Energetics, Disparities, and Lifespan: A Unified Hypothesis

Awardee: David B. Allison
Award: Transformative Research Award
Awardee Institution: University of Alabama at Birmingham

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Much evidence indicates that the evolution and ontogeny of lifespan at the species and individual level, the energetic control of the organism in its environment, the storage of metabolizable energy as body fat, and socioeconomic disparities within populations all may be intricately related. Yet the nature of these interrelations is poorly understood. Fundamental questions remain open as to why organisms age and what controls the rate at which they do so. Subsidiary questions such as why caloric restriction leads to increased lifespan and why lower socioeconomic status is related to obesity in developed countries also remain unanswered. Herein, we propose a unified model informed by evolutionary thinking about life strategies which integrates these phenomena. In this model, aging (or more precisely, senescence) is not something that passively happens as the result of environmental insults or from metabolizing energy, but is something organisms may actively regulate. That is, mortality rate and the rate of aging are seen (partially) as phenomena that may be regulated internally, e.g., the control of body temperature by homeotherms in which the regulated state is responsive to perceptions about the energetic state of the environment. In our model, it is perceptions of the energetic security of the environment that are a key factor in linking these phenomena. We have elaborated this theory and from it have derived seven specific hypotheses, each being tested in a different experiment using model organisms. We have assembled a team of the world's leading researchers, who have begun conducting these seven experiments, with follow-up work to study a priori specified causal mechanisms involving internal clock regulation. We will also conduct exploratory work to reveal new candidate mechanisms that integrate senescence, energy storage, and health disparities. Although very early in our lifespan studies, emerging data are supportive of the hypotheses of effects of various environmental and social conditions on aging and energetics. Results of this work will have profound implications for our understanding of the nature of aging, health disparities, and obesity. Maximizing quality of life for citizens from all social strata and all ages requires an understanding of the mechanisms that lead to disparities in health outcomes, differences in body fat levels, and rates of aging in our increasingly older population. We anticipate the results of these studies will reveal mechanisms that will inform future efforts towards increasing the number of healthy older persons and reducing health disparities.

Highly Evolved Brain Circuits in Primates: Molecular Vulnerabilities for Disease

Awardee: Amy F. T. Arnsten
Award: Pioneer Award
Awardee Institution: Yale University

Disorders of higher cognition such as Alzheimer's disease (AD) and schizophrenia are a tremendous emotional and financial burden on our society, and the costs of AD will only escalate as our society grows increasingly older. These disorders primarily afflict the highly evolved association cortices, with little effect on the primary sensory cortices. What makes the association cortices so vulnerable? Our Pioneer Award will test the hypothesis that the molecular signaling pathways modulating higher cortical connections have evolved to be fundamentally different from those found in the evolutionarily older, sensory cortices, and that dysregulation of these signaling pathways following genetic or environmental insults predisposes these higher circuits to dysfunction and degeneration, e.g., through hyperphosphorylation of tau. This work must be done in nonhuman primates, as rodents do not have higher association cortices. Our data have revealed that primate prefrontal association circuits show evidence of feedforward Ca^{2+}-cAMP-PKA signaling and Ca^{2+}-cAMP-regulated K^+ channels near their network connections. These pathways normally serve to gate inputs, coordinate cognitive and arousal states, and enhance mental flexibility. However, these actions require precise regulation, and even small insults to regulatory processes impair cognition, and may also increase risk for degeneration. A striking number of regulatory proteins in these synapses are genetically altered in schizophrenia, and show changes with advancing age. We hypothesize that primate cortical circuits will have differing sensitivities to Ca^{2+}-cAMP signaling based on their evolutionary status, with highly evolved association cortices being most responsive to Ca^{2+}- and cAMP-K^+ actions, and primary sensory cortex being least responsive. The research will study the nonhuman primate cortex using two complementary methods: (1) multiple-label immunoelectron microscopy (immunoEM) to reveal the constellations of interacting Ca^{2+}-cAMP-related proteins near cortical-cortical synapses, with focus on proteins linked to disease; and (2) physiological recordings coupled with iontophoretic application of drugs to observe physiological interactions and responsiveness to elevated Ca^{2+}-cAMP signaling as monkeys perform higher cognitive tasks. Finally, we will compare young adult to aged cortices to see if there is evidence of Ca^{2+}-cAMP dysregulation and abnormal tau phoshorylation in the aged association cortex, but not in the aged sensory cortex. We hope that this research will transform our view of cognitive disorders, revealing key vulnerabilities that will provide informed therapeutic targets for prevention and treatment of these crippling, complex diseases.

