
The fruit fly *Drosophila melanogaster* is our primary experimental system. We work on a variety of problems, mostly concerning the molecular mechanisms by which synaptic connections are formed during development. In the embryo, we examine signaling systems involved in motor and CNS axon guidance. In the larva, we study synaptogenesis and synaptic plasticity in the neuromuscular system. We have also begun a new project to study the mechanisms involved in defining the geometries of tubes in the tracheal (respiratory) system. Our approaches combine genetics, molecular biology, electrophysiology, biochemistry, and cell biology. The current group members include four postdocs (Kaushiki Menon, Anna Salazar, Ed Silverman, and Peter (Hyung-Kook) Lee), a graduate student (Ashley Wright), a lab manager/technician (Violana Nesterova), a technician working on motor axon targeting (Amy Cording), and a technician participating in various projects by performing dissections and *Drosophila* crosses (Elena Armand). Another postdoc (Mili Jeon) is continuing her work on RPTPs in Matthew Scott's lab at Stanford. Bader Al-Anzi, a postdoc from the Benzer lab, is also associated with our group, and Elena Armand works with him.

Motor axon guidance and muscle targeting
The *Drosophila* motor axon network has provided one of the best systems in which to study axonal pathfinding mechanisms. The network is simple: 32 motoneurons innervate 30 body wall muscle fibers in each abdominal segment. Each motoneuron axon is targeted to a specific muscle fiber, and very few projection errors are made during normal development. Thus, the motor axon network is a genetically hard-wired map, and is an ideal system in which to study how genes control the formation of specific synaptic connections. In much of our work, we have focused on the roles of tyrosine phosphorylation in regulating motor axon guidance decisions. We are now conducting genetic screens to determine the mechanisms by which cell surface proteins label specific muscle fibers for recognition by motor axon growth cones.

Targeting of motor axons to specific muscle fibers
Despite the advances in characterizing molecules that regulate motor axon pathfinding, we still understand little about how specific muscle fibers are recognized as targets for synapse formation by these axons. Many mutations affect pathfinding decisions, leading to aberrant wiring of the neuromuscular system, but no single loss-of-function (LOF) mutations are known that block recognition of specific muscle targets. These results are most easily explained by invoking genetic redundancy in target labeling. If each muscle fiber were defined by a combination of several cell surface labels, removing one of the labels might not have a major effect on targeting of axons to that fiber. This would explain why targeting molecules have not been identified in conventional LOF genetic screens.

Studies of gain-of-function (GOF) phenotypes by other groups are consistent with the redundancy hypothesis. For example, the homophilic cell adhesion molecule Fasciclin III (Fas III) is expressed on only two muscle fibers, 6 and 7, and on the growth cone of the RP3 neuron that innervates these two fibers. Fas III appears to be a functional target label, because when it is ectopically expressed on other muscle fibers near 6 and 7, the RP3 neuron makes abnormal synapses on these Fas III-expressing fibers. However, when Fas III is removed by a LOF mutation, there is no effect on targeting of RP3 to 6 and 7. These results imply that Fas III can be used for muscle targeting, but that targeting of 6 and 7 can still proceed in its absence, presumably because these fibers are also labeled by other surface molecules that can be recognized by the RP3 growth cone when Fas III is not present.

These findings suggest that cell-surface proteins that label specific targets in the motor axon system might be identifiable by a GOF genetic screen in which candidate labels are ectopically expressed on all muscle fibers. If these proteins are functional labels, their misexpression might produce alterations in target recognition, as observed in the Fas III experiments described above. By identifying genes encoded in the *Drosophila* genome that can confer GOF phenotypes in which targeting of specific muscle fibers is altered, we will acquire the tools to understand the mechanisms involved in target recognition in this system. This type of screen should allow us to overcome the redundancy problem. For example, suppose one could identify three different cell-surface proteins that are normally expressed on a specific muscle fiber, but whose misexpression on other muscle fibers produces targeting errors. One might then predict that removing all three of these proteins by making a triple LOF mutant (through conventional or RNAi techniques) would now prevent targeting of this muscle fiber. Through these kinds of experiments, we could begin to understand the combinatorial code for muscle targeting. Insights into the motor axon targeting code would be likely to facilitate an understanding of targeting in other neuronal systems e.g., the antennal lobe, optic lobe, and mushroom body), since candidate target

labels are usually expressed by a variety of neuronal and non-neuronal cell types.

To conduct this GOF screen, we first created a database of all cell-surface and secreted (CSS) proteins in *Drosophila* that are likely to be involved in specific cell-cell interactions. The database was generated by database mining and reiterative computational screening. We defined all fly genes encoding proteins that contain domains known to be present in CSS proteins in other eukaryotes (including all of the 240 domains in the 'extracellular' portion of the SMART database, http://smart.embl-heidelberg.de/browse.shtml, which are represented in flies). We then eliminated several hundred genes that we thought were unlikely to be important for cell recognition, and defined a CSS cell recognition candidate collection of 976 genes.

To drive expression of these genes in muscles, we used the 'EP' system, in which a P element containing a block of UAS sequences that are responsive to the yeast transcription factor GAL4 is jumped around the genome. Like other P elements, EPs usually land upstream of genes. If a line bearing an EP upstream of a gene is crossed to a 'driver' line expressing GAL4 in all muscle fibers, the gene will now be expressed at high levels in muscles in the resulting progeny embryos and larvae. To find EP-like elements upstream of the CSS genes, we searched through about 40,000 different insertions that have been maintained in collections of *Drosophila* lines. These include the original EP set generated by Pernille Rorth, the EY insertion lines generated in the Bellen lab, the GS lines developed in Japan, insertions generated by Exelixis, Inc., and maintained at Harvard, and the GE lines developed by GenExel, Inc. We were able to identify insertions that can confer expression of 410 of the 976 CSS genes in our database, representing about 40% of the repertoire and including members of all CSS protein families. This database has since been employed for a variety of projects.

To screen for genes encoding potential targeting molecules, we crossed each of these insertions to a strong pan-muscle GAL4 driver and visualized motor axons and neuromuscular junction synapses in the resulting F1 progeny larvae by immunostaining. We have already identified 30 genes that cause synaptic mistargeting on muscles 12 and 13, and 55 genes that cause synaptic morphology phenotypes.

We focused initially on the analysis of the mistargeting genes, as this is our primary interest. In a paper published last year in *Neuron* (Kurusu, Cording *et al.*, 2008) we showed that one class of receptor, the leucine-rich repeat (LRR) family, is overrepresented within the mistargeting set. Using a combination of loss-of-function and gain-of-function analysis, we demonstrate that four of these LRR proteins participate in the decision of the RP5 motor axon to choose muscle 12 as its target (Kurusu *et al.*, in press.). These proteins are: Tartan (Trn), Capricious (Caps), CG14351/Hattifattener (Haf), and CG8561/Als (Kurusu/Cording abstract).

Since publication of this paper, Amy Cording has continued to evaluate other members of the LRR family for potential roles in axon guidance and synapse formation. We have also collaborated with Liqun Luo's group at Stanford, who used our collection of lines to identify proteins involved in formation of synaptic connections in the antennal lobe. This work has led to the submission of a paper showing that Caps and Trn are also involved in targeting of projection neuron dendrites to specific glomeruli of the antennal lobe.

Receptor tyrosine phosphatases

In the 1990's, we showed that 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 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 (LAR, PTP10D, PTP69D, PTP99A, PTP52F, PTP4E), and we have generated or obtained mutations in all six of the genes encoding these proteins.

We have now performed a detailed characterization of the genetic interactions among all six RPTPs. We find that each growth cone guidance decision in the neuromuscular system has a requirement for a unique subset of RPTPs; thus, in a sense, there is an "RPTP code" for each decision. 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. The patterns of redundancy are summarized in a paper (Jeon *et al.*) published in 2008.

A genetic approach to identification of RPTP ligands

The ligands recognized by RPTPs *in vivo* 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.

One of our current approaches to identifying ligands is based on our observation that fusion proteins in which the extracellular domains of RPTPs are joined to human placental alkaline phosphatase (AP) can be used to stain live-dissected *Drosophila* embryos. Each of four fusion proteins (LAR-AP, PTP69D-AP, PTP10D-AP, PTP99A-AP) binds in a specific manner. Each fusion protein stains a subset of CNS axons and also binds to other cell types in the periphery. To identify the genes

encoding the RPTP ligands, we are screening deficiency (Df) mutations that remove specific portions of the genome.

Using the Df screen, we found a Df that contains a gene encoding a ligand that binds to LAR-AP, and have identified this ligand as Syndecan (Sdc). Sdc is a heparan sulfate proteoglycan (HSPG) that binds to Lar with nanomolar affinity and participates in its axon guidance functions. This work was published in 2005. We have continued the Df screen, and have identified three regions required for 99A-AP staining, and identified the corresponding genes. Analysis of these indicates that a novel glial-neuronal interaction is required to specify expression of the 99A ligand.

Our approach is general, and can be used to identify ligands for any 'orphan receptor' that has a *Drosophila* ortholog. We also used the method to define genomic regions required for expression of selected cell surface antigens, including those recognized by the 1D4 and BP102 monoclonal antibodies (mAbs). We began with the Bloomington Df kit, but have replaced many of the lines with other Dfs for which homozygotes have more normal development. As part of the analysis, we have defined a new Df kit for embryonic screening, which uses alternative Bloomington Dfs to allow screening of regions of the genome whose removal in the normal Df kit causes early developmental failure. This new kit contains about 450 lines, and covers about 89% of polytene chromosome bands. It can be used to analyze any region of the genome for the desired embryonic phenotype. We have already analyzed about half of the genome for regions necessary for motor axon guidance by staining Df embryos with 1D4 (Wright abstract).

A gain-of-function screen for RPTP ligands

Despite the success of the Df screen (an LOF approach), it is clearly not capable of identification of all RPTP ligands, and may not even be capable of finding most of them. First, about 11% of the genome still cannot be screened, either because no Dfs exist there or because embryos homozygous for those regions do not develop. Second, and most important, the four RPTP-AP probes all stain subsets of CNS axons, in addition to other patterns outside the CNS. If multiple ligands for an RPTP were all expressed on CNS axons, removal of one ligand gene by a Df might not perturb staining enough to detect a difference from wild type. We already know that this is the case for LAR: Sdc is expressed both on CNS axons and in the periphery, but only peripheral staining is eliminated in an *Sdc* mutant. CNS axons in *Sdc* mutants continue to stain with LAR-AP, and are also stained by a mutant version of LAR-AP that cannot bind to Sdc[7]. These data show that there is at least one non-HSPG ligand for LAR that is expressed on CNS axons together with the HSPG ligand Sdc. Because of these limitations, we have developed a new GOF approach to ligand screening that allows direct identification of proteins that bind in embryos to an RPTP probe, regardless of whether such proteins are normally expressed in patterns that overlap with those of other ligands. This approach is also general and can be applied to any orphan receptor of interest that has *Drosophila* orthologs. It is based on observations made by Fox and Zinn (2005), who showed that when Sdc is ectopically expressed on muscle fibers, this produces ectopic muscle staining with LAR-AP, which normally does not bind to muscles. Thus, if one were able to express ligand genes in new patterns in the embryo, one would expect to be able to see additional staining with RPTP-AP probes and identify ligands in this manner.

Our approach is a directed EP screen. It uses the collection of EP element lines described above to ectopically express CSS proteins in new patterns in the embryo. To screen for new RPTP ligands, we are crossing each line in our CSS EP collection to GAL4 driver lines that confer ectopic gene expression in cells that normally do not stain with RPTP-AP fusion proteins. If I detect new staining patterns in embryos derived from such a cross, this may indicate that the gene driven by that EP-like element encodes a protein that can bind to the RPTP. Peter Lee has already found many such lines, and these define more than 20 new RPTP ligands (Lee abstract).

Searching for RPTP substrates

It is difficult to identify PTP substrates biochemically because PTPs usually do not display strong specificity *in vitro*. To find substrate candidates, we performed yeast two-hybrid screens with 'substrate-trap' mutant versions of PTP10D, PTP69D, PTP52F, and PTP99A. These 'trap' proteins form stable complexes with tyrosine-phosphorylated substrates because they bind normally but cannot catalyze dephosphorylation. We introduced a constitutively activated chicken Src tyrosine kinase into yeast together with the PTP trap constructs and the cDNA library, in the hope that it would phosphorylate relevant substrate fusion proteins made from cDNA library plasmids. We identified Tartan (Trn) in this screen, and have published a paper showing that Trn is in fact a Ptp52F substrate and that Trn and Ptp52F participate in the same signaling pathway in motor neurons (Bugga abstract).

Tracheal development: Regulation of EGFR tyrosine kinase activity by the Ptp10D and Ptp4E RPTPs regulates lumen formation

In the process of examination of double mutants lacking expression of the closely related proteins Ptp4E and Ptp10D, we noticed that that the tracheal network exhibits a unique phenotype in these embryos. Unicellular and seamless tracheal tubes develop bubble-like cysts in place of tubular lumen. The double mutation affects the sculpting of lumen into a tubular shape, a process that has not been studied before using molecular genetics. Cyst formation is partially due to hyperactivation of the Egfr tyrosine kinase, and Ptp10D and Egfr directly associate in cultured cells. We have a paper in press describing this work (Jeon abstract).

Genes controlling synaptogenesis in the larval neuromuscular system

Motor growth cones reach their muscle targets during late embryogenesis and then mature into presynaptic terminals that are functional by the time of hatching. The pattern of Type I neuromuscular junction (NMJ) synapses in the larva is simple and highly stereotyped, with boutons restricted to specific locations on each muscle fiber. 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. This growth represents a form of synaptic plasticity, because it is controlled by feedback from the muscle to the neuron. Studies of NMJ synapses in flies are relevant to an understanding of synaptic plasticity in the mammalian brain, because the fly NMJ is a glutamatergic synapse, organized into boutons, that uses ionotropic glutamate receptors homologous to vertebrate AMPA receptors.

Control of synaptic local translation by Pumilio and Nanos

Our recent work on synapses has focused on control of synaptic protein translation. Local translation at synapses has been studied in *Aplysia*, mammalian, and arthropod systems. It has attracted interest because it is a mechanism that allows neurons to separately adjust the strengths of individual synapses.

To identify genes involved in synaptogenesis in larvae, including those that regulate local translation, we devised and executed a GOF screen of live third instar larvae. In the screen, we identified *pumilio* (*pum*), which encodes an RNA-binding protein that shuts down translation of specific mRNAs by binding to their 3' untranslated regions. Translational repression by Pum controls posterior patterning during embryonic development. In a 2004 paper, we showed that Pum is an important mediator of synaptic growth and plasticity at the NMJ. Pum is localized to the postsynaptic side of the NMJ in third instar larvae, and is also expressed in larval neurons. Neuronal Pum regulates synaptic growth. In its absence, NMJ boutons are larger and fewer in number, while Pum overexpression increases bouton number and decreases bouton size. Postsynaptic Pum negatively regulates expression of the essential translation factor eIF-4E (the cap-binding protein) at the NMJ, and Pum binds selectively to the 3'UTR of *eIF-4E* mRNA. These data suggest that Pum is a direct regulator of local eIF-4E translation, and that eIF-4E (which is normally limiting for translation) in turn switches on translation of other synaptic mRNAs. Pum also directly regulates the GluRIIa glutamate receptor. These results, together with genetic epistasis studies, suggest that postsynaptic Pum modulates synaptic function via direct control of local synaptic translation.

In our current work, we have studied the Pum cofactor Nanos, which works together with Pum to repress translation in the early embryo, as a participant in Pum regulation of targets at the NMJ. In *nos* mutants (or transgenic *nos* RNAi larvae), GluRIIB is downregulated, while the alternative subunit GluRIIB is upregulated. Thus, the phenotypes of nos and pum are opposite in this system. We also show that Pum represses Nos expression. Regulation of GluRIIA and Nos by Pum involves direct binding of Pum to the 3' UTRs of their mRNAs. A paper describing this work was recently published (Menon *et al*.).

Assembly of Pumilio into ordered aggregates as a regulatory switching mechanism

We are also studying Pum in another context: its potential role as switch that could control synaptic translation via regulated assembly into an ordered aggregate. This project emerged from a computational search we performed to identify switch proteins that might have the capacity to form ordered aggregates. This is relevant to human disease, as well, since proteins involved in many human neurodegenerative diseases share a propensity to form amyloid aggregates. One class of sequences that can form amyloids are domains rich in glutamine (Q) and asparagine (N). These are present in many metazoan proteins, including ~450 in *Drosophila*. Q/N domains are found in all yeast prions, and these domains have been positively selected during evolution, perhaps in order to allow reversible switching of the functional domain of the prion into an inactive aggregated state. We wondered if this type of selection might also maintain Q/N domains in metazoans. To examine this question, we devised a computational search strategy to identify candidates for nucleic-acid binding prion switches in metazoan proteomes.

One of the two strong *Drosophila* candidates identified in this search is Pum. As described above, work by our group had shown that Pum is localized to the postsynaptic side of the larval NMJ, where it acts as a regulator of local mRNA translation. We found that a Q/N-rich domain (denoted NQ1) from Pum exhibits prion-like behavior in budding yeast, including heritable phenotypic switching and reversibility by guanidine hydrochloride. NQ1 purified from *E. coli* forms amyloid fibrils *in vitro*. To test whether NQ1 aggregate formation can perturb Pum's function in the nervous system, we created transgenic fly lines in which NQ1 expression is driven by GAL4. Our results show that postsynaptic NQ1 expression generates alterations in the NMJ that phenocopy the *pum* loss-of-function phenotype and interact genetically with *pum* mutations. Postsynaptic Pum overexpression is lethal, but co-overexpression of NQ1 rescues this lethality, suggesting that NQ1 can inactivate endogenous Pum. We have submitted a paper describing these findings (Salazar/Silverman/Menon abstract).

117. Obesity-blocking neurons in *Drosophila*

Bader Al-Anzi, Viveca Sapin, Christopher Waters, Kai Zinn, Robert J. Wyman[1], Seymour Benzer[2]

In mammals, fat store levels are communicated by leptin and insulin signaling to brain centers that regulate food intake and metabolism. By using transgenic manipulation of neural activity, we report the isolation of two distinct neuronal populations in flies that perform a similar function, the c673a-Gal4 and fruitless-Gal4 neurons. When either of these neuronal groups is silenced, fat store levels increase. This change is mediated through an increase in food intake and altered metabolism in c673a-Gal4 silenced flies, while silencing fruitless-Gal4 neurons alters only metabolism. Hyperactivation of either neuronal group causes depletion of fat stores by increasing metabolic rate and decreasing fatty acid synthesis. Altering the activities of these neurons causes changes in expression of genes known to regulate fat utilization. Our results show that the fly brain measures fat store levels and can induce changes in food intake and metabolism to maintain them within normal limits.

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118. The cell surface receptor Tartan is a potential *in vivo* substrate for the receptor tyrosine phosphatase Ptp52F

Lakshmi Bugga[1], Anuradha Ratnaparkhi[2]

Receptor tyrosine phosphatases (RPTPs) are essential regulators of axon guidance and synaptogenesis in *Drosophila*, but the signaling pathways in which they function are poorly defined. We identified the cell-surface receptor Tartan (Trn) as a candidate substrate for the neuronal RPTP Ptp52F using a modified two-hybrid screen with a substrate-trapping mutant of Ptp52F as "bait." Trn can bind to the Ptp52F substrate-trapping mutant in transfected *Drosophila* S2 cells if v-Src kinase, which phosphorylates Trn, is also expressed. Coexpression of wild-type Ptp52F causes dephosphorylation of v-Src-phosphorylated Trn. To examine the specificity of the interaction *in vitro*, we incubated Ptp52F-GST fusion proteins with pervanadate-treated S2 cell lysates. Wild-type Ptp52F dephosphorylated Trn, as well as most other bands in the lysate. GST "pulldown" experiments demonstrated that the Ptp52F substrate-trapping mutant binds exclusively to phospho-Trn. Wild-type Ptp52F pulled down dephosphorylated Trn, suggesting that it forms a stable Ptp52F-Trn complex that persists after substrate dephosphorylation. To evaluate whether Trn and Ptp52F are part of the same pathway *in vivo*, we examined motor axon guidance in mutant embryos. *trn* and *Ptp52F* mutations produce identical phenotypes affecting the SNa motor nerve. The genes also display dosage-dependent interactions, suggesting that Ptp52F regulates Trn signaling in SNa motor neurons.

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119. Receptor tyrosine phosphatases control tracheal tube geometries through negative regulation of EGFR signaling

Mili Jeon

The formation of epithelial tubes with defined shapes and sizes is essential for organ development. Here we describe a unique tracheal tubulogenesis phenotype caused by loss of both *Drosophila* type III receptor tyrosine phosphatases (RPTPs), Ptp4E and Ptp10D. Ptp4E is the only widely expressed *Drosophila* RPTP, and is the last of the six fly RPTPs to be genetically characterized. We recently isolated mutations in Ptp4E, and discovered that, although Ptp4E null mutants have no detectable phenotypes, double mutants lacking both Ptp4E and Ptp10D display synthetic lethality at hatching due to respiratory failure. In these double mutants, unicellular and terminal tracheal branches develop large bubble-like cysts that selectively incorporate apical cell surface markers. Cysts in unicellular branches are enlargements of lumen that are sealed by adherens junctions, while cysts in terminal branches are cytoplasmic vacuoles. Cyst size and number are increased by tracheal expression of activated Egfr tyrosine kinase, and decreased by reducing Egfr levels. Ptp10D forms a complex with Egfr in transfected cells. Downregulation of EGFR signaling by the RPTPs is required for construction of tubular lumens, whether extracellular or intracellular, by cells that undergo remodelling during branch morphogenesis. The Ptp4E Ptp10D phenotype represents the first evidence of an essential role for RPTPs in epithelial organ development. These findings may be relevant to organ development and disease in mammals, because DEP-1 (PTPRJ), an ortholog of Ptp4E/Ptp10D, interacts with the hepatocyte growth factor receptor tyrosine kinase. PTPRJ corresponds to the murine Scc1 (suppressor of colon cancer) gene.

120. A screen of cell-surface molecules identifies leucine-rich repeat proteins as key mediators of synaptic target selection

Mitsuhiko Kurusu[†], Amy Cording[*], Misako Taniguchi[†], Kaushiki Menon[*]*

In *Drosophila* embryos and larvae, a small number of identified motor neurons innervate body wall muscles in a highly stereotyped pattern. Although genetic screens have identified many proteins that are required for axon guidance and synaptogenesis in this system, little is known about the mechanisms by which muscle fibers are defined as targets for specific motor axons. To identify potential target labels, we screened 410 genes encoding cell-surface and secreted proteins, searching for those whose overexpression on all muscle fibers causes motor axons to make targeting errors. Thirty such genes were

identified, and a number of these were members of a large gene family encoding proteins whose extracellular domains contain leucine-rich repeat (LRR) sequences, which are protein interaction modules. By manipulating gene expression in muscle 12, we showed that four LRR proteins participate in the selection of this muscle as the appropriate synaptic target for the RP5 motor neuron.

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121. Identification of ligands for neural receptor tyrosine phosphatases

Hyung-Kook (Peter) Lee

There are about 1000 *Drosophila* cell-surface and secreted (CSS) proteins that may be involved in cell-cell recognition events. Most of these are "orphans" that is, the ligands/receptors with which they interact are unknown. "Deorphanizing" the CSS proteins will be necessary in order to understand the mechanisms by which cells interact during development. This is a major challenge for the future. We previously developed a deficiency screen method, based on staining of live embryo dissections with receptor-human placental alkaline phosphatase (receptor-AP) fusion proteins, to find regions containing ligand genes. This led the identification of Syndecan as an *in vivo* ligand for the receptor tyrosine phosphatase (RPTP) Lar (Fox and Zinn, 2005). We have now developed a new screening method, based on the collection of "EP-like" P element lines described in Kurusu *et al*., 2008, that allows us to directly identify ligand genes. These lines allow us to ectopically express more than 400 of the CSS proteins, or >40% of the total repertoire, in any desired pattern. To screen for orphan receptor ligands using this collection, we cross each line to a strong pancellular GAL4 driver and stain live embryos with receptor-AP fusion proteins, looking for ectopic staining patterns. If such a pattern is observed, it provides evidence that the receptor-AP protein can bind to the protein encoded downstream of the EP element. We conducted this screen for four of the six neural RPTPs, which are regulators of axon guidance and synapse formation during embryonic and larval development. The screen has identified more than 20 CSS proteins as potential RPTP ligands. For one ligand, stranded at second (Sas), which interacts with Ptp10D, we have already used genetics to demonstrate that Sas is required for Ptp10D's functions in regulation of axon guidance across the midline of the embryo.

122. The translational repressors Nanos and Pumilio have divergent effects on presynaptic terminal growth and postsynaptic glutamate receptor subunit composition

Kaushiki Menon, Shane Andrews[*], Mala Murthy, Elizabeth R. Gavis[*]

Pumilio (Pum) is a translational repressor that binds selectively to target mRNAs and recruits Nanos (Nos) as a corepressor. In the larval neuromuscular system, Pum represses expression of the translation factor eIF-4E and the glutamate receptor subunit GluRIIA. Here we show that Nos, like Pum, is expressed at the neuromuscular junction (NMJ) and in neuronal cell bodies. Surprisingly, however, Nos and Pum have divergent functions on both the pre- and postsynaptic sides of the NMJ. In *nos* mutant and *nos* RNAi larvae, the number of NMJ boutons is increased, while loss of Pum reduces bouton number. On the postsynaptic side, Nos acts in opposition to Pum in regulating the subunit composition of the glutamate receptor. NMJ active zones are associated with GluRIIA- and GluRIIB-containing receptor clusters. Loss of Nos causes downregulation of GluRIIA and increases the levels of GluRIIB. Consistent with this finding, the electrophysiological properties of NMJs lacking postsynaptic Nos suggest that they employ primarily GluRIIB-containing receptors. Nos can regulate GluRIIB in the absence of GluRIIA, suggesting that the effects of Nos on GluRIIB levels are at least partially independent of synaptic competition between GluRIIA and GluRIIB. Nos is a target for Pum repression, and Pum binds selectively to the 3' UTRs of the *nos* and *GluRIIA* mRNAs. Our results suggest a model in which regulatory interplay between Pum, Nos, GluRIIA, and GluRIIB could cause a small change in Pum activity to be amplified into a large shift in the balance between GluRIIA and GluRIIB synapses.

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123. Regulation of synaptic Pumilio function by an aggregation-prone domain

Anna M. Salazar, Edward J. Silverman, Kaushiki P. Menon

We identified Pumilio (Pum), a *Drosophila* translational repressor, in a computational search for metazoan proteins whose activities might be regulated by assembly into ordered aggregates. The search algorithm was based on evolutionary sequence conservation patterns observed for yeast prion proteins, which contain aggregation-prone glutamine/asparagine (Q/N)-rich domains attached to functional domains of normal amino acid composition. We examined aggregation of Pum and its nematode ortholog PUF-9 by expression in yeast. A domain of Pum containing the Q/N-rich sequence, denoted as NQ1, the entire Pum N-terminus, and the complete PUF-9 protein localize to macroscopic aggregates (foci) in yeast. NQ1 and PUF-9 can generate the yeast *Pin+* trait, which is transmitted by a heritable aggregate. NQ1 also assembles into amyloid fibrils *in*

vitro. In *Drosophila*, Pum regulates postsynaptic translation at neuromuscular junctions (NMJs). To assess whether NQ1 affects synaptic Pum activity *in vivo*, we expressed it in muscles. We found that it negatively regulates endogenous Pum, producing gene dosage-dependent *pum* loss-of-function NMJ phenotypes. NQ1 coexpression suppresses the severe NMJ phenotypes caused by overexpression of Pum in muscles, and it also rescues the lethality conferred by this overexpression. Negative regulation of Pum by NQ1 might be explained by formation of inactive aggregates of the type we observed in yeast, but we have been unable to demonstrate that NQ1 or Pum form aggregates in *Drosophila*. Alternatively, NQ1 could regulate Pum *in vivo* by a "dominant-negative" effect, in which it would block the interaction of Pum with itself or cofactors required for translational repression.

124. Using gain-of-function and deficiency screens to identify orphan receptor ligands and define new synaptic targeting and axon guidance phenotypes

Ashley Wright, Hyung-Kook (Peter) Lee, Amy Cording, Kai Zinn

For the past ten years, we have been interested in two interlocking problems: how to identify ligands for axon guidance/synaptic receptors such as the receptor tyrosine phosphatases (RPTPs), and how to identify and characterize cell surface proteins that label specific cells as synaptic targets for motor neurons and interneurons. We have taken a variety of approaches to these problems. First, we used baculovirus-expressed dimeric RPTP fusion proteins as probes to localize RPTP ligands in live-dissected embryos. Having done this, we developed a deficiency (Df) screen to find regions of the genome containing genes required for ligand synthesis (Fox and Zinn, 2005). This screen can also be used to systematically survey about ½ of the genome for genes that control CNS and motor axon guidance, muscle development, PNS development, tracheal development, and other processes. As a ligand screen, however, this method has limitations. Our data suggest that the RPTPs have multiple ligands that are expressed in overlapping patterns, and removing one such ligand may not affect staining sufficiently to allow gene identification. Accordingly, we developed a gain-of-function (EP screen) approach, in which we can express about 400 of the ~1000 cell-surface and secreted (CSS) proteins that are likely to be relevant to cell recognition during development. We screened this collection for ligands by ectopically expressing each CSS protein outside the CNS and looking for new RPTP probe staining patterns. We have identified about 25 putative ligands for four of the six fly RPTPs, thus far. The same collection of CSS EPs can also be used to search for proteins that can redirect motor axons when they are expressed on all muscles. Such proteins may represent ligands that are normally used for targeting to specific muscles (Kurusu *et al*., 2008). The screen-defined proteins containing leucine-rich repeat (LRR) extracellular domains as of particular interest, and we are continuing to examine the remainder of the large LRR family for new synaptic targeting molecules.

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Developmental and Regulatory Biology

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Summary: This laboratory's research centers on the early formation of the nervous system in vertebrate embryos. The peripheral nervous system forms from two cell types that are unique to vertebrates: neural crest cells and ectodermal placodes. We study the cellular and molecular events underlying the formation, cell lineage decisions and migration of these two cells types. 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. In addition to their specific neuronal and glial derivatives, neural crest cells can also form melanocytes, craniofacial bone and cartilage and smooth muscle. Placodes are discrete regions of thickened epithelium that give rise to portions of the cranial sensory ganglia, as well as form the paired sense organs (lens, nose, ears). Placodes and neural crest cells share several properties including the ability to migrate and to undergo an epithelial to mesenchymal transition. Their progeny are also similar: sensory neurons, glia, neuroendocrine cells, and cells that can secrete special extracellular matrices.

Our laboratory concentrates on studying the cellular and molecular mechanisms underlying the induction, early development and evolution of the neural crest and placodes. 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 and identification of gene regulatory regions. These studies shed important light on the mechanisms of neural crest and placode formation, migration and differentiation. In addition, the neural crest and placodes are unique to vertebrates. In studying the evolution of these traits, we hope to better understand the origin of vertebrates.

Because these cell types are involved in a variety of birth defects and cancers such as neurofibromatosis, melanoma, neuroblastoma, and 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.

125. Comprehensive spatiotemporal analysis of early chick neural crest network genes

Jane Khudyakov, Marianne Bronner-Fraser

Specification of neural crest progenitors begins during gastrulation at the neural plate border, long before migration or differentiation. Neural crest cell fate is acquired by progressive activation of discrete groups of specifier genes, which are thought to be temporally distinct. Expression domains of molecules in the putative neural crest gene regulatory network are highly conserved in vertebrates; however, comprehensive analysis of their expression is lacking in chick, an important model system for neural crest development. We analyzed expression of ten transcription factors that are known specifiers of neural plate border and neural crest fate and compared them across chick development from gastrulation to neural crest migration. Surprisingly, we find that most neural crest specifiers are expressed during gastrulation, concominant with and in similar domains as neural plate border specifiers. This suggests that interactions between these molecules may occur much earlier than previously thought, an important consideration for functional studies.

126. Birth of ophthalmic trigeminal neurons initiates early in the placodal ectoderm

Kathryn McCabe, John Sechrist, Marianne Bronner-Fraser

The largest of the cranial ganglia, the trigeminal ganglion relays cutaneous sensations of the head to the central nervous system. Its sensory neurons have a dual origin from both ectodermal placodes and neural crest. We show that birth of neurons derived from the chick ophthalmic trigeminal placode begins prior to their ingression (HH11), as early as HH8, and considerably earlier than previously suspected (HH16). Furthermore, cells exiting the cell cycle shortly thereafter express the ophthalmic trigeminal placode marker Pax3 (HH9). At HH11, these post-mitotic Pax3+ placode cells begin to express the pan neuronal marker, neurofilament, while still in the ectoderm. Analysis of the ectodermal origin and distribution of these early post-mitotic neurons reveals that the ophthalmic placode extends further rostrally than anticipated, contributing to neurons that reside in and make a significant contribution to the ophthalmic trigeminal nerve. These data redefine the timing and extent of neuron formation from the ophthalmic trigeminal placode.

127. Fate map and morphogenesis of presumptive neural crest and dorsal neural tube

Maxellende Ezin, Scott E. Fraser, Marianne Bronner-Fraser

In contrast to the classical assumption that neural crest cells in chick are induced as the neural folds elevate, recent data suggest that they are already specified during gastrulation. This prompted us to map the origin of the

neural crest and dorsal neural tube in the early avian embryo. Using a combination of focal dye injection and time-lapse imaging, we find that neural crest and dorsal neural tube precursors are present in a broad, crescent-shaped region of the gastrula, juxtaposed to the future neural plate. Surprisingly, static fate maps together with dynamic confocal imaging reveal that the neural plate border is considerably broader and positioned more caudally than expected. Some degree of rostrocaudal patterning, albeit incomplete, is already evident in the gastrula. Time-lapse imaging studies showed that the neural crest and dorsal neural tube precursors undergo choreographed movements that follow a tight spatiotemporal progression from lateral to medial and from anterior to posterior. These movements include convergence and extension, reorientation, cell intermixing, and motility deep within the embryo. It is through those rearrangement and reorganization movements that, gradually, the neural crest and dorsal neural tube precursors become regionally segregated, coming to occupy predictable rostrocaudal positions along the embryonic axis. This regionalization occurs progressively and appears to be fixed in the neurula by stage 7 at levels rostral to Hensen's node. Interestingly, we find that the position of the presumptive neural crest broadly correlates with BMP4 expression domain from gastrula to neurula stages.

128. Dissecting early regulatory relationships in the lamprey neural crest gene regulatory network

Natalya Nikitina, Tatjana Sauka-Spengler, Marianne Bronner-Fraser

The neural crest, a multipotent embryonic cell type, originates at the border between neural and non-neural ectoderm. After neural tube closure, these cells undergo an epithelial-mesenchymal transition, migrate to precise, often distant locations, and differentiate into diverse derivatives. Analyses of expression and function of signaling and transcription factors in higher vertebrates has led to proposal that a neural crest gene regulatory network (NC-GRN) orchestrates neural crest formation. Here, we interrogate the NC-GRN in the lamprey, taking advantage of its slow development and basal phylogenetic position to resolve early inductive events, one regulatory step at the time. To establish regulatory relationships at the neural plate border, we assess relative expression of six neural crest network genes and effects of individually perturbing each on the remaining five. The results refine an upstream portion of the NC-GRN and reveal unexpected order and linkages therein; e.g., lamprey AP-2 appears to function early as a neural plate border rather than a neural crest specifier and in a pathway linked to MsxA but independent of ZicA. These findings provide an ancestral framework for performing comparative tests in higher vertebrates in which network linkages may be more difficult to resolve due to their rapid development.

129. Isolation and characterization of neural crest stem cells derived from *in vitro* – differentiated human embryonic stem cells

X. Jiang, Y. Gwye, Sonja McKeown, Marianne Bronner-Fraser, C. Lutzko, Elizabeth Lawlor

The neural crest is a transient structure of vertebrate embryos that initially generates neural crest stem cells (NCSC) that then migrate throughout the body to produce a diverse array of mature tissue types. Unfortunately, due to the rarity of adult NCSC, as well as ethical and technical issues surrounding isolation of early embryonic tissues, biologic studies of human NCSC are extremely challenging. Thus, much of what is known about human neural crest development has been inferred from model organisms.

We report that NCSC can be rapidly generated and isolated from *in vitro* – differentiated human embryonic stem cells (hESC). Using the stromal-derived inducing activity (SDIA) of PA6 fibroblast co-culture we have induced hESC to differentiate into neural crest. Within one week migrating cells that express the early neural crest markers p75 and HNK1, as well as numerous other genes associated with neural crest induction such as *SNAIL, SLUG and SOX10* are detectable. FACS-based isolation of the p75 positive population enriches for cells with genetic, phenotypic and functional characteristics of NCSC. These p75-enriched cells readily form neurospheres in suspension culture, self-renew to form secondary spheres, and give rise under differentiation conditions to multiple neural crest lineages including peripheral nerves, glial and myofibroblastic cells. Thus, this SDIA protocol can be used to successfully and efficiently isolate early human NCSC from hESC *in vitro.* This renewable source of NCSC provides an invaluable source of cells for studies of both normal and disordered human neural crest development.

130. Semaphorin3A/neuropilin-1 signaling acts as a molecular switch regulating neural crest migration during cornea development

Peter Lwigale, Marianne Bronner-Fraser

Cranial neural crest cells migrate into the periocular region and later contribute to various ocular tissues including the cornea, ciliary body and iris. After reaching the eye, they initially pause before migrating over the lens to form the cornea. Interestingly, removal of the lens leads to premature invasion and abnormal differentiation of the cornea. In exploring the molecular mechanisms underlying this effect, we find that semaphorin3A (*Sema3A*) is expressed in the lens placode and epithelium continuously throughout eye development. Interestingly, neuropilin-1 (*Npn-1*) is expressed by periocular neural crest but down regulated, in a manner independent of the lens, by the subpopulation that migrates into the eye and gives rise to the cornea endothelium and stroma. In contrast, *Npn-1* expressing neural crest remain in the periocular region and contribute to the anterior uvea and ocular blood vessels. Introduction of a peptide that inhibits Sema3A/Npn-1 signaling results in premature

entry of neural crest cells over the lens that phenocopies lens ablation. Furthermore, Sema3A inhibits periocular neural crest migration *in vitro*. Taken together, our data reveal a novel and essential role of Sema3A/Npn-1 signaling in coordinating periocular neural crest migration that is vital for proper ocular development.

131. N-cadherin acts in concert with Slit1-Robo2 signaling in regulating aggregation of placode-derived cranial sensory neurons

Celia Shiau, Marianne Bronner-Fraser

In vertebrates, cranial sensory ganglia have a dual origin from the neural crest and ectodermal placodes. In the largest of these, the trigeminal ganglion, Slit1-Robo2 signaling mediates neural crest-placode interactions important for proper ganglion assembly. We demonstrate a critical role for the cell adhesion molecule N-cadherin during this process. In the chick embryo, N-cadherin is expressed by placodal neurons in the surface ectoderm and ganglionic anlage of all cranial sensory ganglia but not by migrating cranial neural crest loss of N-cadherin function in placodal cells results in formation of dispersed ganglia, similar to that observed after loss of Robo2. Conversely, coexpression of full-length N-cadherin partially rescues the Robo2 loss-of-function phenotype, suggesting they may function in concert. Furthermore, perturbing Slit1-Robo2 signaling alters the localization pattern of N-cadherin in placode cells. Like the trigeminal, epibranchial placodes express Robo2 whereas the hindbrain neural crest expresses Slit1. Together, the data suggest a novel mechanism whereby N-cadherin appears to work together with Slit-Robo signaling between neural crest and placodal cells in mediating placodal cell adhesion, required for proper ganglion formation.

132. Myosin-X is critical for migratory ability of *Xenopus* cranial neural crest cells

Shuyi Nie, Yun Kee, Marianne Bronner-Fraser

The neural crest is a highly migratory cell population, unique to vertebrates, that forms much of the craniofacial skeleton and peripheral nervous system. In exploring the cell biological basis underlying this behavior, we have identified an unconventional myosin, myosin-X (Myo10) that is required for neural crest migration. Myo10 is highly expressed in both premigratory and migrating cranial neural crest (CNC) cells in *Xenopus* embryos. Disrupting Myo10 expression using antisense morpholino oligonucleotides leads to impaired neural crest migration, but only a slight delay in induction. *In vivo* grafting experiments reveal that Myo10-depleted CNC cells migrate a shorter distance and fail to segregate into distinct migratory streams. Finally, *in vitro* cultures and cell dissociation-reaggregation assays suggest that Myo10 may be critical for production of cell protrusions and cell-cell adhesion. These results demonstrate an essential role for Myo10 in normal cranial neural crest migration and suggest a link to cell-cell interactions and formation of processes.

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Summary: The major focus of research in our laboratory is the systems biology of the gene regulatory networks (GRNs) that control development, and the evolution of these networks. Most of our research is done on sea urchin embryos, which provide key experimental advantages. Among these are: an easy gene transfer technology, which makes this a system of choice for studying the genomic regulatory code; advanced molecular biology technologies for high throughput perturbation of gene expression in the embryo; a novel high throughput method co *cis*-regulatory analysis; multiple means of visualizing and measuring gene expression; availability of embryonic material at all seasons of the year; an optically clear, easily handled embryo that is remarkably able to withstand micromanipulations, injections 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). There is also a rich collection of arrayed cDNA and BAC libraries for many other species of sea urchin, at various degrees of relatedness to this one. The genome of *S. purpuratus* has been sequenced at HGSC (Baylor) and annotated. We utilize additional experimental echinoderm models for evolutionary GRN comparisons, viz. the starfish *Asterina miniata* also of local provenance, and the primitive "pencil urchin" *Eucidaris tribuloides*. The embryos of both these animals prove to be as excellent subjects for gene regulation molecular biology as is that of the sea urchin.

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. It has become apparent that the only level of analysis from which causal explanations of major developmental phenomena directly emerge, is the GRN system level.

The main research initiatives in our laboratories at the present time are as follows: ***i. Analysis of the gene regulatory network underlying endomesoderm specification in* S. purpuratus *embryos***: At present over 60 regulatory and signaling 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 perturbation data obtained by gene expression knockouts and other methods, combined with experimental embryology. A predictive model of the GRN has emerged which indicates the inputs and outputs of the *cis*-regulatory elements at its key nodes. This model essentially provides the genomic regulatory code for specification of the endomesodermal territories of the embryo, up to gastrula stage. 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 was 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 the required specific regulatory inputs and outputs linking the genes within the GRN. These predictions are subject to direct experimental *cis*-regulatory test, and correction. We have now authenticated the predicted *cis*-regulatory inputs into genes at a majority of the key nodes of the current GRN. At these nodes are regulatory genes into which there are multiple regulatory inputs from genes elsewhere in the GRN, and multiple outputs to other genes in the GRN. For some regions of the GRN the analysis is approaching maturity, in that it extends convincingly from maternal inputs to cell-type differentiation. The best example is the GRN subregion determining skeletogenic micromere specification. Overall, the results of these experiments are converting the GRN from a model proposition into a hard-wired map of the genomic control logic for this portion of development. At present among *cis*-regulatory systems that are the subject of experimental analysis are those of the following genes: *alx1, tgif, hex, foxa, brachyury, pmar1, hesC, gcm, hox11/13b, tbr, evenskipped.* ***iii. Completion of the repertoire of regulatory genes engaged in the endomesoderm GRN:*** We used the 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 were determined, and for those genes sufficiently expressed in the embryo, the spatial patterns, as well. Regulatory genes were identified in this manner that evidently play a role in endomesoderm specification, because they are expressed specifically in the endomesodermal territories at the relevant times, but that had not yet been incorporated into the GRN. All of these genes are now being linked into the GRN by perturbation and *cis*-regulatory analysis; this project has been completed for the skeletogenic micromere lineage and is in process for the non-skeletogenic mesoderm and the endoderm. The results include a new understanding of the processes of endoderm specification and of establishment of the fate boundaries between endoderm and mesoderm that will shortly be included in a revised GRN. ***iv. Evolution, viewed as a process of change in GRN architecture:*** Starfish and sea urchins shared a last common ancestor about 500 million years ago. Thus, analysis of the GRN controlling endomesoderm specification 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. Examples of both have now been documented. The underlying processes are of course change, or alternatively, conservation, of functional *cis*-regulatory features. To study this we are examining starfish/sea urchin GRN differences at the *cis*-regulatory level. A second ongoing project is an attempt to reprogram the development of the skeletogenic cell lineage in a primitive sea urchin, *Eucidaris tribuloides*, by inserting regulatory apparatus from *S. purpuratus*. These echinoids diverged from a common ancestor in the Middle Triassic and generate their embryonic skeleton in different ways. We term this Synthetic Experimental Evolution. We have already been successful using this approach in causing the *Eucidaris* skeletogenic lineage to invaginate precociously as does that of *S. purpuratus*. ***v.* cis*-Regulatory evolution and interspecific recognition of* cis*-regulatory modules:*** We are carrying out a sequence level evolutionary analysis of experimentally authenticated *cis*-regulatory modules from nine different genes in four species of sea urchin. At a 50 mya divergence distance (*S. purpuratus* and *Lytechinus variegatus*) *cis*-regulatory modules and exons are the only conserved sequence elements. Surprisingly, of authenticated transcription factor target sites, about a third are either novel additional occurrences of given sites in one or the other species, or are sites that have changed position within the conserved module. However, some proximal site pairs recur repeatedly. At the closer distance (~15-20 mya) represented by two congeners for which genomic sequence exists (*Allocentrotus fragilis* and *Strongylocentrotus franciscanus*), *cis*-regulatory modules are marked by sharply decreased frequencies of large indels. These are otherwise a major mechanism of divergence in unselected sequence. On the other hand at the divergence distance of 150 mya represented by the species pair *Arbacia punctulata* and *S. purpuratus*, *cis*-regulatory sequence is not conserved except for target sites. Interspecific gene transfer tests show that the regulatory landscape has changed so much over this interval that only about half of the *cis*-regulatory systems of one species works when introduced (as recombineered BACs) into the other. ***vi. Oral and aboral ectoderm GRNs:*** We have recently attained draft GRNs for oral and aboral ectoderm specification including about 30 more regulatory genes. This is part of an effort to extend the same kind of causal, system level GRN analysis to the whole embryo, and represents a major step toward that goal. There is only one additional early embryonic territory, the apical neurogenic region, which is being studied in other sea urchin laboratories. The aboral ectoderm generates a single cell type, but the oral ectoderm gives rise to several distinctly functioning domains: mouth, columnar "facial" epithelium, neurogenic ciliary band, and the ectodermal signaling stripes which determine the location of the skeletal rods. The approach is to obtain all the regulatory players expressed in oral and aboral ectoderm, and ciliary band, from the analysis of all genes encoding transcription factors predicted in the genomic sequence, and engage them in a provisional network by carrying out a matrix of perturbation experiments. The network is anchored at the onset of the ectodermal specification process, of which the initial gene zygotically expressed on the oral side is *nodal*. The *cis*-regulatory module controlling early oral ectoderm expression of *nodal* has been thoroughly characterized: its target sites provide the direct links between the initial cytoplasmic anisotropy by which the future oral and aboral

sides of the embryo are distinguished, i.e., differences in redox potential, and differential zygotic gene expression. The ciliary band is also specified early by a nodal independent pathway at top of which is the regulatory gene hnf6, which is the initial component of a ciliary band-specific GRN. ***vii. Various explorations by new methods and approaches:*** As always, we are trying to expand knowledge by use of novel technologies for analysis of the GRN and the genome. Current applications of new technology include increasingly widespread use of *in vitro* reengineered BAC recombinants, which we are supplying to the whole sea urchin field; use of these in first attempts to "redesign" the process of embryonic development, by introduction of altered regulatory subcircuits in novel spatial domains; and extensive application of two-color gene transfer experiments in which the control version of a *cis*-regulatory construct drives expression of a reporter detectable in one color and a mutated version injected with it and incorporated in the same cells drives expression of a reporter detected in a different color. Most importantly, we have also developed a completely novel method for blocking expression of any gene whenever and wherever desired, by injection of morpholino antisense oligonucleotides into blastocoel whenever desired. This method allows us to determine the function of regulatory genes that have multiple activity phases in one of the later phases, in embryos that develop normally up to that point. Another new technology that has had a major impact is utilization of NanoString technology, a new instrument that permits direct automated counting of transcripts of one-several hundred genes in control and experimentally perturbed lysates of small numbers of embryos with high accuracy efficiency. ***viii. Revolutionizing* cis*-regulatory analysis:*** We are seeking to improve the efficiency of *cis*-regulatory analysis by large factors. First, we have shown that high coverage Solexa reads of *Lytechinus* BACs containing given genes can be used to identify conserved *cis*-regulatory modules by mapping them on the *S. purpuratus* genome sequence, using an elegant display apparatus, "Cis-Browser," developed by our colleague Sorin Istrail (Brown University). Second, we have found that FACS sorted, disaggregated embryos expressing regulatory constructs producing GFP provide populations of expressing cells that can be analysed by QPCR to determine spatial and temporal domain of *cis*-regulatory expression. This obviates time consuming microscopic screening and can be done on many samples at once. Third, we have developed multiple "bar-coded" tags for use as reporters so that many samples can be injected into embryos at once and analysed together by NanoString or QPCR. There are two systems now in use in our lab, a 13-tag and a 129-tag system allowing simultaneous analysis of these numbers of individual constructs. ***ix. Computational approaches to regulatory gene network analysis:*** The GRN visualization software BioTapestry, developed by our collaborators Hamid Bolouri and Wm. Longabaugh at ISB, is now in wide use, and we are further expanding its capacities so that it will automatically generate allowed network architectures from machine readable time and space of expression data plus results of perturbation analysis. A second-generation version with much enhanced capacities has been published. Many additional computational genomics and other projects are summarized below. ***x. New genomics projects:*** HGRI has recently agreed to support genomic sequencing of additional echinoderm species, and the initial targets include *Lytechinus variegatus* and the sea star referred to above, *Asterina miniata*. The leaders in this project are Richard Gibbs and Kim Worley at the Baylor College of Medicine Human Genome Sequencing Center (BCM-HGSC) in Houston, and together with them we are working on utilization of the new sequencing platforms to obtain these and other high quality genomic sequences. ***xi. GRN structure:*** As we delve more deeply into the functional organization of the sea urchin embryo GRNs several apparent principles of their organization are emerging. (a) They are composed of modular subcircuits with discrete logic functions. (b) These subcircuits evolve at different rates within the same GRN and may have diverse evolutionary origins. (c) Multiple, overlayered subcircuits are brought to bear on given developmental processes to ensure that they function accurately and reproducibly: the "wiring" can be described as fail-safe, and is clearly not parsimonious in design. (d) Fail-safe wiring accounts for the resilience of the developmental process, including the regulative ability of the embryo to recover from potential regulatory disaster, as we show explicitly in one of the projects described below.

The Center for Computational Regulatory Genomics

R. Andrew Cameron, Director

Eric H. Davison, Principal Investigator

The mission of the Center for is to initiate and support comparative studies designed to identify and characterize regulatory information in the genome. In particular, the Resource focuses on the genomic information of lower deuterostomes especially echinoderms. This focus emerged from the production of a draft genome sequence for the purple sea urchin, *Strongylocentrotus purpuratus* (Sp), and continues as genome resources for other species in this group become available. Activities of the Center include production of materials and data structures to support the genome sequencing enterprise and efforts to utilize this genomic information effectively. The latter goal is centered on the elucidation of gene regulatory networks in development and the evolutionary analysis of the genomic information within this clade. These goals are accomplished through a variety of approaches that include automated molecular biology, recombinant DNA and gene transfer, and broadly based computational efforts.

This mission is realized in three overlapping areas of activity. The Genomics Facility is a high-throughput library arraying and printing operation that supplies macroarray libraries and clones to the community. The molecular biology branch tests and refines techniques associated with molecular developmental biology, gene

transfer, and the routine aspects of *cis*-regulatory analysis developed in the Davidson laboratory. The computational branch supplies software to the sea urchin developmental biologist and maintains databases fundamental to the Sea Urchin Genome Project, an initiative that began in the Davidson laboratory and at the Genomics Technology Facility.

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 macro-array libraries and filters. We currently maintain in -80°C freezers 40 different echinoderm libraries comprising a total of approximately four 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. The existing genomic DNA and cDNA libraries that were so extensively employed for the annotation of the sea urchin genome are stably maintained in our freezers. We print new filters for these as needed. In addition, we have made and arrayed an *Arbacia punctulata* mixed-stage larval cDNA library and another *Lytechinus variegatus* BAC library to complement sequencing efforts. Our complement of libraries is listed at SpBase (http://spbase.org). During the past year we have arrayed a total of 120,000 colonies and printed a total of 317 macroarray filters. Research materials have been provided to research laboratories ranging from University of Washington to Stazione Zoologica Anton Dohrn (Italy).

Our collaboration with the BCM-HGSC continues on several fronts. We provided material for, and later computational analysis of a skim-sequencing project in two related species, *Strongylocentrotus franciscanus* and the deep water species, *Allocentrotus fragilis*. The 2X coverage provides a glimpse into the genomic parameters between these and our reference species, *Strongylocentrotus purpuratus*. These sequences will be used to confirm our model that predicts a constraint for large insertions and deletions in *cis*-regulatory modules.

As the elucidation of gene regulatory networks proceeds, more genes become candidates for comparative sequence analysis correlated to function. Genomic sequences from species that diverged from the reference at times from 20-50 mya are most useful for this analysis. At the Center we are responsible for identifying and characterizing BAC clones containing genes that play a role in gene regulatory networks in early development of *Strongylocentrotus purpuratus* (Sp), our reference species. BACs from *Lytechinus variegatus* (Lv; 50 mya diverged) representing a majority of these genes have been recovered. From libraries for two species that are 20 mya diverged from our reference, *Strongylocentrotus franciscanus* (Sf) and *Allocentrotus fragilis* (Af), we are now recovering BACs for these same genes. We have performed in-house Illumina/Solexa sequencing of BACs for the Sf and Af genes. We are combining the 32 bp Solexa reads and the 454 reads from Baylor to give gap-free sequences across known *cis*-module regions for a four-way sequence comparison (Sp, Lv, Sf, and Af).

Research Center

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.

One aspect of the Center is the Sea Urchin Genome Resource that maintains information resources that are used widely in the sea urchin research community. We provide sequence information through the Sea Urchin Genome Project web site (http://spbase.org/). With the advent of the web resources for annotation established at the Human Genome Sequencing Center, Baylor College of Medicine and the Sea Urchin Genome Resources at NCBI, we have not seen the need to expand our local databases. However, we have refined the cross-index between our library clones and sequences stored in public databases at NCBI. Since so many of our libraries were used for the sequencing project, and the library location for the clones was preserved in the sequence information, we can provide a searchable sequence database from which the user can obtain clone information and order the clone. This "clone by computer" method renders our arrayed libraries extremely useful and readily accessible for the working molecular biologist.

Beowulf Cluster Hardware and Configuration

Our 40-unit Beowulf cluster continues to operate with a minimum of downtime. The Rocks software stack of software components has indeed proven to be an efficient system with which to build, operate and maintain the cluster. We are processing the entire genome sequence on the cluster in various ways as part of the genome database. We further expect to queue search jobs from the genome database through this cluster.

133. Regulative recovery in the sea urchin embryo, and the stabilizing role of fail-safe gene network wiring

Joel Smith

Gene regulatory networks for development are organized to ensure reproducible and invariant embryonic process, and one means is "fail-safe" design of the subcircuits constituting these networks. *cis*-Regulatory studies on a network subcircuit activated early in the development of the sea urchin embryo reveal a sequence of encoded fail-safe regulatory devices. These ensure maintenance of fate separation between skeletogenic and

non-skeletogenic mesoderm lineages. An additional unexpected design feature enables the process of embryogenesis to "recover" from a regulatory interference that has catastrophic effects if this feature is disarmed. Genomically encoded back-up control circuitry thus, provides the mechanism underlying a specific example of the regulative development for which the sea urchin embryo has long been famous.

134. Regulatory logic of endoderm development in pre-gastrula-stage sea urchin embryos

Isabelle Peter, Jina Yun

The sea urchin embryonic gut is formed by derivatives of two cell lineages that become separated during cleavage stages. The contribution of these cell lineages to the gut is biased along the anterior/posterior axis. The veg2 cell lineage constitutes to the foregut and part of the midgut, whereas veg1-derived cells form part of the midgut, as well as the hindgut. To analyze the regulatory states present in the nuclei of these cell lineages, we have determined the expression pattern of transcription factors by double fluorescent *in situ* hybridizations. We found that the combination of regulatory factors present in veg2 and veg1 cells is different at all stages examined. At early blastula stages, most of the transcription factors involved in endoderm specification are expressed in the veg2 lineage, whereas only *even-skipped* (*eve*) showed expression in veg1-derived cells. However, at mesenchyme blastula stage, some of the regulatory factors initially expressed in the veg2 lineage are turned off in these cells and become restricted to the veg1 lineage. The architecture of the underlying gene regulatory network was analyzed by perturbing the expression of each regulatory factor and determining the resulting change in gene expression levels of all other genes. Consistent with the spatial expression pattern, perturbation of Eve expression did not affect the expression of any other regulatory gene in the endoderm GRN at early blastula stages. A few hours later, however, Eve is responsible for activating the expression of additional regulatory genes in the veg1 lineage, including the posterior hox gene encoding Hox11/13b, which in turn controls the expression of *brachyury*. Endoderm specification in the veg2 and veg1 cell lineages is therefore, driven by different gene regulatory networks, which might be the cause of anterior-posterior polarity in the future larval gut. The regulatory network driving endomesoderm specification leads to the formation of five territories, each expressing a specific combination of regulatory factors. We are now in the position to describe a comprehensive network for pre-gastrular endoderm regionalization.

135. Solving sea urchin ectodermal gene regulatory network

Enhu Li, Yi-Hsien Su[*]

Gene regulatory networks (GRNs) consist of interacting regulatory genes and provide a mechanistic understanding of developmental processes. Previous efforts had been dedicated to solve the sea urchin endomesodermal GRN, while here we extended gene regulatory network analysis to the adjacent oral and aboral ectoderm territories. Genes involved in the ectodermal formation were first identified by whole mount *in situ* hybridization. To see the interaction among regulatory genes, we carried out large-scale perturbation assays in which expression of each gene was interrupted by introduction of morpholino antisense oligonucleotide (MASO). Resultant expression changes were quantified by QPCR, and by nCounter Analysis System. Perturbations data and spatiotemporal expression pattern were combined to infer network linkages, and construct GRN model. Our current ectodermal GRN includes 22 genes of transcription factors, four genes encoding known signaling ligands, and three genes that are yet unknown but are predicted to perform specific roles.

A well-established GRN provides predictive power that can be used to generate detailed descriptions of missing components. Previously a putative repressor (Repressor A) was proposed in the ectodermal GRN downstream of *gsc*. *Gsc* had been shown as a key oral determinant and *gsc* knockdown results in complete loss of the oral ectoderm. The expression profile of *gsc*-MASO-treated embryos also showed broad downregulation of oral ectodermal genes. However, previous studies indicated that *gsc* is a committed repressor therefore, its function in defining the oral ectoderm should be better explained though repressing another putative repressor (Repressor A). Based on the current network model, we predicted the properties pertinent to this putative repressor. A subsequent screening was performed to identify upregulated genes upon gsc MASO perturbation. The promising candidate *sip1* gene was further characterized using perturbation assay combined with expression analysis. WMISH revealed that *sip1* gene expression is pan-ectodermal but soon becomes aboral. Knocking down *sip1* caused upregulation of *foxg*, while overexpression of *sip1* was able to suppress ectodermal *bra* expression. Evidence acquired in this report, justified *sip1* as the Repressor A, and *sip1* and *gsc* constitute a double-negative gate that specifies oral ectoderm.

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136. Mesoderm specification in sea urchins

Stefan C. Materna

The mesoderm in the sea urchin gives rise to different cell types including pigment cells and blastocoelar cells both of which have immune function. Initially, the endomesoderm forms a ring around the

skeletogenic lineage at the center of the vegetal plate. A Delta/Notch signal originating in the skeletogenic cells activates the *glial cells missing* (*gcm*) gene in endomesodermal cells. After endo- and mesodermal lineages split, *gcm* expression is maintained in the ring of mesodermal cells. This at the outset homogenous ring is subdivided into an oral region (blastocoelar cells) and an aboral region (pigment cells) by the mesenchyme blastula stage. A number of genes whose vertebrate orthologs are employed in hematopoiesis, including *gatac*, *scl*, *ese*, and *prox*, become activated in the oral mesodermal patch while *gcm* expression becomes confined to the opposite, aboral area.

Following exhaustive genetic perturbation of potential upstream activators we monitored changes in transcription of 180 (regulatory) genes that cover the mesoderm and other territories in the embryo with the Nanostring nCounter, a novel high throughput method that allows accurate measurements of low abundance transcripts. These experiments show that the initial Delta/Notch (D/N) signal is necessary for the establishment of both oral and aboral mesodermal regions. *gcm* expression, albeit panmesodermal in the beginning, is important only for the establishment of aboral or pigment cell fate. Instead, ectodermal genes involved in oral/aboral axis establishment appear to play a significant role in mesoderm subdivision. Thus, the activation of oral mesodermal genes may be best described as an AND logic between the D/N signal and an as yet to be identified signal from the oral ectoderm.

We are currently investigating what ectodermal gene(s) lie upstream of the inferred signal. Also, we are characterizing the relationships among the newly identified mesodermal genes to understand the regulatory architecture that underlies the lock down of mesodermal cell fates.

137. The information processing at the *foxa* node of the sea urchin gene regulatory network

Smadar Ben-Tabou de-Leon

The transcription factor *foxa* plays a central and evolutional ancient role in the endoderm specification. In the sea urchin embryo *foxa* is essential for the gut formation and for exclusion of mesodermal fate in the endoderm. The early expression pattern of *foxa* is broad in the endomesoderm progenitor field, however in about 12 hours it resolves to specific endodermal sub-domain. We utilize the advanced state of the sea urchin endomesoderm gene regulatory network and combined quantitative experimental methodologies with kinetic modeling to study the *cis*-regulatory device that controls *foxa* expression. We find that no fewer than five *cis*-regulatory modules interact with each other and switch their dominance in controlling *foxa* expression in different spatial domains and at different times. We conducted a detailed mutation and perturbation analysis to study the inputs to each of the modules. We identified the ectoderm and mesoderm repressor and the early and late activators of *foxa*. The binding sites of these inputs are located at different *cis*-regulatory modules, which indicate that the modules interact with each other to produce the correct spatial pattern and expression level. *foxa* is downstream of both Wnt-βcatenin and Delta-Notch signaling pathways and its complex and dynamic expression is regulated by a combination of excluding repressors, permissive switches and localized activators. A critical question that came out of our study was the lingering effect of transient activators on *foxa* expression level. An example of such gene is the transcription factor Brachyury that overlaps with *foxa* for few hours and then turns off in the *foxa* expressing cells. We applied a kinetic model and were able to explain how transient inputs affect the level of their downstream target hours after they had turned off in the target expressing cells. We were therefore, not only able to decipher the specific genomic code controlling a key regulatory gene but also gained insights into the general design principles of the regulatory genome.

138. *cis*-Regulatory analysis of *Strongylocentrotus purpuratus Alx*1 gene

Sagar Damle

The *SpAlx1* gene encodes a homeodomain transcription factor that is deployed during embryonic skeletogenesis in the purple sea urchin. Its expression pattern is biphasic with expression peaks at midblastula stage (roughly 11 hpf) and mesenchyme blastula stage (24 hpf). It is expressed initially in the large micromeres at early blastula stage (7 hpf) and continues as these cells develop into primary mesenchyme (PMC) and ultimately to skeletogenic cells. The *Alx1* gene contains six exons spanning roughly 50kb and sits in a gene-poor region of the genome. When expressed, Alx1/Cart1 family members are thought to homodimerize and heterodimerize with other family members while binding regulatory DNA. In the sea urchin embryo, Alx1 is necessary for ingression of PMCs into the blastocoel, migration, and skeletogenesis through the production of skeletal matrix proteins.

The *cis*-regulatory modules controlling both early and late expression are contained 6kb upstream of the start site of transcription. GFP reporter constructs containing this region are capable of recapitulating spatially the endogenous Alx1 expression pattern. This sequence contains three conserved *cis*-regulatory modules roughly 500 bp in length. The proximal module contains HesC binding sites and restrict expression to domains that lack the Hes-c repressor namely the large micromeres at early blastula stage and primary and secondary mesenchyme prior to hatching blastula stage (16 hpf). The middle and distal modules contain binding sites for amplifiers of expression and are necessary for producing the 11-hour expression peak. Morpholino knockdown of Otx, Ax1 and ets1 affect Alx1 expression. The distal and proximal modules contain putative binding sites for these inputs. Currently their functions are being validated through mutational analysis of reporter constructs

139. *cis*-Regulatory analysis of *SpTgif*

Qiang Tu

Positive feedback subcircuits are used as a dynamic state stabilization device, frequently found within developmental gene regulatory networks (GRNs). A three-gene positive feedback loop composed of *erg*, *hex* and *tgif*, has been identified in a large-scale perturbation analysis for primary mesenchyme cell (PMC) specification in the sea urchin embryo. Ultimately, the goal of the present study is to understand this subcircuit by identifying all *cis*-regulatory modules (CRMs) functioning in the feedback.

Phylogenetic footprinting is a reliable way to identify putative CRMs. One major difficulty is that this method requires high quality genome sequences from more than one species appropriately distant from each other, evolutionarily speaking. Deep sequencing, an emerging technology, provides a quick solution for phylogenetic footprinting. The reads obtained in deep sequencing are generally too short to be assembled into larger segments. However, they might be sufficient to recognize conserved patches by mapping reads of one species onto a well-assembled genome of another species. To test this idea, we applied this method in the present study, specifically by employing the Solexa (Illumina) sequencing platform, and have successfully identified a PMC-specific CRM for *tgif*.

This CRM has been refined to a 665 bp DNA fragment, showing it is sufficient to drive the PMC specific expression as endogenous *tgif* does. We also tested the necessity of this module by knocking it out from a GFP-knockin BAC construct that contains 30 kb of gene sequence and 70 kb of flanking sequence. The knockout construct losses its PMC specific expression. According to the PMC GRN architecture, *tgif* has four inputs: *ets*, *erg*, *hex*, and *tgif* itself from mid-blastula to the mesenchyme blastula stage (18-24 hpf), although the input from *hex* at 24 hpf is marginal. To corroborate this, constructs were generated in which three types of biding sites (canonical ETS family, HEX, and TGIF sites) are individually disrupted. Once mutated, both the Ets and Tgif sites virtually loss all regulatory ability. Constructs with mutant Hex sites remain to be evaluated.

140. Regulatory gene interactions that drive development of the neurogenic ciliated band in the sea urchin embryo

Julius C. Barsi

Ectoderm specification in the sea urchin embryo has recently been subjected to large-scale gene regulatory network (GRN) analysis (*). This particular project, attempts to push beyond 30 hpf. At around this time a distinct embryonic territory emerges, known as the neurogenic ciliated band (nCB). It is unique in that it lies adjacent to almost all other embryonic territories, and that it will foreshadow the location of neuroblasts outside of the apical domain. The earliest regulatory gene expressed in the nCB is Hnf6 (Figure 1). In contrast to most transcription factors (TFs) found in the oral ectoderm, Hnf6 expression does not require Nodal activity. Data obtained thus far, suggests that this TF drives the GRN module underlying nCB identity. Next generation gene expression analysis combined with a novel perturbation approach, was used to resolve the regulatory gene interactions at work within this module. FoxG lies immediately downstream of Hnf6. It is clear that FoxG in turn, drives but a single TF within the nCB, this being OtxB1/2. Interestingly enough, we find that OtxB1/2 reciprocally regulates FoxG expression. This direct, dynamic transcriptional feedback circuit, can be interpreted as a lockdown of the nCB regulatory state. Hnf6 not only drives development of the nCB, but also serves to generate a sub-domain characterized by the expression of Nk1. Lim1 is expressed along the ectoderm/vegetal-endomesoderm border that runs perpendicular to the nCB. In what appears to be a classical case of AND logic, Nk1 is activated precisely where Hnf6 and Lim1 expression overlap. Furthermore, perturbation of either results in Nk1 down-regulation. The GRN module responsible for nCB formation has been identified, the main features of which have been outlined above. Perhaps equally interesting, will be to determine the direct input required for Hnf6 expression.

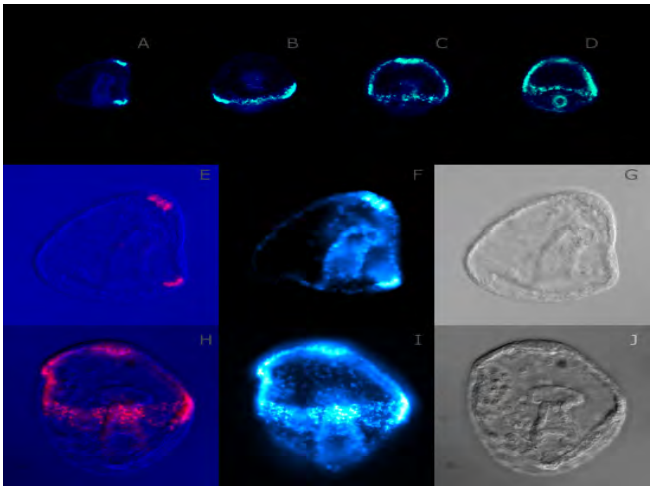

Figure 1. Hnf6 expression highlights the nCB in *Strongylocentrotus purpuratus* 35 hpf as detected by fluorescent RNA *in situ* hybridization. All images pseudo-colored in such a way as to maximize the contrast with respect to their background. (A, E, F, G) Lateral view, (B) Apical view, (C, H, I, J) Anterior view, (D) Vegetal view, (G, J) Differential interference contrast microscopy. (A, B, C, D, F, I) Fluorescent microscopy. (E, H) Merge between F, G and I, J, respectively. (A-D) 200x magnification, (E-J) 400x magnification.

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141. Novel approaches for stage-specific gene perturbation in the sea urchin embryo

Julius C. Barsi

The sea urchin embryo has remained a premier model organism for studying animal development, in large part because it is amenable for gene specific loss-of-function. The method of choice, exploits the ability of morpholino-substituted antisense oligonucleotides (MASO) to block translation of transcripts in a sequence dependent manner. This approach, in as far as our model organism is concerned, carries with it two inherent constraints.

The first has to do with MASO delivery. This is achieved through pressure-controlled microinjection. The window of time for which this is possible is limited to a short period soon after fertilization. The second concerns the mechanism by which a MASO functions. That is to say, it becomes effective the moment it is introduced. Therefore, current methods do not allow gene-specific perturbation to be carried out with spatial and temporal resolution. In order to address later stages of embryonic development, it becomes necessary to distinguish amongst the different phases of expression from a same gene. This problem has been addressed before successfully (1). Nevertheless, such DNA-based methods suffer from mosaic incorporation. This project takes a two-pronged approach, each aimed at relaxing one of the two constraints described above. We have developed a protocol that revolves around microinjection into the blastocoel (http://www.its.caltech.edu/~barsi/protocol).
This makes use of a novel peptide, with the ability to mediate endocytic take-up and subsequent release in order to deliver MASO to the surrounding tissue (2). This procedure has been shown to work, and relies on COPAS BIOSORT instrumentation in order to obtain a homogenous population of perturbed embryos. Currently under development is a light-inducible system, which would allow us to inhibit the function of a MASO until activated.

142. A novel experimental method for the multiplex and quantitative measurement of *cis*-regulatory module activity *in vivo*

Jongmin Nam

The need for high throughput *in vivo* functional assays of *cis*-regulatory modules (CRMs) has been increasing: to better understand the role and evolution of noncoding regions; to better build and authenticate gene regulatory networks among many other reasons. To accelerate *cis*-regulatory analysis, we recently developed a novel multiplex, quantitative method for the simultaneous measurement of CRM activity *in vivo*. A key aspect of this method is to use barcoded sequence tags to track the activities of many co-transformed CRMs. As a demonstration of this method using the sea urchin system, an initial set of sequence tags was successfully applied to measure temporal activities of ~400 putative CRMs that were predicted by a heuristic version of phylogenetic footprinting for the regions within or nearby 35 regulatory genes scattered throughout the genome. Immediate findings were as follows: on average ~3 CRMs per gene were found active during early embryogenesis and their temporal activities overlapped significantly; 5'-proximal regions were found active for >80% of the genes tested; the locations of the remaining CRMs varied. We currently have the power to track the activities of >100 CRMs simultaneously.

143. Comparative study of delta gene *cis*-regulatory modules across 500 million years of evolution

Feng Gao

The intercellular signals are one of the most common developmental GRN switches affecting the spatial location of GRN sub-circuit activity during development. An evolutionary signaling change high up in the GRN hierarchy was discovered by comparing the embryonic developmental GRNs of two echinodermal species, a sea urchin and a sea star, which share a common ancestor 500 mya. Delta-Notch signaling has been deployed in strikingly different ways in the two organisms. This provides a cardinal case of evolutionary flexibility in use of these signal-driven regulatory switches.

Since the Notch receptor during the time studied is relatively ubiquitously expressed, the function of the system primarily depends on the use of the Delta ligand. There are two aspects of difference in delta gene utilization. First, the delta gene of the sea urchin has a special *cis*-regulatory module controlling its expression in the skeletogenic domain of the embryo, which responds to the pmar1 micromere system not present in the sea star. Second, though in both species specification of the mesoderm is followed by expression of the delta gene, its activation in the sea star depends on endomesodermal tbr expression while tbr is not even expressed in the mesoderm in the sea urchin. While the discovery of the skeletogenic micromere module of the sea urchin delta gene provides the genomic basis of the first difference, the second is enigmatic.

The sea urchin mesodermal expression module has been recently isolated and analyzed as a subset of two broadly expressed TFs with Runt1 as the activator and HesC as the inhibitor. The principal goal is to now perform a *cis*-regulatory analysis of the sea star module and then compare to the sea urchin one. Of 15 clones isolated from BAC library screening, a sea star delta BAC of 150 kb was studied with GFP incorporated in the place of its first exon by homologous recombination and its sequence determined by the Genomic Sequencing Center of Washington University. A new method for high throughput *cis*-regulatory analysis was used to screen and analyze the sea star delta mesodermal modules. Constructs consisting of candidate modules and GFP and unique Tag sequences were built and injected into sea star embryos, and the *in vivo* regulatory functions of these modules were then analyzed through QPCR and fluorescence. A region of 3 kb is so far nailed down to reside all the necessary

elements to recapitulate its endogenous mesodermal expression. Presumable binding sits for HesC, Runt1 and Tbr were found within this 3 kb region from Cluster Buster analysis. Current studies are designed to authenticate the functions of these binding sites.

144. The sea urchin genome database, SpBase

R. Andrew Cameron, Qiu Autumn Yuan, Dong He, Ung-Jin Kim, Manoj Samanta, David Felt

The goal of the Sea Urchin Model Organism Database (http://spbase.org) is to provide genomic information about the purple sea urchin, *Strongylocentrotus purpuratus*, in a form convenient for the experimentalist. It incorporates the extensive manual annotations and gene models emerging from the work of the Sea Urchin Genome Sequencing Project as managed by BCM-HGSC and pursued by the members of the Sea Urchin Genome Annotation team. The database is a permanent home for these manual annotations and other meta-information that can be associated with the genome sequence including expression data and array analyses. The present instance of SpBase uses GMOD components to organize and present the sea urchin genome information. These open source components are accessed through high level links will likely not change as the web system elaborates. Most additions will occur under these headings. For example, we have added a PubMed link to each gene page if it is available. The genome browsers have been upgraded several times by adding additional track of sequences aligned to the reference genome. The latest addition is the alignments of *Allocentrotus fragilis* and *Strongylocentrotus franciscanus* 454 reads to the version 2.1 genome assembly. We have installed a Biomart search engine on our development version and it will be included in the next public build.

The average number of visitors remains consistent at about 50 per day over the year and a quick scan of the URLs that were logged show that connected sites included ones from Japan, Europe and the USA.

145. Improving the *Strongylocentrotus purpuratus* genome sequence assembly

R. Andrew Cameron, Manoj Samanta, Dong He

The BCM-HGSC has undertaken additional genome sequencing using the ABI SOLID sequencing system. They generated 500 million SOLID mate pair reads from which about 27 million were uniquely mapped to the 2.1 genome assembly and also had both ends mapped. We have been analyzing this additional sequence information to improve the assembly of the draft genome sequence. Of these 24 million had both ends on the same scaffold, and only three million fell on different scaffolds. The value of these high quality mate pair reads lies in their capacity to join existing scaffolds thus, reducing gaps. There are 1823 scaffolds longer than 100 Kb and we were able to order and orient 421 of these with other scaffolds longer that 20 Kb. At most we could chain together four scaffolds. About 9800 of the genome sequence is in scaffolds less that 3 Kb in length. We were able to place approximately 36,000 of these short scaffolds either in gaps in the large one or at the ends of the larger ones. In sum we reduced the total number of scaffolds by almost 25%. We continue to collaborate with the computational staff of the BCM-HGSC to organize these data and present another genome assembly.

146. Further annotation of the purple sea urchin gene set

R. Andrew Cameron, Qiu Autumn Yuan, Ung-Jin Kim

The great majority of genes in *S. purpuratus* have been identified by protein sequence homologies to putative orthologs in various non-sea urchin species. Approximately 10,000 gene candidates have been identified from the collection of 28,944 GLEAN3 gene models. Since *S. purpuratus* is estimated to have 23,300 genes, additional 13,300 genes have yet to be identified, mainly from the remaining ~19,000 gene models originally predicted from the draft genome sequence. In addition to the electronic, automated annotation process, we have undertaken a manual approach comparing the gene models to closely related species. Each of the predicted proteins is queried by BLAST against nonredundant protein set at the NCBI protein database, followed by visual analysis of the graphic output. Conserved protein domains and ortholog-like proteins are identified, and putative gene identity is assigned to the queried gene model after carefully inspecting the extent (span) of the matches and the degree of homologies between query and target sequences. Currently, we have annotated more than 7,000 additional gene models, of which roughly 40% have been matched to be homologs to known genes, 30% assigned to "hypothetical" protein group, 20% assigned to anonymous proteins named after their structural motifs, human open reading frame number or other arbitrary cDNA identifiers. A small fraction of newly annotated gene models (approximately 3%) appear to be spurious since they have no match at all or are potentially a chimera of two or more unrelated open reading frames.

To add further evidence supporting the gene predictions we have begun to work through the literature on sea urchin cell and developmental biology to link research reports to existing annotated genes. To date, 4,942 gene models have been linked to articles in PubMed, the National Library of Medicine Literature Archive.

147. Network gene annotation project

Qiu Autumn Yuan, R. Andrew Cameron

One of the strengths of the sea urchin embryo research model is the ease with which one can describe gene regulatory networks. As this experimental approach matures it is becoming clear that a gene annotation knowledge base is indispensible for designing experiments. Gene sequences, expression patterns and responses to perturbation are the central classes of information to be used here. Our previously built and

currently maintained database of sequence and expression data for sea urchin regulatory genes fills that role. The data housed in this suite of databases continues to grow as additional genes, expression patterns and network linkages are added to the existing information. The new ectoderm regulatory network studies from the Davidson laboratory have added a unique chunk of data to this effort. These unpublished data are available to a registered user group through a private Caltech web site. Our client base continues to grow as new users add more biological data. The latest addition is a function that graphically displays gene expression time-courses during embryogenesis.

148. The evolution of *cis*-regulatory module sequence at 50 mya divergence

R. Andrew Cameron

The echinoderm taxa make an unusually good set of species in which to examine the evolution of *cis*-regulatory modules (CRM). We have used genomic sequence comparisons covering a range of divergences from 20-540 million years (mya) in order to address a variety of questions about CRMs. Conserved sequence patches across 50 mya between *Strongylocentrous purpuratus* (Sp, our reference species) and *Lytechinus variegatus* (Lv) reveal candidate CRM sequences for gene regulatory network analysis. A number of CRMs identified by the comparison to Lv have been functionally characterized by perturbation studies and the gene transfer of mutated CRM sequences to reveal the specific input transcription factor binding sites. Thus, the input transcription factor binding sites could be similarly compared in the Lv sequence producing a fine-grained picture of the evolution of transcription factor binding sites within modules. We have considered eight *cis*-regulatory modules from seven genes, which on the *Sp* side total about 6.5 Kb of regulatory sequence. The sequence identities in the individual modules varies from 38% to 93% with a median value at 75.5%. The combined total is 72%. Binding sites for ten different transcription factors were included in the analysis. In the *Sp* gene regulatory networks, these appear as 19 qualitatively distinct, authenticated inputs interacting at 46 authenticated target sites. The majority of the sites remained relatively unchanged within their respective CRM, and the repertoire of inputs remained unchanged in every case. However, eight (17%) of the binding sites are in different relative positions in the orthologous CRMs, and 16 (30%) more either appeared *de novo* or were lost since divergence of the two species from their common ancestor about 50 mya. Given this overall picture of extensive plasticity in CRM organization even at this relatively close evolutionary distance, it is striking that there are seven closely apposed or overlapping site pairs that are perfectly conserved with respect to position and distance, *viz*. an Otx/Blimp site pair; three Tcf/Blimp site pairs; a Hesc/Runx site pair; and two Otx/Gatae site pairs. While the necessity for input transcription factor binding sites is conserved, the order and position is free to change unless there is an interaction between factors that occupy the sites.

149. The evolution of *cis*-regulatory modules revealed through cross-species gene transfer studies

R. Andrew Cameron, Eve Helguero, Qiu Autumn Yuan, Ping Dong, Julie Hahn

The lower deuterostome clade of which sea urchins are a member, is a rich source of material for cross-species comparisons aimed at understanding the structure and evolution of *cis*-regulatory modules (CRM). Our collection of genomic BAC libraries and the ease of BAC recombineering provide another route to understand CRM evolution. The present state of knowledge permits analysis of 12 genes compared among five species. To date, we have prepared recombinant GFP BAC clones for 34 of the 60 gene/species combinations in the matrix. We are continuing to screen BAC libraries for the missing genes from which BAC recombinants will be prepared. Our set of Ap recombinant BACs offers an opportunity to examine sequence changes at about 150 mya compared to Sp. Of six recombinant Ap BACs in hand only those containing the genes for *delta* and *gcm* yielded a significant proportion of Sp embryos expressing the reporter. The endogenous *delta* expression pattern is complex, but the pattern seen for the Ap reporter gene is very similar to it. The *Ap-gcm* recombinant BAC also recapitulates the endogenous pattern in Sp. Thus, the control of these two genes is conserved over a divergence of ~150 mya. We have recently obtained ordered and oriented sequence for the *Ap-otx* BAC, one for which the recombinant was negative when transferred into Sp. The *cis*-regulatory analysis of otxb1-2 in Sp identified Blimp1, Gatae and Otx sites that were necessary (Yuh *et al*., 2004). The Ap sequence has a mutation in the Blimp1 site and lacks two of the gatae sites in Lv and Sp. The lack of these sites explains the lack of expression in Sp but not how the CRM works in Ap. The most probable explanation is a more distant Blimp1 site and indeed there are two Blimp1 sites just outside of the three-way conserved region.

150. Transcriptional control of the sea urchin Brachyury gene

Lydia Dennis, R. Andrew Cameron

Previously we have identified a minimal enhancer for *brachyury (Sp-Bra)*, a gene intermediate in the endomesoderm specification gene regulatory network. Up to the peak of embryonic expression at 24 hrs, it recapitulates, in time and space, the expression of the endogenous gene. This enhancer contains binding sites for Sp-Tcf, a transcription factor that is activated by the Wnt pathway and Sp-Gatae, the output of the endomesoderm kernel subcircuit. An Otx binding site in the larger intronic sequence that contains the minimal enhancer also plays a role in activating this gene. These experiments have taken advantage of BAC clones recombineered with either GFP or RFP reporters. Our current hypothesis for

brachyury early transcriptional control is: a wnt activity in veg2 endoderm precursors leads to the nuclearization of beta-catenin and the activation of *Sp-Tcf* in those cells. Thus, brachyury transcription elevates beginning at about 10 hrs postfertilization. Later as *Sp-Gatae* and *Sp-Otx* begin to be expressed in these cells the expression is pushed to the maximum at 24 hrs.

To confirm a recent perturbation study that places upstream of *Sp-Bra*, we have employed a morpholino-antisense oligonucleotide (MASO) that abolishes the translation of the Hox11/13b protein. When co-injected with the SP-Bra GFP BAC, this MASO greatly reduces the expression of GFP indicating that the input from *Sp-Hox11/13b* is likely to be direct. We are now conducting experiments to see if the intron enhancer or its' minimal fragment are responsive to *Sp-Hox11/13b.*

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Postdoctoral Scholars: Kristin Branson[3], William Dickson, Wyatt Korff, Gaby Maimon, Akira Mamiya, Andrew Straw, Allan Wong[1]
Graduate Students: Gwyneth Card, Sawyer Fuller[2], Alice Robie, Jasper Simon, Marie Suver, Peter Weir, Floris van Breugel[2], Francisco Zabala[2]
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Summary: Complex and intellectually challenging problems can be so commonplace that they escape our attention. The research in my lab focuses on one such everyday phenomenon - the motion of a fly through the air. While the buzz of fly wings is more likely to elicit a sense of annoyance than wonder, insect flight behavior links a series of fundamental processes within both the physical and biological sciences: neuronal signaling within brains, the dynamics of unsteady fluid flow, the structural mechanics of composite materials, and the behavior of complex nonlinear systems. The aim research in my lab is to elucidate the means by which flies accomplish their aerodynamic feats. A rigorous mechanistic description of flight requires an integration of biology, engineering, fluid mechanics, and control theory. The long-term goal, however, is not simply to understand the material basis of insect flight, but to develop its study into a model that can provide insight to the behavior and robustness of complex systems in general. The following projects in my lab, some well underway, others nascent and moving in the direction of a deeper understanding of the genetic control and brain of this insect, are helping move us in the direction of reverse engineering a fly.

151. Multimodal sensory control of *Drosophila* locomotor behavior

Alice Robie

How organisms sense and respond to their environment is a fundamental question of neuroscience. We are studying this question in walking *Drosophila melanogaster* due to their relatively complex behavior while walking and the ease with which their sensory environment can be controlled in the laboratory. We developed a large, free-walking arena and to determine how a topologically complex environment influences the exploratory behavior of flies, we tracked individual flies for 10 min in the arena with four cones of equal area but differing height and slope (Figure 1A, B). The presence of the cones in the arena qualitative altered the overall exploration behavior in the arena. Flies appear to orient towards cones from a distance and once encountered climb on top of them. The flies spent significantly more time on the tallest-steepest object in the arena than any other object, more time than even the arena floor when it is normalized for area. We termed this behavior the cone response. By analyzing the locomotor activity of the flies, we found that the cone response is due to an increase in the duration of stops preformed on the tallest-steepest cone (upper left of Figure 1C). To investigate what sensory modalities underlie the cone response we impaired the visual and gravitational senses of flies. We impaired vision simply by running experiments in complete darkness (flies could still be seen by the tracking camera because of near-IR lighting), and we impaired gravitational sense by gluing the joint between antennal segments 2 and 3, a manipulation that disrupts the function of the Johnston's organ. We found that the cone response was maintained in flies with single sensory modality impairments, but when both modalities are impaired the cone response is lost (Figure 1C). This result implies the either sensory modality is sufficient to compensate for the loss of the other to produce the change in locomotor behavior we call the cone response.

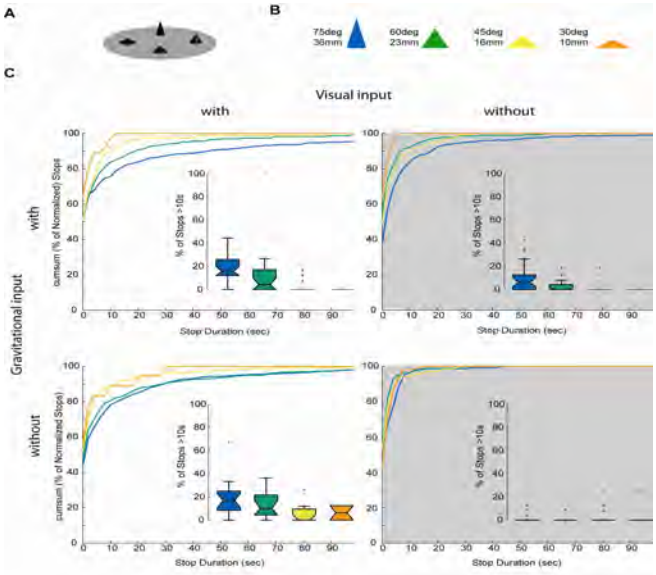


Figure 1. (A) A schematic of object arrangement in the d = 25 cm arena. (B) The color code for cones in part C. C: "Punnet square" of visual and gravitational sensory ablations. Each panel shows the cumulative sum of the percentage of normalized stop durations for all stops for all flies with an insert showing the percentage of each flies' stops that were longer than 10 sec.

152. Flight mechanics of turns about the yaw axis during hover in *Drosophila*

Will Dickson

The flight mechanics of fruit flies are quite complex and the basic mechanics of how flies modulate the forces and moments in order to maintain stable flight and maneuver is at present not well understood. A common maneuver performed by fruit flies is the 'body saccade' in which they change their heading by approximately 90 degrees in around 50 ms. A typical saccade can be idealized as a rapid turn about the functional yaw axis. Using a dynamically scaled robotic model of *Drosophila* melanogaster we examined the flight mechanics of turns about the yaw axis during hovering flight. The robotic model consisted of a pair of three degree of freedom flapping wings mounted on a motorized yaw axis (Figure A). The model was equipped with a sensor to measure torque about the yaw axis. Four parameterized actuation modes, which deform the flapping wing kinematics to generate yaw torque, were examined. For each actuation mode the stroke averaged yaw torque was measured as a function of yaw velocity and the control input. The stroke averaged yaw torque was found to be well approximated by a linear function of both the control input and the yaw velocity (Figure B1-B4).

In order to examine the flight mechanics of turns the robot was equipped with a real-time torque feedback mechanism that enabled us to use the measured yaw torque to determine the systems yaw velocity. In this manner the robotic model was capable of turning itself based on changes to the underlying wing kinematics. The yaw velocity was determined, in real-time, by integrating the equations of motions for the system. For each actuation mode the open loop system dynamics where measured by performing an input/output analysis using sinusoids. The gain and phase as a function of frequency for the open loop system where found to closely approximate that of a linear system (Figure 2, C1-C4 and Figure 2, D1-D4).

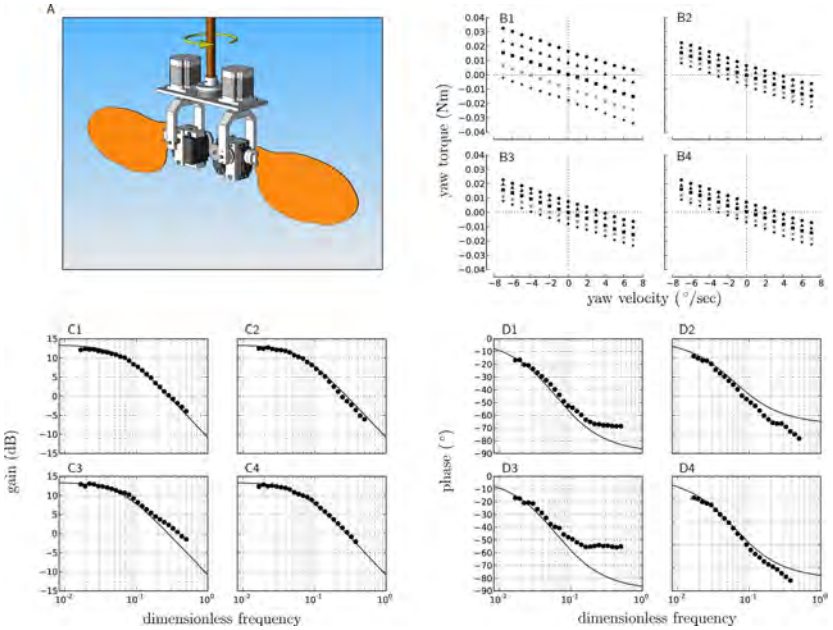


Figure 2. (A) Dynamically scaled robotic mechanism. (B1-B4) Yaw toque as a function of yaw velocity and control input for the four-actuation modes. (C1-C4) Gain as a function of dimensionless control input frequency for the four-actuation modes. (D1-D4) Phase as a function of control input frequency for the four-actuation modes.

153. Evidence for non-Lévy flight distribution in *Drosophila* search behavior

Rosalyn W. Sayaman, Andrew Straw

In recent years, several studies have pointed to the preponderance of Lévy-flight characteristics in the search behavior of animal species in nature. However, Lévy-flight studies of animals in nature are confounded by a number of difficulties and limitations, leading to challenges to the robustness of some of the original Lévy-flight data. Methodologically, difficulties of tracking animal movement in nature remain the same as studies are normally done with tracking devices that record only the animal's location absent its actual behavior or interaction with the environment. More problematic is the underlying assumption that the recorded movements are random searches of the environment rather directed movements driven by environmental stimuli. On another front, the interpretation of Lévy-flight behavior based on the graphical representation of a logarithmically transformed histogram of movement length data has also been challenged. Here we propose an alternative approach that addresses the some of these challenges by tracking the flight behavior of different species of the fruit fly, *Drosophila*, in a controlled environment. Previous

research has shown that flies explore their environment using a combination of straight flight and rapid turns, called saccades. We use a magnetic tethered-flight arena to track the spontaneous turns generated by the fly around its yaw axis in a low-threshold homogenous background. We plot the distribution of the inter-saccade intervals, the straight-flight sequences in between saccades and look for evidence of a Lévy-flight distribution. Experiments in the tethered-flight arena give the advantage of allowing us to track the actual behavior continuously while removing any emergent environmental cues or stimuli that could bias the results. Tethered-flight arena experiments also give clearer resolution to the distribution of the longer inter-saccade interval durations that are truncated in the earlier free-flight experiments in *D. melanogaster* due to the limited size of the arena. We useD the LBN technique proposed by Sims in analyzing the data for six species of *Drosophila* - four cosmopolitan species (*D. melanogaster*, *D. ananassae*, *D.pseduoobscura*, *D.hydei*) and two cactophilic species (*D. mojavensis*, *D. arizonae*), and show that the distributions of the inter-saccade intervals show no evidence of Lévy-flight behavior. Results also show that the distribution extends to longer straight-flight intervals for the two-cactophilic species, a behavior and search strategy that correlates well with the sparse resources of desert environment in which they live.

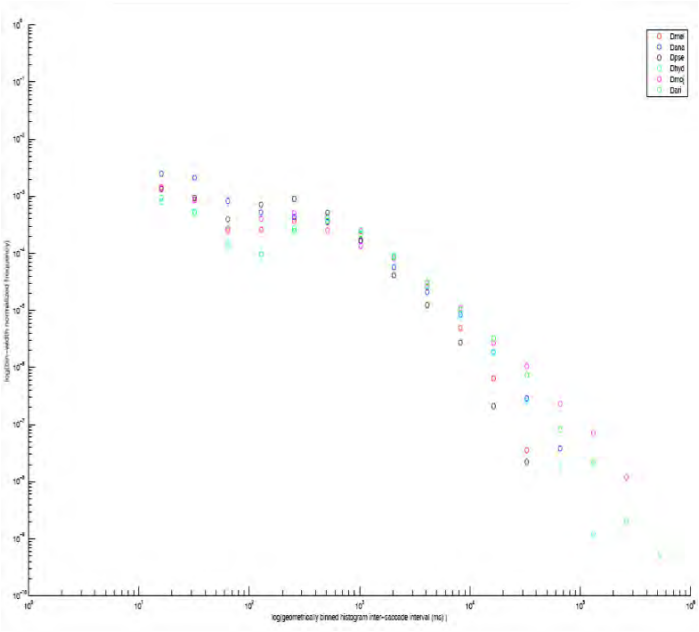


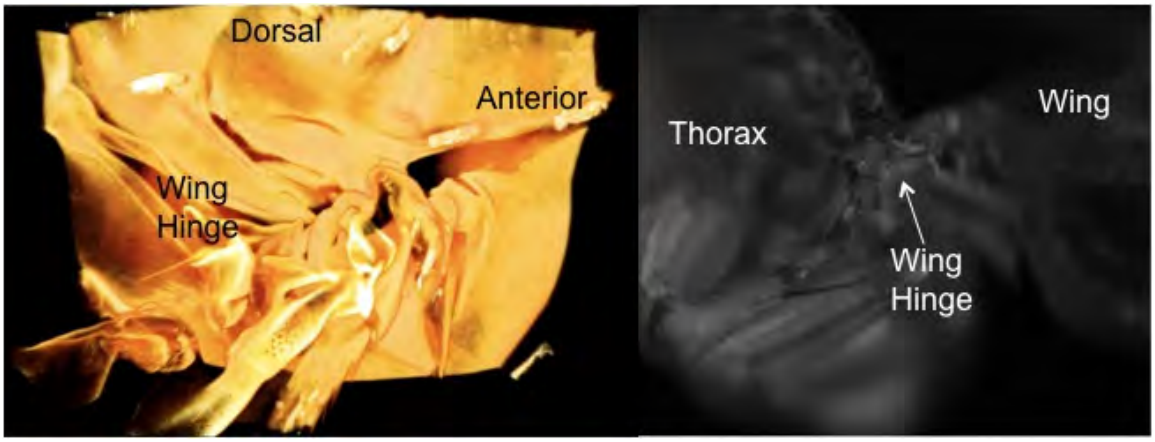
Figure 3. Log bin normalization transformation of a histogram of inter-saccade intervals in six species of *Drosophila*. Straight line fit with a slope, $\mu = 2$, characteristic of Lévy-flight behavior is not observed.

154. Wing hinge dynamics in *Drosophila*

Michael Elzinga, Wyatt Korff

The primary objective of this project is to understand how small strains generated by indirect power muscles within the thorax of a flying insect are transmitted via the wing hinge into controlled wing motions capable of sustaining flight. Recent advances in high-speed video technology provide both the spatial and temporal resolution necessary to reconstruct the time-dependent strain field of the exoskeleton through the use of image processing techniques, as well as track the motion of individual components involved the operation of the wing hinge. These kinematic measurements coupled with detailed knowledge of the 3D morphology, achieved through imaging techniques such as two photon excitation laser scanning microscopy (TPLSM), provide the data necessary for constructing dynamic finite element models of the thorax and wing hinge. The ultimate goal is to provide a significant contribution to the foundation required for the design of biologically inspired flexure-joints that could have significant durability, manufacturing, and efficiency advantages over traditional transmission mechanisms for small-scale applications such as micro and nano air vehicles. Two photon laser scanning microscopy of *Drosophila hydei* specimens was used to acquiring 3D morphological data that will be required for future use in a dynamic finite element model (FEM). To prepare the specimens for imaging, a standardized mounting and clearing procedure was developed to allow for greater transmission through the cuticle with less scatter, resulting in greater image clarity and deeper penetration limits. Image post-processing was conducted using the 3D reconstruction software package, Amira (VisageImaging). High-speed video sequences of tethered flight were recorded and analyzed to make kinematic measurements of the wing hinge and thorax. A high-speed video test rig was fabricated that consists of a series of prisms and mirrors mounted to a stage that rotates around the optical axis of the camera and an integrated LED panel virtual reality (VR) flight arena. The rotating stage allows for alternative viewing angles for unobstructed visualization of the wing hinge throughout the entire wing beat cycle. The VR arena displays programmed visual stimuli to elicit turning and pitching maneuvers, allowing for the study of how wing hinge kinematics are modulated to accomplish such maneuvers.

Figure 4 (next page): Left: 3D rendering of the thorax and wing hinge, reconstructed from serial TPLSM images. Right: Still frame from a high-speed video sequence (8kHz) of the wing hinge (image size: ~ 1 mm x .8 mm).



155. Multiple visual pathways control altitude in flying *Drosophila*

Andrew Straw, Serin Lee

Regulation of altitude is essential for flying animals to fly in appropriate wind conditions, to migrate, to find food and mates, to avoid hazards such as lakes and to land safely. Because of these varying tasks, this regulation is likely to consist of several sensory-motor neural circuits. The model organism *Drosophila melanogaster* is an ideal animal in which to study these behaviors and their neural basis due to the rapid, precise and highly visual control of flight and amenability to many levels of experimentation. Using an automated, high-throughput 3D fly tracker and a high speed digital image projector system, we developed a 'virtual reality' arena for flies. By clamping a visual stimulus at a precisely specified velocity to an individual fly as it flew through our control volume (a virtual open loop condition), we tested several models for the control of altitude. We found that longitudinal motion underneath the animal has little effect on altitude, contrary to the predictions of a recent model proposed by Franceschini, This same stimulus elicited velocity following behavior. Furthermore, we found that lateral motion in the ventral visual field elicited upward motion, and vertical motion in the lateral visual regions elicited same-direction responses. Finally, high-contrast horizontal edges caused flies to adjust their altitude to that of the edge. Together, these visual-motor responses show that flies regulate altitude using velocity and position sensitive visual circuits across large parts of the field of view.

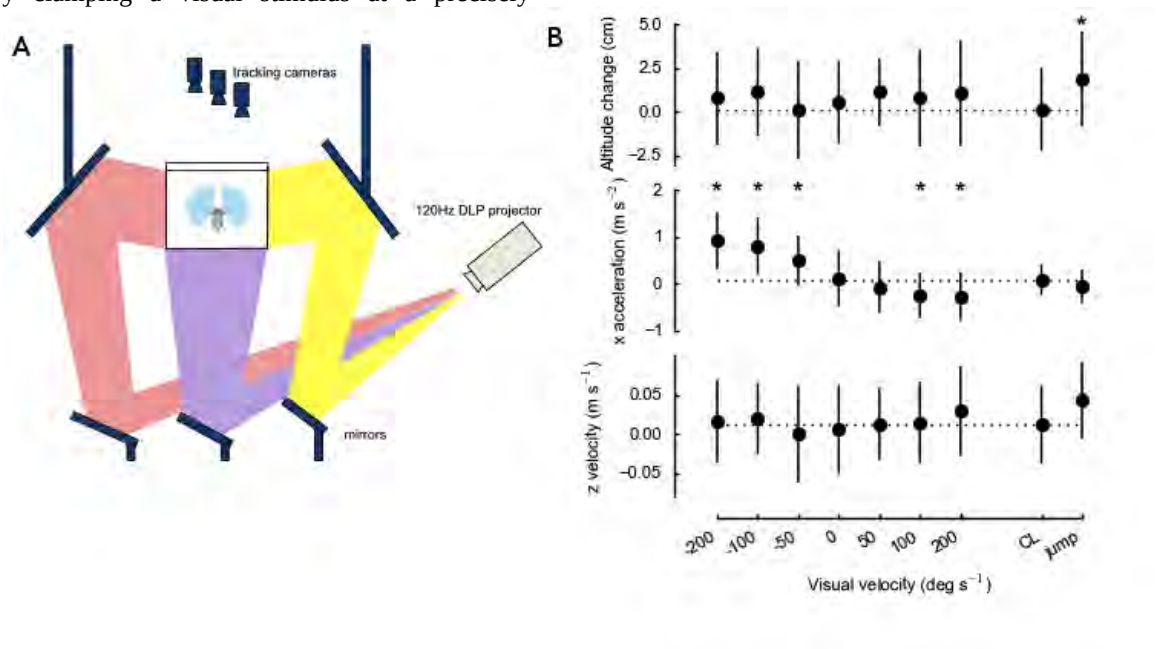


Figure 5. (A) Schematic view of virtual reality arena in which flies are tracked using cameras from above and the visual surround is displayed using computer graphics projectors on the walls and floor. (B) Regulation of several variables by flies indicates that ventral motion is used to regulate horizontal, but not vertical, velocity.

156. Patch-clamp recordings from central neurons during tethered-flight in *Drosophila*

Gaby Maimon

We have successfully developed a new recording apparatus, in which we can obtain whole-cell patch clamp measurements from single cells while simultaneously making behavioral measurements in the same fly. Critical to our success was developing a novel computer-designed microscope stage (Figure 6A-C). Ultraviolet-activated glue is applied to the head and front of the thorax of an anesthetized fly, and the animal is carefully attached to a small opening at the bottom of this stage. A small amount of cuticle is removed from the back of the fly's head, overlying the fly's brain, and neurons can be visually targeted with a microelectrode. The stage is designed such that the exposed brain is bathed in a perfused saline solution for electro-physiological recordings, while the rest of the animal's body is dry and unrestrained. Genetic tools available in *Drosophila* make it possible to express GFP in subsets of neurons, which allow one to target the same cells across preparations. We are currently recording from motion-sensitive interneurons within the lobula plate, a third order optic neuropil in the fly's brain. These cells show direction selective responses to visual motion (Figure 6D) and it is believed that these cells may serve an important role in visually-guided flight behavior. We are probing the function of the vertical-system cells by comparing the responses of these cells during quiescence and tethered-flight. Initial observations suggest that activity in vertical-system cells may be strongly boosted during active flight as compared to quiescence (Figure 6D). After recordings, the identity of neurons can be verified through fluorescence imaging of a dye present in the electrode which diffuses throughout the cell's cytoplasm (Figure 6E). More generally, this preparation will allow us to examine the electrophysiological basis for a variety of visually guided behaviors in *Drosophila*.

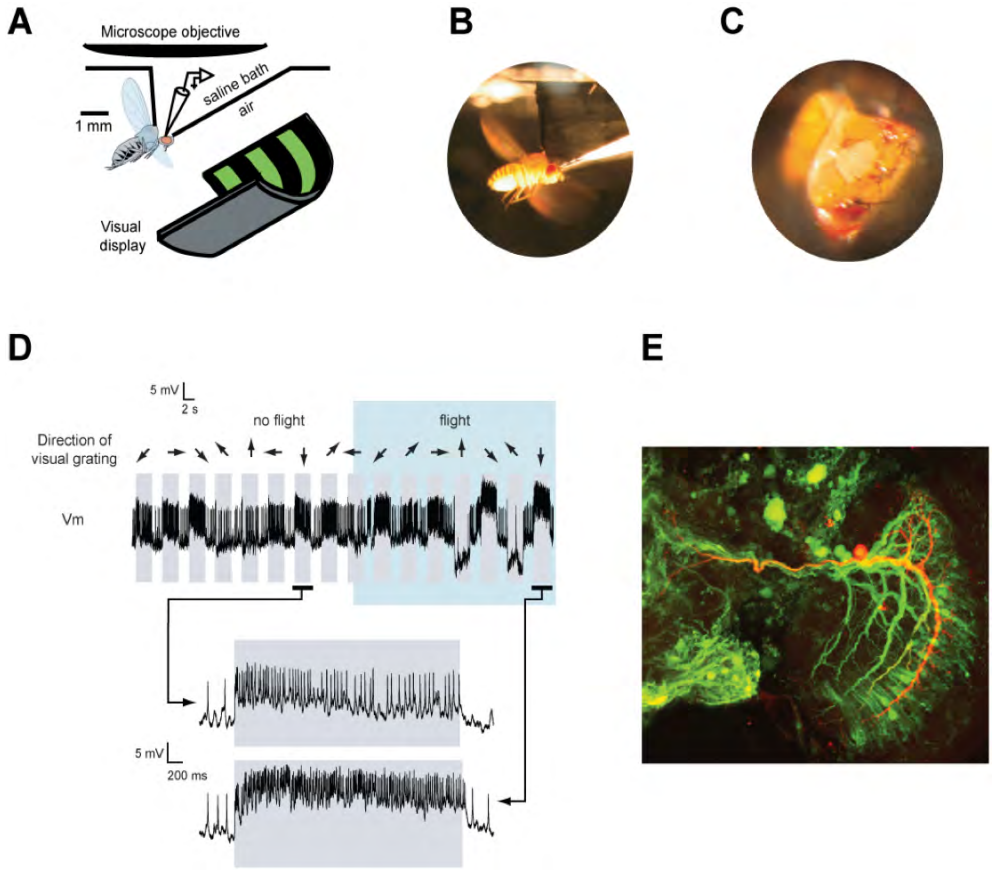


Figure 6. (A) Schematic of the apparatus. A fly is attached to a custom stage, such that the wings can flap freely in air with the brain exposed to saline. We perform patch-clamp recordings from single neurons as the animal responds to stimuli presented on a visual display. (B) Photograph of a tethered, flying fly, attached to the apparatus. (C) View from above, exposing the brain. (D) Patch-clamp recording from a vertical-system neuron. This cell was sensitive to the direction of motion of a grating. (E) Flattened z-stack of fluorescence signals. In green is the native GFP fluorescence in the line of flies used for recordings (3a-Gal4 x UAS-eGFP). In red is the immuno-labeled biocytin signal within a recorded neuron.

157. Identification of descending neurons

Allan Wong

A fundamental goal of the project is to understand where in the brain different stimuli are integrated and how the signals propagated to the muscles that move the appendages. In particular, we wish to understand how descending neurons transmit signals from the brain to the thoracic ganglia: whether they carry multimodal commands from prior integrated sensory information, or if they carry unimodal commands that represent discrete channels for sensory to motor action. In the past year, we pursued three different avenues to address this question. First, we conducted a pilot behavioral screen in which we silenced subsets of neurons in the fly brain and analyzed the fly's locomotor trajectories with an automated tracking program. We found that when we silence the neurons in a line which labels primarily the fan-shaped body in the central brain, the flies exhibit novel encounter behaviors where the flies collide with one another instead of the usual tendency to avoid such occurrences. Second, we also performed concurrent behavior analysis and neural activity imaging using various genetically-encoded calcium dyes (GCaMP1.3, GCaMP1.6, TNXXL) in central neurons of the fly. So far, we have not been able to see correlated neural activity with behavior.

We are currently trying a third generation of calcium dye with greater dynamic range and sensitivity. Third, we have been anatomically mapping and categorizing descending neurons. We use various Gal4 lines to express photoactivatable-GFP in descending interneurons and then photo convert the pa-GFP molecules using a two-photon microscope. We see approximately 200 pairs of descending neurons in the fly brain. One pair, in particular, stands out. Using the pa-GFP labeling technique, we discovered a pair of giant descending neurons with dendrites in the antenna that sends its axons through fly brain and into the thoracic ganglia (Figure 6).

Currently, we are trying to understand the function of this unique pair of giant descending sensory neuron. We are also making images of expression patterns in publicly available GAL4 driver lines in order to identify candidate neurons to target with either electrophysiological or imaging techniques. Figure 6b shows four different GAL4 lines of interest crossed to UAS-CD8GFP in order to visualize expression patterns.

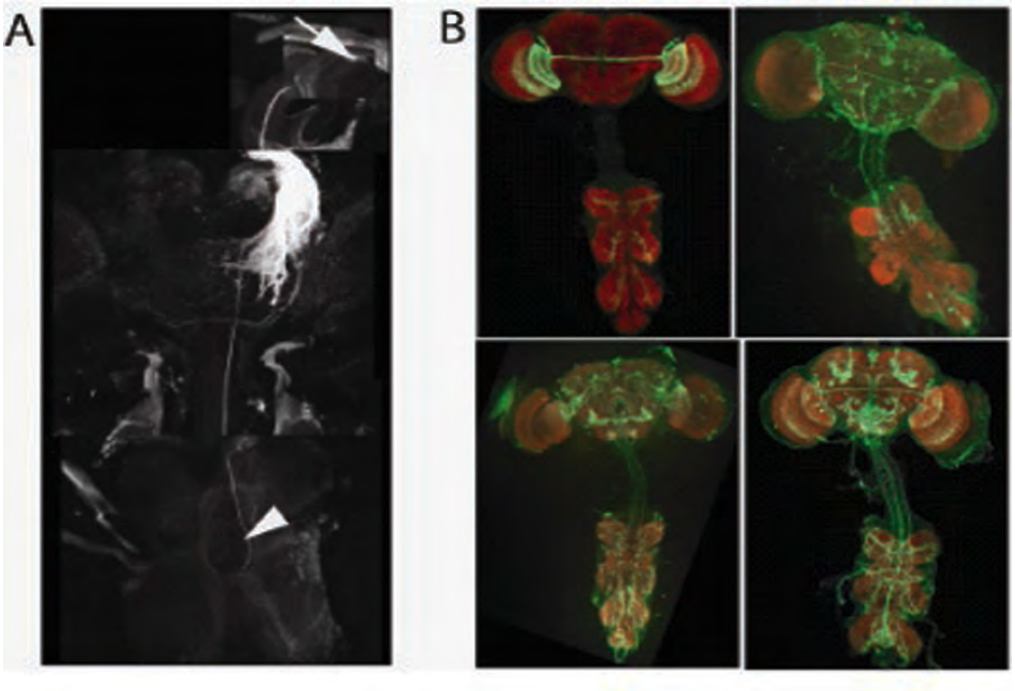


Figure 7. (A) Composite image of showing morphology of giant antennal descending neuron. The image was made by selectively activating antennal and cervical nerves of a fly expressing photoactivable-GFP pan-neuronally. When the cervical nerve was activated the cell body in the second antennal segment is revealed (arrow). When the antennal nerve was activated the Johnston's organ and olfactory sensory neurons, as well as a descending neuron that terminates in first and second thoracic neuromeres (arrowhead). (B) Confocal images of GAL4 driver lines crossed to UAS-CD8GFP. Red indicates neuropil, green the GAL4 expression pattern.

158. Visually-guided programs in escaping flies

Francisco Zabala, Gwyneth Card

Previously, we found that fruit flies can control the direction in which they take off during escape from a rapidly approaching object. We determined that the fly only needs visual input from the threatening object and can plan and execute its takeoff in less than 200 ms. These observations established fly escape behaviors as a model system in which to study rapid, visually-mediated motor planning in a genetically accessible organism. We are pursuing these studies at the behavioral and neuronal levels. We find that flies appear to have two distinct mechanisms with which they control their initial escape trajectory. The first mechanism is composed of postural adjustments of the fly's middle (jumping) legs relative to its center of mass that occur before the fly initiates flight. The amplitude of these leg and body motions depends in a graded fashion on the stimulus approach direction (Figure 7A,B). Conversely, the fly uses the second mechanism during the rapid kick-off that initiates flight (Figure 7C). Our data show that the fly does not push off the ground with both middle legs equally, but rather extends the leg closer to the stimulus much more that the other. The result is that the fly is directed away from the threat. Unlike the pre-jump leg motions, however, this uneven extension appears to be an all-or-nothing fixed action pattern. The fly either extends the joint fully (to 80-90% of full extension) in the case of the closer leg, or minimally (to only 20-30% of full extension) in the case of the far leg, regardless of stimulus direction. We are currently developing a physiological preparation that will help us to determine key neural components of the sensory-to-motor transform involved in these two directional jumping behaviors.

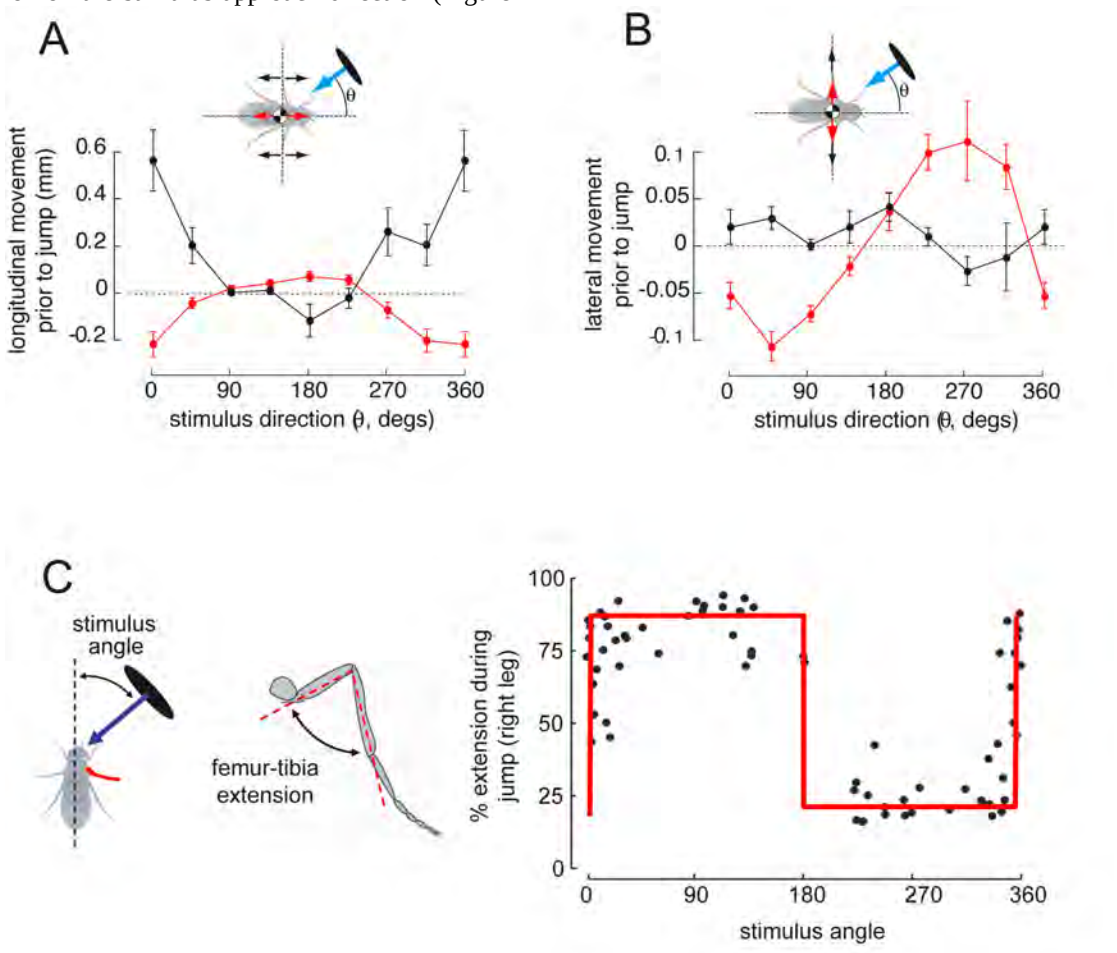


Figure 8. Towards the neural substrate of directed escape takeoffs. (A and B) When a physical black disk falls on a collision course towards a fly, its initial response is to adjust the relative posture of its mesothoracic (jumping) legs (black) and its center of mass (red). (A) and (B) plot the magnitude of these adjustments (mean +/- SEM) either longitudinally along the body (A) or lateral to the body (B) as a function of stimulus direction. (C). Extension of the right jumping leg as a function of stimulus direction (black dots) during the escape jump. Overlaid red square wave suggests leg extension during the jump is "all-or-nothing."

159. Polarization vision in *Drosophila*

Marie Suver, Peter Weir

Understanding how relevant sensory information is used to regulate locomotor behaviors is one of the central questions in our project. It has been observed that many species of insects use detection of linearly polarized light to aid in navigation. For instance, dung beetles use the pattern of polarization present in sky light to chart a straight course away from areas of high resource competition. In almost all species studied thus far, researchers have identified a region of the insect compound eye, the Dorsal Rim Area (DRA), which has specializations that implicate a role in polarized light vision. Despite this body of work, very little is known about the role of polarized light vision in *Drosophila*. We have undertaken a series of behavioral experiments aimed at determining if and how *Drosophila* uses this sensory modality during flight. In a tethered flight simulator we have developed a paradigm in which individual wild-type flies show a robust polarization-sensitive response (Figure 9a,b). Using this paradigm we have shown that only UV sensitive receptors are involved in the behavior (Figure 9c). Additionally, by blocking parts of the compound eyes with opaque wax, we have shown that these receptors are not exclusively located in the DRA. Now that we have characterized this robust response to polarized light, we are planning to initiate a physiological study to identify the underlying circuits in the central brain.

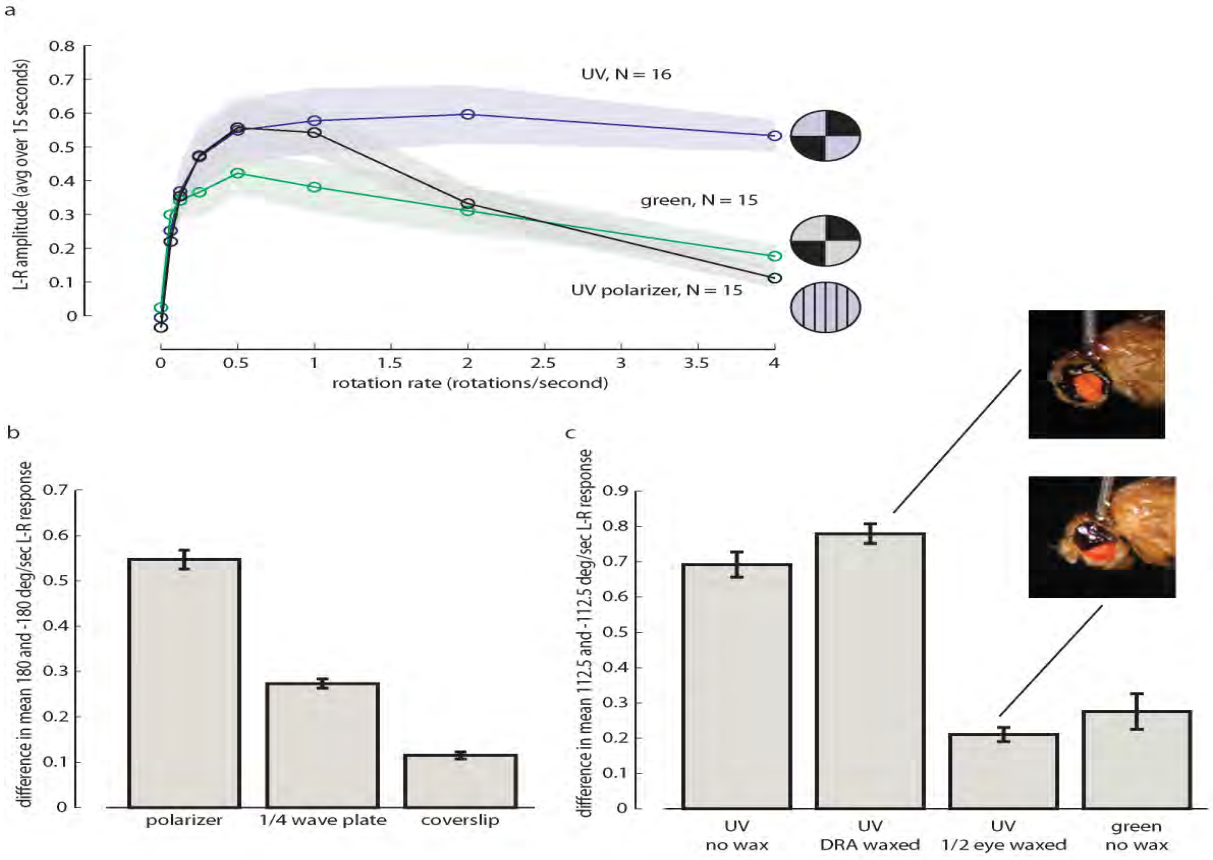


Figure 9. (A) Turning response (left minus right wingbeat) measured at nine rates of rotation under different conditions: Polarizing filter with UV light (black line), filter with opaque quarter pattern and UV light (blue line), filter with opaque quarter pattern and green light (green line), all with standard error of the mean (shaded regions). (B) Turning response of flies presented with different filters in ultraviolet light with standard error of the mean (error bars). Polarizer response is about twice the strength of the unpolarized light response. A rotation control confirms that the polarized light response it not due to the rotating holder. (C) Wavelength and eye region comparisons of response to rotating polarizer with standard error of the mean. Black wax applied to the dorsal rim area (DRA) does not decrease the response. Wax applied to the entire dorsal half of the eye greatly reduces the response. Green light also produces a much smaller effect.

160. High-throughput ethomics in large groups of *Drosophila*

Kristin Branson, Alice Robie

Our research has focused on creating tools for and performing quantitative behavior analysis on *Drosophila*. We have created a freely available program called Ctrax: The Caltech Multiple Fly Tracker, which uses machine vision algorithms to accurately track many interacting individuals without swapping identities. We have also developed a behavior analysis toolbox that operates on the trajectories output by Ctrax. This toolbox can be used to represent the trajectories as an ethogram that plots the time course of behaviors exhibited by each fly, or as a vector that concisely captures the statistical properties of all behaviors displayed within a given period. We showed that behavioral differences across individuals are consistent over time and are sufficient to accurately predict gender and genotype. In addition, we showed that the relative positions of flies during social interactions vary according to gender, genotype, and social environment. We hope that the Ctrax software, which permits high-throughput screening, will complement existing molecular methods available in *Drosophila*, facilitating new investigations into the genetic and cellular basis of behavior.

We have two directions of current research. First, we observed that female flies maintain a buffer between each other, performing stereotypical avoidance responses to another approaching female. We are working to discover the program that female flies use when interacting with other female flies. The second direction of research is characterizing whether and how individuals within a genetically homogeneous population differ in behavior. We have found effects of individuality on a number of statistics describing behavior for individuals over 6-12 hour periods. Initial analyses indicate that flies differ in their level of activity and how their behavior changes with hunger.

Publication

Branson, K., Robie, A., Bender, J., Perona, P. and Dickinson, M.H. (2009) High-throughput Ethomics in large groups of *Drosophila*. *Nature Meths*. **6**:451-457. (http://www/dickinson.caltech.edu/ctrax)

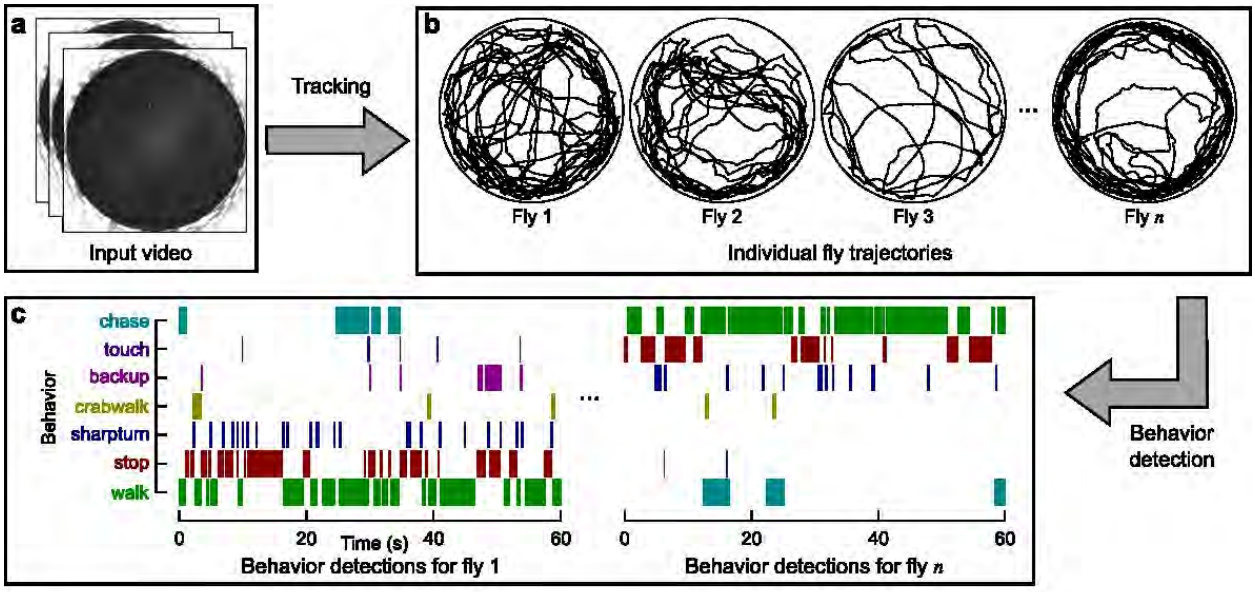


Figure 10. Flow chart illustrating our quantitative behavior analysis tools. (A) Input video sequence of a group of flies behaving. (B) Trajectories saved by Ctrax. In each circle, we plot the center position of a tracked fly in the arena. (C) Segments of video in which a given fly is performing a given behavior computed with our learned behavior detectors. The x-axis indicates time, the y-axis behavior. Colored in regions indicate that the fly is performing the behavior in the corresponding frame.

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Summary: Cells process information, interact with one another, and exhibit patterned development using circuits composed of interacting genes and proteins. Although many of these components and their interactions are now known, it remains unclear how the circuits they compose function reliably within cells. In order to understand how genetic circuits operate at the single-cell level, we are applying experimental and theoretical techniques to key model systems:

First, we construct synthetic genetic circuits and study their behavior in individual cells. These synthetic circuits are simpler counterparts to the complex circuits one finds in nature. This approach, often called "synthetic biology," allows one to analyze how various circuit designs might work, and begin to understand what is special about the specific circuit architectures observed in organisms. We have constructed circuits that exhibit oscillations and other dynamic phenomena, (e.g., Elowitz and Leibler, 2000). We have used synthetic circuits to analyze the dynamics and variability of gene regulation at the single-cell level, (e.g., Elowitz *et al*., 2002, and Rosenfeld *et al*., 2005). We also make use of 're-wiring' perturbations to alter the architecture of natural genetic circuits, as in our recent studies of the genetic competence system of *Bacillus subtilis* (Süel *et al*., 2006; Süel *et al*., 2007).

Second, we analyze the dynamics of specific natural genetic circuits in order to understand basic principles of their operation. We have developed the ability to acquire and quantitatively analyze large time-lapse movie datasets. These movies allow tracking of circuit dynamics in individual cells as they grow and develop. By incorporating several distinguishable fluorescent protein reporter genes in these organisms, we can track multiple circuit components simultaneously. The results constrain models of the corresponding circuits and provide insight into basic principles of their operation. A recent example of this approach is our work on regulation of genetic competence in *Bacillus subtilis* (see Süel *et al*., 2006 and Süel *et al*., 2007), as well as our studies of frequency modulated nuclear localization bursts in yeast (Cai *et al*., 2008).

Third, we are analyzing the generation of variability within cell populations. Genetically identical cells appear to actively generate variability, even in homogeneous environmental conditions. We focus specifically on two complementary questions: How do cells use intrinsic "noise" (stochasticity) in their own components to make effectively random cell fate decisions? And how do they suppress noise in order to operate reliably despite of variability. Current projects are examining these issues in *Bacillus subtilis,* a very simple prokaryote that exhibits both differentiation and development, as well as in more complicated mammalian cell culture systems. Recently, we have examined the role that noise plays in enabling an alternative mode of evolution through partially penetrant intermediate genotypes (Eldar *et al*., 2009). We have also studied the way in which dynamic correlations of fluctuations in gene network dynamics can help identify active regulatory interactions (Dunlop *et al*., 2008).

Projects in the lab make extensive use of relatively simple mathematical models of genetic circuits. We are also developing software and tools to improve gene circuit construction and quantitative analysis of movie data.

161. Partial penetrance facilitates developmental evolution in bacteria

Avigdor Eldar, Vasant Chary, Panagiotis Xenopoulos, Michelle E. Fontes, Oliver C. Loson, Jonathan Dworkin, Patrick Piggot, Michael B. Elowitz

Development normally occurs similarly in all individuals within an isogenic population, but mutations often affect the fate of individual organisms differently. This phenomenon, known as partial penetrance, has been observed in diverse developmental systems. However, it remains unclear how the underlying genetic network specifies the set of possible alternative fates and how the relative frequencies of these fates evolve. We identified a stochastic cell fate determination process that operates in *Bacillus subtilis* sporulation mutants and showed how it enables the gradual evolution of a novel developmental fate through states of intermediate penetrance. Mutations that attenuate cell-cell signaling produced a set of discrete alternative fates not observed in wild-type cells, including the relatively infrequent formation of two viable "twin" spores, rather than one, within a single cell. We systematically tuned the penetrance of each mutant fate by genetically perturbing cellular processes such as chromosome replication, septation, and signaling. In

particular, we obtained strains that formed twin spores at high penetrance. These strains exhibited similar features to natural twin sporulation in the anaerobic spore-former *Clostridium oceanicum*, suggesting that they could represent evolutionary intermediates. A mathematical model showed how twin sporulation can evolve through gradual changes in fate penetrance, and how specific experimentally observed features of the system facilitate this transition. Together these results show how noise can facilitate developmental evolution by enabling the initial expression of discrete morphological traits at low penetrance, and allowing their stabilization by gradual adjustment of genetic parameters.

In a follow-up to this work, we are studying the natural development and evolution of twin sporulation, which may have evolved through genetic assimilation on frequency. Using RNA FISH we will follow gene expression pattern of the naturally twin forming species, *C. oceanicum*. We have sequenced the bacteria and are currently characterizing genes that may explain the formation of twins under some conditions and the formation of mono-spores under other conditions. We hope that this research into the environmental plasticity of twin frequency will shed more light on its evolution and the relation between partial penetrance, environmental plasticity and evolution.

162. Global regulatory dynamics of *B. subtilis* stress response

James Locke, Jon Young, Michael B. Elowitz

Clonal populations of cells contain the same genome, and may be grown in the same environment. However, individual cells are frequently found to exhibit a heterogeneous set of cellular states. A fundamental question in biology is how this heterogeneity is generated. Recent work suggests that stochasticity, or noise, in underlying reactions is used by cells for probabilistic differentiation. Thus, a major problem is to understand, first, how such noise enables heterogeneous decision-making in cell populations and, second, how genetic circuits within the cell constrain variability to generate specific cellular states.

We are using *B. subtilis* as a model system to study this problem. *B. subtilis* exhibits an extremely wide range of alternative states and stress responses, such as chemotaxis, competence, sporulation, chaining, and biofilm formation. Although the genetic circuitry for many of these pathways have been mapped out, it is not clear how much variability there is in the occupation of these states at the single cell level. We have created a library of reporter strains, containing chromosomally integrated reporters representing key transcriptional pathways. We have screened these strains using snapshots and time-lapse microscopy under a variety of conditions to examine the variation in gene expression. Several pathways displayed a great deal of noise, even bi-modality, at the single cell level. Our work has revealed a rich and varied structure to the transcriptional state space of *B. subtilis*. We are developing mathematical models to explain and characterize this variability from a network perspective. In particular we are using the general stress response sigma factor sigB as a model system to understand how heterogeneous states can be generated.

163. Cis interactions between notch and delta generate mutually exclusive signaling states

David Sprinzak, Lauren LeBon, Amit Lakhanpal, Michael B. Elowitz

The Notch-Delta signaling pathway is the canonical pathway for communication between neighboring cells during development. It plays a critical role in the formation of 'fine-grained' patterns, generating distinct cell fates among groups of initially equivalent neighboring cells, and in sharply delineating neighboring regions in developing tissues. The Delta ligand has been shown to have two activities: it trans-activates Notch in neighboring cells, and cis-inhibits Notch in its own cell. However, it remains unclear how Notch integrates these two activities and how the resulting system facilitates pattern formation. To address these questions, we developed a quantitative time-lapse microscopy platform for analyzing Notch-Delta signaling dynamics in individual mammalian cells. By controlling both cis- and trans-Delta levels, and monitoring the dynamics of a Notch reporter, we measured the combined cis-trans input-output relationship for the Notch-Delta system. The data revealed a striking difference between the response of Notch to trans- and cis-Delta: While the response to trans-Delta is graded, the response to cis-Delta exhibits a sharp, switch-like response at a fixed threshold, independent of trans-Delta. We developed a simple mathematical model that shows how these behaviors emerge from the mutual inactivation of Notch and Delta proteins. This interaction generates an ultrasensitive switch between sending (high Delta / low Notch) and receiving (high Notch / low Delta) signaling states. Critically, the two states are mutually exclusive: cells predominantly send or receive, but cannot do both simultaneously. At the multicellular level, this switch can amplify small differences between neighboring cells even without transcription-mediated feedback. This Notch-Delta signaling switch facilitates the formation of sharp boundaries and lateral inhibition patterns in models of development, and provides insight into previously unexplained mutant behaviors.

164. Single-cell analysis of genetic circuits in mouse embryonic stem cells

Julia Tischler, John Yong, Michael B. Elowitz

Embryonic stem (ES) cells provide a powerful model for the study of self-renewal and cell fate decision making. However, while specific genes and feedback structures governing the maintenance of pluripotency and differentiation into specific fates have been elucidated, ES cells appear to display substantial variability in the expression of certain key transcription factors. Moreover, under most conditions, ES cells differentiate into a heterogeneous set of cell types. This poses a major obstacle for the use of stem cells in therapeutic

applications. Thus, an important problem in stem cell biology is to gain a better understanding of the types of variability present in ES cell populations, to determine how that variability is created, how it controls metastablity of ES cells, and how it influences the outcome of cell fate decisions.

To address these questions, we aim to (1) investigate fundamental dynamics of genes and genetic circuits underlying the self-renewal and cell fate decision-making behaviors of individual ES cells and (2) to develop the ability to understand overall dynamics in terms of quantitative models. These efforts will give us insights into the stability and dynamics of genetic circuits underlying self-renewal and cell fate decision-making and will provide a foundation for more extensive analysis and control of stem cell behavior.

165. Nuclear localization bursts and signal encoding in yeast

Chiraj Dalal, Long Cai, Kasra Rahbar, Michael B. Elowitz

In yeast, the transcription factor Crz1 is dephosphorylated and translocates into the nucleus in response to extracellular calcium. Using time-lapse microscopy, we found that Crz1 exhibited short bursts of nuclear localization (~2 minutes) that occurred stochastically in individual cells and propagated to the expression of downstream genes. Strikingly, calcium concentration controlled the frequency, but not duration, of localization bursts. Using an analytic model, we found that this frequency modulation (FM) of bursts ensures proportional expression of multiple target genes across a wide dynamic range of expression levels, independent of promoter characteristics. We experimentally confirmed this theory with natural and synthetic Crz1 target promoters. Another stress response transcription factor, Msn2, exhibits similar, but largely uncorrelated, localization bursts under calcium stress. These results suggest that FM regulation of localization bursts may be a general control strategy utilized by the cell to coordinate multi-gene responses to external signals.

In this work, we observed that the yeast transcription factor Crz1 exhibits frequency-modulated bursts of nuclear localization in response to its stimulus, calcium. This behavior enables proportional regulation of Crz1 target genes in response to calcium, revealing a novel regulatory strategy cells may use to optimally tune expression levels of groups of promoters (Cai *et al*., 2008). These observations provoke the question of what other related regulatory strategies might be employed by the cell. Taking advantage of the yeast GFP library, we are using high-throughput time-lapse microscopy to identify other localization dynamic regulatory systems.

166. Sporulation initiation dynamics in *B. subtilis*

Joseph H. Levine, Shaunak Sen, Michael B. Elowitz

We are investigating the initiation of sporulation in the genetic model bacterium *Bacillus subtilis*. In response to various stress conditions, *B. subtilis* initiates expression of sporulation initiation genes in a highly noisy and heterogeneous manner. This gene expression continues for multiple cell cycles until the cell eventually begins morphological transformation into a resistant spore. Gene expression is regulated by the master regulator of sporulation, Spo0A, a two-component response regulator. Spo0A is embedded in an elaborate signal transduction cascade, and also feeds back to both positively and negatively regulate its own expression and phosphorylation state. We are working to ascertain the genetic mechanisms behind these heterogeneous gene expression dynamics using quantitative time-lapse microscopy and specific rewiring and genetic perturbations to the phosphorelay circuit.

167. Embryonic stem cell circuit dynamics

Fred Tan, Michael B. Elowitz

Mouse embryonic stem cells (mESCs) can self-renew indefinitely and differentiate into almost any cell type. In addition to their key role in regenerative medicine research, mESCs have also become a well-characterized developmental model for dissecting pluripotency and cellular decision making. While much progress has been made at identifying critical regulators that support pluripotency or the initial stages of differentiation, knowledge of how these factors functionally interact is lacking. A transcriptional network supported by a triad of transcription factors (Oct4, Sox2 and Nanog) reinforces the expression of pluripotency-associated genes while repressing downstream developmental markers. And in concert with an extensive and coupled signaling environment including exogenous LIF and BMP4, pluripotency is maintained and differentiation is restricted. We employ a variety of genetic and pharmacological perturbations to analyze the correlation and possible mechanistic connection between diverse signaling factors and transcription factors in circuit dynamics and function.

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Dunlop, M.J., Cox III, R.S., Levine, J.H., Murray, R.M. and Elowitz, M.B. (2008) Regulatory activity revealed by dynamic correlations in gene expression noise. *Nature Genetics* **40**(12):1493-1498. PMID:19029898.

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Summary: Our laboratory has dedicated itself to an integrative approach to defining the cell and molecular basis of embryonic patterning, in which *in vivo* imaging tools play a central role. Our approach has been to develop imaging tools that are sufficiently robust so that the movements, lineages and gene expression in living cells can be analyzed as an adjunct to the techniques more typically employed by systems biology. Systems biology provides a means to organize the explosion of data from molecular approaches and the dramatic progress from *in vitro* culture assays, but it requires some means to test the proposed linkages. Our goal is to test these proposed mechanisms in the intact embryo, with the hope of moving forward to an understanding of which of the potential mechanisms operate in the natural biological context. There are many challenges to such tests, including 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. Solutions to these challenges require the coordinated efforts of researchers spanning the life and physical sciences.

In the past year we have made significant advances in imaging fast events in the developing embryo, ranging from the movements of cilia in the developing ear and left-right organizer and the flows these cilia generate, to the workings of the beating heart as it takes shape. This has required the development of new image acquisition and processing tools that offer quantitative analyses of the key events. These tools are proving to be generalizable to other systems such as the *Drosophila* embryo. As a result, we have great hope for exploring events that have previously been too difficult to address.

In parallel with the refinement of new imaging tools, we have been creating new and more efficient means for creating embryos with genetically-encoded fluorescent tags. Through random insertion into the genome, this approach permits the creation of functional fusions between a host protein and a fluorescent protein, permitting cells and developmental questions to be posed in normally developing embryos. The creation of these new lines is dramatically more efficient than previous approaches, making it possible for even modest sized facilities to embark on large-scale screens. This increased efficiency of creating marked strains requires a parallel increase in imaging technologies, and the refinement of *in toto* image acquisition and analysis tools answers this challenge.

There have been dramatic advances in other areas, as well ranging from the refinement of new sensor technologies with researchers in the Kavli Nanoscience Institute, to improved tools for acquiring information from MRI images and collecting 4D light microscopic images. Our combination of modern and classical technologies continues to offer surprises, including the discovery of a novel sensory pathway in the mammalian olfactory system and the development of a molecular sensor with unprecedented sensitivity.

168. Screening for developmentally expressed genes by FlipTrap: An approach to fluorescently tag zebrafish proteins at their endogenous loci

Le A. Trinh, Sean G. Megason, Tatiana Hochgreb, Chathurani Jayasena, Frederique Ruf, Ankur Saxena, Rasheeda Hawk, Aidyl Gonzalez-Serricchio, Alana Dixson, Elly Chow, Constanza Gonzales, Ho-Yin Leung, Ilana Solomon, Marianne Bronner-Fraser, Scott E. Fraser

A fundamental goal in biology is to identify genes involved in biological processes and study their protein function. To this end, we have developed a gene trapping approach to generate endogenously expressed fluorescent fusion proteins and create Cre-mediated conditional alleles at the same loci. The gene trapping vector, termed FlipTrap, consists of an artificial exon encoding the yellow fluorescent protein, citrine, flanked by a splice acceptor and donor in the forward orientation, the red fluorescent protein, mCherry and a polyadenylation signal in the reverse orientation, and two pairs of heterotypic *lox* sites. Insertion of the citrine exon into the intron of an actively expressed gene by Tol2 transposition leads to the splicing of the citrine sequence into frame with the endogenous mRNA and hence, the formation of a full-length functional fluorescent fusion protein. We have integrated the FlipTrap vector throughout the genome of zebrafish and screened for the presence of Citrine expression. To date, we have isolated 148 FlipTrap lines with distinct expression patterns. Cloning of the trap transcripts indicate that 24.1% of the trap transcripts match cDNA and ESTs that have not been characterized in previous studies and identified as hypothetical genes. Our results offer experimental verification for these hypothetical genes. Interestingly, 9.3% of the trap transcripts are novel and do not match cDNA or ESTs in the existing collections of zebrafish libraries. These results demonstrate that the FlipTrap strategy is an alternative experimental approach for the identification of novel genes and can complement existing genomic efforts in the zebrafish field.

169. α-catenin dynamics during myocardial epithelia morphogenesis in heart tube formation

Le A. Trinh, Scott E. Fraser

A fundamental challenge in epithelial biology is to understand how epithelial cells undergo collective cell migration as they participate in organ formation. To this end, we are using the migration of myocardial precursors of the embryonic heart tube as a model to study the dynamic molecular and cellular interactions of epithelial cells as they undergo morphogenesis. The myocardial precursors form bilateral epithelial tubes that move coordinately toward the embryonic midline to form the linear heart tube. Previous studies using static imaging of the migration process have shown that these cells form a maturing epithelia as they migrate. The cadherin-catenin complexes have also been shown to restrict to the basolateral domain of the myocardial epithelia. To study the dynamic properties of junctional maturation in the embryonic heart, we are performing 4-dimensional confocal imaging and fluorescence recovery after photobleaching (FRAP) in transgenic embryos that express an α-catenin::citrine fusion protein. α-catenin is a component of the cadherin-catenin complex, co-localizing with E-cadherin and β-catenin. The α-catenin::citrine fusion was generated using a transposon-mediated gene trapping strategy that we previously developed in which we fluorescently tag zebrafish proteins at their endogenous loci with an internal exon encoding citrine. Using this gene trap line and confocal time-lapse microscopy, we are able to detect the dynamic changes in α-catenin localization during migration of the myocardial precursors. Using FRAP analysis, we find differences in α-catenin dynamics in the myocardial precursors compared to static epithelia cells such as the dermis. We are currently using this data in combination with analysis of α-catenin dynamics in mutant embryos that affect epithelial formation to provide a genetic framework into which these dynamic junctional behaviors can be placed. The analyses of α-catenin localization in wildtype and mutant cells during heart tube formation should further facilitate our understanding of the complex molecular and cellular interactions during cardiac development, as well as the mechanisms underlying epithelial morphogenesis.

170. Spatiotemporal analysis of GABA (A) receptors in the developing zebrafish with a FlipTrap insertion

Rasheeda Hawk, Eduardo Rosa-Molinar, Scott E. Fraser, Marianne Bronner-Fraser*

Spatiotemporal analysis of GABAergic expression was studied in the developing and adult zebrafish, *Danio rerio*. From the FlipTrap screen, a small population of zebrafish labeled as FT99b began expressing the citrine reporter gene in neuronal cells of the forebrain at approximately 72hpf. Gene isolation, cloning, and sequencing have confirmed that the citrine-labeled protein is a GABA (A) receptor. To understand the citrine expression pattern for this flip-trap in detail, we analyzed the expression with laser scanning confocal microscopy (LSCM) and found expression in the pallium and prethalamus. This along with the use of neuroanatomical tract tracing and immunofluorescence against GABA(A) receptor in the adult fish suggests that GABAergic projections descend to the mesencephalic locomotor region (MLR); a region that has a significant role in the control of locomotor behavior. In the adult zebrafish, several GABAergic projections to the MLR were also identified in the telecephalon and diencephalon.

Little is known about the role of GABA (A) receptors in neurogenesis. The goal of this proposal is to use new technology in zebrafish to identify, dynamically follow and ultimately mutate GABAergic receptors in living and developing embryos to adults.

A recent application of fluorescent microscopy known as fliptrapping has been used in conjunction with molecular biology to study gene expression and tissue and

organ development. This integrative science technique approach allows us to identify proliferating and migration of Gabanergic cells during neurogenesis in zebrafish.

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Aim 1: Dynamic spatiotemporal analysis of the protein expression pattern and function of GABA (A) receptor in the developing zebrafish. Embryos will be imaged from day 2 to day 7 to look at the pattern of expression of GABA(A) receptor proteins in real time and the formation neuronal connections.

Aim 2: Effects of loss-of-function of GABA (A) receptors using cre-mediated knock-down. Taking advantage of the fliptrap vector, I will perform both global and tissue-specific knock-downs of the receptor in particular populations.

Aim 3: To investigate the effects of environmental toxins on the expression of GABA (A) receptor in the developing zebrafish. Studying the changes in the expression of critical genes is one of the ways to understand any morphological malformation in developmental toxicology. Through the fliptrap technology, these changes can be continuously recorded and quantified.

171. Imaging somitogenesis and myogenesis in FlipTrap and muscular dystrophic zebrafish

Frederique Ruf-Zamojski, Sean Megason, Le Trinh, Scott E. Fraser

Somitogenesis and myogenesis are highly conserved mechanisms in vertebrate species that give rise to skeletal formations. These processes have not been investigated with single-cell resolution during the development of living embryos. It is important to observe these mechanisms with high resolution to understand how muscles form, are maintained, or degenerate, and to measure if there are any variations in single-cell behaviors when comparing wild-type and muscle-affected mutant development. To that end, we started to combine two powerful methods in living zebrafish embryos to study muscle formation and degeneration with high-resolution and non-invasively: 1) *in toto* single-cell imaging using "embryo arrays," individual cell labeling, confocal microscopy and 3D reconstructions (Figure 1); and 2) a FlipTrap genetic screen to isolate lines with citrine fusion protein expression in muscles and a possibility to flip this expression and create cre-lox conditional mutants. This novel screening approach has so far identified ten lines with functional fluorescent protein fusions to endogenous muscle proteins and is used to assess *in vivo* the specific functions of the trapped genes, before and after disrupting their function with cre-lox recombination (Figure 2). Preliminary results show that the trapped proteins recapitulate endogeneous protein expression using immunostaining when antibodies are available (Figure 3), and we compare the distribution of the specific mRNAs by combining imaging of the trapped protein with *in situ* hybridization. Time-lapse microscopy of the fluorescent proteins allows us to watch the process of muscle formation and the distribution of the trapped proteins with unprecedented resolution in living zebrafish embryos. Together, these two approaches enable us to gain more insights into the roles played by specific proteins in muscle formation and maintenance. In parallel we are imaging with high-resolution and early in development known zebrafish mutants and morphants exhibiting muscular dystrophies phenotypes to compare and understand the effects and roles of specific proteins in this muscle disease.

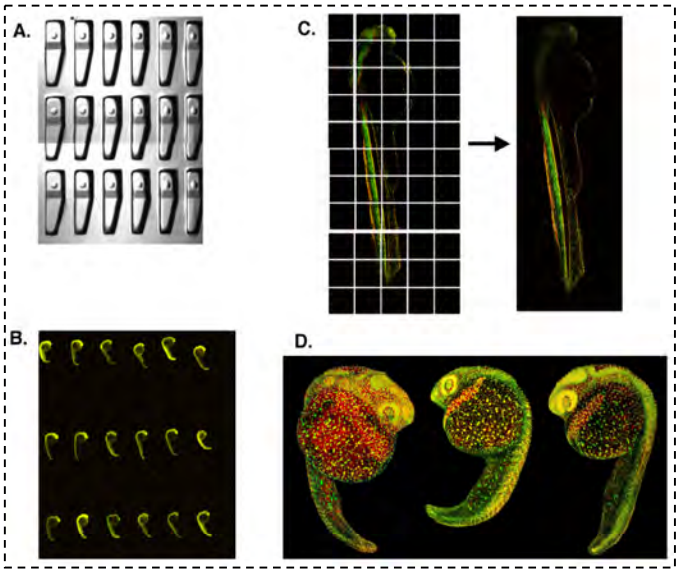

Figure 1: Imaging zebrafish embryos using an *in toto* imaging approach.
A. Embryo arrays agarose molds, specially designed by Dr. Megason to hold the yolk, growing head and tail. **B.** H2B-GFP / membrane-cherry expressing embryos (after RNA injection) placed in the embryo array and imaged at 5X magnification. **C.** Imaging a whole zebrafish with single-cell resolution: Left: Tiling example of a 48 hpf embryo performed with MegaCapture to cover the whole embryo at each z-plane; Right: 2D reconstruction at one specific z plane using MegaMontage, 40X magnification. **D**. 3D reconstructions of 24 hpf embryos using Imaris software, 40X magnification.

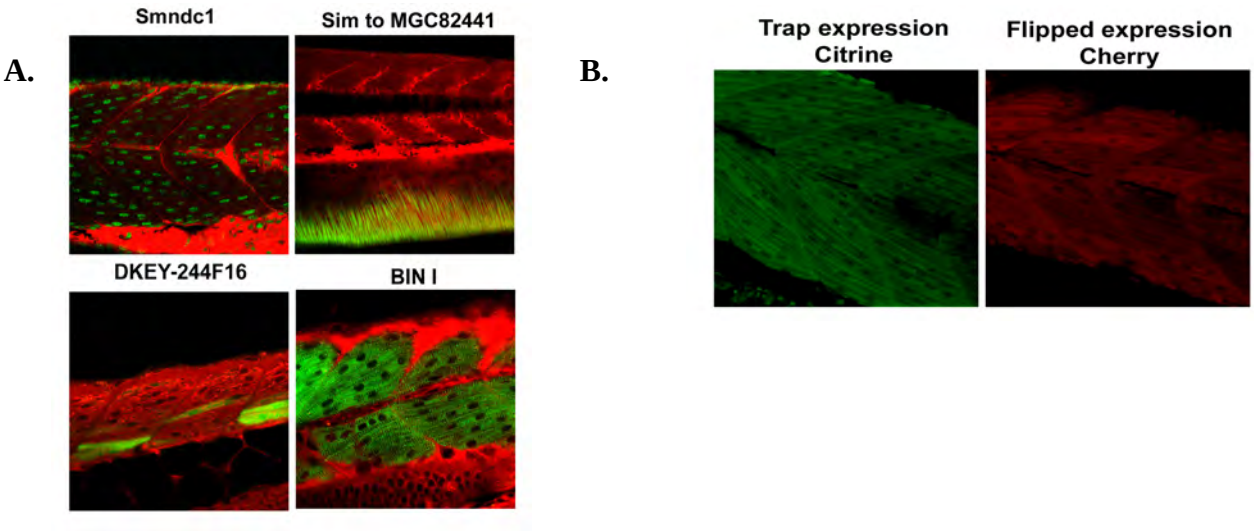

Figure 2: A. Examples of FlipTrap lines showing muscle/trunk expression (green; red: Bodipy, counterstain). B. Example of expression of a muscle trap in the citrine (left, green) and its flipped (right, cherry) conformations showing same localization.

Figure 3: Example of colocalization (yellow, left) of the trapped protein (green, right) and the antibody against that protein (red, middle) in this case dystrophin.

172. Protein kinase C alpha (PKC-α) in embryonic and larval zebrafish spinal cord neurons

Alana Dixson, Scott E. Fraser

The FlipTrap vector is a transposon-based gene trap designed to track the endogenous temporal and spatial expression of developmentally important genes in zebrafish embryos. When the vector randomly inserts into an intronic region its yellow fluorescent protein (YFP) component is transcribed in frame to create a fluorescent fusion protein with the endogenous gene's product. In this study, I characterized one FlipTrap line, CT 54a (N = 50) via confocal laser scanning microscopy (CLSM) over the first 30 days post-fertilization (dpf).

In this transgenic line, Protein Kinase C alpha (PKC-α) is trapped and the YFP-PKC-α fusion protein can be visualized with low-magnification fluorescence microscopy beginning at ~20 hours post-fertilization (hpf). YFP-PKC-α expression was found consistently in dorsal spinal cord neurons, including some Rohon-Beard cells (R-B), which are mechanosensory primary sensory neurons. Several brain regions, the lens and the trigeminal ganglion are also fluorescently labeled. As found previously by other workers, by 3-4 dpf a small number of cells of the dorsal root ganglion (DRG) also contained the fusion protein up to 30 dpf. Beyond this time point further analysis has not been performed.

As late as 14 dpf R-B like neurons appear to persist as identified by morphology and location. These are the largest and most superficial neuronal cells in the dorsal spinal cord. Using anti-zn-12 (N = 15) and anti-Islet-1 antibodies (N = 20), I further confirmed the identity of R-B neurons during the first 5 dpf. Interestingly, both antibodies labeled substantially more R-B neurons than the fusion protein. From 6 dpf onward, reliable immunohistochemical phenotyping has not been possible.

Additionally, *in vivo* time-lapse imaging captured axonogenesis deriving from R-B neuronal somata as early as ~28-31 hpf and continuing over the ensuing 24 hours (N= 4). Peripheral axonal arbors in the skin and muscles, and what appears to be the dorsal lateral tract (DLT), expressed the YFP-PKC-α fusion protein. These axons also co-labeled with anti-zn-12 antibody during the same time period and as late as 6 dpf.

In conclusion, YFP-PKC-α-positive neurons of CT 54a appear to be a subset of the larger population of both Islet-1 and zn-12-labeled R-B neurons. These neurons coexist with at least some cells of the DRG even as late as 14 dpf, providing some evidence that the appearance of this ganglion may not coincide with the complete disappearance of all R-B neurons.

173. Filopodia characterization in the zebrafish embryos

Luca Caneparo, Periklis Pantazis, Scott E. Fraser

During vertebrate gastrulation, a series of well conserved signalling events and morphogenetic movements are required, to set up the correct formation of the body plan and to guarantee future cell diversity. In zebrafish, the optical clarity of the embryo allows the *in vivo* visualization of the gastrula movement, and the availability of multiplex fluorescent tags consent to highlight the gastrula cytoarchitecture, with single cell resolution. This approach allowed *in vivo* visualization of cell shape and morphology, often indicators of changes in cell function. Cellular protrusions, for example, are characteristic of exploratory behavior and used for cell-cell communication, these mechanisms are ubiquitous and share among animals. Well-studied examples of short-range membrane cell protrusions are microvilli, lamellipodia and ruffles.

We describe for the first time the presence of filopodia reaching up to 250μm in length in the developing zebrafish embryo during gastrulation. We characterized their growth and we topologically mapped the filopodia distribution in the embryos *in vivo*; we quantitatively measured length and persistence of these extensions and we analyzed the cell membrane dynamic along the projection during gastrulation *in vivo*.

Cell protrusion extending over a distance of more than 50μm such as filopodia, cytonemes and nanotunneling have been previously described, respectively, in sea urchin, during gastrulation, in *Drosophila*, in the imaginal disc and in different cell types in the vertebrate immune system. During vertebrate development only short filopodia have been previously reported in the mouse blastocysts, with range and extension only up to 20μm and not comparable to what we described in zebrafish or to what has been reported in invertebrates.

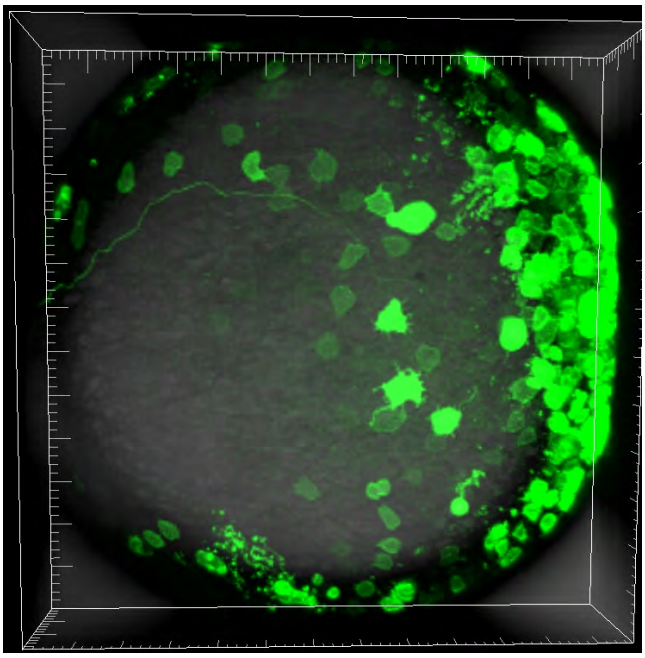

Figure 1: Animal pole view of 60% epiboly zebrafish embryo reconstruction showing cell projection extending along the epiblast germ layer. Cells are labeled using mDendra2.

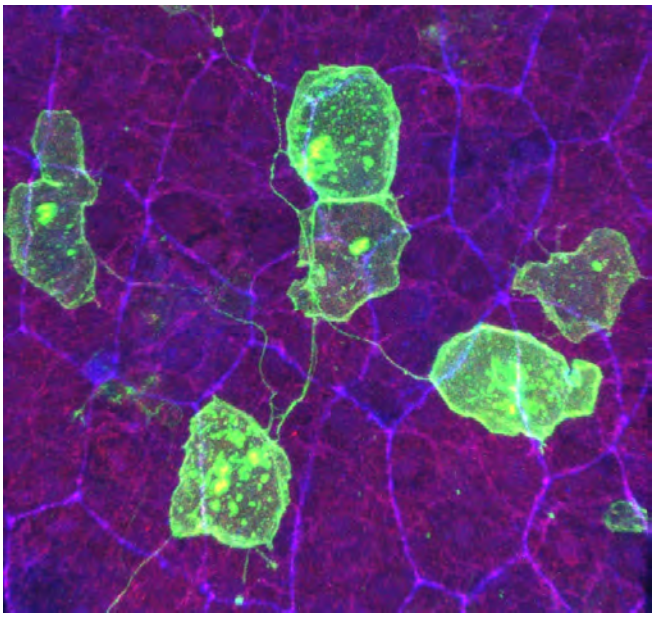

Figure 2: Cell projections in the zebrafish neural plate at 75% epiboly. In green mDendra2, in blue falloidin and in red β-ca.

174. Oriented cell division in the zebrafish neural plate: Towards a statistical model

Luca Caneparo, Elena Quesada Hernandez[*], Carl-Philipp Heisenberg[*], Michael Liebeling[#], Scott E. Fraser

Oriented cell division is a fundamental process used to generate cell diversity in plants and animals. In metazoans this approach is used throughout the lifespan of the animal: during development (e.g., in mouse cortex formation and in the zebrafish neural plate); in stem cell maintenance and in certain disorders like polycystic kidney disease or cancer development. In recent years, much has been discovered about the molecular cues and the signal transduction pathways that mediate stereotypical orientation of cell division during embryonic development. In contrast, such molecular approaches have been less thoroughly defined with a quantitative and systematic analysis. What is required is a set of new portable algorithms that permit *in vivo* measurements of angles at which cell divisions occur independently of the model system used. This will permit key questions to be addressed; the characterization of oriented cell divisions in different embryonic layers, the study of the roles played by different pathways in cell mitosis alignments and the formulation of a statistical model for the orientation of cell divisions in vertebrates.

We chose the zebrafish as our vertebrate model due to its mutant availability, the ease to generate gain and loss of function and its optical clarity. Recent work demonstrates that oriented cell divisions in the gastrula align along the antero-posterior (AP) axis in one of the embryonic layers: the epiblast. This event has been previously described to rely on Wnt non-canonical signaling. Perturbations of this pathway generate a randomization in the orientation of the mitotic division, which are no longer aligned with the AP axis during gastrulation. We extended this analysis to both neural plate layers: the hypoblast and the previously described epiblast. At gastrula stage we analyzed the polarity of cell divisions with respect to the main embryonic axes: the antero-posterior and the dorso-ventral (DV) axes.

To make this analysis feasible on a statistically significant number of cell divisions and embryos, we designed a semi-automatic algorithm to measure the angles at which cell divisions occur with respect to the AP and the DV axes. We further adapted and implemented a statistical test (chi-square) to compare the angular distribution of cell divisions in embryos where different developmental pathways were perturbed. The analysis was extended by comparing the cell division behavior at the level of different embryonic layers for each alteration. Exploiting the algorithm characteristics by comparing different scenarios, we found different degrees of randomization in the orientation of mitosis in the zebrafish neural plate. These methods only require limited computational power, which allows for the use of standard personal computers to routinely perform this sort of analysis.

We generated a flexible and portable computational pipeline to reconstruct and analyze oriented cell divisions and to automatically perform statistical tests on the measured angular distributions. Since our approach to study the orientation of cell divisions is systematic and in large part automated, we can envision analyzing large amounts of data, as well as the possibility to formulate and verify statistical models of cell divisions during zebrafish gastrulation.

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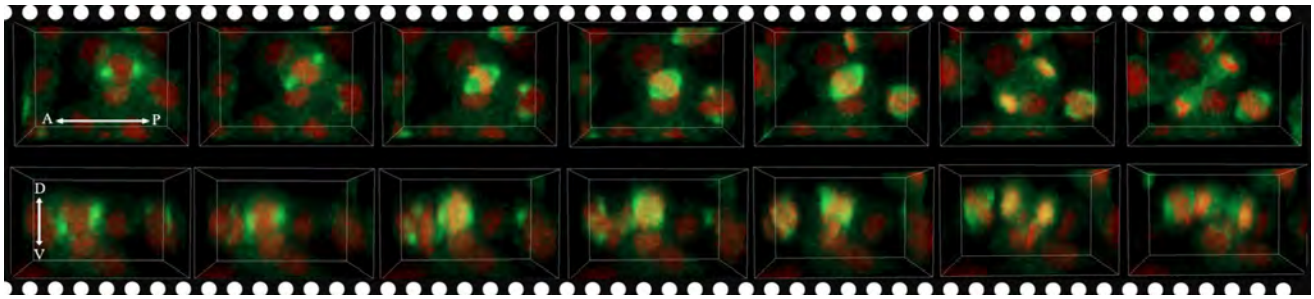


Figure 1: Cell division in the zebrafish neural plate. The first and second rows show a 3D reconstruction of the same mitotic event from two different perspectives. The spindle formation is shown in green using α-tubulin- and in red H2B-mRFP show the nuclear changes occurring during mitosis.

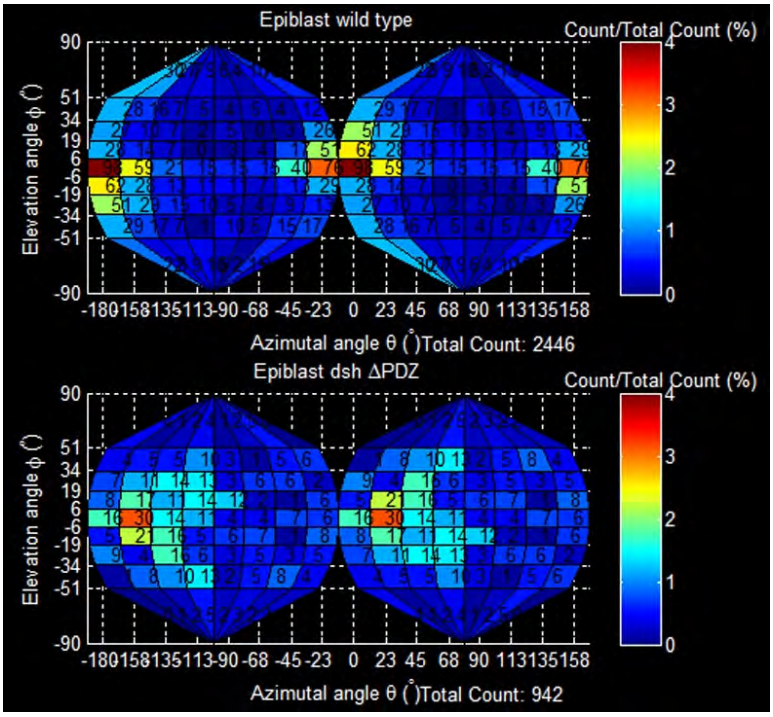


Figure 2: Quantification of the angle at which cell division occurs in the zebrafish neural plate. Both graphs show the angle of division of epiblast cells along the antero-posterior axis (Azimutal angle) and the angle along the dorso-ventral axis (Elevation angle). The top panel represents wild-type embryos and the bottom panel represents embryos in which the Wnt pathway has been perturbed.

175. Quantitative imaging of the collective cell movements shaping an embryo

Willy Supatto, Amy McMahon, Angelike Stathopoulos[*]*, Thai Truong, Scott E. Fraser*

The combination of advanced imaging and image analysis techniques enables the investigation of large, dynamic cell populations within a developing embryo. These imaging approaches provide a unique opportunity to study embryonic morphogenesis from the level of cellular processes to the scale of an entire tissue or organism. Gastrulation in the *Drosophila melanogaster* embryo is an excellent model system for the study of embryonic morphogenesis. In less than two hours of development, ~6000 cells undergo stereotypical morphogenetic events, such as tissue invagination, convergence-extension, planar cell intercalation, radial cell intercalation, epithelial-to-mesenchymal transition, synchronized waves of cell division, and collective cell migration.

We developed an experimental strategy combining 4D *in vivo* imaging using 2-photon excited fluorescence (2PEF) microscopy, 3D-cell tracking using image processing, and automated analysis of cell trajectories using computational tools [1]. This quantitative approach decomposes 3D cell movements, generating a precise description of morphogenetic events. The reproducibility of morphogenetic events among wild-type embryos can be tested and mutant phenotypes can be dynamically analyzed. The quantitative investigation of the collective nature of cell movements provides a method to study complex or even subtle mutant phenotypes, such as the ability to distinguish cell populations that exhibit different behaviors. We recently applied this approach to gain insights into the control of collective cell migration during mesoderm spreading in *Drosophila* embryos [2].

We are currently working on the improvement and the generalization of this approach to other morphogenetic events, embryonic stages, or model organisms. This work includes the study of phototoxicity and its limitation to improve the *in toto* imaging of large cell populations; or the optimization of automated cell trajectory analysis to quantify the stereotypical processes of morphogenesis (cell intercalation, cell division, tissue invagination, etc.).

[*]*Assistant Professor, Division of Biology*

References

[1] Supatto, W., McMahon, A., Fraser, S.E. and Stathopoulos A. (2009) *Nature Protocols.* In press.

[2] McMahon, A[*]., Supatto, W[*]., Fraser S.E. and Stathopoulos, A. (2008) *Science* **322**:1546-1550. [*]Equal contribution

176. Probing cilia-driven flow in living embryos using femtosecond laser ablation and fast imaging

Willy Supatto, Scott E. Fraser, Julien Vermot

Embryonic development strictly depends on fluid dynamics. As a consequence, understanding biological fluid dynamics is essential since it is unclear how flow affects development. For instance, the specification of the left-right axis in vertebrates depends on fluid flow where beating cilia generate a directional flow necessary for breaking the embryonic symmetry in the so-called left-right organizer. We developed an all-optical strategy to investigate flow dynamics *in vivo* that is compatible with both normal biology and *in vivo* imaging [1,2]. First we used sub-cellular femtosecond laser ablation to generate fluorescent micro-debris to label the flow. The non-linear effect used in this technique allows a high spatial confinement and a low invasiveness, thus permitting us to target sub-cellular regions deep inside the embryo. Then, we used fast confocal microscopy and 3D-particle tracking to image and quantify the seeded flow. This approach enabled us to investigate the flow generated within the zebrafish left-right organizer, a micrometer-scale ciliated vesicle located deep inside the embryo and involved in breaking left-right embryonic symmetry. We mapped the velocity field within this vesicle (called the Kupffer's vesicle) and surrounding a single beating cilium, and showed that this method can address the dynamics of cilia-driven flows at multiple length scales. We validated the flow features as predicted from previous simulations. We are currently using such detailed descriptions of fluid movements to investigate the relationship between cilia-driven flow and signal transduction.

Publications

[1] Supatto, W., Fraser, S.E. and Vermot, J. (2008) An all-optical approach for probing microscopic flows in living embryos. *Biophys. J.* **95**:L29-31.

[2] Supatto, W., Fraser, S.E. and Vermot, J. (2009) Probing cilia-driven flow in living embryos using femtosecond laser ablation and fast imaging. *SPIE Proceedings*, invited paper. In press.

177. Extended volume imaging of aqueous samples

John Choi, David Koos, Thai Truong, Scott E. Fraser

Genetically encoded fluorescent protein tags are important tools for directly visualizing and highlighting specific cell populations and their cellular components. These labels are ideally suited for studying multi-cellular events such as tissue differentiation, organ growth and the establishment of neural connectivity dispersed over large regions. To gain insight into the interactions and interdependencies throughout the sample it is necessary to image a macroscopic sample in 3-dimensions with microscopic, cellular resolution. Unfortunately, the depth of imaging is severely restricted in current high-resolution imaging tools: in the best cases about a hundred micrometers for confocal microscopy and about a millimeter for 2-photon microscopy. In our approach, we combine high-resolution laser scanning microscopy with aqueous microtoming techniques in an automated way to enable extended 3-dimensional volume imaging of static samples. We use a mosaic reconstruction method that involves imaging multiple, adjacent, and overlapping tissue volumes. By restricting our approach to aqueous compatible microtoming, we retain compatibility with fluorescent proteins, an advantage over existing methods that rely on organic solvents during the embedding process. Thus, we avoid damaging the sample (distortion, shrinkage, and extraction of cellular components) and preserve fluorescent protein fluorescence.

178. Completion of multi-photon raster-scanning microscope

Thai Truong, Scott E. Fraser

Construction of the multi-photon laser-scanning microscope (MPLSM) has been completed. Figure 1 shows an overview picture of the completed instrument, while Figure 2 shows a sample 3-dimensional image taken with the system. The system is capable of excitation with wavelength range 690-1080 nm, with one detection channel in epi, capturing a field of view of 1024x1024 pixels with one frame per second, reaching approximately the diffraction-limited resolution. This puts the system at about the same capability as a standard commercial multiphoton microscope. We are presently continuing with further development of the MPLSM: adding two epi detection channels and one transmitted channel (for a total of four channels), adding more lasers to allow simultaneous excitation with two or three different wavelengths, spanning the entire excitation spectra of cyan to red fluorescent proteins. We are also working to add flexible and user-friendly features to the imaging setup, allowing for easier optical alignment optimization and for the eventual usage of the system by more users.

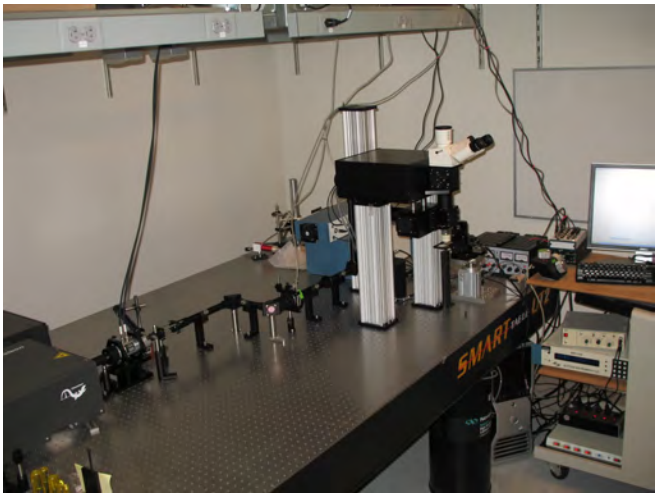


Figure 1. Completed 2-photon imaging station. Exploration of various imaging methodologies will be carried out using the same hardware platform and lab room facility.

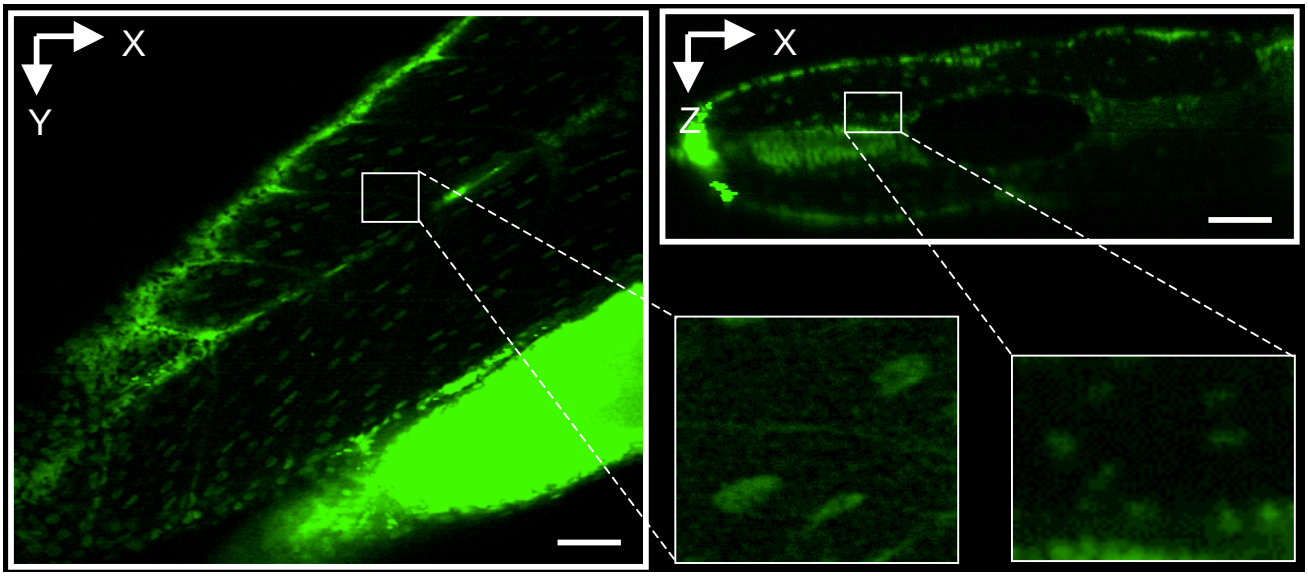


Figure 2. Sample image taken by newly constructed multi-photon raster-scanning microscope. Sample is a live zebrafish embryo, of the flip-trap line Ft80a, at ~2.5 days post fertilization, showing the ubiquitous expression pattern of nuclear-labeled citrine. The image of a xyz-volume of 500x500x210 microns^3 was taken. Left panel shows the view of a xy-slice at ~50 microns deep into the sample. Right top panel shows an xz-cross-sectional view. The achieved resolution, as shown in the zoomed-in boxes, is more than adequate for segmentation of the cell nuclei. Scale bar = 50 microns.

179. Extended volume imaging using combination of two-photon microscopy and microtome sectioning

John Choi, Thai Truong, Scott E. Fraser, David Koos

Direct optical imaging allows access to tissues/organisms that are at most 1 mm thick. To image anything larger, it is necessary to cut the sample into smaller parts, image them separately, and reconstruct the separate images into a whole 3-dimensional image. We are working on constructing an automated system where the cutting and imaging can be carried out serially without manual repositioning of the sample. This system will be built off of the MPLSM imaging platform.

180. Imaging the organizational structure and composition of neuronal receptors

Larry Wade, D. Lo, Henry Lester, Scott E. Fraser*

Nicotinic acetylcholine receptors (nAChRs) are found in many central nervous system and nerve-skeletal muscle postsynaptic membranes. The nAChR is a (pseudo) symmetric pentameric structure comprised of homologous subunits. This straightforward structure is complicated by there being five major groups of subunits that can be arranged in a variety of permutations: α, β, γ, δ, and ε. Furthermore, the α subunits exist in at least ten different subtypes (α1 through α10) and the β subunits exist in at least four subtypes (β1 through β4). Therefore, a remarkable variety of specific assemblages are possible with a corresponding variety of chemical reaction rates.

We have initiated an effort that combines several novel techniques to directly image the microorganization of nAChRs expressed in N2a cells. By expressing different color XFPs in specific AChR α and β subunits, we expect to resolve individual receptors. Furthermore, we hope to discretely identify individual receptors over the field-of-view. This will enable us to simultaneously characterize the large-scale distribution of such receptors, to identify local structures within an imaged membrane surface, and to assay variation of receptor subtypes.

The techniques being combined in this experiment include a novel method for repeatedly aligning the atomic force microscope probe and excitation laser with specific substrate locations to within a few 10's of nm, and an ~10 nm resolution, single-molecule sensitive near-field optical microscope. Receptor subunit imaging is enabled by the Lester group's development of specific XFP-labeled AChR subunits.

By developing the ability to directly image each subunit of each AChR expressed in the membrane of N2a cells, we lay the groundwork for future studies of transmembrane proteins, receptors and membrane-embedded proteins in many different types of cells. In particular we will substantially advance our ability to image the microorganization of nicotinic acetylcholine receptors at neuronal synapses.

Professor, Division of Biology, Caltech

This work is supervised by Professor Scott E. Fraser in collaboration with Professor Henry A. Lester.

Support: JPL R&TD and BioNano programs, NASA-PRSGC IDEAS-ER grant, Caltech President's fund, NIH, NSF and Pharmagenomix grants, NS-11756, DA-17279, and Philip Morris External Research Program.

181. Characterization of pulse-splitting technique in imaging of zebrafish embryo

Thai Truong, William Dempsey, Scott E. Fraser

Pulse splitting is an optical method used in multiple-photon imaging for reducing photobleaching and phototoxicity. We have conducted preliminary characterizations of pulse splitting in imaging of developing fish embryos. Details of our experimental test are described in Figure 3 and its caption. The preliminary conclusion is that the use of multiple pulses, while less damaging than single pulse, still inflicts observable damaging effects to the developing zebrafish embryo. We are in the process of verifying this preliminary result, testing with more embryos and with various experimental conditions. If this result is confirmed, then the pulse-splitting technique cannot be used to improve the current two-photon-fluorescence in order to meet in toto imaging requirements – alternative strategies have to be explored.

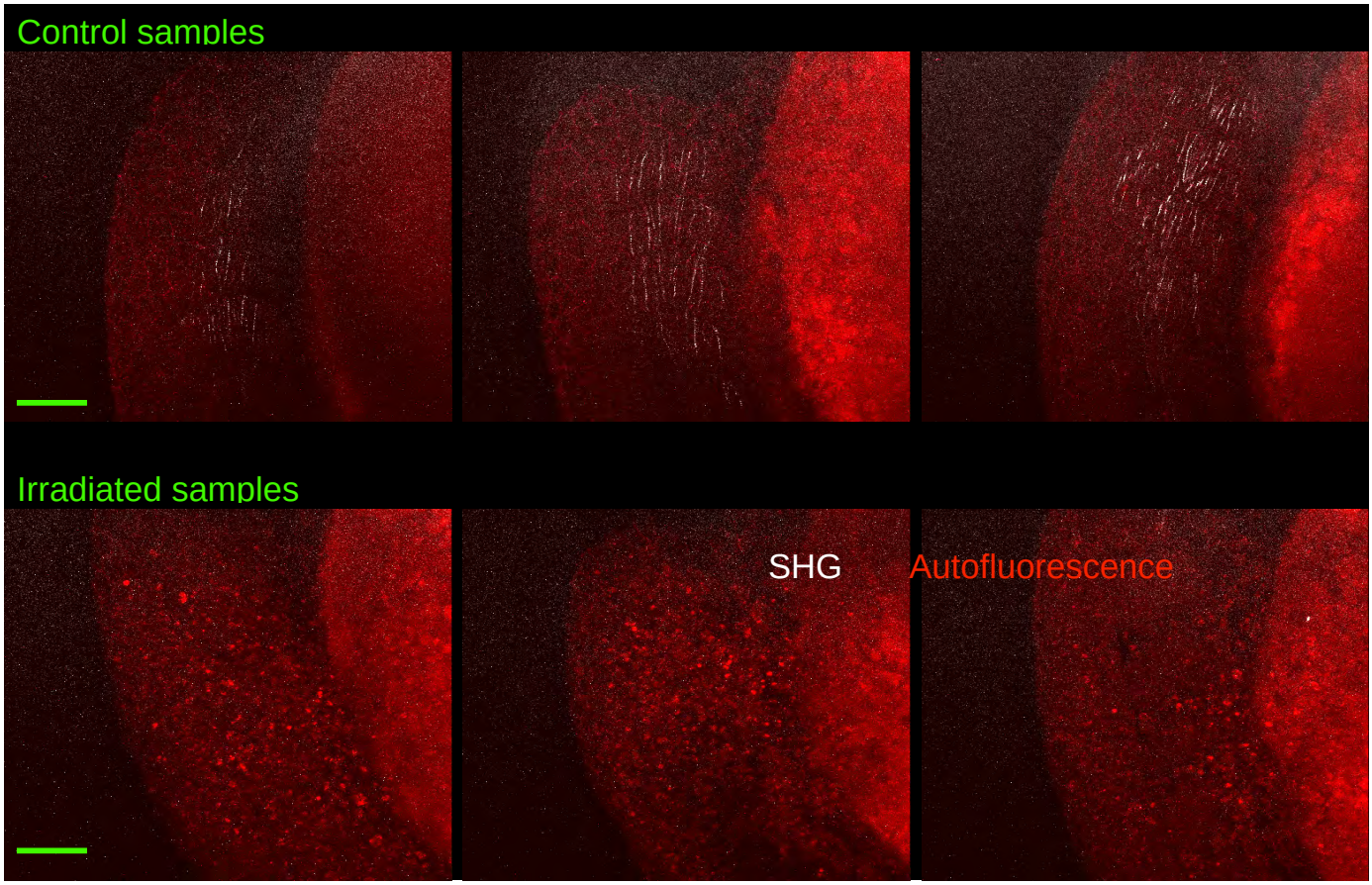


Figure 3. Use of multiple pulses in multi-photon imaging affects development of zebrafish embryos. In our test, we looked at the development of skeletal muscle tissues in the trunk area of embryos as a readout of their viability after being subjected to imaging. Embryos were irradiated with laser pulses that are each split into 16 pulses, for ~3 hrs @ 32 degree C (10 to 18-somite stage), at the intensity level approximately 1/5 of what is needed for in toto imaging. After irradiation, the samples were looked at using second-harmonic generation (SHG) (white color) and endogenous 2-photon-fluorescence (red color), to see the progress of skeletal muscle development (which would yield positive SHG signal) and the overall cellular morphology. Control samples are embryos mounted in the same imaging slide, but not subjected to irradiation. As shown in the figure, the irradiated samples did not show SHG signals that are seen as white streaks in the control samples, signifying that development of muscle tissue had been affected. Also, the autofluorescence of irradiated samples showed a puntated pattern, possibly evident of cell deaths. Scale bars = 50 microns.

182. Characterization and visualization of the FcRn-dependent transcytotic pathway using high-resolution fluorescence confocal microscopy

Galina V. Jerdeva, Pamela J. Bjorkman[*]*, Scott E. Fraser*

Specific delivery of proteins across polarized epithelia is controlled by receptor-mediated transcytosis. Based on studies of the trafficking of model receptors such as the polymeric immunoglobulin receptor (pIgR), the pathways for receptor-mediated transport of protein ligands in the basolateral to apical direction are relatively well understood. Neonatal Fc receptor (FcRn), transports maternal immunoglobulin G (IgG) in the opposite direction across intestinal or placental epithelial barriers to provide immunity to fetal or newborn mammals and serves as a protection receptor for IgG. To investigate FcRn-mediated transport of IgG, we are using Madin-Darby Canine Kidney (MDCK) cells stably expressing FcRn (MDCK-FcRn). The transfected cells specifically transcytose IgG and Fc across polarized cell monolayers, therefore, presenting an *ex vivo* system that mimics the *in vivo* FcRn-dependent transport system. Using high-resolution confocal microscopy, we are able to identify intracellular compartments involved in transcytosis of labeled Fc and IgG in MDCK-FcRn cells by colocalization studies with organelle-specific markers. Comparing the intracellular trafficking of FcRn and its ligands with trafficking of a basal to apical model receptor, pIgR, which transports dimeric dIgA (dIgA), provided information about common trafficking pathways of IgG and dIgA, such as traffic through early endosomal and common endosomal compartments. To compare FcRn- and pIgR-dependent intracellular pathways**,** we are using MDCK-FcRn cell line transiently expressing pIgR. Fluorescently-labeled Fc and dIgA can be internalized apically and basolaterally, respectively, by polarized MDCK-FcRn-pIgR monolayers, and the intracellular locations of internalized ligands is investigated by confocal microscopy followed by colocalization analysis in 3D of internalized probes with different endogenous markers. pIgR receptor traffic is used as a reference in colocalization experiments. Increase in colocalization in early chase points (5-10 min) with markers, EEA1 and rab11, indicate that FcRn-Fc travels trough early and sorting endosomes, respectively, while its later enrichment (15-20 min) with transferrin-positive compartments indicate that complex travels through common sorting and basolateral endosomal compartments. To investigate dynamics of Fc trafficking, we are using a high speed synchronized spinning disk Confocal Imaging System (UltraVIEW ERS) with a sensitive EMCCD camera, resulting in near real-time high resolution time-lapse microscopy, performed on living polarized cells at acquisition speed of 5-10 frames per sec. Mobility characteristics of FcRn- and pIgR-positive structures in live MDCK cells, such as vesicle-track displacement and vesicle track straightness, were similar for these two vesicle populations and were similarly affecter by microtubule-destabilizing agent, Nocodazole.