Systems Analysis Reveals Regulatory Pathways Maintaining the Self-Renewal State of Human Embryonic Stem Cells

Awardee: Ipsita Banerjee
Award: New Innovator Award
Awardee Institution: University of Pittsburgh

Co-authors: Shibin Mathew, Sankaramanivel Sundararaj, Hikaru Mamiya
Co-authors' Institution: University of Pittsburgh

Propagation of human embryonic stem cells (hESCs) is a critical first step before inducing lineage-specific differentiation into desired cell and tissue types. The process of self-renewal differentiation is under the control of complex and non-linearly interacting signalingpathways. The insulin-mediated PI3K/AKT pathway has been identified to be critical in maintaining self-renewal and influencing differentiation of hESCs. It is known that the levels of the molecule p-AKT correlate well with self-renewal (pAKT ON) and differentiation (pAKT OFF). However, there is less information on specific regulatory pathways that maintain the level of pAKT. The PI3K pathway involves several regulatory modules; namely,(i) insulin receptor activation and trafficking;(ii) receptor-mediated activation of intracellular IRS1/PI3K/AKT;(iii) negative feedback by serine phosphorylation of IRS1 by molecules like PKC-ζ (iv) negative regulators like PTEN, SHIP, and PTP; and (iv) positive feedback by AKT.

We have developed an integrated experimental and systems analysis technique to analyze a population of self-renewing hESCs. The dynamics of the key components of the PI3K/AKT pathway in hESCs was first analyzed by experimentally stimulating H1 cells with insulin after growth factor starvation. The dynamics werethen compared to *in silico* simulations ofa detailed mechanistic model of the insulin-mediated PI3K/AKT pathway. The model was analyzed using global sensitivity analysis (GSA) to identify key interactions in the pathway that affect the levels of p-AKT. We utilized a computationally efficient algorithm called random sampling-high dimensional model representation (RS-HDMR) to capture global sensitivity between the large numbers of model parameters.

The negative feedback and regulators of the pathway were identified to be more sensitive regulators of the pAKT levels. We validated our prediction by targeted perturbation of the pathway, where it was found that inhibition of negative feedback by p-PKC-ζ increased p-AKT levels considerably. Thus our detailed mechanistic model and experimental analysis identified an important role of negative feedback to maintain the PI3K/AKT pathway in hESCs under homeostatic conditions, which was previously unappreciated.

RNA Exosome Regulated, Divergently Transcribed Loci are

AID Target Sites Genome-Wide

Awardee: Uttiya Basu
Award: New Innovator Award
Awardee Institution: Columbia University

Co-authors: Evangelos Pefanis, Jiguang Wang, Gerson Rothschild, Jaime Chao, Raul Rabadan, Aris Economides
Co-authors' Institutions: Columbia University, Regeneron Pharmaceuticals

Prior to the discovery of non-coding RNA (ncRNA) as a major subclass of eukaryotic genome regulators, the presence of noncoding germline transcripts in the immunoglobulin (Ig) locus had already attracted the attention of many molecular biologists and immunologists. Accumulating over the last four decades, ample evidence had unequivocally established that the synthesis of long noncoding germline transcripts in the Ig locus play a pivotal role in recruiting B cell-specific DNA mutator factors recombination activation genes (RAG-1 and RAG-2) and activation-induced cytidine deaminase (AID) to their target DNA sequences. A major part of the mammalian genome has the potential to express ncRNA. From work performed in our laboratory and elsewhere we know that the 11 subunit RNA exosome complex is the main source of cellular 3'->5' RNA exoribonucleolytic activity and can potentially regulate the mammalian noncoding transcriptome. To evaluate the role of ncRNA processing in the Ig locus we generated mouse models in which RNA exosome activity and other co-factors of the ncRNA processing complex can be tissue-specifically deleted. Using these mouse models we have identified the mechanism using which the RNA exosome complex regulates the level and function of various noncoding RNA families in B cells. We find that the transcriptome of RNA exosome-deficient B cells includes a subset of transcription start site (TSS)-associated transcripts, xTSS-RNA, which can exceed 500 base pairs in length and are transcribed divergently—in the antisense orientation—to cognate coding gene transcripts. Strikingly, xTSS-RNA are expressed at genes which accumulate AID-mediated somatic mutations and/or are frequent translocation partners of DNA double strand breaks generated at either Myc or IgH loci in B cells. We conclude that divergent transcription generates DNA structures that attract AID and links ncRNA transcription with overall maintenance of B cell genomic integrity. Indeed, lack of processing of xTSS-RNAs leads to defective class switch recombination and somatic hypermutation, two AID-dependent DNA alteration mechanisms that are essential for development of mammalian adaptive immunity.

Immune Response to Optogenetic Gene Therapy for Blindness

Awardee: Jean Bennett
Award: Pioneer Award
Awardee Institution: University of Pennsylvania

Co-authors: Rhoda A. Chang, Ru Xiao, Rajani Shelke, and Luk H. Vandenberghe
Co-authors' Institutions: Harvard University, University of Pennsylvania

Therapeutic approaches based on optogenetic molecules rely on the gene transfer of optogenetic tools to specific neural cell populations that subsequently permit exogenous control of individual neurons in living organisms by conferring light sensitivity to specific neurons. One of the most immediate applications of this strategy is the restoration of vision in blindness caused by retinal degenerations such as retinitis pigmentosa. While gene augmentation approaches have moved forward clinically with great success, the optogenetic approach has two main advantages: (1) the treatment paradigm would be independent of disease etiology, and therefore a single therapeutic would be useful for a spectrum of blinding disorders; and (2) in late stages of the disease, following the loss of photoreceptors, optogenetic strategies permit other retinal cell types to be reprogrammed to become photosensitive.

Here, adeno-associated virus (AAV) is injected subretinally to deliver channelrhodopsin-2 (ChR2) or halorhodopsin (NpHR) to specific retinal cells to depolarize or hyperpolarize them, respectively. While in early studies these foreign molecules have been well-tolerated, the potential immune response to optogenetic transgenes or AAV using optogenetics has not yet been investigated.

To map the dominant T cells epitopes of ChR2, NpHR, and AAV capsid proteins, the anterior tibialis muscle of mice was injected with immunogenetic AAV2/rh32.33 encoding these transgenes in order to raise a potent cellular and humoral immune response. Peptides spanning the length of these proteins are used in an ELISPOT assay to determine the dominant T cell epitopes of these proteins. Next, mice will be injected subretinally to express NpHR in cone photoreceptors or ChR2 in ON bipolar cells. Activation of T cells in the retina and peripheral lymph nodes will be determined using peptides of the dominant epitopes in an ELISPOT assay. Levels of peripheral antibodies will also be measured.

We aim to investigate these potential immune responses elicited by optogenetic gene therapy in treating blindness caused by retinal degeneration. Such studies are crucial for the advancement of this approach to clinical trials.

A Novel Mouse Model of Kabuki Syndrome Demonstrates a Hippocampal Neurogenesis Defect Attenuated with Histone Deacetylase Inhibition

Awardee: Hans T. Bjornsson
Award: Early Independence Award
Awardee Institution: Johns Hopkins University

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Co-authors' Institutions: Johns Hopkins University, Howard Hughes Medical Institute

Kabuki syndrome is an autosomal dominant disorder, characterized by intellectual disability, and shown to be caused by two different chromatin modifying genes, MLL2 and KDM6A. MLL2 is responsible for adding histone 3 lysine 4 trimethylation (H3K4me3, an open chromatin mark) while KDM6A removes histone 3 lysine 27 trimethylation (H3K27me3, a closed chromatin mark). We hypothesized that the pathogenesis of Kabuki syndrome is a manifestation of a relative deficiency of open chromatin states, and that by promoting open chromatin we might be able to ameliorate disease phenotypes. To test this, we have characterized a novel mouse model of Kabuki syndrome that has a heterozygous loss of function mutation in Mll2 ($Mll2^{+/\beta Geo}$). This model has yielded some insight into the pathogenesis of Kabuki syndrome, as we have demonstrated decreased H3K4me3 and reduced expression of doublecortin, a marker of neurogenesis, in the granule cell layer (GCL) of the dentate gyrus, associated with hippocampal memory defects. Furthermore, after treatment with 10mg/kg/day of the histone deacetylase inhibitor AR-42 for 2 weeks we see recovery of H3K4me3 and expression of doublecortin in the GCL associated with a recovery of the hippocampal memory defects. This work not only suggests that a deficiency of gene expression and neurogenesis are critical to the pathogenetic sequence of the intellectual disability seen in Kabuki syndrome, but also demonstrate that these deficiencies can be reversed by targeting the epigenetic system at postnatal time point, yielding some optimism for future therapeutic development for this group of disorders.

Trans-Cellular Activation of Transcription in Biological Systems

Awardee: Josh Bonkowsky
Award: New Innovator Award
Awardee Institution: University of Utah

Visualization and manipulation of neural circuitry have remained vexing problems in neurobiology. A more general related issue is how to induce expression of a transgene in a vertebrate system when two cells make contact.

We have made a genetic method for visualizing and driving expression in two cells that make contact. TCAT, or trans-cellular activation of transcription, is based on components from the receptor/ligand pair of Notch/Delta. Upon ligand binding to receptor, the intracellular domain of Notch is cleaved and translocates to the nucleus. We replaced the intracellular domain of Notch with the yeast transcriptional activator Gal4, so that we can express transgenes at the Gal4-binding site UAS. For TCAT we used the homologs LAG-2 (Delta) and LIN-12 (Notch) from the nematode *C. elegans* to prevent cross-reactivity with the endogenous zebrafish proteins. Expression from the UAS occurs when Gal4 is targeted to the nucleus, and this only occurs in the presence of ligand-receptor (cell-cell) LAG-2 to LIN-12 binding.

We overcame species-incompatibility of the protease cleavage reaction necessary for TCAT by developing a chimeric system: receptor-ligand binding specificity is maintained using *C. elegans* LAG-2 and LIN-12 binding domains, but with substitution of the zebrafish Delta and Notch signal sequences and transmembrane domains. In proof-of-principle experiments, we found that chimeric LAG-2/Delta (LAD) and LIN-12/Notch (LINch) activate transcription in different cell types in transient injections. In stable transgenic animals expressing LINch in motor neurons and an RFP-tagged LAD (LADR) in muscles, we achieved specific activation of GFP transcription in motor neurons when they make contact with muscles. Control experiments demonstrated that both components (LAD and LINch) must be present for TCAT; expression of either component alone is unable to activate transcription even when expressed at high levels. To overcome low expression levels of LINch we designed and tested a series of constructs with 5' and 3' elements to bolster translation. To demonstrate the wide applicability of TCAT for a range of biological questions we have generated transgenic animals expressing LINch or LADR in different cell types and organs including the heart and vasculature, retinal ganglion cells and the tectum, dermal progenitor cells and the epidermis, and dopaminergic neurons and subpallial interneurons.