[*]*Professor, Division of Biology, Caltech*

183. Manganese-enhanced MRI for studying spinal cord functional projection

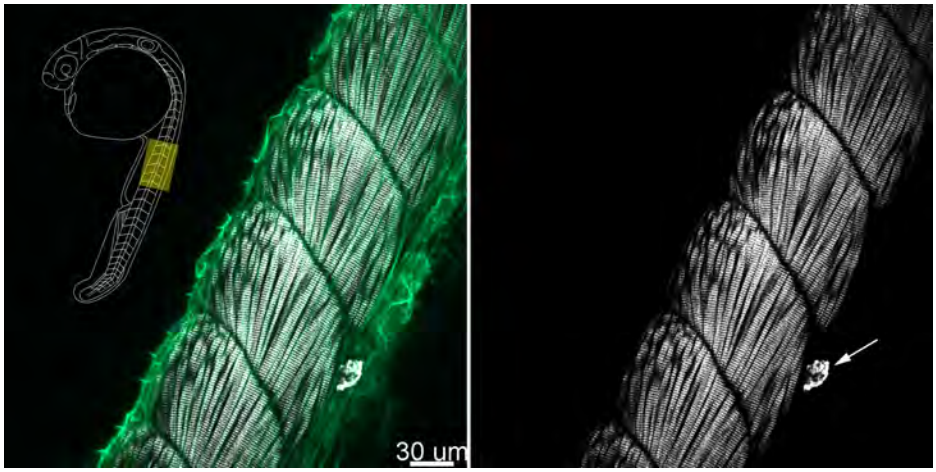
Xiaowei Zhang, Russell E. Jacobs
Biological Imaging Center, Beckman Institute, Caltech

The present study provides a sensitive and rapid experimental approach for MRI visualization and analysis of spinal cord (SC) projection in normal animals. This approach is based upon neuronal manganese (Mn^{2+}) uptake after focal SC injection of $MnCl_2$, and subsequent *in vivo* manganese-enhanced magnetic resonance imaging (MEMRI) of the SC pathway. The experiments were conducted on three C57LB mice. Under general anesthesia, 1 μL of 200 mM $MnCl_2$ was directly infused into SC via a burr hole drilled in the T2 right lamina. MR images were acquired with a T_1 weighted 3D FLASH sequence at 30 min, 8, 24, 48 and 72 hrs post-injection. The time-lapse MRI shows Mn^{2+} enhanced intensity spread from the injection site along the SC in views with different time points. At 30 min, Mn^{2+}-induced intensity changes are obvious near the injection site. At later time points, 8, 24, 48 and 72 hr, Mn^{2+}-induced intensity has progressed further along the SC. By 72 hr, the cerebral peduncle and pyramidal tract of the brain had discernible increased intensity. These preliminary results collectively demonstrate that MEMRI is a sensitive and objective tool for *in vivo* visualization and quantification of functional projection in the SC.

184. Second harmonic generation (SHG) imaging microscopy

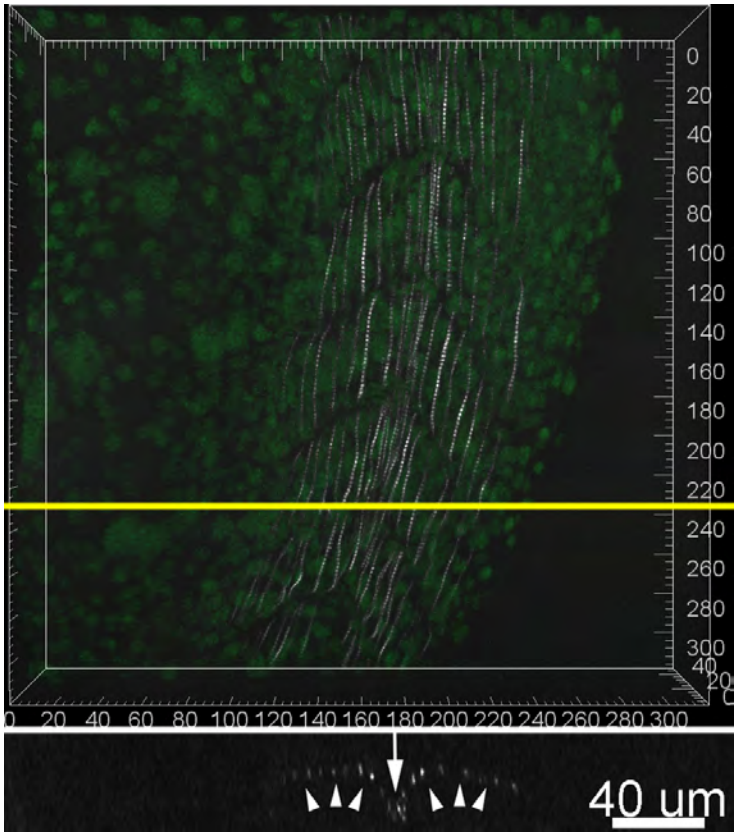
Periklis Pantazis, William Dempsey, Robert Kaspar, Scott E. Fraser

Second harmonic generation (SHG) imaging microscopy is an emerging microscopic technique for a wide range of biological and medical imaging. SHG is a second-order nonlinear optical process in which two photons at the frequency (w) interacting with non-centrosymmetrical media (i.e., material lacking a generalized mirror symmetry) are combined to form a new photon with twice the energy, and therefore, twice the frequency (2w) and half the wavelength of the initial photons. In zebrafish, a vertebrate model system, the tissues that produce SHG signal and the conditions required for imaging have not been established. We determined which zebrafish tissues produce SHG, at which developmental stages, and at which wavelengths. The generated optimum wavelength map for various organs at early embryonic stages offers us the possibility to perform long-term *in vivo* imaging without the use of fluorescent markers. This way, mutations affecting developmental processes, as well as function of SHG zebrafish tissue can be analyzed.



Longitudinal section of a 2dpf zebrafish embryo.

Left: SHG (white) and membrane marker (green) channel merge. Inset is the general imaging location within the embryo. Right: SHG (white) channel depicting skeletal muscle SHG (striated tissue within the somites) and a pigment cell (arrow).



A single SHG layer is observed in WT somites by 20 hpf.

Top: Nuclear label (green) and SHG (white) channel merge. Inset is the general imaging location within the embryo. The yellow line depicts the general location of the cross section shown below (this line shows the general location of the cross section in the inset, as well). (Units: μm).

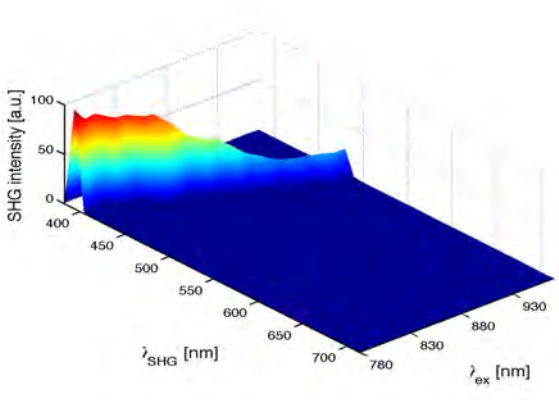
Bottom: Cross-sectional view of single SHG layer (white) from slow-muscle tissue (arrowheads). The arrow depicts the so-called horizontal myoseptum, which separates dorsal and ventral somites.

185. Second harmonic generation (SHG) nanoprobes for *in vivo* imaging

Periklis Pantazis, Ye Pu[1,2], James Maloney, David Wu, Chia-Lung Hsieh[1,2], Rachel Grange, Demetri Psaltis[1,2], Scott E. Fraser

Fluorescence microscopy has profoundly changed how cell and molecular biology is studied in almost every aspect. However, the ultimate need of characterizing biological targets is largely unmet due to fundamental deficiencies associated with the use of fluorescent agents. Dye bleaching, dye signal saturation and tissue autofluorescence can severely limit the signal-to-noise ratio (SNR). Here, we demonstrate that second harmonic generating (SHG) nanoprobes are suitable for *in vivo* imaging and eliminate most of the inherent drawbacks encountered in classical fluorescence systems. These nonlinear nanocrystals of less than 100 nm are capable of generating second harmonic signals because they do not possess an inversion symmetry and can be detected by conventional two-photon microscopy. Unlike commonly used fluorescent probes, SHG nanoprobes neither bleach nor blink, and the signal does not saturate with increasing illumination intensity. SHG nanoprobes provide a superb SNR in live imaging of zebrafish embryos, and unlike endogenous second harmonic generation their signal can be readily detected both in trans- and in epi-direction**.**

[1&2]Caltech and PFL, Switzerland



Displayed is the 3D normalized SHG signal data profiles of SHG nanoprobes (signal ranging from 380 to 710 nm) generated by conventional two-photon excitation (excitation ranging from 760 to 970 nm). Note that the SHG signal peaks are always half the wavelength of the incident excitation wavelength. Color code: dark blue corresponds to zero intensity and dark red corresponds to maximal intensity.

186. Field resonance enhanced second harmonic (FRESH) signaling biosensors

Periklis Pantazis, Nathan Hodas, Ye Pu, Demetri Psaltis[*], Scott E. Fraser

Signaling regulates embryonic development by providing positional information to cells so that they differentiate properly as they proliferate to build up the final shape. However, defects leading to abnormal activation of signal pathways often underlie most tumorigenic events. In recent years, much has been discovered about the molecular and biochemical characteristics of a variety of signal transduction pathways. In contrast, the cell biology of such signaling events is starting now to be studied. In particular, we are interested in the following questions: 1) When does signaling occur? 2) Where does it take place? and, 3) for how long? We aim to address these questions by developing new biosensors based on field resonance enhanced second harmonic (FRESH) for following *in vivo* signaling events with large sensitivity increase and high spatial and temporal resolution. We plan to use such biosensors to monitor signal transduction through visualization of conformational changes and protein-protein interaction of components of signaling pathways that will enable us to follow various signaling events in real time. Ultimately, the established biosensors will be used to investigate signaling during zebrafish development and in the manifestation of cancer.

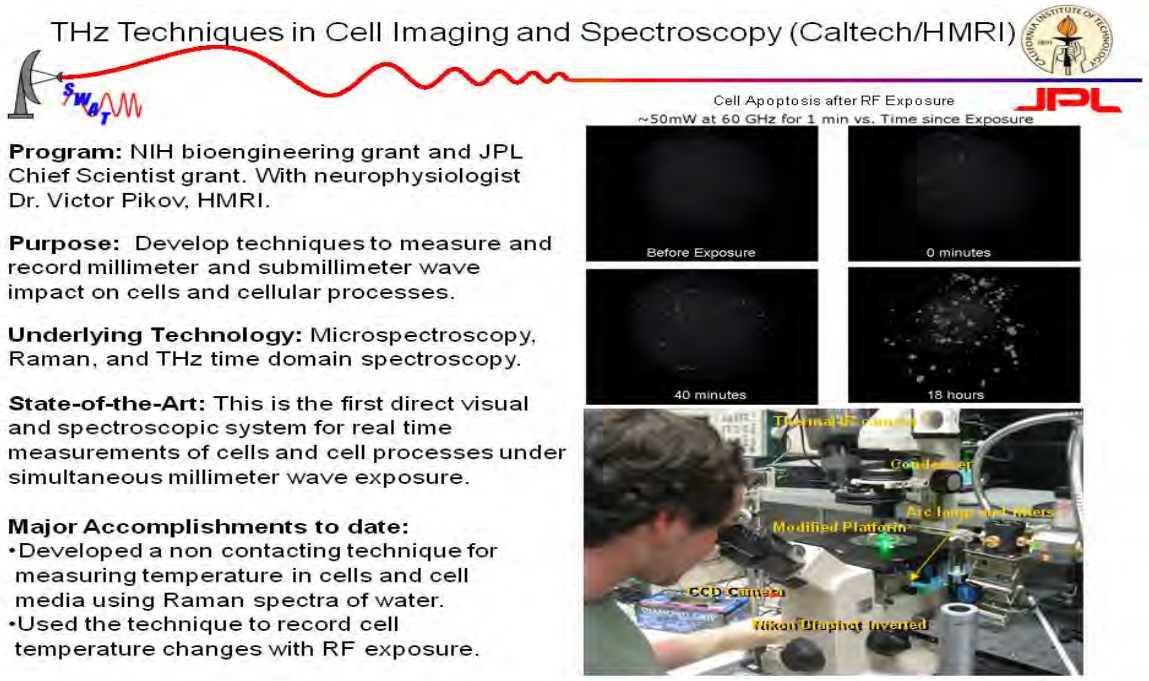
[*]EPFL, Switzerland

187. Terahertz interactions with cells

Peter H. Siegel, Scott E. Fraser, Victor Pikov[*]

This program explores the mechanisms and impacts of high frequency electromagnetic radiation on cells and cellular processes. It blends biological, optical and RF instrumentation in a novel way to examine RF dosimetry effects while directly monitoring cell lines. It establishes one of the first IR Raman/optical/RF test platforms for microscopic evaluation of thermal and chemical processes at the cellular level. This year a new experimental procedure for remotely monitoring the absolute temperature of cells in media without any dyes or genetic markers was developed. The technique is based on recording the Raman signatures of a temperature-sensitive hydrogen stretch mode in water at 3100 cm-1. The technique was used to make the first measurements of the heat dissipation in cells in media due to millimeter wave exposure. Several proposals were submitted to expand and continue the work, which was begun last year. The work is being conducted in conjunction with neurophysiologist, Dr. Victor Pikov, at the Huntington Medical Research Institute, Pasadena.

[*]Huntington Medical Research Institute, Pasadena, CA, USA

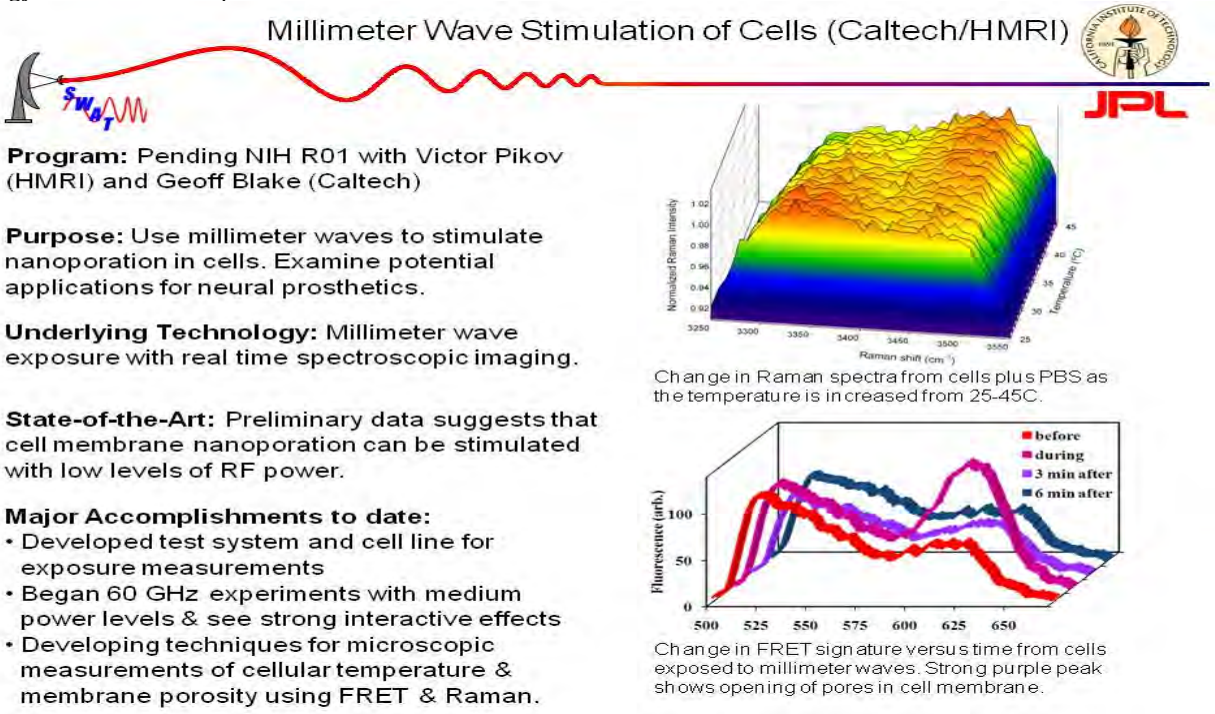


188. Using millimeter waves to stimulate cell depolarization

Peter H. Siegel, Scott E. Fraser, Victor Pikov[*]

Low levels of radio frequency energy at and above 60 GHz are being used as a non-contact probe to stimulate depolarization of the cell membrane, remotely triggering the onset of nanopores that might be used for injection of drugs or dyes into the intercellular fluid. A series of FRET experiments on GFP transfected perpetual human lung cells were performed which indicated the appearance of nanopores after illumination with 60 GHz RF energy and the subsequent return to normal state without cell damage. The first experiments were very preliminary and are being repeated under a variety of new conditions and with several new cell lines over the next fiscal year. A proposal has been submitted to NIH to expand and continue this work, which is being conducted at Caltech with the help of neurophysiologist, Dr. Victor Pikov, at the Huntington Medical Research Institute, Pasadena.

[*]*Huntington Medical Research Institute, Pasadena, CA, USA*



189. THz low loss flexible waveguide

*Peter H. Siegel, Nuria Llombart, Scott E. Fraser, Agnese Mazzinghi**

This program involves analysis, fabrication and testing of several types of new waveguide media specifically tailored for the submillimeter bands: ribbon guide, photonic-band gap waveguide and a new Bragg guide, based on quartz fiber and low-loss biocompatible plastic technology. Applications include simple flexible piping of energy between radio frequency components, inside instruments such as spectrometers and transceivers, as well as possible use in endoscopy to perform THz imagery *in vivo.*

Dr. Nuria Llombart Juan, a Caltech post-doctoral fellow, and Agnese Mazzinghi, a visiting student from University of Florence are working on the task. The task ends in September, 2009.

**Visiting Student, University of Florence, Italy*

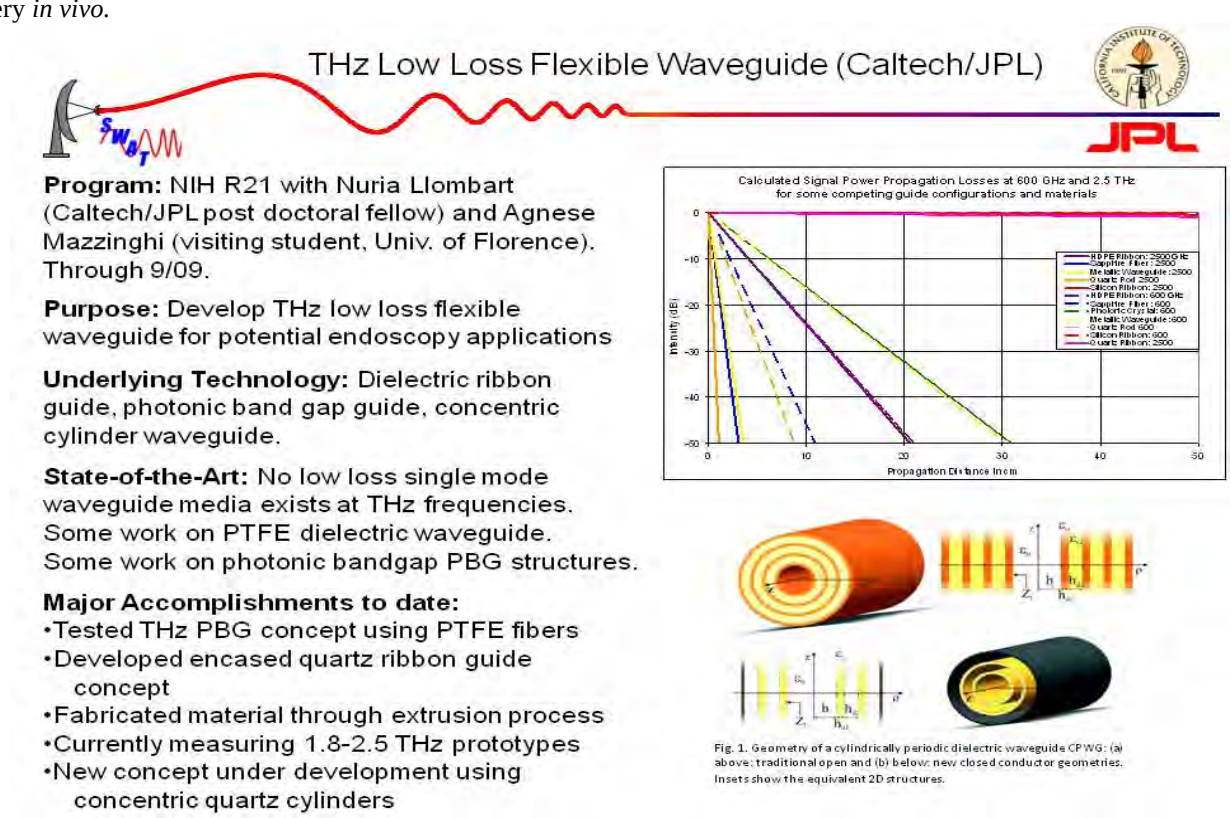


Fig. 1. Geometry of a cylindrically periodic dielectric waveguide CPWG: (a) above: traditional open and (b) below: new closed conductor geometries. Insets show the equivalent 2D structures.

190. Regeneration of the cardiac neural crest in chick

Akouavi M. Ezin, Angela Zah, John W. Sechrist, Scott E. Fraser, Marianne Bronner-Fraser

The cardiac neural crest is a unique subpopulation that contributes to septation of the cardiac outflow tract and formation of aortic arches. Removal of this population after chick neural tube closure results in severe septation defects (Kirby), mimicking human birth defects. We asked whether the cardiac neural crest, like other cranial crest populations, will initially have regenerative capacity that diminishes with time. Because the cardiac crest first resides at a fixed axial level at stage 7, we tested regenerative ability at this stage.

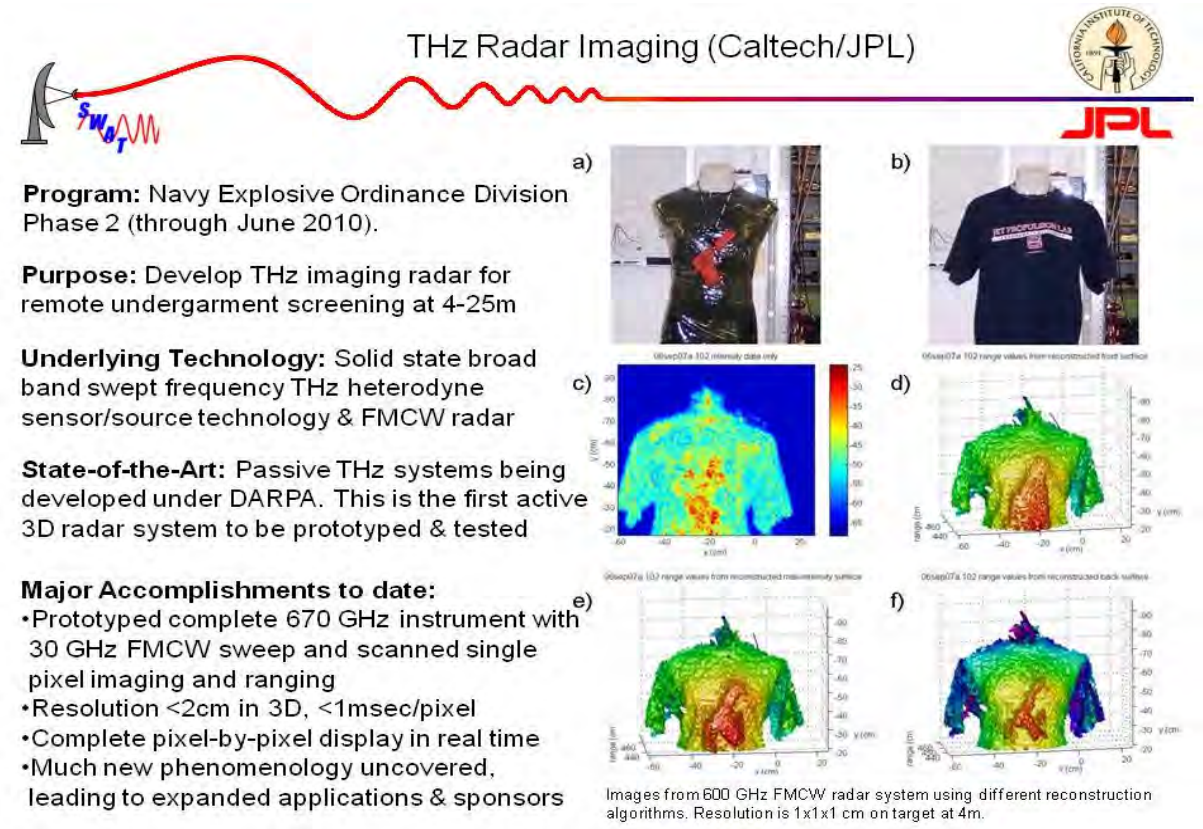
Interestingly, bilateral ablation resulted in formation of a new cardiac crest population by stage 13. The regenerating cells derived from neural ectoderm ventral and rostral to the ablation site. Septation of the heart appeared normal, albeit delayed by 1.5 days compared with unoperated controls. In contrast, formation of aortic arch arteries and enteric ganglia was unaffected. However, regenerative capacity was lost by stage 9. These results show that the cardiac neural crest can regenerate with some delay when its axial identity is first established, but this ability becomes restricted with time.

191. THz radar imaging

Peter H. Siegel, Tomas Bryllert, Nuria Llombart, Scott E. Fraser, Ken Cooper, Robert Dengler**

This program has been developing a new imaging concept based on THz frequency modulated continuous wave (FMCW) radar. Using terahertz sensor and source technology we developed at JPL for NASA Earth, planetary and astrophysics applications, we have designed, fabricated and demonstrated a 32 GHz chirped heterodyne radar imager operating between 655 and 685 GHz with 3 msec per pixel data acquisition rate. The imager has been used to perform 3D reconstruction of objects at 4 and 25 meters with unprecedented dynamic range and axial resolution. Since THz waves can pass through many dielectrics, the system is being applied to undergarment threat detection. Significant phenomenologic breakthroughs have already been established which are changing the way RF imagers are being used. The ultimate goal of the program is to demonstrate near video rate imaging over a modest angular scene scale. The work is being supported by the DoD, although applications include medical screening. Most of the instrumentation and staff reside at JPL. The principle program participants are JPL SWAT team members Dr. Ken Cooper and Mr. Robert Dengler and Caltech post doctoral fellows Dr. Nuria Llombart Juan and Dr. Tomas Bryllert. Other JPL SWAT team members who have been involved include Dr. Imran Mehdi, Dr. Goutam Chattopadhyay, Dr. Erich Schlecht, Dr. Anders Skalare, Mr. Robert Lin, Dr. Choonsup Lee and Dr. John Gill.

**Submillimeter Wave Advanced Technology (SWAT) team, JPL, Pasadena, CA, USA*



Images from 600 GHz FMCW radar system using different reconstruction algorithms. Resolution is 1x1x1 cm on target at 4m.

192. Ultra-high-Q microtoroid resonators for single cell proteomics

Judith Su, Scott E. Fraser

A single cell assay is needed to fundamentally understand protein function and expression; however, current detectors cannot sense the small amount of protein present in one cell, which is typically on the order of zeptomoles. Recently, novel optical devices known as microtoroid resonators (**Figure 1**) have been shown to be capable of achieving label-free single molecule detection. We propose a microfluidic toroid based assay as a new and simpler (fewer processing steps) means to achieve single cell proteomics and have replicated in the Fraser lab all the equipment needed to conduct such measurements. Currently we are working on integrating the toroid into microfluidic channels for high throughput experiments (in collaboration with S. Vyawahre**)** and validating the binding data taken with the toroid by direct comparison with industry standard instrumentation, Biacore Surface Plasmon Resonance (in collaboration with A. Goldberg, J. Vielmetter, P.J. Bjorkman, and B. Stoltz). In addition, we are working on using the toroid as a faster and more sensitive tool to study the flexibility of DNA and peptides (in collaboration with D. Wu) and have refined the toroid to create a new way to sense the binding of molecules to the sensor that is far more robust than has been previously reported (in collaboration with K. Vahala).

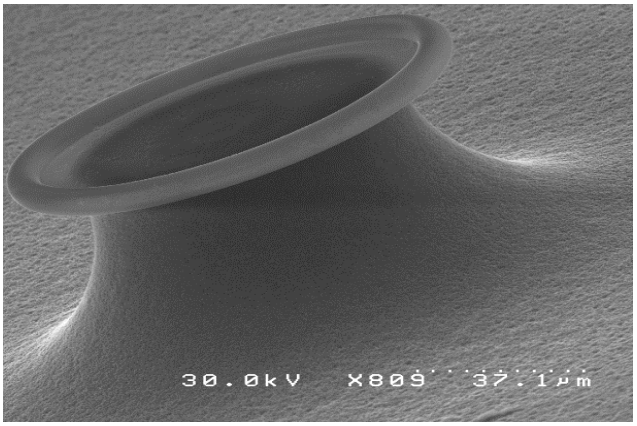


Figure 1. SEM image of an ultra-high-Q microtoroid resonator.

193. Dynamic imaging of primordial kidneys in transgenic avians

Christie Canaria, Scott E. Fraser, Rusty Lansford

The scientific community's understanding of organ formation will have profound impacts on both human tissue engineering and medical science. Discerning the events that yield fully functioning organs and tissues will better guide researchers to recapitulate these processes *in vitro* for the purposes of regenerating and replacing damaged human organs such as the liver, kidney, and pancreas.

Newly generated lines of transgenic quail now allow us to readily track and study cellular movement within the developing embryo. Inspection of an embryonic quail embryo (embryonic day 3) *in ovo* reveals that a number of tissues are readily accessible; among them are blood vessels, heart, brain, and primordial kidney. Of particular interest is the primordial kidney, as its development may serve as a model for both organ formation and cell senescence [1].

The accessibility of the avian embryo makes it amenable to tissue transplantation and time-lapse videomicroscopy, unlike mice. However, the genetic techniques that have been so powerfully exploited in mice have not been developed for avians, until recently. Our research will generate, genetically manipulate, and dynamically image transgenic quail expressing fluorescent protein in order to dynamically study vertebrate organ development. We have chosen Coturnix quail because of their smaller eggs, their moderately sized breeding adults, their short generation time, and their transgenic feasibility [2].

In avian development, as in mammals, the kidneys form in stage [3]. The pronephric kidneys appear at 30 hours, disappear at 64 hours, and are at succeeded in overlap by mesonephric tissue formation (48-96 hours). Lastly the metanephric kidneys appear (96 hours) and persist into adulthood. The primordial kidneys (pronephric and mesonephric) arise and differentiate from the intermediate mesoderm, which lie between the somites and lateral plate; vascularize with glomeruli, peaking in development at 72 hours; and fall dormant within a relatively short time span, making them ideal organs for investigation, especially within the context of our transgenic quail system. Kidney development studies are commonly performed on murine samples, but those tissues are explanted and cultured away from paracrine signals [4-7]. Specifically, studies carried out on urino-genital/blood vessel development were performed by *in vitro* co-culture, as opposed to *in vivo* [7]. However, all stages of kidney formation are accessible to study in living animal transgenic quail without the disruptive efforts that are required to excise and image mouse tissue.

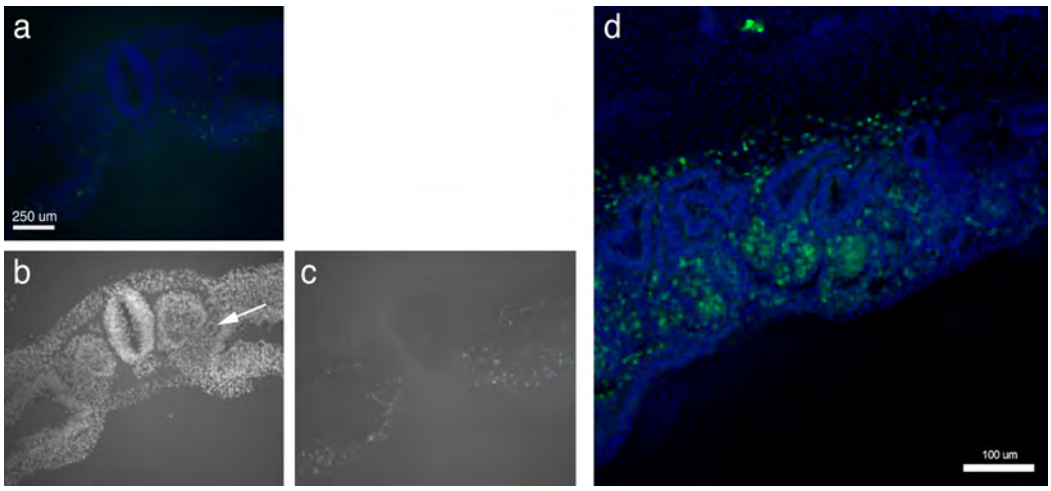


Figure 1. Static images a-c) Transverse section of an E4 *Tie1.H2B-EYFP* quail embryo. **a**) Composite image of **b**) DAPI stained tissue and **c**) EYFP signal. The white arrow in (**b**) indicates a nephric tubule. **d**) Sagittal section of a different E4 embryo, 5 μm thick. Tubules have branched and open out into the Wolffian duct. Endothelial cells in green are seen adjacent to individual tubules, creating the glomeruli. (Data by C. Canaria)

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194. Dynamic imaging analysis of blood vessel formation in transgenic quail embryo

Yuki Sato, David Huss, Greg Poynter, Scott E. Fraser, Rusty Lansford

Blood vessels help to establish the circulatory system, which is essential for exchanging gas, nutriens, and waste. The blood vessels also play a vital role in formation of the nervous system and visceral organs during development. Although numerous molecules required for blood vessel formation have been identified, a detailed understanding of normal blood vessel formation and direct effects of genetic abnormalities is lacking due to the difficulty of imaging living embryos. To take advantage of the easy accessibility to developing quail embryos, we generated a transgenic line- tg(tie1:H2B::eYFP)- that expresses H2B-tagged EYFP in endothelial cells under the control of the Tie1 promoter gene. The tg(tie1:H2B::eYFP) quail enables us to image the nuclei of every blood vessel endothelial cell in the living embryo with the use of confocal laser microscopes. Time-lapse imaging of the transgenic quail embryo determined that the endothelial cell differentiation starts in the extraembryonic area at HH stage 6, and the endothelial cells migrate through nascent vascular plexus unidirectionally from lateral to medial, eventually giving rise to blood vessels inside the embryo. We are studying dorsal aorta formation to better understand various types of dynamic morphogenetic events, including cell migration/ sprouting, cell division, mesenchymal-to-epithelial transition, and rearrangement of luminal structure of the forming blood vessels. Cell-tracking analysis revealed that the dorsal aorta endothelial cells move from posterior to anterior against blood flow. The endothelial cells aligned in the dorsal and ventral walls of the dorsal aorta exhibit different migratory behaviors (Figure 1).

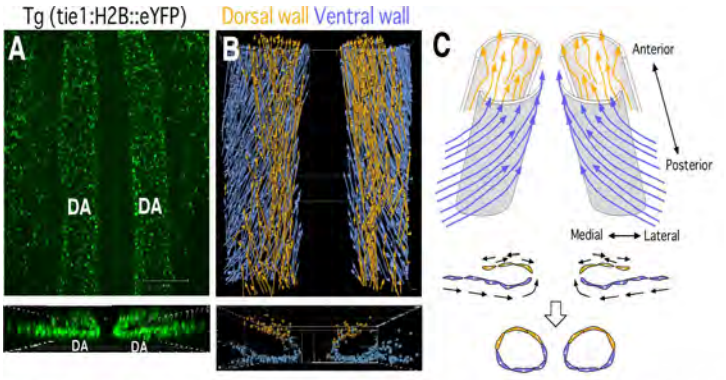

Figure 1. Endothelial cell behavior in forming dorsal aortae. (A) Ventral and 3D-reconstructed transverse views of dorsal aortae in a tg(tie1:H2B::eYFP) embryo at HH stage 11(upper and lower panels). (B) Displacement pattern of dorsal and ventral wall cells of the dorsal aorta. (C) Schematic representation of different migratory behaviors of the dorsal and ventral wall cells.

Such cell behaviors seem to be correlated to differences in their origins. To understand the molecular mechanisms underlying these endothelial cell behaviors, we are performing experimental perturbations of specific genes in the transgenic quail embryo. This will provide us new insights into the endothelial cell behaviors during blood vessel formation.

195. Characterizing cardiogenesis through dynamic fluorescent imaging in the quail embryo

Jennifer Yang, Michael Liebling, Scott E. Fraser, Rusty Lansford

Recent technological advances in dynamic confocal microscopy and four-dimensional reconstruction have shown that the embryonic heart tube in the developing zebrafish functions more as a dynamic suction pump than a peristaltic pump, disputing a theory cardiac physiologists had long held. Along with technological advances, recent research has shown the impact of flow in heart morphogenesis and valve development. As scientific methods and tools improve, previous conclusions of cardiogenesis need to be revised to incorporate new data at the molecular level.

The Japanese quail is an ideal system to study the pump mechanics of the four-chambered heart because it is a warm-blooded vertebrate with easily accessible embryos. All stages of the developing heart during looping and chamber formation are accessible *in ovo* and *ex ovo*, allowing for ease of manipulation of the embryo for dynamic imaging. Knowledge obtained from an avian four-chambered heart can then be applied to the less accessible mammalian and human hearts.

The mechanics of the developing embryonic heart are still poorly understood. Characterization of the interactions of the different layers of the heart will give us information on the contractile wave moving along the heart muscle, the offset of the contractile wave as it transverses the cardiac jelly to appear in the endocardium, and how this affects fluid flow in the heart. In order to characterize the heart, we are utilizing two independent transgenic lines of quail: tg(tie1:H2B::eFYP) and tg(pgk:H2B::mCherry). The Tie1 promoter localizes the eYFP signal to the nuclei of endothelial cells while the PGK promoter ubiquitously expresses mCherry in all cells. By crossing the two lines, we will have a novel transgenic with the myocardium labeled red and the endocardium doubly labeled yellow and red.

With the use of high-speed dynamic imaging and innovative computer algorithms, we can reconstruct the live heart while it is beating. By dynamically imaging the endocardium, myocardium, and blood cells, we will be able to study the interplay between hemodynamics and

genetics by offering unsurpassed visual access to the morphogenetic events of cardiogenesis and angiogenesis. Future experiments will involve the addition of pharmaceutical drugs to determine their effects on morphology and hemodynamics in cardiogenesis and heart valve development.

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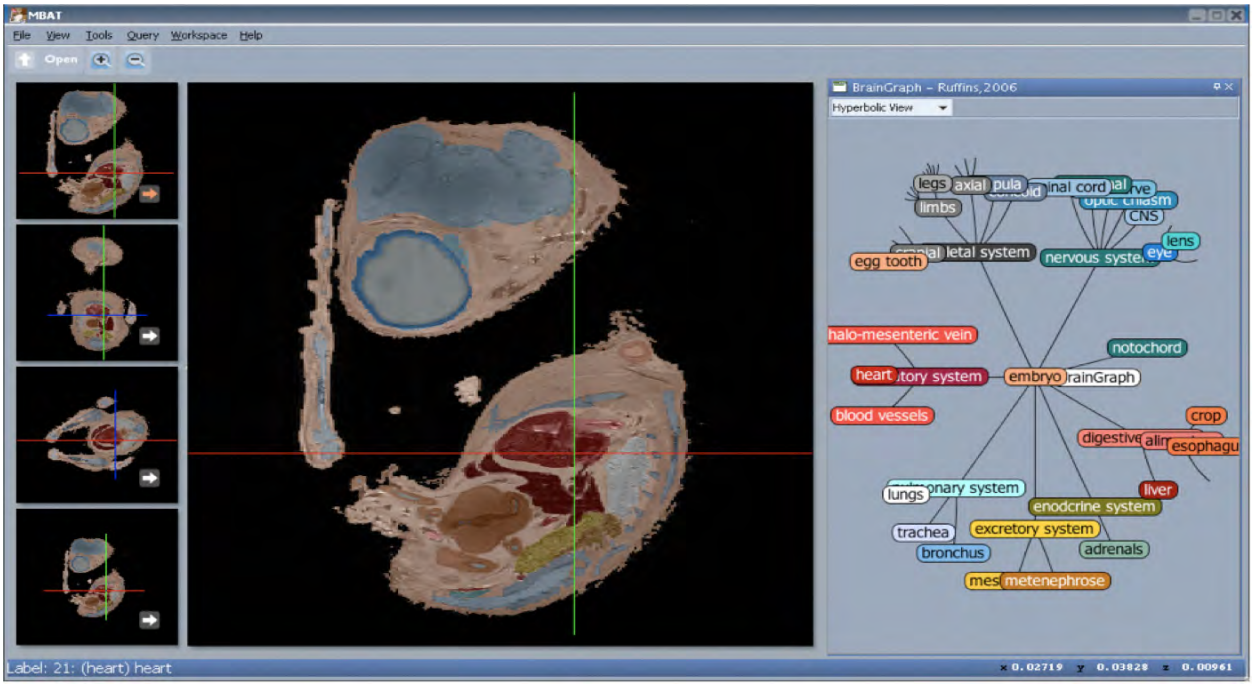
196. Quail developmental atlas

Seth W. Ruffins, Melanie Martin, Lindsey Keough, Salina Truong, Scott E. Fraser, Russell E. Jacobs, Rusty Lansford

Development is a complex process involving a vast number of events occurring over temporal scales from seconds to days, and spatial scales from nanometer to millimeters. The embryonic form provides an intuitive framework for integrating the vast quantities of data generated in the quest to understand development. In the effort to integrate developmental events, we are constructing a series of 3-D embryonic atlases of the Japanese quail (*Coturnix japonica*) based on microscopic Magnetic Resonance Imaging (μMRI). The atlas series contains six *ex ovo* embryos ranging in age from 5 to 10 days of incubation and three *in ovo* embryos at 10, 11 and 13 days of incubation. Currently, the atlases are strictly anatomical but provide a framework for spatially mapping developmental events such as gene expression patterns, the distribution of extra-cellular matrix, fate maps, cell migration patterns, etc. I will discuss the construction of these atlases, how they are being linked to other data sources and how they can be used in education and research.



197. Imaging cell dynamics in early mammalian embryos

Nicolas Plachta, Periklis Pantazis, Carol Readhead, Shirley Pease[*]*, Juan Silva*[*]*, Scott E. Fraser*

To better understand the mechanisms of development in mammals we focus our work on performing imaging analyses of live mouse embryos. Our main goal is to perform quantitative analyses of the cell motions that pattern the earliest tissues and organs of the embryo, and to compare these dynamics in normal versus genetically altered embryos.

In order to get a dynamic view of how genes affect the motion of cells and the overall shaping of tissues, we established techniques for culturing mouse embryos *ex utero* and imaging them using laser scanning confocal microscopy. We label all cell nuclei and membranes in the embryos by injecting RNA encoding for different fluorescent proteins.

By acquiring several optical slices that cover the entire embryo every 5 minutes we can reconstruct a three dimensional time series of embryonic development. We are currently using computational methods to analyze the imaging data obtained from several embryos. We pay particular attention to the cellular events that occur after the 8-cell stage, which is roughly by the 3rd day after zygotic fertilization. Until this stage, all the cells of the embryo are pluripotent, meaning that they have the potential to become virtually any cell type of the mature organism. This special state is lost over the following days, when the cells accelerate their rate of division and

the daughter cells start to be sorted into the diverse tissues of the body.

Unlike other species, mammalian embryos are thought to show a high level of variability during early development and we expect that our experiments will allow us to determine if this variability is also observed at the cellular level in live embryos, and if it is related to the aberrant development typically observed in mutant embryos.

[*]*Genetically Engineered Mouse Services, California Institute of Technology*

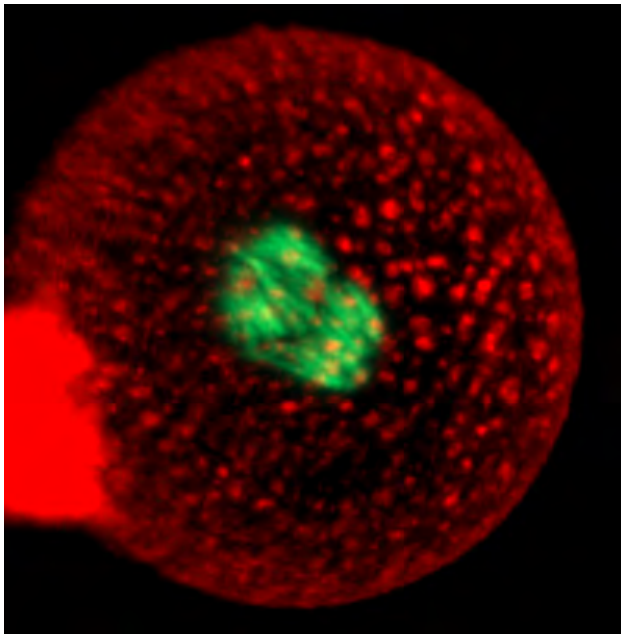


Figure legend: Cultured mouse embryo at the 1-cell stage injected with RNAs encoding for nuclear-targeted green fluorescent protein and for a membrane-targeted red fluorescent protein.

198. Redefining the olfactory necklace in terms of the Grueneberg Ganglion

David S. Koos, Scott E. Fraser

The Grueneberg Ganglion (GG) has recently been recognized as an olfactory organ in most mammals, distinctly located at the tip of the nose where it is ideally situated to be the first olfactory subsystem to interrogate inhaled chemicals. In the mouse, the GG projects to the olfactory necklace domain, an unusual sub-region of the olfactory bulb containing interconnected glomeruli that appear like beads on a string encircling the entire caudal olfactory bulb. The olfactory necklace domain also receives afferent input from another olfactory subsystem comprised of GC-D expressing neurons. A major mystery is how these two distinct populations interact and contribute to those unusual necklace glomeruli structures, and whether or not individual necklace glomeruli are innervated by both populations or are exclusively innervated by one or the other. To address this fundamental question, we are microscopically dissecting the olfactory necklace glomeruli. By combining state of the art imaging technologies with genetic labeling, inducible axon tract tracing and whole-mount immunolabeling, we generate large-scale, high-resolution, extended-volume datasets that represent the olfactory necklace axons and their cognate glomeruli. 3D reconstructions of these data allow us to explore the physical relationships between the two different classes of necklace axons, and demonstrate the amount of overlapping glomerular innervation (or lack thereof) among these populations. Our preliminary results indicate that the olfactory necklace domain contains multiple, genetically-defined axon necklaces of glomeruli. There appears to be no overlapping glomerular innervation between these populations, even though these necklaces of interconnected glomeruli occupy the same region of the olfactory bulb. This suggests that the GG necklace and the GC-D necklace have their own exclusive glomeruli. This high-resolution connectivity map of the olfactory necklace glomeruli lays the foundation for functional analysis of the olfactory necklace domain's response to odorants and its relation to animal behavior.

199. Characterizing the receptive structures of the Grueneberg Ganglion

David S. Koos, Cambrian Y. Liu, Scott E. Fraser

The Grueneberg Ganglion (GG) is a mammalian olfactory subsystem located just inside of the nostrils and far forward of the other olfactory subsystems. Morphologically, the GG is unusual because, unlike the sensory neurons of the other olfactory subsystems, GG neurons do not appear to project a dendrite with receptive structures to the luminal surface of the nasal cavity. Furthermore, within the sub-epithelium of the nasal vestibule, the GG neurons are completely ensheathed by glial satellite cells. Given this unusual arrangement, it is a major mystery where the GG's receptive structures are located and the classes of ligands they can access. To address this fundamental question, we are using semi-serial EM, optical imaging, genetic labeling and immunolabeling to characterize the mouse GG in detail. We find that the neurons of the GG are often decorated with two large distinct bundles of cilia. Each ciliary bundle is composed of several individual aligned cilia. The cilia originate from basal bodies located deep within the GG soma and extend to the cell surface where they follow the surface contour of the soma and appear to remain underneath the ensheathing glia. Although their arrangement is unusual, the ciliary bundles on the GG neurons are suggestive of receptive structures. Providing further support for this proposal, we find that specific particulate guanylyl cyclase receptors are preferentially localized to the ciliary bundles. These findings provide strong support that the ciliary bundles located on the GG soma represent receptive structures. We propose that the unique sub-epithelial location and glial encasement of the GG and its receptive structures may serve as a selective filter that controls the type of chemicals that can access and be detected by this atypical olfactory subsystem.

200. The Grueneberg Ganglion olfactory subsystem employs a cGMP signaling pathway

Cambrian Y. Liu, Scott E. Fraser, David S. Koos

The mammalian olfactory sense employs several olfactory subsystems situated at characteristic locations in the nasal cavity to detect and report on different classes of odors. These olfactory subsystems use different neuronal signal transduction pathways, receptor expression repertoires, and axonal projection targets. The Grueneberg Ganglion (GG) is a newly appreciated olfactory subsystem with receptor neurons located just inside of the nostrils that project axons to a unique domain of interconnected glomeruli in the caudal olfactory bulb. It is not well understood how the GG relates to other olfactory subsystems in contributing to the olfactory sense. Furthermore, the range of chemoreceptors and the signal transduction cascade utilized by the GG have remained mysterious. To resolve these unknowns, we explored the molecular relationship between the GG and the GC-D neurons, another olfactory subsystem that innervates similarly interconnected glomeruli in the same bulbar region. We found that mouse GG neurons express the cGMP-associated signaling proteins phosphodiesterase 2a, cGMP-dependent kinase II, and cyclic nucleotide gated channel subunit A3 coupled to a chemoreceptor repertoire of cilia-localized particulate guanylyl cyclases (pGC-G and pGC-A). The primary cGMP signaling pathway of the GG is shared with the GC-D neurons, unifying their target glomeruli as a unique center of olfactory cGMP signal transduction. However, the distinct chemoreceptor repertoire in the GG suggests that the GG is an independent olfactory subsystem. This subsystem is well suited to detect a unique set of odors and to mediate behaviors that remained intact in previous olfactory perturbations.

Reference

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201. Watching axons wire up the olfactory bulb

Nakul Reddy, David Koos, Scott E. Fraser

In the mammalian olfactory system, structures called glomeruli encode the odor information received at the nose and relayed to the brain. A glomerulus is formed in the olfactory bulb by the convergence of thousands of axons, but with high molecular specificity. How these axons grow in such an organized fashion is unknown. Since they differ highly between individuals, and even between left and right halves of the olfactory bulb in the same animal, chronic imaging is required to characterize axon behavior over time to elucidate mechanisms of glomerular convergence. Our goal is to characterize formation of a single glomerulus in mice *in vivo* via two-photon laser scanning microscopy. We can then understand mechanisms like axon growth, branching and bundling that take place during glomerular formation.

Molecular signaling by odorant receptors have been implicated the most in directing glomerular wiring, but there is little precedent for G-protein coupled receptors in axon guidance. Moreover, very few mutants completely disrupt glomerular formation, suggesting lots of redundant mechanisms for axon wiring. Thorough characterization allows us to establish wild-type behavior and detect subtle changes in mouse mutants with disrupted axon guidance molecular pathways.

Non-invasive *in vivo* imaging of neuronal development will give us realistic cell behaviors we cannot see in two-dimensional *in vitro* cultures. Understanding mechanisms for highly efficient axon guidance like those behind glomerular convergence can be valuable in developmental and regenerative pathways.

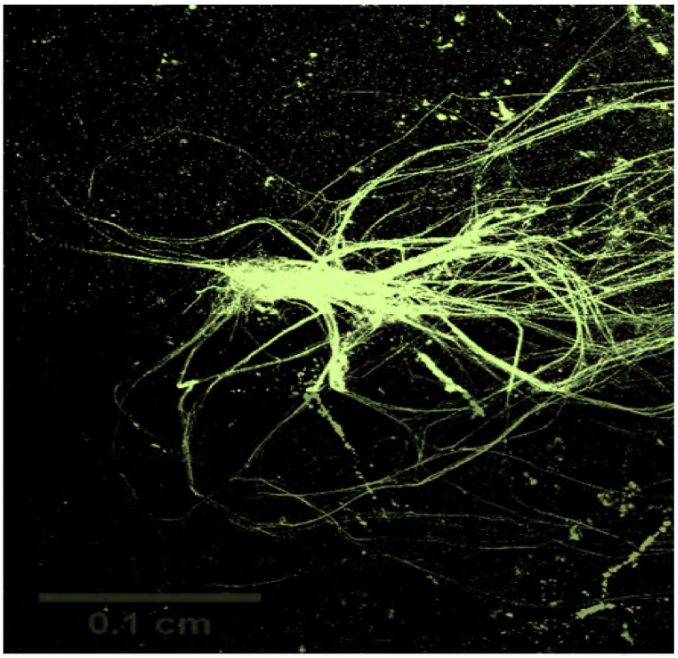

Figure 1: A two-photon image of an olfactory glomerulus in an adult mouse.

202. *In vivo* quantitative topography of optic nerve head in mouse models for MS

Reza Motaghiannezam, Carol Readhead, David Koos, Scott E. Fraser

Multiple sclerosis (MS) is an autoimmune disease often recognized by axon demyelination. While recent studies abundantly confirmed gray matter is affected, as well as white matter in MS, fundamental mechanisms of neuronal injury are still not fully explored. MS disease is becoming better understood through assessment of gray and white matter health using advanced imaging techniques. However, there remains an important unmet need for novel *in vivo* ophthalmic morphological monitoring techniques to provide indirect assessments of the central nervous system (CNS) status. Our long-term goal is to apply and validate a novel technique to monitor optic nerve head (ONH) topography and evaluate it as a separate assay for identifying the pathogenesis of gray and white matter and test the efficacy of drug treatment in mouse models for MS.

We have been successful in developing sensitive non-invasive swept source optical coherence tomography (SS-OCT) techniques thus, providing the tools for *in vivo* longitudinal studies of different mouse models for multiple sclerosis (MS). SS-OCT enabled us to visualize *in vivo* the 3D structure of the retina in myelin oligodendrocyte glycoprotein experimental allergic encephalomyelitis (MOG-EAE) and MOG-TCR transgenic mice, as well as untouched control mice. For the first time, we performed quantitative topography of the ONH in MS. The quantitative topography of ONH was evaluated in MOG-EAE and MOG-TCR transgenic mice. Significant differences were observed between the proposed quantitative morphological biomarkers in different mouse models. The goal is to extend this study by monitoring the ONH topography and optic nerve electrophysiological responses simultaneously, evaluating combination of these biomarkers as surrogate assays of abnormalities in the central nervous system (CNS) at different EAE stages, and testing the efficacy of a drug treatment within these paradigms. In order to do that we perform longitudinal *in vivo* studies of different mouse models, which develop optic neuritis and/or EAE.

203. Reduced cerebral perfusion in mouse model of glutaric acidemia type I

Jelena Lazovic, William Zinnanti, Ellen Wolpert, David Antonetti, Russell E. Jacobs

Glutaric acidemia type I (GA-1) is due to deficiency of glutaryl-CoA dehydrogenase (Gcdh), an enzyme involved in lysine, tryptophan, and hydroxylysine catabolism. The clinical presentation includes retinal and cerebral hemorrhages, encephalopathy and irreversible bilateral striatal injury in neonates and young children (between 0-36 months of life). We hypothesized that frequent hemorrhages and cerebral edema may result as a consequence of increased VEGF expression, further contributing to reduced cerebral perfusion. To test this hypothesis we used our previously described GA-1 mouse model, where Gcdh-/- mice develop encephalopathy at 4 weeks of age after exposure to lysine-enriched diet (Zinnanti *et al.*, 2006). Magnetic resonance imaging (MRI) was performed on a 7.0 T Bruker system, and arterial spin labeling (ASL) method (Williams *et al.*, 1992) was used to acquire a single-slice perfusion-weighted image at the level of the striatum. MR images were obtained before the lysine diet onset (N=11 Gcdh-/- mice, N=4 WT mice), and then on the 2nd, 3rd and 4th day after diet onset for N=4 Gcdh-/- and on the 7th day after onset for N=4 WT mice. Additional Gcdh-/- (N=4 on high lysine and N=3 on normal diet) and WT mice (N=3 on a normal diet and N=3 on high lysine diet) were processed for Western blot analysis. WT mice remained asymptomatic on the high lysine diet during the course of the study with no significant changes in perfusion values. In contrast, Gcdh-/- mice experienced encephalopathy and reduced cortical and striatal perfusion following high lysine diet onset that was significantly different by 4th day ($p<0.005$), **Fig. 1B-D**. Western blot analysis revealed significantly increased cortical and striatal VEGF expression after 4 days of high lysine diet in Gcdh-/- compared to WT mice ($p<0.05$), **Fig. 2**. Additional post-mortem high resolution MRI revealed highly distended vasculature, **Fig. 3**.

In conclusion, our results demonstrate how continuous metabolic derangement can quickly lead to severe global perfusion deficits, and strongly imply perfusion-weighted MRI as an important monitoring technology for individuals affected with GA-1.

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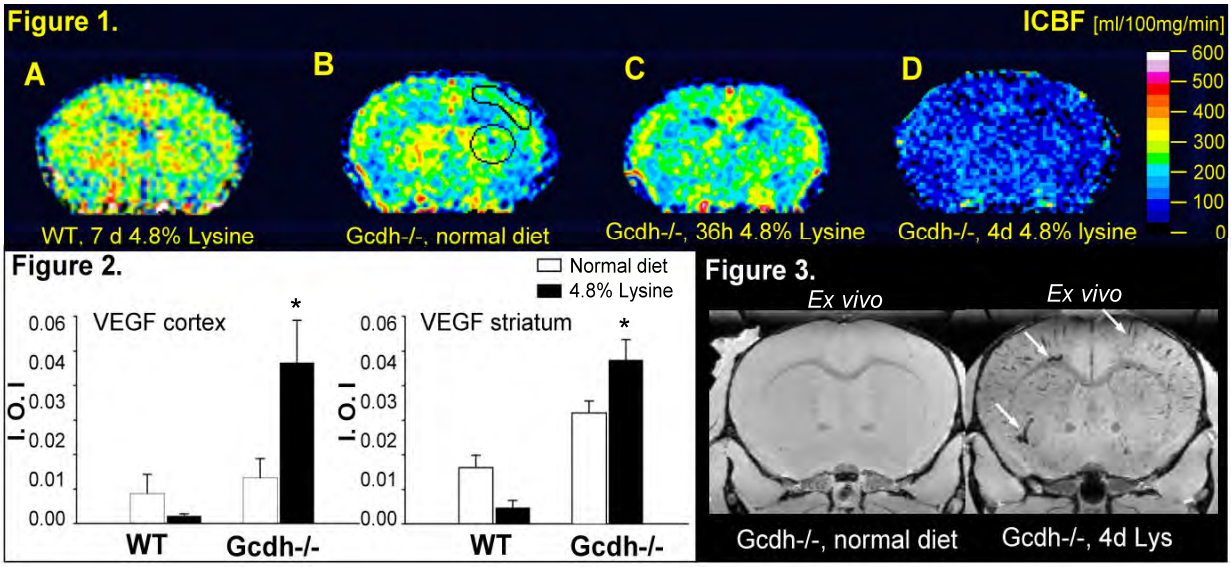

Figure 1. Index of cerebral blood flow (ICBF) at the level of the striatum in (A) WT and Gcdh-/- mice (B-D). Color scale represents different ICBF values. The ROIs used for statistical analysis are outlined in B (ROI was bilateral).

Figure 2. VEGF levels in cortex and striatum expressed as integrated optical density (I. O. I.), and after being normalized for actin expression, *p<0.05 (WT on high lysine diet (N=3) *vs*. Gcdh-/- on high lysine diet, (N=4)).

Figure 3. High resolution 3D FLASH MRI reveals multiple distended blood vessels (white arrows) in Gcdh-/-mice on high lysine diet for 4 days.

204. Magnetization transfer MRI in a mouse model of glutaric acidemia type 1 (GA-1)

Jelena Lazovic, Russell E. Jacobs

A mitochondrial enzyme glutaryl-CoA dehydrogenase (Gcdh-/-) is necessary for the catabolism of three essential amino acids: lysine, tryptophan and hydroxylysine. The Gcdh deficiency leads to glutaric acidemia type I (GA-1), a disorder with accumulation of glutaric acid and 3-OH glutaric acid in the serum, tissue and urine. The clinical presentation and severity of this disorder is variable among individuals affected. The most common finding is encephalopathy during infancy, resulting in devastating bilateral striatal necrosis, while later in life white matter disease is observed (Bahr *et al*., 2002; Kulkens *et al*., 2005). The white matter abnormalities are consistent with leukoencephalopathy, and a recent report suggests oligodendrocyte precursor cells and more mature oligodendrocytes to be susceptible to glutaric acid and 3-OH glutaric acid in culture (Gerstner *et al*., 2005).

In this work we explore the use of magnetization transfer magnetic resonance imaging (MRI) to non-invasively determine the presence of myelin in the striatum of older Gcdh-/- mice on a long-term high lysine diet. Magnetization transfer MRI can be used to differentiate free water vs. water bound to the macromolecules (including myelin) and provide a quantitative parameter, the magnetization transfer ratio. Utilizing Gcdh-/- mice and increased dietary lysine we were able to show increased signal on T2-weighted images in the striatum, consistent with white matter lesions (Zinnanti *et al*., 2006). In the same study, histologic examination revealed intense vacuolation of white matter patches in the striatum. Here we hypothesize reduced myelin content in the striatum of Gcdh-/- mice as a consequence of a long-term lysine diet. Imaging was performed on 11.7 T Bruker Biospin scanner. We compared N=6 age matched male Gcdh-/- mice on regular or increased lysine diet (1.8% vs. 4.8% total lysine content). A significantly decreased magnetization transfer ratio was found in all Gcdh-/- mice on the high lysine diet compared to Gcdh-/- on a normal diet ($p<0.05$, t-sample t-test), Figure 1 A-B.

These findings strongly suggest long-term lysine dietary restrictions for affected GA-1 individuals may prevent leukoencephalopathy later in life.

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Figure 1.

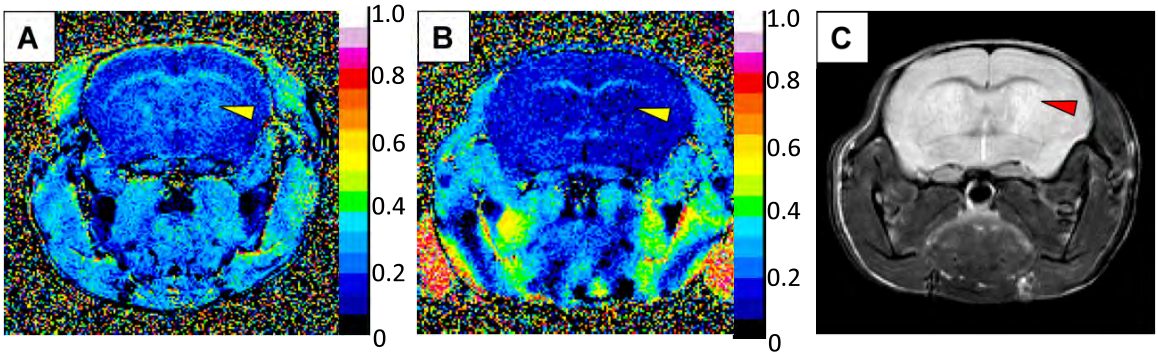

Figure 1. **A**) Magnetization transfer ratio (0-1) in 8-month old Gcdh-/- mouse on a normal diet. Notice increased magnetization transfer ratio in the myelin rich areas: corpus callosum and striatum (yellow arrow). **B**) Magnetization transfer ratio in 8-month old Gcdh-/- mouse on a high lysine diet for 4 months. Notice decreased magnetization transfer ratio in the striatum (yellow arrow), and partially in the corpus callosum. **C**) A T2-weighted image of the same mouse as shown in B). The increased signal intensity in the striatum (red arrow) is consistent with leukoencephalopathy.

205. Mapping dopaminergic pathways by pharmacological MRI

A.T. Perles-Barbacaru, D. Procissi, A.V. Demyanenko, Russell E. Jacobs

The specific aim of this project is to study monoamine neurotransmission abnormalities after cocaine administration in transgenic monoamine transporter knock out mouse models using non-invasive cerebral blood volume (CBV) weighted pharmacological magnetic resonance imaging (MRI).

All procedures are carried out in a 7T Bruker Biospec/Avance 30 cm horizontal bore magnet in the Caltech Brain Imaging Center under general anesthesia using isoflurane. The MR signal is made sensitive to the CBV change accompanying neuronal activity, by overpowering the blood oxygenation level-dependent effect [1] on T_2*-weighted images of the brain with the intravenous injection of a superparamagnetic contrast agent [2, 3]. P904, a new superparamagnetic contrast agent from Guerbet Laboratories in France has been used at a dose of 25 mg/kg. Figure 1 shows a coronal relative CBV map (a) and the corresponding pre-contrast fast gradient echo image of the mouse head (b).

Neuronal activity in specific pathways is elicited by the pharmacological challenge with cocaine. Figure 2 shows the time course of the CBV-weighted signal from the nucleus accumbens (N acc) of a dopamine transporter-deficient mouse, illustrating a CBV decrease after a 30 mg/kg cocaine dose injected intraperitoneally 30 min after P904 injection. Figure 3 shows maps of the vascular response at different time points after cocaine administration depicting spatial differences. No spatial or quantitative differences of the hemodynamic response to cocaine were found between dopamine transporter knock out mice and healthy control mice, which is probably due to the adaptations that occur in the monoamine neurotransmitter systems in these mice. Contrary to the vascular response in rats [4], acute intravenous cocaine administration leads to a general CBV decrease that has also been observed in monkeys [5] and humans [6] and is believed to reflect neuronal deactivation [7]. However, it is also possible that increased levels of dopamine have a direct vascular effect.

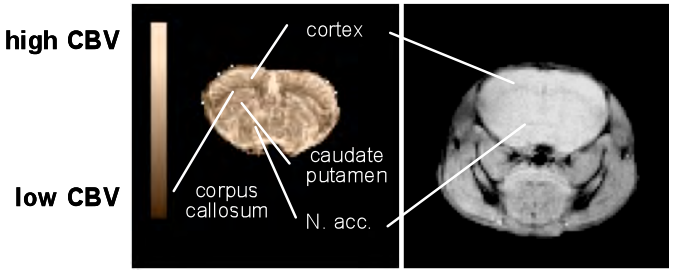

Figure 1: Relative cerebral blood volume (CBV) map of a coronal slice through a mouse brain before cocaine injection (a) and the corresponding T_1 weighted slice through the mouse head (b)

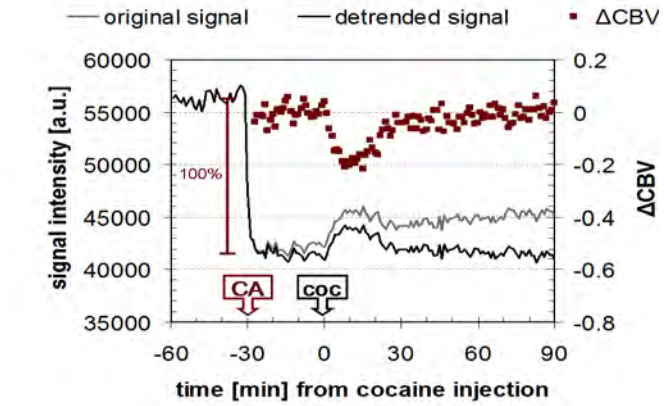

Figure 2: Signal time course in a pharmacological MRI experiment involving contrast agent (CA) injection and a pharmacological challenge (cocaine). The gray plot is the original signal, the black plot is corrected for CA elimination (half life 5 hours). This signal is converted to relative CBV and the CBV change (ΔXBV) can be quantified by setting the signal drop upon CA injection to 100% (red plot, right axis). It shows the CBV decrease after cocaine administration with respect to the pre-cocaine baseline blood volume.

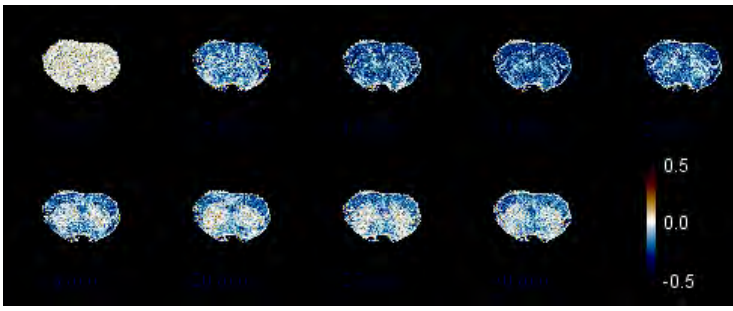

Figure 3: Maps of the CBV change at different time points after cocaine administration. The blue color corresponds to a CBV decrease. The vascular response in brain structures differ in amplitude and duration.

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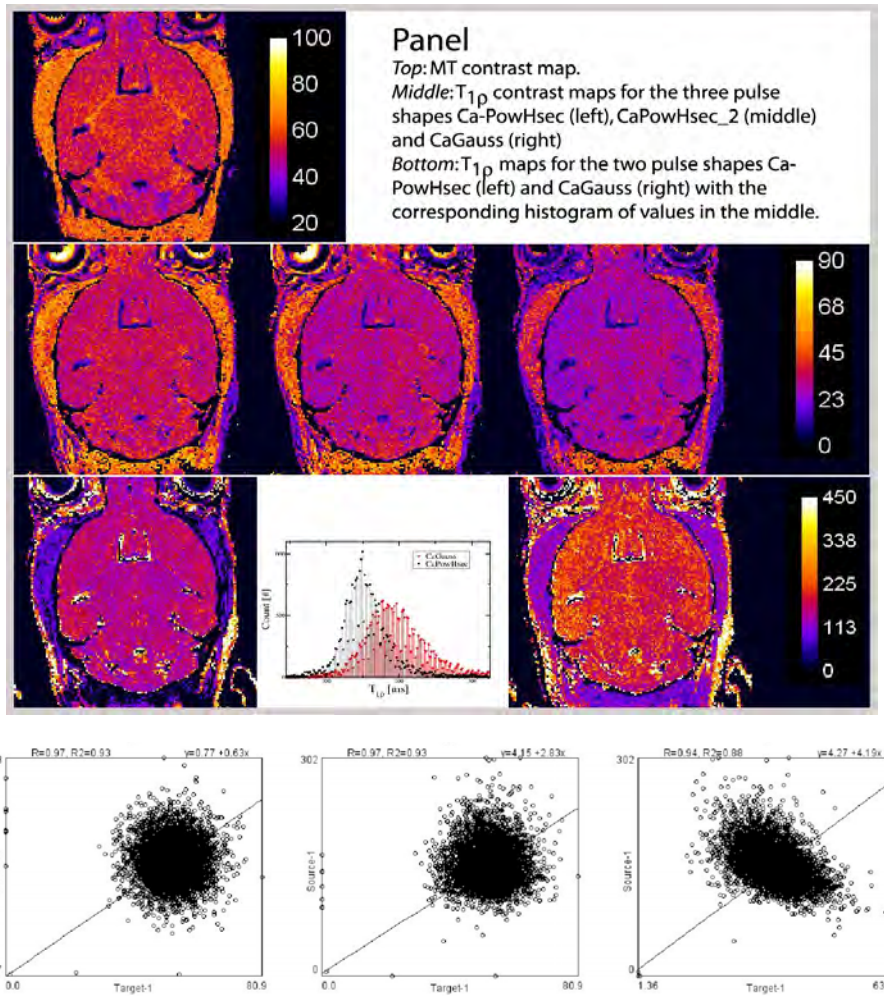
206. Magnetization transfer and adiabatic T_{1r} in the live mouse brain

Benoit Boulat, P.T. Narasimhan, Russell E. Jacobs

Magnetization transfer (MT) takes advantage of interactions between water molecules and semi-solid protein matrix or cell membranes to reveal a wealth of information about the macromolecular distribution in living tissue or the appearance of unusual body like tumors within the tissue. Longitudinal relaxation in the rotating frame (T_{1r}) is known to potentially unveil the existence of slow molecular motions in the kilohertz range. The T_{1r} method can be performed with a spin locking train of adiabatic RF pulses. In this manner, differential assessments of low frequency motional regimes in living tissue can be obtained, by manipulating the time-dependent amplitude of the adiabatic RF pulse. We compared the results obtained through MT techniques and adiabatic T_{1r} ones in the live mouse head using a 11.7Tesla MR imager. For this purpose we mapped the quantity that is used to present evidence of MT contrast:

S_0-$S(w_{rad},t)$ / S_0, for both cases of MT and adiabatic T_{1r}; S_0 is the intensity of the image recorded without prior manipulation of the spins, while $S(w_{rad},t)$ is the intensity of the image obtained after an irradiation of the MT or adiabatic T_{1r} type has been conducted. The variable w_{rad} is the frequency offset with respect to the frequency of the free water at which the RF irradiation is placed and t is its duration.

In the figure below a typical mapping of the quantity defined above is presented in one coronal slice, 200 mm thick and with an in-plane resolution of 56x56mm, through the head of a C57 wild-type mouse. Using RF trains of different duration, T_{1r} maps were obtained by fitting the intensity of each pixel to a single exponential (third row in the figure). For one particular RF pulse shape (Ca-PowHsec) the pixel-by-pixel correlation plots between MT contrast map and T_{1r} contrast map (bottom left), MT contrast map and T_{1r} map (bottom middle) and T_{1r} contrast map and T_{1r} map (bottom right) show a very good correlation between the quantities involved.



207. Development of simultaneous PET/MR technology for small animal imaging

Thomas Ng, Daniel Procissi, Yibao Wu[*]*, Andrey Demyanenko, Simon R. Cherry*[*]*, Russell E. Jacobs*

The study of biological processes *in vivo* is vital for their proper elucidation. Positron Emission Tomography (PET) and Magnetic Resonance (MR) imaging are two non-invasive imaging modalities that offer complementary functional and anatomic information [1]. While retrospective fusion of individual PET and MR datasets has shown useful correlations of the multi-modal datasets [2], simultaneous acquisition of PET/MR images allows time-sensitive biology to be studied from multiple perspectives without difficult and complex spatial co-registration of the multi-modal data. Building upon our lab's previous work of developing the first MR-compatible PET insert instrumentation for small animal imaging [3, 4], we have developed and characterized the integrated PET/MR system for use in robust *in vivo* studies. A simple yet accurate spatial alignment and processing pipeline was developed to ensure good registration of the PET and MR images. The PET and MR electronics operating systems were integrated to allow synchronous dynamic imaging. This combined with parallel computing algorithms enable time-sensitive *in vivo* studies to be performed in an efficient manner. For example, this integrated system setup allowed us to compare PET and MR metabolic signatures of a tumor cell mass in real time. Rapid processing and analysis of fluorodeoxyglucose (FDG) PET/ MR image datasets guided the acquisition of magnetic resonance spectra from differential regions of glucose uptake. High regions of glucose uptake correlated with those of high choline signal, which suggests regions of high cellular activity.

Ongoing work aims to improve the combined PET/MR technology. We are harnessing recent advancements in solid-state photodiode technology and mechanical engineering designs to improve the efficiency of the detector hardware. Studies characterizing the current integrated system and novel detector setups will be used to guide the design of next generation of PET/MR integrated systems. To maximize the flexibility and power of this system, we are also developing protocols to enhance the range of complementary information that can be obtained using the PET/MR, through developing novel imaging sequences coupled to the use of interesting contrast agents.

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208. Understanding kinetics and dynamics of nano-therapies within *in vivo* cancer models

Thomas Ng, Daniel Procissi, James Bading[*]*, Hargun Sohi, Andrew A. Raubitschek*[*]*, Russell E. Jacobs*

Nanoparticle [1] and antibody [2] cancer therapies are promising because of their ability to target within a specific site in the body, the tumor mass. Increased uptake of the drug within the tumor site reduces systemic toxicity while concentrating localized response. It is likely that both classes of therapies have specific therapeutic dosage windows, which maximize their efficacy. Current methods to understand and evaluate the efficacy of these therapies include tumor size measurements, *in vitro* biochemical assays and intra-vital microscopy. PET and MR imaging complement these studies, allowing high resolution, real-time and longitudinal studies of drug uptake and response within the tumor environment. Moreover, these techniques are clinically translatable. This past year, we have developed PET and MR imaging techniques to understand the kinetics of antibody uptake within the context of a heterogeneous tumor. For example, Figure 1 shows a changing pattern of tumor localizing antibodies over the period of 2 days, suggesting a heterogeneous tumor uptake of the antibody over time and space. This correlates with previous *ex vivo* and *in vitro* studies showing heterogeneous tumor uptake of antibodies [3] and highlights the utility of the simultaneous PET/MR technology. Apart from this, we explored PET/MR methods to follow the response of tumors to nano-particle treatments. By examining the diffusion status of the tumor with MR, it is possible to determine the cellular density of the tumor mass[4]. Over the course of a week, we demonstrated that diffusion MR was sensitive to changes in tumors treated with a nano-particle chemotherapy [5] compared to controls, which correlated with anticipated size changes (Figure 2). Furthermore, heterogeneous patterns of response were observed within tumors *in vivo*, a finding not possible with traditional assays.

Ongoing work aims to further explore how the tumor bio-distribution of these classes of drugs affects tumor response *in-vivo*. Longitudinal *in vivo* and *ex vivo* assays of tumor histology will allow us to understand the efficacy of such theranostics, feeding back to guide better drug design, while providing a set of tools that can be used clinically to tailor personalized treatment regimens.

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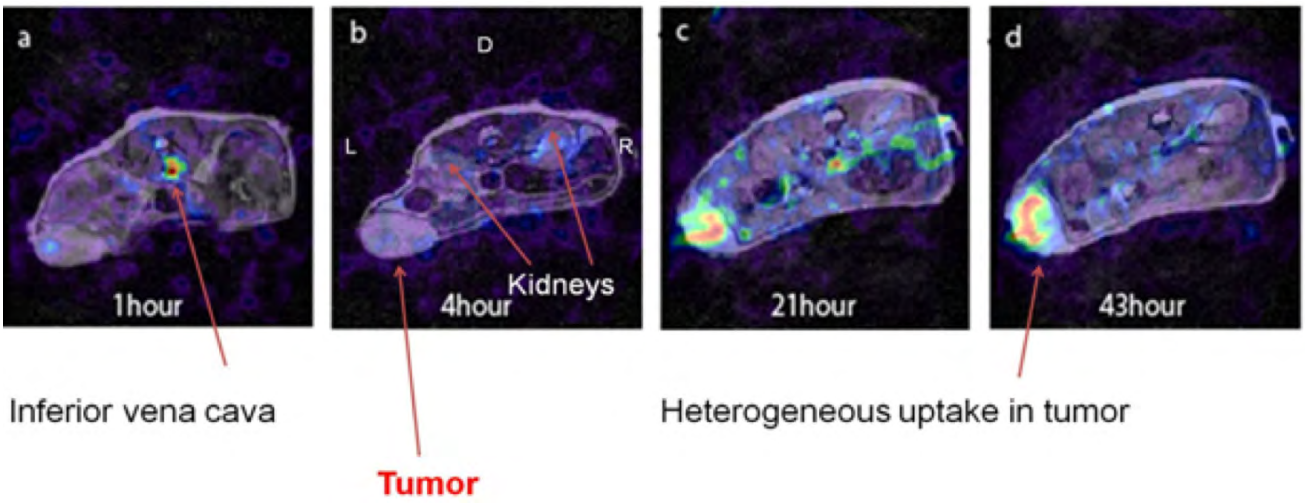


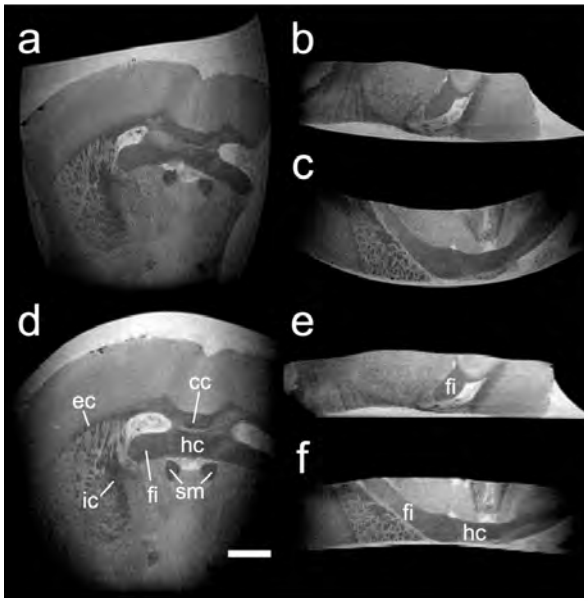
Figure 1: PET/MR shows heterogeneous 64Cu-Herceptin uptake. Mice implanted with HER2 receptor expressing MCF cancer cells subcutaneously were treated with herceptin intravenously. Simultaneous PET/MR images were obtained at the timepoints shown. PET images were decay corrected and aligned to the MRI. At 1 hour, most of the antibody remains in the circulatory systems, with some uptake in the tumor, suggesting primarily a blood vessel distribution. Over the course of 2 days, there is a steady increase of the antibody uptake in the tumor. Moreover, the pattern of the antibody uptake was heterogeneously within the tumor mass shown by the MR images (c, d). This suggests that the tumor microenvironment may modulate the drug's tumor distribution. (L:Left, D:Dorsal, R:Right).

209. A magnetic resonance stage microscope for developmental biology

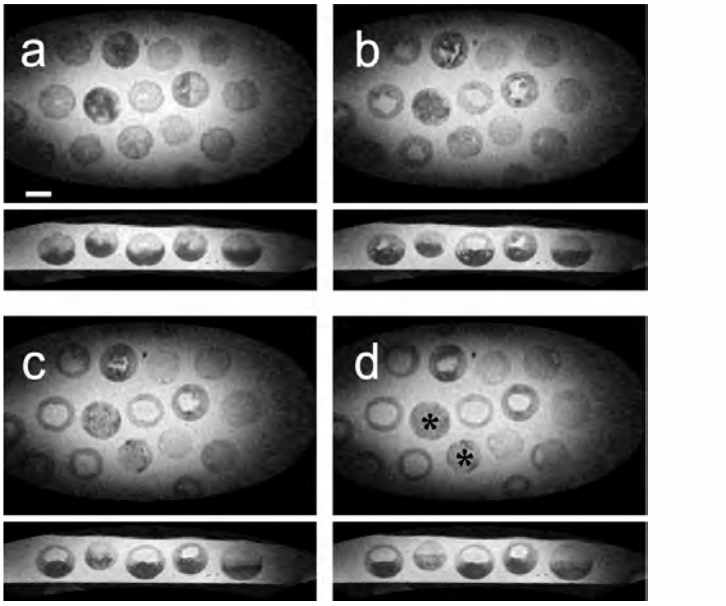
Andrey V. Demyanenko, Lin Zhao, J. Michael Tyszka

A uniplanar magnetic resonance microscope has been developed to address directly the needs of developmental biologists studying live embryos. This instrument replaces the conventional cylindrical gradient set of a horizontal bore high field magnetic resonance imaging system with a one-sided planar gradient set and radiofrequency coil and is similar, conceptually, to an inverted optical stage microscope. A variety of engineering problems, including adequate insulation of the sample embryo from heat generated by the gradient coils, correction for geometric image distortion and electromechanical robustness under typical imaging conditions were addressed during recent development. The current prototype is capable of generating field gradients as high as 4.5T/m at 2% duty cycle and is well suited for high-speed data acquisition including echo planar imaging. Current applications include *in utero* imaging of mouse embryos, high-performance mouse brain imaging and high resolution time-series imaging of externally developing amphibian embryos. Future goals include integration with an MR-compatible optical microscope for dual-mode imaging and construction of a biplanar gradient set exploiting the engineering developments of the uniplanar set.

This work is funded in part by a grant from the National Science Foundation (DBI 0552396).



MR stage microscope image of a 2mm thick coronal slice from a perfusion fixed C57BL/6 mouse brain, without (a-c) and with distortion correction (d-f). A variety of myelinated structures are visualized, including the internal capsule (ic), external capsule (ec), hippocampal commisure (hc), stria medullaris (sm), corpus callosum and fimbria (fi). The reconstructed voxel size in (d)-(f) is 39μm, scale bar = 1mm.



Four of 30 distortion corrected 3D images from a serial study of live developing *Xenopus* laevis embryos acquired using the uniplanar gradient set at experimental times of (a) 0:00 hours (b) 3:30 hours (c) 7:30 hours and (d) 11:30 hours. Abnormally or slowly developing embryos are easily identified (examples indicated by asterisk in d). The formation of the blastocele can be followed in the majority of embryos (asterisks). Volumes were reconstructed with an isotropic voxel size of 47μm from an original nominal voxel size at the center of the FOV of 76μm. Scale bar =1mm.

210. Diffusion magnetic resonance imaging of *in utero* mouse embryos

Lin Zhao, Scott E. Fraser, Julian M. Tyszka

Mouse embryos have been widely used as mammalian models for studying human developmental disorders. However, methods for imaging *in utero* mouse embryo brain development at late stages (E13+) are lacking. The goal of this research is to use diffusion magnetic resonance imaging (MRI) to noninvasively image *in utero* mouse embryo development at late stages. As water molecules diffuse across different length scales, they probe the microstructure of tissue and offer rich information on the microenvironment. The distance water molecules travel depends on the travel time (the so-called diffusion time). When the diffusion time is long, water molecules in tissue diffuse a long distance and offers structural information at cellular scale. When the diffusion time is short, tissue contrast mainly depends on the subcellular microenvironment. We will follow *in utero* mouse embryo development with diffusion-weighted imaging qualitatively at a range of diffusion times.

Figure 1 shows multi-slice diffusion-weighted images of an E15.5 *in utero* mouse embryo with a diffusion time of 7 ms. We will further use fast imaging techniques and multiple diffusion imaging modalities to improve the resolution and the contrast of *in utero* mouse embryo images. The successful development and validation of *in utero* diffusion MRI can offer a unique method to monitor mouse embryo development.

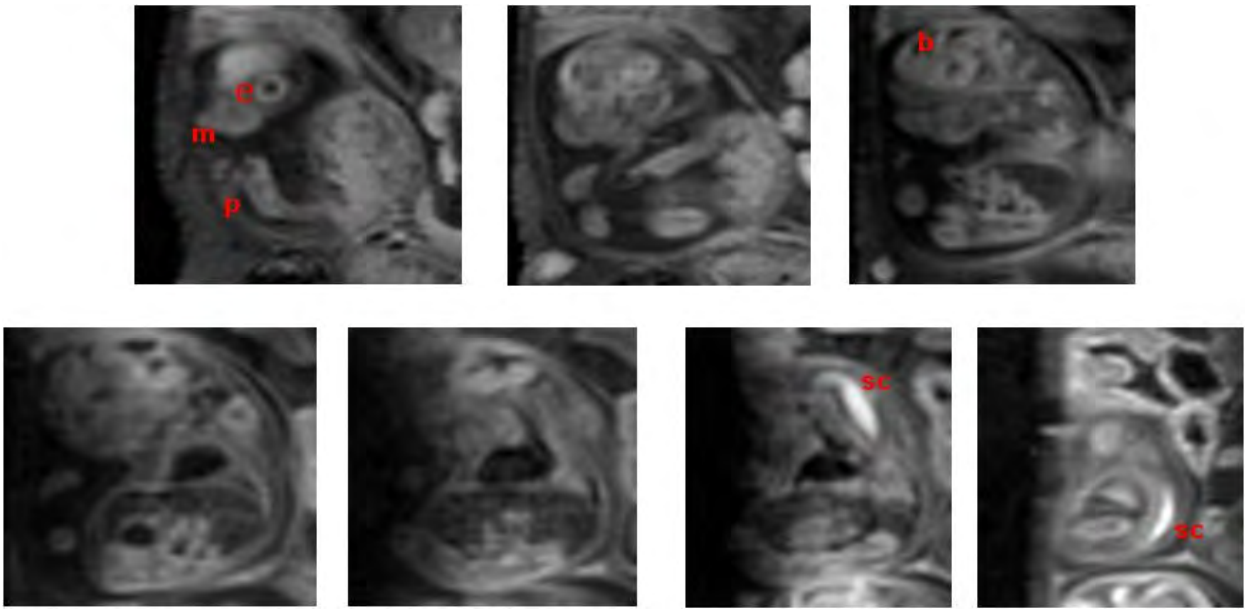


Figure 1. Diffusion-weighted MRI images (multiple slices) of an E15.5 *in utero* mouse embryo. The labels are – e: eye; m: mouth; p: paw; b: brain; sc: spinal cord. Image acquisition parameters: slice-thickness: 500 um; in plane resolution: 312 x 234 um. TE/TR: 20/1800 ms, DELTA: 8 ms, delta: 3 ms, b value: 500 s/mm^2, averages:16.

211. Zebrafish inner ear motile cilia hydrodynamically sculpt the otolith

David Wu, John Freund, Scott E. Fraser, Julien Vermot

The inner ear of the zebrafish contains two otoliths for sensing acceleration and sound. They are situated orthogonally to each other to help the fish navigate its three-dimension spatial environment, and each are above a patch of hair cells. However, the mechanism by which the otoliths are precisely and reliably placed is unknown. What is known is that otolith precursor particles are secreted into the developing inner ear by surrounding cells and that the particles accrete atop so-called tether cilia; over time the otolith forms from these aggregated particles. At the same time, accessory motile cilia provide a continuous flow, which is thought to concentrate the precursor particles in a region near the tether cilia, promoting aggregation. In fact, previous studies have shown that by knocking out dynein-controlling proteins, the position of otoliths becomes randomized and the shape variegated, which is consistent with the proposed mechanism but not proof of it. Here, we first show with a simple model that randomly diffusing precursor particles inside a sphere will tend to aggregate into otoliths in a random distribution, but by introducing motile cilia, the aggregation is concentrated along the axis of rotation of the cilia. We next show that we can obtain coarse flow data by using optical traps to seed the flow streams of the cilia, allowing us to measure the breadth of the proposed focusing effect. In short, cilia organize the flow field such that the growth is a controlled process, and not subject so much to random variegations of a diffusion-limited process, consistent with the simulation. Next, by using blinking optical traps, we obtain fine-grained flow data that allows us to precisely measure the diffusive/flow components that modulate the precursor aggregation pattern induced by cilia. This is used to demonstrate that the interaction length that drives otolith formation is on the order of a few microns. Finally, we ablate half the motile cilia in the inner ear and show that the otolith grows in a shortened fashion and becomes irregular in shape – the aggregation process is now driven by diffusion exclusively, without the sculpting effect of the motile cilia.

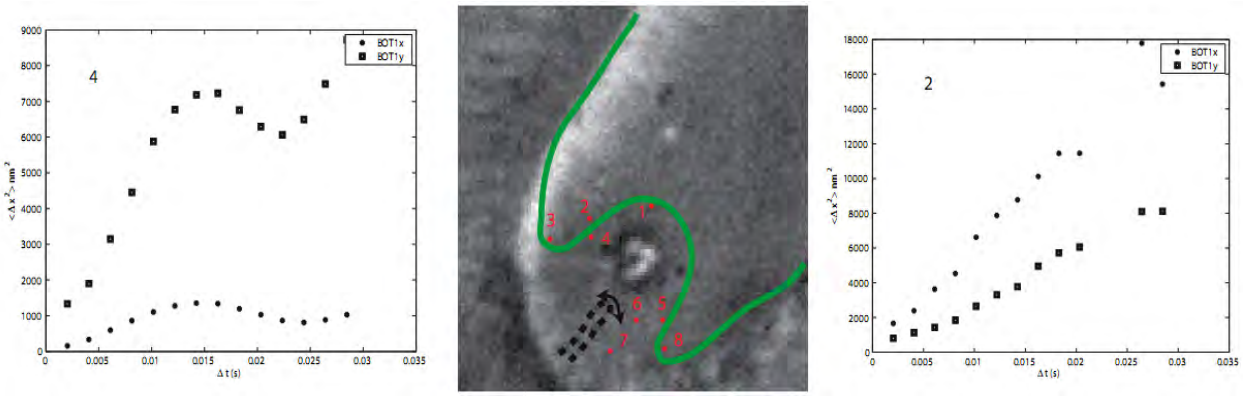


Figure: Measurements of the local diffusion coefficient were performed using blinking optical tweezers. The green line demarcates a boundary between primarily diffusive dynamics and flow driven dynamics due to the cilia (black outline). Spots nearer the otolith (such as 4) display nonlinear behavior in the second moment of displacement, whereas spots farther away (such as 2) display linear behavior, which is characteristic of diffusing species.