TCAT is a significant technical innovation for mapping and understanding brain circuits. Further, because TCAT can be used to drive expression of any desired gene, the method allows both labeling and manipulation in a variety of biological systems.

A Nuclear F-Actin Scaffold Stabilizes RNP Droplets against Gravity in Large Cells

Awardee: Cliff Brangwynne
Award: New Innovator Award
Awardee Institution: Princeton University

The size of a typical eukaryotic cell is on the order of ≈10 μm. However, some cell types grow to very large sizes, including oocytes (immature eggs) of organisms from humans to starfish. For example, oocytes of the frog *X. laevis* grow to a diameter ≥1 mm. They contain a correspondingly large nucleus (germinal vesicle, GV) of ≈450 μm in diameter, which is similar to smaller somatic nuclei, but contains a significantly higher concentration of actin. The form and structure of this nuclear actin remain controversial, and its potential mechanical role within these large nuclei is unknown. We use a microrheology and quantitative imaging approach to show that GVs contain an elastic F-actin scaffold that mechanically stabilizes these large nuclei against gravitational forces, which are usually considered negligible within cells. We find that upon actin disruption, RNA/protein droplets, including nucleoli and histone locus bodies, undergo gravitational sedimentation and fusion. We develop a model that reveals how gravity becomes an increasingly potent force as cells and their nuclei grow larger than ≈10 μm, explaining the requirement for a stabilizing the nuclear F-actin scaffold in large *X. laevis* ooctyes. All life forms are subject to gravity, and our results may have broad implications for cell growth and size control.

Cyclotides: A Novel Ultrastable Microprotein Scaffold for Targeting Protein-Protein Interactions

Awardee: Julio Camarero
Award: Transformative Research Award
Awardee Institution: University of Southern California

High-throughput assays are indispensable for comprehensive functional proteome research. The development of these techniques has been driven by the complete mapping of many genomes, including the human. Of great importance for achieving this goal is the development of new protein capture tools for the detection and identification of specific proteins as well as for the development of novel protein-based therapeutics. New capture reagents should be stable to thermal and proteolytic degradation, have high affinity, be easy to produce, and present low cross-reactivity. In response to this challenge, we propose the use of cell-based libraries of cyclotides for selecting specific cyclotide sequences against particular protein targets.

Cyclotides are a new, emerging family of large plant-derived backbone-cyclized polypeptides (≈28–37 amino acids long) that share a disulfide-stabilized core (3 disulfide bonds) characterized by an unusual knotted. Cyclotides contrast with other circular polypeptides in

that they have a well-defined three-dimensional structure, and despite their small size, can be considered as mini- or microproteins. The main features of cyclotides are therefore a remarkable stability due to the cystine knot, a small size making them readily accessible to chemical synthesis, and an excellent tolerance to sequence variations. For example, the first cyclotide to be discovered, kalata B1, is an orally effective uterotonic, and other cyclotides have been shown to cross the cell membrane through macro-pinocytosis. Cyclotides thus appear as promising leads or frameworks for design of peptide-based diagnostic and therapeutic compounds.

I will present our ongoing efforts in the development of high-throughput methods for the screening and selection of novel cyclotides with new biological properties as well as recent results on the rational design of cyclotides using molecular grafting to specifically target extra- and intracellular protein-protein interactions with therapeutic value.

Early Specification of CD8+ T Lymphocyte Fates during Adaptive Immunity Revealed by Single-Cell Gene Expression Analyses

Awardee: John T. Chang
Award: New Innovator Award
Awardee Institution: University of California, San Diego

Co-authors: Janilyn Arsenio, Boyko Kakaradov, Patrick J. Metz, Stephanie H. Kim, Gene W. Yeo
Co-authors' Institution: University of California, San Diego

T lymphocytes responding to microbial infection give rise to both terminally differentiated effector cells that mediate acute host defense and self-renewing memory cells that provide long-lived immunity. However, the process by which an individual lymphocyte transitions from a naïve state to one of these differentiated fates, as well as the identity and temporal expression of the molecular regulators that control these transitions, is poorly understood. Although transcriptome studies using measurements of bulk cell populations have begun to elucidate the transcriptional networks that control lymphocyte fate specification, they have been unable to discern the fate decisions made by individual cells. Importantly, whether lymphocytes progress along a linear differentiation pathway or diverge early during an immune response, owing to asymmetric cell division, has been a longstanding question in biology that remains to be resolved. Technological advances in single-cell transcriptional profiling have enabled recent studies to probe the heterogeneity within human colonic tumors and examine hematopoiesis, embryonic development, and cellular reprogramming of induced pluripotent stem cells. Here, we combine high-throughput single-cell gene expression analyses with machine-learning approaches to trace the transcriptional roadmap of individual CD8+ T lymphocytes throughout the course of an immune response to a microbial pathogen in vivo. Surprisingly, we observed that CD8+ T lymphocytes as early as the first cellular division had

already acquired gene expression signatures predictive of their eventual fates as effector or memory cells. We constructed a computational model that predicts the temporal expression pattern of key genes that together orchestrate each of these cellular fates. Finally, we demonstrate that asymmetric partitioning of the interleukin-2 receptor alpha chain (IL2R⍺) during the first CD8+ T lymphocyte division in vivo may be critical in driving distinct gene expression profiles that lead to disparate cellular fates. Together, these results underscore the importance of single-cell analyses in understanding fate determination and provide new insights into the specification of divergent lymphocyte fates early during an immune response to microbial infection.