212. Synthetic *cis*-regulatory modules

Roee Amit, Hernan Garcia, Rob Phillips, Frances Arnold, Scott E. Fraser

I would like to introduce a new paradigm for synthetic biology – Synthetic Cis Regulatory Modules (SCRM). Namely, rather than design circuits, design and implement non-protein coding regions of DNA that regulate gene-expression in a precise 5-D pattern (time, space, and intensity). In nature, CRMs are the large non-coding genomic regions that occupy broad segments of the genome (sometimes tens of kb), and are implicated in the regulation of many genes. The regulation is due to a handful of transcription factors (typically 5-10), DNA binding proteins, and chromatin remodeling complexes that bind at several (typically clustered) binding sites each. Recently, detailed *cis*-regulatory analysis carried out on several developmentally important genes in fly and sea urchin model organisms has provided evidence that regulatory function carried out by CRMs are akin to complex computational operations.

In order to address this question, my goal is to construct synthetic CRMs (SCRM) *de novo* with binding sites for commonly used transcription factors that will show emergent computational behavior. In order to control the number of variables, I chose to use a bacterial system consisting of a minimally active promoter with the poised alternative sigma factor σ^{54}, and an associated upstream minimal enhancer. The SCRM sequences designed thus far, are divided into two classes: 1- cassettes that alter the spacing between the enhancer and minimal promoter; and 2- cassettes of either TetR or TraR binding sites added to the enhancer.

Our results divide into three levels of increasing complexity. First, we measured reporter expression as a function of the length separating the minimal promoter and enhancer and found that expression levels depend on both DNA helicity and probability of DNA looping. Second, we introduced a single binding site for TetR 22bp upstream of the activator binding site, and found that the looping dependence induces a repression function which has two distinct states: one fully repressed and one only partially inhibited. Finally, we constructed multiple binding site cassettes of either TetR or TraR, which exhibited a step like response of reporter expression levels to an input of variable amounts of externally supplied ligand (aTc or OOHl respectively). Thus, showing the potential to convert a chemical gradient input to an output step function. In conclusion, our simple synthetic enhancer system was able to recapitulate complex regulatory behavior that has been previously observed for natural CRMs.

213. Developmental dynamics of the sea urchin embryo

Mat E. Barnet, Eric H. Davidson, Scott E. Fraser*

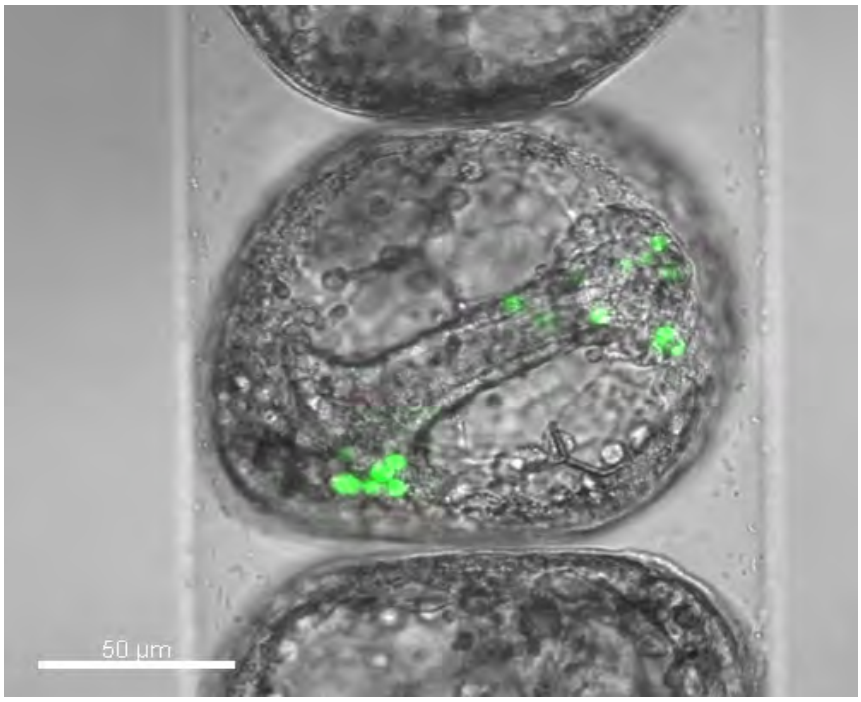
The fundamental question of developmental biology is how a single fertilized egg cell gives rise to a complex organism containing thousands, millions, or billions of highly integrated cells. As large-scale genome projects continue to reveal the striking genetic similarities among different species, it is becoming more and more clear that interspecies variation results not from major differences in the sets of genes different organisms possess, but from differences in the regulation and expression of those genes. To better understand how gene regulatory networks govern embryonic development, we are collaborating with Eric Davidson's lab, using sea urchins as a model system. The size, shape, and optical transparency of sea urchin embryos make them ideal for study by light microscopy.

We have recently developed a technique for multi-hour time-lapse imaging of living sea urchin embryos, revealing details of their development inaccessible to traditional techniques. For example, by tracking the migration of each cell in a single embryo over time, we are investigating the roles of specific genes involved in gastrulation.

Traditionally, studies of dynamic gene expression have been performed by combining snapshots of different embryos that were collected at different developmental times. By comparing expression of a gene in one embryo to expression of the gene in a different embryo at a different developmental time, conclusions have been drawn about the dynamic expression of that gene. Such conclusions, however, are necessarily limited by the fact that, although different embryos share stereotypical cell types, a *different* embryo is viewed at each point in time, making direct comparisons between embryos difficult.

Our technique removes this limitation by examining dynamic gene expression in a single embryo over time. Through such live imaging, we aim to track the fate of each individual cell involved in gastrulation, while simultaneously gaining insight into the regulatory states of these cells.

[*]*Professor, Division of Biology, Caltech*



Live late-gastrula-stage sea urchin embryos. Expression of the transcription factor *foxa* is shown in nuclei of the foregut and hindgut, via injection of a *dendra2-foxa* fusion BAC.

214. Age-related macular degeneration diagnostic improvement using optical coherence tomography

Jeff Fingler, Dan Schwartz[*], Scott E. Fraser

Age-related macular degeneration is the leading cause of vision loss in the Western world. Current diagnostics of this disease are limited and development of improved diagnostics can better track the progress of the disease, improve effectiveness of current treatments, and assist in the development of new therapeutic techniques. Optical coherence tomography (OCT) is an optical imaging technique, which allows three-dimensional reconstructions of the structural information of tissues like the retina. Our research is based on developing contrast improvements to OCT in order to visualize the smallest blood vessels in the retina, features that are critical to track the progression of the disease. Recent data has demonstrated the visualization of the three-dimensional microvasculature in the human retina, separating out several different layers of microvasculature in a visualization method never before demonstrated.

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Summary: We are interested in multiple questions in basic and applied biology. For further information on Hay lab research consult our web page (http://www.its.caltech.edu/~haylab/). One goal of our work is directed towards understanding the genetic and molecular mechanisms that regulate cell death, proliferation, innate immunity, microRNA function, and spermatogenesis. We use *Drosophila melanogaster* as a model system to identify genes that function to regulate these processes. Important cellular regulatory pathways are evolutionarily conserved; thus, molecules identified as regulators of these processes in *Drosophila* are likely to have homologs in vertebrates and the pathways that link these molecules are likely to be regulated similarly. A second goal of our work addresses three questions in population biology. 1) Can we bring about reproductive isolation (speciation) between populations of plants or animals that otherwise freely interbreed? Answers to this question have application to the growing number of situations in which plants and animals are engineered to show specific pharmaceutical or agricultural traits. In brief, we would like to be able to limit gene flow between engineered organisms and their wild counterparts. 2) Can we engineer the genetics of populations so that they drive themselves to local extinction? For example, invasive non-native plants and animals cause substantial economic losses. A number also cause substantial environmental damage, leading in many cases to extensive range reduction and/or extinction of unique, endemic species. Our goal is to develop genetic tricks that drive local extinction of invasive species and disease vectors. 3) Can we drive genes into wild populations so that all individuals express a trait of interest. With regard to this last aim, we are particularly interested in developing transgenic insects that will prevent transmission of mosquito-borne diseases such as malaria and dengue fever. More than 500 million people are infected with the malaria parasite each year, resulting in 1-3 million deaths. Dengue, a mosquito-borne virus infects more than 100 million people each year, resulting in more than 25,000 deaths. Effective vaccines for these diseases do not exist, and in the case of malaria, the causative agent, the parasite *Plasmodium falciparum* has acquired resistance to many drugs. Vector suppression through the release of sterile males, the use of insecticides, or modification of the environment provides an important tool for limiting mosquito-borne disease. However, each approach has limitations. Release of sterile males provides only transient population suppression, insecticides affect many non-target species and mosquitoes often evolve resistance to these compounds, and wholesale modification of the environment may not be feasible, or desirable in many situations based on ecological concerns. Our goals are two-fold: to develop transgenic insects that lack the ability to transmit these pathogens; and to develop genetic tools for driving these genes into wild populations of insects, thereby blocking disease transmission.

215. *Drosophila* models of human neuro-degenerative diseases

Ming Guo (and the Guo lab), Haixia Huang, Bruce A. Hay, Gal Barak, Kenneth Chan

In collaboration with the Guo lab at UCLA we are studying *Drosophila* models of the two most common neurodegenerative diseases, Alzheimer's disease and Parkinson's disease.

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

Chun Hong Chen, Haixia Huang

We have carried out several screens for cell death regulators in the fly and have identified a number of new molecules. Among these are multiple microRNAs, small noncoding RNAs that function by inhibiting translation of target transcripts. We are interested in determining when and where these molecules regulate death, as well as the nature of their targets. We are also designing microRNAs that target known cell death regulators as a way of probing the function of these proteins in specific contexts.

217. Cell death, caspases and IAPs

H. Arno J. Müller, Bruce A. Hay, Chun-Hong Chen

In flies and vertebrates most, if not all, cells can undergo apoptosis in the absence of new gene expression, indicating that the components required to carry out apoptosis are present and ready for activation. The core of the cell death machine consists of members of a family of proteases known as caspases, which become activated in response to many different death signals. Active caspases then cleave a number of different cellular substrates that ultimately lead to cell death and corpse phagocytosis. Most if not all cells constitutively express caspase zymogens (inactive precursors) sufficient to bring about apoptosis. Thus, the key to cell death and survival signaling revolves around controlling the levels of active caspases in the cell. Several basic strategies are used to regulate caspase activity, and the core proteins that drive caspase-dependent death are evolutionarily conserved. In *Drosophila* many cells experience chronic activation of the apical cell death caspase Dronc. If unrestrained, active Dronc cleaves and activates downstream effector caspases that bring about cell death. Cells survive because they express the IAP DIAP1, which suppresses Dronc activity, as well as that of caspases activated by Dronc. One major pathway through which caspase-dependent cell death in flies is induced is through the regulated expression of pro-apoptotic proteins that disrupt DIAP1-caspase interactions through several different mechanisms, each of which has the effect of unleashing a cascade of apoptosis-inducing caspase activity. We are interested in several questions. 1) What are the signals that lead to caspase activation in cells that would normally live? 2) How do IAPs regulate caspase activity and when and where does this regulation define points of control? 3) How is IAP activity regulated? 4) And finally, as discussed further below, how do caspases, IAPs and their regulators work to regulate non-apoptotic processes? We are using both genetic screens and biochemical approaches to identify the critical molecules.

218. Caspases and their regulators in a non-apoptotic process, spermatid differentiation

Haixia Huang, Shamili Allam, Joy Chen, Geoffrey Pittman

We have found that multiple caspases, acting through distinct pathways, acting at distinct points in time and space, are required for spermatid individualization, a process in which spermatids (which develop in a common cytoplasm) become enclosed in individual plasma membranes and shed most of their cytoplasm*. Spermatid individualization is an evolutionarily conserved process, but little is known about how it is brought about. Several questions are of interest to us: 1) What are the upstream signals that drive caspase activation? 2) What are the nonapoptotic targets that facilitate differentiation? 3) How is cell death prevented in the face of high levels of caspase activity that would normally be associated with cell death? 4) Do caspases play similar roles in promoting spermatid differentiation in mammals? 5) Can we manipulate the biology of spermatogenesis so as to bias gamete production so that males produce gametes carrying the Y chromosome, but not the X chromosome? Elements with these characteristics, if they are located on the Y chromosome, are predicted to drive a population to extinction through the generation of male-only populations.

Reference

Huh, J. *et al*. (2004) *PLoS Biology* **1**:E15.

219. Cell death and the innate immune system

Chun-Hong Chen, Ming Guo, Bruce A. Hay

As discussed above, many IAP family proteins inhibit apoptosis. IAPs contain N-terminal BIR domains and a C-terminal RING ubiquitin ligase domain. *Drosophila* DIAP1 protects cells from apoptosis by inhibiting caspases. Apoptosis initiates when proteins such as Reaper and Hid bind a surface groove in DIAP1 BIR domains via an N-terminal IAP-binding motif (IBM). This evolutionarily conserved interaction disrupts IAP-caspase interactions, unleashing apoptosis-inducing caspase activity. DIAP2 overexpression also inhibits Rpr- and Hid-dependent apoptosis, but little is known about DIAP2's normal functions. We generated *diap2* null mutants, which are viable and show no defects in developmental or stress-induced apoptosis. Instead, DIAP2 is required for the innate immune response to Gram-negative bacterial infection. DIAP2 promotes cytoplasmic cleavage and nuclear translocation of the NF-κB homolog Relish, and this requires the DIAP2 RING domain. Increasing the genetic dose of *diap2* results in an increased immune response, while expression of Rpr or Hid results in down-regulation of DIAP2 protein levels. Together these observations suggest that DIAP2 can regulate immune signaling in a dose-dependent manner, and that DIAP2 is regulated by IBM-containing proteins. Therefore, *diap2* may identify a point of convergence between apoptosis and immune signaling pathways.

Reference

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220. Driving genes for disease refractoriness into wild pest insect populations

Chun Hong Chen, Haixia Huang, Catherine Ward, Jessica Su, Nikolai Kandul, Geoff Pittman, Omar Akbari, Arun Kumar, Daniel Leighton, Bruce A. Hay

An attractive approach to suppressing mosquito-borne diseases involves replacing the wild-insect population with modified counterparts unable to transmit disease. Mosquitoes with a diminished capacity to transmit *Plasmodium* have been identified in the wild and created in the laboratory, demonstrating that endogenous or engineered mosquito immunity can be harnessed to attack *Plasmodium*. However, a critical unanswered question is

how to spread these effector genes throughout the areas inhabited by disease-transmitting insects. Epidemiological and modeling studies suggest that it will be necessary to rapidly replace a large percentage of the wild mosquito population with refractory insects in order to achieve significant levels of disease control. Because insect disease vectors are spread over wide areas and can migrate significant distances, mass release of refractory insects associated with simple Mendelian transmission of effector-bearing chromosomes is unlikely to result in a high enough frequency of transgene-bearing individuals. Compounding this problem, enhancement of immune function in insects is often costly, requiring tradeoffs with other life history traits such as longevity and fecundity that decrease fitness. Therefore, it is likely that insects carrying effector transgenes will be less fit than their wild counterparts, resulting in a decrease in the fraction of individuals carrying genes for refractoriness over time. These observations argue that population replacement will require coupling of genes conferring disease refractoriness with a genetic mechanism for driving these genes through the wild population at greater than Mendelian frequencies.

Maternal-effect lethal selfish genetic elements have been described as genetic entities in the flour beetle *Tribolium casteneum*. The molecular nature of these elements (known as *Medea* elements) is unknown, but their genetic behavior makes them attractive candidates to mediate drive. This is because when present in a female, they must be inherited in the next generation in order for the offspring to survive.

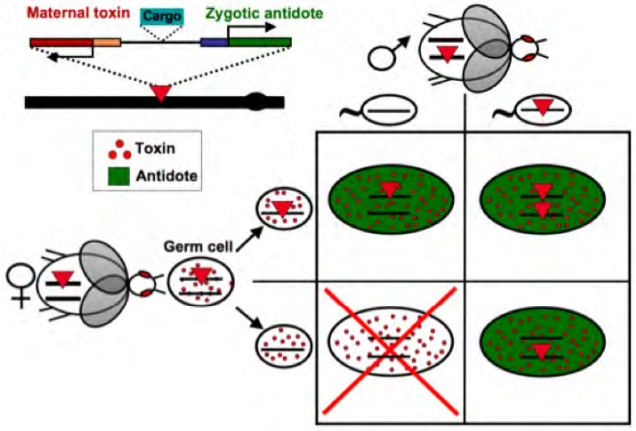

Figure 1. *Medea* is a "spiteful" selfish genetic element that enhances its transmission from generation to generation by causing the death of offspring that fail to inherit it. Mothers that carry a *Medea* element express a toxin (red dots) that is inherited by all oocytes (small ovals). Embryos (large ovals) that do not inherit *Medea* die because toxin activity (red background) is unimpeded (lower left square). Embryos that inherit *Medea* from the mother (upper left square), the father (lower right square) or both (upper right square), survive because expression of an antidote early during embryogenesis (green background) neutralizes toxin activity. We imagine that *Medea* is comprised of two closely linked genes (upper left). One consists of a maternal germline-specific promoter that drives the expression of an RNA or protein that is toxic to the embryo. The second locus consists of a zygotic (early embryo) promoter that drives expression of an antidote.

This behavior is predicted to lead to rapid spread of the element within the population even if it carries an associated fitness cost because the chromosome that carries it gains a transmission advantage relative to counterparts that do not. Since the molecular biology of endogenous *Medea* elements is unknown, we created synthetic elements in *Drosophila* that can drive population replacement and that are resistant to recombination-mediated dissociation of drive and effector functions. The genetic and cell-biological principles utilized, which utilize microRNA-mediated silencing of a maternally-expressed gene essential for embryogenesis, coupled with early zygotic expression of a rescuing transgene, should be generally applicable to a number of other animal and plant species and have the potential to allow for iterative cycles of population replacement. We are now expanding this work into the mosquito system.

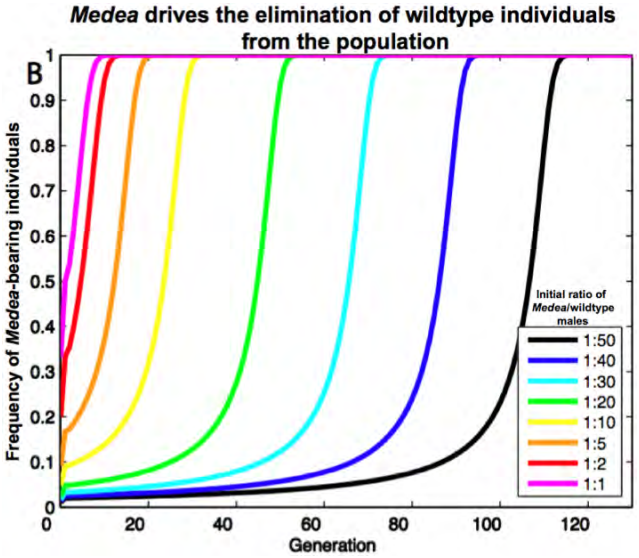

Figure 2. When *Medea*-bearing males are introduced into a population consisting of wildtype males and females, wildtype individuals are eliminated from the population. The greater the initial ratio of *Medea* to wildtype males, the more rapidly this elimination occurs.

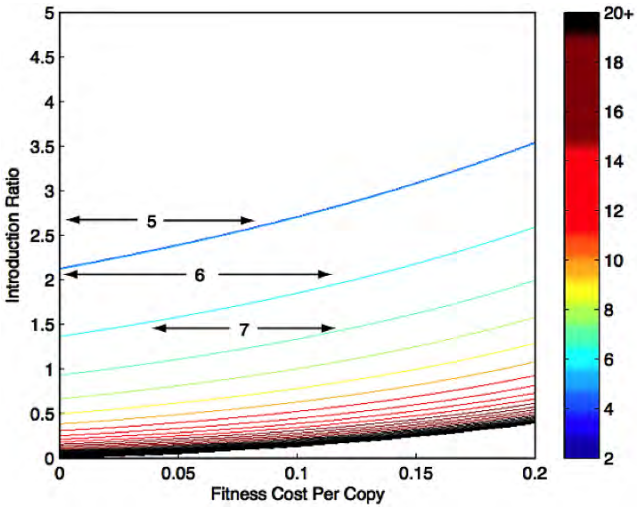

Figure 3. Medea's ability to spread, and the time it takes to become present in all individuals, is a function of fitness cost and introduction frequency. The plot describes the number of generations required for *Medea* to be present in 99% of individuals, for a *Medea* element with an embryonic fitness cost (resulting from the presence of a cargo transgene designed to protect from disease, for example). Homozygous *Medea*:non-*Medea* introduction ratios are indicated on the Y axis, and embryonic fitness cost on the X axis. Area between lines indicates regions of parameter space within which a specific number of generations (indicated by numbers and arrows) are required for the frequency of *Medea* individuals to reach a frequency of 99% or greater. Line color, shown in the heat map at right, provides a measure of how many generations are required. Black lines (50+) indicate that fifty or more generations are required. The border between the black-lined region and the lower unlined region defines the critical *Medea*:non-*Medea* introduction ratio, below which *Medea* will be eliminated from the population.

Reference

Chen *et al*. (2007*) Science* **316**:597-600.

221. Sensing and killing dengue and yellow fever virus-infected cells in their insect host

Kelly J. Dusinberre, Gal Barak

Dengue and yellow Fever virus infect mosquitoes during a blood meal. The virus must enter and replicate inside mosquito midgut cells, disseminate throughout the body and ultimately infect the salivary gland (7-14 days later), in order to be transmitted to a new individual during a subsequent blood meal. Our goal is to develop transgenes that are phenotypically neutral when expressed in uninfected individuals, but that kill virus-infected cells and/or the mosquitoes themselves. The virus encodes several activities that are not present in uninfected host cells. These include a viral polyprotein protease, and RNA-dependent RNA polymerase. We are developing molecules that sense these activities and cause the death of cells and insects in which they occur, thereby preventing disease transmission to humans.

222. Engineering reproductive isolation and population replacement using a synthetic underdominance system

Kelly Dusinberre, Katie Kennedy, Margaret Chiu, Jessica Su

The Medea system detailed above is very good at spreading genes into populations distributed over large areas, provided that modest levels of migration occur. This is ideal for situations in which the goal is to carry out population replacement in large regions. However, some communities may favor an approach in which population replacement is restricted to a local environment (Lets see how it does in your back yard, before trying it in mine). This creates a challenge: how to spread genes within a local environment, but maintain a barrier to migration-driven spread and fixation in surrounding regions. To address this need we are developing the synthetic underdominance system illustrated below. In this system homologous chromosomes carry toxin-antidote pairs in which the toxin present on chromosome A (Killer 1) is linked to an antidote (Rescue 2) that represses Killer 2. Killer 2 is located at the same position on the homologous chromosome B, linked with an antidote (Rescue 1) that represses Killer 1 (Figure 4). In such a system, organisms can only survive if they carry A and B chromosomes (in A/B individuals), or only wildtype (+) chromosomes (in +/+ individuals). A/+ and B/+ individuals die. A and B chromosomes will also carry genes that confer resistance to disease transmission. Such a system has two interesting features.

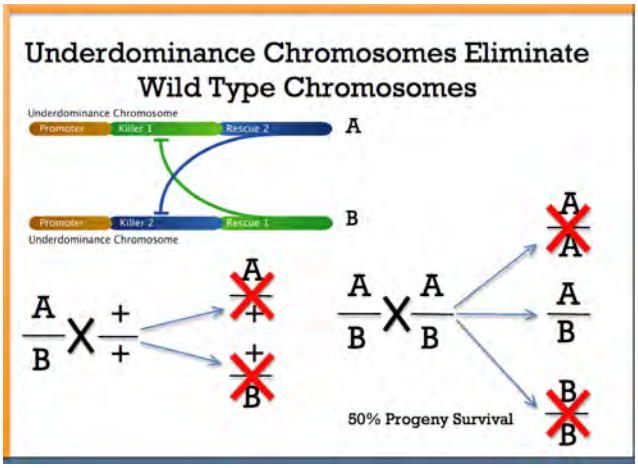

Figure 4. A single-locus underdominance system can be used to engineer reproductive isolation and population replacement.

First, it constitutes a simple method for engineering reproductive isolation (speciation). Matings between +/+ individuals produce viable progeny, as do matings between A/B individuals. However, mating between +/+ and A/B individuals produce only A/+ and B/+ progeny, which all die. This simple technology has a number of potential applications and provides a platform

from which to explore some of the evolutionary consequences of reproductive isolation.

Second, it provides a method for driving genes into a local environment in such a way that they are unlikely spread to fixation in surrounding regions through migration. In brief, for underdominance, as with Medea elements that carry a fitness cost, a threshold frequency must be achieved in order for spread to occur at all. With underdominance this threshold is quite high. But once the threshold is crossed, the underdominant system drives the wildtype chromosomes out of the population by causing their death in heterozygotes. A and B chromosomes also die in heterozygous progeny, but so long as A/B individuals make up greater than 66% of the population, more + chromosomes and thus, +/+ individuals are eliminated than are A and B chromosomes, in A/B individuals. The A/B genotypes have great difficulty in spreading into surrounding regions through migration because as these individuals migrate into areas composed largely of +/+ individuals, they are more likely to mate with +/+ individuals than with A/B individuals, resulting in the likely death of the A and B chromosomes in heterozygous progeny. We are developing several versions of underdominance in *Drosophila* and are working to move these systems to mosquito species.

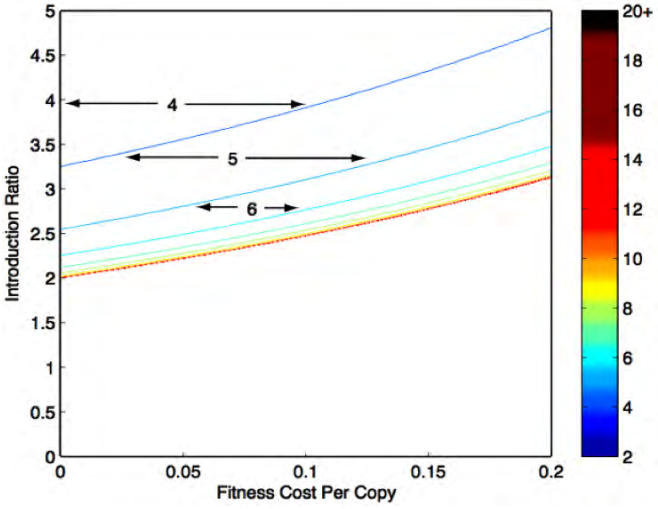

Figure 5. A single locus underdominant system's ability to spread, and the time it takes to become present in all individuals, is a function of fitness cost and introduction frequency. The plot describes the number of generations required for underdominant chromosomes to be present in 99% of individuals, for a situation in which the presence of the underdominant chromosomes is associated with an embryonic fitness cost (resulting from the presence of a cargo transgene designed to protect from disease, for example). Homozygous A/B:+/+ introduction ratios are indicated on the Y axis, and embryonic fitness cost on the X axis. Area between lines indicates regions of parameter space within which a specific number of generations (indicated by numbers and arrows) are required for the frequency of underdominant chromosome-bearing individuals to reach a frequency of 99% or greater. Line color, shown in the heat map at right, provides a measure of how many generations are required. Black lines (50+) indicate that fifty or more generations are required. The border between the red-lined region and the lower unlined region defines the critical A/B:+/+ introduction ratio, below which underdominant chromosomes will be eliminated from the population.

223. Sensing and responding to normal and abnormal microRNA expression

Nikolai Kandul, Alan Li

MicroRNAs (miRNAs) are small, non-coding RNAs that regulate gene expression by suppressing the translation or promoting the degradation of transcripts to which they hybridize. Importantly for our purposes, when miRNAs are perfectly complementary to their target transcripts, transcript cleavage and degradation results. It is clear that miRNA expression is deregulated in many disease states. In addition, many viruses encode miRNAs that promote viral replication and/or suppress host defense systems. Our goal is to develop methods for sensing the expression of a particular miRNA, and then transducing this signal into changes in gene or protein expression. This will allow us to monitor the levels of miRNA expression in living animals. It will also allow us to regulate cellular physiology in response to the levels of particular miRNAs.

224. Predicting the fate of gene drive systems and their cargos in the wild

Catherine Ward, John Marshall

As we develop gene drive strategies we need to be able to predict how they are likely to behave. A number of question arise. Under what ecological and population genetic conditions will drive chromosomes spread? What are the likely epidemiological consequences of spread in terms of disease prevention? What are the likely functional lifetimes of these elements in the wild? What are the possibilities for removal and replacement of first-generation elements with second-generation elements? We are using mathematical modeling and computer simulations to address these issues for a number of different drive strategies.

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Graduate Students: Janet Chow, Arya Khosravi, Sung-Eun Lee, Alex C. Romero, Yue Shen
Undergraduate Students: Gloria Tran, Vivian Yang
Research and Laboratory Staff: Taren M. Johnson, Sara W. McBride

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Summary: The Western world is experiencing a growing medical crisis. Epidemiologic and clinical reports reveal a dramatic increase in immune disorders: inflammatory bowel disease, asthma, type 1 diabetes, and multiple sclerosis. Emboldened by the 'hygiene hypothesis' proposed two decades ago, scientists have speculated that lifestyle changes (vaccination, sanitation, antibiotics) have predisposed developed societies to these disorders by reducing bacterial infections. However, the hypothesis remains without explanation as our exposure to most bacteria does not result in disease. Mammals are colonized for life with 100 trillion indigenous bacteria, creating a diverse ecosystem whose contributions to human health remain poorly understood. In recent years, there has been a revolution in biology toward understanding how (and more importantly, why) mammals harbor multitudes of symbiotic bacteria. We have recently demonstrated for the first time that intestinal bacteria direct universal development of the immune system; thus fundamental aspects of mammalian health are inextricably dependent on microbial symbiosis. Furthermore, it is now clear that all of the diseases in question astonishingly involve a common immunologic defect found in the absence of symbiotic bacteria. As we have co-evolved with our microbial partners for eons, have strategies used against infectious agents reduced our exposure to health-promoting bacteria, ultimately leading to increased disease? We propose that the human genome does not encode all functions required for health, and we depend on crucial interactions with products of our microbiome (collective genomes of our gut bacterial species). Through genomics, microbiology, immunology and animal models, we wish to define the molecular processes employed by symbiotic bacteria that mediate protection from disease. Advances in the past year have now made it possible to mine this untapped reservoir for beneficial microbial molecules. Ultimately, understanding the immune mechanisms of these *symbiosis factors* may lead to natural therapeutics for human diseases based on entirely novel biological principles.

225. Dynamic surface variation by symbiotic bacteria is required for host colonization

Sung-Eun Lee, Vivian Yang

Bacterial surfaces represent functional organelles decorated with molecules that mediate critical interactions between the microbes and their milieu. These environments may be on or within another organism; not uncommonly, that organism is a mammal. Capsular polysaccharides are abundant external structures of bacteria, and the capsules of many prokaryotic pathogens have been found to be important virulence factors during mammalian infection. Unlike pathogens, commensal bacteria establish a life-long co-habitation with their mammalian hosts. However, the molecular mechanisms employed to establish this beneficial relationship remain almost entirely undescribed. The unique identification of multiple surface polysaccharides in the important human symbiont *Bacteroides fragilis* raised the critical question of how these molecules contribute to commensalism. Herein, we report that mutation of the master regulator of *B. fragilis* polysaccharide expression results in a global reduction of capsule. Surprisingly, attempts to completely eliminate expression of capsule are not tolerated and result in abrogation of bacterial growth. Subsequently, the organism acquires a spontaneous mutation that restores growth and production of at least one capsular polysaccharide. We identify an alternative pathway by which *B. fragilis* is capable of re-establishing capsule production. Most importantly, mutants expressing single, defined surface polysaccharides remain defective for intestinal colonization of animals compared to bacteria that express a complete polysaccharide repertoire, a process mediated by specific interactions between bacteria and intestinal mucus. The extensive surface diversity and multiple layers of regulation suggest a profound evolutionary requirement for capsular polysaccharide during host-bacterial symbiosis.

226. Host-bacterial mutualism by a microbial symbiosis factor prevents inflammatory disease

June L. Round, Arya Khosravi, Alex C. Romero, Gloria Tran

Colonization of humans with multitudes of commensal species creates an ecosystem harboring members of five of the six kingdoms of life. Bacteria in particular dominate this ecologic niche; the gastrointestinal tract is resident to an astounding $>10^{14}$ microorganisms with a diversity of approximately 2,000 species. This consortium of gut bacteria represents an integral factor in mammalian biology. Germ-free animals, born and raised under sterile conditions, exhibit profound defects in the development of intestinal tissues. Many reports have shown that both gastrointestinal and systemic immune responses are deficient in the absence of commensal microoroganisms. Surprisingly, however, the gut is stably colonized by both beneficial and potentially pathogenic microorganisms; the reasons for this phenomenon remain unclear. Moreover, imbalances in the composition of the

bacterial microbiota, known as dysbiosis, are thought to be a major factor in human disorders such as inflammatory bowel disease (IBD). We report herein that the ubiquitous human symbiont, *Bacteroides fragilis*, protects animals from experimental colitis induced by the pathogenic commensal, *Helicobacter hepaticus*. Most importantly, this beneficial activity requires a single bacterial molecule (Polysaccharide A or PSA). Animals harboring *B. fragilis* not expressing PSA develop disease and produce pro-inflammatory cytokines in colonic tissues similar to *H. hepaticus* colonization alone. Purified PSA administered to animals protects from experimental colitis and wasting disease by inducing anti-inflammatory responses, both *in vivo* and *in vitro*, and activation of interleukin 10-producing $CD4^+$ T cells. These results reveal the first molecule of intestinal commensal bacteria that mediates the critical balance between health and disease. As incidence of IBD have dramatically increased over the last several decades, harnessing the immunomodulatory capacity of *symbiosis factors* such as PSA may ultimately provide novel therapeutics for human inflammatory disorders.

227. Affect of intestinal colonization with *Bacteroides fragilis* on autoimmune diabetes in NOD mice

Mary A. Yui, Sara W. McBride, Taren M. Johnson

Humans, and other organisms, are hosts to extremely complex, diverse and highly co-evolved microbial communities living at mucosal surfaces, especially in the gastrointestinal tract. Recent evidence has been accumulating which suggest that these microbial communities may play a key role in maintaining host health and avoiding autoreactivity, although mechanisms by which this occurs are as yet poorly understood. Correlations have been noted between the alarming rise in developed nations in the rates of autoimmune disorders, including Type 1 diabetes (T1D), and the decrease in human infectious diseases due to improvements in sanitation, vaccination, and the widespread use of antibiotics. While susceptibility to T1D has a strong genetic component in humans and rodent models, several lines of evidence in the rodent models suggest that manipulation of the microbiota, with pathogenic or non-pathogenic organisms, antibiotics, or diet, may reduce the incidence of T1D in animals genetically predisposed to develop diabetes. We are testing the hypothesis that a non-pathogenic human intestinal microbe, *Bacteroides fragilis*, can alter autoreactivity the non-obese diabetic (NOD) mouse model of T1D. *B. fragilis* is a prominent member of the human intestinal microbiota, and was the first symbiotic bacteria experimentally shown to affect the development of the mammalian immune system. *B. fragilis* has recently been shown by the Mazmanian lab to protect animals from experimental autoimmune colitis by inducing anti-inflammatory regulatory T cells through the production of a single product (Polysaccharide A or PSA). Induction of regulatory T cells has also been shown to be a powerful means of preventing T1D in NOD mice, so we are testing the hypothesis that mucosal colonization with this PSA-producing microbe can change the balance between pathogenic and protective T cells and prevent pancreatic inflammation and the development of diabetes in NOD mice. Groups of NOD mice have been colonized with *B. fragilis*, with and without PSA, using several different protocols and beginning at several different ages, to determine if there is any effect on the timing or incidence of diabetes. These studies are ongoing. In addition, we are testing if *B. fragilis* and/or PSA have direct effects on the types of cytokines secreted by dendritic cells and T cells isolated from NOD mice.

228. Immune modulation by microbial outer membrane vesicles (OMVs) during *Bacteroides fragilis* colonization

Yue Shen

Previous studies in the lab have shown *Bacteroides fragilis*, an important and numerically abundant commensal bacteria of the human gut, modulates host immune responses and prevents animals from experimental colitis. More importantly, the molecule that is required and sufficient for this immuno-modulatory activity has already been identified as one of the eight known capsular polysaccharides of B. *fragilis*, Polysaccharide A (PSA). In order to study the molecular mechanism for how PSA is delivered from *B. fragilis* to host immune cells, we proposed to investigate the potential role of outer membrane vesicles (OMVs), which are naturally produced by growing *B. fragilis*, in this delivery process. We have visualized OMVs produced by *in vitro* cultured *B. fragilis* via electron microscopy. And by immunoblot and immunogold labeling studies, and discovered that PSA is indeed associated with *in vitro* purified OMVs. Furthermore, our data show PSA-containing OMVs could induce specific immuno-regulatory response *in vitro* and could also protect animals from experimental colitis *in vivo* in a PSA-dependent manner, which suggested OMV-associated PSA has the same immuno-modulatory activity as purified PSA does. And the ongoing studies are aimed to further confirm these results, but more importantly, to test the necessity of OMVs in the delivery process as well as to investigate the detail processes of how OMV-associated PSA affects the physiology of the immune cells and eventually lead to the immuno-regulatory responses both *in vitro* and *in vivo*. A better understanding of the function of PSA associated with *B. fragilis* OMVs will not only help us to determine the role of OMVs in delivering PSA, but also shed light on the clinical application of *B. fragilis* OMVs in treating human inflammatory bowel disease.

229. Type VI secretion by *Helicobacter hepaticus* limits bacterial colonization and host intestinal inflammation during symbiosis

Janet Chow

Symbiotic bacteria colonize mammals by creating a molecular discourse with their hosts. Evolution has created numerous mechanisms that mediate these interactions, and bacterial secretion systems represent an important component for how microbes network with eukaryotic cells. Recently characterized, type VI secretion systems (T6SS) present a new means of forging microbial-host interactions. Interestingly, T6SS genes are present in over 25% of all bacterial genomes sequenced to date, yet specific knowledge of the function and substrates for these secretion systems remain largely unknown. Although mostly implicated in a role for virulence, a growing number of studies have shown type VI secretion may also mediate non-pathogenic relationships between microbe and host. Our study focuses on the role of the T6SS in *Helicobacter hepaticus*, a Gram-negative bacterium that stably colonizes the murine gastrointestinal tract for long-term without leading to intestinal disease. We find that mutants defective in T6S display higher intracellular and cell-associated numbers of bacteria upon incubation with intestinal epithelial cells *in vitro.* In addition, T6S mutants show increased colonization of the gastrointestinal tract *in vivo*. Furthermore, *in vivo* colonization with a T6SS mutant leads to exacerbated host inflammatory responses in an experimental murine model of colitis. Based on these findings, we propose that type VI secretion in *H. hepaticus* may function to maintain balance of a commensal relationship within the host gastrointestinal tract by limiting bacterial colonization and host inflammatory responses.

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Summary: Our lab studies shoot apical meristems, which are the collections of stem cells found at the tip of each growing shoot in plants. In our case the plant studied, for the most part, is the small mustard *Arabidopsis thaliana*, which we have chosen because of its small size, rapid growth, and fully sequenced genome. In *Arabidopsis*, similarly to most flowering plants, the shoot apical meristem is a collection of a few hundred cells that is first formed during embryogenesis, and after seed germination provides the entire above-ground part of the plant, including stems, leaves, and flowers. It is divided into functional regions (the ultimate stem cells at the tip, beneath them the multipotent stem cells that will make up the stem, and surrounding them the cells that will make leaves and flowers) and contains many overlapping domains of gene expression. This indicates that the meristem is highly organized, and the patterns of gene expression, and therefore the organization, are fairly constant, even though cells divide and are displaced from one region to another. The cells therefore know their positions in the meristem, and as these positions change after addition of cells due to cell division, the cells change their patterns of gene expression.

One central unanswered question about these (and other) stem cells, is how they assess their positions, and thereby regulate their genes and their activities. We study several different modes of communication between the meristematic cells, which appear to allow for the dynamic activities of the meristematic cells. One mode of communication is chemical. The ultimate stem cells at the tip secrete a peptide, CLV3, which regulates the behavior of the cells beneath through a set of transmembrane receptor kinases and related proteins, including CLV1 and CLV2. All of the cells in the top layer of cells of the meristem actively transport the plant hormone auxin to their neighbors, and feedbacks that regulate the direction and amount of transport establish the pattern of leaves and flowers around the stem. The cells in the center of the meristem respond to the plant hormones called cytokinins, and this establishes the gene expression domain that characterizes the cells that will become parts of the stem. A second mode of communication is physical. We, with a large international group of collaborators, have established that meristem cells can sense the principle direction of physical stress, and that they realign their cortical microtubules, part of the cellular skeleton, in response to it. We are now testing if this mode of cell-cell communication is involved in regulation of auxin transport, and in planes of cell division.

Shoot apical meristems form not only in embryogenesis, but also in masses of cultured cells, showing that they are self-organizing. Some of our current studies are directed to understanding the nature of this process, and include studies of the states of gene expression and of cellular communication in cultured cell masses (callus) before, during, and after the *de novo* formation of meristems.

When flowers form on the flanks of the shoot apical meristem a number of different patterning processes occur, including formation of floral organs, whose cells differentiate in specific patterns, formation of boundaries between organs, which requires activation of specific genes, and patterned expression of genes that direct flower development. Abstracts below also describe new research in each of these areas.

Two critical types of research form the foundation of all of our studies – computational modeling of cell behavior as cells grow, divide, and communicate with each other, an area we call Computational Morphodynamics; and detailed analysis of gene expression in specific cell types. Both of these topics are also covered in the following abstracts.

230. Cell biological, genetic and biochemical approaches to studying the Clavata1 receptor kinase

Zachary L. Nimchuk, Elliot M. Meyerowitz

Plants grow via the constant proliferation of cells from specific regions termed meristems. The shoot meristem gives rise to all above ground tissues. In the shoot meristem the rate of cell proliferation within the meristem is balanced with the allocation of cells into lateral organs. Thus the net exit of cells from the meristem is balanced with new cell production in order to maintain the meristem proper. Several classes of mutants exist in *Arabidopsis* that affect in this balance. Mutations in *CLV1* lead to a hyper-accumulation of stem cells in both shoot and floral meristems resulting in stem fasciation, club-shaped siliques and extra floral organs. *CLV1* encodes a receptor-like kinase (RLK) with extracellular leucine-rich repeats and a cytoplasmic serine threonine, (ser-thr)

signaling domain that may interact with the putative peptide ligand CLV3. Although the *CLV* class-of-mutants have been around for many years, little is known about CLV1 regulation or signaling. We are taking several complementary approaches to study CLV1 function. Using a range of tagged CLV1 alleles we define roles for different domains in CLV1 function at the genetic and cellular level. Our analysis indicates that the perception of CLV3 by CLV1 results in alterations in CLV1 subcellular localization. We are in the process of defining both cis and trans pathway mutations which affect this process.

231. Functional analysis of CLV2 in SAM maintenance

Xiang Qu, Elliot M. Meyerowitz

Located at the growing tip of stems, shoot apical meristems (SAMs) are actively dividing, embryonic tissues responsible for all the aerial organs in plants. A balance between meristematic cell division and differentiation is required to maintain a functional SAM. In *Arabidopsis*, the *CLAVATA* (*CLV*) genes encode important elements in SAM maintenance. Single loss-of-function mutations of the *CLV* genes (*clv*) result in a progressive enlargement of shoot and floral meristems. Among those, the *CLV2* gene contains a single exon that encodes a 720-amino acid LRR-RLP (leucine-rich repeat receptor-like protein). It was hypothesized that CLV2 forms a heterodimer with CLV1 so as to stabilize the CLV1 signaling complex at the plasma membrane. However, the recent finding that *clv1-11 clv2-1* double mutants closely resemble *clv3*-2 single mutant, that has a much stronger phenotype than the loss-of-function *clv1-11* single mutant, predicates against this possibility. We have now been using a combination of methodologies, including biochemical, genetic, and cell biological approaches, to gain information in molecular detail how the receptor-like protein CLV2 functions as an element to maintain a functional SAM. We have generated constructs that allow us to visualize the CLV2 protein directly in the confocal microscope and/or detect it indirectly by chemi-luminescence. We are also now testing functions of various domains of CLV2 during SAM maintenance using both the tobacco transient expression system and stable *Arabidopsis* transgenic lines.

232. Identification of additional signaling components within the CLV pathway

Xiang Qu, Elliot M. Meyerowitz

Genetic evidence indicates that the *CLAVATA* genes act in concert to restrict the size of the SAM (*POL, PLL1* and *KAPP* seem to play important roles, as well). At the molecular level of detail, however, little is known of the signal transduction mechanisms by which CLAVATA1 and CLAVATA2/CORYNE, the central receptor kinases in the shoot apical meristem, transduce the signal from the CLAVATA3 ligand to WUSCHEL expression level. Signal transduction in this system requires protein-protein interaction. However, little is known of the composition of the CLV signaling complex, other than the direct interaction between the peptide ligand CLV3 and the receptor kinase CLV1. CLV1 has been previously reported to form two distinct complexes with respective molecular masses of 185 kD and 450 kD. The presence of CLV3 is required for CLV1 to form the 450-kD complex. In addition, CLV1 monomer elutes upon treatment with reducing agent, suggesting that intermolecular disulfide linkages might be important for formation of the CLV1 signaling complexes. However, these data contradict our recent findings on the stability of CLV1, which show that CLV1 is rapidly degraded and undetectable in a wild-type genetic background. In addition, CLV1 and CLV2 act independently to regulate the SAM based on the recent study of CRN and our *clv1;clv2* double mutant analysis. Thus, we are reanalyzing the CLV signaling complex by use of both genetic and biochemical approaches. We are conducting a sensitized genetic screen for CLV1-interacting genes. We are also conducting a series of experiments to examine the CLV1/CLV2 protein complexes in the SAM and further to identify novel components that directly interact with CLV1/CLV2 by *in vivo* protein complex purification and mass spectrometry.

233. Pattern formation by hormonal interactions: A recurring theme in regenerating tissue and the shoot apical meristem

Sean Gordon, Vijay Chickarmane, Elliot M. Meyerowitz

Recent experiments in which regenerating tissue which was grown in shoot-inducing medium, led to the formation of roots, as well as shoots at specific locations. We are interested in the mechanisms that lead to these spatial patterns, as a result of hormonal interactions. From confocal imaging and RT-PCR studies, we have hypothesized that a reaction-diffusion mechanism due to interactions between the hormones auxin and cytokinin must exist, which ultimately determines the formation of roots and shoots. Assuming that auxin is an activator and cytokinin, an inhibitor, robust patterns emerge in a simple mathematical model. However, unlike most models that involve passive transport, our model will involve active transport of auxin due to PIN proteins, which should have the consequence of changing the effective diffusion. Recent experiments have suggested that pattern formation in the shoot apical meristem could also emerge due to interactions between auxin and cytokinin. We are developing models on a 2-dimensional fixed template, which would later be extended to 3-D. Our research shows therefore a recurring theme in which hormones mediate pattern formation.

234. How does the shoot apical meristem achieve homeostasis of stem cell numbers?

Vijay Chickarmane, Sean Gordon, Paul Tarr, Henrik Johnsson, Eric Mjolsness, Elliot M. Meyerowitz

Within the shoot apical meristem, which is located at the tip of the shoot, reside stem cells, which provide differentiated cells to form lateral organs, such as leaves and flowers. We are interested in knowing how their numbers are conserved throughout the life time of the plant as a result of signaling and regulatory interactions between stem cells and the other differentiated cell populations. Previous studies have implicated a negative feedback loop by which stem cell numbers are maintained. Stem cells negatively control another group of organizing cells, which are located below the stem cells, and these cells in turn maintain the stem cells through signaling by an unknown factor. Our laboratory has previously elucidated one aspect of control through live imaging, which cells can de-differentiate to stem cells. This aspect of re-programming cells to become stem cells leads to a very interesting picture, namely that homeostasis of stem cells is a result of fine tuning of respecification events that are controlled by neighboring cell populations, as well as by the stem cells themselves. We have developed population-based models that explore several mechanisms by which cells get respecified from one cell type to the other, which seek to identify core regulatory interactions which can give rise to homeostasis. We have also been developing a 2-dimensional spatial model, which is representative of a longitudinal slice of a confocal section of the shoot apical meristem, in which we track the fate of individual cells. Hence through a modeling approach we seek to identify the consequences of the hypothesized interactions as to how these cell numbers are maintained in a spatially well defined way.

235. Dynamics of two-component systems in cytokinin signal transduction in the shoot apical meristem

Sean Gordon, Vijay Chickarmane, Elliot M. Meyerowitz

The hormone cytokinin is known to be a key activator of several biological processes. The biochemical network that senses cytokinin, and transduces its effect to several target genes, is essentially composed of a two-component signaling network involving histidine kinases, and the type A & B *Arabidopsis* response regulators. The network responds to cytokinin by using several negative and positive feedback loops, ultimately providing a threshold-dependent response to cytokinin concentration. We seek to uncover functional principles of this network, through a theoretical exploration of a computational model. One result that emerges from a mathematical analysis of the dynamics of the network, suggests that the cytokinin perception circuit functions as a multilevel switch, such that at varied threshold levels of the input concentration of cytokinin, we have different stable levels of output. We also model bidirectional phosphorelay transfer that occurs due to both kinase, as well as phosphatase activity, of one member of the cytokinin receptor family that is dependent on cytokinin concentration levels.

236. Activator inhibitor model of plant patterning

Sean P. Gordon, Vijay S. Chickarmane, Elliot M. Meyerowitz

Higher plants maintain continuous development throughout their life by closely regulating the process of cell differentiation. Cell differentiation is partly controlled by hormonal cues, which interface with gene function. Based on characterization of hormone signaling and patterning of gene expression during *de novo* shoot meristem initiation from tissue culture, we propose a novel activator/inhibitor model by which auxin and cytokinin interact to regulate patterning of cell differentiation. In this model, the activity of auxin, the activator of cell differentiation, is regulated by cytokinin, an inhibitor of cell differentiation. Computational models of these interactions lead to self-organized patterning of hormone response and cell differentiation as observed in experiments.

237. Multiple feedback loops through cytokinin signaling control stem cell number within the *Arabidopsis* shoot meristem

Sean P. Gordon, Vijay S. Chickarmane, Elliot M. Meyerowitz

A central unanswered question in stem cell biology, both in plants and in animals, is how the spatial organization of stem cell niches are maintained as cells move through them. We address this question for the shoot apical meristem (SAM) that harbors pluripotent stem cells responsible for growth of above ground tissues in flowering plant. We find that localized perception of the plant hormone cytokinin establishes a spatial domain in which cell fate is respecified through induction of the master regulator *WUSCHEL* as cells are displaced during growth. Cytokinin-induced *WUSCHEL* expression occurs through both CLAVATA-dependent and independent pathways. Computational analysis shows that feedback between cytokinin response and genetic regulators predicts their relative patterning, which we confirm experimentally. Our results also may explain how increasing cytokinin concentration leads to the first steps in reestablishing the shoot stem cell niche *in vitro*.

238. Analysis of LOG gene function in the patterning and maintenance of the shoot apical meristem

Paul Tarr, Elliot M. Meyerowitz

An essential part of plant and animal development is the establishment and maintenance of undifferentiated pluripotent stem cell populations within specialized proliferative tissues called stem cell niches. The stem niche represents a defined anatomical structure that serves as a reservoir of stem cells, which are responsible for the growth, homeostasis, and repair of tissues in an organism.

In *Arabidopsis thaliana*, the two primary stem cell niches reside in tissues called meristems, one in the root and the other found at the apex of the shoot. In the shoot apical meristem (SAM) cell identity is not an inherent cellular property but a function of the positional cues a cell receives in the zone in which it resides. The stem cell niche of the shoot apical meristem is divided into three distinct zones of activity largely defined by their gene expression profiles and rates of cell division. The 20-30 cells in the center of the first two to three cell layers (L1-L3, primarily L1-L2) at the tip of the SAM constitute the Central Zone (CZ) and constitute the pool of pluripotent stem cells and are the ultimate source for all the cells in the above ground parts of the plant. Recently a new family of phosphoribohydrolases, the *LONELY GUY* (*LOG*) gene family, has been characterized in rice (*Oryza sativa*) that are expressed in the CZ and required for SAM maintenance. The *LOG* genes function as cytokinin-activating enzymes, by liberating the isoprenoid N^6 substituted adenine base (the active phytohormone) from the phosphopentose moiety of AMP. Cytokinins are implicated in a host biological processes including control of cell division and shoot initiation. The focus of this project will be on charactering the contribution of LOG produced cytokinin in the CZ on the patterning, specification, and maintenance of the stem cell populations in the SAM of *Arabidopsis thaliana.*

239. The nature of callus tissue in *Arabidopsis*

Kaoru Sugimoto, Elliot M. Meyerowitz

Unlike most animal cells, plant cells have been recognized as totipotent because they can regenerate the full array of tissues in culture. The mechanism behind the totipotency of plant cells is largely unknown. Even the basic questions regarding what differentiation status cells go through during regeneration and whether common processes drive regeneration from different tissues are yet to be answered. We have characterized the initial process of *Arabidopsis in vitro* regeneration, where a pluripotent cell mass termed callus is induced. We first observed fluorescent reporters in callus induced from roots, cotyledons, and petals. Although roots, cotyledons, and petals are distinct in their position, origin, and differentiation in plant development, we find a quite similar reporter expression pattern in the callus derived from each of these tissues: The calli from all three organs differentiate to express root tissue markers and form a somewhat organized tissue like lateral root primordium. As it has been thought that callus is an undifferentiated tissue, this will come as a surprise to the regeneration field. We also verified this up-regulation of root genes in callus derived from the three organs using whole-genome microarrays. By comparison to the previously reported gene expression profiles in various *Arabidopsis* tissues, we conclude that callus resembles the root tip meristem more than other tissues, such as shoot meristems or embryonic tissues, which give rise to new organs in normal plant development. These findings demonstrate that formation of shoot meristems from callus is a trans-differentiation process.

240. The link between callus formation and lateral root formation

Kaoru Sugimoto, Elliot M. Meyerowitz

From the characterization of callus tissue (mentioned in the previous abstract), we find that callus formation resembles lateral root formation. This finding raises the additional questions of whether callus forms under the same genetic controls as lateral root formation in just the same way as a developing root, and if so, which processes are shared between callus and lateral root formation. To answer these questions, we tested the effects on callus formation of mutation or growth conditions that prevent lateral root formation. We find that callus formation reproduces the initial developmental program of lateral roots, but does not continue with the program followed after lateral root emergence. The processes involved in the initial cell division of pericycle cells during the formation of lateral roots occur in callus formation. Although, to date, the term pericycle has only been used to describe a cell layer in the roots of higher vascular plants, our live imaging and mutant analysis suggest that the cells equivalent to root pericycle cells are present around the vasculature of multiple organs throughout the plant body and proliferate and differentiate when callus is formed. From these results we concluded that the ectopic activation of a lateral root development program from pericycle-like cells is the common mechanism of callus formation from various organs. In this case, the pericycle-like cells are functionally analogous to animal tissue stem cells, although the cell types of animal tissue stem cells vary depending on their tissue of origin, and are generally limited to differentiating into the original lineage of cell types.

241. Cell division in the *Arabidopsis* shoot apical meristem

Cory Tobin, Marcus Heisler, Bruce Shapiro

Developmental decisions made by the first layer of cells (L1) in the *Arabidopsis* shoot apical meristem are critical to the plants' above ground architecture. During division, these cells make a decision as to the location of the new wall that will separate the daughter cells. The aim of this project is to create a model that accurately predicts the division plane that will be chosen by any given cell in the L1. Our hypothesis as to which input parameters are important is based on the observation that prior to division a nucleus will send out nuclear-radiating microtubules to each wall in a fashion that minimizes the distance from the nucleus to the wall. One pair of these "spokes" will become the phragmosome and mark the site of the next cell wall. To achieve our goal we are determining the positions of the cell walls and the nuclei using fluorescent protein reporters, fluorescent dyes and confocal microscopy. From these data we are extracting various parameters such as the angle between spokes and the total length of a pair of spokes. These parameters are analyzed

with a neural network to obtain a model that can then be used to anticipate other L1 cells' choice of division planes. The eventual result is expected to be a predictive model tied to known mechanisms of cell growth and environmental perception.

242. Sepal development in *Arabidopsis*: Pattern formation due to stochastic regulation of the endoreduplication decision

Vijay Chickarmane, Adrienne H.K. Roeder, Elliot M. Meyerowitz

In the plant *Arabidopsis thaliana*, the sepal, which is the outermost green leaf-like floral organ, has provided us with a unique opportunity to study the relationship between single cell developmental decisions and the overall patterning of an organ with different cell types. Through a combination of experiments involving confocal imaging and the individual tracking of single cells over time, and computational analysis, we have discovered that the single most important descriptor of the patterning mechanism is stochastic control of the decision of a cell to either divide, or to endoreduplicate (a specialized cell cycle in which a cell replicates DNA, but does not divide). This decision that is inherently probabilistic and is dependent on the history of a cell, determines the range of cell sizes. The earlier a cell enters endoreduplication, the larger it becomes relative to its dividing neighbors. In a local region of the sepal, which is expanding due to proliferating cells, the timing of endoreduplication of cells determines which cells stop dividing, while their neighbors continue to divide. This creates a pattern of different cell types (ploidy). At the global level, in a developmental context, live imaging has revealed that a group of cells at the base of the sepal proliferates, and each daughter cell which emerges enters a replication program: namely, it has a fixed number of cell cycles before cell growth ceases. During this fixed life time, at each round of division, each cell can either enter endoreduplication or continue to divide. In addition to the randomness in the endoreduplication decision, there is a high degree of variability in the cell cycle time of mitotically dividing cells, as well as asymmetries in the divisions, both of which add to the variability in cell size distribution. The results of our combined live imaging and computational approaches reveal that regulation of developmental decisions through control of endoreduplication/cell division, can lead to novel pattern formation.

143. Determining the ploidy distribution of a specific cell type

Adrienne Roeder, Rochelle Diamond, Elliot M. Meyerowitz

Plant cells often undergo a specialized cell cycle called endoreduplication in which they replicate their DNA but bypass mitosis and fail to divide. Thus the cells increase their DNA content or ploidy to varying extents. The DNA contents and inferred endoreduplication states of cells in a tissue are measured through flow cytometry. The standard protocol calls for chopping up the organ, straining the nuclei through a nylon mesh, staining the DNA with propidium iodide, and quantitating of the fluorescence of each nucleus on the flow cytometer. However, there are many different cell types in a single tissue and the differences in endoreduplication between different cell types are completely obscured by this procedure. Furthermore, if there are many nuclei from the wrong cell types, those from the correct cell types can be lost in the noise. For example, if the ploidy of the whole *Arabidopsis* sepal is analyzed, only 2, 4, and 8C cells are detected and the few 16C giant cells of the epidermis are lost in the background. To circumvent this problem, we have labeled the nuclei of interest by expressing a nuclear localized HISTONE 2B-GFP fusion protein under the control of a cell type specific promoter, such as the epidermal specific *ATML1* promoter. Then as before, the tissue is chopped, nuclei are filtered, and DNA is stained with propidium iodide. Now, the flow cytometer records both the GFP and PI fluorescences and gating is used to separate the GFP positive cells of interest from the GFP negative cells. Then PI fluorescences of the GFP positive cells are recorded to determine the proportion of cells in each ploidy. Using this new procedure we have determined that the sepal epidermis contains 1% 16C giant cells, 6% 8C cells, 32% 4C cells, and 61% 2C cells.

244. Asynchronous cell cycles contribute to cell size variability in *Arabidopsis* sepals

Adrienne Roeder, Alex Cunha, Vijay Chickarmane, Cory Tobin, Elliot M. Meyerowitz

As biologists we can use computational models to sharpen our hypotheses. We have made a model to predict how the timing of cell division determines the cell size pattern in the sepal epidermis. The basis for the model was the observation by Melaragno *et al*., that cell size is proportional to DNA content and the corresponding hypothesis by Traas *et al.*, that the ultimate size of a cell is controlled by the time at which it undergoes the specialized endoreduplication cell cycle in which it replicates its DNA without dividing (Melaragno, Mehrotra *et al.*, 1993; Traas, Hulskamp *et al*., 1998). Our model produces a pattern of cell sizes with four distinct categories, one for each ploidy. To test whether this pattern matches the *in vivo* sepal, we used semi-automated image processing to determine the cell size distribution of mature sepals. Mature sepals expressing an epidermal-specific plasma membrane marker were imaged to show the cell outlines. The plasma membranes were automatically segmented using an edge detection method in combination with mathematical morphology. The segmented membranes had some errors often due to background in the images, which were corrected by hand before the areas were measured. The histogram of extracted cell areas shows that the *in vivo* sepal has a wide range of cell sizes, not four distinct classes. DNA content appears to set the mean cell size, but cells within that DNA content vary around the mean. To identify the source of variability, we examined the cell division patterns of cells

in the sepal as determined by live imaging experiments. We observe that the cell cycles are highly asynchronous and there is a wide distribution of cell cycle times. The cells with longer cell cycles grow more in the interval, are larger when division occurs, and consequently produce larger daughter cells. We also observe that cells do not divide precisely in half adding to the variability of daughter cell sizes. Quantitating these two phenomena and adding them to the model produces a wide distribution of cell sizes similar to the *in vivo* sepal. We conclude that asynchronous cell cycles and unequal divisions are largely responsible for the variability in cell sizes in the sepal.

In collaboration with: Boguslaw Obara and B.S. Manjunath, UC Santa Barbara

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245. CYCLIN D3;2 promotes formation of smaller cells in the sepal

Adrienne Roeder, Lisa Yee, Elliot M. Meyerowitz

How does regulation of the cell cycle contribute to pattern formation? We have previously shown that the cell size pattern in the sepal epidermis determined primarily by the time at which cells enter endoreduplication. The *LOSS OF GIANT CELLS FROM ORGANS* (*LGO*) gene encodes a cell cycle inhibitor in the SIAMESE family that promotes the early entry of pavement cells into endoreduplication to produce giant cells. The *lgo* mutant lacks giant cells whereas constitutive ectopic expression of *LGO* promotes additional giant cell formation in the sepal. To determine how LGO interacts with cell cycle regulators to promote endoreduplication, we assayed the interaction through Bimolecular Fluorescence Complementation (BIFC) in a transient tobacco expression system. In this system, LGO can interact with CYCLIN D3;2, CYCLIN 3;3 and CDKA;1 suggesting that LGO may inhibit CYCLIN D3 activity. Similarly, the related protein SIAMESE has been shown to interact with CYCLIN Ds and CDKA;1 (Churchman, Brown *et al.*, 2006). CYCIN D3s have been shown to promote mitotic division instead of endoreduplication (Dewitte, Scofield *et al*., 2007). To test whether expression of CYCLIN D3;2 is sufficient to promote division and inhibit endoreduplication, CYCLIN D3;2 was expressed ectopically throughout the epidermis. The sepals of these CYCLIN D3;2 overexpression plants had fewer giant cells or were entirely lacking giant cells similar to *lgo* mutants. We conclude that CYCLIN D3;2 promotes the formation of small cells by driving mitosis where as LGO promotes giant cell formation and endoreduplication by inhibiting CYCLIN D3s.

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246. Dissection of flower initiation pathway using genetic and genomic approaches

Wuxing Li

Understanding of the regulation of fate determination and patterning in organisms including plants and animals requires insight into their genetic regulatory networks. The shoot apical meristem in *Arabidopsis* provides an excellent system to study processes in cell division, cell differentiation, and cell fate determination. Knowledge of the regulation of flowering processes is of significant agricultural importance and also answers fundamental questions in biology. I propose to systematically characterize the process of floral transition utilizing both genetic and genomic approaches. Transcriptional networks will be examined during the floral transition using expression profiling and dynamic changes in patterns of global transcription will be analyzed. Genes that either promote or repress floral transition will be incorporated to build mathematical models to explain the floral transition process. Chromatin status during this process will also be examined and the involvement of genes, either the regulators of chromatin status, or genes that whose expression is regulated at the chromatin level will be determined.

247. Genetic studies of the expression pattern of LEAFY

Wuxing Li

Recent studies have identified an *Arabidopsis* gene *LEAFY* as a master regulator in specifying floral meristem identity. I propose in detail characterization of this gene: (1) factors that control the temporal and spatial pattern of *LEAFY* expression; (2) upstream components of *LEAFY* function; and (3) genes that interact genetically or biochemically with *LEAFY*. The expression pattern of *LEAFY* has been observed in different mutant backgrounds and an auxin function-related gene was found to potentially regulate *LEAFY* spatial expression. Through a mutagenesis study of a weak *lfy-5* allele, I have obtained several putative genetic modifiers, and further characterization of the interaction between *LEAFY* and these modifiers is providing new information toward understanding the floral initiation pathway. In addition, the function of chromatin status regulatory components in the flower transition and floral organ development is being investigated by genetic and genomic approaches. It is also expected that yeast hybrid experiments would elucidate new components in the pathway of LEAFY function. This research will advance our understanding in fundamental questions such as cell fate determination and will impact on research in other multicellular mechanisms such as humans.

248. Boundary specification and maintenance between the shoot apical meristem and organ primordia

Xiaolan Zhang, Elliot M. Meyerowitz

Boundaries serve the purpose of delimiting regions of gene activity and of separating distinct organs as they develop. M-O boundaries (meristem-organ) are formed to separate plant organ primordia from the shoot apical meristem (SAM), whereas O-O boundaries (organ-organ) develop between individual floral organs to create space between them. Loss of boundaries can result in abnormal organ fusion, failure of SAM initiation and defects in SAM maintenance. However, little is known of when and how the boundaries are specified and maintained during plant development. There are three hypotheses for M-O boundary formation: (1) cells that downregulate the meristematic marker *SHOOT MERISTEMLESS (STM)* immediately acquire boundary identity, which acts as a barrier allowing their progeny to gain organ primordial identity; (2) juxtaposition of *STM* and primordial marker *ASYMMETRIC LEAVES1 (AS1)*-expressing cells is required to form a boundary; and (3) *STM* and *AS1* expression transiently overlap, and the overlapping cells become the boundary. To test above hypotheses, fluorescent tags were used to label cells expressing STM (*pSTM::STM-VENUS*), AS1 (*pAS1::AS1-CFP*) and the boundary gene *LATERAL SUPPRESSOR (pLAS:LAS-GFP*) simultaneously, and tracked their expression dynamics during new primordium initiation using live imaging. I found that new primordia are first marked by weak AS1 expression, and then upregulation of STM directs new boundary formation. In addition, transient overexpression of STM leads to aberrant boundary specification, while AS1 ectopic induction has no obvious effect. Similar experiments will be performed using inducible inactivation lines *(p35S::STM ds RNAi-GR, p35S::AS1 ds RNAi-GR* and *p35::LAS ds RNAi-GR)* to validate results from the above experiments. The effect of plant hormones on boundary formation has also been explored. Despite both cytokinin and auxin controlling the position and the shape of boundaries, cytokinin leads to more severe defects when applying with the same concentration as auxin. Future research directions will focus on the mechanism of how boundaries are maintained, specifically, the relationship between LAS, STM and cytokinin during plant development.

249. Exploration the mechanism of how HANABA TARANU affects floral development and SAM organization

Xiaolan Zhang, Elliot M. Meyerowitz

HAN (*HANABA TARANU*) encodes a GATA transcription factor that is expressed at the boundaries between meristem and organ primordia, and at the boundaries between different floral organs. *HAN* knockouts display small and flat SAMs, fused sepals and reduced numbers of floral organs, whereas *HAN* overexpression leads to delayed plant growth, disturbed cell division and lose of meristem activity. However, it is completely unknown how boundary-expressing *HAN* affects floral development, SAM organization and cell division. In order to understand the underlying mechanisms, we first observed the dynamic change of *WUSCHEL (WUS)* and *CLVATA3 (CLV3)* upon HAN induction using *pWUS::GFP-ER*; *pCLV3::mGFP-ER* in a *p35S::HAN*-GR line. The WUS domain is greatly expanded after 3 days of DEX treatment, the same phenomenon as shown in *han* mutant lines by *in situ* hybridization. We next performed time-course whole genome oligonucleotide microarrays using floral buds after 4 hours, 12 hours and 3 days of DEX treatment. Our data indicated that induction of HAN causes repression of HAN itself and three additional HAN family genes (At4g36620, At4g26150 and At5g56860), and this repression has been verified either by real-time RT-PCR or by Western blotting. Double, triple and quadruple mutants of HAN and HAN family genes are in progress to try to recapitulate the *HAN* overexpression phenotype, and ChIP-PCR and BiFC will be performed to see whether the transcriptional and protein interactions of HAN and its relatives are direct. In order to identify the protein location, translational fluorescent tags and immunolocalization of HAN are in progress. Additionally, the genetic interaction of HAN with other boundary genes and ten known genes involving in meristem or organ formation will be explored by making double mutants, and reverse genetic analysis of 50 candidates from microarray data are in progress to screen for important downstream targets of HAN.

250. Next generation cellular resolution profiling of the transcriptome and epigenome

Yuling Jiao

We are in the process of developing and applying methods to sequence translating mRNA, miRNA, and active chromatin from specific cell types. The principal deficit in current microarray and chromatin immunoprecipitation experiments on *Arabidopsis* shoots, including our own, is that the RNA and DNA used comes from a mixture of different cell types. As cell type-specific data are necessary to infer gene regulatory networks, current types of data are inadequate to understand a complex organ. Using developing flowers as the system, we will adapt existing methods for expressing epitope-tagged ribosomal proteins, will develop new constructs and methods to drive cell-type-specific expression, and will apply methods we have developed for obtaining large amounts of floral tissue at defined developmental stages, followed by immunoprecipitation of polysomes, to perform cell- and stage-specific mRNA-seq analysis of different floral cell types at multiple critical developmental stages. Progress to date with cell types defined by expression of the AP1 and AP3 genes is promising.

251. Translating profiling reveals post-transcriptional regulation of the transcriptomes

Yuling Jiao

Our understanding for post-transcriptional regulation has lagged behind our knowledge for transcriptional control, especially at the genome scale. By deep sequencing of polysome-binding mRNA in *Arabidopsis*, we were able to identify post-transcriptional regulation at the intron-splicing level and at the translational level. We found that intron splicing is regulated by several sequence features and, U2-type splicing is clearly more efficient than U12-type splicing. Moreover, splicing efficiency significantly affects mRNA abundance. A large variation in translation efficiency was found in genes of various functions. Our analysis suggests that coding-sequence mRNA folding and codons at translation initiation sites influence translation. Finally, we identified novel translated mRNAs, which were previously un-annotated or annotated as non-coding RNA.

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Summary: The Rothenberg group studies the gene regulatory basis for development of T lymphocytes from hematopoietic stem cells. We are most interested in the early stages of this process, when initially multipotent cells gradually discard other developmental options and commit to a fate as some kind of a T cell. Although T-lineage signaling and receptor genes become activated in this process, commitment is essentially complete before the T-cell receptor for antigen is assembled and before T-cell receptor signaling can itself participate in developmental choices. The regulatory state of the cells is highly dynamic throughout this process, with stepwise silencing of a large cohort of stem-cell-inherited regulatory genes accompanied by stepwise induction of a select group of T-lineage regulatory genes. We are trying to determine what the key factors are those that push the cells forward to a T-cell fate and exactly how they exert this impact at a molecular level.

For at least five years, the lab has focused mostly on two transcription factors acting in this process, PU.1 and GATA-3, and on their interaction with environmental signaling through the Notch/Delta pathway that is essential for T-cell commitment. We have formalized the regulatory relationships involving this trio of factors in a gene regulatory network model that has recently been published and is available on our lab web site. In the past year, however, there have been two new areas of emphasis. One is on a new transcription factor that appears to have a vital role in this process, the bifunctional zinc finger factor Bcl11b. The other area of new emphasis has been mapping of potential regulatory sites throughout the whole genome that are "in play" during the T-lineage specification process. This has been carried out by deep sequencing of DNA enriched by chromatin immune precipitation for particular histone modifications, through collaboration with the Barbara Wold lab and with Ali Mortazavi in the Sternberg lab.

Bcl11b is turning out to be one part of the answer to a central enigma of early T-cell development, namely, what enables the cells at the key point of commitment to shed their stem-cell regulatory inheritance. This factor appears to play an essential role in the precisely staged repression of many progenitor-cell regulatory genes. As a factor that is sharply upregulated just before commitment but then sustained throughout T-cell differentiation into maturity, it may have a number of different roles as the developmental context changes. However, this early role supplies a logically needed function in our gene regulatory network that had lacked a specific molecular candidate until now. It is also of considerable interest that Bcl11b is a tumor suppressor, since it may regulate a shift in growth control at this early point that is needed to prevent malignant transformation.

The genome-wide mapping of regulatory sites is proving to be a robust and deeply informative way to track a developmental progression as it is "felt" at the genome level. Different modes of positive and negative regulation are vividly revealed through shifts in post-translational histone modifications at promoters and at intronic and distal potential enhancer sites. This approach is particularly informative in light of the data we have already accumulated about the expression patterns of >100 different genes of interest throughout the progression to commitment, since these benchmark loci now provide a key to relate expression to chromatin marks. New potential regulatory candidates of interest emerge, as a few "new" genes are identified with highly stage-dependent patterns of modification. These features stand out clearly against the remarkably steady and reproducible background patterns of marking at the overwhelming majority of genes at all stages in the process. We are now using a number of approaches to determine how we can use these data to identify sites of specific regulatory inputs, both known (e.g., PU.1 and GATA-3 binding sites) and as yet unknown. We are also collaborating closely with a group led by Dr. Hamid Bolouri, who are applying a battery of computational approaches to our data to clarify the network relationships of all genes under active regulation during T-lineage commitment.

At the same time, we are moving forward with our understanding of the competitive and collaborative roles of PU.1 and Notch signaling in early T-cell development, and in our fine-mapping of the precise timing and molecular context of lineage commitment. These projects continue in the context of our other broader examinations of lymphocyte development and evolution. A distinct research program in our group, run by Dr. Mary Yui, continues to address the genetic and molecular basis

for the derangements in T-cell development that may underlie a predisposition to autoimmunity in a mouse model of insulin-dependent diabetes. This project has already shed light on an unexpected difference in programming of αβ and γδ T-cell subsets in the autoimmune-prone animals that maps back to the earliest stages of commitment. In our most ambitious look at the broader context of lymphocyte development, we have also continued to learn about the regulatory genes that control immune receptor gene expression in the most basal vertebrate with true adaptive immunity, the lamprey.

252. Transitional stages in T-lineage specification and commitment

Mary Yui, Ni Feng

T-cell development from bone marrow derived progenitors is marked by the loss of alternative lineage choices accompanying specification and commitment to the T-cell lineage. The developmental sequence is defined by changes in a series of cell surface markers, which subdivide these cells into CD4/CD8-double negative (DN) cell populations (DN1, DN2, DN3, DN4). Although much is known about these processes, the precise timing and order of events is not clear. To more precisely define the sequence of events and to correlate them with gene expression changes we have further divided the DN2 stage into c-Kit-high and c-Kit-intermediate populations (designated DN2a and DN2b respectively). We sorted these populations and (1) cultured them in the absence of Notch signals to assess their non-T-cell developmental potential; (2) cultured them in the presence of Notch signals to determine their αβTCR vs. γδTCR T-lineage choices; and (3) extracted RNA for quantitative real-time PCR analysis of gene expression changes. When placed in culture with cytokines and OP9 stromal cells lacking Notch ligands, we found that dendritic cell (DC) potential is greatest in the earliest DN1 T-cell progenitors, declining 10X in the DN2a cells, while natural killer (NK) cell potential is maintained in DN2a cells. However, the ability of early T-cell precursors to divert to both lineages is essentially lost by the DN2b stage. Under culture conditions with DL1 Notch ligand signals, DN2a cells are predominantly bipotent, individual cells capable of generating both γδT and αβT cells, while DN2b cells are more likely to be committed to the αβT-cell lineage, although a significant number of these cells remain bipotent. We have also assessed the major gene expression changes in T-cell and non-T cell genes through this time. Notably, our results suggest that loss of alternative lineage fates precedes the full upregulation of most T-cell genes characteristic of the DN3 gene expression pattern. Furthermore, we found that the transcription factor PU.1, which is required for DC cell differentiation and must be turned off for T-cell commitment, changes little from DN1 to DN2a and declines rapidly between the DN2a and DN2b stage, suggesting that PU.1 expression is not the sole rate-limiting factor for maintaining the dendritic cell potential of these progenitor cells, while its loss correlates with T-cell commitment.

253. Regulatory mechanisms underlying T-cell specification in haematopoietic progenitor cells: key nodes characterized by single-cell time-lapse imaging

Hao Yuan Kueh

Haematopoietic progenitor cells maintain plasticity to alternative developmental fates during early stages of T-cell development, but eventually decide to exclude alternate fates and commit to a T-cell fate. Commitment involves a regulatory network of interacting signaling pathways and transcription factors that integrates differentiation signals, decides between alternative cell fates, and maintains stable expression of specific genes after a fate decision has been made. Feedback regulatory interactions within gene regulatory networks are thought to play a central role in cell fate specification and commitment; however, it is still unclear how specific regulatory interactions function to enforce stable commitment of haematopoietic progenitors to the T-cell fate.

In this project, we aim to elucidate the architecture of a key nexus in the gene regulatory network underlying T-cell specification and commitment. Our general approach will be to observe single haematopoietic progenitors undergoing T-cell development *in vitro* using timelapse imaging. We have been able to track these progenitors and their descendants throughout the period required for commitment to the T lineage *in vitro* in cocultures with OP9-DL1 stromal cells. We plan to measure the expression dynamics of key T-cell regulatory genes in the network, and determine how these regulatory genes respond to changes in Notch signaling, a key instructive signal for T-cell development, and to perturbations of regulatory genes in the network. Specifically, we will measure the expression dynamics of two important T-cell transcription factors Bcl11b and PU.1: Bcl11b is sharply up-regulated during early T-cell development and plays a key role in promoting T-cell specification, whereas, PU.1 is sharply down-regulated during development and inhibits expression of many T-cell genes when present. We will use transgenic PU.1-GFP reporter mice developed by Stephen Nutt and colleagues, and are currently constructing a fluorescent Bcl11b expression reporter mouse strain (see accompanying report). Using these fluorescent reporters, we will determine how the expression of Bcl11b or PU.1 changes in individual cells in response to Notch signaling, or to network perturbations. In particular, we can test whether Bcl11b (or PU.1) participates in a regulatory feedback loop by over-expressing it, or knocking it down, and then determining the effects on the expression of its own locus. We can also test for bi-stable behavior generated by feedback regulatory interactions using hysteresis experiments, which involve measuring the response of a system to the addition and subsequent withdrawal of a signal such as Notch-Delta interaction.

To gain further insight into experimental observations, we will use mathematical modeling to investigate the dynamic behavior of regulatory networks with different feedback architectures. Such models will enable us to impose constraints on network architectures and parameter regimes, and allow us to generate quantitative predictions that are testable by imaging experiments. These mathematical models will also allow us to gain insight into more complex regulatory interactions whose dynamic behaviors may not be readily predicted from intuition alone. In particular, currently hypothetical regulatory interactions in the T-cell specification network involve inter-locking feedback loops, as well multiple connections from a single source with opposite signs. We will use mathematical modeling to understand these regulatory interactions and connect them to experimental observations.

254. Bcl11b is an enforcer of T-lineage commitment

Long Li

Hematopoietic stem cells commit to the T-lineage by gradually losing stem-ness and gaining T-cell identity. A recently discovered transcription factor Bcl11b is expected to be a key regulator in this process because of its unique expression pattern. The expression of Bcl11b is restricted to the T lineage, and its mRNA level increases dramatically around the time that HSCs commit their fate to T lineage. Its expression pattern is opposite to that of PU.1, which roughly correlates with potential to differentiate into DC and myeloid lineages, and to that of other stem cell genes encoding transcription factors such as SCL, Id2 and GATA2. It is known that Bcl11b is crucial for αβ T-cell development and for survival of double-positive cells specifically. However, little is known about how Bcl11b works in the very early stages of T-cell development.

We have recently adapted an *in vitro* growth and differentiation system that enables us to investigate the critical role of Bcl11b in this process. We have been able to use retrovirally transduced Cre to infect cells from a conditional Bcl11b knockout mouse model to study synchronized T-cell development from Bcl11b-/- hematopoietic progenitor cells. We have found that under certain conditions Bcl11b-deficient pro-T cells fail to pass the DN2/DN3 transition, which is linked to the lineage commitment step in T-cell development. Instead, Bcl11b-deficient cells continuously grow at DN1-like and DN2A-like stages for more than twenty days, while wild-type controls develop normally to DN3 and DN4 cells. Bcl11b-/- progenitors may develop to cells resembling DN2B pro-T cells. However, when these mutant DN2B cells were sorted and recultured, they again generated DN1 and DN2A-like populations of cells in this condition. Unlike their wild-type counterparts, the long-term Bcl11b-deficient cells still possess developmental plasticity with an NK-like population growing out from the cells. Gene expression profile reveals that Bcl11b-deficient cells express T-cell identity genes including *Cd3e* and *Lck,* and continue to silence some important genes associated with major developmental alternatives, such as B-cell factors; however, they fail to turn off stem cell genes and some alternative lineage-regulators, such as *Scl, Lyl1, Hoxb4, Gfi1b, Id2, Tbx21, Il2rb, Zbtb16, Sfpi1* and *Bcl11a*.

These results indicate that Bcl11b controls the correct exit of T-cell precursors from a stem cell state. No other factor has been shown to have this distinctive function. Our research suggests that Bcl11b plays a major role in T-lineage commitment.

255. Role of the transcription factor PU.1 in early T-cell development

Ameya S. Champhekar, Ellen V. Rothenberg

T-cell development progresses through a step-wise process in which precursor cells sequentially lose their ability to differentiate into other cell types and finally commit to T-cell lineage. Precursor cells that enter the thymus progress through the so-called double negative stages (DN1-4) and in the process become committed to the T lineage between the DN2 and DN3 stage of development. A number of transcription factors participate in this process and are important for T-cell development to proceed smoothly. PU.1, a member of the Ets family of transcription factors, is expressed in T-cell precursors that enter the thymus, and previous work from our lab has shown that its expression must be shut off by the DN2 stage for T-cell development to proceed normally. However, PU.1 function may also be required until as late as the DN2 stage, for germline knockout mice have severely reduced thymocyte numbers (> 30 fold reduced as compared to wild-type thymi) and display slower progression through T-cell development as compared to wild-type cells. Development of B cells and myeloid lineages is also completely blocked in the absence of PU.1. Since most published studies used a germline knockout model of PU.1 it is not clear whether the abnormal T-cell development observed in these mice was a result of lack of PU.1 function after these cells entered the T-cell pathway or due to a defect in a precursor cell. To study the role of PU.1 once precursor cells have entered the T-cell pathway, we chose to delete PU.1 from E14.5 c-Kit^{+} CD27^{+} fetal liver precursors immediately before they are primed to enter the T-cell pathway. This was done by infecting FL cells carrying PU.1$^{fl/fl}$ alleles with Cre containing retroviruses and then sorting c-Kit^{+} CD27^{+} cells after two days of culture. The sorted cells were then cultured for an additional 10 days and assayed for progression through early stages (DN1-4) of T-cell development at 2-day intervals. Our results indicate that, starting at day 4 of culture, on any given day fewer cells progress through the early DN1 to DN2 progression in the absence of PU.1. Also, cells that have deleted PU.1 are lost from the culture faster than wild-type Cre-expressing cells, hinting at a role of PU.1 in early T-lineage progenitor expansion or maintenance. Future experiments will be aimed at studying the changes in gene expression patterns in wild type versus cells lacking PU.1 to delineate the function of PU.1 in the early stages of T-cell development.

256. Modulation of the transcriptional impact of PU.1 by Notch signaling

Marissa Morales Del Real

T-cell development is dependent on the combinatorial expression of transcription factors and environmental signals. Early pro-T cells retain lineage plasticity and can be diverted to myeloid/dendritic and NK lineages by the enforced expression of certain transcription factors, including PU.1. PU.1 expression is necessary for early T-development, but enforced expression of PU.1 beyond the DN2 can divert cells from the T-lineage to the myeloid lineage. However, the ability of PU.1 to cause a block in T development and diversion to the myeloid fate is attenuated by Notch signaling, which modifies the activities of PU.1 so that T-lineage fidelity can be conserved. Elucidating the mechanism by which Notch can mediate this modulation can identify key players in the early decisions of pro-T cells to restrain diversion even while the cells still require high expression levels of PU.1.

There are several possibilities as to how Notch signaling could be modifying the activity of PU.1 such that pro-T-cells retain lineage fidelity. The first set involves changes to the PU.1 protein itself including degradation, modifications that would interfere with PU.1's ability to bind to co-factors or gene regulatory regions, and cellular localization. So far the question of PU.1 degradation has been addressed by Western blots and intracellular staining detecting the abundance of PU.1 protein in sorted cells from a pro-T-cell line, with and without forced expression of PU.1, in the presence and absence of a Notch signaling inhibitor. Results from the Western blots and intracellular staining suggest that the effects of Notch signaling are not driven by PU.1 protein degradation or modification. The question of cellular localization of PU.1 in samples cultured in the presence or absence of Notch signaling still needs to be addressed.

The cell line model system reveals that different responses can be elicited by the same, or similar, levels of PU.1 even in cells of the same clonal background. This confirms that some additional regulatory input, or removal of an inhibitory input, is required for PU.1 to alter the identity of the cells. Our results in this system show that one of the features that consistently distinguish cells that do switch fates in response to overexpression of PU.1 is a general depression of Notch pathway-dependent genes. The effect in the cells beginning diversion in response to PU.1 is more severe than in control cells treated with strong doses of a direct Notch signaling inhibitor. This suggests that PU.1, under some circumstances, can reciprocally antagonize Notch signaling. We are now seeking to determine the pathway through which PU.1 interacts with the Notch pathway in both the cell line system and in primary T-cell precursors.

257. Extreme GATA-3 dose dependence during early T specification

Deirdre D. Scripture-Adams, Koorosh J. Elihu

Commitment to the T-lymphocyte lineage, defined by loss of the ability to generate other hematopoietic lineages, occurs after many cell cycles in the thymus but is completed by the $CD4^-$ $CD8^-$ $CD44^{low}$ $CD25^+$ (DN3) stage of T-cell development. GATA-3 protein is specifically upregulated to its highest levels during commitment. We used RNA interference technologies to downregulate GATA-3 protein at specific stages of T-cell differentiation *in vitro*. Although GATA-3 protein and RNA expression was only reduced by 2-4 fold, we found that sub-optimal GATA-3 levels produced at least two powerful blocks in T-cell development. The first was a defect in survival or expansion at the earliest, DN1 stage, while a second, separable effect was to arrest the differentiation of the cells at the DN2 stage. The block to T-lineage developmental progression was cell-autonomous and was not bypassed by a Bcl2 transgene. The DN2-stage developmental progression block was also mimicked by using retroviral transduction of Cre to induce deletion of a floxed *Gata3* allele in precursors that then underwent abortive differentiation *in vitro*. This same modest reduction of GATA-3 caused by RNA interference also resulted in specific upregulation of PU.1 in T-lineage precursors, against a background of otherwise relatively normal T-lineage gene induction. This suggested a role for GATA-3 in a pathway involved in PU.1 repression that could also be implicated in lineage commitment itself. To examine the roles of GATA-3 in exclusion of alternative lineages, we used retroviral transduction to force expression of obligate repressor and tamoxifen-inducible GATA-3 forms in fetal liver precursors that were cultured under B-cell and myeloid lineage-permissive conditions. These results showed that GATA-3 blocked B-cell development under conditions which were still permissive for myeloid development, i.e., at lower doses of GATA-3 and when GATA-3 is converted to an obligate repressor. Thus, GATA-3 is not only required for at least two phases of early T-cell progression to commitment, but also it restricts two different lineage alternatives by distinct mechanisms.

258. *cis*-Regulatory analysis of the murine PU.1 gene

Mark Zarnegar

Differentiation of hematopoietic stem cells into committed T cells is a highly regulated process whereby sequence specific transcription factors drive differentiation through activation of lineage specific developmental programs and by restricting or repressing alternative developmental choices. PU.1, an Ets family transcription factor, has been shown to be an essential regulator of multiple cell fate decisions, and is differentially regulated in the hematopoietic compartment. Critically, PU.1 must be present in early T cells, but silenced at the correct stage to facilitate T-cell development. We set out to identify novel regulatory elements contributing to PU.1 expression

in multiple lineages with the specific goal of elucidating the mechanism by which PU.1 is shut off in pro-T cells.

We have identified multiple novel *cis*-regulatory elements. Some of the regulatory sequences have been revealed as lineage-specific enhancers contributing to high expression of PU.1 in myeloid cells. We are investigating what factors bind to these enhancers to drive high PU.1 expression. Through the use of ChIP assays with cell lines of multiple hematopoietic lineages, specifically myeloid, B, and T cells, we have observed cell type-specific occupancy of the *cis*-regulatory elements by members of the Ets family. Most intriguingly, PU.1 itself can bind to most *cis* elements in myeloid cells, a lineage in which PU.1 is thought to be autoregulatory, but PU.1 is unable to occupy the same *cis* elements in B cells or PU.1-transfected T cells. Differences in PU.1 expression levels or differences in chromatin modifications are not thought to be sufficient to explain the inability of PU.1 to bind regulatory regions in non myeloid cells. Instead, evidence suggests context-dependent recruitment mechanisms exist to recruit PU.1 to *cis* elements, possibly with differing mechanisms in place not only between lineage types, but also within a given cell type for specific recruitment to particular *cis* elements. What these recruitment mechanisms are remains to be discovered.

A novel *cis*-regulatory element capable of silencing reporter activity in DN3 like pro-T cells, the stage coincident with shutting off PU.1 in developing thymocytes, was also discovered in our regulatory analysis. We have dissected this silencing element and discovered numerous sites upon which silencing activity is dependent. Most of these sites have been shown to be non-consensus Runx target sequences. Multiple nonconsensus sites appear to be used as a quantitative sensor of Runx1 protein levels, making pro-T cells sensitive to the dynamic regulation of Runx1 itself. Sensitivity to Runx1 protein levels may also be a critical aspect of Runx1-mediated silencing of other developmentally regulated T-cell genes, including Rag1/2, Th-POK, and CD4. We have shown that Runx1 is able to bind the PU.1 silencer sites *in vivo* and that Runx1 is essential for the stage specific silencing of PU.1. However, while Runx1 is necessary for turning off PU.1, it may not be sufficient. Work continues in order to discover accessory factors that aid Runx1 in terminating PU.1 expression in developing pro-T-cells.

259. Determining the transcriptional regulation of *Bcl11b* in T-lineage commitment

Long Li, Hao Yuan Kueh, Ruzbeh Mosadeghi

The expression pattern of Bcl11b is unique: it is silent in HSCs, B cells and myeloid cells; it is activated in DN2A pro-T cells and up-regulated during the transition from DN2A to DNB. The timing of its activation and up-regulation coincides with T-lineage commitment. It is the only gene we have found so far that showed such a developmental stage-specific, as well as T-lineage specific expression pattern. Understanding of the inputs that activate Bcl11b in early DN2 cells will dramatically increase our knowledge of T-lineage commitment.

We have so far mapped DNA methylation of the promoter region of Bcl11b using bisulfite-DNA sequencing. Apparently, there is an unmethylated window from -900 to -300 bp with respect to the transcriptional start site of the *Bcl11b* locus, both in Bcl11b expressing P2C2 cells (DN3 like pre-T cells) and in Rag1 knockout thymocytes. We have made several promoter-luciferase reporter constructs, but stable transfections of P2C2 cells and Raw264.7 cells (myeloid cells) with these constructs were unable to recapitulate Bcl11b expression.

A genome-wide mapping of the chromatin modifications in pro-T cells is currently being performed in our lab by Jingli Zhang (see below). This study has identified a genomic region >500 kb downstream of the Bcl11b locus that shares the same stage-specific chromatin modifications as the Bcl11b promoter. Clinically, the region is involved in activating genes that translocate to *Bcl11b* locus in a subset of T-acute lymphocytic leukemias. Based on this evidence, we hypothesize that this downstream region could participate in regulating Bcl11b expression. To test this hypothesis, we have first used chromatin conformation capture (3C) to map physical interaction between Bcl11b and the downstream region. The results revealed a T cell-specific interaction between the region and Bcl11b promoter/exon1, suggesting that our hypothesis might be true. We then cloned the region to a Bcl11b promoter-luciferase construct and stably transfected P2C2, EL4, Raw264.7 and NIH3T3 cells with the construct. Preliminary results are encouraging because the downstream region showed enhancer activity in P2C2 and EL4 cells.

To study *cis*-regulatory elements within this downstream genomic region, and to further understand the transcriptional regulation of Bcl11b in general, we are currently developing fluorescent Bcl11b expression reporters in haematopoietic cells using two complementary strategies: 1) We are making transgenic mouse strains with either an insertion of IRES-mCitrine (or mCherry) into the endogenous Bcl11b 3'UTR, or else a replacement of Bcl11b exon1 with mCitrine (or mCherry). The former acts solely as an expression reporter, whereas the latter also acts concurrently as a gene knockout. We are testing preliminary constructs with both fluorescent protein reporters. 2) We are transfecting into various haematopoietic cell lines a bacterial artificial chromosome (BAC) containing the Bcl11b locus with an mCitrine insertion, as well as a knock-in of the putative downstream regulatory region. We will see whether this BAC recapitulates T cell-specific Bcl11b expression. If so, we will map out *cis*-regulatory elements within the downstream region by modifying its sequence and measuring subsequent reporter expression levels. To make these DNA constructs, we will modify a BAC containing the Bcl11b locus using recombineering, a molecular biology technique involving homologous recombination in *E. coli*. We have inserted fluorescent protein cassettes into the Bcl11b BAC using recombineering, and will further

perform recombineering on these fluorescent reporter-containing BACs to insert the downstream regulatory region as a starting point for *cis*-regulatory studies, and to retrieve the fluorescent reporter-containing region into a gene-targeting vector for making the Bcl11b-reporter transgenic mouse.

260. Genome-wide mapping of histone modifications in early T-cell development

Jingli Zhang

T-cell-lineage commitment is a process of steadily acquiring T-cell characteristics and gradually reducing of other lineage potential. While DN1 and DN2 cells retain the potential to develop into DCs or NK cells, DN3 cells are fully committed to be T cells. Genetic expression studies demonstrate that, as cells progressing from DN1 to DN3 stages, up-regulation of T-cell lineage-specific genes is tightly coupled with down-regulation of genes of alternative fates. How this regulation is prosecuted genome-wide remains unclear.

Histone modifications are major epigenetic factors influencing gene expression. Among the different histone modifications, Histone 3 (H3) Lysine 9 and Lysine 14 acetylation (AcH3) is known to be coupled with open chromatin and active gene transcription, while Polycomb-associated H3K27 trimethylation (H3K27me3) promotes a condensed chromatin structure, therefore is closely linked with transcriptional silencing. Dimethylation on H3K4 (H3K4me2) has been shown to correlate with accessibility or competence for transcriptional activation, whether or not the gene is actually being transcribed. The different combinations of these three chromatin marks can provide insights into molecular mechanisms that determine how genes are regulated at different developmental stages.

We use ChIP-Seq (chromatin immuno-precipitation combined with high throughput sequencing), in collaboration with Ali Mortazavi, members of the Wold lab, and the Millard and Muriel Jacobs Genetics and Genomes Laboratory, to survey stage-specific genome-wide epigenetic modifications as they change during T-lineage specification. We analyze genome-wide distributions of histone modifications in purified DN1 and DN3 cells that have been differentiated *in vitro* from fetal liver cells (FLDN1 and FLDN3, respectively), and in DN3 and DP cells purified from adult mouse thymus (ThyDN3 and ThyDP, respectively). There is a major developmental event, β-selection, occurring between DN3 stage and DP stage, that may be important to "confirm" lineage commitment, and thus, including DP in our study may help us better understand the full trajectory of histone modifications along the developmental process. We have obtained 10-15 million successful reads per histone marker from each population and the results from independent biological replicates give highly concordant patterns.

Our preliminary data largely agree with the available gene expression profiles and provide additional insight. For example, in FLDN1 cells we know that B-lineage developmental potential is already lost whereas some myeloid potential remains. In these cells, whereas two B-cell-specific regulatory gene loci, *Pax5* and *Ebf1*, are "closed" with close-to-background levels of AcH3 and H3K4me2 and highly enriched H3K27me3, the myeloid-specific *Cebpa* locus is still open but "poised" for expression or closure with all three markers being enriched. On the other hand, corresponding with its expression pattern, the locus of T lineage-specific gene Bcl11b is marked with enriched H3K27me3 in FLDN1 cells but with enriched AcH3 and H3K4me2 and a loss of H3K27me3 in DN3 and DP cells. On the other hand, some T-lineage regulatory genes, such as Myb and Ikaros, which are expressed at all stages, are open and accessible in all three different stages. Combining histone modification data with normal gene expression and perturbation studies, we plan to predict *cis*-regulatory modules of target genes using computational methods.

261. Genome-wide mapping of PU.1 targets and GATA3 targets in early T-cell development

Jingli Zhang

In order for prethymic progenitors to successfully pass through the highly ordered T-specification and lineage commitment process and become mature T cells, the whole developmental pathway must be tightly regulated by multiple distinct transcriptional programs, especially at lineage decision branchpoints. Two such transcription regulators are GATA-3 and PU.1. GATA-3 plays a crucial role in multiple stages of T-cell development and functional differentiation. It is required for pre-thymic precursor entering into T-lineage pathway and for T-lineage commitment. Studies have shown that the level of GATA-3 during early T-cell development is tightly controlled. While overexpression of GATA-3 in DP thymocytes favors CD4SP over CD8SP, overexpression of GATA-3 in E14.5 fetal liver precursors or DN thymocytes neither bypasses the requirement for upstream factors nor increases entry into the T-cell lineage. Instead, too much GATA-3 in DN1 or DN2 blocks further development, and more strikingly, under certain circumstances diverts DN1 cells into another hematopoietic lineage. The dosage-dependent character of GATA-3 makes it difficult for functional study of GATA-3 using loss-of-function or gain-of-function. The ETS family transcription factor PU.1 is another indispensable T-development regulator. The requirement for PU.1 in T-cell development is stage-dependent. It is vital for the generation of ETP from HSC. As a cell matures, the expression of PU.1 starts to diminish, and must be silent at DN3. If over-expressed at the DN2b or DN3 stage, PU.1 instead becomes antagonistic to T-lineage commitment. To investigate and compare the potential targets of PU.1 at DN1 and DN2 stages may help us to elucidate this unique "dual-functionality" of PU.1.

Using ChIP-Seq, we have generated genome-wide maps of physical binding sites for PU.1 in FLDN2 cells and for GATA3 in FLDN3 cells. Over 4000 sites are enriched for PU.1 binding in FLDN2, and about 700 sites are positive for GATA-3 binding in FLDN3. Our data suggest that PU.1 may be involved in regulating multiple

signaling pathways, including Notch/Delta, TGFβ, and cytokine signaling cascades. Our results suggest that it also plays a direct role in regulating some stem cell regulatory genes, such as *Tal1* and *Ikaros*, and other T-lineage regulators, such as *Runx1*, 2 and 3. GATA-3 maybe involved in regulating Rag1/2 and TCRβ expression, which are essential for β-selection and the generation of the T-cell signature, TCR.

We will continue to map genome-wide PU.1 binding sites in FLDN1 cells, and GATA-3 binding in FLDN2 cells. Once we have the comprehensive maps of PU.1 or GATA-3 direct binding targets at these overlapping developmental stages under normal conditions, CHIP-Seqs will be performed to cells that are under different perturbation conditions to help us fully understand the rules that govern the real functions (e.g., activation, repression or inactive binding) of individual physical binding events.

262. High throughput genomic and epigenetic data integration for predicting the T-cell regulatory interactome

Constantin Georgescu, Hamid Bolouri

Ongoing research on the regulatory network controlling T-cell development has mostly focused on a set of about 140 transcription factors and cell type-specific genes identified as key regulatory players by experimental work both in the Rothenberg lab and outside. Stage-specific diagrams, including known regulatory interactions among this core set of genes curated from the literature, have been assembled into a draft gene regulatory network using BioTapestry, which is updated periodically and available at: http://www.its.caltech.edu/~tcellgrn/TCellMap.html. Integrating related high-throughput information from various data repositories available on the Internet, the computational component of the group attempts to place the effort of discovering new regulatory interactions into a genome-wide context, and to generate reliable predictions that can enhance the design and increase the efficiency of the experiments.

To obtain a global view of potentially regulatory interactions we computationally combined interactome data from Transfac, PreMod and MIPS, gene expression data from the GEO repository, and annotations from the Ensembl database. The resulting network was explored using Cytoscape functionality. Overrepresentation analysis procedures were adapted to work on graphs to identify high connectivity motifs, especially those involving regulatory genes from our initial set of 140 index genes. In a first phase, we sought the identity of some crucial regulatory inputs stated as "placeholder" nodes in our initial network, the existence of which was required by developmental expression data. We also modeled the structure of a potential feedback circuit including some previously unknown intermediate links that could explain the GATA-3–PU.1 relationship in T-cell differentiation. We are exploring the applicability of recent graphical model analyses to our system (ARACNE, DEAL package), to develop models that account for the observed data on effects of PU.1, GATA-3, and Notch-Delta signaling, obtained previously in the lab.

Most recently we have been exploring the feasibility of using ChipSeq epigenetic data from fetal liver-derived pro-T cells (by Jingli Zhang, see previous abstracts) to predict directly cell type-specific *cis*-regulatory elements. The histone markers used, H3K4 methylation, H3 acetylation and H3K27 trimethylation, were chosen to enable a variety of *cis* elements to be identified by combinatorial patterns of the three histone modifications. H3K4me2 is useful in conjunction with "positive" marker H3Ac and the "negative" marker H3K27me3 because it can mark sharp boundaries of a range of both positively and negatively acting regulatory elements. We developed a novel method for simultaneous study of the abundance of the three markers in two cell types (DN1-DN3), linking the patterns observed to the fold change in directly measured expression values. Based on applying a partial least squares regression on top of a correspondence analysis-transformed output, we have trained a model on the known QPCR data of the 140-core set of index genes. This model now seems to give very promising results both in predicting new cell type-specific genes, and in using cell type-specifically enriched histone marks to identify regions of the genome with high potential of hosting transcription factor binding sites. As a next step, we are attempting to predict the set of transcription factors most likely to be acting at these dynamically modified regions. We have mapped 868 consensus transcription factor binding site strings from Transfac to the selected regions, filtering the matches based on spatial clustering of binding sites, accuracy of the motif matches and to some extent their sequence conservation. The resulting predicted binding sites are provisionally assigned to the nearest gene as a likely target. This is enabling us to construct a global network model including many potential regulatory interactions. Currently we are exploring the potential of using spectral analysis techniques based on canonical component decomposition of the graph Laplacians, for relating the global network to the "gold standard" small network described above, and to the microarray data retrieved from GEO repository. These techniques should enable us to filter the huge number of regulatory predicted connections into a small set of high confidence links that are worth exploring experimentally.

263. Web-based resource development for transcriptome data analysis

Michael Angerman, Hamid Bolouri

Many transcriptome analysis methods - such as Principal Component Analysis (PCA), Partial Least Squares Regresssion (PLSR), K means clustering, and Multi Dimensional Scaling (MDS) - are freely available as packages written in the statistical language R (http://www.r-project.org/). However, the use of such R packages requires some statistical expertise, and familiarity with R syntax and the R computing

environment. These requirements often make the use of R transcriptome analysis resources difficult for bench biologists.

To address this challenge, we are developing a menu-driven web-based resource for visualization and analysis of gene expression data using standardized R packages. Users can select and configure analysis options through point-and-click menus. Our server (CRdata.org, due for initial release in fall 2009) translates user-selections into R code, executes the code, and returns the results to the user's web-browser in graphical form. In essence, CRdata.org provides a web-based graphical user interface to sophisticated R resources. In the coming year, we plan to extend the range of analysis tools available to include high-throughput short-read sequencing data.

264. Exploratory approaches for improved T-cell gene network manipulation

Amy A. Ross, Rochelle A. Diamond, Diana Perez, Ellen V. Rothenberg

To validate our gene network models, it would be useful to improve three aspects of our standard conditions for genetic manipulation of T-lineage precursors developing *in vitro*. First, we would like to replace the stromal cell line OP9-DL1 with a defined source of the critical Delta-like Notch ligand, so that we can control dosage and uniformity of ligand presentation. This would also have the advantage of improving longitudinal time-lapse imaging of developing T cells without interference from the stromal cells. Second, we need to improve our tools for acute downregulation of genes of interest in the developing cells. Third, we would like to optimize conditions in which precursors from adult mouse bone marrow or thymus could be used routinely instead of relying on precursors from fetal tissues. Most previous attempts to manipulate the gene expression patterns in T-cell precursors have used fetal thymus or fetal liver-derived cells, which can readily be transduced with retroviral vectors, but this advantage comes with substantial disadvantages. The ability to transfect T-cell precursors from adult mice will help solve the logistical issues of using fetal-derived thymocytes in our studies.

(1) Stromal-free T-cell development cultures. The Notch signaling pathway is important in hematopoietic development, especially lymphoid differentiation. Notch ligands Delta1 (DLL1) or Delta4 (DLL4) are required for early T-cell differentiation. At least one of these ligands must be presented bound to a surface, and the ability to control which Notch ligands are present in *in vitro* systems may allow for selective T-cell differentiation[1,2]. Our laboratory has traditionally used the stromal feeder cell line OP9-DL1 for Notch signaling in the study of early-stage T-cell development. However, this makes it difficult to control the dose and uniformity of Notch ligand presentation across the whole tissue culture area. Substitution of immobilized Notch Delta ligands for feeder cells has been used by others to activate lymphopoiesis from hematopoietic precursors[1-3]. We are therefore exploring the use of an *in vitro* system that uses biotinylated Delta ligands bound to avidin-coated tissue culture plates in lieu of OP9-DL1 feeder cells to promote differentiation of early-stage T cells.

We have tested the immobilized Delta ligand method with DN1 and DN2 thymocytes from pre-weaned or young adult (6-8 week) mice. However, in comparison to the control OP9-DL1 stromal cocultures, the immobilized DLL1 and DLL4 cultures showed slower cell proliferation and drastically lower overall cell numbers. Cell morphologies varied from lymphoid to myeloid-like to dendritic-like. We are in the process of retesting this system with fetal liver and fetal thymus-derived precursor cells to determine whether the T-cell precursors present from earlier ontogenic stages will be more robust in this system.

(2) Exploration of the effect of Toll-like receptor genotype on gene transfection of early-stage thymocytes from adult mice. Although retroviral vectors are effective for ectopic expression or overexpression of regulatory genes, we need additional methods to cause loss of function of specific regulatory genes in these proliferating, developmentally dynamic cells. We have therefore tested acute methods for transfecting morpholino-substituted antisense oligonucleotides and siRNAs. Differentiating pro-T cells were obtained from *in vitro* differentiation of sorted DN1 and DN2 stage thymocytes from adult (four to six week-old) mice. Our earlier data *in vitro* differentiated thymocytes indicated that we could transfect DN1-derived DN3 cells using the Amaxa Nucleofection® protocol. However, fewer surviving DN2 transfectants were found and it was unknown if effective knockdown could be obtained using transfected siRNA in this system. Early attempts to use siRNAs in T-lineage precursors had appeared to result in massive cell loss.

We hypothesized that one problem could be an innate immune system-based suicide response that might be triggered by siRNA in transfected precursors through the Toll-like receptor system, specifically TLR3. We therefore compared DN1-derived *in vitro* differentiated DN2-DN3 thymocytes from TLR3-deficient and C57BL/6 control adult mice in their responses to both morpholino and siRNA transfection. Both genotypes yield good *in vitro* T-cell development in nontransfected controls. A co-transfected plasmid encoding GFP was used to track the fate of cells taking up nucleic acid generally through the Amaxa protocol. Unfortunately, the results showed that irrespective of the use of siRNA, morpholinos, or plasmid alone, transfection using the Amaxa Nucleofection® protocol results in low transfection rates and is fatal to early-stage T-lymphocytes derived from adult mouse thymus of both genotypes.

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265. Genome-wide QTL mapping of the early T cell checkpoint breakthrough in NOD mice

Mary Yui, Ni Feng, Justine Chia

Type 1 diabetes (T1D) is a complex polygenic autoimmune trait in humans and in rodent disease models. More than 20 diabetes susceptibility loci have been mapped in NOD mice, which spontaneously develop T1D. Several of the loci have been reported to impact NOD T cells, although the gene and their roles in autoimmunity are not yet determined. NOD early T cells, which are the progenitors of all T-cell lineages, exhibit several developmental defects. One defect is a breakthrough at the first T-cell receptor (TCR)-dependent checkpoint, beta-selection. We previously reported that thymocytes from NOD mice that are unable to rearrange a TCR, due to the *Prkdc*^{scid} mutation or *Rag*-deficiency, do not arrest at this TCR-dependent checkpoint, as they should. To determine if this trait is related to a fundamental T-cell phenotype that maps to T1D susceptibility loci in NOD mice we are conducting a linkage analysis. A preliminary PCR-based analysis of an F2 cross and an N2 backcross, using breakthrough-prone NOD.*Rag*$^{-/-}$ mice and breakthrough-resistant B6.*Rag*$^{-/-}$ mice, showed that this trait maps to more than one gene and confirmed partial mapping to the telomeric chromosome (chr) 4 region, which contains diabetes susceptibility loci *Idd9* and *Idd11*. Based on preliminary results, congenic mice were constructed by breeding the *Rag1*-deficiency onto NOD.*B10chr4(Idd9)* mice with a T1D protective *Idd9* region. These congenic mice do not differ in breakthrough incidence from NOD.*Rag*$^{-/-}$ mice, showing that the trait maps outside the *Idd9* region, possibly mapping to the somewhat more centromeric *Idd11* region. A genome-wide QTL (quantitative trait locus) analysis using SNP-based genotyping is currently underway to more closely map the trait within the chr4 region and to find additional genetic regions that impact this trait.

266. Skewed αβ- versus γδ-T lineage decisions by pro-T cells from non-obese diabetic (NOD) mice

Mary Yui, Ni Feng

All T cell lineages originate from the same progenitor cells that migrate from bone marrow to the thymus, where they undergo TCR-independent development, followed by TCR-dependent differentiation, selection events, and lineage choices. T cells are required for the development of Type 1 diabetes (T1D) in rodents and humans, and alterations in numbers and functions of various T-cell lineages have been proposed to contribute to autoimmunity. To determine if T-cell progenitors from NOD mice exhibit intrinsic defects in development we studied their differentiation in the Notch-ligand presenting OP9-DL1 co-culture system, as well as by analysis of developmentally regulated changes in cell surface receptors *in vivo*. We found that relative to two other strains of mice, cultured precursor NOD CD4 and CD8 double negative (DN) cells exhibit major defects in the generation of CD4 and CD8 double positive (DP) αβ T cells, while γδ T-cell development from bipotent DN precursors is enhanced. These defects are most pronounced in the earlier DN1 and DN2 subsets in long-term culture but are also found in cultures from more mature DN subsets. In addition, NOD and B6 early T-cell subsets show different patterns of expression of activation-induced receptor molecules, before and in response to preTCR or γδ TCR signals. Thus, NOD T-cell precursors reveal lineage-specific differentiation abnormalities before and after the first TCR-dependent developmental choice point.

267. Origins of vertebrate lymphocytes

Jonathan Moore

Until recently, the origin of the vertebrate adaptive immune system could be seen as an evolutionary explosion. With a few scattered exceptions, immunoglobulins, TCRs, MHCs, and the RAGs are not found outside of the jawed vertebrates, and the small evolutionary distances between the jawed vertebrates and their close relatives suggest rapid evolutionary invention or cooption. Five years ago, a plausible intermediate step in the evolution of the adaptive immune system was discovered: a novel somatically recombinant immune receptor molecule, the VLRB, was found in the jawless vertebrates, the sister taxon to the jawed vertebrates who also possess an adaptive immune system. This suggests an obvious question: did these systems evolve independently or do they share a single common origin? In many diverse jawed vertebrate clades, the set of transcription factors (TFs) involved in immune function is conserved. We are seeking to discover which TFs regulate the *Vlrb* gene complex, since if these are shared in common with jawed-vertebrate immune genes, this could be evidence of a common origin to both adaptive immune systems. To this end, we have used a variety of approaches, including bioinformatics, heterologous assays, and homologous assays.

Previously, we had identified two regions upstream of the *Vlrb* transcriptional start site which were: (a) more conserved than neighboring segments; (b) had more TF binding site predictions than would be expected from a random distribution of predictions; and (c) affected the regulation of transfection reporters in heterologous assays in catfish B- and T- cell lines. Functional assays were pursued first in the heterologous catfish system because neither lamprey cell lines nor a developmentally suitable *in vivo* gene transfer system have yet been developed. Lymphocytes from catfish, though they use conventional immunoglobulin or T-cell receptors for their own function, appear to be able to drive transcription dependent on the *Vlrb* promoter region, and the question is whether they do so using regulatory mechanisms homologous to those used by the lamprey themselves. This past year we nailed down the transcription reporter results with catfish lines, both to establish clear statistical significances and to finely map the sites that affect transcription. EMSAs with catfish nuclear extracts performed on the *Vlrb* promoter show binding to a 70-bp

core containing bound Ets sites, Octamer sites, and an E4F/CREB site. Intriguingly, the sites that have the greatest impact on expression in catfish lymphocytes lie between the Ets sites in the putative E4F/CREB site.

When DNA binding experiments were performed with extracts from sorted lamprey cells expressing and not expressing VLRB, we found that the Octamer sites were bound by VLRB- nuclear extracts but not by VLRB+, while the Ets sites were bound by VLRB+ cell nuclear extracts but not by VLRB- cell extracts. Unlike the catfish results, the E4F/CREB site showed only slight evidence of binding in the lamprey extracts. Thus, the Ets sites themselves may be targets for cell type-specific binding of a positively acting transcription factor in the native lamprey context. In order to determine the exact Ets factors involved in the EMSA binding, we recently utilized supershift experiments. These point to several possible Ets factors, but in both VLR+ and the catfish cell lines, Erg seems to be the strongest binder. To narrow the possibilities for factors that may be driving *Vlrb* expression in the native VLRB+ cell context, RT-PCR experiments were performed on samples from VLRB- and VLRB+ cells from three different tissues and two different life stages. The most striking changes are the much lower levels of GATA2/3 in VLR+ cells and higher levels of a Runx family factor in VLRB+ cells. Other notable factors higher in VLRB+ cells are NFkB, cRel, Oct1, and the two Ets family factors Spi and Erg.

This summer we have begun testing our reporter constructs in a homologous system *in vivo* by injecting them into lamprey embryos with the help of Tatjana Sauka-Spengler and her colleagues in Marianne Bronner-Fraser's lab. These experiments have begun to yield the first evidence of reporter expression from our *Vlrb* constructs in the embryos. We plan to optimize this system over coming seasons for pursuit into later stages of development, to visualize *Vlrb* reporter expression as the lymphocyte lineages emerge.

Publications

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Summary: The main focus of the research program is the analysis of the dorsal-ventral axis gene regulatory network (GRN) functioning in the early *Drosophila* embryo. In particular, we are interested in the morphogenetic movements that are controlled by this network during gastrulation. Our working hypothesis is that transcriptional inputs help to coordinate groups of cells during collective cell movements. Therefore, we believe that the analysis of *cis*-regulatory mechanism will impact our analyses of gastrulation cell movements. Our goal is to extend our imaging approaches to assay all the cells within a developing *Drosophila* embryo, in the context of defined genetic and molecular perturbation, in order to understand the movements of each and every cell as an output of the genomic regulatory code. In addition, we will strive to develop additional methods that will allow us to visualize transcription and the activation of signaling pathways in a live developing embryo. To describe development of an organism as a sequence of molecular events is our ultimate goal.

One unifying goal of the studies being conducted in my laboratory is to understand *how* genes are orchestrated to control development. To date, the basis for our studies relates to a GRN, which describes what is known of dorsal-ventral patterning in the early *Drosophila* embryo. Within this GRN, information regarding genetic interactions and *cis*-regulatory control of ~60 genes is detailed. These genes interact to specify patterning along the *Drosophila* dorsal-ventral axis, to control cell movements that drive gastrulation, as well as to influence the subsequent differentiation of cells into different tissue types. We have used this extensive knowledge base to provide mechanistic insights into the development of embryos: (1) to understand how genes are expressed with proper spatial precision to pattern the embryo; and (2) to define the functions of these differentially expressed genes in controlling morphogenesis and differentiation.

Cis-Regulatory design: dynamic interpretation of transcription factor levels

We have assembled a provisional network, which describes how ~60 genes interact during gastrulation to specify dorsal-ventral patterning and subsequently to control differentiation of cells in *Drosophila*. However, even after such an extensive analysis, it remained unclear how the transcription factor Dorsal can regulate the expression of genes in a broad lateral domain. We have determined that the levels of this factor decrease dramatically within this domain (Liberman *et al*., in review). To this end, we have conducted an analysis of the *cis*-regulatory sequences supporting expression in the broad lateral domain of embryos, and found evidence that cooperation between Dorsal and a ubiquitous activator is required for this threshold response (i.e., specification of a broad lateral stripe domain) (Liberman and Stathopoulos, *Dev. Biol*., 2009). Our approach combined evolutionary analysis, site-directed mutagenesis, and synthetic construct design to support this model. Furthermore, we provide evidence for flexibility in the composition and organization of sites required to support expression within this domain.

ChIP-chip and ChIP-seq analyses will be analyzed to identify *cis*-regulatory sequences, not yet identified, for genes that are differentially expressed along the dorsal-ventral axis. Our prediction would be that different examples of *cis*-regulatory design will be identified, which would explain why we had not been able to identify these regions in the past using standard bioinformatic methods and our prior knowledge (Ozdemir *et al*., in preparation).

Furthermore, one of the most striking properties of some developing systems is the ability to re-organize their developmental program and apparently give rise to normal adults when the size or shape of the embryo is altered. We are currently using genetic and computational approaches to understand how patterning is controlled by morphogen gradients (e.g., Nahmad and Stathopoulos, *PLoS Biol*., 2009).

Cell movement coordination during migration of cells: high-level spatial organization

The function of many genes differentially expressed along the dorsal-ventral axis of *Drosophila* embryos is to coordinate the cell movements that are driving gastrulation. We are analyzing the mechanism by which mesoderm spreading is accomplished. With technical advances in imaging and novel quantitative analyses, we have shown that mesoderm migration is a directed process; that cells move from ventral-most to dorsal-most regions of the ectoderm in a coordinate fashion (McMahon *et al*., *Science*, 2008). High-level spatial organization within the moving population of cells was visualized. Cells at the leading edge originate from a particular position within the invaginated mesoderm; cell divisions are regulated temporally and spatially; and intercalation events contribute to monolayer formation of the migrating collective. We aim to determine whether such spatial

organization is required for collective cell migration in general, a process that makes essential contributions to embryonic development.

We also investigate FGF signaling mechanisms using *Drosophila* as a model system. We demonstrate that the FGF-8 homologous proteins, Pyramus and Thisbe, are not redundantly functioning ligands but instead these genes have distinct functions, due in part to differential range of action and in part to differential expression (Kadam *et al*., *Development*, 2009). In addition, our work has suggested that FGF signaling is important for collective cell mesoderm migration during gastrulation, but that it is not absolutely required. In the absence of FGF signaling, those cells in contact with the ectoderm are competent to migrate in a directional manner; those that cannot contact the ectoderm exhibit random movements, and lose the ability to move directionally. In the future, we will determine if these ligands differ in range of action and, to this end, are characterizing the protein profiles for each protein.

268. Quantitative imaging of the Dorsal nuclear gradient reveals limitations to threshold-dependent patterning in *Drosophila*

Louisa M. Liberman, Gregory T. Reeves*, Angelike Stathopoulos*

The NF-κB related transcription factor, Dorsal, forms a nuclear concentration gradient in the early *Drosophila* embryo patterning the dorsal-ventral axis to specify mesoderm, neurogenic ectoderm and dorsal ectoderm cell fates. These patterning events are thought to be determined by the concentration of nuclear Dorsal; however, the actual levels of nuclear Dorsal have not been quantified. Furthermore, existing models for Dorsal-dependent germ layer specification and patterning consider steady-state levels of Dorsal relative to target gene expression patterns, yet Dorsal gradient formation is dynamic as is gene expression. We devised a quantitative imaging method to characterize the dynamics of Dorsal nuclear gradient formation while simultaneously examining Dorsal target gene expression in nuclei along the dorsal-ventral axis. Unlike what has been observed in other insects such as *Tribolium*, we find that the Dorsal gradient maintains a constant bell-shaped distribution during embryogenesis. We also find that some genes that require Dorsal for activation fall outside the graded localization of Dorsal, raising the question whether these genes are direct Dorsal targets. Additionally, we show that Dorsal levels change in time during embryogenesis such that steady state is not reached even at cellularization. These results suggest that the multiple gene expression outputs observed along the dorsal-ventral axis do not simply reflect a steady-state Dorsal nuclear gradient. Instead we propose that the Dorsal gradient supplies positional information throughout nuclear cycles 10 through 14 and that compensatory combinatorial interactions between Dorsal and other factors effect differential gene expression along the dorsal-ventral axis.

**These authors contributed equally to this work.*

269. Design flexibility in *cis*-regulatory control of gene expression

Louisa Liberman, Angelike Stathopoulos

In early *Drosophila* embryos, the transcription factor Dorsal regulates patterns of gene expression and cell fate specification along the dorsal–ventral axis. How gene expression is produced within the broad lateral domain of the presumptive neurogenic ectoderm is not understood. To investigate transcriptional control during neurogenic ectoderm specification, we examined divergence and function of an embryonic *cis*-regulatory element controlling the gene short gastrulation (sog). While transcription factor binding sites are not completely conserved, we demonstrate that these sequences are bona-fide regulatory elements, despite variable regulatory architecture. Mutation of conserved sequences revealed that putative transcription factor binding sites for Dorsal and Zelda, a ubiquitous maternal transcription factor, are required for proper sog expression. When Zelda and Dorsal sites are paired in a synthetic regulatory element, broad lateral expression results. However, synthetic regulatory elements that contain Dorsal and an additional activator also drive expression throughout the neurogenic ectoderm. Our results suggest that interaction between Dorsal and Zelda drives expression within the presumptive neurogenic ectoderm, but they also demonstrate that regulatory architecture directing expression in this domain is flexible. We propose a model for neurogenic ectoderm specification in which gene regulation occurs at the intersection of temporal and spatial transcription factor inputs.

Reference

Liberman, L.M. and Stathopoulos, A. (2009) *Dev. Biol.* **327**(2):578-589.

270. Advanced approaches to analyze *cis*-regulatory sequences in *Drosophila*: Insights into embryonic patterning

Anil Ozdemir, Leslie Dunipace, Katherine Fisher, Shirley Pepke, Manoj Samanta[1], Barbara Wold[2], Angelike Stathopoulos

Whole-genome methods have greatly improved our understanding of how *cis*-regulatory sequences contribute to animal development. Here we discuss experimental approaches that can assay more rigorously the function of these sequences. We show how high-throughput sequencing of chromatin-immunoprecipitations (ChIP-seq) can identify the DNA sequences occupied *in vivo*, at binding site resolution. Next, we employ recombineering methods to manipulate large DNA fragments and assay stable transgenic fly stocks for the expression of a reporter gene in its native environment. Each of these technical advances has the potential to elucidate important new insights, in general, into the *cis*-regulatory mechanisms that control gene expression. To demonstrate, we show how ChIP-seq can define the *in vivo* binding site consensus for the transcription factor Twist and how recombineering methods can be utilized to

demonstrate that enhancers for the *brinker* gene function autonomously within the early embryo, to support expression that is both spatially and temporally distinct.

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271. Dynamic interpretation of hedgehog signaling in the *Drosophila* wing disc

Marcos Nahmad, Angelike Stathopoulos

Morphogens are classically defined as molecules that control patterning by acting at a distance to regulate gene expression in a concentration-dependent manner. In the *Drosophila* wing imaginal disc, secreted Hedgehog (Hh) forms an extracellular gradient that organizes patterning along the anterior-posterior axis and specifies at least three different domains of gene expression. While the prevailing view is that Hh functions in the *Drosophila* wing disc as a classical morphogen, a direct correspondence between the borders of these patterns and Hh-concentration thresholds has not been demonstrated. We found evidence that the interpretation of Hh signaling depends on the history of exposure to Hh and propose that a single concentration threshold is sufficient to support multiple outputs. Using mathematical modeling, we predict that at steady-state only two domains can be defined in response to Hh, suggesting that the boundaries of two or more gene expression patterns cannot be specified by a static Hh gradient. Computer simulations suggest that a spatial 'overshoot' of the Hh gradient occurs, this is, a transient state in which the Hh profile is expanded compared to the Hh steady-state gradient. Through a temporal examination of Hh-target gene expression, we observe that the patterns initially expand anteriorly and then refine, providing *in vivo* evidence for the overshoot. The Hh gene network architecture suggests this overshoot results from the Hh-dependent upregulation of the receptor, Patched (Ptc). In fact, when the network structure was altered such that the *ptc* gene is no longer upregulated in response to Hh-signaling activation, we found that the patterns of gene expression, which have distinct borders in wild-type discs, now overlap. Our results support a model in which Hh gradient dynamics, resulting from Ptc-upregulation, play an instructional role in the establishment of patterns of gene expression.

272. Size regulation of dorsal-ventral patterning in *Drosophila* embryogenesis

Marcos Nahmad[*]*, Gregory T. Reeves*[*]*, Angelike Stathopoulos*

There is broad interest in the molecular mechanisms allowing animals to preserve proportions with respect to size. Despite the increasing knowledge on the formation of morphogenetic patterns, the molecular and genetic relationships between pattern formation and size remain poorly understood: how do patterns accommodate variation in the size of a developmental field? We investigate the patterning of the embryonic dorsal-ventral (DV) axis with respect to its proper scaling despite large variations in the size of the DV domain. The *Drosophila* NF-κB homologue, Dorsal (dl), is the only known maternal source of dorsal-ventral information in the embryo. As such, the ability to generate a well-proportioned insect initially depends on whether or not patterning by dl – and factors downstream of it – scales with respect to the size of the DV axis.

Using fluorescent *in situ* hybridization, we quantify the expression domains of known dl target genes in both wild type and mutant genetic backgrounds and have observed scaling with respect to natural variations in the size of the embryo for all genes investigated. We also employ a system in which an ectopic dl gradient is generated along the anteroposterior (AP) axis in embryos that lack the endogenous DV patterning. Since the AP axis is about 2-fold larger than the DV axis, this artificial system provides an opportunity to study mechanisms of size-dependent scaling in development. Previous models have suggested that opposing gradients may provide size-dependent positional information. If such an opposing gradient exists in the artificial system, then it should depend on dl itself. In addition, we investigate whether the dl gradient itself scales by analyzing the spatial distribution of the dl localization in wild-type and mutant embryos.

As a complementary approach, we are using mathematical models to uncover the possible network architectures that can support scaling in this system. The output of these theoretical models can be used to guide future experiments and to predict new molecular candidates responsible for the accurate establishment of positional information.

[*]*These authors contributed equally to this work*

273. The role of localized repressors in *Drosophila* dorsal ventral patterning

Mayra Garcia, Angelike Stathopoulos

The *Drosophila* pre-gastrula embryo is patterned by a nuclear gradient of the transcription factor Dorsal, which sets the boundaries of the presumptive mesoderm, neurogenic ectoderm, and non-neurogenic ectoderm. Targets in the neurogenic ectoderm have high affinity Dorsal binding sites and can be activated by both high and intermediate levels of Dorsal but are repressed in ventral regions by other Dorsal targets. Targets expressed in ventral regions have low affinity Dorsal sites and can only be activated by the highest levels of Dorsal. Current models postulate that limiting amounts of Dorsal delineate the dorsal borders of the target genes, although recent *cis*-regulatory analysis and the sharpness of the borders suggest that this may not be the case. It is still unclear how the dorsal borders of Dorsal target genes are established. Our goal is to show that localized repressors set the dorsal borders of Dorsal target genes. This will change our current understanding of how the dorsal-ventral axis of the *Drosophila* embryo is patterned. Previous synthetic enhancer analysis of the *intermediate neuroblast defective (ind)* enhancer, located a short 12 base pair repetitive sequence that mediates repression in dorsal regions, when in the context of 100 base pairs. We

conducted further analysis of this element and found that it is sufficient to mediate repression in dorsal regions. We believe that a repressor, which is expressed in dorsal regions, binds to this sequence and sets the dorsal border of *ind* and possibly other Dorsal target genes. We have undertaken a biochemical approach coupling DNA affinity chromatography with mass spectrometry to identify transcription factor that may bind this site. We have several candidates upon which we are conducting further studies.

274. Dynamic analyses of *Drosophila* gastrulation reveal spatial organization of cell behaviors

Amy McMahon, Willy Supatto, Scott E. Fraser, Angelike Stathopoulos*

The collective movement of distinct groups of cells contributes to morphogenesis during development, yet it remains unclear how the coordinated movement of many cells is accomplished. We define an imaging approach and quantitative methods that now make it possible to obtain and analyze the trajectories of hundreds of cells deep within *Drosophila* embryos during gastrulation. We find evidence for coordinated movements of mesoderm and ectoderm cells.

In addition, we define autonomous movements of the mesoderm cells that are directed and exhibit high-level spatial organization. Such dynamic imaging studies provide insights into the mechanisms guiding collective cell movement and provides a framework that allows us to interpret mutant phenotypes dynamically. Thus, we are able to determine that FGF signaling is required during mesoderm spreading for collapse of the invaginated mesoderm and provide evidence that a distinct signal must promote the directed movement of mesoderm cells within *Drosophila* embryos. We contend that decomposing complex cell movements is required to dissect the behavior of cell cohorts to obtain mechanistic insights into the underlying biology of collective cell migration.

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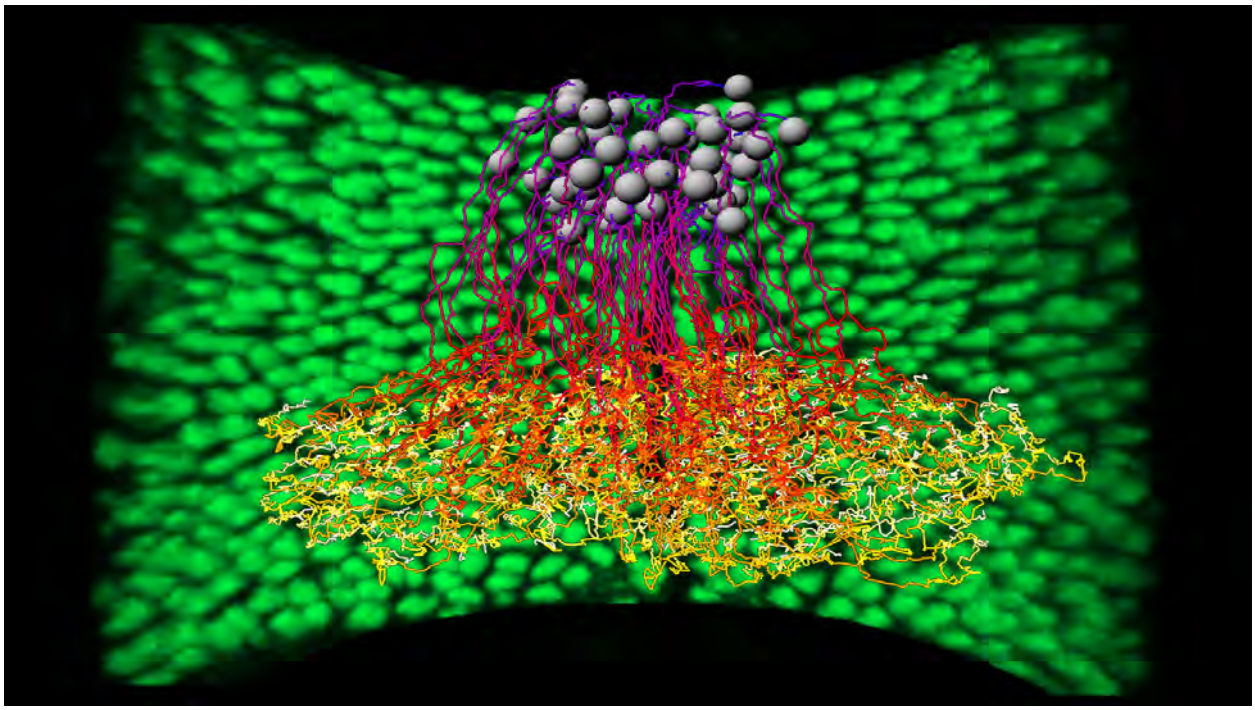
Reference

McMahon, A., Supatto, W., Fraser, S.E. and Stathopoulos, A. (2008) *Science* **322**(5907):1546-1550.



275. Signaling mechanisms of collective cell migration during *Drosophila* gastrulation

Young-Kyung Bae, Angelike Stathopoulos

Collective cell migration is at the basis of developmental morphogenesis, wound healing, and immune response. During *Drosophila melanogaster* gastrulation, the mesodermal cells migrate dorsally after invagination, a movement that is critical for differentiation of cells to diverse mesodermal cell fates. We are using *Drosophila* mesoderm migration as a model system to understand how collective cell migration is regulated during early development. Recently, we have shown that mesodermal cell organization is prevalent during their 2-hour migration and that the FGF signaling is responsible for directing motility of cells at the leadering edge but not of those cells that follow (McMahon *et al*., 2008). Major signaling pathways and membrane receptors have been identified as key players in diverse systems in which cell migration occurs. However, whether signaling pathways in addition to FGF support *Drosophila* mesodermal migration is currently unknown. Regulation of dynamic cellular behavior and collective organization by signaling cascades is likely.

The long-term goal of this project is to identify signaling pathways that influence mesoderm spreading, elucidate their cellular function, and ultimately build a comprehensive transcriptional and signaling network supporting mesoderm migration. We hypothesize that distinct and multiple signaling pathways actively coordinate cellular behaviors by controlling functional modules: cell motility, directed migration, cell-cell coordination, collective organization, or cell density. To identify new signaling pathways regulating collective cell migration *in vivo*, we plan to take three complimentary approaches: candidate gene RNAi, gene expression profiling of migrating mesoderm, and a genetic modifier screen. Our preliminary data suggest that the JNK pathway plays a role in *Drosophila* mesoderm migration. We are currently investigating that which cellular output is controlled by JNK pathway and if FGF and JNK signaling regulate each other.

276. Investigating the coordination of the migrating mesoderm collective

Nathanie Trisnadi, Angelike Stathopoulos

Collective cell migration exhibits high spatiotemporal coordination among a large population of cells and between multiple cellular processes. Although there is much research in understanding the mechanics of individual cell behaviors, it remains unclear how groups of cells coordinate these events. Our understanding of collective migration has previously been limited to static images. These "snapshots" cannot provide the complete story of such a dynamic process. With our recent advancement of imaging techniques and new cell tracking analysis, we can now obtain "movies" that describe the behavior of collective cell migration. In addition, we are developing tools to selectively label specific cells within a migrating population. This allows us to dissect the differences between cell groups such as leader vs. follower cells and to easily track cells originating from certain regions of interest.

We study the mesoderm in *Drosophila* embryos that undergoes collective cell migration during gastrulation. The FGF pathway has long been known to be a required component for proper mesoderm development. However, its role was unclear until recently when work from our lab revealed that FGF is responsible for only a subset of mesoderm cells and that another signal is also functioning in mesoderm migration. Efforts by others in the lab are investigating this elusive pathway. We are also interested to learn how cell membranes play a role in collective migration, specifically in protrusions for directional sensing and in maintaining cell-cell contacts through adhesion molecules. In addition, migration and mitosis cannot occur simultaneously due to the fact that these processes share cytoskeletal components. We believe there is a switch that instructs cells to either migrate or divide. We plan to examine various mutant embryos with known migration defects using live imaging. Analysis of cell tracks will determine multiple cellular processes are coordinated during collective migration. Our strategy uses novel tools that will allow us to probe into the behavior of the migrating cell population as a whole and begin to integrate the different aspects of collective migration.

277. FGF ligands in *Drosophila* have distinct activities required to support cell migration and differentiation

Snehalata Kadam, Amy McMahon, Phoebe Tzou, Angelike Stathopoulos

Fibroblast growth factor (FGF) signaling controls a vast array of biological processes including cell differentiation and migration, wound healing and malignancy. In vertebrates, FGF signaling is complex, with over 100 predicted FGF ligand-receptor combinations. *Drosophila melanogaster* presents a simpler model system in which to study FGF signaling, with only three ligands and two FGF receptors (FGFRs) identified. Here we analyze the specificity of FGFR [Heartless (Htl) and Breathless (Btl)] activation by each of the FGF ligands [Pyramus (Pyr), Thisbe (Ths) and Branchless (Bnl)] in *Drosophila*. We confirm that both Pyr and Ths can activate Htl, and that only Bnl can activate Btl. To examine the role of each ligand in supporting activation of the Htl FGFR, we utilize genetic approaches that focus on the earliest stages of embryonic development. When pyr and ths are equivalently expressed using the Gal4 system, these ligands support qualitatively different FGFR signaling responses. Both Pyr and Ths function in a non-autonomous fashion to support mesoderm spreading during gastrulation, but Pyr exhibits a longer functional range. pyr and ths single mutants exhibit defects in mesoderm spreading during gastrulation, yet only pyr mutants exhibit severe defects in dorsal mesoderm

specification. We demonstrate that the *Drosophila* FGFs have different activities and that cell migration and differentiation have different ligand requirements. Furthermore, these FGF ligands are not regulated solely by differential expression, but the sequences of these linked genes have evolved to serve different functions. We contend that inherent properties of FGF ligands make them suitable to support specific FGF-dependent processes, and that FGF ligands are not always interchangeable.

Reference

Kadam, S., McMahon, A., Tzou, P., Payne, S. and Stathopoulos, A. (2009) *Development* **136**(5):739-747.

278. FGF ligands pyramus and thisbe are required for intercalation of mesoderm cells during gastrulation in the *Drosophila* embryo

Amy McMahon, Angelike Stathopoulos

The concerted movement of groups of cells contributes to morphogenesis during development, yet it remains unclear how the coordination of many cells is accomplished. Using live-imaging and quantitative analysis, we have found that the fibroblast growth factor (FGF) ligands pyramus and thisbe are required for intercalation of the mesoderm collective. We are further characterizing potential downstream targets of the FGFs to find out how intercalation is controlled during gastrulation.

279. Structure/function analysis of *Drosophila* FGF ligands pyramus and thisbe

Sarah Payne, Angela Stathopoulos

Drosophila has three FGF ligand genes and two of them, pyramus (pyr) and thisbe (ths), represent ancient gene duplication, and are present in all Drosophilids. Pyr and Ths are most closely related to FGF8 in vertebrates. The vertebrate FGFs are 18-30kD in size, whereas the *Drosophila* FGFs are 79-82kD, with no other predicted domains besides the homologous FGF domain. The longer C-terminus of *Drosophila* FGFs may serve a regulatory purpose where the function of the FGF domain is activated or attenuated by protein processing. Processing by proteases is a common mechanism for the regulation of ligand proteins and the highly dynamic expression pattern of pyr and ths suggests that such regulation is needed. Using modified constructs of Pyr and Ths we have undertaken a structure/function analysis to understand which part of the proteins are required for function and which part is available for processing by regulatory proteases.

We investigated whether the FGF domain of Pyr and Ths was sufficient for function and found that for Ths it is and for Pyr it is not. We are still examining the function of the C-terminus and although it is still unclear, it could be used for regulation of activation by protein processing. Pyr and Ths are cleaved into smaller N-terminal fragments in *Drosophila* S2 cells, where the FGF domain remains intact. One of the vertebrate FGFs, FGF23, is also thought to be processed by proprotein convertases.

280. Role of FGFs during LVMF cell migration

Snehalata Kadam, Angelike Stathopoulos

During migration the founders of the longitudinal muscle fibers (LVMFs) express the Fgf receptor Heartless. It has also been shown that Thisbe, and not Pyramus, is expressed in the founders of the circular muscle fibers (CVMs) (Stathopoulos *et al.*, 2004). The CVM cells are poised to serve as a migration substratum for the LVMFs. The CVMs express the FGF ligand Thisbe that may provide chemo-attractive cues to the LVMFs, which express the FGF receptor Heartless. The surrounding ectoderm expresses FGF ligand pyramus which may provide a permissive cues to the migrating LVMFs.

Our live imaging and fixed embryo staining analyses suggest that pyramus and thisbe double mutants exhibit a disrupted migration phenotype: loss of coordination and direction of migration resulting in the activation of apoptosis. The FGF receptor heartless mutants also exhibit similar phenotype. In thisbe and pyramus single mutants the LVMF migration appears to be partially disrupted suggesting both the FGF ligands are required for normal migration. Ectopic expression of thisbe in LVMFs appears to partially rescue the migration defects. Interestingly ectopic expression of pyramus in the LVMFs results in partial rescue of migration phenotype but the cells lose the directionality of their migrating path.

These experiments have the potential to identify a unique function of Thisbe and Pyramus for Hearltess receptor activation using LVMF cell migration model system. These studies also have the potential to provide insights into vertebrate function of Fgf ligand/receptor interactions, and specifically in the case of LVM/CVM migration to provide insights into human disease. FGFs have been implicated in DiGeorge syndrome and mutations in this gene results in a severe congenital heart disease.

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Collaborators: Igor Antoshechkin, Martin Chalfie, Miriam Goodman, Lorian Schaeffer, Brian Williams, Barbara Wold
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Summary: We seek to understand how the genome controls nematode development, behavior and physiology. We use molecular genetics to understand detailed mechanisms, and functional genomics to obtain global views of development and behavior. We try to couple tightly computation and experimental data, in part to use computation to make experimental tests more efficient. Moreover, we study other genomes, genetics, and biology of other nematodes to help us comprehend *C. elegans*, to learn how development and behavior evolve, and to learn how to control parasitic and pestilent nematodes.

In the area of signal transduction, we continue to define pathway interactions and to understand the determinants of signaling specificity: how does the same pathway lead to distinct outcomes in different tissues? For these studies we analyze EGF-receptor signaling, WNT signaling, and G-protein-mediated signaling pathways. We had previously found that the EGF-receptor acts in a single neuron, ALA, during this process, and acts via a phospholipase-C-gamma-mediated diacylglycerol-signaling pathway to control a sleep-like state, lethargus. We have now found two genes necessary for expression of the EGF-receptor in the ALA neuron. We are trying to find the receptor(s) and G proteins necessary for response to *C. elegans* mating pheromones.

Vulval development involves a remarkable series of intercellular signaling events 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. For example, we found that the PAX2/5/8 type homeodomain protein EGL-38 regulates the F cell type while the tx-like protein COG-1 regulates the E cell type. Regulation by the EGF-receptor, WNT and HOM-C pathways impinge not only on vulval development but also the neuroectoblast P12 specification, male hook and spicule development. By comparing these examples with vulval development, we seek to understand the signaling specificity and signal integration. The relative contributions of EGF and WNT differ in each example. Multipotent cells with three fates utilize NOTCH signaling, as well as EGF/WNT signaling.

Our efforts in genomics are experimental and computational. Our experimental genome annotation includes identifying *in vitro* binding sites for transcription factors, testing enhancer function in transgenic worms, and *de novo* assembly of genomes and transcriptomes. We are investigating ways to compare nematode genomes. In particular, we are collaborating with the Genome Sequencing Center of Washington University to annotate three new *Caenorhabditis* genomes (in addition to the two already published) and that of *Heterorhabditis* bacteriophora. We have generated a draft genome for a sixth *Caenorhabditis* species based on sequence data generated at Caltech. Our computational projects involve establishing pipelines for *cis*-regulatory computational analysis, new programs to use orthology and known binding sites or motifs, etc. We have successfully combined information from worms, flies and yeast to predict gene-gene interactions in *C. elegans* and *Drosophila*. This predicted network was used to interpret the relationship among genes expressed in several parasitic nematodes. We are part of the WormBase Consortium, which develops and maintains WormBase, a web-accessible comprehensive database of the genome, genetics and biology of *C. elegans* and close relatives (www.wormbase.org).

We have developed Textpresso (www.textpresso.org), an ontology-based search engine for full text of biological papers. Textpresso is used by *C. elegans* researchers, as well as the WormBase staff; we

have made versions for *Neuroscience* (www.textpresso.org/neuroscience) as part of the Neuroscience Information Framework and *Drosophila* (in collaboration with FlyBase (www.flybase.org). We are also part of the Gene Ontology Consortium (www.geneontology.org), which seeks to annotate gene and protein function with a standardized, organized vocabulary. We have begun to use Textpresso and other computational linguistic approaches to automate the curation process at WormBase; this year we have sped up the rate of associating proteins to Gene Ontology Cell Components about eight-fold.

Our behavioral studies focus on understanding male mating behavior, as well as locomotion of both sexes. Mating behavior, with its multiple steps, is arguably the most complex of *C. elegans* behaviors. Because it is not essential for reproduction, given the presence of internally self-fertilizing hermaphrodites, male mating 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. This year we discovered components of the mating pheromone made by hermaphrodites and sensed by males. Our comparative studies include both analyzing behavioral differences among species, and genetic analysis of *C. briggsae*, *Pristionchus pacificus* (a nematode species we discovered during the 1990s), and two insect-killing nematodes, *Heterorhabditis bacteriophora* and *Steinernema carpocapsae*. We discovered that *H. bacteriophora* uses the same sensory neuron as *C. elegans* to respond to carbon dioxide.

281. The genomes of gonochoristic versus hermaphroditic *Caenorhabditis* species

Erich M. Schwarz and the Caenorhabditis Genome Analysis Consortium

To identify candidate regulatory elements and possible genetic determinants of hermaphroditism, we have undertaken comparative analysis of three newly determined genomes from gonochoristic (dioecious) relatives of *C. elegans* (*C. remanei*, *C. brenneri*, and *C. japonica*) versus two published genomes of hermaphroditic *C. elegans* and *C. briggsae*. Core analyses include refined prediction of protein-coding genes using tools empirically optimized with control analyses of *C. elegans* (nGASP), predictions of small and non-coding RNA genes, repeat predictions, and scans for unresolved heterozygosity found in outbreeding species even after incrossing. Protein-coding genes are being examined for novel protein domains, lineage-specific domains, domain architectures, nematode-specific genes, and accelerated evolution or expansion of protein families. Whole-genome alignments allow global searches for conserved non-coding DNA and reveal syntenic gene order: these are being produced by the well-validated PECAN/ENREDO/GERP pipeline, the newly devised FSA algorithm, and other tools such as OrthoCluster and CYNTENATOR. Finally, detailed analysis of orthologous protein groups and sex-determination genes will be aimed at uncovering traces of the parallel evolution of hermaphroditism in the *C. elegans* and *C. briggsae* genomes.

282. Ultra-high throughput sequencing of amplified transcripts from individually dissected cells

Erich M. Schwarz, Miriam B. Goodman[1], Ali Mortazavi, Brian A. Williams, Lorian Schaeffer, Mihoko Kato, Martin Chalfie[2], Barbara J. Wold[2], Paul W. Sternberg

Gene expression in individual cell types of *C. elegans* has been studied through microarray analysis of populations, either by sorting of GFP-labeled embryonic culture cells or by precipitation of tagged poly(A)-binding proteins. Ultra-high throughput sequencing of cDNA (RNA-seq) allows gene expression data to be quantitated and mapped across the genome with unprecedented precision, and onto unannotated transcripts or splicing isoforms; amplification of transcripts from individually dissected cells allows these data to be generated from cells in their native context (rather than on a culture dish) and from single cells at precise moments of their development (e.g., from a migrating postembryonic cell). We are therefore, developing RNA-seq on postembryonic neurons. Our preliminary data from the AFD, ASER, and PLML neurons indicate that this technique is usable for transcript discovery: ~11,000 protein-coding genes show expression in at least one isolate, and transcripts known to be representative of these neuron types, such as *gcy-8, gcy-19*, and *mec-7* are strongly expressed. Globally, though, our individual data sets are quite noisy: for multiple isolates of a given cell type, activity for only ~500+ genes is repeatedly observed, and overall variability of observed expression is high (r-squared ≤ 0.4). To overcome this, we are testing improved amplification techniques and small pools of cells.

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283. Cell type-specific transcriptome analysis using biosynthetically-labeled RNA in *C. elegans*

Meenakshi K. Doma, Igor Antoshechkin

Cell type-specific gene expression patterns are created and maintained to facilitate cell fate decisions during development in *C. elegans*. Currently, analysis of cell-specific gene expression is limited to microarray-based strategies that include, microarray analysis of RNA from either GFP-tagged embryonic neurons using fluorescence activated cell sorting (FACS) or an mRNA tagging technique used to profile larval cells, body wall muscle, intestine and specific neurons. Both these methods are limited in their applications. To address this, we are developing a new strategy to isolate RNA from specific cells that will enable cell type-specific transcriptome analysis in *C. elegans*.

Recent studies have shown that uracil phosphoribosyltransferase (UPRT) from *Toxoplasma gondii* can be used for biosynthetic labeling of newly synthesized RNA from specific cells in multicellular organisms like *Drosophila*. To adapt this method to *C. elegans*, a primary obstacle is that although *C. elegans* has a UPRT homolog that lacks several key amino acids for activity, the bacteria it feeds on i.e., OP50 (*E. coli*) can incorporate UPRT. To circumvent this problem, we will use an *E. coli* strain lacking the UPRT gene. Preliminary observation indicates that *C. elegans* growth is not significantly affected by feeding on these bacteria. We have now designed transgenes that express TgUPRT (HA-tagged) in a spatially restricted manner using cell type-specific promoters. Single copies of the expression cassettes will be integrated in *C. elegans* using the Mos1-mediated single-copy insertion (MosSCI). On providing the uracil analog 4-thiouracil (TU), it is modified and incorporated in newly synthesized RNA only from cells that express the HA-TgUPRT. The TU-labeled RNA will be biotinylated, purified and subsequently, deep sequenced using RNAseq. The development of this method will provide *C. elegans* researchers a powerful tool for cell-type-specific gene expression studies with several potential applications.

284. Ultra-high throughput sequencing of the genome and transcriptome of *Caenorhabditis* sp. 3 PS1010

Ali Mortazavi, Erich M. Schwarz, Brian Williams, Lorian Schaeffer, Barbara Wold, Paul W. Sternberg

The *de novo* sequencing of nematode genomes has been an arduous process that involves large-scale projects working over multi-year time scales to sequence and annotate genomes. The recent advent of ultra-high throughput sequencers that are moving towards the $1,000 human genome foreshadow the coming of the "$50 worm genome" for sequencing reagents, which will afford a much larger scale whole-genome survey of the nematode phylum. In order to develop tools to analyze and annotate nematode genomes of interest using ultra-high-throughput technology, we have sequenced the genome and the transcriptome of *Caenorhabditis* sp. 3 PS1010 using 2x75 bp reads produced on an Illumina GAII. We have been able to compare our genomic DNA and cDNA sequence data to 417 kb of high-quality, annotated contigs built using traditional Sanger sequencing of PS1010 fosmids. We assembled 49 million paired reads into 65.5 Mb using the Velvet short read assembler with an N50 of 1.1 kb which achieved 95% coverage of our PS1010 contigs, for which the gene-prediction program AUGUSTUS predicted ~30,000 protein-coding genes or segments of genes. We also sequenced a pool of mixed-stage, polyA-selected RNA with over 26 million mappable reads (including 3.7 million splice-crossing reads), and found that we observed reliable signal of at least one or more Reads Per Kb per Million (RPKM) over 75% of the 108,000 AUGUSTUS-predicted exons; this includes developmental control genes expected to be expressed at low levels, such as *lin-3* and *lin-11*. By taking advantage of the paired-end RNA-seq reads, we were able to further improve our assembly using RNA-reads spanning contigs and thus, increase our N50 to 1.6 kb. The combination of ultra-high throughput sequencing of genomic DNA and of the transcriptome along with their complementary assembly provides a straightforward path for the further analysis of key species in the nematode phylum.

285. Identifying components and connections of the muscle differentiation transcription factor network in *C. elegans*

Steven Gregory Kuntz, Lorian Schaeffer, John DeModena, Brian Williams[*], Paul W. Sternberg, Barbara Wold[**]

Several evolutionarily ancient gene regulatory networks (GRNs) control tissue differentiation in *C. elegans*. We want to learn which components and connections of tissue differentiation GRNs are invariant over evolution, which are variable, and what functional consequences come from the variations. Embryonic body-wall muscle differentiation in *C. elegans* is very accessible and several key transcriptional regulators are known, including *hlh-1* (myogenic regulatory factor), *hnd-1* (TWIST/hand protein), and *unc-120* (SRF/MEF protein). Nevertheless, we do not know all the components, nor how they are connected with each other and their target genes. Also, the network appears to be resistant to mutations, continuing to drive differentiation even when core transcriptional regulators are mutated. To expand our knowledge of myogenic factors, we first performed a synthetic lethal screen to identify additional core network transcription factors. By feeding RNAi knock down in an *hlh-1* mutant background, we identified multiple transcription factors required cooperatively for muscle differentiation. We have found a number of potentially interesting new components – including *ceh-51* and *hmg-1.2* – and are asking how these factors interact with each other to drive muscle differentiation, as well as how they affect downstream muscle gene expression. To further understand factor-factor interactions, we are knocking down each core factor (by dsRNA microinjection) in all available core factor mutant backgrounds thus, revealing cross-interactions. For understanding of downstream expression, we are looking fluctuations in transcript level through RNA-seq in the mutant backgrounds. Finally, to understand how these genetic cross-interactions between transcription factors appear to 'buffer' the downstream transcript changes, we are using ChIP-seq to look at differential binding of RNA Polymerase II in mutants for several myogenic transcription factors.

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286. Neuron-specific regulatory motifs in *C. elegans*

Carmie Puckett Robinson, Erich M. Schwarz

Neurons express a largely overlapping set of genes that convey the neuronal phenotype. The individual identity of each neuron or neuron type is determined by the expression of a subset this larger set of genes that are expressed during terminal differentiation and the life of the cell. The regulatory sequences that determine the expression of this terminal gene battery are poorly understood and a challenging problem in both vertebrates and invertebrates due to the cellular complexity of the nervous system. This question has been approached by examining genes expressed in the terminal gene battery to identify cell-specific motifs sufficient to drive expression in select neurons. This approach has been used in conjunction with phylogenetic comparisons of non-coding regions between closely related species to identify conserved regions that can be shown experimentally to be enriched for regulatory motifs.

Potassium channels are widely expressed and set resting potentials in cells and are required for neuronal activity. In *Caenorhabditis elegans*, there are estimated 70 potassium channels with a large subclass of TWK potassium channels that are encoded by 46 *twk* genes. The *twk* genes encode channel subunits that are orthologous to 11 potassium channels in *Drosophila* and 15 potassium channels in humans. In *C. elegans*, TWK potassium channels are expressed in a restricted number of neurons. The only neuron identified in the tail of the animal to express the *twk-16* potassium channel is a single interneuron DVA. This restricted expression is determined by the *twk-16* enhancer element.

Using phylogenetic comparisons and mutagenesis we identified regions of sequence within the *twk-16* enhancer that have positive and negative effects on expression. These sequence regions were contained within a 53 bp region of the *twk-16* intronic enhancer. Within this 53 bp region there were: (1) a 24 bp region that drives expression in DVA and other neurons (2) a 8 bp region which when mutated abolishes expression in DVA and all other neurons; and (3) a 18 bp region of sequence which when mutated de-represses expression in neurons in multiple regions of the nervous system and also produces ectopic expression in non-neuronal cells.

We identified sequence regions in a 53 bp region of the *twk-16* enhancer that both activate and repress expression in DVA and other neurons. Restricted expression of the *twk-16* gene relies on a combination of sequence regions with both positive and negatives effects on expression in DVA and other neurons. These results are also consistent with there being additional unidentified regions within the *twk-16* enhancer not identified by conservation that drive expression in DVA and repress expression in other neurons. We are currently studying a GA-rich motif found in the *twk-16* enhancer that is shared by two other highly conserved regulatory regions in the *nmr-1* (NMDA receptor) and *fax-1* (nuclear hormone receptor) genes that conveys expression in the DVA neuron. These results are consistent with a surprisingly complex architecture to the regulatory sequences within the *twk-16* enhancer that determine the restricted expression of the *twk-16* gene.

287. RNA Pol II accumulates at promoters of growth genes during developmental arrest

L. Ryan Baugh, John DeModena

When *Caenorhabditis elegans* larvae hatch from the egg case in the absence of food, their development is arrested (L1 arrest), and they show increased stress resistance until food becomes available. To study nutritional control of larval development, we analyzed growth and gene expression profiles during L1 arrest and recovery. Larvae that were fed responded relatively slowly to starvation compared with the rapid response of arrested larvae to feeding. Chromatin immunoprecipitation of RNA polymerase II (Pol II) followed by deep sequencing showed that during L1 arrest, Pol II continued transcribing starvation-response genes, but the enzyme accumulated on the promoters of growth and development genes. In response to feeding, promoter accumulation decreased, and elongation and messenger RNA levels increased. Therefore, accumulation of Pol II at promoters anticipates nutritionally controlled gene expression during *C. elegans* development.

288. Metazoan operons accelerate transcription and recovery rates

Alon Zaslaver, L. Ryan Baugh

Existing theories efficiently explain why operons are advantageous in prokaryotes, but their emergence in metazoans is still an enigma. We present a combination of genomic meta-analysis, experiment and theory to explain how operons could be adaptive during metazoan evolution. Focusing first on nematodes, we show that operon genes, typically consisted of growth genes, are significantly up-regulated during recovery from multiple growth-arrested states, and that this expression pattern is anti-correlated to the expression pattern of non-operon genes. In addition, we find that transcriptional resources are initially limited during arrest recovery, and that recovering worms are extremely sensitive to any additional limitation in transcriptional resources. By clustering growth genes into operons, fewer promoters compete for limited transcriptional machinery, effectively increasing the concentration of transcriptional resources and accelerating growth during recovery. A simple mathematical model of transcription dynamics reveals how a moderate increase in transcriptional resources can lead to a substantial enhancement in transcription rate and recovery. We find evidence for this design principle in different nematodes (e.g., *Brugia malayi* and *Pristionchus pacificus*), as well as in the chordate *Ciona intestinalis*. As recovery from a growth arrested state into a fast growing state is a physiological feature shared by many metazoans, operons could evolve as an evolutionary solution to facilitate these processes.

289. Neuronal regulation of ascaroside response during mate response behavior in the nematode *Caenorhabditis elegans*

Jagan Srinivasan, Fatma Kaplan[1], Chirag Pungaliya[2], Arthur S. Edison[1], Frank C. Schroeder[2], Paul W. Sternberg

Small-molecule signaling plays an important role in the biology of *Caenorhabditis elegans*. We have previously shown that ascarosides, glycosides of the dideoxysugar ascarylose regulate both development and behavior in *C. elegans* [1]. The mating signal consists of a synergistic blend of three dauer-inducing ascarosides, ascr#2, ascr#3, and ascr#4. The ascarosides ascr#2 and ascr#3 carry different though overlapping information, as ascr#3 is more potent as a male attractant than ascr#2, whereas, ascr#2 is slightly more potent than ascr#3 in promoting dauer formation. Using differential analysis of NMR spectra (DANS), we have now identified additional ascarosides in the *C. elegans* metabolome [2]. Biological testing of synthetic samples of these compounds revealed additional evidence for synergy and provided insights into structure-activity relationships. Two types of neurons, the ASK neurons and the male-specific CEM neurons, are required for male attraction by ascr#3. We are currently testing neuronal and genetic requirements for the response to the new ascarosides discovered using DANS.

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290. A shortcut to identifying small molecule signals regulating behavior and development in *Caenorhabditis elegans*

Chirag Pungaliya, Jagan Srinivasan, Bennett W. Fox, Rabia U. Malik, Andreas H. Ludewig, Paul W. Sternberg, Frank C. Schroeder

Small molecule metabolites play important roles in *C. elegans* biology, but effective approaches for identifying their chemical structures are lacking. Recent studies revealed that a family of glycosides, the ascarosides, differentially regulate *C. elegans* development and behavior. Low concentrations of ascarosides attract males and thus, appear to be part of the *C. elegans* sex pheromone, whereas, higher concentrations induce developmental arrest at the dauer stage, an alternative, non-ageing larval stage. The ascarosides act synergistically, which presented challenges for their identification *via* traditional activity-guided fractionation. As a result the chemical characterization of the dauer and male attracting pheromones remained incomplete. Here we describe the identification of several additional pheromone components using a recently developed NMR-spectroscopic approach, DANS, which simplifies linking small molecule metabolites with their biological function. DANS-based comparison of wild-type *C. elegans* and a signaling-deficient mutant, *daf-22*, enabled identification of three known and four previously undescribed ascarosides that feature novel structural motifs including a *p*-aminobenzoic acid subunit. Biological testing of synthetic samples of these compounds revealed additional evidence for synergy and provided insights into structure-activity relationships. Using a combination of the three most active ascarosides allowed full reconstitution of the male-attracting activity of wild-type pheromone extract. Our results highlight the efficacy of DANS as a method for identifying small molecule metabolites and placing them within a specific genetic context. This study further supports the hypothesis that ascarosides represent a structurally diverse set of nematode signaling molecules regulating major life history traits.

291. Natural variation and selection in locomotory phenotypes

Adler R. Dillman, Christopher J. Cronin, Charles F. Baer (Florida)

A major motivation behind establishing *C. elegans* as a model system was to understand the influence of genes on behavior. Some behaviors, however, have proven to be difficult to quantitatively evaluate. We used a well-established automated tracking system, which analyzes the body posture of nematodes over time and extracts quantitative information concerning their movement. Quantitatively assessing phenotypic differences in locomotory data provides a unique opportunity to better understand the phylogenetic diversity of *Caenorhabditis* nematode locomotion. Using this system, we evaluated the level of detectable variation in locomotory phenotypes in nine different *Caenorhabditis* species and eight natural isolates of *C. elegans*. Aside from phenotypic variation among and between species, we are interested in understanding the relative roles of mutation and natural selection involved in driving phenotypic variation in locomotion. We report direct measurements of trait variation in natural isolates and trait variation resulting from mutation alone by utilizing seven different mutation accumulation (MA) lines; allowing us to quantify the extent to which purifying selection promotes stability in locomotory behaviors. Our data suggest that strong stabilizing selection acts to shape locomotory behavior in *C. elegans* and that certain subsets of locomotion traits coevolve.

292. Stimulation of jumping behavior in steinernematid nematodes (Steinernematidae) by host associated semiochemicals

Adler R. Dillman

Entomopathogenic nematodes (EPN) are lethal insect parasites that are currently being used and further explored as biological control agents and alternatives to chemical pesticides. Several species of EPN in the genus *Steinernema* are capable of jumping, which is exceptionally rare for soft-bodied, limbless invertebrates, Jumping behavior is thought to serve the dual purposes of acute host finding/predation and dispersal. We explored the predatory aspects of jumping behavior in several species of *Steinernema* and have shown that jumping species can detect and respond to the presence of host associated volatile compounds. We have further shown that this response varies interspecifically and likely reflects niche partitioning. We identified several semiochemicals emitted by known and supposed insect hosts of EPN by GC-MS analysis and have shown differential jumping responses to individual volatiles. Ongoing and future studies will be directed at further exploring the capacity of EPN to distinguish insect hosts by olfaction, elucidating the neural circuitry involved in detection of host volatiles and in jumping, and exploring the evolution and maintenance of jumping within Steinernematidae.

293. Transfer at a thermosensory synapse in *C. elegans*

Anusha Narayan, Gilles Laurent, Paul W. Sternberg

C. elegans is an attractive model organism for neural circuit analysis. In order to characterize the functional dynamics of the circuits that control behavior, it is necessary to understand the synaptic transformations that take place. Thermotaxis is an established behavior in *C. elegans*. We attempt to characterize the transfer function at a prominent synapse within the thermotactic circuit. AFD is the primary thermosensory neuron, and AIY is its principal post-synaptic partner. We drive expression of Channelrhodopsin-2, a light-activated cation channel, solely in AFD using a cell-specific promoter, and use whole cell patch clamp recording techniques to measure the light-evoked synaptic response at AIY. We are able to reliably activate the presynaptic cell AFD with blue light, evoking depolarization of up to 40 mV and inward currents of up to 15 pA, with responses lasting the duration of the stimulus. The postsynaptic response at the AIY cell body is small - less than 5 mV, with inward currents of less than 1 pA, and appears to be graded and tonic, lasting the duration of the stimulus. Our results indicate that this synapse has low gain, and transmits information from AFD to AIY with short latencies and high fidelity. It will be interesting to see how AIY uses this information and integrates it with other incoming streams, and to examine processing further downstream in the thermosensory circuit.

294. Sensory regulation of male-specific motor behaviors of *C. elegans*

Allyson J. Whittaker

To survive and reproduce, animals must modify their motor behavior in response to changes in their environment. To understand how animals modify motor behavior in response to sensory input, we are studying male mating behavior in *C. elegans*. This is a particularly powerful model because, as the hermaphrodite does not play a cooperative role in mating and continues moving, the male must modify his movement and body posture in order to maintain contact and copulate with the hermaphrodite. This requires the coordination of the activity of multiple muscle groups in response to both external passive cues from the hermaphrodite, as well as internal proprioceptive cues.

During mating, the most dramatic changes in male body posture are seen in its tail, which, as it contains almost all of the sensory structures required for copulation, must remain in contact with the hermaphrodite. Thus, a focus of our work is to understand the motor circuits regulating male tail posture. Previous studies have shown that serotonin regulates ventral curling of the male tail, and this requires male-specific muscles. Using genetic and laser ablation analysis, in conjunction with behavioral assays, we have expanded our understanding of neurotransmitters, receptors, neurons and muscles required for the regulation of male tail posture. We find that proper tail posture is maintained by coordination of sex-specific and core muscle groups that bend the tail ventrally and dorsally, a model that fits with the male tail wiring data. We demonstrate that cholinergic neurons, acting, at least in part, independently of serotonin, regulate both dorsal and ventral curling of the male tail and this requires dorsal body wall muscles, and male-specific muscles, respectively. Cholinergic regulation of tail posture requires GABA. Males with mutations in the GABA receptor *unc-49* over curl their tails during mating suggesting that cross inhibition of muscle groups is important to maintain proper tail posture.

We are also interested in the sensory pathways that regulate male motor behaviors. Our results show that the proprioceptive mechanosensory TRPN channel, *trp-4*, is required for males to remain at the vulva, and for efficient turning. We find that *trp-4* is expressed in the postclocal sensilla and hook neurons and male-specific muscles and we are currently determining the site of action.

295. Spatio-temporal regulation of the dauer decision

Oren Schaedel

C. elegans dauer larvae are an alternative third larval stage that are stress-resistant, non-feeding and are analogous to infective juveniles of some parasitic nematodes. The dauer decision integrates food abundance, population density and temperature. Dauer frequency in a population increases as conditions shift from favorable (e.g., high food concentration and low population density)

to unfavorable (e.g., low food concentration and high population density). This decision is coordinated over the whole animal resulting in dauer or L3 fates, as wild-type animals do not display mosaicism of either fate. *daf-9* and *daf-12*, which encode a cytochrome P450 and a nuclear hormone receptor, respectively, act downstream the insulin and TGFΔ signaling pathways. Dafachronic acid, a lipophilic hormone product of DAF-9, works cell non-autonomously directing L3 programs. Dafachronic acid binds to DAF-12, which then upregulates hypodermal expression of *daf-9* via a positive feedback loop. We hypothesize that information from environmental sensing is integrated through cellular and molecular mechanisms and reduced to a single cell non-autonomous regulator, thereby explaining the tight binary nature of the decision. We propose a fate coordination mechanism in which secretion of a small amount of hormone is propagated across the anterior-posterior axis, locking in the L3 fate. Specifically, we show that Δ7-dafachronic acid is necessary for the L2d/L3 fate transition and execution of L4 programs. Using a combination of laser ablations and time-lapse image analysis, we demonstrate that upon L2d to L3 decision, XXXL/R cells act as a source releasing Δ7-dafachronic acid. As a result, hypodermal *daf-9* expression propagates via the DAF-12 positive feedback loop from anterior to posterior, generating high amounts of dafachronic acid. Moreover, we show that high amounts of Δ7-dafachronic acid are required for proper distal tip cell migration, an L4 program, suggesting that this is a fate locking mechanism. This mechanism of positive feedback acting on a small amount of signal has been shown in other cell fate decisions and can help explain how one fate is propagated in a coordinated manor across the whole organism.

296. Development and function of the sleep-inducing ALA neuron of *C. elegans*

Cheryl Van Buskirk

The impact of sleep disorders on human health has underlined the need for a greater understanding of the regulation of sleep. Such studies have largely focused on mammalian systems, but recently sleep-like states have been described in invertebrates, including *C. elegans* (Raizen *et al*., 2008). Importantly, some of the factors regulating sleep behavior appear to be highly conserved, such as signaling through the Epidermal Growth Factor Receptor (EGFR) (Van Buskirk and Sternberg, 2007). In *C. elegans*, EGF-induced sleep is marked by a cessation of feeding and locomotion and is normally restricted to a short period preceding each larval molt. However, this sleep-like state can be induced at any time during development or adulthood through conditional overexpression of LIN-3/EGF. The effects of EGF expression in *C. elegans* are strikingly similar to those seen in response to EGF administration in mammals, pointing to an evolutionarily conserved role for EGF signaling in the regulation of behavior.

We have found that EGF-induced sleep is triggered by activation of the EGF receptor (LET-23) within a single neuron called ALA, and we have identified several EGF-resistant mutants that define components of a signal transduction pathway mediating this effect. In addition, we have identified a class of mutations that render animal's EGF-resistant due to defects in ALA-specific gene expression, including impaired *let-23* transcription. Lastly, using a combination of genetic analyses and laser ablations, we have investigated the mechanism by which the ALA neuron signals to effect sleep behavior. We will present the results of these analyses on the development and function of the ALA neuron.

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297. Looking for ALA-independent sleep

Julie Cho

Both the conservation of sleep across animal species and the impact of disordered sleep on health point to a critical function and highlight a need for greater understanding of sleep regulation. Previous work by Van Buskirk and Sternberg has shown that the ALA neuron in *Caenorhabditis elegans* is involved in inducing a quiescent state in the worm via the EGF/LET-23 signaling pathway. Ablation of the ALA neuron results in hyperactivity during lethargus, the normally quiescent period that precedes each larval molt. In parallel work by Raizen and colleagues, lethargus was shown to satisfy the behavioral criteria of sleep. Furthermore, the quiescence during lethargus was shown to be dependent on the activity of EGL-4, a cGMP-dependent protein kinase (PKG). An *egl-4* loss-of-function mutation results in disruption of sleep-like behavior.

Despite the fact that EGFR activation in the ALA neuron is sufficient to induce a strikingly quiescent state, ALA ablation in wild-type animals leads to a relatively mild loss of quiescence during lethargus. Thus, it is likely that other pathways contribute to the sleep-like state. We are interested in characterizing the EGF-independent signaling pathways regulating sleep in *C. elegans*. In order to determine whether EGL-4 activity contributes to EGF-dependent or EGF-independent sleep, we have studied the interaction between the EGL-4 and ALA-ablated hyperactive phenotypes. We have found that the absence of the ALA did not significantly alter locomotion in the *egl-4(lf)* mutants during lethargus. These results are consistent with *egl-4* and ALA acting in a common pathway. We aim to look for alternative pathways that may be involved in regulating sleep.

298. CO_2 response and host seeking in free-living and parasitic nematodes

Elissa A. Hallem

Parasitic nematodes are a major health concern worldwide, and current strategies for preventing or eliminating nematode infections are insufficient. One possible control strategy is to interfere with the ability of nematodes to locate hosts. Carbon dioxide is an important host-seeking cue for many parasitic nematodes, yet little is known about the mechanism of CO_2 response in nematodes. We found that *C. elegans* displays acute CO_2 avoidance: exposure to CO_2 results in the cessation of forward movement and the initiation of backward movement. We found that multiple signaling molecules affect CO_2 avoidance, including TAX-2/TAX-4, RGS-3, TAX-6, and NPR-1. Nutritional status also modulates CO_2 responsiveness via the insulin and TGF-β pathways. Acute CO_2 avoidance is mediated primarily by the BAG neurons, and TAX-2/TAX-4 are required in the BAG neurons for CO_2 response. Additional ciliated sensory neurons also contribute to CO_2 response. We are now extending this analysis to other nematodes. We have found that the BAG neurons are required for CO_2 avoidance in the necromenic worm *Pristionchus pacificus*, and for CO_2 attraction in the insect-parasitic nematode *Heterorhabditis bacteriophora*. We are now investigating the mechanism by which analogous neurons mediate attraction in parasitic nematodes and repulsion in free-living nematodes. We are also investigating how CO_2 response changes depending on the life stage of the worm. Finally, we are examining behavioral responses to other potential host-seeking odorants in *H. bacteriophora* and a different insect-parasitic nematode, *Steinernema carpocapsae.* We have found that the two species show very different odor response spectra, raising the possibility that some of the tested odorants contribute to host specificity.

299. Toward characterization of bacteria-to-nematodes HGT

Amir Sapir, Alon Zaslaver, John DeModena*

Horizontal gene transfer (HTG) is a fundamental process among unicellular organisms for acquiring new traits. Although initially thought to be extremely rare in metazoans, recent whole-genome sequencing projects reveal extensive gene transfer from prokaryotes to metazoans. This type of gene transfer is particularly relevant for symbiotic organisms that occupy new niches, where survival requires acquisition of new genes not previously present in the organism's gene pool. For example, hemi-cellulose hydrolysis, induced by plant parasitic nematodes, is thought to have been acquired by the transfer of bacterial genes to the plant parasites' bacteriovorous ancestors. In contrast to HGT between bacteria, the sequence of events leading to bacteria-to-nematodes HGT, as well as the molecular details of this process, remain elusive. So far, mechanistic studies of HGT in metazoans have been hindered by its rare occurrence, and the fact that symbiotic organisms are usually not suitable for long in-lab evolutionary studies.

Our aim is to study HGT by combining the powerful genetics of *E. coli* (the donor) and *C. elegans* (the recipient). Specifically, we use the transfer of the *unc-119* rescuing gene from *E. coli* to *unc-119* mutant worms as an indicator for successful HGT. Rescued worms are examined to validate that gene transfer indeed happened, and these worms will be further analyzed to decipher the exact mechanism by which HGT occurred. We predict that the problem of identifying rare HGT events can be overcome by our experimental settings that involve growing multiple generations of worms in large numbers under specific selection. In a pilot experiment, we grew $4x10^6$ worms per generation over seven generations on *E. coli* carrying the *unc-119* rescuing gene and validated that this approach is suitable for HGT studies. If HGT will not be identified, we will employ different conditions and genetic backgrounds that might increase HGT probabilities: **i)** Use of *C. elegans* mutants promoting bacterial propagation in the worm's gut; **ii)** Exposing the cultured worms to various stress conditions; and **iii)** Inducing HGT by *unc-119* gene transposition in the *E. coli* donor. Ultimately, this system will serve as an empirical framework to elucidate the enigmatic process of bacteria-to-nematodes HGT.

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300. *nhr-67/Tailless* regulates a stage-specific program of linker cell migration during male gonadogenesis

Mihoko Kato

Cell migrations are common events during organogenesis, yet little is known about how migration is temporally coordinated with organ development. The linker cell (LC), an individual, male-specific cell, leads the long-range migration of the developing male gonad from the early L2 stage to the mid-L4 stage, as it travels along the bodywall and executes turns to connect the gonad to the developing proctodeum. We have found that *nhr-67* regulates one temporal subset of changes in the LC from the early L3 to the mid-L4 larval stage. Gonad migration up to the early L3 stage is normal in *nhr-67*(RNAi) males, but is subsequently, much slower than in wild-type males. The migrating LC changes its position, gene expression, and cell shape in wild-type males during the L3 and L4 stages; *nhr-67* is required for each of these changes to occur at their normal time. Specifically, *nhr-67* is required to inhibit *unc-5*/netrin receptor expression in the LC in the mid-L3 stage, and to activate *zmp-1*/zinc metalloprotease expression in the L4 stage. In *nhr-67*(RNAi) animals, *unc-5* continues to be expressed after the L3 stage, resulting in a delayed second LC turn, which requires the downregulation of *unc-5*; meanwhile, *zmp-1* is not expressed in L4 stage LCs. The LC normally changes from a round shape in L3 larvae to increasingly polarized arrowhead shapes in L4 larvae, but it remains round throughout most of the L4 stage in *nhr-67*(RNAi) animals.

These LC changes are not induced by spatially restricted cues at the L4 stage LC position. In *nhr-67*(RNAi) animals, however, the LC undergoes late L4 stage changes normally, including developing into an extremely polarized shape and undergoing cell death. Also, genes that are expressed in L3 and L4 larvae throughout LC migration (such as *lag-2*, *him-4*, *gon-1*, and *mig-2*) have unchanged expression in *nhr-67*(RNAi) larvae. We thus, propose that LC migration consists of a basal migration program and stage-specific modifiers, like *nhr-67* and at least one other potential transcription factor, which provide temporally appropriate programs.

301. Wnt and FGF signaling control vulval secondary lineage polarity

Paul Minor, Anand R. Asthagiri, Paul W. Sternberg

The *C. elegans* vulva is formed from divisions of three vulval precursor cells (VPCs): P5.p, P6.p, and P7.p. P5.p and P7.p are induced and divide to form a 2° lineage pattern in which the daughter cells of P5.p and those of P7.p form a mirror. The orientation of these cells is established by the interaction of multiple Wnt signals: Wnts LIN-44 and MOM-2 act through Fz/LIN-17 and Ryk/LIN-18, respectively, to promote the wild-type, anterior-facing P7.p, vulval lineage, termed refined polarity, whereas, Wnt EGL-20 acts through VANG-1 and Ror/CAM-1 to promote posterior-reversed vulval lineage (P-Rvl) of the P7.p daughter cells, termed ground polarity. Here we show that additional components of the planar cell polarity (PCP) pathway, as well as the FGF pathway control vulval orientation. Our data suggest that DSH-1 and DSH-2 (two homologs of the PCP and canonical Wnt pathway protein Dishevelled) work downstream of EGL-20 orienting P7.p to face the posterior. In contrast, the third Dishevelled homolog, MIG-5, as well as FMI-1, the homolog of the PCP protein Flamingo, act in the pathway leading to refined polarity. Finally, preliminary data suggests that the FGF pathway also controls polarity in the vulva. FGFR/EGL-15 and its adaptor protein, SEM-5, both act to orient P7.p to the anterior, based on both single mutant phenotypes, as well as genetic interactions with a *lin-18* mutation. Thus, we implicate a fourth signaling pathway in the orientation of VPC lineages, and raise the question of how Wnt and FGF signaling are integrated in VPC lineages.

302. Semi-automated curation of protein subcellular localization

Kimberly Van Auken, Josh Jaffery, Juancarlos Chan, Hans-Michael Müller, Paul W. Sternberg

Manual curation of experimental data from the biomedical literature is an expensive and time-consuming endeavor. Nevertheless, most biological knowledge bases still rely heavily on manual curation for data extraction and entry. Text mining software that can semi- or fully-automate information retrieval from the literature would thus, provide a significant boost to manual curation efforts. We used our category-based information retrieval and extraction system (Textpresso; http://www.textpresso.org), to explore how we might improve the efficiency with which we manually curate *C. elegans* proteins to the Gene Ontology's Cellular Component Ontology. Using a training set of sentences that describe results of localization experiments in the published literature, we generated three new curation task-specific categories (Cellular Components, Assay Terms, and Verbs) containing words and phrases associated with reports of experimentally-determined subcellular localization. We compared the results of manual curation to that of Textpresso queries that searched the full text of articles for sentences containing terms from each of the three new categories plus the name of a previously uncurated *C. elegans* protein, and found that Textpresso searches identified curatable papers with recall and precision rates of 79.1% and 61.8%, respectively (F-score of 69.5%), when compared to manual curation. Within those documents, Textpresso identified relevant sentences with recall and precision rates of 30.3% and 80.1% (F-score of 44.0%). From returned sentences, curators were able to make 66.2% of all possible experimentally supported GO Cellular Component annotations with 97.3% precision (F-score of 78.8%). Measuring the relative efficiencies of Textpresso-based versus manual curation we found that Textpresso has the potential to increase curation efficiency by at least 8-fold, and perhaps as much as 15-fold, given differences in individual curatorial speed. Textpresso is an effective tool for improving the efficiency of manual, experimentally-based curation. Incorporating a Textpresso-based Cellular Component curation pipeline at WormBase has allowed us to transition from strictly manual curation of this data type to a more efficient pipeline of computer-assisted validation. Continued development of curation task-specific Textpresso categories will provide an invaluable resource for genomics databases that rely heavily on manual curation.

303. WormBook updated and expanded

Jane E. Mendel, Qinghua Wang, Martin Chalfie[*]

WormBook (http://www.wormbook.org/) is a comprehensive, open-access collection of original peer-reviewed chapters covering the biology of *C. elegans* and other nematodes. WormBook also includes WormMethods, a collection of protocols for nematode researchers. Wormbook now includes over 140 published chapters; an additional 46 chapters are currently in preparation. WormBook appears in the NCBI Bookshelf and has been indexed in PubMed since the fall of 2007, greatly increasing its visibility in the research community. In 2008, WormBook expanded its discussion forum so that readers could comment on individual chapters. Our goal in making the discussion boards more prominent and easily accessible was to stimulate an open and lively discussion on WormBook topics. At this time we are soliciting revisions for chapters published in 2005 and 2006, and we are also adding introductory chapters to each section. Major upcoming changes to WormBook include the expansion of our coverage of nematodes other than *C.*

elegans. We are currently launching three new sections covering free-living and plant and animal parasitic nematodes. In addition, a new section on using nematodes in teaching is planned. We are also making major changes to the organization and content of WormMethods to make this section more complete, up-to-date and user-friendly. Lastly, WormBook is excited to announce the return on the 'Worm Breeder's Gazette' in a new on-line form. This informal newsletter will be linked from WormBook and will provide a non-reviewed platform for communicating preliminary results, new methods and tips for researchers using *C. elegans* and other nematodes.

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Summary: Our group is interested in the composition, evolution and function of gene regulatory networks. We often use muscle development, degeneration, and regeneration as a model system. This network is one among a large class whose members govern how cell fates and states are specified, executed and maintained. The approaches we take to these problems make heavy use of genome-wide and proteome-wide assays to extract connectivity relationships and evolutionary relatedness. Evolution of networks is studied in collaboration with Paul Sternberg and his group to compare mammalian and worm networks, and with John Allman and his group to compare selected brain networks of primates and rodents.

A longstanding goal for us is to understand regulatory events that drive the progression of multipotential mesodermal precursor cells or adult stem cells to become determined unipotential muscle progenitors and, finally, fully differentiated cells. We currently study these cell states and transitions using functional genomics tools based on high throughput DNA sequencing methods (RNA-Seq, ChIP-Seq etc., coupled with comparative genomics. In mouse and other vertebrates, the muscle 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 trigger nonmuscle recipient cells enter, and often to execute, the myogenic differentiation program. Our working model emphasizes that MyoD family factors are able to do this because they are highly connected and cross talk within their group and with other collaborating factors (like MEF2 factors) and other regulators that now include microRNAs. It is also clear that negative regulators of skeletal myogenesis are probably just as important for regulating the outcome, as are the positive regulators. 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. This focuses attention on defining the *in vivo* target repertoire for the repressors, and learning their relationship with the positive-acting regulators.

To obtain the genome-wide input and output maps for multiple activating and repressing regulators, we were motivated to develop ChIPSeq and RNASeq methods (Johnson *et al*., 2007; Mortazavi, Williams *et al*., 2008 and below). We have been working to add to and improve these methods and the related informatics, which are increasingly concerned with integrating these diverse datasets. The basic methods produce high resolution *in vivo* protein:DNA interaction maps and quantitative RNA expression profiles. By mapping *in vivo* factor occupancy maps for key regulators and learning how the maps change from one cell state to another, members of the group are trying to address some longstanding puzzles: What distinguishes sequence motifs in the DNA that are actively bound by their cognate regulators *in vivo* from other motif instances that are identical in sequence but are not similarly bound? How does factor occupancy relate to transcriptional output of target genes? What is the target promoter repertoire for a given bound regulator? Work by Brian Williams, Ali Mortazavi, Gordon Kwan, Tony Kirilusha, Georgi Miranov, Katherine Fisher and Gilberto DeSalvo contribute to this series of studies.

The MUSSA/MUSSAGL informatics tools (previous work of Tristan DeBuysscher, Diane Trout and Brandon King) have been used to find candidate conserved regulatory elements in both worm and mammalian phyla. This is part of an ongoing partnership with the Sternberg lab that involves several joint projects (see Steven Kuntz and Ali Mortazavi entries below and in the Sternberg lab section). The ultimate goal is to learn enough about the structure and function of cis-regulatory elements to be able to identify them in the genome computationally and to be able to predict their functional output with minimal new experimental data. At the network level, we seek insights into the evolution ancient gene networks that drive myogenesis across the large phylogenetic distances separating worms, mice and humans

To define protein:protein complexes in the network more comprehensively, we developed a collaborative effort with Ray Deshaies and Sonya Hess in the Beckman Proteome Exploration Lab. This enabled postdoctoral fellow Libera Berghella to identify a repressor activity (encoded by the MSY3/csda gene) that acts on myogenin in mature myotubes (Berghella *et al*., 2008). It operates through a very highly conserved *cis*-acting DNA sequence element in myogenin, which appears to have two distinct functions, one acting in early development to open the gene for transcription, and a second, repressive function that operates later on during muscle innervation and maturation.

Our group is also part of the NIH Project ENCODE, in collaboration the Rick Myers, Wing Wong and Gavin Sherlock groups at Stanford and Hudson Alpha Institute. In this project we contribute measurements of transcription factor:genome interactions and transcriptome structure in human ES cells and other cell lines and develop methods of analysis, including a study of RNA

editing across the transcriptome (see entry by Ali Mortazavi, and SoCAL BSI summer interns, Wendy Lee and Alicia Rogers).

304. Protein:DNA interaction maps by ChIP-Seq

Ali Mortazavi, Ken McCue, Shirley Pepke, Rick Myers (and group)

In vivo protein-DNA interactions connect each transcription factor with its direct targets to form a physical gene network scaffold. Although much is known about transcription factor binding and action at specific genes, the improvement we were able to make with ChIP-Seq (Johnson *et al*., 2007) was to make it relatively routine to map all sites of interaction in large genomes (as well as smaller ones), and to do it with resolution of 50 to 100bp for single point sources. Direct physical interactions between transcription factors or cofactors and the chromosome is detected by chromatin immunoprecipitation. In ChIP experiments, an immune reagent specific for a DNA-binding factor is used to enrich target DNA sites to which the factor was bound in the living cell. The enriched DNA sites are then identified and quantified.