Optical Trapping and Sorting of the Human Immunodeficiency Viruses at the Single-Molecule Level

Awardee: Wei Cheng
Award: New Innovator Award
Awardee Institution: University of Michigan

Co-authors: Yuanjie Pang, Hanna Song, Jin H. Kim, Ximiao Hou
Co-authors' Institution: University of Michigan

Viruses may exist as heterogeneous populations, but the molecular basis of heterogeneity is often elusive due to the lack of sensitive techniques to characterize viruses at the single-molecule level. Analogous to flow cytometry for cells, here we demonstrate a technique, "virometry," to conduct multi-parameter analysis of viruses in culture media under native conditions. This technique relies on optical manipulation of a single virus in culture media, and simultaneous two-photon fluorescence detection of viral proteins associated with the particle at the single-molecule level. Using HIV-1 as an example, we show that individual virions differ in the number of two viral proteins by more than one order of magnitude despite the fact that they are all derived from a single clone. The single-molecule sensitivity allows us to sort HIV-1 into distinct populations. The correlation of viral infectivity with its subpopulations reveals that individual virions differ in their efficiency of infection at single-particle level, and proves the existence of "elite" viruses that can infect host cells better than others. These "elite" viruses may bias viral spread and evolution toward the specific RNA genomes packaged in them. On the contrary, therapeutic strategies may achieve better efficacy by selectively targeting those highly infectious virions in a virus population.

Progress toward Solving the Membrane Protein Expression Problem

Awardee: William M. Clemons, Jr.
Award: Pioneer Award
Awardee Institution: California Institute of Technology

Co-author: Axel Müller
Co-author's Institution: California Institute of Technology

A major goal in biomedical research aims to understand integral membrane proteins (MPs) at a molecular level. This is limited by the ability to produce significant amounts of protein in heterologous expression systems. Our work aims to develop methods that overcome this problem. The general approach relies on interplay between bioinformatic analysis and experimental approaches. The success of genome sequencing gave rise to unprecedented amounts of data. To mine this data we have combined several existing databases with properties calculated for transmembrane protein sequences. We will present a number of queries that illustrate the capacity of our database. At the experimental level, we set out to determine factors crucial for expression by performing tests on chimeras of various differentially expressed homologs. The result was the identification of a feature that controlled expression and was transferable. Finally, we are taking the direct approach of determining the ideal MP-targeting signal empirically. We will present various aspects of this developing work.

Hippocampal Memory Traces Are Differentially Modulated by Experience, Time, and Adult Neurogenesis

Awardee: Christine A. Denny
Award: Early Independence Award
Awardee Institution: Columbia University

Co-authors: Mazen A. Kheirbek, Eva L. Alba, Kenji F. Tanaka, Rebecca A. Brachman, Kimberly B. Laughman, Nicole K. Tomm, Gergely F. Turi, Attila Losonczy, and René Hen
Co-authors' Institution: Columbia University

Memory traces are believed to be ensembles of cells used to store memories. To visualize memory traces, we created a transgenic line that allows for the comparison between cells activated during encoding and retrieval of a memory. Mice re-exposed to a fear-inducing context froze more and had a greater percentage of reactivated cells in the dentate gyrus (DG) and CA3 than mice exposed to a novel context. Over time, these differences disappeared, in keeping with the observation that memories become generalized. Optogenetically silencing DG or CA3 cells that were recruited during encoding of a fear-inducing context prevented retrieval of the corresponding memory. Mice with reduced neurogenesis displayed less contextual memory and less reactivation in CA3, but, surprisingly, normal reactivation in the DG. These studies suggest that distinct memory traces are located in the DG and in CA3, but that the strength of the memory is related to reactivation in CA3.

Essential Gene Discovery in the Malaria Parasite *Plasmodium falciparum*

Awardee: Jeffrey D. Dvorin
Award: New Innovator Award
Awardee Institution: Boston Children's Hospital/Harvard Medical School

Human malaria is a leading cause of death and disease worldwide, resulting in nearly one million deaths each year. The most severe forms of malaria result from infection by the *Plasmodium falciparum* parasite, which causes the vast majority of malaria in Africa. Deaths from malaria disproportionately affect children under 5 years old and pregnant women. Resistance to existing antimalarial medications is a constant and continually emerging hurdle to the effective treatment of malaria. A molecular understanding of the fundamental biological process of *P. falciparum* replication will provide the necessary tools to develop new antimalarial therapeutics. Although the genome of *P. falciparum* has been fully sequenced, the function of more than half of the 5,300 genes in the parasite remains unknown. Many of the genes with unknown function have little or no homology with characterized genes from other organisms. Therefore, existing molecular genetic and bioinformatics techniques cannot be used to efficiently determine the function of many of the genes in the parasite. Furthermore, existing technologies cannot predict which genes are essential for survival of the parasite. We

hypothesize that these essential genes, and the proteins that they encode, will be attractive targets for the rational design of new antimalarial therapeutics.