We first used the NRSF repressor to work out the ChIPSeq method because we had previously generated a detailed genome-wide computational model for its target sites along with a large set of individually measured, validated true positive and true negative sites (Mortazavi *et al*., 2006). The Myers/Wold joint project for NHGRI ENCODE program has added over 30 interactomes. A focus this year has been on developing methods for integrating diverse transcription factor, chromatin mark, and transcriptome information to explain how factor occupancy and chromatin maps are related to - and ultimately regulate - the transcriptome output. Principle components analysis, SOMs (self organizing maps), and regression analysis are all being explored for this purpose. Additional informatics work includes later generations of ChIP signal processing software (Mortazavi) of more advanced methods for detecting ChIPSeq signals and for subdividing compound regions of factor occupancy into their individual contributing sites.

305. RNA-Seq: Transcriptome discovery and quantification

Brian Williams, Ali Mortazavi, Georgi Miranov, Lorian Schaeffer, Gordon Kwan

Transcription profiling is powerful tool for phenotyping cells, tissues, and entire organisms, but it is of greatest interest to us at this time as a measure the output of gene regulatory networks. An ideal transcription profile has a low signal to noise ratio and is a comprehensive survey of all parts of all genes. We developed methods and informatics for transcriptome profiling by high throughput DNA sequencing (called RNASeq) (Mortazavi and Williams *et al*., *Nature Methods*, 2008), because it overcomes many limitations of microarray systems. Using the Solexa/ Illumina sequencing instrument, a second generation of RNA-Seq is being developed. Changes in the type of data include the increase from very short reads of 25-36bp to 100bp dual end reads (i.e., 100bp from each end of a given cDNA fragment). In addition, specific strand information has been obtained. These improvements in the data are driving relevant improvements in the associated software both locally and in a collaboration with Cole Trapnell and Lior Patcher from the Berkeley Department of Applied Mathematics. Our primary software package for short DNA read functional genomics (ERANGE) counts and combines the aligned sequence reads within the known borders of transcription units for message quantification. Immediate applications of transcriptome methods are to correlate the global skeletal muscle RNA measurements with regulatory factor binding and activity in mouse muscle and in *C. elegans* muscle (see Kuntz, Williams, Fisher, Kirilusha and Mortazavi entries) and to map genes expressed in key cortical sites and neuronal populations in human and primate brains (see Tetreault and Abelin entries and Allman section). By mapping the physical connection points in the gene regulatory model with its transcriptional output, we hope to infer pertinent parts of the regulatory logic that drives differentiation of skeletal muscle.

306. Skeletal muscle transcriptional regulatory network

Barbara Wold, Brian Williams, Anthony Kirilusha, Katherine Fisher, Georgi Miranov, Gordon Kwan, Sandy Sharp, Shirley Pepke, Ali Mortazavi

Progression from undifferentiated myoblast precursor cells to differentiated cells is regulated primarily at the level of gene transcription. Genome-scale experimental approaches allow us to assay changes in the transcriptome during the differentiation step, and also to map changes in transcription factor binding patterns on the genome and associated changes in chromatin structure. A first analysis of such data asks how changes in factor binding patterns are related to changes in the RNA population expressed. Transcription factor-site occupancy maps are being made immunoprecipitating chromatin preparations from the C2C12 skeletal muscle cell line using antibodies key regulators of muscle differentiation. Shared analysis work in the project is to integrate occupancy maps and transcription output for factors that now include MyoD, myogenin, E47, Mef2, NRSF, SRF, together with chromatin marks. The dominant sequence motif for MRF binding defined from these data differs from that expected in older literature based on a few instances, and occurs over 2 million times in mouse and human genomes, with less than 1% being occupied in differentiated muscle cells. Most *in vivo* binding sites can bind both MyoD and myogenin, although two interesting and relatively small classes of binding regions are highly preferential for either myogenin or MyoD, but not for both. We also defined the class of MyoD target sites that are occupied in myoblasts. This result argues that the long-standing model in literature, in which MyoD protein is expressed but not available for DNA binding in myoblasts due to its interaction with the Id class HLH inhibitory regulators or other post-translational modifications, cannot be correct. An emerging puzzle is that more than half the

high quality *in vivo* binding sites are not adjacent any gene regulated in a muscle specific manner. Are these sites acting on more distant genes? Are these due to evolution in motion – i.e., sites of no significance functionally that have emerged and have not yet deteriorated? Are they sites being held in check by nearby unrecognized repressors? Testing these and related questions with functional assays coupled with key factor perturbations is underway.

307. The Msy3/Csda repressor mediates transcriptional down-regulation of myogenin during innervation

Libera Berghella, Shirley Pease

Myogenin, one of the four-member MyoD family of bHLH myogenic transcription regulators, is a crucial regulator of myogenesis that is dramatically down regulated at the RNA level during muscle fiber maturation as a consequence of innervation. We previously found that the Y-box protein MSY-3/Csda represses postnatal expression of myogenin by binding at a highly conserved DNA *cis*-acting element located upstream of the myogenin promoter (myogHCE). During muscle maturation when innervation takes place, MSY-3 activity is upregulated, and upon muscle denervation it is downregulated. This is consistent with additional data showing that the MyogHCE/Msy3 is needed to properly control extra synaptic expression of ACHR in postnatal muscle. (Berghella *et al*., 2008).

We are now asking how Csda/MSY3 is regulated during muscle maturation. First, we found that both short and long isoforms of the MSY-3 protein are dephosphorylated during maturation, and the dephosphorylated form accumulates in myonuclei. Further evidence suggests that Akt kinase is involved and that its activity inhibits nuclear localization of CSDA and binding by CSDA at the MyogHCE sequence. In addition to regulation of protein localization and activity by Akt, a second pathway that is induced by Ca++ targets MSY-3/Csda transcription and or RNA turnover also operates in maturing muscle tissue in the mouse.

We are also analyzing the effect of MSY-3 on neuromuscular synapse formation by using transient MSY-3 knockdown and MSY-3 knockout mice. Preliminary observations indicate reduction in the number of AChRs at the synapse and an increase in extra-junctional AchRs. To further characterize the role of MSY-3/CSDA in adult muscle, we are doing an RNA-Seq profile to discover additional regulatory targets and to find out how many activity-dependent genes. The role of CSDA's putative RNA-binding activity in these events is also being examined.

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308. Genome-wide comparative analysis of the NRSF/REST target gene network

Ali Mortazavi

We are investigating the role of a major transcriptional repressor in the evolution of the corresponding gene regulatory network (GRN). We are using a combination of computational prediction and direct experimentation to define the genome-wide set of direct targets of the well-known Neuron-Restrictive Silencer Factor (NRSF/REST). This repressor was originally identified as a global repressor of neuronal genes in non-neuronal tissues in work in the Anderson lab at Caltech in the late 80's. It has more recently been shown to repress neuronal genes in stem cells prior to their differentiation. Roles for it have also been suggested in pancreatic development, cardiac lineages, and lymphocyte lineages, although it is not clear whether these roles differ from repression of neuronal genes in non-neuronal tissues during embryogenesis. NRSF is an ideal model factor for defining a regulatory network from the *trans*-factor point of view, in part because it has a long (21 bp) and relatively well-defined binding motif. Basic network architecture questions include: What are all the *in vivo* targets of NRSF? In what cells are they occupied by the factor and with what consequences? How has this network evolved since its beginnings in the vertebrate lineage?

Our first approach to these questions used Cistematic (see Mortazavi and Aerni above) across human, mouse and dog genomes to leverage evolutionary conservation to refine the motif model and to locate instances and their adjacent genes. The major experimental tests of sites identified computationally, along with sites of the same size and similar base composition that are predicted not to bind NRSF, are ChIP assays (chromatin immunoprecipitation). Initially ~100 predicted sites from the group of ~700 were assayed by Q-PCR. These allowed us to evaluate the predictive success of our model of the human NRSF target repertoire in Jurkat cells (Mortazavi *et al*., 2006). We are finding similar site occupancy patterns in mouse muscle cells. The composition of the computational target gene set was also interesting because it includes multiple microRNAs and regulators of neuron specific splicing. Among these are microRNAs predicted by sequence to be plausible regulators of NRSF and its Co-Repressor, CoREST, suggesting a dynamic feedback. The perfect conservation of the entire zinc-finger set that comprises the NRSF DNA-binding domain throughout all sequenced vertebrates is striking. Coupled with the absence of any identifiable NRSF transcription factor in all sequenced invertebrates, suggests the notion that the emergence of NRSF in the vertebrates may have been needed to permit "reuse" of neuronal genes in non-neuronal, vertebrate-specific regulatory networks.

To further test and refine the computationally-derived model of the NRSF interactome, we developed ChIP-Seq to measure the entire global "NRSFome" experimentally by using microarray and very high throughput sample sequencing (e.g., Solexa) to make

ChIP measurements (see first entry above). We are now using ChIP-Seq to map NRSF interactomes in mouse, horse, and dog genomes, for comparison with the human version. The aims are to determine which target genes, and which detectably conserved binding sites, are used in all interactomes; how many new NRSF/target relationships emerge in each genome, and how many have disappeared. This work will also investigate how the NRSF interactome changes from one cell type to another within the same specie. In the first NRSF ChIP-Seq work, performed in human T cells, we found that NRSF binds to a family of previously unappreciated half-site motifs, as well as to its canonical full site (Johnson *et al*., 2007). This suggested a working hypothesis for the evolution of strong full-length sites from a pool of less optimal half-sites separated by flexible spacing. Patterns of site evolution and use across the four species in the current work should begin to test this idea.

309. High throughput transfection assays of candidate myogenic transcriptional regulatory modules

Gilberto DeSalvo

ChIP-Seq has identified tens of thousands potential binding sites for each key regulator of muscle differentiation (Myogenin, MyoD, Mef2 and SRF). However, we do not know which of these candidate functional elements are actually able to regulate transcription. I first selected a set of 24 previously studied muscle related enhancers associated with genes that are expressed in C2C12 cells and found Myogenin and MyoD binding in more than 90% of these contrasted with less than 10% for enhancers from t-cell or axonal origin. This provides me with a set of literature-vetted control enhancers to compare with newly found candidate elements that I select based on ChIP data. We are testing 90 binding sites that were selected based signal strength, binding of multiple factors, evolutionary conservation (or lack of it) and proximity to genes that are either strongly up-regulated kinetically early in the differentiation of C2C12 cells (B.A. Williams microarray data) or genes that are expressed differently. These elements will be tested in a medium-throughput transfection assay for their ability to drive luciferase transcription in both mouse (10T1/2, C2C12) and human (LHCN) myoblasts and myocytes. We will further test these elements by a mutation screen of the identified ebox and Mef sites and a further round of transfection to dissect the functional sub-elements within each of these 96 identified regions. Working with Georgi Marinov, we are also attempting to develop an improved functional assay that is designed to be more quantitative, more parallel and will have an RNA sequence-based readout. The "bar-coding" of construct transcripts at the time of their synthesis, coupled with use of fluorescent or bioluminescent reporters offer new options for multi-gene transfection and rapid screening based on reporter signals.

310. Generation and vetting of ChIP-grade mouse monoclonal and camelid antibodies

Gilberto DeSalvo, Jost Vielmetter, Susan Ou, Serge Muyeldermans[*]

As part of the NHGRI ENCODE project we are producing >50 Chromatin Immunoprecipitation (ChIP) validated monoclonal antibodies directed against transcription factors that do not currently have available ChIP-grade reagents. We set up a pipeline that involves the Caltech Beckman Protein Expression Center (PEC), the Biology Monoclonal Facility, and outside collaborators to produce both mouse monoclonals and camelid antibodies and to test which of these sources of monoclonal reagents is most effective. The camelid antibody is ultimately prepared as a molecularly cloned single heavy chain domain with a variable region derived from Llamas that offers a highly stable antibody with normal affinity. These are ultimately produced by inducible expression in *E. coli*, which offers significant advantages over classical cell culture of mouse monoclonal reagents. The first phase of this project has been to produce monoclonals from both mouse and camelid systems for the same set of seven antigens from different parts of three transcription factors. We also tested antigens that have been chemically fixed is the same manner as is used in ChIP studies, with the aim of increasing the success rate of usable reagents. We are currently testing the first of these antibodies for both monoclonals and camelids for their ability to ChIP.

[*]*University of Leuwen*

Reference

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311. Analysis of twist DNA occupancy in *Drosophila* embryos

Katherine Fisher, Shirley Pepke[*]*, Angela Stathopoulos*

This is a collaborative project in which our role is the computational analysis of Twist ChIP-Seq data obtained in the Stathopoulos laboratory. The objective of the study is to better understand the function of the transcription factor *twist* in the dorsoventral specification gene network of *Drosophila melanogaster*. For the computational and informatics effort, a second objective is to compare and contrast the overall structure and data characteristics of ChIP-Seq interactomes for bHLH-family factors in the small fruitfly genome, versus large mammalian ones for which we have the greatest body of data, thus far. Twist is involved in dorsal-ventral patterning very early in *Drosophila* development, and much is known about several (approximately 20) twist-responsive genes. To identify the entire repertoire of target genes, ChIP-Seq was performed by Anil Ozdemir, and from these data, we created a genome-wide occupation map. By comparing and calibrating the informatics with occupied sites in regions that have been experimentally shown to be functional twist-binding sites, we identified

over 500 novel twist-binding sites of similar or greater signal strength. Reporter gene functional assays were then performed by Leslie Dunipace and Anil, and they showed that many (but not all) of sites identified by ChIP-Seq and computationally highlighted as candidate driving domains are superior to predictions made by prior standard methods.

We further analyzed twist-occupied sites to determine ask is in common among them and how their factor occupancy relates to the identity and expression of nearby genes. DNA sequence motif analysis of the regions showed that the highest twist occupancy is at the E-box CAYRTG motif, which is highly enriched in bound regions. The dorsal motif was also found in a subset of these twist regions. This supports previous studies that predict that twist binds to hexamer CANNTG E-boxes and that it co-binds sometimes with dorsal. However, we are further investigating the exact variations and spacing of CAYRTG and other motifs, including the possibility that twist favors a larger binding motif. We aim ultimately to determine one or a set of specific of motifs that are explanatory for twist recognition, binding, and regulatory action. We also want to determine which co-regulators and other characteristics distinguish the minority of twist motifs that give high twist occupancy signals from the majority which display little or no ChIP signal. Twist's ortholog in mammals (mTwi) is involved in development of paraxial mesoderm, and Twist has a second role later in *Drosophila* development in myogenic precursors. One goal of future work will be to examine how the network of genes regulated by twist has evolved.

[*]*Caltech Center for Advanced Computing Research*

312. Computational and transgenic *cis*-regulatory analysis of a *C. elegans* Hox cluster

Steven Gregory Kuntz, Erich Schwarz, Paul Sternberg

Laborious *in vivo* promoter dissection is has been the most frequently used way to identify animal *cis*-regulatory modules. Previous studies in the nematode *Caenorhabditis elegans* took advantage of pairwise sequence comparison with the closely related *C. briggsae* genome to highlight putative modules by conservation. Comparisons with additional genomes at appropriate evolutionary distances would be expected to improve the predictive power. The parameters necessary for optimal sensitivity and specificity of *cis*-regulatory predictions have not previously been quantified in nematodes. Due to genome structure differences between the various animal phyla, it is expected that these parameters will differ from similar parameters in chordates or arthropods. By comparing five *Caenorhabditis* genomes (*C. elegans*, *C. briggsae*, *C. brenneri*, *C. remanei*, and *C.* sp. 3 PS1010) to identify candidate non-coding regulatory modules, we were able to empirically test various predictive parameters. For the species *C. brenneri* and *C.* sp. 3 PS1010, the entire genomic assembly was not available, so we sequenced ~0.5% of these genomes. The *ceh-13/lin-39* Hox clusters of these nematodes were computationally scanned for ungapped sequence similarity, and therefore conservation suppressed for insertions and deletions, using the MUSSA (Multi-Species Sequence Analysis) algorithm. Based on an initial computational analysis, the entire intergenic and intronic non-coding sequence was divided into 21 regions, including both regions exhibiting strong conservation and regions lacking notable conservation. Through iterative sequence analysis and transgenic assays of all these regions, we identified MUSSA parameters that yielded 100% specificity and a 77% recovery of all functional regions. In total, 12 regions drove detectible and reproducible expression, with the expression patterns matching the expected native expression patterns observed for *ceh-13* and *lin-39*. We are currently testing whether these parameters are similarly successful in identifying functional *cis*-regulatory sites in other loci of the nematode *C. elegans* that have been previously dissected through *in vivo* promoter dissection. This should provide significant insight into the utility of these computational predictions in nematodes.

313. Muscle tissue differentiation network response to mutation

Steven Gregory Kuntz, Paul Sternberg

Biological networks are sometimes remarkably resistant to insults, being able to achieve their end point even when parts of the network are removed. To understand how different parts of the system interact to compensate for such aberrations requires a thorough understanding of the network's structure under normal and mutated conditions. Striated muscle differentiation is an excellent model for transcription factor network analysis, being an evolutionarily ancient and robust system. We are using the differentiation of bodywall muscle in the late embryo of the nematode *Caenorhabditis elegans* to study the transcription factor network. We want to learn which components and connections of tissue differentiation gene regulatory networks are invariant over evolution, which are variable, and what functional consequences come from the variations. Several important transcriptional regulators have been identified, including *hlh-1* (myogenic regulatory factor), *hnd-1* (TWIST/hand protein), and *unc-120* (SRF/MEF protein). Given the stability of the network in the presence of mutations, understanding the structure is best done within mutants when the network is compromised. For understanding of downstream expression, we are measuring transcript levels by RNA-seq in the mutant backgrounds. To understand how these genetic cross-interactions between transcription factors appear to 'buffer' the downstream transcript changes, we are using ChIP-seq to look at differential binding of RNA Polymerase II in mutants for the known myogenic transcription factors. The identification and description of interactions within the network will help us construct a draft map of the muscle differentiation gene regulatory network and facilitate a more complete understanding of the network architecture, including how it may compensate when components of the network are compromised.

314. Gene expression profiling of primate brain regions and cells that mediate social interaction

*Brian Williams, Nicole Tetreault, Mingshun Liu, Anna Abelin, Virginie Goubert, Brandon King, John Allman**

Von Economo neurons (VENs), are a recently evolved population of specialized neurons found in parts of layer 5 of the human cortex. They are believed to be involved in the perception of social emotions, and intuitive assessment of complex, uncertain circumstances. A working hypothesis is that the brain areas where VENs are concentrated [frontoinsular cortex (FI) and anterior cingulate cortex (ACC)] may be dysfunctional in autistic individuals. These relationships have been deduced from anatomical and pathological evidence, including selective VEN pathology in another disorder that disrupts social interaction capacity (fronto temporal dementia). But little is known about the molecular genetics of FI, ACC and the VENs.

In this collaboration with the Allman lab, we have produced transcriptome profiles from small pieces of FI and ACC gathered from autistic and age-matched control individuals, with the proximate objective of trying to identify groups of genes that differ. We have discovered upregulation of a network of genes related to modulation of the immune response and inflammation, in a subset of these individuals (Autism A group). This suggests that VEN activity may be affected by a subject's immune status. Microglia, known as the macrophages of the brain, in a subset of our samples are more numerous and show the dense cellular processes characteristic of activated microglia that are found in regions of cellular death and damage. Microglia are a source of inflammatory cytokines, and a more extended immunocytochemical survey has found that this is seen in additional autistic brains and is not frequent in control brains.

Additional analysis of differential gene expression using Ingenuity Pathway Analysis has identified 73 upregulated genes in autistic subjects that comprise a coherent network. Seven of these are upregulated in activated microglia. Leptin was also found to be upregulated in our autistic subjects, confirming previous reports that circulating leptin levels are increased in the serum of autistic individuals. Leptin may influence an individual's immune status due to its action as a cytokine that stimulates the secretion of IL6. Our immunocytochemical experiments describe strong immunolocalization of the interleukin 6 receptor (IL6-R) on VENs and related layer 5 neurons in the ACC, providing further support for this hypothesis.

To more precisely define VENs and other neuronal types within FI and ACC we are currently working with laser capture microdissection techniques (LCM) and amplification protocols to produce reliable representative transcriptome measurements from individual cells taken from fresh frozen brain slices of autistic and control individuals. These experiments should expand our understanding of the gene expression regulation network that functions in normal VENs, and may help us learn about the molecular basis for VEN dysfunction in the autism spectrum.

**Professor, Division of Biology*

315. Gene regulatory networks of the skeletal muscle neurovascular bundle

Gilberto Hernandez

My goal is to identify and then learn the function of components of the gene regulatory networks underlying transcriptome changes in the skeletal muscle neurovascular bundle (smnvb) following denervation injury. The molecular events associated with degeneration and regeneration paradigms of the smnvb are partially characterized. However, the explicit transcriptional gene regulatory networks that control the genes and non-protein coding regulatory molecules which function to coordinate the molecular response of the smnvb following denervation injury remain largely unknown.

Brian Williams and I conducted high resolution-global quantitative RNA measurements of the smnvb at multiple timepoints status post denervation injury. Our data include a subset of differentially expressed regulatory, signaling, and cell type specific genes, as well as non-protein coding regulatory molecules that are candidates to be the principal drivers of regulatory change in the smnvb. These candidates are being further assembled into an experimentally testable gene network model, created using BioTapestry (Longabaugh and Bolouri, http://www.biotapestry.org). *In vivo* gene transfer experiments will be utilized to functionally characterize our target genes. High resolution-global quantitative studies of micro-RNAs will also be included soon and differentially expressed small rna regulatory candidates and their predicted putative targets will be added to the aforementioned gene regulatory network model for further validation.

Genes that demonstrate functionality in our model will be further scrutinized via *cis*-regulatory analysis. Working from functional *cis*-regulatory elements, identified partly by their conservation through evolution and partly by experiment, we aim to define components of the transcriptional network underlying skeletal muscle atrophy. Our sequence similarity analysis includes a focus on shared motifs within 3'UTR regions of candidate regulatory genes and has led a focus on specific elements as predicted target sites for transcription factors binding or for microRNA interaction. We hope to use these *cis*-acting regulatory regions, and sequence motifs within them, to identify other genes that share them and are part of the denervation response gene network of the smnvb.

316. Bioinformatics of high-throughput DNA sequencing

Diane Trout, Brandon W. King, Rami Rauch, Timothy E. Reddy*, Gavin Sherlock**

High throughput sequencing technology (HTS) is now widely available, and is being used in many different assays, of genome function and status such as ChIP-Seq, RNA-Seq and Methyl-Seq, as well as in general DNA sequencing of genomes and BAC clone pools. The size of the data-sets generated in these experiments (currently, several gigabases per week per instrument) presents a significant challenge for efficiently managing sample

tracking, acquiring and attaching all relevant covariate information and performing downstream data management, archiving and analysis in an integrated manner.

We are currently using the Solexa/Illumina platform for ChIP-Seq, RNA-Seq and Methyl-Seq as part of the ENCODE project, and have developed a LIMSystem, HTS-Workflow, for addressing the initial capture of sample meta-data through the primary experiment analysis. Specifically, we use our software for: 1) experiment planning; 2) experiment tracking; and, 3) data collecting; and 4) launching the initial data analysis. Our development goal is to provide genome center staff and clients with an intuitive web interface to track the progress of their samples and data through the process. The Web front-end connects with the data processing server, so that the user can define additional data analysis "tasks" and "projects" on the Web to be performed after the instrument specific analysis has finished. We have implemented and deployed HTS-Workflow using a python based Web development framework, Django. Recent work has been to test and integrate a LIMS system for sample tracking in the Wold Lab and in the Jacobs center to track physical samples and associated data and covariate information from the point of sample submission to the final datasets.

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317. Analysis of transcription factor combinations in muscle differentiation

Katherine Fisher

We are interested in how different transcription factors interact in order to convey very specific information about gene activity in development. Using a mouse muscle cell line, I have generated occupancy maps Mef2, and SRF using ChIP-seq. Based on prior studies of several muscle differentiation genes, it is thought that MyoD and/or myogenin (for which Tony Kirilusha and Gordon Kwan have generated occupancy maps) and Mef2 are causal and sufficient for the regulation of muscle-specific genes, but SRF and myogenin are also known to be major players for a subset of muscle-specific genes. I have also generated preliminary data for genome-wide occupancy of the co-activator p300, which identifies a subset of active enhancers. I have analyzed the occupancy data to find regions co-occupied by these factors, but with no occupancy by NRSF, a factor not involved in muscle differentiation. Using this collection of regions, I have derived known and candidate new binding motifs, and have designed reporter gene constructs to test if the regions and motifs within them are functional. Data from the functional assays will be used to refine our model of what comprises a muscle-active regulatory module. We have also analyzed RNA expression data from these cells (Georgi Miranov) and found that in a majority of instances, the nearest coding gene is not expressed is a classic muscle specific pattern. This is a surprise, and suggests that promoter selectivity and regulatory action at greater distances is likely to be playing a larger role than previously appreciated. Tests for more distant interactions are in progress.

318. The Caltech custom UCSC-based functional genomics browser

Henry Amrhein

I have developed a customized version of the UCSC Genome Browser to provide the local community with a higher performance, higher capacity tool to visualize their high throughput sequencing experiments. Currently, this localized genome browser contains the following genomes from UCSC: hg18, mm9, ce6, dm3, as well as TAIR8 (*arabidopsis thaliana*) from arabidopsis.org, and a handful of in-house nematode genome assemblies. The configuration has been heavily customized to allow multiple labs to have their own private view of the browser without disclosing each other's private information. The effect of adding this tool to our toolbox is that researchers can now work with very large sets of ChIP-Seq, RNA-Seq, and novel genome data without the need to upload it to the UCSC site. This not only saves bandwidth, but also allows us to add global custom tracks into custom-defined groups. An additional benefit of this is that the custom track database is never expired, thereby allowing users to have their own custom tracks accessible through a web-based interface.

Publications

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Structural, Molecular and Cell Biology

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Summary: The Baltimore laboratory has many different programs in progress. The largest is a program with the aim of providing a new method of therapy and potentially immunization against HIV/AIDS. This involves solving many independent problems. On the more basic side, we continue to investigate aspects of a key transcriptional regulatory protein we discovered more than 20 years ago, NF-κB. As part of our continuing interest in the immune system, we are also studying the roles of a series of microRNAs in innate and adaptive immune cell function and development. We finally have a few outlier studies that fall under the general rubric of infection and immunity: the evolution of influenza virus and the ability of a fetus to grow in a mother in spite of the immunologic differences between mother and fetus. Together, this work provides a rich set of objectives for an inventive group of postdoctoral fellows, graduate student and technical experts.

319. Engineering immunity to treat HIV and other dangerous pathogens

Xin M. Luo, Ryan Michael O'Connell, Eun Mi Hur, Kenneth Yu, Alejandro Balazs, Yun Yong Margarida Lei, Rana A. Feidi, Christine Kivork, Sonal N. Patel, Angie Frausto, Lili Yang[*]*, David Baltimore*

Supported by the Bill and Melinda Gates Foundation through the Grand Challenges in Global Health Initiative, we are exploring a fundamentally new way of stimulating the immune system to fight off infectious diseases, focusing on HIV as a test of the concept. The premise of this project is that for some infections, including HIV, the immune system's natural responses are inherently inadequate, and therefore, the traditional approach of using vaccines to stimulate and boost these responses is likely to be ineffective. As an alternative, we propose to genetically engineer immune cells that can produce adequate responses. Our work is intended to eventually lead to immunotherapy for people who are infected with HIV. It could also lead to new ways to prevent HIV infection.

Our strategy is to use gene therapy for expression of genes encoding neutralizing antibodies against HIV. Multiple problems will be solved to allow expression of purposely-designed antibody genes in the cells of infected people. We plan to implant genes in blood stem cells and allow the cells to give rise to B cells, the body's natural antibody-producing cells, requiring the solution of particular problems posed by the architecture of antibody genes. We must also design antibodies or antibody-like proteins that can efficiently neutralize the infectivity of HIV. This will be done by design methods or by selection. We must then prove the effectiveness of the design in human cells or in a mouse/human chimera. Finally, we have to drive the cost of the process to a low enough level to use in the less developed world. At low cost, it could even be a vaccine.

With the goal of developing this general method, captured by the phrase "Engineering Immunity" to treat infection by HIV and other dangerous pathogens, we have the following projects:

[*]*Lead Scientist; Project Manager*

Note: This project is a joint effort of three groups: David Baltimore and Pamela Bjorkman's groups at Caltech and, Pin Wang's group at USC.

Project 1: Protective efficacy of 2G12 neutralizing anti-HIV antibody delivered by "backpack" tumors

Xin M. Luo, Yun Yong Margarida Lei, Rana A. Feidi

We are interested in applying an engineering approach in the delivery of neutralizing antibody-based HIV vaccines. During the last year, we have proved that HIV infection could be prevented in humanized mice engineered to have μg/mL level of a broadly neutralizing anti-HIV antibody, 2G12, through s.c. injection of 293T cells expressing this antibody. The 293T cells formed benign tumors (or backpacks) and the size of tumors was controlled by the drug ganciclovir. We adjusted the amounts of 2G12 produced in the mice by injecting different numbers of 293T cells. The backpacking approach, leading to average plasma 2G12 levels of 5.4 ± 2.7 μg/mL (smaller backpacks) and 51.2 ± 12.9 μg/mL (larger backpacks) over 5 weeks, was apparently more effective in restoring the percentage of $CD4^+$ T cells in the blood than passive transfer of the antibody. We also looked closely at the 2G12 concentrations within 1 week of HIV challenge, and found that the mice with initial 2G12 levels over 10 μg/mL had nearly no $p24^+$ cells in the spleen and much lower HIV copies in the plasma than the mice with lower 2G12 levels upon HIV infection. This indicates that the 2G12 titer threshold for prophylactic protection against HIV is around 10 μg/mL. Overall, these results suggest that neutralizing

antibodies delivered via the engineering immunity approach can prevent HIV infection *in vivo*.

Project 2: **To perfect lentiviral expression systems that can impart the anti-HIV specificities to the human antibody repertoire**

Kenneth Yu

We previously reported the preliminary data that demonstrated the "Synthetic Switch" antibody approach could be used to generate tunable ratios of soluble to membrane bound antibodies. We hypothesized that the Synthetic Switch antibody genes will allow us to encapsulate the function of the 1Mb immunoglobulin locus in a compact 10 kb lentivector. We have tested this hypothesis and here we report the demonstration of the Synthetic Antibody genes producing signaling competent B-cell receptors (BCR) that are capable of supporting B cell maturation from early progenitor cells.

Using OCI-Ly7, a B-cell line that expresses surface IgM, we show that the Synthetic Switch genes are able to generate different ratios of secreted to membrane bound antibodies in a B lineage cell. When we stimulated the cells with crosslinking antibodies against the Synthetic Switch receptors, we observed robust calcium signaling, indicating the receptors are fully functional. To further obtain functional evidence that the receptors are capable of supporting B cell development, we used a cell line model of B cell development called EU12. When transduced with lentivectors carrying the Synthetic Switch antibody genes, the EU12 cells developed from a pro-B cell phenotype (CD34+, CD10-) into a definitive B cell phenotype (CD34-, CD10-). These data demonstrate the feasibility of using the Synthetic Antibody gene approach to program human HSCs to differentiate into functional anti-HIV B cells. We are currently evaluating this approach in mouse models.

Project 3: **Improving "Humanized" mice using human cytokines delivered by viral vectors**

Ryan O'Connell, Alejandro Balazs, Christine Kivork

"Humanized" mice, which are mice with human immune cell populations, are valuable preclinical models that can be used to study human cells in a physiological setting, and essential for investigating potential therapies against human pathogens like HIV. Although the current "Humanized" mouse models can generate human lymphocytes within a few months of human CD34+ stem cell delivery to mice, the cellular output is rather low and the overall immune response is defective. Consequently, we have been exploring a new approach to stably deliver specific human cytokines to "humanized" mice in hopes of enhancing human cell output and function. We have begun by delivering human Interleukin-7 (hIL-7), an important factor necessary for T lymphocyte homeostatic proliferation. "Humanized" mice were injected with a lentiviral vector encoding hIL-7, and within a few days we detected steady expression of hIL-7 in the serum in a manner dependent upon the amount of viral vector injected. Within about two months of hIL-7 treatment, we observed expanded T cell numbers in the peripheral blood and spleen compared to mice receiving the control virus. IL-7 also expanded lymphoid follicles in the spleen, and elevated expression of the anti-apoptotic factor BCL2 in lymphocytes. We have also found that "Humanized" mice treated with IL-7 can be infected with HIV, but do not have higher HIV titers in their peripheral blood despite the increase in T cell numbers. Using IL-7 as a prototype, we have established an approach by which human cytokines can be stably delivered to "Humanized" mice and demonstrated that hIL-7 can improve T lymphocyte populations in these chimeric mice. Furthermore, our findings in this model reveal that this IL-7 dependent T cell increase does not substantially alter HIV levels upon infection, which argues in favor of using this as a therapy to rejuvenate T cell numbers in HIV infected individuals. Future studies will investigate additional cytokines and viral vector delivery systems with the potential to further enhance HSC engraftment and myeloid development in "Humanized" mice.

Project 4: **Generation of neutralizing immunoglobulin antibody against HIV-1 carrying B12 V region in humanized mice and epithelial transport to mucosal compartments**

Eun Mi Hur, Sonal N. Patel

Since T lymphocytes located in mucosal sites such as gastrointestinal tract and genital tract are the primary target of HIV initial infection in human, it is important to block viral transmission in mucosal sites. Immunoglobulin A (IgA) plays critical roles in mucosal immune responses by blocking viral attachment and crossing epithelial barrier, as well as neutralization of the virus. Failures in generating antibodies that broadly neutralize primary isolates of HIV-1 in many vaccine candidates suggest that alternative approach beyond active immunization is needed to develop potent HIV vaccine. We have explored a novel approach to generate neutralizing activity in mucosal compartments, as well as in serum. To do that, we used lentivirus vector system to express human recombinant IgA2 that carries those V regions of IgG1 b12, a potent and broadly neutralizing anti-HIV antibody, in human CD34 cord blood hematopoietic stem/progenitor cells (HSPCs). Human HSPCs transduced with IgA2 b12 transgene were then transplanted to $Rag2^{-/-}\gamma C^{-/-}$ mice to generate mice with human immune system producing IgA2 b12. IgA2 b12 transgene under the control of immunoglobulin light-chain promoter were expressed predominantly in human B cells derived in this humanized mice. We tested epithelial transport of expressed human IgA2 b12 to mucosal sites such as genital tract, gastrointestinal tract and respiratory tract. Passively administered purified IgA2 b12 or endogenously produced IgA2 b12 from embedded tumor cells, were detected in mouse genital secretions, intestinal secretions and bronchoalveolar lavage. We also adopted new humanized mouse model, bone marrow/liver/thymus humanized mice (BLT or NOD/SCID hu-BLT mice) to produce b12 IgA *in vivo*. In this animal model, transplantation of autologous human hematopoietic fetal

liver CD34+ cells into NOD/SCID gC mice previously implanted with human fetal thymic and liver tissues results in long-term, systemic human T cell homeostasis. We generated these mice by transducing human hematopoietic fetal liver CD34+ cells with lentiviral vector carrying recombinant b12 IgA construct as described above. Human cell engraftment and b12 IgA expression were investigated in BLT mice transduced with b12 IgA transgene (BLT-b12a). We are currently investigating the prophylactic effect by the neutralizing IgA antibodies produced in these humanized mice to HIV-1 mucosal transmission.

Project 5: **Engineering of immunity through adeno associated vectors**

Alejandro Balazs

Adeno associated virus (AAV) is a small single-stranded parvovirus capable of infecting humans without causing any known illness. AAV has also been found to be capable of producing high levels of transgene expression in a variety of animal models making it an attractive gene transfer vector. We are developing AAV as a tool for the engineering of the humoral response through antibody expression from non-hematopoietic tissues such as liver and muscle. We hope to express well-characterized neutralizing antibodies against HIV using AAV to create a synthetic vaccine capable of providing long-lived protection against infection. To test this approach, we will utilize both a Human immune system (HIS), as well as NOD/SCID/Gamma-HuPBMC (NSG) mouse model that can be engrafted by human hematopoietic cells to simulate a human immune system. These mice will be challenged with live HIV to determine the effectiveness of our AAV-vaccination strategy. This system will be used to determine the minimum quantities of antibody necessary to protect mice from challenge and identify the optimal combination of neutralizing antibodies that can provide protection.

Publication

Luo X.M., Maarschalk E., O'Connell R.M., Wang P., Yang, L. and Baltimore D. (2009) Engineering human hematopoietic stem/progenitor cells to produce a broadly neutralizing anti-HIV antibody after *in vitro* maturation to human B lymphocytes. *Blood* **113**(7):1422-1431.

320. Modulation of NF-κB action by O-GlcNAc glycosylation

Parameswaran Ramakrishnan, David Baltimore

O-GlcNAc glycosylation is an abundant post-translational modification of serine or threonine residues occurring in nuclear and cytoplasmic proteins. Alterations in O-GlcNAcylation are linked to the pathology of diabetes and to neurological disorders through its influence on the activity and stability of proteins, as well as transcriptional regulation. NF-κB represents a family of transcription factors playing a pivotal role in diverse biological processes including immune and inflammatory response, apoptosis and neuronal plasticity. Although NF-κB regulation by phosphorylation, ubiquitination and acetylation is well studied, much less is known about the O-GlcNAcylated forms of NF-κB. We are studying the modification of the NF-κB proteins by O-GlcNAc and its role in transcriptional regulation using cellular systems and animal models. We are focusing on identifying O-GlcNAcylated NF-κB proteins and map the sites of O-GlcNAc attachment by a glyco-proteomic approach. We are investigating the significance of O-GlcNAcylation of NF-κB by mutating the O-GlcNAc target site(s) and ascertaining the impact of these mutations on the stability, localization and activation of NF-κB. We are also examining the influence of O-GlcNAcylation on NF-κB transactivation and target gene specificity. These findings will significantly advance understanding the role of O-GlcNAcylated NF-κB in normal physiology as well as in disease conditions like hyperglycemia and neuronal disorders, where both NF-κB and O-GlcNAc dysregulation have been implicated.

321. Molecular constraints in the evolution of influenza virus

Jesse D. Bloom, Ian Gong, David Baltimore

Our work has focused on molecular characterization of the evolution of influenza virus. The last year has certainly been an exciting time with respect to the influenza evolution, and with the appearance of the swine flu and the ensuing media attention. We certainly now get a lot more general questions about my research. Our research currently has several main focuses, which are outlined individually below.

Molecular constraints governing the appearance of oseltamivir (Tamiflu) resistance in H1N1 viruses

The most widely used antiviral drug against influenza is oseltamivir (sold under the name Tamiflu), which is a viral neuraminidase inhibitor. With the last few years, human H1N1 influenza viruses have begun to exhibit widespread resistance to Tamiflu. This resistance is due to a single mutation, His274 -> Tyr, in the neuraminidase gene. The widespread appearance of the H274Y mutation was considered quite surprising, since earlier studies had shown that this mutation attenuated several H1N1 strains in a variety of contexts (tissue culture, mice, ferrets). These earlier studies had led researchers to conclude that the H274Y mutation would not be of clinical significance since virus carrying this mutation would be too attenuated to spread widely.

We are examining the molecular events that have allowed for the recent spread of the oseltamivir resistance H274Y mutation in human H1N1 viruses. The guiding hypothesis is that some "potentiating" mutation must have occurred to alleviate the deleterious effects of H274Y. We have developed a novel system to label viruses by making them carry either a green or red fluorescent protein. We can then infect cells with both green and red viruses at a low multiplicity of infection, and monitor the relative growth of the two strains. This allows for accurate

measurements of the fitness effects of specific viral mutations. Using this system, we have confirmed that H274Y indeed attenuates several H1N1 strains. We are now examining the basis of this attenuation, and have obtained preliminary results suggesting that the mutation is deleterious to the folding of the neuraminidase. We are also in the process of identifying specific potentiating mutations that alleviate this deleterious effect.

In addition to understanding the historical evolution of oseltamivir resistance in human H1N1 viruses, we are using the same approach to understand the likelihood that the H274Y resistance mutation will spread widely in the "swine flu" pandemic H1N1 strain. We are currently in the process of measuring the viral fitness's of "swine flu" strains carrying this mutation. We hope this research will enable an accurate assessment of the likelihood of widespread oseltamivir resistance in these viruses, and an understanding of the molecular events that are likely to be necessary for this to occur.

Use of the bacterial lectin cyanovirin as an antiviral agent

We are currently working collaboratively with the lab of Dr. Stephen Mayo to characterize the potential of the bacterial protein cyanovirin as an antiviral agent. Cyanovirin with originally isolated in a screen of proteins that inhibited HIV. It has subsequently been characterized as a lectin that binds strongly to the glycans found on the surfaces of many enveloped viruses. Our collaborators in the Mayo lab have been engineering this protein for greater potency and good expression.

We have been working with them to test the antiviral activity of engineered cyanovirins against influenza. We have developed a sensitive luciferase-based neutralization assay that allows for rapid and accurate determination of inhibitory concentrations of antibodies and antibody-like molecules against influenza. We are now using this system to assess the activity of a panel of cyanovirin variants against a panel of different influenza strains. Our preliminary results indicate that some of the engineered cyanovirins have increased potency against influenza.

Role of natural antibodies in targeting the glycans on influenza viruses

Recent studies have indicated that natural antibody secreted by B-1 cells can, in conjunction with complement, neutralize influenza virus. Other work has shown that B-1 cells are an essential component of the murine immune response against influenza. However, the role of natural antibodies in viral immunity tends to be overlooked, possibly because these antibodies do not have strong neutralizing activity that is apparent *in vitro* assays in heat-inactivated serum. We are using a newly developed highly sensitive neutralization assay to probe the role of natural antibodies in limiting influenza virus infection. We are particularly interested in the role of viral variation that can enhance sensitivity to natural antibody. Our preliminary results indicate that increased glycosylation makes influenza more susceptible to neutralization by natural antibody.

322. The stability of mRNA influences the temporal order of the induction of genes encoding inflammatory molecules

Shengli Hao, David Baltimore

Inflammation can be classified into acute inflammation (early stage) and chronic inflammation (late stage) according to the temporal stages the inflammation has gone into. Resolution of inflammation at the acute stage usually ends up with better healing of tissue injuries, while chronic inflammation is associated with extensive tissue damage and various chronic diseases. This suggests that late stage of inflammation is qualitatively different from the early stage. The goal of this study is to explore the molecular basis for the differences. To achieve this, three steps were taken. First, we tested whether different genes were activated in late stage comparing to the early stage. We then analyzed the common properties that are shared by the late genes that distinguish them from the early genes. Lastly, we explored the underlying molecular mechanism(s).

Our study started with microarray analysis of global gene responses to TNF, a critical proinflammatory cytokine, in three time points (0.5h, 2h and 12 h) in cultured mouse fibroblasts. We found that 43 genes were upregulated (mRNA fold change >= 2, $p<0.01$) by TNF by 0.5h and 91 genes were upregulated by 2 hours and 140 genes upregulated by 12 hours of TNF stimulation. There are about 70 genes downregulated (fold change >=2, $p<0.01$) at either of these time points. Here we only focused on those genes that are upregulated for further study. Using agglomerative hierarchical clustering algorithm, we were able to group all the upregulated genes into three groups. Group I (25 genes) peak about 0.5h; Group II genes (57 genes) peak at 2h; and Group III genes (98 genes) peak at 12 hours. The three groups of genes overlapped more or less with each other at any time points. However, Group I genes are the predominant group in the early time (0.5h); Group II dominate at later time (~2h) and Group III genes dominate even later (after 12 hours). We thus referred these three groups of genes as early, intermediate and late genes. This notion is also supported by their correlation with the known temporal inflammatory events with the group mapping of the genes that control them. For example, the recruitment of neutrophils that marks the beginning stage of inflammation neutrophil maps nicely with the Group I genes CXCL1 (KC) and CXCL2 (MIP-2), the neutrophil recruiting chemokines. The subsequent recruitment of monocyte maps well with the monocyte-recruiting chemokine gene CCL2 (MCP-1) and CCL7 in the Group II. Group III genes contain many genes involved in adaptive immune response and tissue remodeling, correlating with the events of the later stages of inflammation.

We then analyzed the common properties that define the early, intermediate and late genes. The only property we have found is the specific activation pattern

each group has. Group I genes are activated quickly and transiently: their mRNA peaks about 30 minutes after addition of TNF and then falls quickly (**Figure 1**).

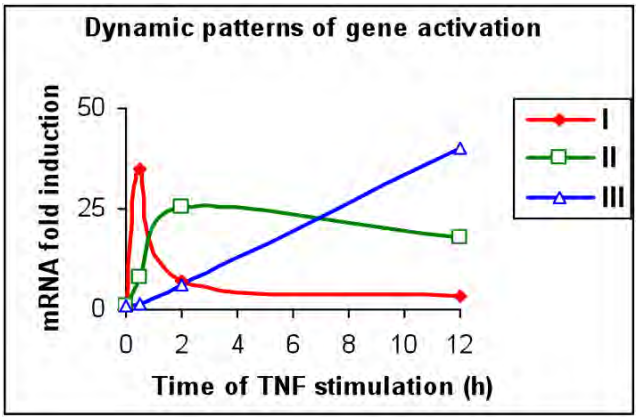

Figure 1. Example of mRNA dynamics of three Groups of genes in response to TNF stimulation.

Group II genes take a longer time to reach maximum (mRNA levels peak about 2h) and their level is then mostly sustained. The Group III genes are activated in a delayed, slow but steady manner. They have not reached peak accumulation even after 12 hours of continual TNF stimulation (Figure 1). We found that the activation pattern for any particular gene is usually constant from fibroblasts to macrophages, from mouse to human and independent of extracellular stimulus (TNF vs. LPS) and signal strength (from 0.1 ng up to 20 ng/ml TNF), suggesting activation pattern is an intrinsic property of the gene.

The well conserved activation pattern for a gene suggests it may encode important biology information. To explore this, we analyzed the different responses of three groups of genes to short pulse of TNF stimulation and termination of TNF stimulation. We found the activation pattern encoded two critical parameters of gene activation, the temporal order and duration of activation. The three groups of genes are activated in this order: group I first, then group II and lastly group III. The duration of three groups are in a similar this order: Group I genes only last shortly and quick inactivated even though the stimulus is still on; The duration of Group II genes correlates to duration of stimulus and they start to decrease only when the stimulus signal was terminated. The group III genes last longest; even though the stimulus was removed they still remain high.

We then analyzed the molecular mechanisms that cause these different activation patterns between the three groups. Interestingly, even differences in activation patterns, genes in these different three groups are dependent on the same transcription factor NF-κb for their activation (our unpublished data). It seems that although NF-κb is critical gene regulator, it does not specify the difference between groups. Since mRNA change is subject to two opposite regulatory process: synthesis through transcription and degradation. We then analyzed mRNA stabilities of genes between groups. We measured the degradation rate of many of the induced mRNAs in the various Groups by either Actinomycin D treatment or removal of TNF. We found that mRNA stabilities are surprisingly elegantly mapped on the Grouping of genes. The mRNAs of Group I genes in fibroblasts are collectively unstable with half life (0.2-0.8h); labile, Group II genes are stable with half life (1 to 8h) and Group III are most stable (half life over 8h). This trend of difference between three groups remains the same in human primary dermal fibroblasts and mouse primary macrophages although the half life of a particular gene may be somewhat different from different types of cells. This was true whether we studied induced or uninduced cells and once again suggest that the mRNA stability is an intrinsic property of the gene. Examination of the number of AU rich elements (AREs) in 3'UTR of the mRNAs showed that Group I genes have most (4 to 10), followed by Group II genes (1 or 2 AREs) and virtually absent in Group III. This provides a strong support that activation patterns are intrinsic to the gene. To further confirm that mRNA stability instead of transcription regulation is the main determinant for the gene activation pattern, we created a series GFP reporter transgenes controlled by different promoters from Cxcl2 (group I), Cxcl10 (group II) and Ccl5 (Group III) gene in combination of the 3'UTR of either c-fos (Group I) and Ccl5. The results confirmed that mRNA stability is the main determinant for the gene activation pattern [1].

This study provides a model of how inflammatory genes are organized and suggests the different roles of transcriptional regulation and mRNA stability in this complicated process. Transcription regulation through combinatorial effects of transcription factors is important because it determine what genes will be involved. However, when and how long a gene comes into play is determined by a rule that the sequential order of genes is set according to their relative mRNA stabilities. As transcriptional regulatory elements and the elements to control mRNA stability are all encoded in genome, the overall gene activation program is actually preset independent of extracellular signals. However, extracellular signals can still modulate this program to a small degree through activation specific transcriptional factors and modulation mRNA stability of certain specific genes to tailor the personalized cellular response to its special scenario.

Publication

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323. Regulation of NF-κB by methylation

Chee-Kwee Ea, David Baltimore

NF-κB undergoes several post-translational modifications upon activation, including phosphorylation, ubiquitination, nitrosylation, and acetylation. These regulatory modifications have distinct functional consequences. For example, acetylation of RelA at K218 and K221 inhibits IκBα binding and enhances DNA-binding; while acetylation of RelA at K122 and K123 inhibits its transcriptional activity. Protein methylation is another interesting post-translational modification. Protein can be post-translational methylated at lysine, arginine, histidine and dicarboxylic amino acids by highly specific methyltransferases. The addition of methyl groups to the e-amine of a lysine residue results in the formation of mono-, di-, and tri-methyl-lysines. This process can be reversed by demethylases. Histone is one of the best-studied proteins that undergo methylation. Specific sites of methylation on histones correlate with either activation or repression of transcription. Recently, several transcription factors, including p53, STAT1, RARα, and ERα , have been shown to be methylated and this modification affected their biological activities as transcription factors. Thus we speculate that NF-κB may also be regulated by methylation.

The main goal of this project is to identify novel mechanism of NF-κB regulation through lysine methylation. Our preliminary results indicated that RelA is methylated *in vitro* by SET9 and Smyd3 methyltransferases at lysine 37. Methylation of RelA at lysine 37 regulates the DNA binding of RelA. We have raised p65K37me1 antibodies to facilitate the study of p65 methylation and found that p65 is methylated at K37 in response to TNFα and IL-1β stimulation. Furthermore, we found that Set9-mediated methylation of RelA is required for the expression of a subset of NF-κB target genes in response to TNFα stimulation. These results set the stage for a detailed study of the biological function of RelA methylation.

324. Maternal immune awareness of the fetus during pregnancy

Daniel Kahn, David Baltimore

A central goal of our research is to better understand the maternal immune awareness of the fetus during pregnancy.

The following sub-objectives were proposed to address the central goal:

1. At which point in the pregnancy does the mother's immune system become aware of the fetus?
2. Are the immune reactions created, typical of those known to favor tolerance?
3. Can the immune reaction to the fetus be modified during pregnancy either by genetic defects in inflammatory activation or by pro-inflammatory treatments?

During the past year, progress has been made in all three areas.

Sub-objective 1:

Our initial model used the immunogenic difference between male offspring and their mothers. It is well characterized from transplant experience, that female inbred mice will ultimately reject a skin graft from a male littermate due the ubiquitous presence of the minor male transplant antigen, H-Y. Using the maternal immunologic awareness of her male fetuses, we have been able to document that by day 12 a detectable immune response was present. Furthermore, we learned that the magnitude of this response is dependent on the relative amount of fetal antigen. That is, with increasing numbers of male fetuses (over which we have no control), the maternal immune response to the male fetuses occurs earlier in the pregnancy.

Sub-objective 2:

Using the maternal response to male fetuses we were able to characterize that the nature of the immune response was indeed tolerogenic. Furthermore, we discovered that the maternal response generated a class of T cells that suppress (limit) immune reactions in general (T regulatory cells). Using the known antigenic difference between the mother and her male antigens, we found the previously unknown feature of T regulatory cells of their requirement for antigen specificity.

Sub-objective 3:

In this objective we sought to manipulate the maternal response to the fetus during pregnancy to change the outcome. This area of investigation is still evolving, but we have made the preliminary observation that in a significantly (immunologic) disparate mating (father and mother different at all major transplant antigens) treatment early in pregnancy with a low dose of proinflammatory compound (LipoPolyScharride; LPS) caused a 50% reduction in T regulatory cells throughout the mother and significantly lighter pups than saline injected control. Furthermore, in pregnant mice in which only the males are different, treatment with a drug that depletes the T regulatory cell population causes an adverse pregnancy outcome for the male pups specifically reflecting their role the maintenance of maternal tolerance during pregnancy.

The future of the project aims to understand the following:

1. What is it about pregnancy that leads to the development of the T regulatory cells? Is it hormonal or is it the kinetics of exposure to the fetal antigens?
2. What are the molecular mechanisms by which the T regulatory cells are functioning? Pregnancy turns out to be an excellent mechanism for generating T regulatory cells. Using this approach provides a unique opportunity to learn about the biology of T regulatory cells.

3. Which fetal antigens are the most significant for the mother to cope with during pregnancy? Presumably the major transplant antigens are more potent, but this is not yet clear. To this end, we have cloned all the major transplant antigens from a Balb/c mouse (H-2 haplotype d) and created cell lines from a C57BL/6 (H-2 haplotype b) mouse that express each individual H-2d antigen. Using these cells a source of antigenic stimulus, we will be able to delineate the relative impact of each individual MHC.
4. To probe the maternal T regulatory response with more fidelity (given the lack of control of the number of male fetuses), we have begun to work with a transgenic mouse (OVA-CAG) that expresses chicken ovalbumin on the surface of every cell. Using this mouse as the father with a wild type mother of the same inbred background, each fetus will express the ovalbumin. This in combination with the well characterized T cell responses to ovalbumin and multiple regents available will allow for even deeper, more rigorous, characterization of the effects of pregnancy on the nature of the immune regulatory response to the fetus.

325. MicroRNAs in B-cell development and transformation

Dinesh S. Rao, David Baltimore

The differentiation of pluripotent progenitors into the various hematopoietic lineages follows an ordered series of molecular events that shape the function of the resulting progeny cells. The molecular regulation of differentiation occurs via developmental stage-specific transcriptional regulation and post-transcriptional regulation of gene expression by microRNA. In B-cell development, neoplasia is postulated to occur as a consequence of mistakes in physiological DNA rearrangement, resulting in the translocation of an oncogene to the transcriptionally active IgH locus. The resulting gene expression changes are thought to transform the B-cells; however, as we now know, the modulation of gene expression by post-transcriptional mechanisms may be just as important in controlling transformation.

By utilizing a target prediction algorithm to identify microRNAs that target the oncogenes, c-MYC, BCL2, and BCL6 (which are those commonly translocated in B-cell lymphoma), miR-34 was recognized as a family of microRNA that may be important in B-cell development and transformation. Shortly thereafter several groups determined that miR-34a was induced by the tumor suppressor p53. We are currently studying the roles that miR-34a plays in B-cell development and transformation by a combination of gain-of-function, loss-of-function, and mechanistic analyses.

Our first study of this miRNA focuses on the role that miR-34a plays in early B-cell development. In addition to its role in cancer, p53 also plays roles in hematopoietic development and specifically in lymphopoiesis. To search for a role of miR-34a in hematopoiesis, we performed a gain-of-function analysis in murine bone marrow. Constitutive expression of miR-34a led to a block in B-cell development at the pro-B cell to pre-B cell transition, leading to a reduction in mature B-cells in the bone marrow and peripheral blood. This block in B-cell development appears to be mediated primarily by inhibited expression of the Forkhead transcription factor, Foxp1. We demonstrate that Foxp1 is a direct target of miR-34a and that a conserved site in its 3'UTR mediates repression. Bone marrow transfer experiments with a siRNA directed against Foxp1 recapitulated many aspects of the B-cell developmental phenotype induced by miR-34a. Co-transduction of miR-34a with Foxp1 lacking its 3'UTR rescued the B-cell developmental phenotype, indicating that Foxp1 is a major target of miR-34a during B-cell development. These findings identify an important role for miR-34a in connecting the p53 network with suppression of Foxp1, a known B-cell oncogene.

A second study involves studying the role that p53 and miR-34a play in promoting the differentiation of plasma cells. These antibody secreting cells are critical to the humoral immune response, and their differentiation from antigen-stimulated B-cells is only partially understood. Both p53 and miR-34a are upregulated upon stimulation and differentiation of murine splenocytes. p53-deficient B-cells show decreased differentiation and immunoglobulin secretion compared with wild-type B-cells. *In vivo*, overall secretion of immunoglobulin (IgM) and the specific response against T-dependent antigens appear to be reduced in p53-deficient animals. Upon stimulation, p53-/- B-cells show a markedly reduced induction of miR-34a after stimulation, suggesting that it may mediate some of the defects of differentiation seen in these cells. We are currently in the process of addressing whether miR-34a itself is involved by a combination of gain- and loss-of-function analyses *in vitro* and *in vivo*.

Lastly, we are trying to determine whether miR-34a, which clearly has effects in B-cell differentiation, also plays a role in B-cell tumorigenesis in p53-deficient animals. For this purpose, bone marrow from p53-/- mice is transduced with miRNA prior to transplantation into lethally irradiated recipients, and formation of tumors then assessed in the recipient mice. In preliminary experiments, the development of tumors was observed in a subset of mice receiving marrow transduced with an empty control vector, but not in mice that received marrow transduced with miR-34a. These results suggest that miR-34a modulates the oncogenic potential of p53-deficiency. If this is the case, therapeutic possibilities are suggested for tumors arising in the context of p53-deficiency, the most common somatic genetic abnormality in human cancer.

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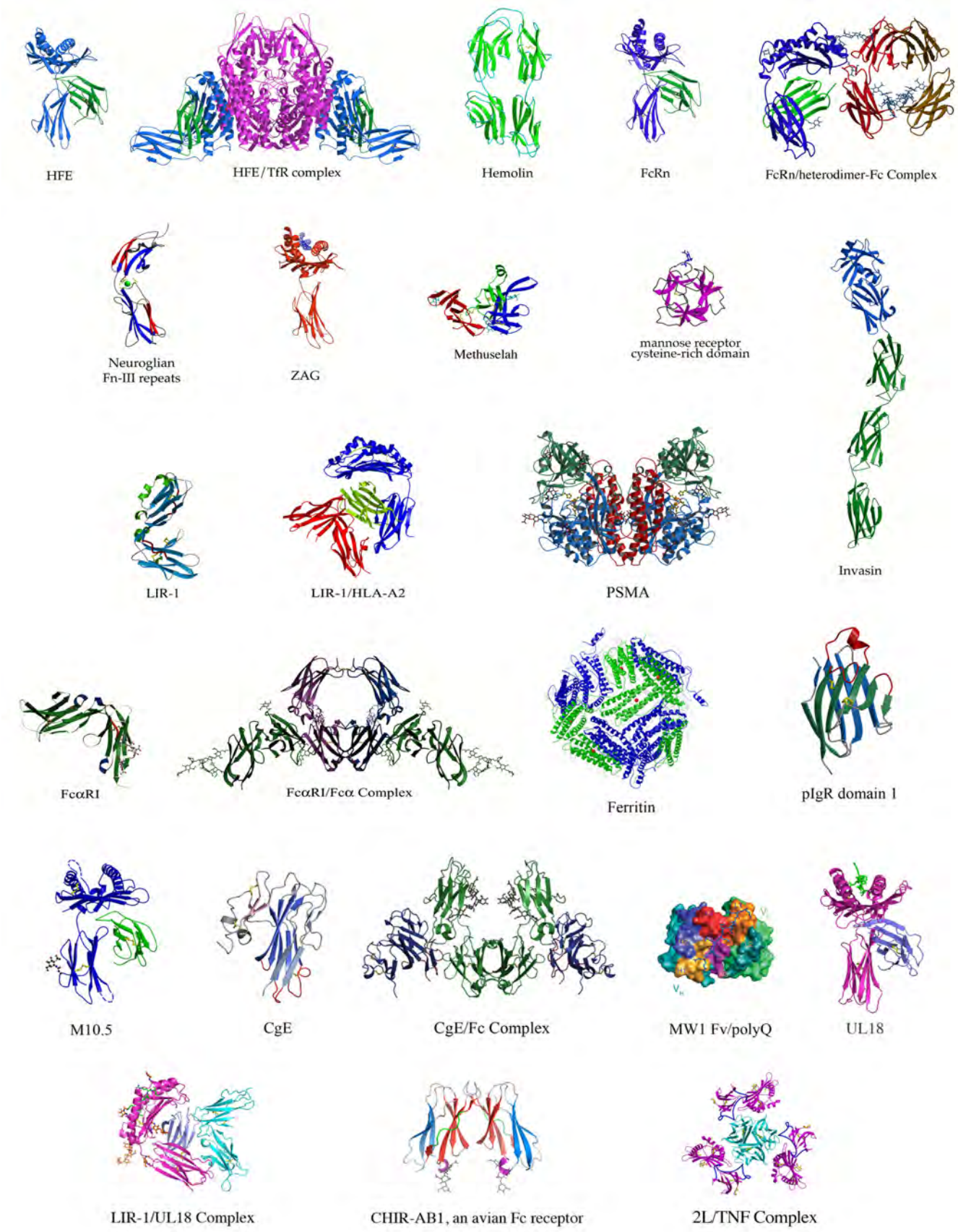
Summary: My laboratory is interested in protein-protein interactions, particularly those mediating immune recognition. We use X-ray crystallography and biochemistry to study purified proteins. Examples of crystal structures determined by our laboratory are shown in Figure 1. Some of our work focuses upon homologs and mimics of class I major histocompatibility complex (MHC) proteins. Classical class I MHC proteins present peptides derived from self and non-self proteins to T cells during immune surveillance. MHC homologs share similar three-dimensional structures with classical MHC molecules but have different functions, including immune functions (IgG transport by FcRn, the neonatal Fc receptor, and evasion of the immune response by viral MHC mimics) and non-immune functions (regulation of iron metabolism by HFE, and serving as a chaperone for pheromone receptors in the case of M10 proteins). We are also comparing the structures and functions of host and viral Fc receptors with FcRn.

We have recently begun to include confocal and electron microcopy to examine protein complexes in cells. Some of our efforts in these areas involve the study of the trafficking pathway by which FcRn transports IgG across polarized epithelial cells, which we are examining by electron tomography and live cell confocal microscopy.

Another recent focus of our laboratory is collaboration with David Baltimore's laboratory to "Engineer Immunity" against HIV. Our portion of the project involves designing, producing, and testing novel anti-HIV protein reagents in an effort to find proteins with increased efficacy in HIV neutralization. Promising candidates could then be administered via a gene therapy approach to HIV-infected individuals.

Figure 1


HFE
HFE/TfR complex
Hemolin
FcRn
FcRn/heterodimer-Fc Complex
Neuroglian
Fn-III repeats
ZAG
Methuselah
mannose receptor
cysteine-rich domain
Invasin
LIR-1
LIR-1/HLA-A2
PSMA
FcαRI
FcαRI/Fcα Complex
Ferritin
pIgR domain 1
M10.5
CgE
CgE/Fc Complex
MW1 Fv/polyQ
UL18
LIR-1/UL18 Complex
CHIR-AB1, an avian Fc receptor
2L/TNF Complex

326. Electron tomographic studies of FcRn-mediated antibody transport across epithelial cells

Mark S. Ladinsky, Kathryn E. Huey-Tubman

The neonatal Fc receptor (FcRn) transports maternal IgG across epithelial barriers, thereby providing the fetus or newborn with humoral immunity before its immune system is fully functional. In newborn rodents, FcRn transfers IgG from milk to blood by apical-to-basolateral transcytosis across intestinal epithelial cells. As milk passes through the neonatal digestive system, maternal IgG is removed by FcRn-expressing cells in the proximal small intestine (duodenum and jejunum). Remaining proteins are absorbed and degraded by FcRn-negative cells in the distal small intestine (ileum). We have developed a new method to enhance and detect individual nanogold-labeled Fc within transport vesicles (ref. 1) and have used this method, in conjunction with electron tomography and immuno-electron microscopy, to directly visualize transcytosis in jejunal cells (ref. 2). We are currently expanding upon this work to draw a more complete picture of FcRn-mediated transcytosis. We are following gold-labeled Fc in the basolateral portions of jejunal cells to characterize the compartments responsible for the exit of IgG from the intestinal epithelium to the bloodstream. We are also comparing jejunal structures with their counterparts in duodenum and contrasting them with FcRn-positive fluid-phase endocytic compartments in the ileum. Additional studies are focusing on FcRn expression and localization in immortalized cell lines (MDCK and IEC-6); model systems that can be experimentally perturbed in ways that are not practical with neonatal animal systems. Our results to date show that Fc moves through complex networks of entangled tubules and irregular vesicles during transcytosis. We have elucidated more than 30 new structural features of transcytosis and demonstrated a dramatic spatial complexity of the process, at both the structural and molecular levels. New studies of transcytosis, using these approaches in other systems, will further expand our understanding of FcRn's role in other intracellular trafficking systems.

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327. Characterization and visualization of the FcRn-dependent transcytotic pathway using high-resolution fluorescence confocal microscopy

Galina Jerdev, Scott E. Fraser[*]

Specific delivery of proteins across polarized epithelia is controlled by receptor-mediated transcytosis. Based on studies of the trafficking of model receptors such as the polymeric immunoglobulin receptor (pIgR), the pathways for receptor-mediated transport of protein ligands in the basolateral to apical direction are relatively well understood. The neonatal Fc receptor (FcRn) transports maternal immunoglobulin G (IgG) in the opposite direction across intestinal or placental epithelial barriers to provide immunity to fetal or newborn mammals and serves as a protection receptor for IgG. To investigate FcRn-mediated transport of IgG, we are using Madin-Darby Canine Kidney (MDCK) cells stably expressing FcRn (MDCK-FcRn). The transfected cells specifically transcytose IgG and Fc across polarized cell monolayers, therefore presenting an *ex vivo* system that mimics the *in vivo* FcRn-dependent transport system. Using high resolution confocal microscopy, we able to identify intracellular compartments involved in transcytosis of labeled Fc and IgG in MDCK-FcRn cells by colocalization studies with organelle-specific markers. Comparing the intracellular trafficking of FcRn and its ligands with trafficking of a basal-to-apical model receptor, pIgR, which transports dimeric dIgA (dIgA), provided information about common trafficking pathways of IgG and dIgA, such as transport through early endosomal and common endosomal compartments. To compare FcRn- and pIgR-dependent intracellular pathways, we are using MDCK-FcRn cells transiently expressing pIgR. Fluorescently-labeled Fc and dIgA can be internalized apically and basolaterally, respectively, by polarized MDCK-FcRn-pIgR monolayers, and the intracellular locations of internalized ligands investigated by confocal microscopy followed by colocalization analysis in 3D of internalized probes with different endogenous markers. An increase in colocalization in early chase points (5-10 min) with EEA1 and rab11 markers indicates that FcRn-Fc travels through early and sorting endosomes, respectively, while its later enrichment (15-20 min) with transferrin-positive compartments indicates that the complex travels through common sorting and basolateral endosomal compartments. To investigate the dynamics of Fc trafficking, we are using a high speed synchronized spinning disk Confocal Imaging System (UltraVIEW ERS) with a sensitive EMCCD camera, resulting in near real-time high-resolution time-lapse microscopy, performed on living polarized cells. The mobility characteristics of FcRn- and pig-positive structures in live MDCK cells were similar for these two vesicle populations and both were affected by a microtubule destabilizing agent.

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328. Nanogold as a specific marker for electron cryotomography

*Yongning He, Grant J. Jensen**

While electron cryotomography provides "molecular" resolution, three-dimensional images of unique biological specimens, sample crowdedness, and/or resolution limitations can make it difficult to identify specific macromolecular components. Here we used a 1.4 nm Nanogold®cluster specifically attached to the Fc fragment of IgG to monitor its interaction with the neonatal Fc receptor (FcRn), a membrane-bound receptor that transports IgG across cells in acidic intracellular vesicles. ECT was used to image complexes formed by Nanogold-labeled Fc bound to FcRn attached to the outer surface of synthetic liposomes. In the resulting three-dimensional reconstructions, 1.4 nm Nanogold particles were distributed predominantly along the interfaces where 2:1 FcRn-Fc complexes bridged adjacent lipid bilayers. These results demonstrate that the 1.4 nm Nanogold cluster is visible in tomograms of typically thick samples (~250 nm) recorded with defocuses appropriate for large macromolecules and is thus an effective marker.

**Associate Professor, Division of Biology, Caltech*

329. Intracellular trafficking of an antibody bipolar bridged complex of HSV-1 gE-gI, IgG, and a viral antigen

Alex Farley

The Herpes Simplex Virus 1 (HSV-1) glycoprotein gE-gI is a transmembrane Fc receptor found on the surface of infected cells and virions that binds host immunoglobulin G (IgG). gE-gI can participate in antibody bipolar bridging, a process by which the antigen-binding fragments (Fabs) of the IgG bind a viral antigen while the Fc binds to gE-gI. The gE-gI interaction with IgG is pH dependent (binding at the basic pH of the cell surface but not at the acidic pH of endosomes), suggesting that IgG bound by cell surface gE-gI would dissociate and be degraded in intracellular compartments if endocytosed. The fate of viral antigens associated with gE-gI-bound IgG in an antibody bipolar-bridged complex was unknown: they could remain at the cell surface or be endocytosed together with the IgG. We used a model system to investigate the trafficking of antibody bipolar-bridged complexes of gE-gI, IgG, and a viral antigen. Here we show that transfected HeLa cells expressing gE-gI endocytosed human IgG into intracellular compartments including early endosomes, recycling endosomes, and lysosomes. When the IgG was specific for a viral antigen expressed in the same cells as gE-gI, the antigen appeared in these intracellular compartments along with IgG only when the IgG could bind to gE-gI, thus demonstrating that antibody bipolar bridged complexes can be endocytosed by gE-gI. These results suggest that gE-gI plays an active role in clearing the infected cell surface of both host IgG and viral antigens, providing HSV-1 with a mechanism to evade IgG-mediated immune responses.

330. Structure of UL18, a peptide-binding viral MHC mimic, bound to a host inhibitory receptor

*Zhiru Yang**

UL18 is a human cytomegalovirus class I MHC (MHCI) homolog that binds the host inhibitory receptor LIR-1, and the only known viral MHC homolog that presents peptides. The 2.2Å structure of a LIR-1/UL18/peptide complex reveals increased contacts and optimal surface complementarity in the LIR-1/UL18 interface compared to LIR/MHCI interfaces, resulting in a >1000-fold higher affinity. Despite sharing only ~25% sequence identity, UL18's structure and peptide binding are surprisingly similar to host MHCI. The crystal structure suggests that most of the UL18 surface, except where LIR-1 and the host-derived light chain bind, is covered by carbohydrates attached to 13 potential N-linked glycosylation sites, thereby preventing access to bound peptide and association with most MHCI-binding proteins. The LIR-1/UL18 structure demonstrates how a viral protein evolves from its host ancestor to impede unwanted interactions, while preserving and improving its receptor-binding site.

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331. Crystal structure of TNFα complexed with a Poxvirus MHC-related TNF binding protein

Zhiru Yang, Anthony P. West, Jr.*

The poxvirus 2L protein binds tumor necrosis factor-α (TNFα) to inhibit host anti-viral and inflammatory responses. The 2.8 Å 2L/TNFα structure reveals three symmetrically arranged 2L molecules per TNFα trimer. 2L resembles class I MHC molecules, but lacks a peptide-binding groove and β2-microglobulin light chain. To compensate, the 2L α3 domain includes a large insertion stabilizing the α1-α2 platform and pre-organizing the TNFα binding surface, which involves residues from all three, 2L domains. Portions of the α3 insertion and the α2 domain helix form a large and complementary interface with the groove between adjacent TNFα subunits. Overlap between the 2L and host TNF receptor binding sites on TNFα rationalizes 2L inhibition of TNFα/TNF receptor interactions and prevention of TNFa-induced inflammation. The 2L/TNFα structure could facilitate rational design of anti-inflammatory therapeutics.

**Current Address: Novo Nordisk China R&D Center (NNST)*

332. Cryo-electron tomography of homophilic adhesion mediated by the neural cell adhesion molecule L1

*Yongning He, Grant J. Jensen**

The neural cell adhesion molecule L1 participates in homophilic interactions important for axon guidance and neuronal development. The structural details of homophilic adhesion mediated by L1 and other immunoglobulin superfamily members containing an

N-terminal horseshoe arrangement of four immunoglobulin-like domains are unknown. We used cryo-electron tomography to study liposomes to which intact or truncated forms of the L1 ectodomain were attached. Tomographic reconstructions revealed an adhesion interface with a regular and repeating pattern consistent with interactions between paired horseshoes contributed by L1 proteins from neighboring liposomes. The characteristics of the pattern changed when N-linked carbohydrates were altered by removing sialic acids or converting from complex to high mannose or oligomannose glycans, suggesting a regulatory role for carbohydrates in L1-mediated homophilic adhesion. Using the results from tomograms and crystal structures of L1-related molecules, we present a structural model for L1-mediated homophilic adhesion that depends on protein-protein, protein-carbohydrate, and carbohydrate-carbohydrate interactions.

[*]*Associate Professor, Division of Biology, Caltech*

333. Biophysical approach to understanding L1-mediated homophilic adhesion

Fan Yang, Tristan Ursell[1], Rob Phillips[2]

L1 is a transmembrane immunoglobulin superfamily (IgSF) molecule known to mediate homophilic and heterophilic adhesion events in neural cell recognition. L1 interacts with various distinct binding partners and plays important roles in neural development as well as in the adult nervous system, including neurite outgrowth, neuronal migration and survival, and synapse organization. Various models have been proposed to explain homophilic adhesion by L1, including a recent carbohydrate-dependent model based on cryo-electron tomography work by Yongning He from our lab (ref. 1). In this study, we aim to address the energetics of L1-mediated homophilic adhesion using a biophysical approach. We developed a confocal microscopy assay using giant unilamellar vesicles (GUV) produced by electroformation as an experimental platform. Recombinant L1 protein containing a 6x-His-tag was reconstituted in these GUVs, which contained lipids with a Ni-NTA functional head group for binding His-tagged proteins and rhodamine-conjugated lipids to facilitate visualization. The prepared GUVs were then incubated on an L1-treated copper-NTA coated glass slide and z-stack images were taken in order to reconstruct the three-dimensional shape of these GUVs adhering to the glass surface. Vesicle deformation was observed due to vesicle-vesicle and vesicle-surface adhesion when L1 was present. A theoretical model was developed based on membrane mechanics in order to evaluate adhesion energy on the basis of vesicle configurations. Simulations showed that over a certain energy range, the adhesion energy could be calculated by fitting confocal data to the model. When more data become available, we also explore issues related to cooperativity in L1-mediated adhesion.

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[2]*Professor of Applied Physics and Mechanical Engineering, Caltech*

Reference

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334. Biophysical and cell biological characterization of ferroportin

Adrian E. Rice, Douglas C. Rees[*]

Mammalian iron homeostasis is maintained by an intricate network of diverse proteins that constantly survey systemic iron levels and carefully regulate the uptake of iron from the diet. Control of this uptake is critically important because once iron is absorbed, mammals have no regulated mechanism for its removal. The portal through which iron enters the body is ferroportin, a multipass membrane protein expressed on the basolateral membrane of epithelial cells in the duodenum. The iron export function of ferroportin is primarily regulated by the serum peptide hormone hepcidin, which is secreted from the liver when systemic iron levels are high. Hepcidin acts as a negative regulator of iron uptake by binding to ferroportin at the cell surface and inducing its internalization and degradation. Genetic defects in ferroportin, hepcidin, or the proteins involved with sensing systemic iron levels lead to iron overload diseases known as hereditary hemochromatosis. Using the tools of biophysics and cell biology, we sought to study ferroportin and its interaction with hepcidin in order to better understand this critical bottleneck in iron uptake and how genetic defects within ferroportin might lead to disease. We developed the first protocols for the overexpression, detergent-solubilization, and purification of recombinant ferroportin. We determined that detergent-solubilized ferroportin is a monomer capable of binding hepcidin *in vitro*. We characterized the expression and subcellular localization of ferroportin in mammalian tissue culture and determined that both the amino- and carboxy-termini of ferroportin are cytosolic. We developed cell-based assays for the hepcidin-induced internalization of ferroportin and used these to characterize the route of internalization from the plasma membrane through early endosomes to degradative lysosomal compartments. Using live-cell imaging techniques, we showed that this internalization depended on intact microtubules. We expanded this cell-biological study to include sixteen disease-related ferroportin mutants and reported that each mutant was expressed on the plasma membrane like wild-type ferroportin, but that only a subset of the mutants were capable of being internalized by hepcidin. These studies form a foundation for future biophysical and cell-biological studies of ferroportin function.

[*]*Roscoe Gilkey Dickinson Professor of Chemistry, Caltech*

335. Structural studies of class C GPCRs

Rich Olson[*]

My research investigates the three-dimensional structure and molecular mechanism of membrane proteins central to health and disease using crystallographic methods. I seek to answer questions relating to the specificity and biological context of protein-protein and protein-ligand interactions, the structural rearrangements in membrane proteins that accompany and facilitate signal transmission across the cell membrane, and the importance of oligomerization and multi-molecular complex formation in the function of integral and peripheral membrane proteins. I have two principal areas of investigation: proteins that mediate host-pathogen interactions involving the human immune system and proteins involved in signal transduction events in the central nervous system. In the first area, I am employing X-ray crystallography to determine high-resolution structures of pore-forming toxins from pathogenic bacteria. By solving toxin structures in water-soluble and fully assembled integral membrane states, I seek details about the mechanism and specificity of these agents against human cells. In the second area, I am studying the structure and function of G-protein coupled receptors involved in olfaction and taste. I aim to understand how the binding of small molecules to the extracellular domain of olfactory receptors is translated into an intercellular signal. Together, these studies seek to delineate the structural underpinnings that allow membrane proteins to accomplish a wide range of important cellular functions.

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336. Three-dimensional structure of the detergent-solubilized *Vibrio cholerae* cytolysin (VCC) heptamer by electron cryomicroscopy

Yongning He, Rich Olson[*]

Vibrio cholerae cytolysin (VCC) is a pore-forming toxin that inserts a lytic water-filled channel into susceptible host membranes. Assembly of the toxin on cell surfaces may be enhanced by two tandem lectin domains, in addition to direct interactions with lipids and cholesterol within the membrane itself. We have used single-particle electron cryomicroscopy (cryoEM) to generate a low-resolution molecular structure of the detergent-solubilized VCC oligomer to 20 Å resolution. After confirming a heptameric arrangement of individual protomers, seven-fold averaging around the central pore was utilized to improve the structure. Docking of the previously determined VCC protoxin crystal structure was possible with rigid-body rearrangements between the cytolytic and lectin domains. A second cryoEM reconstruction of a truncated VCC mutant supported the topology of our model in which the carboxyl-terminal lectin domain forms "spikes" around the toxin core with the putative carbohydrate receptor-binding site accessible on the surface of the oligomer. This finding points to an assembly mechanism in which lectin domains may remain bound to receptors on the cell surface throughout assembly of the cytolytic toxin core and explains the hemagglutinating activity of purified toxin. Our model provides an insight into the structural rearrangements that accompany VCC-mediated cytolysis and may aid in the engineering of novel pore-forming toxins to attack specific cells towards therapeutic ends.

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337. Structural studies of the HIV spike recognition by neutralizing antibodies

Ron Diskin, Rachel Galimidi, Chris Foglesong, Noreen Tiangco[*]*, Maria Suzuki, Anthony P. West, Jr.*

HIV is a retrovirus that cause AIDS, a devastating disease especially prevalent in developing countries. Low awareness for prevention and the inability to purchase modern drug therapies result in the fast spreading of the epidemic and a substantial decrease in life expectancy. Infection by HIV can be prevented in the presence of broadly reactive neutralizing antibodies (NAbs), which target the functional envelope spike protein of the virus. This spike mediates fusion of HIV to CD4-positive cells using a well-masked conserved core. HIV spikes consist of three gp120-gp41 proteins, which are the cleavage products of the gp160 precursor. This protein complex is the only viral derived moiety presented on its surface. Attempts to trigger the immune system to produce NAbs using a vaccine have failed so far. One of the main obstacles impairing these trials is the lack of accurate structural data on the functional form of the envelope spike complex and the mechanism that NAbs exploit to recognize it. Our research is aimed at studying the mechanism that governs the recognition of the functional HIV spike complex by NAbs. To achieve this goal we plan to solve the crystal structure of the functional spike complex (the gp120-gp41 heterotrimer) or a related substructure (gp120 homotrimer) with or without various known and newly designed NAbs. As the crystallization of the functional heterotrimer may present many difficulties, we are also researching toward solving the crystal structures of the gp120 monomers in complex with novel NAbs. To enhance our chances for succeeding, we are utilizing a broad array of proteins derived from many different HIV clones thus, introducing sequence variability to the crystallization trials. The resulting information from our research could bridge the existing gap in our knowledge concerning the neutralization of HIV by NAbs. This may facilitate the production of artificial epitopes that would trigger the immune system to produce effective NAbs. If successful, such epitopes will serve as a much needed vaccine against HIV.

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338. Examination of the contributions of size and avidity to the neutralization mechanisms of the anti-HIV antibodies b12 and 4E10

Joshua S. Klein, Priyanthi N.P. Gnanapragasam, Rachel P. Galimidi, Christopher P. Foglesong, Anthony P. West, Jr., Pamela J. Bjorkman

Monoclonal antibodies b12 and 4E10 are broadly neutralizing against a variety of strains of the human immunodeficiency virus type 1 (HIV-1). The epitope for b12 maps to the CD4-binding site in the gp120 subunit of HIV-1's trimeric gp120-gp41 envelope spike, whereas 4E10 recognizes the membrane-proximal external region (MPER) of gp41. Here, we constructed and compared a series of architectures for the b12 and 4E10 combining sites that differed in size, valency, and flexibility. In a comparative analysis of the ability of the b12 and 4E10 constructs to neutralize a panel of clade B HIV-1 strains, we observed that the ability of bivalent constructs to cross-link envelope spikes on the virion surface made a greater contribution to neutralization by b12 than by 4E10. Increased distance and flexibility between antibody combining sites correlated with enhanced neutralization for both antibodies, suggesting restricted mobility for the trimeric spikes embedded in the virion surface. The size of a construct did not appear to be correlated with neutralization potency for b12, but larger 4E10 constructs exhibited a steric occlusion effect, which we interpret as evidence for restricted access to its gp41 epitope. The combination of limited avidity and steric occlusion suggests a mechanism for evading neutralization by antibodies that target epitopes in the highly conserved MPER of gp41.

339. Design and expression of a dimeric form of human immunodeficiency virus type 1 antibody 2G12 with increased neutralization potency

Anthony P. West, Jr., Rachel P. Galimidi, Christopher P. Foglesong, Priyanthi N.P. Gnanapragasam, Kathryn E. Huey-Tubman, Joshua S. Klein, Maria D. Suzuki, Noreen E. Tiangco, Jost Vielmetter, Pamela J. Bjorkman

The antigen-binding fragment of the broadly neutralizing human immunodeficiency virus type 1 (HIV-1) antibody 2G12 has an unusual three-dimensional (3D) domain-swapped structure with two aligned combining sites that facilitates recognition of its carbohydrate epitope on gp120. When expressed as an intact immunoglobulin G (IgG), 2G12 formed typical IgG monomers containing two combining sites and a small fraction of a higher-molecular-weight species, which showed a significant increase in neutralization potency (50- to 80-fold compared to 2G12 monomer) across a range of clade A and B strains of HIV-1. Here we show that the higher-molecular-weight species corresponds to a 2G12 dimer containing four combining sites and present a model for how intermolecular 3D domain swapping could create a 2G12 dimer. Based on the structural model for a 3D domain-swapped 2G12 dimer, we designed and tested a series of 2G12 mutants predicted to increase the ratio of 2G12 dimer to monomer. We report a mutation that effectively increases the 2G12 dimer/monomer ratio without decreasing the expression yield. Increasing the proportion of 2G12 dimer compared to monomer could lead to a more potent reagent for gene therapy or passive immunization.

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340. Potent neutralization of HIV by monoclonal antibodies is not a predictor of the ability to trigger antibody-dependent cellular cytotoxicity

Joshua S. Klein[*], Alexandre Webster[*], Priyanthi N.P. Gnanapragasam

Antibody-dependent cellular cytotoxicity (ADCC) refers to a mechanism by which antibodies bound to cells can trigger their lysis by certain effector cells of the innate immune system. Current research suggests that ADCC may be important in controlling and/or preventing HIV infections. However, the lack of a quantitative *in vitro* assay has prevented the evaluation of anti-HIV monoclonal antibodies to trigger ADCC. We have developed an ADCC assay using cells that stably expresses HIV gp160 that allows for the evaluation of antibodies directed against this antigen. Using a panel of antibodies directed against gp120 carbohydrate, the CD4-binding site, the V3 loop, and the membrane proximal external region (MPER) of gp41, we observed that all of the antibodies were potent neutralizers of a strain of virus displaying the gp160 variant used in the ADCC assay, with each antibody able to neutralize ~100% of the virus at concentrations at or below ~10 μg/mL. However, the antibodies exhibited wide variation in their abilities to recruit ADCC activity. Monomeric and dimeric IgG1 2G12 were most effective at recruiting ADCC, yielding typical sigmoidal dose-response curves and maximal specific lysis values of 75% and 63%, respectively. By contrast, the anti-CD4-binding site antibody, IgG1 b12, and the anti-V3 loop antibody, IgG1 447-52D, exhibited only modest abilities to recruit ADCC. However, perhaps most striking was the absence of any detectable ADCC activity upon the addition of IgG1 4E10 or 2F5, which target adjacent epitopes of the MPER at the base of the stalk of the trimeric envelope spike. These data suggest that different epitopes on the envelope spike impose unique restrictions on the abilities of IgG1 antibodies to recruit ADCC that do not necessarily apply to the mechanism of neutralization. We are currently examining whether Fc mutations that enhance binding to CD16 and mutations that increase the accessibility of the Fc region of IgG1 antibodies bound to the envelope spike can serve to offset these restrictions.

[*]*Contributed equally to this work.*

341. Engineering immunity against HIV-1: Novel high-avidity neutralizing antibody architectures seek to enhance intra-spike cross-linking

Alexandre Webster, Rachel Galimidi

Cross-linking by bivalent anti-HIV antibodies could contribute neutralization, however HIV generally evades antibody cross-linking by populating its outer membrane envelope with few viral spikes. Conventional antibody architectures subsequently have difficulty cross-linking multiple HIV-1 epitopes because of the low density of epitopes present on the viral surface. Additionally, conventional antibody architectures lack the structural flexibility to bind repeating epitopes within the envelope spike trimer. Novel architectures we are currently engineering will enhance nAb cross-linking potential by specifically targeting repeating intra-spike epitopes. One such approach employs Agnathan Variable Lymphocyte Receptors (VLR) fused to single-chain fragment variable regions (scFvs) from anti-HIV antibodies. VLR are leucine-rich repeat proteins that spontaneously form stable multimeric structures containing up to 10 closely-spaced binding sites. Preliminary structural modeling analysis suggests multimeric VLR-nAb fusion constructs fit dimension restrictions required for intra-spike epitope cross-linking. We will be testing chimeric VLR-nAb constructs for *in vitro* viral neutralization capabilities in the near future and hope to further define the requirements for developing new anti-HIV-1 reagents.

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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. Some of the simulation programs, as Macintosh applications, are available at www.cco.caltech.edu/~brokawc/software.html

342. Dynein-driven oscillation resulting from doublet separation and reassociation

Charles J. Brokaw

When ATP is supplied to partially disintegrated, demembranated flagella, where the outer doublets remain attached at the basal end, sliding of a doublet towards the basal end causes it to bulge out and separate away from the adjacent doublet. Further sliding in the distal region is then accommodated by bending of the sliding doublet into a large loop, and finally by complete separation. This followed by reassociation of the separated doublet, starting from the basal end where it is held close to its partner. The sequence can then be repeated at a regular frequency. This situation, and other examples where doublets separate and reassociate, suggest that dyneins are not only the flagellar motors, but are also responsible for maintaining the proper operating distance between doublets. Although obviously this is a pathological situation, it is the only example of dynein-driven oscillation that can be explained by obvious on and off states, resulting from proximity to or separation from, the partner doublet.

A computer program has been developed to simulate this behavior. Although based on previous simulations of flagellar and microtubular bending, it incorporates the new features of variable distance between two filaments, with an elastic adhesive force as well as modulation of active sliding by the distance between the filaments. Analysis of this model provides some interesting insights. The separation between two doublets is initiated by a buckling instability, which also initiates active sliding distal to the separation. It is therefore a sliding initiation event, similar to the sliding initiation events that occur in normal beating of cilia and flagella. It suggests that sliding initiation events in cilia and flagella may not be initiated by an activation of dyneins, as previously supposed, but by release of an impediment to sliding near the base of the flagellum. Although various hypotheses have been proposed for activation and inactivation of dynein during flagellar bending, they have not explained how parameters such as frequency and amplitude are determined. Whether this problem can be resolved by understanding sliding initiation events as a result of buckling instability awaits further analysis.

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Professor of Biology and Chemistry: Judith L. Campbell
Member of the Professional Staff: Martin Budd, Piotr Polaczek
Postdoctoral Scholars: Kenneth Kimani, Subhash Pokharel
Research Fellow: Hui-Qiang Lou
Graduate Student: Barbara K. Fortini
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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 MRC1, the ortholog of mammalian claspin, a protein involved in mediating the signal from a stalled replication fork to the cell cycle checkpoint apparatus. Huiqiang Lou in the lab has recently shown that the interaction between Mrc1 and ε is regulated by phosphorylation by the master checkpoint kinase, ATM (Mec1 in yeast).

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. We are 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 set of experiments used global synthetic lethal screening to identify a network of 822 genetic interactions that together account for the pathways protecting the eukaryotic genome from rearrangements due to DNA damage.

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 life span. Microarray analysis by Isabelle Lesur shows that the *dna2* mutants age by the same pathway as wildtype cells; they just age faster. Interestingly, the human Bloom and Werner genes complements the replication defect of *dna2* mutants, suggesting that Dna2 works in the same pathway with these genes. We have now shown that the Dna2 helicase works with the yeast BLM ortholog, Sgs1 in the major pathway of double-strand break repair in yeast and are studying the same process in human cells. Dna2 is involved in an early event that results in the production of a single-stranded 3' end that has two functions. It is involved in strand invasion of the homolog and thus the initiation of strand exchange. Perhaps even more important the single-stranded DNA is a key intermediate in the activation of the cell cycle checkpoint that protects the cell from genome instability in the presence of a double-strand break arising from replication fork failure.

343. Distribution of magnetosomes at cell division in *M. magnetotacticum*

Kristen Kozak[*]*, Zhuo Li, Elizabeth Bertani*

The most commonly studied magnetotactic bacteria contain membrane-enclosed crystals of magnetite, termed magnetosomes. The magnetosomes are usually lined up in a single long chain running the length of the cell. As in most bacteria, cell division occurs by elongation of the organism followed by separation at the mid-plate to produce two daughter cells. In most bacteria, cell division is a complicated process involving a tubulin-like structural component, the product of the *fts*Z gene. But, what happens to the magnetosomes? How are they

divided between the two-daughter cells? They might be distributed all to one daughter cell, leaving the other to replenish its supply of magnetosomes, or they might be equally divided between the daughters, which would require a division mechanism. It has even been suggested that the magnetosomal chain is duplicated before cell division and a duplicate is distributed to each daughter.

To distinguish between these suggestions, transmission electron microscopy was used to examine dividing cells (i.e., showing a constricted center) of *M. magnetotacticum*, strain AMB-1. Dividing cells are rare, because the rate of cell division is low, so only a total of 11 dividing cells were examined. In all cases, the chain of magnetosomes was visible on both sides of the midplate and appeared continuous, except across the midplate, itself. No cells containing two parallel chains were seen. Thus, an equal division between the daughter cells is most likely.

AMB-1 has two genes with an ftsZ signature. Gene *fts*Z-1 is clustered together with genes that are involved in normal cell division, but *fts*Z-2 is located in a region of the genome known to be necessary for magnetotaxis. Gene *fts*Z-2, therefore, is a likely candidate to be involved in magnetosome chain division. GFP-tagged derivatives of the *fts*Z-2 protein are being prepared to allow us to visualize the location of the protein during cell division.

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344. Multiple nucleases are involved in double strand break repair and telomere maintenance

Martin Budd, Judith L. Campbell

The appearance of a 3' single-stranded GT tails at a chromosome end is a critical step in the maintenance of telomeres. In yeast, the overhangs can arise due to nucleolytic processing or to failure to complete lagging strand replication and do not appear to require telomerase. Mre11, which has nuclease activity, is involved in resection since 3' GT tails are shorter in *mre11Δ* mutants and fail to appear at DSBs having a short telomere seed sequence in mre11 nuclease deficient mutants. However, other nucleases must also be involved. Dna2 is a 5' to 3' helicase, a exo/endonuclease which processes Okazaki fragments in collaboration with Rad27/FEN1, at a subset of difficult to replicate sites in the genome that, to date, are poorly defined. A second major function of Dna2 is to mediate 5' to 3' resection, along with Mre11 nuclease, during the initial stage of DSB repair. Since telomeres resemble DSBs, we wished to test if Dna2 also acts with Mre11 in formation of overhangs. We found that unlike at DSBs, 3' GT overhangs still appeared in *dna2Δ mre11-nuclease deficient* strains. This could be explained either by the presence of yet another nuclease, such as Exo1, or failure to fill in the lagging strand. To distinguish, single-stranded GT tails were quantified in a *dna2-2 exo1* strain. 3' GT tails accumulated in the *dna2-2 exo1* mutant but not in either the *dna2-2* or *exo1* mutant. The telomere phenotype was the same as that of a rad72D mutant at 37 C. We propose that that the major enzyme for OF, Rad27 is not sufficient for processing of Okazaki fragments at telomeres, and that either Dna2 or Exo1 is required in addition. This identifies telomeres as sites that require Dna2 for OFP. In addition, our studies show that pif1 mutants, that have long telomeres, probably because of increases telomerase processivity, accumulate excess single-stranded DNA. Since the appearance of GT overhangs is not associated with overreplication of telomeres per se, this provides evidence that Pif1 is also involved in lagging strand synthesis at telomeres.

345. Post-translational modification of Dna2 following DNA damage

Barbara K. Fortini, Martin E. Budd, Judith L. Campbell

Saccharomyces cerevisiae Dna2 is an essential enzyme with helicase, nuclease, ATPase, ssDNA annealing and strand exchange enzymatic activities. In addition to an established role in Okazaki fragment processing and telomere maintenance, both genetic and biochemical evidence implicates Dna2 in the repair of double strand breaks and other forms of DNA damage. Both helicase- and nuclease-defective mutants of Dna2 are known to be sensitive to DNA damaging agents. Further, we recently reported that the Dna2 nuclease and Mre11 nuclease are interchangeable in the repair of X-ray-induced double strand breaks.

We find that the modulation of Dna2 activity after a DNA damaging event depends on the signaling of multiple kinases. Prominent among these is the kinase Mec1, which phosphorylates Dna2 after multiple forms of DNA damage. Post-translational modification of Dna2 is important for DNA damage survival as several non-phosphorylatable point-mutants of Dna2 show dramatic sensitivity to treatment with the DNA damaging agent MMS.

446. Mrc1 functions at eukaryotic replication forks

Huiqiang Lou

Mrc1, ortholog of Claspin, is both a central component of normal DNA replication forks and a mediator of the S phase checkpoint. We report that Mrc1 interacts with Pol2, the catalytic subunit of DNA polymerase epsilon, essential for leading strand DNA replication and for the S phase checkpoint. The bimodal interaction between Mrc1 and Pol2 may identify a novel step in converting DNA damage on the leading strand into a molecular signal that activates the S phase checkpoint. Since Mrc1 also interacts with the putative MCM helicase, Mrc1 may modulate coupling of polymerization and unwinding. The N terminus of Mrc1 interacts with the catalytic N-terminal half of Pol2 (Pol2N), suggesting that Mrc1 may be a polymerase accessory factor. Therefore, we carried out the structure and function analysis of Mrc1. Full-length protein was cloned and expressed along with a set of truncated deletion mutants. Proteins were then purified and applied to *in vitro* functional assays as DNA binding and accessory activity for polymerase. Gel shift assay showed Mrc1 itself can bind to DNA in a substrate

specificity manner. It does not bind to dsDNA, and shows bias to substrates mimic fork and flap structures. DNA binding domain locates at the N-terminal half that contains BP1(basic patch). Its modulation function on activities of pol epsilon is currently under investigation. We are also going to test if Mrc1 affects activities of MCM helicase through collaborating with Shwanca's lab.

Publications

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Rotating Students: David Akopian, Rebecca Rojansky, Mike Rome
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Summary: The primary focus of our lab is to understand the role of mitochondrial dynamics in normal cellular function and human disease. Mitochondria are remarkably dynamic organelles that undergo continual cycles of fusion and fission. The equilibrium of these two opposing processes determines not only the overall morphology of mitochondria in cells, but also has important consequences for mitochondrial function.

Our research falls into several broad areas:

(1) What are the cellular and physiological functions of mitochondrial fusion and fission?
(2) What is the molecular mechanism of mitochondrial membrane fusion and fission?
(3) What role do mitochondrial dynamics play in human diseases?

To address these issues, we use a wide range of approaches, including genetics, biochemistry, cell biology, and structural biology.

I. Cellular and physiological functions of mitochondrial fusion and fission

A typical mammalian cell can have hundreds of mitochondria. However, each mitochondrion is not an autonomous organelle, because fusion and fission events mix mitochondrial membranes and contents. As a result, such events have major implications for the function of the mitochondrial population. We are interested in understanding the cellular role of mitochondrial dynamics, and how changes in mitochondrial dynamics can affect the function of vertebrate tissues.

Much of our work focuses on proteins involved in mitochondrial fusion or fission. Three large GTPases are essential for mitochondrial fusion. The mitofusins (Mfn1 and Mfn2) are transmembrane GTPases embedded in the outer membrane of mitochondria, and OPA1 is a dynamin-related protein localized to the intermembrane space. Our recent work indicates that these proteins act at distinct steps during the membrane fusion process (Zhiyin Song). Mitochondrial fission requires the function of Drp1, a dynamin-related protein that recruited to the mitochondrial surface to promote fission.

To understand the role of mitochondrial fusion in vertebrates, we have constructed mice deficient in either Mfn1 or Mfn2. We find that mice deficient in either Mfn1 or Mfn2 die in mid-gestation, indicating an essential function for mitofusins during embryogenesis. In both cases, the embryonic lethality results from placental dysfunction. From mouse models, we have generated cellular systems to dissect mitochondrial dynamics. Embryonic fibroblasts lacking Mfn1 or Mfn2 display fragmented mitochondria, a phenotype due to a severe reduction in mitochondrial fusion. Cells lacking OPA1 or both Mfn1 and Mfn2 have completely fragmented mitochondria and show no detectable mitochondrial fusion activity. Our analysis indicates that mitochondrial fusion is important not only for maintenance of mitochondrial morphology, but also for cell growth, mitochondrial membrane potential, and respiration. In part, these defects arise from a loss of mtDNA nucleoids, suggesting that content mixing due to mitochondrial fusion plays an important protective role for the mitochondrial population within cells.

We have also generated mice with conditional alleles of Mfn1 and Mfn2 and are using these mouse lines to examine the role of mitochondrial fusion in adult tissues. For example, we have discovered that loss of Mfn2 results in a highly specific degeneration of Purkinje neurons in the cerebellum. These studies are highly relevant to our understanding of several human diseases in which defects in mitochondrial dynamics lead to neurodegeneration (see below). We are also developing mouse models to understand mitochondrial fission (Zhiyin Song) and to track mitochondrial dynamics *in vivo* (Anh Pham).

II. Molecular mechanism of membrane fusion and fission

The best understood membrane fusion proteins are viral envelope proteins and SNARE complexes. Viral envelope proteins, such as gp41 of HIV, reside on the lipid surface of viruses and mediate fusion between the viral and cellular membranes during virus entry (a topic we previously studied). SNARE complexes mediate a wide range of membrane fusion events between cellular membranes. In both cases, cellular and crystallographic studies have shown that the formation of helical bundles plays a critical role in bringing the merging membrane together. We would like to understand mitochondrial fusion at a similar level of resolution and to determine whether there are common features to these diverse forms of membrane fusion. Mitofusins are the only conserved mitochondrial outer membrane proteins involved in fusion. Therefore, it is likely that they directly mediate membrane fusion. Consistent with this idea, mitofusins are required on adjacent mitochondria to mediate fusion. In addition, mitofusins form homotypic and heterotypic complexes that are capable of tethering mitochondria. We are trying to

determine how tethered mitochondria, mediated by mitofusins, proceeds to full fusion. It should be noted that mitochondrial fusion is likely to be more complicated than most other intracellular membrane fusion events, because four lipid bilayers must be coordinately fused.

We are also exploring the roles of other proteins, such as OPA1, in mitochondrial fusion (Tadato Ban). We have generated OPA1-null cells to understand the role of OPA1 in mediating membrane fusion and in maintaining mitochondrial function. In addition, we are developing biochemical assays to examine how OPA1 function is activated (Tadato Ban). We are also using biochemical approaches to understand how mitochondrial fission complexes are assembled on the surface of mitochondria (Yan Zhang).

III. Mitochondrial dynamics in human disease

Two inherited human diseases are caused by defects in mitochondrial dynamics. Charcot-Marie-Tooth (CMT) disease is a neurological disorder that affects the peripheral nerves. Patients with CMT experience progressive weakness of the distal limbs and some loss of sensation. A specific type of CMT, termed CMT2A, is caused by mutations in Mfn2 that lead to degeneration of axons in peripheral nerves. We have used mouse and cellular models to understand how disease alleles of Mfn2 lead to neuronal dysfunction.

The most common inherited form of optic neuropathy (autosomal dominant optic atrophy) is caused by mutations in OPA1.

Finally, an understanding of mitochondrial dynamics will be essential for understanding a large collection of diseases termed mitochondrial encephalomyopathies. Many mitochondrial encephalomyopathies result from mutations in mitochondrial DNA (mtDNA). In mtDNA diseases, tissues maintain their mitochondrial function until pathogenic mtDNA levels exceed a critical threshold. Experiments with cell hybrids indicate that mitochondrial fusion, by enabling cooperation between mitochondria, can protect respiration even when >50% of mtDNAs are mutant. We are exploring how mitochondrial fusion might affect the segregation of mtDNA species within a cell (Oliver Loson), and how such segregation would affect phenotypic expression of pathogenic mtDNA (Anne Chomyn).

To understand the pathogenesis of mtDNA diseases, it is critical to explore how mitochondria can be functionally distinct and yet cooperate as a population within a cell. We anticipate that our studies with mice lacking mitochondrial fusion will help to shed light on this group of often devastating diseases (Hsiuchen Chen). In addition, these studies may be relevant to understanding the link between mtDNA mutations, neurodegeneration, and age-related tissue degeneration (Marc Vermulst).

IV. Additional research areas

In addition to mitochondrial dynamics, we have broad interests in other areas of mitochondrial biology. In particular, we have initiated research projects to understand the organization of mtDNA into nucleoids (Huu Ngo) and to develop tools to analyze mtDNA function and dynamics (Yun Elisabeth Wang). We are also developing methods to understand the turnover of mitochondria through autophagy (Nickie Chan), a topic that is likely relevant for understanding Parkinson's disease.

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347. Biochemical and structural studies of OPA1, a dynamin-related GTPase essential for mitochondrial fusion

Tadato Ban

OPA1, dynamin-related GTPase, has been reported to play a central role in mitochondrial membrane fusion. Several dynamin family proteins have well-known functions in tubulation and constriction of lipid membranes in diverse cellular contexts, including endocytosis, vesicular trafficking, and mitochondrial division. Dynamin-related proteins assemble into higher order filamentous structures and interact with lipid membranes. However, it is unknown if OPA1 is similarly capable of constricting and tubulating mitochondrial membranes, and how such activities might contribute to mitochondrial membrane fusion. To address such issues, we have developed methods to purify large amounts of recombinant OPA1. We are developing *in vitro* assays to explore the assembly properties of OPA1 oligomers and whether they interact with lipid bilayers. In addition, we are attempting crystallization trials to determine an atomic structure.

348. A proteomics approach to identify key regulators of mitophagy

Nickie C. Chan

Mitochondria are the sites of numerous essential cellular activities. These activities range from oxidative phosphorylation, metabolism of amino acids and fatty acids, assembly of iron-sulfur clusters, to the regulation of programmed cell death. It is now clear that many human diseases are caused by defects in mitochondrial function, and the accumulation of mitochondrial DNA mutations have been proposed to be a major driving force of aging.

Recent studies have suggested that defective mitochondria, including those with decrease in membrane potential, can be specifically targeted for degradation via a process known as "mitophagy." Mitophagy involves the engulfment of mitochondria by double-membrane-bound structures called autophagosomes, and their subsequent delivery to the lysosomes for degradation. To date, it is unclear how defective mitochondria are recognized for degradation, and elucidating its molecular mechanism will have major implications for mitochondrial biology and diseases.

To investigate if the recognition of defective mitochondria by the autophagic machinery is signalled by
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changes in the mitochondrial proteome, we are establishing a methodology involving stable isotope labelling with amino acids in cell culture (SILAC) and mass spectrometry. In principle, this methodology involves labelling of the entire proteome with non-radioactive, stable isotopes of amino acids, which will be compared to the proteome of the control cells that is labelled with normal isotopes. Upon analysis by mass spectrometry, differences in the expression level of every single detectable protein in the proteome can be quantitatively compared in an unbiased manner between the experimental and the control cells. Using this approach, we will measure changes in the human mitochondrial proteome by isolating mitochondria from HeLa cells that are treated with chemical agents known to induce mitophagy, and comparing it to the mitochondrial proteome of untreated cells. Proteins that show significant changes in their expression level upon induction of mitophagy will serve as candidates for further biochemical characterization.

The mass spectrometry analysis in this project is performed at the Caltech Proteome Exploration Laboratory.

349. Mitochondrial fusion promotes mtDNA stability and tolerance to mtDNA mutations

Hsiuchen Chen, Marc Vermulst, Y. Elisabeth Wang

Mutations in mitochondrial DNA (mtDNA) cause progressive diseases and may be central to the aging process. It is therefore, important to identify factors that safeguard mtDNA stability. Mitochondrial fusion is thought to protect cells from dysfunction by enabling exchange of contents, including mtDNA. Our studies of mitofusin-deficient mice indicates that loss of mitochondrial fusion in muscle results in a 14-fold reduction in mtDNA levels and accumulate mtDNA point mutations and deletions. Moreover, these mice have hallmarks of mitochondrial myopathy. We also found that fusion-deficient mice cannot tolerate mutations arising from an error prone mtDNA polymerase. These results indicate that mitochondrial fusion is important for maintaining levels and fidelity of mtDNA. Furthermore, when mtDNA mutations are present, robust mitochondrial fusion is necessary to preserve cellular health. Therefore, mitochondrial fusion is likely a protective factor in aging and mtDNA diseases.

350. Interaction between mitochondrial dynamics and mitochondrial genetics

Anne Chomyn, Oliver Loson

Mitochondrial DNA (mtDNA) is present in hundreds or thousands of copies per cell. Under certain circumstances, two distinct types of mitochondrial genomes are present in a cell, a state termed heteroplasmy. With time, their relative proportions in the cell can drift over successive generations to one or the other genotype, and can even drift so far as to segregate the genomes. We would like to examine the role of mitochondrial fusion and fission proteins in the process of such genetic drift, and measure the direction and speed of this phenomenon. To this end, we have begun deriving mtDNA-less (ρ^0) cell lines from Mfn1 and Mfn2 conditional knockout mouse cell lines. Exogenous mtDNA of interest will be introduced into ρ^0 cells by fusion with enucleated donor cells. We have had success in isolating ρ^0 cells using ethidium bromide, which inhibits mtDNA replication and was used previously (King and Attardi, 1989) in human cell lines. We have found it necessary to use higher concentrations than was used to obtain human ρ^0cell lines. We are verifying the absence of mtDNA in our putative ρ^0 lines by metabolic assays, by quantitative PCR tests of total cellular DNA, and by anti-DNA immunocytochemistry. ρ^0 cells reconstituted with a heteroplamic mixture of mtDNAs will be used for genetic drift experiments.

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351. Mitochondrial DNA transfection

Oliver Losón

A great hurdle to better understanding mitochondrial genetics, disease and physiology is an inability to introduce manipulated mitochondrial DNA (mtDNA) into a cell. Transfection of mitochondria in whole-cells has been accomplished for *S. cerevisiae* and *C. reinhardtii*, but there are no reports of successful transfection of mammalian mitochondria. To tackle this technical challenge, we have engineered a mitochondrial genome that encodes enhanced green fluorescent protein (EGFP). Our strategy has been to use established transfection techniques and evaluate the success of transfection by screening for EGFP expression. To date, we have attempted transfection with whole-cell electroporation, lipofection and gold particle bombardment, which was the method used for mitochondrial transfection in *S. cerevisiae* and *C. reinhardtii*. To date, these approaches have not succeeded. As an alternative, we are attempting to transfect isolated mitochondria using electroporation. Using quantitative real-time PCR, we find that isolated mitochondria can be transfected by electroporation, but the efficiency is very low. We hope to optimize this procedure, with the hope of ultimately delivering transfected mitochondria to cells through microinjection.

352. Crystallization and X-ray diffraction analysis of mitochondrial DNA nucleoids

Huu Ngo, Jens Kaiser, Harry Gristick

Mitochondrial DNA (mtDNA) is packaged with protein into compact structures termed mtDNA nucleoids. Mitochondrial transcription factor A (TFAM) is essential for mtDNA maintenance and is thought to play a major role in packaging of mtDNA. TFAM is a member of the high mobility group family of DNA-binding proteins, which cause a kink in DNA upon binding. This multi-functional protein was initially identified as a factor that

promotes bidirectional transcription from the mtDNA light strand and heavy strand promoters through bending and unwinding of the promoters. However, TFAM probably also plays an important role in packaging mtDNA into compact structures. When added to naked DNA, TFAM coats and organizes the DNA into nucleoid-like structures. Little is known about how TFAM interacts with mtDNA, and how binding of TFAM with mtDNA leads to the compact nucleoid structure. Here, we employ structural biology and biochemical techniques to elucidate the mechanistic details of the interactions between TFAM and mtDNA, and how the binding interactions result in a change in mtDNA structure. TFAM from *Homo sapiens* has been successfully cloned, expressed, purified, and crystallized. Human TFAM binds a 28-mer double-stranded DNA fragment in the light strand promoter region of the mitochondria as indicated by gel mobility shift assays and surface plasmon resonance. Crystals of TFAM-DNA complexes were grown by hanging-drop vapor diffusion and belong to the space group C2221 with unit-cell parameters a = 58.1, b = 108.5 and c = 149.7 Å. Crystals of dimensions 0.03 x 0.1 x 0.2 mm diffract to at least 3.2 Å resolution. In addition, we have obtained selenomethionyl human TFAM-DNA crystals for multi-wavelength anomalous diffraction (MAD) analysis. These selenomethionyl crystals diffract to 3.1 Å, but give weak anomalous signals for MAD analysis. We will continue optimizing conditions for the selenomethenionyl crystals and soak native crystals with heavy atoms for multiple isomorphous replacement analysis.

353. Structural study of the human mitochondrial transcription machinery

Huu Ngo

Human mitochondrial genes are transcribed by a dedicated mitochondrial RNA polymerase (POLRMT), which requires the simultaneous presence of mitochondrial transcription factor A (TFAM) and either isoforms of mitochondrial transcription factor B1(TFB1M) or B2 (TFB2M). In the presence of TFAM, POLRMT/TFB1M and POLRMT/TFB2M complexes are each capable of initiating transcription at the mitochondrial promoter. However, POLRMT/TFB2M complexes are reported to be at least two orders of magnitude more active than POLRMT/TFB1M complexes. While the basal components of human mitochondrial transcription machinery have been identified, detailed interactions among these basic proteins and the mechanism of transcriptional initiation remain largely unknown. In this study, we attempt to resolve detailed interactions between TFAM/TFB1M and TFAM/TFB2M complexes of the human mitochondrial transcription machinery by X-ray crystallography. We have successfully cloned, expressed, and purified human TFAM and TFB1M. Our preliminary results indicate that recombinant human TFAM and TFB1M are soluble, but TFB2M is very insoluble. Therefore, we have developed a co-expression system for TFAM/TFB1M and TFAM/TFB2M complexes. TFB2M was found to be much more soluble when co-expressed with TFAM. We will screen for an optimal binding conditions between TFAM and either TFB1M or TFB2M. Once TFAM/TFB1M and TFAM/TFB2M complexes are purified, initial crystal screening in the absence and presence of a promoter-containing DNA fragment will be performed.

354. Generation of a mouse model to track mitochondrial dynamics

Anh Pham

Mitochondria are dynamic organelles that continuously undergo cycles of fusion and fission. Processes of fusion and fission are essential for maintaining mitochondrial shape, number, subcellular distribution, and respiratory capacity. Currently, there are few genetic tools for assessing mitochondrial dynamics *in vivo* at the tissue level. As a result, most knowledge regarding mitochondrial fusion or fission has been derived from *in vitro* experiments performed using immortalized cells lines or the labor-intensive cultures of primary cells. Although cell culture models provide a well-controlled environment for dissecting mechanistic pathways and manipulating key parameters in the signaling cascade, *in vitro* results may not be physiologically relevant when extrapolated to the tissue or whole organism level. To facilitate exploration of mitochondrial dynamics *in vivo* and in cell-types that are not compatible with current culturing techniques, we have designed an expression cassette that enables Cre recombinase-regulated expression of a photoconvertible mitochondrial (PCmito) reporter in a broad range of tissues and has single-organelle resolution for quantitative analysis. We have introduced the reporter construct into mouse embryonic stem (ES) cells and have obtained several high-grade chimeras. After germ line transmission, the PCmito mouse will be crossed with several Cre excision lines to generate cell type specific labeling of mitochondria. With this PCmito reporter mouse, we will conduct the first systematic survey of mitochondrial morphology across many tissues during embryonic and postnatal development. This analysis will address whether cell type-specific properties of mitochondrial dynamics may contribute to the tissue selective vulnerability observed in mitochondrial diseases.

355. The role of OPA1 in mitochondrial fusion

Zhiyin Song

Mitochondria in living cells form dynamic tubular networks that undergo continuously fission and fusion events. Mitochondrial fusion requires the coordinated fusion of the outer and inner membranes. Three large GTPases—OPA1 and the mitofusins Mfn1 and Mfn2—are essential for the fusion of mammalian mitochondria. OPA1 is mutated in dominant optic atrophy (DOA), a neurodegenerative disease of the optic nerve. In yeast, the OPA1 ortholog Mgm1 is required for inner membrane fusion *in vitro*; nevertheless, yeast lacking Mgm1 show neither outer nor inner membrane fusion *in vivo*, due to the tight coupling between these two processes. We find that outer membrane fusion can be readily visualized in OPA1-

null mouse cells *in vivo*, but these events do not progress to inner membrane fusion. Similar defects are found in cells lacking prohibitins, which are required for proper OPA1 processing. In contrast, double Mfn-null cells show neither outer nor inner membrane fusion. Mitochondria in OPA1-null cells often contain multiple matrix compartments bounded together by a single outer membrane, consistent with uncoupling of outer versus inner membrane fusion. In addition, unlike mitofusins and yeast Mgm1, OPA1 is not required on adjacent mitochondria to mediate membrane fusion. These results indicate that mammalian mitofusins and OPA1 mediate distinct, sequential fusion steps that are readily uncoupled, in contrast to the situation in yeast.

356. A mouse model of Drp1 function

Zhiyin Song

Drp1, a member of the dynamin family of large GTPases, is essential for the fission of mammalian mitochondria. Drp1 is located mostly in the cytosol but can translocate to punctate spot on mitochondria upon activation of mitochondrial fission. Inhibition of Drp1 function by RNAi-mediated knockdown or by expression of the dominant-negative mutant Drp1-K38A (defective in GTP binding) results loss of mitochondrial fission, resulting in the formation of long tubular mitochondria. Drp1-mediated mitochondrial fission is an early and critical event in apoptosis, in parallel with activation of the proapoptotic protein Bax.

To examine the physiological functions of Drp1, we have constructed a mouse line containing a conditional null allele of Drp1. Using recombineering technology, we generated a conditional DRP1 knockout construct. In this knockout construct, two loxP recombination sites flank exon 2, which encodes critical residues of the GTPase domain of Drp1, including the residue K38 that is essential for nucleotide binding. Deletion of exon 2 would create a premature stop codon after residue 75 (Drp1 has 699 amino acids). After electroporation and selection in mouse embryonic stem (ES) cells, we have identified at least 15 positive ES cell clones by long-range PCR. After injection of these targeted ES cells into mouse blastocysts, germline chimera was obtained. Experiments are in progress to examine the role of Drp1 in a variety of tissues.

357. Structural and functional studies of the mitochondrial fission complex

Yan Zhang

Mitochondria are dynamic organelles that undergo frequent regulated fusion and fission. An imbalance of mitochondrial fusion/fission results in severe cellular consequences. Recent studies demonstrate that mitochondrial fission is an important part of the cell death machinery at the early-stage of apoptosis. In addition, mitochondrial fission is essential for proper mitochondrial distribution in neurons and has been linked to cellular senescence. In human, lack of mitochondrial fission results in neonatal lethality. Therefore, it is necessary to understand the molecular mechanism of mitochondrial fission.

At the mechanistic level, mitochondrial fission is best understood in the budding yeast *S. cerevisiae*. The central player, the dynamin-like protein Dnm1, self-assembles into spiral and ring structures in a nucleotide-dependent manner. The recruitment of Dnm1 depends on an integral mitochondrial outer membrane protein Fis1 and the molecular adaptor proteins Mdv1 and Caf4. Mdv1p and Caf4p are homologous soluble proteins containing an NH2-terminal extension (NTE), a coiled-coil (CC) region, and a COOH-terminal seven-WD repeat domain. Acting as molecular bridges, these proteins bind to Fis1p through the NTE region and to Dnm1p through the WD40 region. Moreover, Mdv1p and Caf4p are capable of forming homotypic and heterotypic interactions probably through interactions in the CC domain.

To understand how Fis1 interacts with the NTE domains of the two adaptor proteins, we determined the atomic structure of a Fis1/Mdv1-NTE and a Fis1/Caf4-NTE complex. These structures reveal, in contrast to previous models, that the adaptor proteins use a helix-loop-helix motif to pack against both the concave and convex surfaces of the Fis1 TPR domain. To extend our findings, we are using X-ray crystallography to study a larger complex containing Fis1 and NTE-CC domains of Mdv1, which will provide crucial information on how the assembly of the mitochondrial fission complex initiates. We have successfully expressed, purified, and crystallized the new complex. Such crystals diffract to 5 Å. We are currently optimizing our crystallization conditions to obtain high-resolution diffraction.

Publications

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Summary: The Deshaies lab works on two basic biological processes: Control of cell division, and regulation of cell function by attachment of ubiquitin or ubiquitin-like proteins to target polypeptides. We are particularly interested in how attachment of ubiquitin to proteins enables their degradation, and how protein degradation via this mechanism is used to regulate cell division.

Defective control of cell division can result in disease, as when unrestrained cell proliferation leads to cancer. Defects of the ubiquitin system can also lead to cancer, as well as neurodegenerative diseases. An understanding of how the cell division machinery and the ubiquitin system operate will thus, provide insight into basic cellular processes essential to the life of eukaryotic cells, and may suggest cures for diseases that affect millions of people.

We are using biochemical, molecular, and genetic approaches in baker's yeast and mammalian cells to investigate cell proliferation and the ubiquitin system. Our long-term goal is to understand how these processes work and how they are controlled. Baker's yeast is an excellent organism for basic cell biological studies because it is easy to work with, and many studies have confirmed that yeast and animal cells largely use the same proteins to regulate basic cellular processes.

The next section contains a brief description of the four major areas of investigation in the lab, followed by thumbnail descriptions of all current projects.

SCF ubiquitin ligases: Mechanism, regulation, and physiology:

Cellular proteins are marked for degradation by attachment of the polypeptide ubiquitin. Ubiquitin is attached to substrates by a cascade comprising ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin ligase (E3) enzymes. Ubiquitination occurs when an E3 enzyme binds to both substrate and E2 ubiquitin conjugating enzyme, bringing them into proximity so that ubiquitin is transferred from the E2 to substrate. Specificity and regulation of ubiquitination are typically imparted by E3s, which are the most diverse components of the system. Once ubiquitin is attached to a substrate, the reaction can either terminate (in which case the ubiquitin serves as a regulatory signal to modulate protein function or localization) or continue, leading to the assembly of a multiubiquitin chain. A chain of four ubiquitins suffices to specify destruction of the substrate by the proteasome.

Mechanism of action of SCF ligases: In 1999, we reported that RING domains underlie ubiquitin ligase activity (Seol *et al*., 1999). This discovery revealed what is now thought to be the largest class of ubiquitin ligases, with 300 members. The progenitor of the RING-based ubiquitin ligases, SCF (Feldman *et al*., 1997), defines a subfamily of multisubunit cullin–RING ligases that may number as many as 350 members, due to combinatorial mixing of subunits. Thus, there may be as many as 650 distinct RING ligase complexes, which would make it the largest-known family of enzymes in human cells (Petroski and Deshaies, 2005; Deshaies and Joazeiro, 2009). As befits such a large family, the cullin-RING ligases have been implicated in a dazzling array of cellular and organismal processes, ranging from circadian rhythms to sensing of glucose. However, despite the biological import of these enzymes, the mechanism of how they work remains unknown.

Over the past few years, we have made substantial progress towards understanding how SCF enzymes work. A key step was to develop a reconstituted system in which a physiological substrate (budding yeast Cdk inhibitor Sic1 assembled into cyclin–Cdk complexes) is ubiquitinated by a complex of SCF and the ubiquitin-conjugating enzyme Cdc34, and subsequently is degraded by the proteasome – work that was carried out by Renny Feldman, Craig Correll, and Rati Verma (Feldman *et al*., 1997; Verma *et al*., 2001). Matt Petroski then constructed Sic1 substrates bearing single ubiquitin acceptor lysines, and used these substrates to characterize the impact of ubiquitin chain position on substrate recognition and degradation by the proteasome (Petroski and Deshaies, 2003). Matt went on to use his single-lysine substrate to show that assembly of a ubiquitin chain can be broken down into distinguishable initiation and elongation reactions (Petroski and Deshaies, 2005).

We are now using a variety of assays that employ reconstituted SCF to address basic questions that are of central importance to understanding the mechanisms that underlie the operation of the ubiquitin-proteasome system (UPS). Although we have made considerable progress over the past year in understanding how SCF works (see abstracts by G. Kleiger, N. Pierce, and A. Saha, as well as Saha and Deshaies, 2009), much remains to be done. For example, we still do not have answers to the following fundamental questions: how does a RING domain activate ubiquitin transfer from ubiquitin-conjugating enzyme to substrate? What is the basis for the synthesis of the lysine

48-linked ubiquitin chains that signal proteolysis? What is the full range of mechanisms that enable processive ubiquitination? None of these questions are resolved for any RING E3, and thus, illuminating the answers will establish paradigms that inform our understanding of how hundreds of ubiquitin ligase enzymes work. The insights that emerge from this effort may also provide clues to the development of drugs that modulate the activity of RING-based ligases.

Regulation of SCF ubiquitin ligases: It was originally thought that SCF ubiquitin ligases are constitutively active, and substrate turnover is regulated by phosphorylation of the substrate. Subsequently, it was shown that the Cul1 subunit of SCF is modified covalently by the ubiquitin-like protein Nedd8, raising the possibility that SCF might be regulated post-translationally. In 2001, two students from the lab, Svetlana Lyapina and Greg Cope, reported that a poorly understood protein complex known as COP9 Signalosome (CSN) binds SCF in animal cells, and promotes the cleavage of Nedd8 from Cul1 via an intrinsic Nedd8 isopeptidase activity (Lyapina *et al.*, 2001). This was the first biochemical function ascribed to CSN, and opened the door to the study of SCF regulation by reversible cycles of 'neddylation.' Subsequently, Greg Cope discovered that the Csn5 subunit harbors a motif that we named 'JAMM' (for JAb1/Mpn domain Metalloenzyme) (Cope *et al.*, 2002). We predicted that JAMM comprises a novel metalloprotease active site. Later, Xavier Ambroggio, who was a joint student with Doug Rees in Chemistry, substantiated this prediction by employing X-ray crystallography to show that the conserved residues of the JAMM motif coordinate a zinc ion in an active site-like cleft of the protein AF2198 from *Archaeglobulus fulgidis* (Ambroggio *et al.*, 2004). We continue to investigate the regulation of SCF by CSN (see abstract by E. Emberley). We hope to understand how CSN itself is controlled, and what role CSN plays in sustaining active SCF complexes and sculpting the repertoire of SCF complexes in a cell.

Mechanism of action and regulation of the 26S proteasome:

Once substrates are ubiquitinated by E3s, they are degraded by the 26S proteasome. The 26S proteasome is a large protein machine that comprises two major subcomplexes: the 20S 'core' proteasome and the 19S regulatory 'cap.' The 20S core forms a cylindrical structure that houses the protease active sites of the proteasome. Each end of the 20S cylinder is decorated with a 19S cap. The 19S cap can be further subdivided into the 'lid' and the 'base.' The base contains six ATPases that are thought to form a ring that abuts the end of the 20S cylinder. The lid, in turn, sits upon the base. The base is thought to control access of substrates into the 20S proteolytic chamber, whereas the lid confers ubiquitin dependence. The 26S proteasome degrades proteins that are linked to a chain of at least four ubiquitins. The tetraubiquitin chain mediates binding of the attached substrate to the proteasome, after which it is disengaged from bound partners, unfolded, deubiquitinated, and translocated into the proteolytic chamber of the proteasome where the denuded substrate is degraded.

A fully reconstituted system to study Sic1 degradation: To harness the power of yeast molecular genetics to enable dissection of the mechanism-of-action of the proteasome, we developed a system wherein ubiquitinated Sic1 generated *in vitro* with recombinant SCFCdc4 is degraded by affinity-purified yeast proteasomes (Verma *et al.*, 2001). Remarkably, purified proteasomes can extract ubiquitinated Sic1 from complexes with S phase cyclin-Cdk, degrade the Sic1 and release active S phase cyclin-Cdk protein kinase. This result emphasized that the proteasome has the intrinsic ability to disassemble protein complexes to selectively degrade ubiquitinated substrates, and set the stage for our subsequent studies on substrate targeting and deubiquitination. We are currently seeking to extend these studies by developing a purified substrate bearing a ubiquitin chain of defined length and linkage. The objective will be to use a chemically-defined substrate to initiate quantitative kinetic studies on the mechanism of action of the proteasome (see abstract by M. Rome).

Role of deubiquitination in the degradation of Sic1: In the course of characterizing the degradation of ubiquitinated Sic1, we noticed that when the 20S protease inhibitor epoxomicin was present, ubiquitinated Sic1 was converted to a completely deubiquitinated species (Verma *et al.*, 2002). Fortuitously, at the same time as this we observed that the CSN – which is related to the lid subcomplex of the proteasome 19S cap – cleaves the ubiquitin-like protein Nedd8 from the Cul1 subunit of SCF. Spurred by this confluence of observations, we demonstrated that the Rpn11 subunit of the proteasome lid contains a putative JAMM metalloprotease active site analogous to that of Csn5, and this motif is essential for the deubiquitination of Sic1 *in vitro* and the degradation of multiple UPS substrates *in vivo*.

Multiubiquitin chain receptors target substrate to the proteasome: Although it has long been clear that a multiubiquitin chain targets an appended substrate to the proteasome for degradation, the mechanism of targeting has remained poorly understood. Genetic studies in yeast have suggested a potential role for multiubiquitin chain-binding proteins, including Rad23, Dsk2, Ddi1, and Rpn10. In contrast, biochemical studies in mammalian systems have emphasized a role for the proteasome ATPase Rpt5 as a multiubiquitin chain receptor, and have suggested that proteins such as Rad23 prevent premature metabolism of substrate-linked ubiquitin chains. We reasoned that our reconstituted system would enable us to address this fundamental question from a functional, mechanistic perspective. We first demonstrated using mutant proteasomes and add-back experiments that Rad23 and Rpn10 play a direct role in targeting ubiquitinated Sic1 to the proteasome for degradation (Verma *et al.*, 2004a).

We then went on to show that the multiubiquitin chain receptor activities of Rad23 and Rpn10 play a redundant role in sustaining turnover of Sic1 *in vivo*. Surprisingly, individual deletion of these and other receptor proteins led to the accumulation of different UPS substrates, suggesting that the receptors define a layer of specificity that resides downstream of the E3s and upstream of the proteasome. This hypothesis opens up a host of interesting questions about how specificity is achieved in the targeting step, and what its biological purpose is. We plan to address these key questions over the next several years using a combination of biochemical, molecular genetic, and proteomic approaches (see abstract by T. Gomez).

Proteasome inhibitors: Small molecules that inhibit protein turnover by the proteasome can selectively kill cancer cells. One inhibitor has already been approved by the FDA for the treatment of the blood cell cancer multiple myeloma, and others are currently in clinical development. We are interested to understand why these inhibitors kill some cancer cells (such as multiple myeloma and other hematological cancers) but are much less effective against solid tumors. As part of the effort, we are investigating how proteasome inhibitors bring about elevated synthesis of proteasome subunits in mammalian cells (see abstract by S. Radhakrishnan).

A couple of years ago, in collaboration with Dr. Randy King at Harvard, we discovered ubistatins, which are novel inhibitors of protein degradation by the proteasome. We demonstrated that ubistatin A binds to the ubiquitin chain in the same intersubunit cleft that is normally bound by the multiubiquitin chain receptors (Verma *et al*., 2004b). This binding prevents the ubiquitin chain from binding to receptors (Rpn10 and Rad23) that link it to the proteasome. In collaboration with Tim Lewis at the Broad Institute, a set of ubistatin-like derivatives has been produced and we plan to investigate these new compounds to identify ubistatin-like molecules that efficiently block protein turnover within cells. We believe that ubistatins will be useful tools for studies on the UPS.

Role of Cdc48 in targeting and degradation of ubiquitinated proteins: Recently, we have become intrigued by a poorly understood protein, Cdc48 (known as p97 in human cells), that, like the ubiquitin chain receptors, operates downstream of ubiquitin ligases to promote degradation of ubiquitinated proteins by the proteasome. The role of Cdc48 in protein turnover was originally thought to be confined to pulling malfolded secretory proteins through the endoplasmic reticulum membrane so that they can be degraded by the proteasome. However, several lines of evidence hint at a far broader role. Interestingly, there may be as many as seven distinct Cdc48 complexes in budding yeast, and at least thirteen distinct p97 complexes in human cells (see abstract by G. Alexandru and Alexandru *et al*., 2008). Why all of this complexity? It is difficult to even begin to answer this question, because we know so little about Cdc48's function apart from its role in translocation across the ER membrane. Whatever Cdc48 is doing, it appears to be a fundamental component of the UPS, and thus, understanding how it works is important. We plan to attack this problem by first identifying substrates whose degradation depends on particular Cdc48 complexes. One strategy is to study candidate substrates such as Hsl1 or Cdc5 in yeast cells (see abstract by R. Verma) or hypoxia-inducible factor-1a in mammalian cells (Alexandru *et al*., 2008; see abstract by W. den Besten). Another strategy is to employ the mass spec-based proteomics technology discussed below, as well as a novel *in vivo* substrate screen (see abstract by T. Chou). We will then reconstitute the degradation of these substrates using defined components. The objective will be to develop a reconstituted system in which turnover of the substrate is dependent upon Cdc48. We will then use this system to establish the mechanism-of-action of Cdc48. Armed with this information, we will be in a position to initiate investigations on how ubiquitin receptors and Cdc48 complexes collaborate to enable degradation of ubiquitinated substrates. Given the diversity of receptors that guide ubiquitinated proteins to the proteasome and the diversity of Cdc48 complexes that appear to act in concert with these receptors, there is clearly much about the targeting and degradation of ubiquitinated proteins that we do not understand, and thus, this topic may be fertile ground for making unexpected discoveries.

Proteomics:

Complex mixtures of proteins can be analyzed directly by trypsinization followed by multidimensional chromatography and mass spectrometry to characterize their protein composition. One such method, known as MudPIT (for multidimensional protein identification technology) was employed by Johannes Graumann and Thibault Mayor in our lab to study ubiquitination in yeast. In our first efforts we employed subtractive comparisons of samples from wild-type and mutant cells to identify the ubiquitinated polypeptides that accumulate when the Rpn10 multiubiquitin chain receptor is absent (Mayor *et. al*., 2005). More recently, we have used differential labeling of mutant and wild-type cells with stable isotopes to obtain quantitative estimates of substrate accumulation in Rpn10-deficient cells (Mayor *et. al*., 2007). By identifying the set of substrates whose abundance is altered when a particular ubiquitin pathway component is mutated or blocked by the action of a drug, we hope to gain insight into enzyme-substrate relationships, which in turn may yield insights into the mechanisms that underlie specificity. Moreover, knowledge of the substrates affected can provide clues to the phenotypes that may occur upon inactivation of a particular component. Finally, the ability to quantify substrate accumulation in whole cell extracts or in chromatin fractions may enable us to see subtle defects, such as those that occur when one member of a redundant pair of enzymes is mutated (see abstracts by N. Kolawa and Kuang-Jung Change).

In a second project, we plan to use crosslinking and stable isotope labeling to track dynamic protein

interactions that occur inside cells but do not survive the immunoprecipitation and washing steps that are normally employed in the affinity purification of protein complexes for analysis by mass spectrometry (see abstract by E. Lee).

A third proteomics-related project is to screen an shRNA library to identify genes of the UPS that influence the differentiation of embryonic stem cells into cardiomyocytes (see abstract by N. Honarpour).

Functions of the RENT complex in cell cycle control and nucleolar biogenesis:

Several years ago, a graduate student, Wenying Shou, discovered the RENT complex, and proposed that the mitotic exit network (MEN) specifies the exit from mitosis in budding yeast by promoting disassembly of RENT (Shou *et al*., 1999). 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 the MEN signaling pathway, 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. Over the past few years, it has become apparent that RENT is disassembled by a two-step mechanism. In early anaphase, Cdc14 is released from Net1 through a novel activity of separase. Separase is a protease that activates chromosome segregation in anaphase by cleaving the cohesin protein that holds sister chromatids together.

Throughout interphase and early mitosis, separase activity is repressed by a tightly bound inhibitor, securin. At the metaphase-anaphase boundary, securin is abruptly degraded, thereby liberating separase to cleave cohesin and initiate chromosome segregation. In addition to being a protease, separase has a second activity that promotes the phosphorylation of Net1 by cyclin B–Cdk. A graduate student in the lab, Ramzi Azzam, had identified this phosphorylation and demonstrated that it induces the dissociation of Cdc14 from Net1 (Azzam *et al*., 2004). Thus, the action of separase links initiation of the exit from mitosis with the initiation of chromosome segregation. In late anaphase the MEN serves to sustain Cdc14 release and enable the released Cdc14 to gain access to the cytoplasm, such that its substrates are dephosphorylated and the cell exits mitosis. During the past year, we established that the MEN enables Cdc14 to be relocated to the cytoplasm during late anaphase by phosphorylating and inactivating a nuclear localization sequence near the C-terminus of Cdc14 (see abstract by D. Mohl and Mohl *et al*., 2009). This causes Cdc14 to accumulate in the cytoplasm, where it dephosphorylates and activates proteins that help drive the cell out of mitosis and into the subsequent G1 phase. We now plan to address how the MEN dislodge Cdc14 from Net1 to initiate the process of Cdc14 activation.

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358. p97 regulation via interaction with UBX domain-containing co-factors

Gabriela Alexandru

p97/Cdc48 is a type II AAA (ATPase associated with a variety of activities) ATPase, highly conserved from archaebacteria to mammals. p97 plays a role in seemingly unrelated cellular activities, such as membrane fusion, endoplasmic reticulum-associated protein degradation (ERAD) and cell cycle regulation. All of these functions involve recognition of ubiquitinated protein-substrates and, at least in some cases, their subsequent degradation by the proteasome. In its active form, p97 forms homohexameric barrel structures in which the N-termini are free to bind substrate-recruiting co-factors. Thus, p97 in complex with p47 is thought to regulate membrane fusion, while p97/NPL4/UFD1 complexes are mainly required for ERAD. In an attempt to further understand the molecular basis for p97's diverse functions we have analyzed p97 immunoprecipitates from human tissue culture cells by MudPIT (Multidimensional Protein Identification Technology), searching for new p97 co-factors (Alexandru *et al*., 2008). This analysis revealed eight p97 binding partners, all having a UBX domain in their C-terminal region. Two of them have been linked to human diseases, such as atopic dermatitis and alveolar soft part sarcoma. However, the biological function for most of these proteins is largely unknown. Comparative analysis of Flag-UBX protein immunoprecipitates from human cells revealed that UBA-UBX proteins bind ubiquitinated proteins and also interact with multiple E3 ubiquitin ligases, suggesting they might be involved in ubiquitin-dependent protein degradation. This analysis was initially aimed to identify substrates interacting specifically with each UBX-domain co-factor of p97. In particular, we found that UBXD7 mediated p97 interaction with the CUL2/VHL ubiquitin ligase and its substrate, the hypoxia-inducible factor 1α (HIF1α). Depletion of p97 by siRNA led to accumulation of endogenous HIF1α and increased expression of the HIF1α target carbonic anhydrase IX. Thereby our work revealed an unexpected role for p97 in functional regulation of HIF1α, which is the key governor of cellular and organismal responses to oxygen tension. Further UBX protein – substrate pairs will be identified and analyzed to better define the role of UBX proteins within the p97 network.

359. Identifying UPS players and their mechanisms in repairing UV-damaged chromosome

Kuang-Jung Chang

The ubiquitin-proteasome system (UPS) is the major pathway for the degradation of protein to regulate protein turnover and antigenic-peptide generation. Recently, it has been shown that it is also involved in regulating gene transcription and DNA repair. Formation of pyrimidine dimers, which is caused by UV irradiation of DNA, triggers nucleotide excision repair to remove the damaged region. However, if pyrimidine dimers in the transcribed strand are not repaired, they can stall RNA polymerase during transcription. This results in activation of the transcription-coupled repair (TCR) pathway to repair the damage and enable transcription to proceed. It has also been shown that stalled RNA polymerase can also be degraded via the UPS; however, the underlying mechanism and how it relates to TCR remains poorly understood. The aim of this project focuses on identifying the connections between UV damaged DNA and the UPS. Our approach involves collecting all the chromosome-binding proteins with or without UV damage by CsCl gradient. Then we use the SILAC (stable isotope labeling with amino acids in cell culture)-quantification mass spectrometry to identify the proteins that are specifically recruited to chromatin by UV. The results of these efforts will help us to begin dissecting the mechanisms and acquire a better understanding of how the UPS relates to transcription-coupled repair.

360. Identification of inhibitors and substrates for Cdc48/p97 AAA ATPase

Tsui-Fen Chou

Cdc48/p97 is an important AAA ATPase not only due to its intriguing diverse cellular functions but also because it has been implicated in mediating turnover of many proteins involved in tumorgenesis. In an effort to develop small-molecule inhibitors for Cdc48/p97 based on its X-ray structure, we first searched for the scaffolds that are likely to bind to the ATP-binding pocket of the D2 domain of murine p97. Of particular interest to us are scaffolds that include an electrophile that can readily react with a natural cysteine near the active site of p97. Inhibition is monitored by using an *in vitro* ATPase activity assay and the exact cysteine residue involved in the inactivation was confirmed by mass spectroscopy. Moreover, we have developed several cell-based assays to

evaluate whether *in vitro* inhibitors would affect p97-dependent reporter substrates in cells but not the p97-independent substrates. The studies we have performed so far have established that it is feasible to identify specific and reasonably potent small molecule inhibitors of p97 that inhibit UPS activity in tissue culture cells. We have finished a larger-scale HTS effort to identify different p97 inhibitors *in vitro* and will carry out structure-activity relationship study of the promising lead compounds to develop inhibitors with not only higher potency but also better specificity for future cell culture and *in vivo* studies aimed at evaluating whether p97 is a good target in cancer.

We are employing a high-throughput proteomic approach by using automated fluorescence microscope and fluorescence-activated cell sorter to identify substrates of the Cdc48-pathway in budding yeast. We are screening a yeast library of 4,159 strains, each of which expresses a GFP-tagged ORF and the C-terminal GFP tag enables us to measure the abundance of 4,159 different yeast proteins, each expressed from its endogenous promoter. The GFP-ORFs accumulated in cells lacking p97/Cdc48 activity are likely to be substrates of p97/Cdc48. The homologous mammalian substrates will be validated by p97 RNAi. Specific inhibitors and substrates of p97 will serve as valuable biological tools for study the physiological function and mechanism of Cdc48/p97.

361. The role of p97 and UBXD7 in the ubiquitination and degradation of HIF-1

Willem den Besten

The abundant hexameric AAA ATPase p97 participates in a wide range of cellular processes and for many of these different pathways, the function of p97 involves the recognition of ubiquitinated protein substrates via specific substrate-recruiting cofactors. The largest group of p97 cofactors consists of proteins that interact with the N-terminus of p97 through a so-called ubiquitin regulatory X (UBX) domain. There are at least 13 UBX proteins encoded in the human genome, five of which also have an ubiquitin-binding (UBA) domain and interact with polyubiquitin conjugates.

P97 has been proposed to function as a "separase," disassembling protein complexes through the application of mechanical force generated by the hydrolysis of ATP. However, studying the mechanism of p97 function in detail has remained difficult because for almost all the UBX adaptors soluble substrates have not yet been discovered. Recent proteomic analysis of the network of p97 co-factors revealed that in addition to binding ubiquitin conjugates, UBA-UBX proteins also interact with dozens of ubiquitin E3 ligases (Alexandru *et al*., 2008). This was especially striking for UBXD7, which binds to many of the components of cullin–Ring E3 ligase (CRL) complexes, and has the strongest binding preference for Cullin2. In addition, UBXD7 links p97 to the E3 ligase complex Cul2/VHL and its substrate Hypoxia-inducible factor 1 alpha (HIF-1α).

The identification of HIF-1α as the first known substrate for UBXD7 opens the door for more detailed analysis of p97 and UBX protein function in the postubiquitination/pre-degradation step of proteolysis. Therefore, by using HIF-1α degradation as a model system, we are looking to answer the following questions:

1. What determines the association between UBXD7 and CRLs?
2. What is the role of UBXD7/p97 complex in the degradation of HIF-1α?
3. What other substrates require the p97 pathway for their efficient proteolysis?

362. Regulation of SCF ubiquitin ligase activity

Ethan Emberley

Ubiquitin-mediated protein degradation has emerged as a pivotal process in many areas of cell biology. The multi-subunit SCF (Skp1/Cul1/F-box protein) ubiquitin ligase enzymes transfer ubiquitin molecules onto target proteins destined for degradation by the 26S proteosome. Hundreds of proteins within the cell are thought to be targeted for degradation by SCF complexes. Thus, inappropriate regulation of SCF activity would be expected to have multiple negative impacts on cellular homeostasis. Despite impressive advancements in our knowledge about the mechanism of action of SCF complexes, we still know relatively little about how these enzymes are independently regulated. We have begun to study the mechanism by which substrate binding to its respective F-box protein controls SCF activity. We propose that substrate binding either positively regulates the conjugation of the ubiquitin-like protein Nedd8 to the SCF subunit Cul1 by Ubc12 (referred to as 'neddylation'), or negatively regulates Cul1 deneddylation by the COP9 signalosome. Neddylation of Cul1 is necessary for SCF ubiquitin ligase activity and constitutes a key mechanism of SCF regulation. The SCF and COP9 signalosome complexes have been previously shown to physically interact with each other and we aim to define the importance of this interaction further by characterizing the biochemical requirements that result in the removal of Nedd8 from Cul1 and the eventual shutdown of SCF's ubiquitin ligase activity. By describing the specific effects of substrate binding on the neddylation of SCF, we will better understand the steps needed for SCF activity, as well as described a mechanism by which the protein to be degraded is influencing its own ubiquitination. This new pathway controlling SCF activity could be the target of therapeutic intervention as deregulated SCF activity has been suggested to be a player in several human malignancies.

363. Binding of 26S proteasome subunits to ubiquitin receptor proteins

Tara Gomez

The mechanism by which ubiquitinated proteins are delivered to the 26S proteasome is poorly understood. The 26S proteasome is composed of a 20S catalytic core particle (CP) and a 19S regulatory particle (RP), which itself is composed of a base and a lid. In *Saccharomyces*

cerevisiae, the base is composed of about eight proteins, one of which, RPN1 is thought to play an important role in binding to ubiquitin receptor proteins such as RAD23, DSK2 and DDI1, all of which contain a ubiquitin-like domain. It is believed that these UBL proteins deliver ubiquitinated substrates to the proteasome to promote degradation, and may bind the same or overlapping sites of RPN1 via their UBL domain. We plan to take a genetic approach to gain a better understanding of the mechanism and regulation of how UBL domain proteins are recruited to the proteasome.

364. Ubiquitination in stem cell differentiation and cardiovascular development

Narimon Honarpour

Stem cell therapy is a developing technology with great potential to treat human disease. A major limitation, however, is that little is known about how stem cells differentiate. Thus, it is not currently possible to reliably generate tissue that could be used for cell-based therapy. Because the ubiquitin-proteosome system (UPS) plays a central role in regulating intracellular signaling, we have hypothesized that key switches governing differentiation pathways are also controlled by the UPS. We propose to test this hypothesis by seeking UPS genes that influence embryonic stem cell differentiation into cardiovascular tissue. To date our approach has involved the generation of mouse embryonic stem cell lines that express lineage-specific reporter genes, transfecting these cells with a siRNA library, and screening for spontaneous or accelerated differentiation. Preliminary results of our screen suggest a role for several UPS genes; however, further analysis is needed for more definitive results. Screening protocols using stem cell lines which express reporter genes specific for pluripotential cardiac precursors will now be developed.

365. Dynamics and structure of the Cdc34-SCF interaction

Gary Kleiger

Protein degradation by the ubiquitin proteasome system (UPS) is fundamental to cellular homeostasis. Degradation requires assembly of a polyubiquitin chain upon substrate, which is thought to occur in a processive manner. However, the structural and mechanistic features that enable template-independent processivity of ubiquitylation enzymes are unknown. We show that chain assembly by ubiquitin ligase SCF and ubiquitin-conjugating enzyme Cdc34 is facilitated by the unusual nature of Cdc34–SCF transactions: Cdc34 binds SCF with nanomolar affinity, nevertheless the complex is extremely dynamic. These incongruent properties are enabled by rapid bimolecular association driven by electrostatic interactions between the acidic tail of Cdc34 and a basic 'canyon' in the Cul1 subunit of SCF. *De novo* docking between Cdc34 and Cul1 predicts an extensive molecular interface between the tail and the basic canyon, an arrangement confirmed by cross-linking and kinetic analysis of acidic tail and basic canyon mutants. Residues in this region on Cul1 are conserved in both Cul1 paralogs and orthologs, suggesting that the same mechanism underlies processivity for all cullin-RING ubiquitin ligases (CRLs).

366. Identifying substrates of the proteasome using quantitative mass spectrometry

Natalie Kolawa

The complexity of ubiquitin proteasome system (UPS) has made it difficult to study. As such, there are still many substrates yet to be discovered and for many proteins there is still no consensus on whether they are true substrates of the UPS.

In the last couple of years, there have been major advances in shotgun mass spectrometry techniques and data analysis software that have enabled quantitative analysis of changes in protein level on a proteome-wide scale. Our goal is to use these new technologies, coupled with stable isotope labeling of amino acids in cell culture (SILAC) to identify and quantify known and novel substrates of the proteasome in yeast on a global scale. This work will establish a catalog of high-confidence substrates and will better elucidate the cellular pathways that the UPS is involved with, allowing for a clearer understanding of the breadth of the UPS's role in the cell.

367. Identifying molecular components involved in Parkinson's disease

J. Eugene Lee

Parkinson's disease is the most prevalent neurodegenerative movement disorder. Recent genetic findings have provided important insights into the pathogenetic mechanism of this disease. In a large number of familial Parkinson's disease cases, *parkin* was found to contain deletion and missense mutations. *Parkin* encodes a 465-residue ubiquitin ligase. With the aid of ubiquitin-conjugating enzymes, ubiquitin ligases catalyze the synthesis of a polyubiquitin chain on the target substrate. Currently, it is unclear which proteins are modified by *parkin* in the cell. Moreover, the enzymatic function of *parkin* is controversial. Certain lines of evidence suggest that *parkin* mediates the typical K48-linked polyubiquitination for substrate degradation, while others implicate K63-linked polyubiquitination. K63-linked polyubiquitination signals cellular events that are distinct from substrate degradation. To elucidate how *parkin* functions, we will develop a sensitive protein identification method composed of *in vivo* cross-linking and quantitative mass spectrometry. Using this method, we will identify the substrates, and the ubiquitin-conjugating enzyme for *parkin*, which directs the topology of the polyubiquitin chain synthesized. The successful completion of this proposal will present a new strategy for identifying dynamic protein–protein interactions, and could aid in the development of novel therapeutics for Parkinson's disease.

368. Small molecule inhibitors of the proteasome's regulatory particle

Eric Miller

Proteolysis via the ubiquitin proteasome system (UPS) involves the attachment of multiple ubiquitin (Ub) molecules to a target protein and degradation of that protein via the proteasome. The proteasome is a multicatalytic proteinase complex that consists of a cylindrical, proteolytic core particle (CP) capped on one or both ends by a regulatory particle (RP). The RP performs several key functions including recognition and binding of ubiquitinated substrates, unfolding of the substrate, enzymatic substrate deubiquitination, opening of the access pores in the CP and transfer of the unfolded protein into the CP where it is degraded.

The overall aim of this research is to completely characterize the biological activity of a small molecule derivative of ubistatin B (Verma *et al*., 2004 *Science* **306**:117-120) known as TLI-258. TLI-258 blocks the RPs ability to recognize and bind ubiquitinated substrates. We will use this information to formulate more active derivatives of TLI-258 and determine how it could best be used to study the UPS. We propose to conduct multiple *in vitro* and *in vivo* experiments to completely understand the biological activity and specificity of TLI-258. We will utilize the information gleaned from these experiments to guide a collaborative medicinal chemistry effort geared toward formulating more cell-permeable, potent or selective derivatives of TLI-258. We will also use the information gleaned from the initial experiments to ascertain how TLI-258 could best be used as a research tool. Ultimately we plan to employ TLI-258 in conjunction with mass spectral-based proteomic analyses to more accurately determine the total number and identity of cellular proteins that are subject to ubiquitination. Proteasome inhibition is a validated approach in anticancer chemotherapy. Unlike conventional targets, the proteasome represents a unique opportunity for the development of 2nd and 3rd generation inhibitors because it is a multi-enzyme, multi-functional complex. Inhibition of equally essential aspects of proteasome function that are distinct from CP activity such as Ub recognition and binding, should have the potential to exhibit considerable differences in therapeutic index and side-effect profile while maintaining efficacy.

369. Regulation of *Saccharomyces cerevisiae* Cdc14

Dane Mohl

Our work hopes to illuminate the mechanisms that allow the cell to sense the duplication of the DNA genome, and link completion of chromosome segregation to the initiation of cell division. In my research, I have used the model yeast system *Saccharomyces cerevisiae* to look more closely at the regulation of a key cell cycle phosphatase, Cdc14. Our work has demonstrated that a protein kinase complex called Dbf2/Mob1, that becomes active when each of the two new nuclei are segregated to opposite compartments of a pre-divisional cell, directly regulates Cdc14 phosphatase, thereby linking genome duplication to cytokinesis.

The aim of my project has been the identification of *cis*-acting sequences within Cdc14 and its inhibitor Net1p that are required for late mitotic regulation. We have also been searching for the *trans*-acting factors that act upon those epitopes. Live cell imaging and GFP fluorescence localization led us to conclude that a small portion of Cdc14-GFP visited the daughter bound spindle pole bodies of anaphase cells and could therefore, be a direct substrate of Dbf2/Mob1. Our research has, therefore, focused on identifying sequences within Cdc14 that could be sites of Dbf2/Mob1 phosphorylation. Mass spec analysis of *in vitro*- and *in vivo*-phosphorylated Cdc14p has illuminated the importance of key C-terminal sequences within Cdc14. Genetic analysis and GFP fluorescence studies have strengthened our conclusion that Cdc14 is a direct substrate of Dbf2/Mob1 and that phosphorylation of Cdc14 within a tightly regulated nuclear localization signal (NLS) deactivates the NLS and thereby enables Cdc14 to accumulate within the cytoplasm upon its release from the nucleolus.

Our most recent work hopes to find additional Dbf2/Mob1 substrates, as well as identify factors that may act as Cdc14 substrates or factors that partner with Cdc14 to facilitate its activity in both the cytoplasm and nucleus.

370. Integrated dynamics of Sic1

Nathan Pierce

Attachment of a polyubiquitin chain with at least four ubiquitins targets proteins to the proteasome for degradation. A cascade of three enzymes carries out the synthesis of polyubiquitin chains: an ubiquitin activating enzyme (E1), an ubiquitin conjugating enzyme (E2), and an ubiquitin ligase (E3). RING (really interesting new gene) E3s can processively catalyze the direct transfer of ubiquitin from an E2 to a lysine on a target protein. The pathway by which ubiquitin chains are generated on substrate remains unclear and multiple distinct models involving chain assembly on E2 or substrate have been proposed. However, the speed and complexity of the reaction have prevented direct experimental tests to distinguish between potential pathways. We have developed new theoretical and experimental methodologies to address both limitations. A quantitative framework based on product distribution predicts that the E3 SCF^{Cdc4} and the E2 Cdc34 build polyubiquitin chains on substrates by sequential transfers of single ubiquitins. Direct measurements with millisecond time resolution carried out on a quench-flow device demonstrate that substrate polyubiquitylation proceeds sequentially. A quantitative model of the processive reaction yield individual rate constants for substrate dissociation and ubiquitin transfer at each step of chain assembly. Our results present an unprecedented glimpse into the mechanism of RING ubiquitin ligases and provide a universal framework from which the complete mechanistic dissection of RING ubiquitin ligases is possible.

371. Proteasome inhibition as an anti-cancer strategy

Senthil K. Radhakrishnan

Recent studies have indicated that proteasome inhibition is a useful strategy for the treatment of certain cancers. However, one potential problem with inhibiting the proteasome is the existence of a feedback loop in which proteasome inhibitors induce elevated expression of proteasome subunits resulting in recovery of activity. We hypothesize that blocking this feedback loop by down-regulating proteasome resynthesis could increase the efficacy of proteasome inhibitor therapy. We are currently investigating the covalent, irreversible proteasome inhibitor YU101 in combination with a transcriptional or a translational inhibitor (to block proteasome resynthesis) in cancer cell culture models. If promising, this approach could be extended to a mouse model harboring human xenograft tumors. As a part of this project we also intend to test if pulse treatment with YU101 (which mimics the clinical situation where proteasome inhibitors are rapidly cleared from the plasma) followed by inhibition of proteasome resynthesis can effectively sustain proteasome inhibition as a whole. Finally, we seek to discover the transcription factor that mediates elevated expression of proteasome genes upon treatment of cells with proteasome inhibitors. Overall, our approach could lead to rational drug combinations and hence effective therapies against cancer.

372. Mechanism of substrate ubiquitination and degradation by ubiquitin-proteasome system

Anjanabha Saha

Protein turnover by the ubiquitin-proteasome system (UPS) involves substrate ubiquitination by ubiquitin conjugation machinery followed by degradation by the 26S proteasome. Protein turnover is often regulated at the level of substrate ubiquitination by cullin-RING ligases (CRLs). We are utilizing the human SCF complex, the prototype of CRLs, to investigate the mechanism of ubiquitination of substrates like p27 and IkB. In addition, we are examining how reversible modification of CRLs by neddylation stimulates substrate ubiquitination. Current studies indicate that neddylation imparts conformational flexibility within the SCF, which brings the thioesterified E2 and the bound substrate in close proximity enabling increased rate of ubiquitin transfer (Saha and Deshaies, 2008). Additionally, we are investigating the mechanism of degradation of ubiquitinated substrates (p27 and IkB) and role of additional factors involved in substrate turnover.

373. Receptor pathways of the Ubiquitin Proteasome System (UPS)

Rati Verma, Robert Oania

Labile substrates of the 26S proteasome are earmarked for proteolysis by the covalent attachment of a polyubiquitin (polyUb) chain on acceptor lysines. Our prior work has shown that although the Ub chain is a universal degradation signal, there is specificity in the receptor pathway that is preferentially deployed to target the ubiquitinated protein to the proteasome. Currently, there are about ten different polyUb-binding receptors known in budding yeast. Although all known receptors have a polyUb-binding domain (UBD), only a subset of them have a proteasome-binding domain (PBD). The prototype of the latter is Rpn10, which is an intrinsic subunit of the 26S proteasome. Other receptors such as Rad23 and Dsk2 are present in substoichiometric amounts in the 26S proteasome preparations, leading to the "shuttle hypothesis" of delivery of ubiquitinated substrates. Rpn10 is postulated to be the obligate terminal step in this mechanism. For substrates such as the ubiquitinated Cdk inhibitor Sic1, this may indeed be the case. However, Rpn10 is dispensable for viability. Moreover, Rpn10 is not required for the degradation of substrates such as misfolded CPY* which is degraded at the endoplasmic reticulum (ER). Instead, Cdc48 and its adaptors are needed for degradation of CPY*. The questions that follow from these, and other, observations are: 1) Are there other examples of ubiquitinated substrates that are preferentially recognized by Cdc48 and its adaptors? 2) If yes, are they all localized at the ER, or are there also cytoplasmic and nuclear substrates? 3) How are these ubiquitinated proteins delivered to the 26S proteasome, given that Cdc48 and its adaptors have no *bona fide* PBD? 4) How is Rpn10, an intrinsic subunit of the 26S proteasome, bypassed in such pathways? These are some of the questions that we are attempting to answer in studies on the different receptor pathways of the UPS.

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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:

- What controls the timing of MPF activation so that it occurs at a defined interval following the completion of DNA replication?
- How do various checkpoint or feedback controls influence the Cdc2/cyclin B complex?
- 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 that 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.

374. Roles of replication fork-interacting and Chk1-activating domains from Claspin in a DNA replication checkpoint response

Joon Lee, Daniel A. Gold, Anna Shevchenko, Andrej Shevchenko, William G. Dunphy

Claspin is essential for the ATR-dependent activation of Chk1 in *Xenopus* egg extracts containing incompletely replicated DNA. Claspin associates with replication forks upon origin unwinding. We show that Claspin contains a replication fork-interacting domain (RFID, residues 265-605) that associates with Cdc45, DNA polymerase epsilon, RPA, and two RFC complexes on chromatin. The RFID contains two basic patches (BP1 and BP2) at amino acids 265-331 and 470-600, respectively. Deletion of either BP1 or BP2 compromises optimal binding of Claspin to chromatin. Absence of BP1 has no effect on the ability of Claspin to mediate activation of Chk1. By contrast, removal of BP2 causes a large reduction in the Chk1-activating potency of Claspin. We also find that Claspin contains a small Chk1-activating domain (CKAD, residues 776-905) that does not bind stably to chromatin, but is fully effective at high concentrations for mediating activation of Chk1. These results indicate that stable retention of Claspin on chromatin is not necessary for activation of Chk1. Instead, our findings suggest that only transient interaction of Claspin with replication forks potentiates its Chk1-activating function. Another implication of this work is that stable binding of Claspin to chromatin may play a role besides the activation of Chk1.

375. Phosphorylation of Chk1 by ATR in *Xenopus* egg extracts requires binding of ATRIP to ATR but not the stable DNA-binding or coiled-coil domains of ATRIP

Soo-Mi Kim, Akiko Kumagai, Joon Lee, William G. Dunphy

ATR, a critical regulator of DNA replication and damage checkpoint responses, possesses a binding partner called ATRIP. We have studied the functional properties of *Xenopus* ATR and ATRIP in incubations with purified components and in frog egg extracts. In purified systems, ATRIP associates with DNA in both RPA-dependent and RPA-independent manners, depending on the composition of the template. However, in egg extracts, only the RPA-dependent mode of binding to DNA can be detected. ATRIP adopts an oligomeric state in egg extracts that depends upon binding to ATR. In addition, ATR and ATRIP are mutually dependent on one another for stable binding to DNA in egg extracts. The ATR-dependent oligomerization of ATRIP does not require an intact coiled-coil domain in ATRIP and does not change in the presence of checkpoint-inducing DNA templates. Egg extracts containing a mutant of ATRIP that cannot bind to ATR are defective in the phosphorylation of Chk1. However, extracts containing mutants of ATRIP lacking stable DNA-binding and coiled-coil domains show no reduction in the phosphorylation of Chk1 in response to defined DNA templates. Furthermore, activation of Chk1 does not depend upon RPA under these conditions. These results suggest that ATRIP must associate with ATR in order for ATR to carry out the phosphorylation of Chk1 effectively. However, this function of ATRIP does not involve its ability to mediate the stable binding of ATR to defined checkpoint-inducing DNA templates in egg extracts, does not require an intact coiled-coil domain, and does not depend on RPA.

376. TopBP1 activates the ATR-ATRIP complex

Akiko Kumagai, Joon Lee, Hae Yong Yoo, William G. Dunphy

ATR is a key regulator of checkpoint responses to incompletely replicated and damaged DNA, but the mechanisms underlying control of its kinase activity are unknown. TopBP1, the vertebrate homolog of yeast Cut5/Dpb11, has dual roles in initiation of DNA replication and regulation of checkpoint responses. We show that recombinant TopBP1 induces a large increase in the kinase activity of both *Xenopus* and human ATR. The ATR-activating domain resides in a conserved segment of TopBP1 that is distinct from its numerous BRCT repeats. The isolated ATR-activating domain from TopBP1 induces ectopic activation of ATR-dependent signaling in both *Xenopus* egg extracts and human cells. Furthermore, *Xenopus* egg extracts containing a version of TopBP1 with an inactivating point mutation in the ATR-activating domain are defective in checkpoint regulation. These studies establish that activation of ATR by TopBP1 is a crucial step in the initiation of ATR-dependent signaling processes.

377. Site-specific phosphorylation of a checkpoint mediator protein controls its responses to different DNA structures

Hae Yong Yoo, Seong-Yun Jeong, William G. Dunphy

The checkpoint mediator protein Claspin is indispensable for the ATR-dependent phosphorylation of Chk1 in response to stalled DNA replication forks in *Xenopus* egg extracts. We show that Claspin also participates in the detection of chromosomal double-stranded DNA breaks (DSBs) in this system. Significantly, removal of Claspin from egg extracts only partially abrogates the activation of Chk1 in response to chromatin with DSBs, whereas depletion of both Claspin and BRCA1 completely abolishes this activation. The function of Claspin in this DSB-triggered pathway depends upon phosphorylation of T817 and S819 by ATR. Conversely, neither phosphorylation of Claspin on these sites nor the presence of BRCA1 is necessary for activation of Chk1 in response to stalled replication forks. Thus, site-specific phosphorylation of a checkpoint mediator protein is a crucial determinant in the discrimination between various checkpoint-inducing structures. Furthermore, checkpoint mediator proteins exhibit functional overlap that varies depending on the nature of the checkpoint-triggering DNA signal.

378. Ataxia-telangiectasia mutated (ATM)-dependent activation of ATR occurs through phosphorylation of TopBP1 by ATM

Hae Yong Yoo, Akiko Kumagai, Anna Shevchenko, Andrej Shevchenko, William G. Dunphy

ATM is necessary for activation of Chk1 by ATR in response to double-stranded DNA breaks (DSBs) but not to DNA replication stress. TopBP1 has been identified as a direct activator of ATR. We show that ATM regulates *Xenopus* TopBP1 by phosphorylating S1131 and thereby strongly enhancing association of TopBP1 with ATR. *Xenopus* egg extracts containing a mutant of TopBP1 that cannot be phosphorylated on S1131 are defective in the ATR-dependent phosphorylation of Chk1 in response to DSBs but not to DNA replication stress. Thus, TopBP1 is critical for the ATM-dependent activation of ATR following production of DSBs in the genome.

379. The Rad9-Hus1-Rad1 checkpoint clamp regulates interaction of TopBP1 with ATR

Joon Lee, Akiko Kumagai, William G. Dunphy

TopBP1 serves as an activator of the ATR-ATRIP complex in response to the presence of incompletely replicated or damaged DNA. This process involves binding of ATR to the ATR-activating domain of TopBP1, which is located between BRCT domains VI and VII. TopBP1 displays increased binding to ATR-ATRIP in *Xenopus* egg extracts containing checkpoint-inducing DNA templates. We show that an N-terminal region of TopBP1 containing BRCT repeats I-II is essential for this checkpoint-stimulated binding of TopBP1 to ATR-ATRIP. The BRCT I-II region of TopBP1 also binds specifically to the Rad9-Hus1-Rad1 (9-1-1) complex in *Xenopus* egg extracts. This binding occurs via the C-terminal domain of Rad9 and depends upon phosphorylation of its Ser-373 residue. Egg extracts containing either a mutant of TopBP1 lacking the BRCT I-II repeats or a mutant of Rad9 with an alanine substitution at Ser-373 are defective in checkpoint regulation. Furthermore, an isolated C-terminal fragment from Rad9 is an effective inhibitor of checkpoint signaling in egg extracts. These findings suggest that interaction of the 9-1-1 complex with the BRCT I-II region of TopBP1 is necessary for binding of ATR-ATRIP to the ATR-activating domain of TopBP1 and the ensuing activation of ATR.

380. The MRN complex mediates activation of TopBP1 by ATM

Hae Yong Yoo, Akiko Kumagai, Anna Shevchenko, Andrej Shevchenko, William G. Dunphy

TopBP1 serves as an activator of the ATR-ATRIP complex in response to the presence of incompletely replicated or damaged DNA. This process involves binding of ATR to the ATR-activating domain of TopBP1, which is located between BRCT domains VI and VII. TopBP1 displays increased binding to ATR-ATRIP in *Xenopus* egg extracts containing checkpoint-inducing DNA templates. We show that an N-terminal region of TopBP1 containing BRCT repeats I-II is essential for this checkpoint-stimulated binding of TopBP1 to ATR-ATRIP. The BRCT I-II region of TopBP1 also binds specifically to the Rad9-Hus1-Rad1 (9-1-1) complex in *Xenopus* egg extracts. This binding occurs via the C-terminal domain of Rad9 and depends upon phosphorylation of its Ser-373 residue. Egg extracts containing either a mutant of TopBP1 lacking the BRCT I-II repeats or a mutant of Rad9 with an alanine substitution at Ser-373 are defective in checkpoint regulation. Furthermore, an isolated C-terminal fragment from Rad9 is an effective inhibitor of checkpoint signaling in egg extracts. These findings suggest that interaction of the 9-1-1 complex with the BRCT I-II region of TopBP1 is necessary for binding of ATR-ATRIP to the ATR-activating domain of TopBP1 and the ensuing activation of ATR.

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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 finished. While this is of course still just a dream, we are developing electron cryomicroscopy-based technologies to do this for at least the largest structures, hoping to show both how individual proteins work together as large "machines" and how those machines are organized into "assembly lines" within living cells.

The principle technique we're developing is electron cryotomography (ECT). Briefly, purified proteins, viruses, or even cell cultures are spread into thin films across EM grids and plunge-frozen in liquid ethane. Quick-freezing causes the water to form vitreous ice around the proteins and other macromolecules, preserving their native structure but solidifying the sample so it can withstand the high vacuum within an electron microscope. Projection images are then recorded through the sample as the sample is tilted incrementally along one or two axes. The microscope we use is one of only a few like it in the world: a 300 kV, helium-cooled, energy-filtered, dual-axis tilting, FEG cryo-TEM with a lens-coupled 4k x 4k CCD. Three-dimensional reconstructions, or "tomograms," are then calculated from the images. In this way we can produce 3-D structures of heterogeneous proteins, viruses, and even whole bacterial cells in near-native states to "molecular" (~4-7 nm) resolution.

The first cells we've begun imaging are small bacteria. Now that nearly a thousand bacterial genomes have been sequenced, a variety of "omic" technologies are being used to document which genes are transcribed and when, which macromolecules are synthesized and how many of each type are present in the cell, and how they interact in pathways to mediate metabolism and regulate gene expression. Despite this encouraging progress, however, our persistent ignorance about many of the fundamental physical and mechanical processes that occur in a bacterial life cycle is sobering. We still don't know, for instance, how bacteria generate and maintain their characteristic shapes, establish polarity, organize their genomes, segregate their chromosomes, divide, and in some cases move. Thus, in some sense the "omics" technologies are giving us lists of parts and reactions, but bacterial cells are not merely bags of enzymes. Structural and mechanical details are also needed. This is where ECT is poised to make an important contribution.

In recent years we have used ECT to show by direct visualization that bacteria do indeed have an elaborate cytoskeleton. We have documented structural details of different cell motility mechanisms, chemoreception apparati, flagellar motors, and metabolic microcompartments. We continue to work on all these subjects and hope also to begin shedding light on the structure and management of the nucleoid and cell wall. In addition, we are also imaging the smallest known eukaryote, *Ostreococcus tauri*.

We have also worked to apply the power of ECT to the structure and maturation of the human immunodeficiency virus type 1 (HIV-1). HIV-1 is an interesting structural story: following its discovery in the mid-1980's, thousands (!) of different structures of its 15 different proteins and pieces of its RNA genome have been solved. Nevertheless, we still don't know just how these proteins fit together to form intact, infectious virions, or how their organization changes during assembly, maturation, and infection. The main technical obstacle is that like people, while all HIV-1 virions have the same basic features, each virion is unique in its details. Techniques like X-ray crystallography or NMR spectroscopy, which require a large number of identical objects, have not therefore been able to reveal "supramolecular" details. So far we have imaged HIV-1 in its immature and mature states, and are now analyzing these at higher resolution and endeavoring to image HIV-1 structures in living host cells, as well as host factors involved in the HIV-1 life cycle.

Technologically, we are working on optimizing sample preservation, recording better images through improved instrumentation, obtaining more images through automation, and extracting as much biological insight as possible from the images through more sophisticated image processing. For more information, see http://www.jensenlab.caltech.edu.

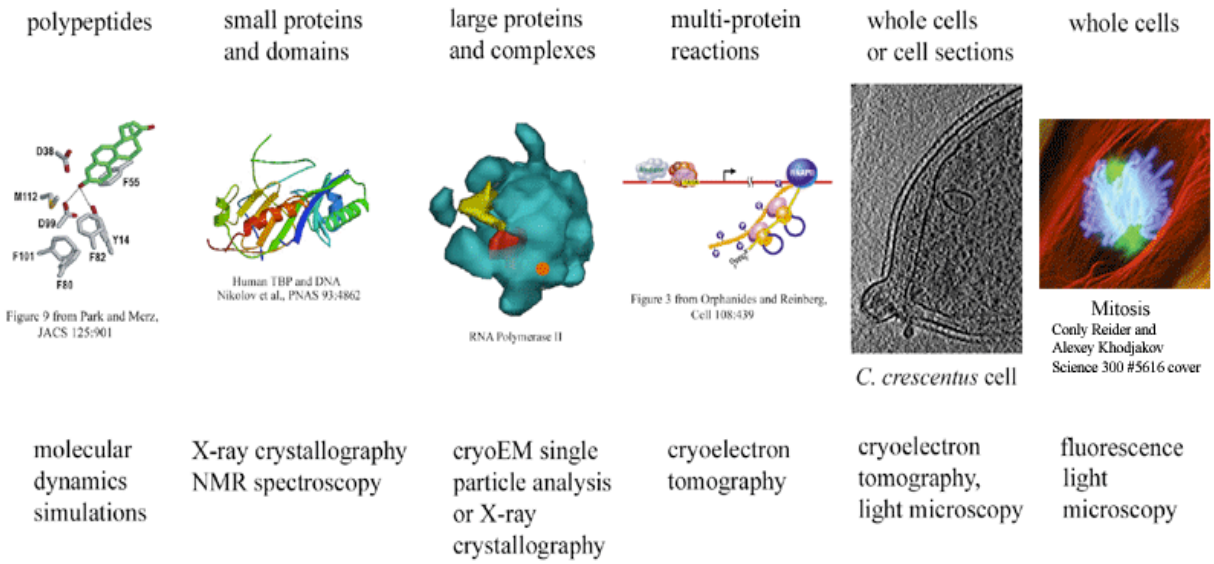

Figure 1. Electron cryotomography completes the "structural biology continuum."

381. Fully automated, sequential tilt-series acquisition with Leginon - C

Christian Suloway, Jian Shi, Anchi Cheng[1], James Pulokas[1], Bridget Carragher[1], Clinton S. Potter[1], Shawn Q. Zheng[2,3], David A. Agard[2,3], Grant J. Jensen

Electron tomography has become a uniquely powerful tool for investigating the structures of individual cells, viruses, and macromolecules. Data collection is, however, time consuming and requires expensive instruments. To optimize productivity, we have incorporated one of the existing tilt-series acquisition programs, UCSF Tomo, into the well-developed automatic electron microscopy data collection package Leginon to enable fully automatic, sequential tilt-series acquisition. Here we describe how UCSF Tomo was integrated into Leginon, what users must do to set up a data collection session, how the automatic collection proceeds, how archived data about the process can be accessed and used, and how the software has been tested.

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Publication

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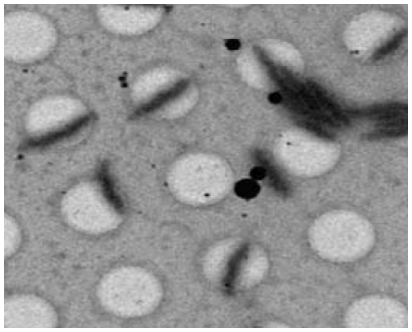

Figure 2: Low magnification image of several *Caulobacter crescentus* cells frozen across an EM grid. The EM grid is covered with a thin layer of carbon with a regular pattern of circular holes. The *Caulobacter* cells are the dark, rod-shaped streaks just longer than the diameter of a hole. Images like this are used to identify target cells for the sequential imaging procedure.

382. Molecular organization of Gram-negative peptidoglycan

Lu Gan, Songye Chen, Grant J. Jensen

The stress-bearing component of the bacterial cell wall-a multi-gigadalton bag-like molecule called the sacculus-is synthesized from peptidoglycan. Whereas, the chemical composition and the 3-D structure of the peptidoglycan subunit (in at least one conformation) are known, the architecture of the assembled sacculus is not. Four decades' worth of biochemical and electron microscopy experiments have resulted in two leading 3-D peptidoglycan models: "Layered" and "Scaffold," in the cell surface, respectively. Here we resolved the basic architecture of purified, frozen-hydrated sacculi through which the glycan strands are parallel and perpendicular to electron cryotomography. In the Gram-negative sacculus, a single layer of glycans lie parallel to the cell surface, roughly perpendicular to the long axis of the cell, encircling the cell in a disorganized hoop-like fashion.

Reference

Gan, L., Chen S.Y. and Jensen, G.J. (2008) *Proc. Natl. Acad. Sci. USA* **105**(48):18953-18957. http://www.pnas.org/content/105/48/18953.

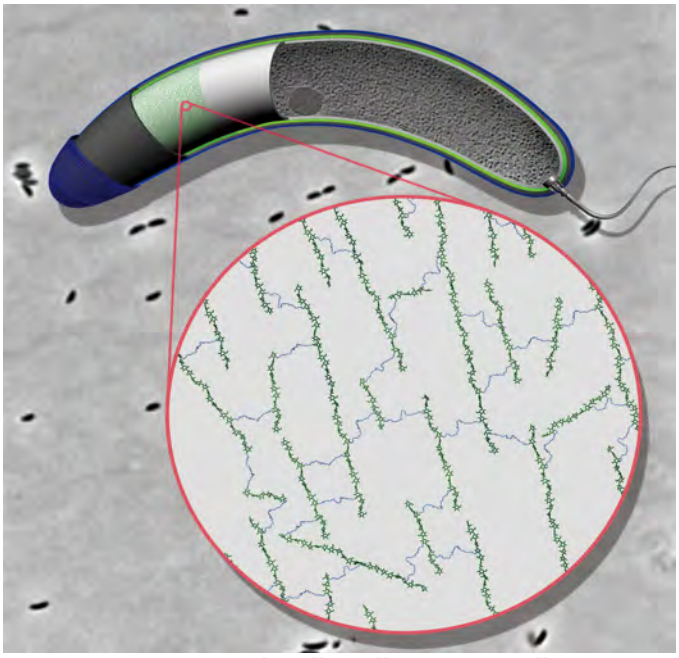

Figure 3: Bacterial cell walls protect cells against mechanical stress and help maintain cell shape. The stress-bearing layer of the cell wall is called the sacculus (green layer) and is built from peptidoglycan. In Gram-negative cells, the sacculus is sandwiched between the outer (dark gray) and inner membranes (light gray). Electron cryotomographic analysis of purified sacculi showed that peptidoglycan is arranged as a "disorganized layer" (inset). Three-dimensional model of *C. crescentus* cell, courtesy of Everett Kane (SuperSoft Design, New York, NY). Phase-contrast light micrograph of *C. crescentus* cells in the background, courtesy of Dr. Ariane Briegel (Caltech, Pasadena, CA).

383. An improved cryogen for plunge freezing

William F. Tivol, Ariane Briegel, Grant J. Jensen

The use of an alkane mixture that remains liquid at 77 K to freeze specimens has advantages over the use of a pure alkane that is solid at 77 K. It was found that a mixture of methane and ethane did not give a cooling rate adequate to produce vitreous ice, but a mixture of propane and ethane did result in vitreous ice. Furthermore, the latter mixture produced less damage to specimens mounted on a very thin, fragile holey carbon substrate.

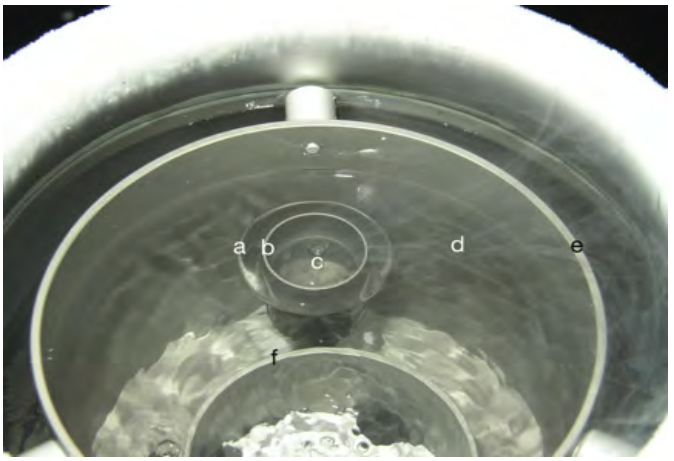

Figure 4: Gravity plunger showing a droplet of liquid propane-ethane floating on the top of a liquid nitrogen bath.

Reference

Tivol, W.F., Briegel, A. and Jensen, G.J. (2008) *Microsc. Microanal.* **4**(5):375-379.

384. A self-associating protein critical for chromosome attachment, division, and polar organization in caulobacter

Gitte Ebersbach[1], Ariane Briegel, Grant J. Jensen, Christine Jacobs-Wagner[1,2]

Cell polarization is an integral part of many unrelated bacterial processes. How intrinsic cell polarization is achieved is poorly understood. Here, we provide evidence that *Caulobacter crescentus* uses a multimeric pole-organizing factor (PopZ) that serves as a hub to concurrently achieve several polarizing functions. During chromosome segregation, polar PopZ captures the ParB*ori complex and thereby anchors sister chromosomes at opposite poles. This step is essential for stabilizing bipolar gradients of a cell division inhibitor and setting up division near midcell. PopZ also affects polar stalk morphogenesis and mediates the polar localization of the morphogenetic and cell cycle signaling proteins CckA and DivJ. Polar accumulation of PopZ, which is central to its polarizing activity, can be achieved independently of division and does not appear to be dictated by the pole curvature. Instead, evidence suggests that localization of PopZ largely relies on PopZ multimerization in chromosome-free regions, consistent with a self-organizing mechanism.

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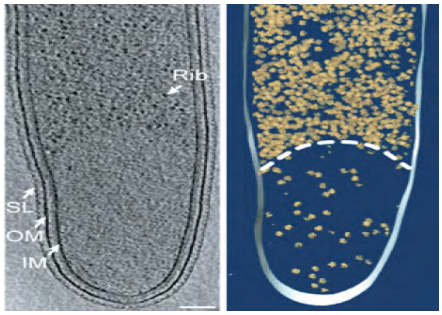

Figure 5: Electron cryo-tomogram of CB15N/pJS14Pxyl-PopZ grown in the presence of xylose for 6.5 hr to overproduce PopZ. Left image: 15 nm thick slice from the median filtered tomogram. Right image: 58 nm thick section of the segmentation including the region shown on the left. SL, surface layer; OM, outer membrane; IM, inner membrane; Rib, probable ribosome (yellow in right panel). The scale bar represents 100 nm.

Reference

Ebersbach, G., Briegel, A., Jensen, G.J. and Jacobs-Wagner, C. (2008) *Cell* **134**(6):956-968.

385. Radiation dose reduction and image enhancement in biological imaging through equally-sloped tomography

Edwin Lee[1], Benjamin P. Fahimian[1,2], Cristina V. Iancu, Christian Suloway, Gavin E. Murphy, Elizabeth R. Wright, Daniel Castaño-Díez[3], Grant J. Jensen, Jianwei Miao[1]

Electron tomography is currently the highest resolution imaging modality available to study the 3D structures of pleomorphic macromolecular assemblies, viruses, organelles and cells. Unfortunately, the resolution is currently limited to 3-5 nm by several factors including the dose tolerance of biological specimens and the inaccessibility of certain tilt angles. Here we report the first experimental demonstration of equally-sloped tomography (EST) to alleviate these problems. As a proof of principle, we applied EST to reconstructing frozen-hydrated keyhole limpet hemocyanin molecules from a tilt-series taken with constant slope increments. In comparison with weighted back-projection (WBP), the algebraic reconstruction technique (ART) and the simultaneous algebraic reconstruction technique (SART), EST reconstructions exhibited higher contrast, less peripheral noise, more easily detectable molecular boundaries and reduced missing wedge effects. More importantly, EST reconstructions including only two-thirds the original images appeared to have the same resolution as full WBP reconstructions, suggesting that EST can either reduce the dose required to reach a given resolution or allow higher resolutions to be achieved with a given dose. EST was also applied to reconstructing a frozen-hydrated bacterial cell from a tilt-series taken with constant angular increments. The results confirmed similar benefits when standard tilts are utilized.

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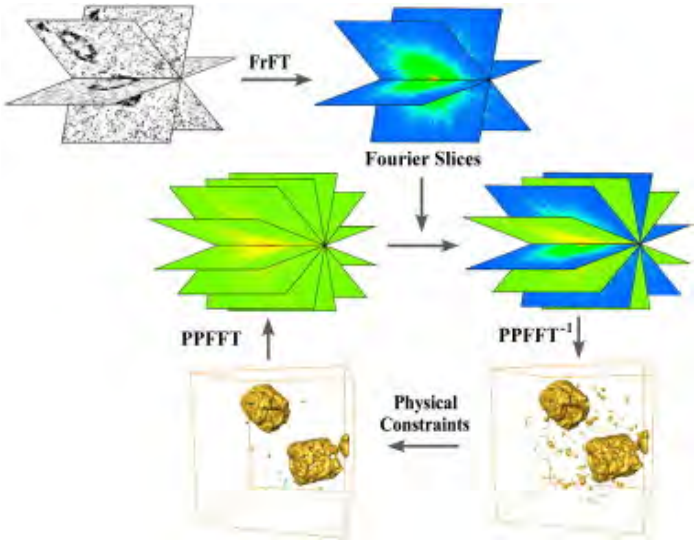

Figure 6: Schematic layout of the iterative EST method. The algorithm iterates back and forth between Fourier and object space. In each iteration, the calculated slices are updated with the measured (experimental) slices in Fourier space and the physical constraints are enforced in object space.

Reference

Lee, E., Fahimian, B.P., Iancu, C.V., Suloway, C., Murphy, G.E., Wright, E.R., Castano-Diez, D., Jensen, G.J. and Miao J.W. (2008) *J. Struct. Biol.* **164**(2):221-227.

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Gan, L., Chen S.Y. and Jensen, G.J. (2008) Molecular organization of Gram-negative peptidoglycan. *Proc. Natl. Acad. Sci. USA* **105**(48):18953-18957. http://www.pnas.org/content/105/48/18953.

Lee, E., Fahimian, B.P., Iancu, C.V., Suloway, C., Murphy, G.E., Wright, E.R., Castano-Diez, D., Jensen, G.J. and Miao J.W. (2008) Radiation dose reduction and image enhancement in biological imaging through equally-sloped tomography. *J. Struct. Biol.* **164**(2):221-227.

Suloway, C., Shi, J., Cheng, A., Pulokas, J., Carragher, B., Potter, C.S., Zheng, S.Q., Agard, D.A. and Jensen, G.J. (2009) Fully automated, sequential tilt-series acquisition with *Leginon*. *J. Struct. Biol*. **167**(1):11-18.

Tivol, W.F., Briegel, A. and Jensen, G.J. (2008) An improved cryogen for plunge freezing. *Microsc. Microanal.* **4**(5):375-379.

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Research Fellows: Swathi Amere, Roberto A. Chica, Jennifer R. Keeffe, Irene Maier
Graduate Students: Benjamin D. Allen, Mohsen Chitsaz, Toni Lee, Matthew M. Moore, Yun Mou, Alex Nisthal, Heidi K. Privett
Research and Laboratory Staff: Marie L. Ary, Rhonda K. Digiusto, Erin Drez
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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.

386. An iterative method for *de novo* computational enzyme design

Heidi K. Privett[*]*, Leonard Thomas, Stephen L. Mayo*

A general method for the design of highly efficient protein catalysts for arbitrary chemical transformations has been an attractive but elusive goal since the advent of computational protein design. In cases where designed enzymes were shown to be inactive, few studies have been performed to determine the cause of this inactivity. We have developed an iterative method for computational enzyme design that involves extensive theoretical and experimental evaluation of several designs that did not initially show activity. These analyses helped identify the causes of the inactivity and led to adjustments of our design procedure, which resulted in the design of multiple enzymes with activity towards our test reaction, the Kemp elimination. Currently, we are pursuing crystal structures of these active designs and are collaborating with a group that is applying directed evolution on one of the most active designs in an effort to further increase the catalytic activity. In addition, we have demonstrated that molecular dynamics simulations can accurately predict the activity of designed Kemp elimination enzymes and can be used as a reliable prescreening step, allowing us to focus our experimental efforts on designs that are most likely to be active. Other enzyme design efforts in the Mayo lab are focused on designing enzymes for additional reactions, including the Claisen rearrangement, in order to demonstrate the generalizability of this method for any arbitrary chemical transformation.

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387. Development and validation of methods for multi-state and library design

Benjamin D. Allen[1]*, Alex Nisthal*[2]*, Stephen L. Mayo*

The stability, activity, and solubility of a protein sequence are determined by a delicate balance of molecular interactions in a wide variety of conformational states, including competing states and native conformational states. Even so, most computational protein design methods model sequences in the context of a single conformation representing the native state. Despite the potential for improved simulation accuracy when the native state is represented by an ensemble of related structures, such calculations have not been attempted due to the lack of sufficiently powerful optimization algorithms for multi-state design. Here, we have applied our multi-state design algorithm to study the potential utility of various forms of input structural data for design.