A forward-genetic system to investigate the function of essential genes does not exist currently. We propose to establish a much needed forward-genetic system in *P. falciparum*. Our forward-genetic analysis relies upon a robust and tightly controlled inducible expression system that I have designed. This inducible system will be used to perform saturating transposon-mediated mutagenesis in *P. falciparum*. I will apply next-generation sequencing to parasites following saturating mutagenesis to identify essential genes. In addition, I will follow mutated parasites for several generations using deep sequencing to assign a relative fitness effect to disruption of the remaining nonessential genes. The immediate goal of this proposal is to generate a complete list of all essential genes in the blood stage of *P. falciparum*. This list, together with a preliminary molecular characterization of gene function, will be an important resource for the malaria research community. The long-term objectives and public health implications of these studies are to identify novel targets for new antimalarial therapeutics. This long-term goal will be achieved as a direct result of our identification of novel essential genes in *P. falciparum* parasites.

From Structure to Systems: Compensatory Interactions through Contact Networks

Awardee: James S. Fraser
Award: Early Independence Award
Awardee Institution: University of California, San Francisco

Studies that explicitly combine data from systems and structural biological approaches are having a profound effect on our ability to predict how mutations and small molecules affect atomic-level mechanisms, disrupt systems-level networks, and ultimately lead to changes in organismal fitness. A shared framework for analysis of nonadditive genetic and thermodynamic responses to perturbations is emerging that will accelerate the integration of reductionist and global approaches. To dissect the structural basis of these responses, we have developed a new algorithm, CONTACT, that identifies contact networks of conformationally heterogeneous residues directly from high-resolution X-ray crystallography data. Contact networks predict long-range pattern of NMR chemical shift perturbations and reveal how mutations that alter coordinated motions can impair catalytic function in enzymes. To examine the systems-level consequences to specific amino acid substitutions, we have been exploiting point mutant epistatic miniarray profiles (pE-MAP) of quantitative genetic interactions in *Saccharomyces cerevisiae*. A recent study of 53 RNAPII point mutants demonstrated surprising new connections between specific RNAPII mutations and protein complexes involved in RNA processing and chromosomal segregation. Interestingly, the importance of dynamic contact networks observed at the atomic level is paralleled at the systems level. We are now performing similar pE-MAP experiments and analyzing new structural data focusing on the ubiquitin proteasome system.

Pollen Grains as Trojan Horses for Oral Vaccination

Awardee: Harvinder Singh Gill
Award: New Innovator Award
Awardee Institution: Texas Tech University

Oral vaccination is painless and is an attractive alternative to painful needle-based injections. Furthermore, it can be self-administered, and it can induce both systemic and mucosal immune responses. However, oral vaccination is challenging because vaccines degrade in the stomach and poorly pass through the intestinal lining. Our research proposes to harness the natural toughness of pollens to help transport vaccines into the human body through the oral route. By first removing the native plant biomolecules, which are the root cause of pollen allergies, a nonallergenic empty shell is created, which can subsequently be filled with vaccines. This "Trojan horse"-like system is postulated to safely ferry vaccines through the harsh environment of the stomach into the intestines for improved uptake into the body.

To develop this novel concept we have used *Lycopodium clavatum* (club moss) spores as a representative of pollens. Our results show that native plant matter from lycopodium spores can be removed to create allergen-free empty microcapsules that can be filled with vaccine antigens. In vivo evaluation of this novel delivery approach using ovalbumin as a model vaccine antigen has shown that high levels of anti-ovalbumin antibodies can be induced in mouse serum and fecal matter, indicative of systemic and mucosal immune responses, respectively. Importantly, the anti-ovalbumin antibody levels induced by lycopodium spores were found to be superior to that induced by the use of cholera toxin (CT) as a control adjuvant. This finding is important because CT, although considered a gold standard among oral adjuvants, is inherently toxic to humans. Lycopodium spores on the other hand are potentially safe and more effective. The immune response was not affected by neutralizing the gastric acid in the stomach, and a durable long-term immune response was observed. To expand our studies to pollens of other species we have recently succeeded in developing a new chemical treatment process that enables us to clean pollens of other species without damaging them, and surprisingly this process also selectively opens the pollen apertures. This is exciting because now we can readily add vaccines and even nanoparticles inside pollens to expand upon the potential of pollens for oral vaccination. Overall these results lay the foundation and facilitate further development of pollens as an innovative approach for painless oral vaccination.