To facilitate this analysis, we developed new methods for the design and high-throughput stability determination of combinatorial mutation libraries based on protein design calculations. The application of these methods to the core design of a small model system produced many variants with improved thermodynamic stability, and showed that multi-state design methods can be applied to large structural ensembles without requiring the use of different rotamer libraries, energy functions, or design strategies. Stabilized variants were found in libraries based on each type of structural data we tested. Our library design method produced degenerate codon libraries that represented the underlying design calculations, and exhaustive screening of these libraries helped to clarify several sources of error in our designs that would have otherwise been difficult to ascertain.

The lack of correlation between our experimental and simulated stability values shows clearly that a design procedure need not reproduce experimental data directly to generate many successful variants. This surprising result suggests potential new directions for the improvement of protein design technology.

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[2]*Graduate Option in Biochemistry and Molecular Biophysics, Caltech*

388. High-throughput combinatorial library analysis of structural ensembles

Alex Nisthal[1], Benjamin D. Allen[2], Stephen L. Mayo

Our work was motivated by a desire to address one of the major approximations of computational protein design (CPD): the reliance on a single, rigid main-chain conformation. In order to overcome this limitation, we have investigated the degree to which X-ray crystallographic structures, NMR solution structures, and ensembles derived from molecular dynamics simulations can serve as useful sources of structural information for CPD. A previously explored problem, core repacking of streptococcal protein G, was employed as the test bed. Selection and stability determination of the designed sequences was made possible by the development of new methods for the computational design and high-throughput experimental stability determination of combinatorial protein libraries. All three sources of structural data resulted in designed libraries with multiple stabilized variants. The designed libraries based on an NMR ensemble were extremely similar, whether a single representative structure or all 60-ensemble members were used for modeling. The most promising results by far were achieved using a constrained 128-member molecular dynamics ensemble, which produced a designed library with no significantly destabilized and many stabilized variants. The results provide simultaneous experimental validation for (1) the application of multi-state protein design methods to large conformational ensembles; (2) the transformation of arbitrary CPD results into combinatorial mutation libraries; and (3) the experimental stability determination of these libraries by high-throughput gene assembly, protein expression, purification, and screening. Despite the apparent success of this design, there was no correlation observed between the simulation energies and the experimental stabilities of any of these variants, suggesting that dramatic improvements in CPD may not come from improvements in ranking ability.

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389. Modifying the spectral properties of a red fluorescent protein

Roberto A. Chica, Matthew M. Moore[*], Benjamin D. Allen[*], Stephen L. Mayo

Fluorescent proteins have found widespread application in cell biology as reporters for the cellular localization of various proteins. Those that emit in the red portion of the visible spectrum are of particular interest because longer wavelengths are less damaging to cells and because cells are more transparent to red light. One such red fluorescent protein, mCherry, has an emission maximum of 610 nm. Our goal is to generate mCherry mutants with improved spectral properties such as a red-shifted emission maximum, higher quantum yield, and increased brightness. We have been using a computational design method to generate focused combinatorial libraries of mCherry. The protein sequences in these libraries have a high probability of folding properly since our method helps to eliminate mutations that would destabilize the protein fold. A total of 13 residues surrounding the chromophore were designed, yielding four libraries of 240-540 mutants each, a size that can easily be screened for the desired properties using a 96-well plate-based assay. We have thus far identified several mutants displaying fluorescence emission red shifts of up to 25 nm. The quantum yields and extinction coefficients of these mutants have been determined. In addition, exhaustive sequencing of all the fluorescent mutants was done to provide information on structure/function relationships in mCherry. Lastly, crystal structures of the most red-shifted mutants have been obtained at high resolution (< 2.0 Å). Analysis of these structures has led to validation of the original hypotheses that enabled bathochromic shifting of emission and has sparked novel rational design schemes for improving fluorescence quantum yield.

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390. A novel high-resolution protein structure refinement algorithm

Mohsen Chitsaz[*], Stephen L. Mayo

The refinement of low-resolution protein models to generate structures with atomic-level accuracy has been a major challenge for computational structural biology. Energy-based refinement depends on two interrelated components: (1) a sufficiently accurate energy function, and (2) an efficient algorithm to search the enormous conformational space. Focusing on the latter, we developed a new high-resolution protein structure relaxation algorithm called Grid search. Grid search walks through the protein chain calculating energies and finding the optimal configuration for each residue given a three-dimensional search domain of $\Delta\phi$, $\Delta\varphi$, and side-chain conformation; the entire process is repeated until there is no further improvement in energy. Domain parameters were altered to find those producing the best results. The best set of parameters was then used and results were compared with those obtained with Backrub, an algorithm that achieves improvements in modeling by incorporating the backbone flexibility associated with correlated backbone side-chain motions [1-2]. Refinements were performed on 11 high-resolution crystal structures from four different protein structural classes. Measurements included energy of the final refined structure, run time required to execute the calculations, and total computational effort (processor time). On average, the best refined structure by Grid search showed about 50% more energy improvement than the best Backrub structure. Grid search was also about 50 times faster than Backrub and used far less computational resources (by more than three orders of magnitude). In addition to protein structure refinement, we expect the dramatically improved efficiency provided by Grid search to be useful in computational protein design and protein structure prediction studies.

*Graduate Option in Biochemistry and Molecular Biophysics, Caltech

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391. Engineering cyanovirin-N for enhanced viral neutralization

Jennifer R. Keeffe, Priyanthi Peiris, Jesse Bloom, Stephen L. Mayo

Cyanovirin-N (CVN), a protein originally isolated from the cyanobacterium *Nostoc ellipsosporum*, has been shown to bind specifically to glycosylated envelope proteins on many enveloped viruses, including HIV, influenza, and Ebola, preventing viral fusion. We have successfully created dimeric and trimeric variants of CVN to investigate the effect of avidity on CVN-mediated HIV neutralization. The proteins were expressed and purified to homogeneity, then assessed for their ability to prevent HIV infection in a cell culture-based neutralization assay [1]. We found that covalently linking two CVN monomers (CVN_2) through a flexible polypeptide linker (LX, where X is the number of amino acids in the linker) decreased the IC_{50} of neutralization ten-fold compared to wild-type CVN. The addition of a third CVN monomer (CVN_3), however, showed minimal further neutralization enhancement. We then tested wild-type CVN, CVN_2 L0, and CVN_2 L10 against 33 strains of HIV from three clades and found that 100% of the strains were neutralized by CVN and the variants. In addition, CVN_2 L0 neutralized 28 out of 31 strains at a lower concentration than any of the four broadly neutralizing HIV antibodies tested. Crystallographic studies show that CVN_2 L0, CVN_2 L1, and CVN_2 L10 are domain-swapped, indicating that we have successfully shifted the equilibrium from a monomer in solution to a domain-swapped dimer. We are currently investigating whether this domain-swapping is responsible for the increase in activity. In addition to the HIV and structural work, we have also successfully developed a plate-based assay for testing influenza neutralization. We showed that the dimeric CVN variants neutralize influenza at significantly lower concentrations, similarly to the HIV results; we are currently testing whether there is a linker-length dependence. We are also investigating a chimeric CVN-Fc fusion protein, which would possess the viral neutralization properties of CVN and contain the ability to recruit the natural immune system through Fc-mediated effector function. Initial experiments indicate that CVN-Fc expressed in and purified from HEK-293 cells is able to neutralize HIV with an IC_{50} similar to wild-type CVN. Future work is aimed at optimizing expression, performing structural studies, and continuing the functional assays.

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392. An assay to assess cyanovirin-N binding to carbohydrates on viral glycoproteins

Irene Maier, Alexandria H. Berry, Jennifer R. Keeffe, Stephen L. Mayo*

Transmembrane-linked glycoproteins on the surface of enveloped viruses such as influenza, Ebola, and human immunodeficiency virus (HIV) initiate viral attachment and fusion to host membranes. These glycoproteins contain the epitopic structures recognized by neutralizing antibodies and are thus the primary targets for the design of anti-viral vaccines and viral entry inhibitors. The Ebola virus genome contains seven genes that direct the synthesis of eight proteins. Transcriptional editing of the fourth gene results in the expression of the 676-residue transmembrane-linked glycoprotein (GP) responsible for receptor-mediated endocytosis [1]. Ebola GP is similar to hemagglutinin, the corresponding glycoprotein expressed on the surface of influenza, in that it is composed of several domains covered by a thick layer of complex oligomannose and hybrid-type glycans, and assembles as a metastable trimer of heterodimers.

A pseudo immunoassay in microtitration plate format is being developed using cyanovirin-N (CVN), a cyanobacterial lectin [2], to monitor protein-carbohydrate binding on Ebola GP. Therefore, recombinant full-length Ebola GP minus the transmembrane domain (GP 1-632) will be expressed in baculovirus insect cells. CVN binds to N-linked high-mannose oligosaccharides and has been shown to bind to glycosylated envelope proteins, including those on the surface of Ebola and gp120 on the surface of HIV, thus inhibiting viral fusion and infection. Its anti-viral activity and binding to sugars under physiological conditions is likely due to a monomeric form of the wild-type protein, which has two carbohydrate binding sites. Although these carbohydrate-binding domains show no sequence homology, they are symmetrically placed, similar to the antigen binding sites in an immunoglobulin, and therefore may bind with similar affinities. Once the assay has been validated, studies will be aimed at enhancing the protein-carbohydrate interaction with computationally designed variants of CVN and screening for improved binding to Ebola GP.

*Graduate Option in Biochemistry and Molecular Biophysics, Caltech

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393. Computational design and characterization of a hyperthermostable cellulase

Toni M. Lee[*]*, Stephen L. Mayo*

The degradation of lignocellulose into monomeric glucose units constitutes an essential step in the efficient synthesis of ethanol-based biofuels. Lignocellulose exists as a crystalline lattice of polymeric glucose chains wrapped in highly stable lignin. Upon disruption of the lignin shell and crystalline lattice, cellulases cooperatively catalyze the hydrolysis of cellulose polymers into monomers. Harsh pretreatment conditions used to expose free cellulose polymers, however, can prematurely denature any enzymes present. As such, one strategy for improving the efficiency of cellulose ethanol production involves the use of cellulases capable of withstanding the high temperature or low pH environment introduced during pretreatment. Computational protein design provides a means of producing such celllulases. To this end, we plan to utilize our design methods to enhance the ability of a highly utilized industrial cellulase, the catalytic domain of Cel7A from *Trichoderma reesei*, to withstand high temperatures.

Validating computationally generated results hinges on the efficient production and characterization of several highly ranked designs. A fungal protein, Cel7A is currently inexpressible in model organisms that are ideal for high-throughput protein expression and purification. It is possible that differences in *T. reesei* and bacterial glycosylation systems may generate different glycosylation patterns in produced proteins, leading to degradation in the bacterial case. As a preface to the computational design process, we are currently expressing Cel7A in *Escherichia coli* as a fusion protein to a variety of well-characterized soluble domains to prevent the degradation of the improperly glycosylated cellulase.

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394. Experimental system to study mechanistic theories of backbone cyclization

Matthew M. Moore[*]*, Stephen L. Mayo*

Peptide backbone cyclization is a rare and quite versatile post-translational modification among proteins. Backbone cyclizations are known to occur in green fluorescent protein (GFP) and histidine ammonia-lyase, where their products are used for bioluminescence and chemical reactivity, respectively. This chemical modification is unique for GFP in that it occurs autocatalytically, and thus, no external cofactors or enzymes are needed to obtain fluorescence during heterologous expression. The biosynthesis of a fluorophore in GFP by way of backbone cyclization is therefore completely encoded in the primary structure of this protein. The mechanism of this fluorophore formation has been intensely studied, and the knowledge gained has been successfully applied toward the rational engineering of fluorescent protein variants with novel properties. In the hopes of recapitulating the requirements for fluorophore formation in a new scaffold, we identified a protein containing the same topological fold present in all known fluorescent proteins—an 11-stranded β-barrel with a coaxial helix. This fold is formed by residues 400-623 of the murine nidogen-1 G2 domain. A 6X-His tag was appended to this protein segment to facilitate purification by immobilized metal ion chromatography. So far, we have developed a high-yield (19 mg per 1.0 L culture) expression system in *Escherichia coli* and have characterized the recombinant protein by circular dichroism, liquid chromatography-mass spectrometry, surface plasmon resonance, and X-ray crystallography. This work confirmed that the protein has the correct tertiary structure (as seen in the 1.90 Å crystal structure), and that it binds to collagen-IV and laminin with affinities consistent with those reported in the literature. The goal of this project is to use the recombinant protein as a "blank-slate scaffold" to test a variety of mechanistic theories on the causes of autocatalytic backbone cyclization. Computational protein design software developed in-house will be used to guide the selection of mutations that may shed light on this process.

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395. Library designs to expand calmodulin binding diversity

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Calmodulin (CaM) is a small Ca^{2+}-binding protein that binds to and regulates a number of different protein targets. Its ability to tightly bind to a diverse set of small peptides makes it an ideal system for searching for new binding targets. Hundreds of sequences are already known to bind CaM, and analysis of the interactions involved has led to hypotheses regarding the basic elements required for binding [1]. Several high-resolution CaM-peptide complexes have been solved by solution NMR and X-ray crystallography. Further, the ORBIT computational design software has been successfully used to generate CaM variants with increased specificity toward a particular target [2]. Using this abundant knowledge of CaM binding affinity and specificity, we intend to design libraries of CaM variants to bind novel peptides with high affinity. We focused on viral proteins as our novel targets, in hopes that the variants obtained could eventually be developed for therapeutic use. A BLAST search of the viral genome database was performed to find novel peptide sequences that contain a CaM-binding consensus sequence. Eight sequences were found that satisfy this criteria, and X-ray crystallographic structures are available for two of them. To experimentally validate the CaM libraries, a high-throughput assay was developed to determine CaM-peptide binding. The assay is based on fluorescence resonance energy transfer (FRET), a state-of-the-art method to characterize interactions between molecules. Color variants of green fluorescent protein (GFP) can be attached to a host protein (CaM) and a target peptide. FRET can be used to determine protein-protein interaction in cell lysates, thus allowing the efficient identification of CaM-binding peptides. Using a 96-well plate-based assay, two 100-member libraries designed to bind peptide sequences of HIV-1 gp120 were tested experimentally. No hits have been found so far.

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396. Engineering lac repressor to respond to non-native ligands

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Lac repressor (LacI), a DNA-binding transcriptional regulatory protein, is widely used in biotechnological applications, including serving as a regulatory component of protein expression vectors. Typically, isopropyl-β-D-thiogalactoside (IPTG) is used as an inducer for recombinant protein expression in place of LacI's native ligand allolactose. Binding of allolactose and other substrate analogs to the repressor protein results in an allosteric transition. Our goal is to engineer and identify LacI variants that will respond to readily available monosaccharides such as glucose, arabinose, and galactose. This will be accomplished via stepwise site-saturation mutagenesis of individual ligand-contacting residues and the *in silico* design of sequence libraries, accompanied by phenotypic screening for variants with enhanced target ligand selectivity at each stage of engineering. The successful identification of highly specific ligand-LacI variant pairs will allow us to selectively regulate gene expression for a wide range of tissue engineering and biomedical applications.

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Summary: Our main subject is the ubiquitin (Ub) system. The field of ubiquitin and regulated protein degradation was created in the 1980s, largely through the complementary discoveries by the laboratory of A. Hershko (Technion, Israel) and by my laboratory, then at MIT. These discoveries revealed three sets of previously unknown facts:

1. ATP-dependent protein degradation involves a new protein modification, ubiquitin conjugation, which is mediated by specific enzymes, termed E1, E2 and E3.

2. The selectivity of ubiquitin conjugation is determined by multipartite degradation signals (degrons) in short-lived proteins, including degrons that give rise to the N-end rule.

3. Ubiquitin-dependent processes play a strikingly broad, previously unsuspected part in cellular physiology, primarily by controlling the *in vivo* levels of specific proteins. Ub conjugation was shown by us to be required for protein degradation *in vivo*, for cell viability, and also, specifically, for the cell cycle, DNA repair, protein synthesis, transcriptional regulation, and stress responses. We also cloned the first Ub genes (discovering their divergent functions), the first specific E3 Ub ligase (Ubr1), the first deubiquitylating enzymes (DUBs), and identified the first physiological substrate of the Ub system, the MATalpha2 transcriptional repressor. We also discovered that Ub-dependent proteolysis involves an essential, substrate-linked polyubiquitin chain of unique topology. In addition, the Ub system was discovered to possess the critical property of *subunit selectivity*, i.e., the ability to destroy a specific subunit of a multisubunit protein, leaving the rest of the protein intact and thereby making possible *protein remodeling*. This fundamental process underlies the cell cycle (the replacement of cyclin subunits in cell-cycle kinases), the activation of transcription factors such as, for example, NF-kappaB, and a legion of other biological pathways.

The Hershko laboratory produced the first of these fundamental advances (item 1), and my laboratory produced the other two (items 2 and 3). Our function-based studies in the 1980s yielded the overall discovery of the *biological regulation by intracellular protein degradation and its central role in cellular physiology*. The complementary "chemical" and "biological" insights by Hershko's and my laboratories caused a massive expansion of the ubiquitin field in the 1990s. It became one of the largest arenas in biomedical science, the point of convergence of many disparate disciplines. Because perturbations of the cell cycle, DNA repair and stress response pathways are hallmarks of malignant transformation, our 1987-88 discoveries with Cdc34 (Ub-conjugating enzyme critical for the cell cycle), Rad6 (Ub-conjugating enzyme required for DNA repair) and Ubi4 (a poly-Ub precursor of Ub that is required for stress responses) opened up Ub-based studies in cancer research as well. For accounts of the early history of the ubiquitin field, see Hershko *et al*. (2000); Varshavsky (2006, 2008).

The above insights in the 1980s yielded the modern paradigm of cellular physiology, in which regulated proteolysis is of central importance. These advances, together with later studies by many groups, revealed that the control through regulated protein degradation rivals, and often surpasses in significance the classical regulation through transcription and translation. This altered understanding of the design of biological circuits is of major importance for medicine, given the astounding functional range of the Ub system and the multitude of ways in which Ub-dependent processes can malfunction in disease or in the course of aging, from cancer and neurodegenerative syndromes to perturbations of immunity and many other illnesses, including birth defects. Our work at Caltech continues to focus on the Ub system and regulated protein degradation.

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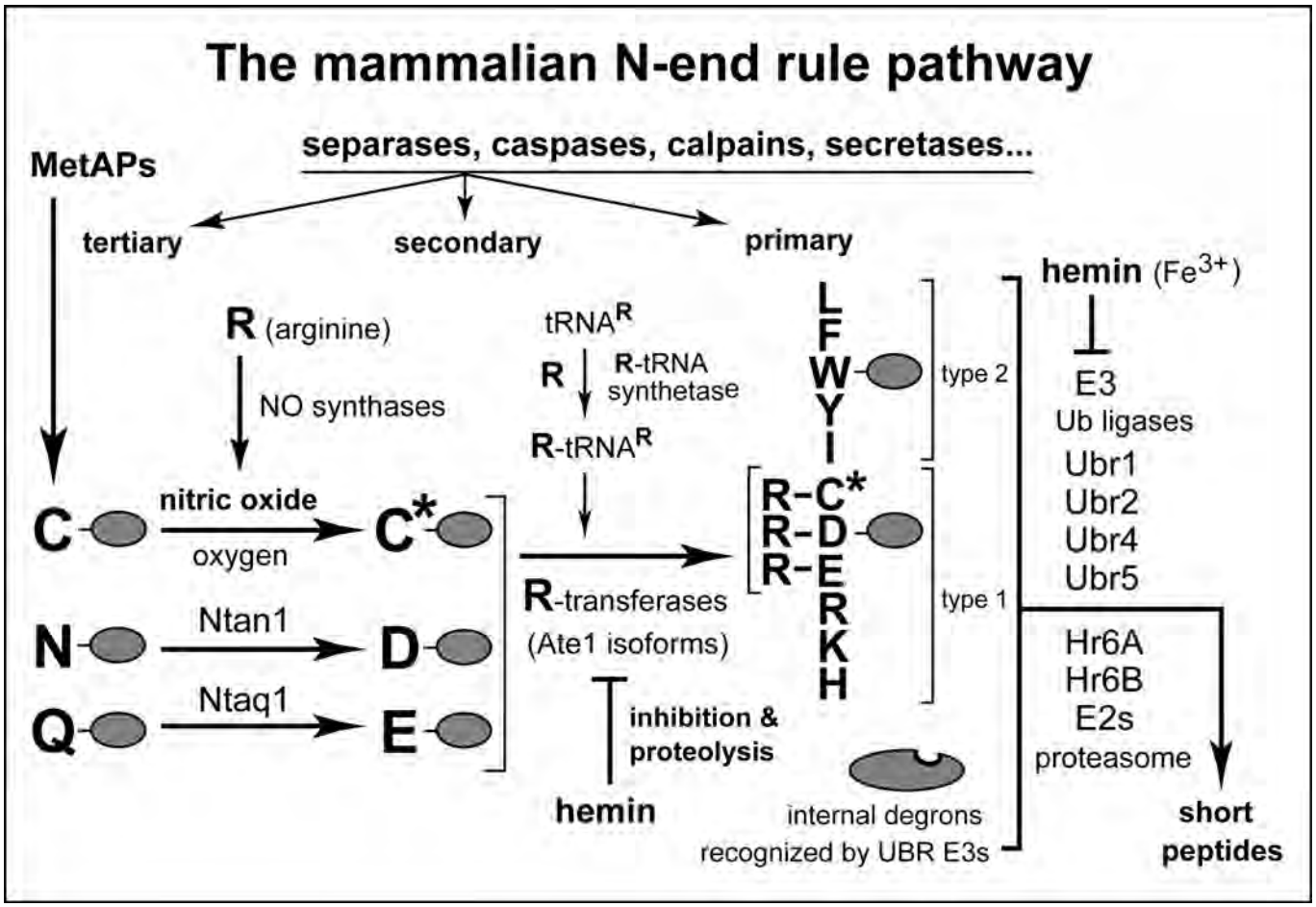

Fig. 1. The mammalian N-end rule pathway. N-terminal residues are indicated by single-letter abbreviations for amino acids. Grey ovals denote the rest of a protein substrate. A sign, above "*hemin*" in the middle of diagram, is a modified "downregulation" sign that denotes a downregulation mediated, at least in part, by target's degradation. MetAPs, methionine aminopeptidases. C* denotes oxidized Cys, either Cys-sulfinate or Cys-sulfonate, produced in reactions mediated by nitric oxide (NO), oxygen (O_2) and their derivatives. Oxidized N-terminal Cys is arginylated by *ATE1*-encoded isoforms of arginyl-transferase (R-transferase). Type-1 and type-2 primary destabilizing N-terminal residues are recognized by the pathway's E3 Ub ligases, called N-recognins. Through their other substrate-binding sites, these E3s also recognize internal (non-N-terminal) degrons in other substrates of the N-end rule pathway, denoted by a larger oval. As shown in the diagram, hemin (Fe^{3+}-heme) interacts not only with Ate1 (R-transferase) but with UBR-family Ub ligases as well, and downregulates at least some E3s of this family.

Ubiquitin (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 Ub-activating enzyme, forms a thioester bond between the C-terminal Gly of Ub and a 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. 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 degradation signals (degrons) of their substrates. Ub has nonproteolytic functions, as well.

One pathway of the Ub system is the N-end rule pathway (Fig. 1). The N-end rule relates the *in vivo* half-life of a protein to the identity of its N-terminal residue. The N-end rule pathway recognizes several kinds of degradation signals, including a set called N-degrons (Fig. 1). Although prokaryotes lack the Ub system, they contain the N-end rule pathway, albeit Ub-independent versions of it. In eukaryotes, an N-degron consists of three determinants: a destabilizing N-terminal residue of a protein substrate, one (or more) of its internal Lys residues (the site of formation of a substrate-linked poly-Ub chain), and a nearby disordered region. The N-end rule has a hierarchic structure (Fig. 1). In eukaryotes, N-terminal Asn and Gln are tertiary destabilizing residues in that they function through their enzymatic deamidation, to yield the secondary destabilizing N-terminal residues Asp and Glu. The destabilizing activity of N-terminal Asp and Glu requires their conjugation to Arg, one of the primary destabilizing residues, by Arg-tRNA-protein transferase

(R-transferase). In mammals and other eukaryotes that produce nitric oxide (NO), the set of arginylated residues contains not only Asp and Glu but also N-terminal Cys, which is arginylated after its oxidation to Cys-sulfinate or Cys-sulfonate. The *in vivo* oxidation of N-terminal Cys requires NO, as well as oxygen (O_2) or its derivatives (Fig. 1). The N-end rule pathway is thus a sensor of NO, through the ability of this pathway to destroy proteins with N-terminal Cys, at rates controlled by NO, O_2 and their derivatives.

E3 Ub ligases of the N-end rule pathway, called N-recognins, bind to primary destabilizing N-terminal residues, including Arg (Fig. 1). (The term "Ub ligase" denotes either an E2-E3 holoenzyme or its E3 component.) At least four N-recognins, including Ubr1, mediate the N-end rule pathway in mammals. The known N-recognins share a ~70-residue motif called the UBR box. Mouse Ubr1 and Ubr2 are sequelogous (similar in sequence) 200-kD RING-type E3 Ub ligases that are 47% identical. Several other UBR-containing N-recognins, either confirmed or putative ones, are HECT-type or SCF-type E3 Ub ligases that share the UBR motif with the RING-type Ubr1/Ubr2 but are largely nonsequelogous to them otherwise. (*A note on terminology*: "sequelog" and "spalog" denote, respectively, a sequence that is similar, to a specified extent, to another sequence, and a 3D (spatial) structure that is similar, to a specified extent, to another 3D structure (Varshavsky, 2004). Besides their usefulness as separate terms for sequence and spatial similarities, the rigor-conferring advantage of "sequelog" and "spalog" is their *evolutionary neutrality*, in contrast to interpretation-laden terms such as "homolog," "ortholog" and "paralog." The latter terms are compatible with the sequelog/spalog terminology and can be used to convey understanding about functions and common descent, if this (additional) information is available.

The N-end rule pathway of the yeast *S. cerevisiae* is mediated by a single N-recognin, Ubr1, a 225-kD sequelog of mammalian Ubr1 and Ubr2 (Fig. 1). *S. cerevisiae* Ubr1 contains at least three substrate-binding sites. The type-1 site is specific for basic N-terminal residues of protein substrates (Arg, Lys, His). The type-2 site is specific for bulky hydrophobic N-terminal residues (Trp, Phe, Tyr, Leu, Ile). The third substrate-binding site of UBR1 targets proteins through their internal (non-N-terminal) degrons, and is allosterically "activated" through a conformational change that is caused by the binding of short peptides to the Ubr1's other two binding sites, type-1 and type-2. One known substrate of the third binding site of Ubr1 is Cup9, a transcriptional repressor whose regulon includes *PTR2*, a gene-encoding transporter of di- and tripeptides. The reversal of Ubr1 autoinhibition by imported peptides accelerates the Ubr1-dependent ubiquitylation of Cup9, leads to its faster degradation, and thereby causes a derepression of *PTR2*. The resulting positive-feedback circuit allows *S. cerevisiae* to detect the presence of extracellular peptides and to react by increasing their uptake.

The functions of the N-end rule pathway were discovered largely over the last decade. These functions include the sensing of heme, owing to inhibition of the pathway's ATE1 R-transferase, in both yeast and mammals, by hemin (Fe^{3+}-heme), which also inhibits N-recognins, the latter at least in yeast; the sensing of NO and oxygen, and the resulting control of signaling by transmembrane receptors, through the conditional, NO/O_2-mediated degradation of G-protein regulators RGS4, RGS5 and RGS16; regulation of import of short peptides, through the degradation, modulated by peptides, of Cup9, the import's repressor; fidelity of chromosome segregation, through degradation of a separase-produced cohesin fragment; regulation of DNA repair, through the degradation of Mgt1, a DNA alkylguanine transferase; regulation of apoptosis, through degradation of a caspase-processed inhibitor of apoptosis; a multitude of processes mediated by the transcription factor c-FOS, a conditional substrate of the N-end rule pathway; regulation of the human immunodeficiency virus (HIV) replication cycle, through degradation of HIV integrase; regulation of meiosis, spermatogenesis, neurogenesis and cardiovascular development in mammals; the functioning of specific organs, in particular the brain and the pancreas; and regulation of leaf senescence and seed germination in plants. Mutations in human Ubr1 (Fig. 1) are the cause of Johansson-Blizzard Syndrome (JBS), which comprises mental retardation, physical malformations, and severe pancreatitis (Varshavsky, 2008, and refs. therein).

Functional and mechanistic studies of the N-end rule pathway in yeast and mammals are a major theme of our current work.

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397. Ablation of arginylation in the N-end rule pathway: Loss of fat, increased metabolic rate, damaged spermatogenesis, and neurological perturbations

Christopher Brower, Alexander Varshavsky

In the N-end rule pathway of protein degradation, the destabilizing activity of N-terminal Asp, Glu or (oxidized) Cys residues requires their conjugation to Arg, which is recognized directly by pathway's ubiquitin ligases. N-terminal arginylation is mediated by the Ate1 arginyltransferase, whose physiological substrates include the Rgs4, Rgs5 and Rgs16 regulators of G proteins. In this study (Brower and Varshavsky, 2009), we employed the Cre-lox technique to uncover new physiological functions of N-terminal arginylation in adult mice. We showed that postnatal deletion of mouse *Ate1* (its unconditional deletion is embryonic lethal) causes a rapid decrease of body weight and results in early death of ~15% of Ate1-deficient mice. Despite being hyperphagic, the surviving Ate1-deficient mice contain little visceral fat. They also exhibit an increased metabolic rate, ectopic induction of the Ucp1 uncoupling protein in white fat, and are resistant to diet-induced obesity. In addition, Ate1-deficient mice have enlarged brains, an enhanced startle response, are strikingly hyperkinetic, and are prone to seizures and kyphosis. Ate1-deficient males are also infertile, owing to defects in *Ate1*$^{-/-}$ spermatocytes. The

remarkably broad range of biological processes that are shown here to be perturbed by the loss of N-terminal arginylation will facilitate the discovery of specific circuits that involve Ate1 and either its known substrates, such as Rgs4, Rgs5 and Rgs16, or those currently unknown.

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398. The N-end rule pathway as a sensor of heme

Rong-Gui Hu[1], Haiqing Wang, Alexander Varshavsky

Heme is an iron-containing protoporphyrin IX. Two major species of heme are ferrous (Fe^{2+}) heme and its ferric (Fe^{3+}) counterpart, called hemin. Intracellular proteins whose functions depend on their binding to heme include hemoglobins, cytochrome oxidases, NO synthases, cGMP cyclases, and catalases, as well as specific kinases, transcription factors, ion channels, and regulators of iron metabolism. A major aspect of heme is its ability to interact with physiologically relevant gases such as O_2, NO and carbon monoxide (CO). Over the last decade, it became clear that reactive oxygen species (ROS), if they are present at "signaling" (i.e., sufficiently low) levels, can act as regulators of circuits that underlie not only stress responses but other functions as well, including the cell cycle, transcription and differentiation. The proximal sensors of H_2O_2 and analogous compounds are cysteine-containing proteins, some of which contain heme, as well. ROS-mediated formation of disulfides or other Cys derivatives changes the activity of sensor proteins, which transduce their altered states into the outputs of circuits they control. In some hemoproteins of these circuits, it is the heme moiety, rather than Cys residues, that functions as a redox sensor.

We discovered (Hu *et al*., 2008) that the arginyl-transferase Ate1, which mediates the arginylation branch of the N-end rule pathway (see Fig. 1 and Introduction), is inhibited by hemin (Fe^{3+}-heme), via a specific redox mechanism that involves the formation of disulfide between cysteine-71 and cysteine-72 of Ate1. Remarkably, hemin also induces degradation of R-transferase *in vivo* thus, acting as both a "stoichiometric" and "catalytic" down-regulator of the N-end rule pathway. This proteolytic circuit, a known sensor of short peptides, nitric oxide and oxygen, is now a sensor of heme, as well. One function of the N-end rule pathway may be to coordinate the activities of small effectors, both reacting to and controlling the redox dynamics of heme, oxygen, nitric oxide and thiols, in part through conditional degradation of specific transcription factors and G-protein regulators.

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399. Enzymatic N-terminal addition of noncanonical amino acids to peptides and proteins

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The production of well-defined protein conjugates is essential for many therapeutic and biochemical technologies. To achieve site-specific modification of proteins, the N-terminus has been targeted by a variety of chemical and enzymatic methods. We developed a fully enzymatic *in vitro* method for the N-terminal addition of non-canonical amino acids to peptides and proteins. The *Escherichia coli* leucyl, phenylalanyl-transferase (Aat), encoded by the *aat* gene, catalyzes the conjugation of leucine (Leu), phenylalanine (Phe) or methionine (Met) from an aminoacylated tRNA to any protein that bears N-terminal arginine or lysine (Connor *et al*., 2008). The transfer of Leu or Phe to the N-terminus of a protein in wild-type *E. coli* cells results in a decrease of the protein's *in vivo* half-life through degradation by ClpAP, an ATP-dependent protease. Aat, ClpAP, and the adapter protein, ClpS, comprise the *E. coli* N-end rule pathway. Aat is tolerant of structural variation in its amino acid substrates and is known to accept Phe analogs through chemically aminoacylated-tRNAs. We used a simple chromatographic assay to demonstrate Aat-mediated transfer of non-canonical amino acids to the acceptor peptide lysylalanyl-7-amino-4-methylcoumarin. Bio-orthogonal reactive functional groups, such as alkenes, alkynes, azides and ketones, can be transferred and used to prepare bio-conjugates in high yield. The method introduced here allows new approaches to the engineering of therapeutic proteins through pegylation; to the study of protein interactions through crosslinking; and to the immobilization of proteins for use in sensors, microarrays, and catalytic systems. Furthermore, the HPLC assay introduced here for monitoring Aat activity can be applied to any non-canonical amino acid.

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400. Substrate-binding sites of Ubr1, the ubiquitin ligase of the N-end rule pathway

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Substrates of a ubiquitin-dependent proteolytic system called the N-end rule pathway include proteins with destabilizing N-terminal residues. N-recognins, the pathway's ubiquitin ligases, contain three substrate-binding sites. The type-1 site is specific for basic N-terminal residues (Arg, Lys, His). The type-2 site is specific for bulky hydrophobic N-terminal residues (Trp, Phe, Tyr, Leu, Ile). We show here that the type-1/2 sites of Ubr1,

the sole N-recognin of the yeast *Saccharomyces cerevisiae*, are located in the first ~700 residues of the 1,950-residue Ubr1. These sites are distinct in that they can be selectively inactivated by mutations, identified through a genetic screen. Mutations inactivating the type-1 site are in the previously delineated ~70-residue UBR motif characteristic of N-recognins. Fluorescence polarization and surface plasmon resonance were used to determine that Ubr1 binds, with K_d of ~1 microM, to either type-1 or type-2 destabilizing N-terminal residues of reporter peptides, but does not bind to a stabilizing N-terminal residue such as Gly (Xia *et al*., 2008). A third substrate-binding site of Ubr1 targets an internal degron of Cup9, a transcriptional repressor of peptide import. We show that the previously demonstrated *in vivo* dependence of CUP9 ubiquitylation on the binding of cognate dipeptides to the type-1/2 sites of Ubr1 can be reconstituted in a completely defined *in vitro* system. We also found that purified Ubr1 and Cup9 interact nonspecifically, and that specific binding (which involves, in particular, the binding by cognate dipeptides to the Ubr1's type-1/2 sites) can be restored either by a chaperone such as Ef1A or through macromolecular crowding.

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401. Amino acids induce peptide uptake via accelerated degradation of Cup9, the transcriptional repressor of the Ptr2 peptide transporter

Z. Xia[1], G.C. Turner[2], Cheol-Sang Hwang, C. Byrd[3], Alexander Varshavsky

Multiple pathways link expression of Ptr2, the transporter of di- and tripeptides in the yeast *S. cerevisiae*, to the availability and quality of nitrogen sources. Previous work has shown that induction of *PTR2* by extracellular amino acids requires, in particular, *SSY1* and *PTR3*. Ssy1 is structurally similar to amino acid transporters, but functions as a sensor of amino acids. Ptr3 acts downstream of Ssy1. Expression of the Ptr2 peptide transporter is induced not only by amino acids but also by dipeptides with destabilizing N-terminal residues. These dipeptides bind to Ubr1, the ubiquitin ligase of the N-end rule pathway, and allosterically accelerate the Ubr1-dependent degradation of Cup9, a transcriptional repressor of *PTR2*. Ubr1 targets Cup9 through its internal degron. In the present study, we found that the repression of *PTR2* by Cup9 requires Tup1 and Ssn6, the corepressor proteins that form a complex with Cup9 (Xia *et al*., 2008). We also show that the induction of *PTR2* by amino acids is mediated by the Ubr1-dependent acceleration of Cup9 degradation that requires both Ssy1 and Ptr3. The acceleration of Cup9 degradation is shown to be attained without increasing the activity of the N-end rule pathway toward substrates with destabilizing N-terminal residues. We also found that Gap1, a general amino acid transporter, strongly contributes to the induction of *PTR2* by Trp. While several aspects of this complex circuit remain to be understood, our findings establish new functional links between the amino acids-sensing SPS system, the Cup9-Tup1-Ssn6 repressor complex, the Ptr2 peptide transporter and the Ubr1-dependent N-end rule pathway.

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402. Regulation of peptide import through phosphorylation of Ubr1, the ubiquitin ligase of the N-end rule pathway

Cheol-Sang Hwang, Alexander Varshavsky

Ubr1 is the N-recognin of the yeast *S. cerevisiae* (see Introduction). Extracellular amino acids or short peptides upregulate the peptide transporter gene *PTR2*, thereby increasing the capacity of a cell to import peptides. Cup9 is a transcriptional repressor that downregulates *PTR2*. The induction of *PTR2* by peptides or amino acids involves accelerated degradation of Cup9 by the N-end rule pathway. We found that the Ubr1 N-recognin, which conditionally targets Cup9 for degradation, is phosphorylated *in vivo* at multiple sites, including Ser300 and Tyr277 (Hwang *et al*., 2008). We also found that the type-I casein kinases Yck1 and Yck2 phosphorylate Ubr1 on Ser300, and thereby make possible ("prime") the subsequent (presumably sequential) phosphorylations of Ubr1 on Ser296, Ser292, Thr288 and Tyr277 by Mck1, a kinase of the glycogen synthase kinase 3 (Gsk3) family. Phosphorylation of Ubr1 on Tyr277 by Mck1 is the first example of a cascade-based tyrosine phosphorylation by a Gsk3-type kinase outside of autophosphorylation. We show that the Yck1/Yck2-mediated phosphorylation of Ubr1 on Ser300 plays a major role in the control of peptide import by the N-end rule pathway. In contrast to phosphorylation on Ser300, the subsequent (primed) phosphorylations, including the one on Tyr277, have at most minor effects on the known properties of Ubr1, including regulation of peptide import. Thus, a biological role of the rest of Ubr1 phosphorylation cascade remains to be identified.

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403. Two proteolytic pathways regulate DNA repair by co-targeting the Mgt1 alkylguanine transferase

Cheol-Sang Hwang, Anna Shemorry, Alexander Varshavsky

Since the 1987 discovery that a key DNA repair protein Rad6 was a ubiquitin (Ub)-conjugating enzyme (Jentsch *et al*., 1987), there have been great strides in understanding the massive, multi-level involvement of the

Ub-proteasome system in the DNA damage response. A major aspect of this response is the repair of damage caused by alkylating agents such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and methyl methane sulfonate (MMS), which produce both mutagenic and cytotoxic lesions in DNA. One functionally severe lesion in double-stranded DNA is O^6-methylguanine (O^6meG), which is demethylated by the O^6-alkylguanine-DNA alkyltransferase (AGT). This protein is called Mgmt (or MGMT) in mammals and Mgt1 in the yeast *S. cerevisiae*. Compounds that produce O^6meG in DNA are common environmental carcinogens. Some of these compounds are also formed as a part of normal cellular metabolism. The repair of O^6meG in DNA is downregulated in many cancers, usually because of lower than normal levels of Mgmt in cancer cells. Consequently, some anticancer drugs are DNA alkylating agents whose targets include O^6 in guanine. An acquired or pre-existing resistance of cancer cells to such drugs often involves an upregulation of Mgmt. AGT proteins remove methyl and other alkyl groups from alkylated O^6 in guanine by transferring an adduct to an active-site Cys residue. The resulting S-alkyl-Cys residue of AGT is not restored back to Cys, so repair proteins of this kind can act only once. In mammals, the alkylated (inactive) Mgmt, and possibly also the unmodified Mgmt are short-lived proteins, degraded by an unknown pathway.

In this study (Hwang *et al*., 2009), we discovered that *S. cerevisiae* Mgt1 is co-targeted for degradation, through a degron near its N-terminus, by two ubiquitin-mediated proteolytic systems, the Ubr1/Rad6-dependent N-end end rule pathway and the Ufd4/Ubc4-dependent UFD (ubiquitin fusion degradation) pathway. The co-targeting of Mgt1 by these pathways is synergistic, in that it increases not only the yield of polyubiquitylated Mgt1, but also the processivity of polyubiquitylation. The N-end rule and UFD pathways co-mediate both the constitutive and MNNG-accelerated degradation of Mgt1. Yeast cells lacking the Ubr1 and Ufd4 ubiquitin ligases were hyperresistant to MNNG but hypersensitive to the toxicity of overexpressed Mgt1. We consider ramifications of this discovery for the control of DNA repair and mechanisms of substrate targeting by the ubiquitin system.

The N-end rule and UFD pathways were the first specific pathways of the Ub system to be discovered (Bachmair *et al*., 1986). Studies of these pathways have been proceeding largely in parallel, until the discovery of their functional and mechanistic connection, as described in the present work.

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404. The N-end rule pathway controls multiple functions during *Arabidopsis* shoot and leaf development

Emmanuelle Graciet[1], Franziska Walter[1], Diarmuid Ó Maoiléidigh[1], Stephan Pollmann[2], Elliot M. Meyerowitz[3], Alexander Varshavsky[3], Frank Wellmer[1]

Regulated proteolysis by the Ub system underlies just about every cellular and organismal function in eukaryotes. In plants, Ub-dependent processes play major and diverse roles, including the regulation of signaling by phytohormones such as auxin, gibberellins and jasmonic acid. In both the yeast *S. cerevisiae* and the mouse an R-transferase is encoded by a single gene (Fig. 1), whereas the model plant *Arabidopsis thaliana* contains two closely related R-transferases, AtATE1 (At5g05700) and AtATE2 (At3g11240). Primary destabilizing residues are recognized by E3 Ub ligases of the N-end rule pathway, called N-recognins. A single N-recognin, Ubr1, is present in *S. cerevisiae*. In contrast, mammalian genomes encode at least four distinct N-recognins, while in plants, two N-recognins, termed PROTEOLYSIS 1 (PRT1) and PRT6, have been identified in *Arabidopsis*, but other N-recognins are likely to be present, as well.

In the present study, we showed that the *Arabidopsis* R-transferases AtATE1 and AtATE2 regulate various aspects of leaf and shoot development. We also showed that the previously identified N-recognin PROTEOLYSIS6 (PRT6) mediates these R-transferase-dependent activities. We further demonstrated that the arginylation branch of the N-end rule pathway plays a role in repressing the meristem-promoting *BREVIPEDICELLUS* (*BP*) gene in developing leaves. *BP* expression is known to be excluded from *Arabidopsis* leaves by the activities of the ASYMMETRIC LEAVES1 (AS1) transcription factor complex and the phytohormone auxin. Our results suggest that AtATE1 and AtATE2 act redundantly with AS1, but independently of auxin, in the control of leaf development.

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405. Glutamine-specific N-terminal amidase, a component of the N-end rule pathway

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The N-end rule has a hierarchic structure (Fig. 1). N-terminal Asn and Gln are tertiary destabilizing residues in that they function through their enzymatic deamidation, to yield the secondary destabilizing N-terminal residues Asp and Glu. Destabilizing activity of N-terminal Asp and Glu requires their conjugation to Arg, one of the primary destabilizing residues, by R-transferase. In *S. cerevisiae*,

the deamidation branch of the N-end rule pathway is mediated by the Nta1 $Nt^{N,Q}$-amidase, which can deamidate either Asn or Gln at the N-termini of polypeptide substrates (Baker and Varshavsky, 1995). In mammals and other multicellular eukaryotes, N-terminal Asn and Gln are deamidated by N-terminal amidases (Nt-amidases) of two kinds (Fig. 1A). One of them, the previously characterized Ntan1 Nt^{N}-amidase, is specific for N-terminal Asn (Grigoryev *et al*., 1996). In part through analyses of *Ntan1*$^{-/-}$ mice, which could not deamidate N-terminal Asn (Kwon *et al*., 2000), it has been inferred that there also exists a Gln-specific Nt^{Q}-amidase.

In the present work (Wang *et al*., 2009), we detected the activity of Nt^{Q}-amidase, termed Ntaq1, in mouse tissues, purified Ntaq1 from bovine brains, identified its gene, and began studies of this previously undescribed enzyme (Fig. 1). The sequence of mouse Ntaq1 (Nt^{Q}-amidase) is highly conserved among animals, plants and some fungi, but is dissimilar to sequences of other amidases, including the N-terminal amidases Ntan1 (Nt^{N}-amidase) and Nta1 ($Nt^{N,Q}$-amidase). A *tungus* mutant in the previously uncharacterized *Drosophila melanogaster Cg8253* gene was found to have defective long-term memory (Dubnau *et al.*, 2003). We show here that this *Drosophila* gene encodes the counterpart of mouse Ntaq1. In addition, previous proteomic studies identified ~15 putative protein ligands of an uncharacterized human protein encoded by *C8orf32* ((Lim *et al*., 2006). We show here that C8orf32 is the human Ntaq1 Nt^{Q}-amidase. Remarkably, "high-throughput" crystallographic studies of human proteins have recently solved the crystal structure of C8orf32 (Ntaq1) (Bitto *et al*., 2008). In conjunction with its crystal structure, our site-directed mutagenesis of Ntaq1 indicates that the active site and catalytic mechanism of Nt^{Q}-amidase are similar to those of transglutaminases. Thus, the discovery and study of Nt^{Q}-amidase as a component of the N-end rule pathway (Fig. 1) were "instantly" complemented by a crystal structure of this enzyme, a set of its putative protein ligands, and evidence for its role in memory processes.

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Facilities

Flow Cytometry and Cell Sorting Facility
Genetically Altered Mouse Production Facility
Millard and Muriel Jacobs Genetics and Genomics Laboratory
Monoclonal Antibody Facility
Nucleic Acid and Protein Sequence Analysis Computing Facility
Protein Expression Center
Protein Microanalytical Laboratory

Flow Cytometry and Cell Sorting Facility
Facility Manager: Rochelle Diamond
Faculty Supervisor: Ellen V. Rothenberg
Sorting Operator: Diana Perez
Optics and Maintenance Specialist: Patrick Koen

The Caltech Flow Cytometry/Cell Sorting Facility is located in Kerckhoff 020 and 026. This multi-user facility provides expert assistance and advanced instrumentation to researchers for analyzing and separating various types of cells and micro-organisms according to their measurable properties of light scatter and fluorescence.

The facility is equipped with two research grade flow cytometer cell sorters and one analyzer:

◊ BD FACSAria IIu, capable of analyzing at least nine colors utilizing three lasers (407nm, 488nm, and 633nm), and of carrying out 4-way sorting up to 10,000 cells per second with reliable efficiency and recovery, or 1-way sorting, such as for single-cell cloning, into various cell culture plate configurations. Up to 11 colors could be analyzed on this instrument if additional filters were purchased. This instrument was upgraded this year by the manufacturer at no cost to the facility.

◊ iCyt Mission Technology Reflection 5-laser/9color (UV, 405, 488, 561, and 633nm) cell sorter with one Highly Automated Parallel Sorting (HAPS) module contained in a Baker Sterilguard Advance Biosafety cabinet.

◊ BD FACSCalibur, a four-color analyzer, together with an offline workstation, which are available to researchers for self-service analysis provided that they demonstrate competence to use the instrument or take training provided by the facility.

The facility provides consultation services to all researchers on issues relating to flow cytometry, cell sorting, and cell separation techniques. In addition, the facility makes Treestar's FlowJo off-line analysis program available to its clients through a network license.

This past year the facility provided service to 21 laboratories from the Divisions of Biology, Chemistry and Chemical Engineering, Applied Physics, Geology and Planetary Science, and JPL. In all, the projects of 63 individual users were supported. Eighteen researchers were trained in flow cytometry and the use of the BD FACSCalibur analyzer. Money was received from internal sources to replace our FACSVantage SE with a new iCyt Mission Technology 5-laser cell sorter that was received and installed in March/April of this year. The facility is now training and validating this new instrument which is much more flexible (and as such technically more complicated) than the FACSAria IIu. This new iCyt Mission Technology Reflection Cell Sorter is housed in a Baker Sterilguard III Advance biosafety cabinet that is BSL2 rated, which will now allow us to sort bacteria, yeast, and any human cell lines that have been certified as pathogen free.

Research applications
In the past year, the Facility has been used by multiple groups for diverse applications. This is a representative sample of the projects under way.

Jongmin Nam and Eric Davidson engaged in a major effort to transform experimental technology for high-throughput *cis*-regulatory analysis. Sea urchin embryos are developed from eggs injected with sets of >100 barcoded *cis*-regulatory constructs to be tested, and also a fluorescent marker gene that identifies a given regulatory domain of the embryo. These embryos are then disaggregated and sorted for cells expressing the fluorescent marker. Those of the uncharacterized constructs that are expressing in the marked domain can be identified by PCR or Nanostring® analysis of the enriched barcodes following isolation of the active cells by FACS.

Danielle Brown in the Fraser Group is examining the programming of induced pluripotent stem cells from fibroblasts. She is using flow cytometry to track the dynamics of reprogramming by analyzing the cells expression of transcription factors tagged with fluorescent proteins in combination with phenotypic markers.

Henry Lester's lab has begun preliminary studies using flow cytometry to study Förster resonance energy transfer (FRET) between subunits of nicotinic acetylcholine receptors (nAChRs) and other molecules. These studies exploit the emerging theme that nicotine serves as a pharmacological chaperone for acetylcholine receptor number and stoichiometry. They are also performing fluorescence complementation assays on α-synuclein, which is deeply implicated in Parkinson's disease. Initial experiments suggest that FACS analysis will be useful in mechanistic investigations of these effects.

John Phillips and Peter Dervan have been exploring the inhibition of androgen receptor signaling in hormone refractory prostate cancer cells. They have been looking at growth arrest via cell cycle analysis, annexin V binding, and activated caspase assays.

Katie Brenner in the Frances Arnold Group has studied how cell-cell communication coordinates population behavior in *E. coli* biofilms. She utilized different fluorescent proteins (CFP and YFP) to mark two kinds of bacteria. One type formed biofilms by themselves and the other was unable to form a biofilm by itself. Mixing the two together both made biofilms. Cell sorting of the mixed biofilm into purified, isolated bacteria validated the transfer of the film forming property from one form to another when recultured.

The Mazmanian Group researches the interaction of bacteria and the immune system. They have been sorting CD4+CD45Rb^{high} or CD4+CD45Rb^{low} T cells for injection into recipient mice to assess whether mice treated with a unique carbohydrate molecule Polysaccharide A from *B. fragilis* are protected from inflammatory bowel

disease and colitis. They also make extensive use of the self-service analyzer provided by the facility to assay for Tregs and pro-inflammatory cytokine production.

The Elowitz group is focused on understanding how the signaling pathways and gene regulatory dynamics work together. They are designing new developmental circuit designs using the signals for Notch and Delta regulation during development using fluorescent reporters as a read-out for Notch activation by a plate-bound Delta protein. They continue to use the cell sorting facility to sort newly designed single cells to create stable cell lines of CHO and MDCK cells expressing the fluorescent reporters, mCherry, CFP, and YFP. In addition the lab is exploring the regulatory pathways that maintain mouse embryonic stem cells in their pluripotent state. They perturb the pathway and use the sorter to purify cells and analyze the molecular transitions.

Adrienne H.K. Roeder and Elliot M. Meyerowitz are using flow cytometry to study regulation of the distribution of DNA contents in the cells of the epidermis of *Arabidopsis* sepals. These *Arabidopsis* sepals are a good model system for understanding the formation of a ploidy distribution because cells range in DNA content from 2C to 16C. The Meyerowitz group has identified a cell cycle inhibitor, LGO, which regulates this ploidy distribution. Using flow cytometry, these investigators have shown that loss of *LGO* function results in a shift in the distribution toward lower DNA contents; however, polyploidy is not completely lost. Conversely, overexpression of a cell cycle inhibitor, KRP1, results in a shift in the ploidy distribution toward higher DNA contents, but likewise does not block the formation of cells with lower DNA contents. These results indicate that regulation of the cell cycle by inhibitors controls the ploidy distribution.

Mary Yui of the Rothenberg Group is studying early T cell development in a mouse model of Type 1 diabetes to determine the genetic, molecular and cellular basis of specific T-cell defects that may lead to autoimmune disease in these animals. She utilizes the single cell sorting and multiple fluorescence capabilities of the FACSAria sorter to purify numerically rare early T-cell populations for two main purposes: (1) To study the developmental potential and lineage choices of these precursor cells in a cell co-culture system; and (2) to determine gene expression patterns of these specific early T-cell populations in the context of gene regulatory networks in T cell development.

Jonathan Moore of the Ellen Rothenberg group has been investigating which transcription factors regulate the variable lymphocyte receptor gene of a jawless vertebrate, to see if these factors are shared with those known to be involved in jawed vertebrate immune gene regulation. The flow cytometry facility provides a way to enrich lamprey cells expressing the variable lymphocyte receptor, as well as negative control cells. These cells are used to create nuclear extracts for gel shift experiments, RNA for qRT-PCR analysis, and DNA-protein complexes for chromatin immunoprecipitation experiments.

Three other projects in the Rothenberg lab also depend on high speed, multiparameter cell sorting. Both Deirdre Scripture Adams and Long Li have been studying the effects of reducing the activity of particular transcription factor genes at defined stages of T-cell development *in vitro*. To do this, they initiate differentiation cultures with progenitor cells sorted from murine fetal liver, and then at particular time points re-sort the cells to induce loss of transcription factor function, either by retroviral introduction of shRNA or by retroviral introduction of Cre, to excise a floxed transcription factor gene. They then return the cells to culture for defined periods and re-sort the resulting developmental subsets from normal and control cells, based on 4-6 color staining, to characterize the alterations in RNA expression and further developmental potential. In the third of these projects, Jingli Zhang has initiated a collaboration with the Wold group to develop a comprehensive genome-wide map of the changes in epigenetic markings across the early stages of T-cell development. To do this, she has optimized conditions for *in vitro* differentiation and expansion of early T-cell precursors, and she has been sorting ~10^7 cell subsets representing defined stages. These are then used for chromatin immune precipitation with a range of histone modification- or transcription factor-specific antibodies. These samples are then converted to Solexa sequencing libraries and sequenced to track the dynamics of histone marking and transcription factor binding across the genome through the developmental process.

Martin Budd of the Judy Campbell laboratory is examining the generation of single-stranded DNA at telomeres in *dna2 mre11* mutants as a function of cell cycle position. The hypothesis is the double *dna2 mre11* mutant will be defective in appearance of single-stranded DNA in S phase. Flow cytometry is used to assay the position in the cell cycle as a correlation with the single-stranded DNA.

Victoria Orphan and Abbie Green in the Geological and Planetary Sciences Division are using FACS sorting for high throughput isolation of fluorescently labeled bacterial and archaeal populations and interspecies symbiotic consortia from heterogeneous environmental samples. Pilot studies using the FACSAria have successfully sorted individual microbial populations stained with a single fluorescent tag.

Roberta Hansman and Kaitlyn Lucey in the Alex Sessions Group in the Geological and Planetary Sciences Division are looking at specific populations of marine picoplankton and fresh-water environmental samples based on a combination of size and fluorescence characteristics. While in some cases the autofluorescence of phytoplankton pigments can be used to distinguish between groups, a variety of dyes, nucleic acid stains, and fluorophore-linked probes are required to identify heterotrophic bacteria and phylogenetically-specific populations for sorting for analysis of ^{13}C content using a specialized microcombustion interface attached to an isotope-ratio mass spectrometer.

The James Heath lab relies heavily on the flow cytometry to provide validation of new approaches and biological methods that are currently under development. These include (1) Developing intracellular signaling network hypotheses for helping understand the tumor microenvironment; and (2) Strategies for the multiplexed sorting of CD8+ antigen-specific Tcells for applications that include cancer immunotherapy. Most of the group's developed methods are chemical approaches that are reduced into microfluidics environments. FACS is one of the primary assay techniques for validating their research approaches.

Flow Cytometry/Cell Sorting Facility Publications

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Genetically Engineered Mouse Production Facility
Director; Member of the Professional Staff: Shirley Pease
Cryopreservation and Microinjection: Juan Silva
Embryonic Stem Cell Culture: Simon Webster
Mouse Colony Manager: Jennifer Alex

In June 2005, the Genetically Altered Mouse Core and the Office of Laboratory Animal Resources (OLAR) combined to form the Caltech Laboratory Animal Services (CLAS). CLAS consists of two subdivisions, OLAR, which is headed by Dr. Janet Baer and Genetically Engineered Mouse Services (GEMs) that is headed by Shirley Pease. The purpose of the merger was to refine, streamline and standardize procedures for laboratory animal care and use on campus. GEMs continues to provide microinjection, cryopreservation, re-derivation and tissue culture services. In addition, we offer services in the form of rodent colony management and use, where required, in all animal Facilities.

Gene addition in the mammalian system is accomplished by injecting DNA into the pronucleus of a fertilized egg (Gordon *et al.*, 1980). This is a non-targeted event. 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 (Zijlstra *et al.*, 1989). 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 to produce descendants that are entirely transgenic, resulting from the ES cell contribution to the germline of the chimeric mouse. (The Nobel Prize in Physiology or Medicine was awarded this year to the pioneers of this technology, Mario Capecchi, Martin Evans and Oliver Smithies.) The facility, in collaboration with Anderson, Baltimore, Kennedy, Lester, Patterson, Rothenberg, Simon, Varshavsky and Wold laboratories, has generated multiple transgenic, knockout and knockin mouse strains, amounting to nearly 170 mouse strains. 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 *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. Since the lentiviral vector method was established, 79 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 mouse facility, which operates on restricted access as part of a barrier system designed to safeguard the microbiological status of the animals. A room outside the facility has been allocated by the Division to be used primarily for teaching graduate 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 Engineered Mouse services.

At the merger, a new position was created, that of Colony Manager. An experienced technician has been appointed to the post and is available now to assist investigators with all colony management questions, primarily assisting investigators in making sure their experimental needs are met as economically as possible. GEMs Facility staff are currently working with IMSS to develop software that will assist technicians and investigators in the management of their mice. Amongst its features, this inter-relational system will track the breeding history of each strain and have the ability to generate family trees. The system will also report on production levels for each strain. Users will access the system to enter genotype results and work requests. An electronic signal will be sent to CLAS staff when work requests are made, helping us to manage work requests in a timely manner. The system will be basic but easy to use. We anticipate this will be a very useful animal management tool.

In tissue culture and the use of embryonic stem (ES) cells, the Facility has, in the past, participated in the derivation of new ES cell lines derived from genetically altered mice (see Simon laboratory Annual Report, 2001). This year, the Facility generated over forty new and as yet untested, embryonic stem cell lines, the majority of which are from C57BL/6 mice. This was a by-product of our wish to determine the most efficient approach to deriving such cell lines, since we anticipate that investigators may wish to use ES cells derived from their own genetically altered strains of mouse. Indeed, five such new ES cell lines have been derived for the Rothenberg lab. Several investigators are using these pluripotent cells in research that involves pushing the cells down specific developmental pathways, and also to investigate the incorporation of extraordinarily large pieces of DNA into the mouse genome. The Facility is able to offer investigators a choice between working with ES cells on a 129 background, a C57BL/6 background or an F1 background, which is a mix between these two strains. We are able to manipulate and obtain germline transmission from all these ES cell types. C57BL/6 ES cells provide a significant advantage in that the mutation will be established initially on this well understood genetic background, instead of undertaking a two-year breeding program to reach the same point, having initially established the mutation on a sub-optimal genetic background. Hybrid ES cells have been reported to be useful for their vigor. Unlike ES cells from an inbred background, (e.g., C57BL/6 and 129), it is possible to

derive from hybrid ES cells live pups that are wholly of ES cell origin. Tetraploid embryos, (embryos with twice the normal number of chromosomes), are able to develop and contribute to extra-embryonic membrane cell lineages, but **not** to the development of any fetal tissues. Thus, a tetraploid embryo at blastocyst stage, injected with hybrid ES cells, will result in the production of an animal that is wholly of ES cell origin. ES cells from inbred strains such as C57BL/6 or 129 require a contribution to the developing fetus from the injected host blastocyst itself, for the production of viable pups. We recently established the production and use of tetraploid embryos at Caltech and have our first pups born from their use in combination with hybrid ES cells.

Once a new mouse model has been characterized, it may be cryopreserved, or sent to the Mutant Mouse Resource Center, to be made available to the research community in general. We currently have 99 mouse models cryopreserved. For each line, between 200 and 500 embryos at eight-cell stage have been preserved in liquid nitrogen. There are currently 30,768 embryos frozen in total. We shall continue to preserve embryos from mouse strains. 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.

During 2006, Facility staff received training on the culture of human embryonic stem cells. We are about to establish the culture of H1 and H9 hES cells at Caltech. Initially, our goal will be to expand the cells and to cryopreserve stocks that may be made available to Caltech investigators at a later date. There are already a few investigators in the Biology Division who will wish to use the cells as soon as they can be made available.

Presently, thirteen principal investigators and their postdoctoral fellows or graduate students use GEMs services. In addition to the maintenance of nearly 100 different targeted and non-targeted 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 currently maintain nearly 200 separate strains of mouse. Some of these strains are immune-deficient and require specialized care to protect them from bacteria commonly present 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 investigator ability to obtain reliable experimental results.

Listed below are the names of the eleven principal investigators and their postdoctoral fellows or graduate students who are presently using the transgenic facility.

David Anderson
Ben Deneen, Wulf Haubensak, Christian Hochstim, Walter Lerchner, Li Ching Lo, Agnes Lukaszewic, Sophia Vrontu

David Baltimore
Mark Boldin, Shengli Hao, Lili Yang

Mark Davis - (Chemistry and Chemical Engineering)
Derek Bartlett, Eric Kowel

Ray Deshaies
Narimon Harnapour

Michael Elowitz
Fred Tan, Julia Tischler

Scott Fraser
David Koos, Carol Readhead, Nicholas Plachta

Mary Kennedy
Eduardo Marcora, Andrew Medina-Marino, Leslie Schenker, Laurie Washburn

Henry Lester
Purnima Deshpande, Princess Imoukhuede, Herwig Just, Raad Nashmi

Paul Patterson
Ben Deverman, Ali Koshnan, Natalia Malkova, Limin Shi, Stephen Smith

Ellen Rothenberg
Deirdre Scripture-Adams, Chase Tydell, Mary Yui, Mark Zarnegar

Melvin Simon
Valeria Mancino, Sang-Kyou Han

Alexander Varshavsky
Christopher Brower, Jun Sheng

Barbara Wold
Brian Williams

References

Gordon, J.W., Scangos, G.A., Plotkin, D.J., Barbosa, J.A. and Ruddle, F.H. (1980) *Proc. Natl. Acad. Sci. USA* **77**(12):7380-7384.

Lois, C., Hong, E.J., Pease, S., Brown, E.J. and Baltimore, D. (2002) *Science* **295**:868-872.

Zijlstra, M., Li, E., Sajjadi, F., Subramani, S. and Jaenisch, R. (1989) *Nature* **342**(6248):435-438.

Millard and Muriel Jacobs Genetics and Genomics Laboratory
Director: Igor Antoshechkin
Staff: Brandon King, Vijaya Kumar, Lorian Schaeffer

Support: The work described in the following research reports has been supported by:
Millard and Muriel Jacobs Family Foundation

Summary: The goal of the Millard and Muriel Jacobs Genetics and Genomics Laboratory in the Division of Biology is to provide a suite of cutting edge genomic research tools to all interested Caltech scientists, with an emphasis on large-scale gene expression profiling and high throughput sequencing (HTS). The Laboratory 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 platforms. HTS is also used for gene expression analysis (RNA-Seq), as well as for other applications such as *de novo* genome sequencing, mutation discovery, genome-wide studies of protein-DNA interactions and epigenetic modifications, etc. During the period of this report, the Laboratory continued to provide support for genomics research to the Caltech community at large, including groups from the Division of Biology, the Division of Chemistry and Chemical Engineering and the Division of Engineering and Applied Science.

Research Support
Division of Biology
The Laboratory worked with the groups of Professors David Anderson, David Baltimore, Eric Davidson, Elliot Meyerowitz, Angela Stathopoulos, Michael Elowitz, Grant Jensen, Mary Kennedy, Ellen Rothenberg, Sarkis Mazmanian, Paul Sternberg and Barbara Wold. Microarray experiments were performed using Affymetrix, Agilent and in-house manufactured microarrays on various organisms (human, mice, *Drosophila*, *C. elegans*, *Arabidopsis*, *S. cerevisiae*) for a wide variety of research projects, such as studies of the influence of dietary restriction on the lifespan of *Drosophila*, transcriptional control of developmental arrest in *C. elegans*, regulation of mRNA degradation in *Arabidopsis* and many others.

High throughput sequencing experiments were carried out utilizing Illumina Genome Analyzer platform to address a large number of biological problems including transcriptome analysis during flower development in *Arabidopsis* (Elliot Meyerowitz), identification of transcription factor targets in *Drosophila* (Angela Stathopoulos), sea urchin genome sequencing and transcriptome analysis (Eric Davidson), studies of genetic networks in mouse myoblasts (Barbara Wold), *de novo* sequencing of nematode genomes (Paul Sternberg), etc.

Division of Chemistry and Chemical Engineering
The Laboratory worked with the groups of Professors Linda Hsieh-Wilson, Judith L. Campbell, Peter Dervan and William A. Goddard. The Laboratory manufactured carbohydrate microarrays for the Hsieh-Wilson group, performed Affymetrix gene expression profiling experiments for the laboratories of Peter Dervan and William A. Goddard, used Illumina high throughput sequencing to screen for mutations in *S. cerevisiae* for the group of Judith L. Campbell.

Division of Engineering and Applied Science
The Laboratory performed genome sequencing and transcriptome analysis of multiple *Treponema* strains for the laboratory of Jared R. Leadbetter using Illumina HTS technology.

Infrastructure and capabilities
The Laboratory is well equipped to perform gene expression analyses using multiple microarray platforms, including Affymetrix and Agilent. The Laboratory is also capable of manufacturing custom microarrays that have been used for projects ranging from gene expression analysis in *Arabidopsis*, to studies of glycosaminoglycan-protein interactions and of the dynamics of DNA hybridization in real time. The major equipment used in microarray work include Affymetrix GeneArray scanner and fluidics station, Agilent and GenePix (Molecular Devices/Axon Instruments) scanners, MicroGrid II array printer (BioRobotics), BioAnalyzer (Agilent Technologies), LightCycler 480 real-time qPCR system (Roche), hybridization ovens, etc. High throughput sequencing experiments are performed using two Illumina Genome Analyzer IIx sequencers. Library amplification and flowcell preparation are done on a Cluster Generation station. The extensive computational infrastructure that the Laboratory has developed allows us to carry out primary data analyses, such as the extraction of sequence data from raw images generated by the Genome Analyzers and provides sufficient storage capacity for the vast amounts of data generated by these experiments. We also are capable to perform some computation-intensive secondary analyses, such as genome assembly, identification of binding sites of DNA-interacting proteins, transcriptome analysis, etc. Resolver software suite (Rosetta Biosoftware) is available to our users for the analysis of microarray-based gene expression data. The system is accessible through client stations using a web-based interface. We also have at our disposal additional microarray and HTS software tools and analysis packages, both public and commercial.

Publications acknowledging the laboratory:

Baugh, L.R., Demodena, J. and Sternberg, P.W. (2009) RNA Pol II accumulates at promoters of growth genes during developmental arrest. S*cience* **324**(5923):92-94.

Jiao, Y., Riechmann, J.L. and Meyerowitz, E.M. (2008) Transcriptome-wide analysis of uncapped mRNAs in *Arabidopsis* reveals regulation of mRNA degradation. *Plant Cell* **20**:2571–2585.

Muzikar, K.A., Nickols, N.G. and Dervan, P.B. (2009) Repression of DNA-binding dependent glucocorticoid receptor-mediated gene expression. *Proc. Natl. Acad. Sci. USA* **106**:16598-16603.

Zid, B.M., Rogers, A.N., Katewa, S.D., Vargas, M.A., Kolipinski, M.C., Lu, T.A., Benzer, S. and Kapahi, P. (2009) 4E-BP extends lifespan upon dietary restriction by enhancing mitochondrial activity in *Drosophila*. *Cell* **139**(1):149-160.

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), ascites fluid or other related tissue culture services. We also produce polyclonal ascites Abs by immunizing mice with antigen and then inducing the mice with sarcoma cells to obtain high titer, polyclonal ascites fluid. This method can provide 10-18 ml polyclonal ascites fluid per mouse while using small amounts of antigen. In addition to these services, the Facility also conducts research on the development of novel immunological techniques.

In its service capacity, the Facility produced Abs for the following groups during the past year. The Dealwis lab (Case Western University) obtained mAbs against the N-terminus of amyloid-beta peptide. Transmembrane Bioscience obtained mAbs and polyclonal ascites against recombinant *rickettsia prowazekii* and Rhesus proteins expressed through a proprietary membrane protein overexpression system. They also obtained mAbs and polyclonal ascites against *odontoglossum ringspot* virus surface glycoprotein, *coxiella burnetii* OMP A (intracellular Q-Fever pathogen). The Patterson lab obtained mAbs against wild-type huntingtin exon 1. The Zipursky lab (UCLA) obtained mAbs against two isoforms of *Drosophila* Dscam. The Chan lab obtained mAbs against Mfn1 (mitofusins, transmembrane GTPases embedded in the outer membrane of mitochondria) and OPA1 (mitochondria protein localized at the intermembrane space). The Wold lab obtained mAbs against human transcription factors NRSF N250, NRSF C250, and GABPA N180.

We are currently working with the following groups: Wold lab is trying to obtain mAbs against human transcription factors NRSF N150, FOX P2 Ag1, FOX P2 Ag2, and GABPA 200-310 inclusion body. The Bjorkman lab is trying to obtain mAbs against Ferritin. The Transmembrane Bioscience company is trying to obtain mAbs against Bartonella, Orientia tsutsugamushi, Coxiella AdaA, and ESAT 6 (antigen from Mycobacterium tuberculosis).

Publications

Gardberg, A.S., Dice, L.T., Pridgen, K., Ko, J., Patterson, P.H., Ou, S., Wetzel, R. and Dealwis, C. (2009) Structures of Aβ-related peptide-monoclonal antibody complexes. *Biochem*. In press.

Gardberg, A.S., Dice, L.T., Ou, S., Rich, R.L., Helbrecht, E., Ko, J., Wetzel, R., Myszka, D.G., Patterson, P.H. and Dealwis, C. (2007) Molecular basis for passive immunotheraphy of Alzheimer's disease. *Proc. Natl. Acad. Sci. USA* **104**:14659-15664.

Publications utilizing work performed by the Facility

Jeon, M., Nguyen, H., Bahri, S.M. and Zinn, K. (2008) Redundancy and compensation in axon guidance: genetic analysis of the *Drosophila* Ptp10D/Ptp4E receptor tyrosine phosphatase subfamily. *Neural Develop.* **3**:3.

Kurusu, M., Cording, A., Taniguchi, M., Menon, K., Suzuki, E. and Zinn, K. (2008) A screen of cell-surface molecules identifies leucine-rich repeat proteins as key mediators of synaptic target selection. *Neuron* **59**:972-985.

Legleiter, J., Lotz, G.P., Miller, J., Ko, J., Ng, C., Williams, G.L., Finkbeiner, S., Patterson, P.H. and Muchowski, P.J. (2009) Monoclonal antibodies recognize distinct conformational epitopes formed by polyglutamine in a mutant huntingtin fragment. *JBC*. In press.

Menon, K.P., Andrews, S., Murthy, M., Gavis, E.R. and Zinn, K. (2009) The translational repressors nanos and pumilio have divergent effects on presynaptic terminal growth and postsynaptic glutamate receptor subunit composition. *J. Neurosci.* **29**:5558-5572.

Ralston, K.S. and Hill, K.L. (2008) The flagellum of *Trypanosoma brucei*: new tricks from an old dog. *Intl. J. Parasitol.* **38**:869-884.

Ralston, K.S., Kabututu, Z.P., Melehani, J.H., Oberholzer, M. and Hill, K.L. (2009) The *Trypanosoma brucei* flagellum: moving parasites in new directions. *Ann. Rev. Microbiol*. In press.

NUCLEIC ACID AND PROTEIN SEQUENCE ANALYSIS COMPUTING FACILITY
Supervisor: Professor Stephen L. Mayo
Staff: David R. Mathog

The Sequence Analysis Facility (SAF) provides software, computers, and support for the analysis of nucleic acid and protein sequences. Current SAF hardware consists of a Sun Netra running Solaris, a small 20 node Beowulf cluster, a file server, a 26 ppm duplexing laser printer, and a 16 ppm duplexing color laser printer. The PCs that comprise the "structure analysis facility" are also located in our facility.

Most common programs for sequence analysis are available on the primary server http://saf.bio.caltech.edu/. 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 EMBOSS-Explorer web interfaces. Other programs, custom written programs, or special databases are available on request. The PCs support hardware stereo under both Linux and Windows. Under Linux the programs Coot, O, PyMol, Molscript, CCP4, and Delphi are available. Under Windows WinCoot, Swiss PDB Viewer, O, PyMol, POVray, and various drawing and animation programs may be used. The searchable documentation for these programs is available on the SAF web server. The lecture notes and homework from the introductory course "Fundamentals of Sequence Analysis" are also available on the SAF web server. A web interface allows common compute intensive jobs to run locally on the SAF Beowulf cluster. BLAST executes in a parallel mode so that searches complete faster than they do at the NCBI server. An enhanced parallel HHMER server offers the full set of HMMER programs plus the unique ability to search any of the installed BLAST databases with an HMM. Personal BLAST sequence databases up to 50Mb may be uploaded and searched. The multiple sequence alignment programs T-COFFEE, POA, Probcons, MAFFT, and Muscle are also available. Traces from any DNA sequencing facility may be uploaded and analyzed. The SAF also distributes these site licensed programs for PCs and Macs: DNASTAR, Gene Construction Kit, and, ChemSketch.

Protein Expression Center
Supervisor: Barbara J. Wold
Director: Jost G. Vielmetter
Faculty Advisors: Pamela J. Bjorkman, Mary B. Kennedy
Staff: Michael Anaya, Chris P. Foglesong, Inderjit K. Nangiana

The Protein Expression Center (PEC) was established in 1996 to provide protein expression and purification mostly for Caltech researchers, but also for outside clients. The expression systems currently used in the center are: bacterial expression of soluble and insoluble proteins; expression using the insect cell based baculovirus system; and transient expression using mammalian (HEK293) cells in suspension culture.

Protein expression optimization experiments have become a routine to ensure optimal and efficient expression in the baculovirus and mammalian expression systems. This is critical to ensure high and consistent protein yields and as a result we have increased our rate of success with the expression projects coming to our center.

In addition the PEC is equipped with a Biacore T100 instrument and offers support including hands-on training in the application of Surface Plasmon Resonance (SPR)-based measurements of bio-molecular interactions on the Biacore Instrument. The interest and use of this instrument has steadily increased since its acquisition and has become a very valued asset in the Caltech research community for the study of bio-molecular interactions.

We continued to produce active human antiviral antibodies and engineered antibody derivatives and viral glycoprotein antigens (influenza and HIV) in the context of a DARPA funded project (Steven Mayo) and the Engineering Immunity project (Pamela Bjorkman) resulting in co-authorship of a publication (see below) and at least 12 publications acknowledging the PEC in 2008/2009.

We made significant progress in the production of "CHIP-able" mAbs for the ENCODE project in collaboration with Barbara Wold's research group. "CHIP-able" mAbs are monoclonal antibodies capable of genome-wide extraction and characterization of transcription factor specific DNA control fragments. We have developed a production pipeline to generate the transcription factor antigens for the generation of the antibodies using mice and camel antibodies and screen for candidate antibodies using a new liquid handling robot that was acquired by the Steve Mayo group and partly, by the Wold group. As a proof of concept we have so far been able to generate "CHIP-able" mAbs against the human transcription factor NRSF.

Publication

West, Jr., A.P., Galimidi, R.P., Foglesong, C.P., Gnanapragasam, P.N.P., Klein, J.S., Suzuki, M., Tiangco, N.E., Vielmetter, J. and Bjorkman, P.J. (2009) Design and expression of a dimeric form of the anti-HIV antibody 2G12 with increased neutralization potency. *J. Virology* **83**:98-104.

Protein Microanalytical Facility (Ppmal)
Director: Jie Zhou
Faculty Advisor: Professor Mary Kennedy
Associate Biologist: Felicia Rusnak

ACTIVITY

Mass spectrometry of large biomolecules and small organic molecules
Proteomics (In-gel enzymatic protein digestion; LC/MS/MS and data base search)
Protein (Edman) chemical sequencing
Development of Os-based IMAC column for enrichment of specific peptides

EQUIPMENT

Quadrupole time-of-flight mass spectrometer (ABI QstarXL)
Triple quadrupole mass spectrometer (MDS Sciex API 365)
MALDI-TOF mass spectrometer (ABI Voyager de.str)
Capillary Protein sequencer (Procise cLC, ABI 492)
HPLC nanoflow, 2D (Eksigent)
HPLC (ABI microbore 140D pump, PE UV monitor)
MASCOT server

NEW DEVELOPMENT

We have been working on the development of Osmium-based IMAC (immobilized metal-ion affinity chromatography) column. We found that cross-linked $[Os(dmebpy)_2Cl]^{+/2+}$-derivatized copolymer of acrylamide and vinylimidazole could be used as IMAC column packings for selective adsorption of Arg, Lys N-terminus, histidine- and tryptophan-containing peptides at near-neutral pH conditions. The adsorbed peptides are bump eluted at acidic conditions of pH <3.5 for immediate electrospray mass analysis. The hybrid column configuration of Osmium-complexed polymer and C_{18} packings has been employed for second dimension reverse-phase separation after adsorbed peptides are eluted onto the C_{18} column. Intact protein myoglobin has also been successfully adsorbed onto the Os-complexed polymer column, but it eluted with a broad time window. Those initial data will be submitted for publication. The new column has the potential to help identifying Arg N-terminus proteins, which play key role in N-end rule pathways.

SERVICES

During the first six months of fiscal 2008 PPMAL interacted with 11 laboratories. Samples were analyzed from the Division of Biology, and Chemistry and Chemical Engineering (see list). A total of 1057 samples were analyzed. In addition to our work for campus faculty and staff, work was also performed for Nanogen and Harvard Children's Hospital (149 samples analyzed).

MASS SPECTROMETRY

In six months, 1034 proteins, peptides, oligonucleotides, and carbohydrates, small organic compounds have been analyzed. Our off-campus activity recorded 148 samples.

PROTEOMICS

For the period covering this report, 23 digests had been analyzed. This extrapolates to an annual throughput of about 46 samples.

PROTEIN AND PEPTIDE SEQUENCE ANALYSIS

The lab has sequenced proteins and peptides for 38 cycles. The average number of residues per sample was about 4.

PROTEIN MICROANALYTICAL FACILITY (PPMAL) - OCTOBER, 2008 – MARCH, 2009
LIST OF FACILITY USERS AND ACTIVITY

On-Campus	Samples	Mass Spectrometry	Proteomics	Sequences	Sequenced Cycles
Barton	530	530			
D. Chan	4	1		3	14
Gray	76	76			
Grubbs	1	1			
Heath	228	228			
Hsieh-Wilson	137	137			
Elowitz	2		2		
Mayo	28	28			
Patterson	5		5		
Tirrell	7	32		7	21
Varshavsky	14		14		
TOTALS	**1057**	**1034**	**23**	**10**	**35**
Off-Campus					
Calando Pharmaceuticals	1	1			
Harvard Medical School	1			1	3
Nanogen	148	148			
TOTALS	**149**	**148**		**1**	**3**

Graduates

DOCTOR OF PHILOSOPHY - 2009
DIVISION OF BIOLOGY

MEGHAN SARA ADAMS, PH.D.
BIOLOGY
B.S., University of California, Los Angeles 2000
Thesis: Uncovering Molecular Properties of Neural Crest Cells.

MEGAN JO ANDERSON, PH.D.
Biochemistry and Molecular Biophysics
B.A., B.S., University of California, San Diego 2003
Thesis: Microfluidics-Based Strategies for Protein Crystallography.

JESSICA ROSE ESCOBEDO
INTEGRATED NEUROBIOLOGY
B.A., Kenyon College 2000
Thesis: Investigating Moral Events: Characterization and Structure of Autobiographical Moral Memories.

SEAN GORDON, PH.D.
Biochemistry and Molecular Biophysics
B.S., University of Kansas 2001; M.Sc., Max Planck Research School for Molecular Biology 2003
Thesis: Hormone and Gene Feedback during Development and Regeneration in *Arabidopsis thaliana*.

JANE IGOR KHUDYAKOV, PH.D.
Biology
B.S., University of North Carolina at Chapel Hill 2003
Thesis: Role of Bmi-1 in Epigenetic Regulation during Early Neural Crest Development.

JOSHUA S. KLEIN, PH.D.
BIOCHEMISTRY AND MOLECULAR BIOPHYSICS
A.A., College of Maine 2000; B.A., University of California, Berkeley 2002
Thesis: investigations in the Design and Characterization of HIV-1 Neutralizing Molecules.

LOUISA M. LIBERMAN, PH.D.
BIOLOGY
B.A., Mount Holyoke College 2002
Thesis: Regulation of Neurogenic Ectoderm Specification in *Drosophila melanogaster*.

ANDREW MEDINA-MARINO, PH.D.
BIOLOGY
B.A., Swarthmore College 2000; M.S., California Institute of Technology 2003
Thesis: Construction and Initial Characterization of the Densin Knockout Mouse.

JULIEN MUFFAT, PH.D.
CELLULAR AND MOLECULAR NEUROBIOLOGY
D.E.A., Ecole Normale Supérieure 2001; Licence 1998; Maîtrise 1999
Thesis: Role of Apolipoprotein D and Its Homologs, in Normal and Pathological Aging, in *Drosophila melanogaster*.

ELIZABETH ANN OTTESEN, PH.D.
BIOLOGY
B.A., Grinnell College 2002
Thesis: The Biology and Community Structure of CO_2-Reducing Acetogens in the Termite Hindgut.

ALEXA MARI PRICE-WHELAN, PH.D.
BIOLOGY
B.A., Barnard College, Columbia University 2002
Thesis: Physiology and Mechanisms of Pyocyanin Reduction in *Pseudomonas aeruginosa*.

ADRIAN EDWARD RICE, PH.D.
BIOCHEMISTRY AND MOLECULAR BIOPHYSICS
B.S., University of Washington 2001
Thesis: Biophysical and Cell Biological Studies Characterizing the Vertebrate Iron Exporter Ferroportin.

TED OLIN RIRIE, PH.D.
BIOLOGY
B.S., Brigham Young University 1999
Thesis: A Multipartite Approach to Mapping the Gene Network Directing *Caenorhabditis elegans* Vulval Organogenesis.

ADELINE SEAH, PH.D.
BIOLOGY
B.S., University of California, Berkeley 2001
Thesis: EGF, WNT & HOX Interactions during Patterning of *Caenorhabditis elegans* Equivalence Groups.

CELIA EENJING SHIAU, PH.D.
BIOLOGY
B.S., University of California, Davis 2003
Thesis: Formation of Cranial Sensory Ganglia: Role of Neural Crest-Placode Interactions, Slit-Robo and Cadherins.

AMBER L. SOUTHWELL, PH.D.
CELLULAR AND MOLECULAR NEUROBIOLOGY
B.S., (Biochemistry), The University of Texas at Austin 1999; M.S., (Molecular Biology), 2001
Thesis: Intrabodies as Theraputics for Huntington's Disease.

DEVIN TRENT TESAR, PH.D.
BIOLOGY
B.A., B.S., University of Missouri-Columbia 2001
Thesis: Investigations of the Mechanisms of Receptor-Mediated Immunoglobulin Transport in Mammals and Birds.

BACHELOR OF SCIENCE, BIOLOGY - 2009

Yezdan Sher Hadi Badrakhan
Tania Banerji
Helen Maria Bermudez
Omer Durak
Erin Paul Flagin*
Calyani Ganesan
Nicholas Scott Goeden
Ellen Hsu, Biology and English
Kristen Kozak
Jerry G. Kwong
Jinwoo Lee*
Lauren Lee
Daniel Leighton
Chen Yee Liaw*, Biology and Business Economics and Management
Hondau Peter Lui*
Yvonne Pao
Soyoung Park*
Corinne Pender*
David Jacob Rosenman*
Ilya Y. Shadrin*
Susan Qi Shen*, Biology and English
Christina Vicky Theodoris*
Harish Vasudevan*
Natalie Dawn Vernia*
Tyler James Volkoff*
Yang Yang*
Calvin Ga Yu
Angela Zah, Biology and English

*Students whose names are followed by an asterisk are being graduated with honor in accordance with a vote of the faculty.

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American Heart Association
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Arnold and Mabel Beckman Foundation
Arrowhead Grubstake Fund
Atazoa Ltd.
Autism Speaks Foundation

BAE Systems/ARL
Balzan Foundation
Beckman Fellowship
Beckman Institute
Beschorman Memorial Fund
Betty and Gordon Moore Fellowship
Anne P. and Benjamin F. Biaggini Professorship of Biology
Bill and Melinda Gates Foundation
Binational Science Foundation
Bing Professorship in Behavioral Biology
Biological Sciences Initiative
Ethel Wilson and Robert Bowles Professor of Biological Sciences
James G. Boswell Foundation
James G. Boswell Professor of Neuroscience
Donald Bren Foundation
Bren Professorship of Biology

California Institute of Regenerative Medicine (CIRM)
Callie McGrath Charitable Trust
Caltech Brain Imaging Center Discovery Grant
Caltech Center for Biological Circuits Design
Caltech President's Fund
Camilla Chandler Frost Fellowship
Cancer Research Fund of the Walter Winchell-Damon Runyon Foundation
Carl Zeiss Jena
Norman Chandler Professorship in Cell Biology
Charles B. Corser Fund
Christopher Reeve Foundation
Cline Neuroscience Discovery Grant
Colvin Fund for Research Initiatives in Biomedical Sciences
Crohn's and Colitis Foundation of America
Croucher Foundation
Albert and Kate Page Crutcher

Damon Runyon Cancer Research Fund
Damon Runyon-Walter Winchell Fund
Dana Foundation
David and Lucile Packard Foundation
Allen and Lenabelle Davis Professorship of Biology
Defense Advanced Research Projects Agency (DARPA)
Della Martin
Della Martin Foundation
Department of Defense (National Security Science and Engineering Faculty Fellowship)
Department of Energy (DOE)
DNA Sequencer Patent Royalty Funds
Donald E. and Delia B. Baxter Foundation

Edelman Discovery Fund
Edelman Foundation
Edelman L.B. Discovery Foundation
Elizabeth Ross Fellowship
Ellison Medical Foundation
ERATO
European Molecular Biology Organization (EMBO)
Evelyn Sharp Fellowship

John and Ellamae Fehrer Endowed Biomedical Discovery Fund
Ferguson Fund for Biology
Lawrence L. and Audrey W. Ferguson
Fletcher Jones Foundation
G. Louis Fletcher
Fling Charitable Trust
Foundation for the NIH
Foundation for NIH Research/Bill and Melinda Gates Foundation
Frank P. Hixon Professorship
French Association for Cancer Research
John Douglas French Alzheimer's Foundation
Ms. Laura Frlan
Mrs. Camilla C. Frost

Louis A. Garfinkle Memorial Laboratory Fund
Gimbel Discovery Fund
Gimble Neuroscience Fund

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
Grubstake Presidents Fund

William D. Hacker Trust
Hanson Fund
Lawrence A. Hanson, Jr. Professorship of Biology
Thomas Hartman Fund for Parkinson's Research, Inc.
The Helen Hay Whitney Foundation
Hicks Fund for Alzheimer Research
Hixon Professorship of Psychobiology
Dr. Norman Horowitz
House Ear Institute
Howard Hughes Medical Institute
Human Frontiers Science Program (HFSP)
Huntington Hospital Research Institute

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Life Sciences Research Foundation Fellowship supported by the Howard Hughes Medical Institute
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Moore Foundation
Moore Fund
Moore Foundation
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NARSAD
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National Aeronautics and Space Administration (NASA)
National Alliance for Research on Schizophrenia and Depression
National Cancer Institute
National Eye Institute
National Heart Lung and Blood Institute
National Human Genome Research Institute
National Institute for Biomedical Imaging and Bioengineering
National Institute of Child Health and Human Development
National Institute of Diabetes and Digestive and Kidney Diseases
National Institute of General Medical Science
National Institute of Mental Health, NRSA, USPHS
National Institute of Neurological Disorders and Stroke
National Institute on Aging
National Institute on Deafness and Other Communication Disorders
National Institute on Drug Abuse
National Institutes of Health, BIST, DE, NHGRI, NIDCR, NINDS, USPHS, NIGMS - OK
National Research Service Award, National Institutes of Health
National Science Foundation (NSF)
Norman & Annemarie Davidson Fund for Research in Biology
Norman Chandler Professorship in Cell Biology
Norman Davidson Lectureship
Norman W. Church Fund

The Packard Foundation
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Ernest D. Zanetti Fund

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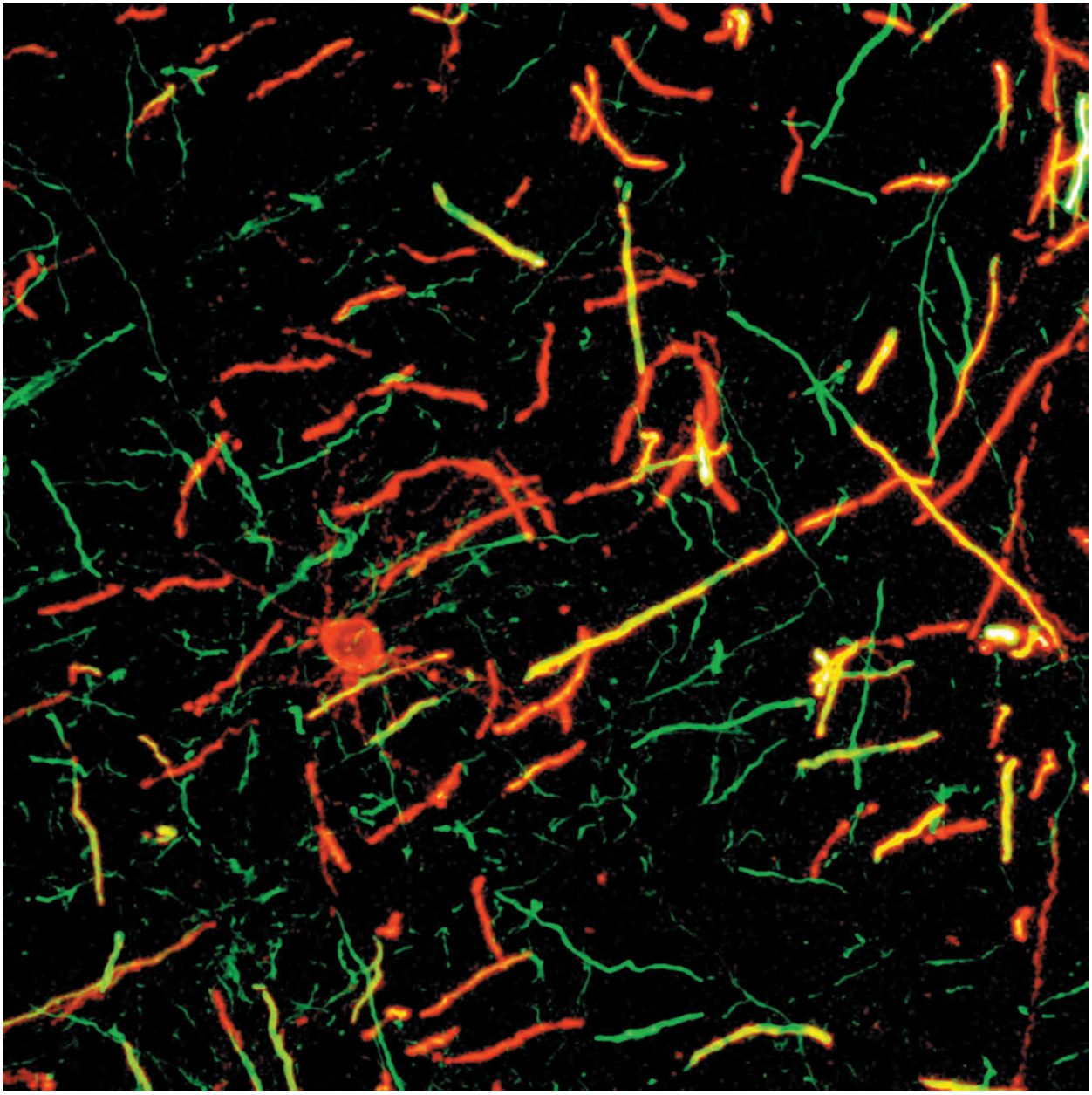

This is an image of an oligodendrocyte (red) in the brain contacting axons (green) provided by Ben Deverman in the Paul Patterson laboratory.
See abstract 88.