Anticipating Sudden Transitions in Biological Populations

Awardee: Jeff Gore
Award: New Innovator Award
Awardee Institution: Massachusetts Institute of Technology

Natural populations can shift suddenly in response to small changes in environmental conditions. Examples of such sudden transitions include the collapse of fisheries in response to over-fishing and disease outbreaks in response to falling vaccination rates. Given that these population transitions can have substantial economic and health implications, it would be valuable to obtain advance warning that such a "tipping point" is approaching. Theory from nonlinear dynamics argues that these tipping points should be associated with potentially universal changes in the dynamics of the system resulting from an increase in the time to recover from perturbations. We have used laboratory microbial ecosystems to study early warning signals of impending population collapse. Yeast cooperatively break down the sugar sucrose, meaning that below a critical size the population cannot sustain itself. We have demonstrated experimentally that changes in the fluctuations of the population size can serve as an early warning signal that the population is close to collapse. In particular, we find that the population fluctuations become both larger and slower near a tipping point leading to collapse. In addition, we have demonstrated that in spatially extended populations it may be possible to use the emergence of spatial patterns to anticipate an impending collapse. The cooperative nature of yeast growth on sucrose suggests that the population may be susceptible to cheater cells, which do not contribute to the public good and instead merely take advantage of the cooperative cells. We have confirmed this possibility experimentally and found that such social parasitism reduces the resilience of the population.

The Xist Large Noncoding RNA Exploits Three-Dimensional Chromosome Architecture to Spread across the X Chromosome

Awardee: Mitchell Guttman
Award: Early Independence Award
Awardee Institution: California Institute of Technology

Co-authors: Jesse M. Engreitz, Amy Pandya-Jones, Patrick McDonel, Alexander Shishkin, Klara Sirokman, Christine Surka, Sabah Kadri, Eric S. Lander, and Kathrin Plath
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Mammalian genomes encode thousands of large noncoding RNAs (lncRNAs), many of which regulate gene expression, interact with chromatin regulatory complexes, and are thought to play a role in localizing these complexes to target loci across the genome. A paradigm for this class of lncRNAs is Xist, which orchestrates mammalian X-chromosome inactivation (XCI) by

coating and silencing one X chromosome in females. Despite the central role of RNA-chromatin interactions in this process, the mechanisms by which Xist localizes to DNA and spreads across the X chromosome remain unknown.

To explore this question, we developed a biochemical method to map the localization of a lncRNA across the genome. Using this approach, we show that during the maintenance of XCI, Xist binds broadly across the X chromosome, lacking defined localization sites, and excludes genes that escape XCI. At the initiation of XCI in mouse embryonic stem cells, Xist initially transfers to distal regions across the X chromosome that are not defined by specific sequences. Instead, Xist RNA identifies these regions using a proximity-guided search mechanism, exploiting the three-dimensional conformation of the X chromosome to spread to distal regions in close spatial proximity to the *Xist* genomic locus. Initially, Xist accumulates on the periphery of actively transcribed regions and requires the A-repeat, the RNA domain of Xist responsible for interacting with PRC2 and silencing gene expression, to spread across actively transcribed regions and access the entire chromosome.

Our data suggest a model for how Xist can integrate its two functions—localization to DNA and silencing of gene expression—to coat the entire X chromosome. In this model, Xist exploits three-dimensional conformation to identify and localize to initial target sites and leads to repositioning of these regions into the growing Xist compartment. These structural changes effectively pull new regions of the chromosome closer to the *Xist* genomic locus, allowing Xist RNA to spread to these newly accessible sites by proximity transfer. This localization strategy capitalizes on the abilities of a lncRNA to act while tethered to its transcription locus and to interact with chromatin regulatory proteins to modify chromatin structure. Beyond Xist, we will present evidence that other lncRNAs utilize a similar strategy to locate regulatory targets in three-dimensional proximity and alter chromatin structure to establish local nuclear compartments containing co-regulated targets.

Illuminating the in Vivo Biochemistry of Cyclic Dinucleotide Second Messengers Using Riboswitch-Based Fluorescent Biosensors

Awardee: Ming C. Hammond
Award: New Innovator Award
Awardee Institution: University of California, Berkeley

Cyclic dinucleotides are an emerging class of second messengers that originally were found in bacteria, but more recently have been discovered in diverse eukaryotes from social amoeba to humans. Cyclic di-GMP regulates important physiological processes including biofilm formation, motility, and virulence responses in bacteria and stalk differentiation in the amoeba *Dictyostelium discoideum*. Very recently, cyclic di-AMP and two structural isomers of cyclic GMP-AMP (cGAMP) have been discovered as novel signaling molecules in different organisms, including humans, but many questions remain about their dynamic regulation, recognition by

effectors, and potential for crosstalk. Here we will describe the development of selective riboswitch-based fluorescent biosensors for live cell imaging of cyclic dinucleotides. The latest progress toward applying these biosensors for monitoring in vivo signaling dynamics, predicting crosstalk, and discovering novel effectors and enzymes involved in cyclic dinucleotide signaling will be discussed.

Generating Extracellular Amyloid Aggregates Using *E. coli* Cells

Awardee: Ann Hochschild
Award: Pioneer Award
Awardee Institution: Harvard University

Co-author: Viknesh Sivanathan
Co-author's Institution: Harvard University

Diverse proteins are known to be capable of forming amyloid aggregates, self-seeding fibrillar assemblies that may be biologically functional or pathological. We sought to develop a cell-based assay that would facilitate the study and identification of amyloidogenic proteins. In designing such an assay, we took advantage of the natural ability of *E. coli* cells to elaborate surface-associated amyloid fibrils known as curli. We found that the curli export pathway can support amyloid fibril formation by heterologous amyloidogenic proteins. In particular, experiments with several different yeast prion proteins and the human huntingtin protein (Htt) indicate that protein secretion via this specialized export pathway promotes acquisition of the amyloid fold specifically for proteins that have an inherent amyloid-forming propensity. In the case of Htt, our system recapitulated the well-known relationship between amyloidogenicity and the number of glutamines within the protein's so-called polyQ region.

Our findings establish the potential of this *E. coli*-based system to serve as a platform for the identification of amyloidogenic proteins and modulators of amyloid aggregation. The system also facilitates investigation of amino acid sequence determinants of amyloid aggregation. Current work will be discussed that is aimed at identifying mutations that modulate Htt aggregation in the context of a fixed number of glutamines in the polyQ region.

Sequence-Specific Labeling and Imaging of DNA in Living Cells

Awardee: Bo Huang
Award: New Innovator Award
Awardee Institution: University of California, San Francisco

We have created a new approach to fluorescently label and image arbitrary, endogenous DNA sequences in living cells. Our approach is based on the CRISPR system, which was originally developed for genome engineering and transcription regulation. It consists of the dCas9 protein and a small guide RNA to bind complementary DNA sequences in the nucleus. Utilizing a dCas9 mutant lacking nuclease activity and fused to GFP, together with an optimized sgRNA design, we were able to label endogenous gene loci as well as telomeres in mammalian cells. This tool allowed us to examine the copy number of genes and followed telomere dynamics in living cells.

The Search for Bacteria-Specific Imaging Agents

Awardee: Sanjay K. Jain
Award: New Innovator Award
Awardee Institution: Johns Hopkins University

Co-authors: Alvaro Ordoñez, Edward A. Weinstein, Allison M. Murawski
Co-authors' Institution: Johns Hopkins University

Early accurate diagnosis of infection is essential for effective therapy, but traditional diagnostic methods are invasive, labor-intensive, and time-consuming, as well as subject to the uncertainties of incorrect sampling and contamination. CT and MRI detect anatomic changes that occur late in a disease process and are neither sensitive nor specific for the diagnosis of bacterial infections. Moreover, though more sensitive, nuclear medicine imaging (^{99}Tc-tagged WBC or [^{18}F]FDG-PET) have poor specificity in differentiating between sterile inflammation and infection. Therefore, bacteria-specific imaging tracers are required to discriminate infection from other disease processes, and to monitor treatment efficacy.

We hypothesize that small prokaryote-specific molecules can be identified and developed for use as radiotracers. To our knowledge, a systematic approach has never been attempted before. We screened a commercial library of over 400 random ^{14}C and ^{3}H radio-labeled small molecules looking for low molecular weight compounds with excellent penetration into diseased tissues and scored these molecules according to our selection criteria: metabolized by prokaryote-specific pathways, evidence for prokaryote accumulation or antimicrobial activity, and absence of known eukaryotic accumulation or metabolism. Of the library, 3% (n = 7) passed all three selection criteria and were tested for intracellular bacterial accumulation in model bacteria representing three important pathogen classes: *Staphylococcus aureus* (gram-positive),

Escherichia coli (gram-negative), or *Mycobacterium smegmatis* (mycobacteria). Intracellular bacterial accumulation was determined by percentage of cell-associated radioactivity measured at different time points using a scintillation counter.

Our results show six of the seven compounds were accumulated within *E. coli*, with D-xylose selectively retained by this organism with 74% ± 2% cell-associated radioactivity at 120 minutes. 4-Aminobenzoic acid (PABA) and D-mannitol were noted to accumulate in all species of bacteria significantly and rapidly. Follow-up testing of PABA with *M. tuberculosis* revealed 96% ± 15% cell-associated radioactivity at 18 hours of incubation. The percentage of cell-associated radioactivity of these compounds was further tested with eukaryotic cells with no significant uptake after 4 hours of incubation.

We have developed an innovative approach for screening bacteria-specific imaging tracers with promising results. These tools would be useful in both preclinical and clinical settings for a broad variety of bacterial infections, including tuberculosis, in which they could be a key component for decision making and appropriate treatment.

Pain Control by the Specialized Pro-Resolution Lipid Mediators: Resolvins and Protectins

Awardees: Ru-Rong Ji and Charles N. Serhan
Award: Transformative Research Award
Awardee Institution(s): Duke University; Brigham and Women's Hospital, Harvard Medical School

Prevalence of chronic pain after major surgeries, such as amputation, chest, and breast surgery, is very high as a result of nerve injury. Current perioperative pain management does not prevent neuropathic pain after surgeries. Thus, it is urgent to develop new therapeutics for preventing and treating neuropathic pain associated with surgery and traumatic injuries. It is generally believed that chronic pain is driven by neural plasticity (hyperactivity of nociceptive neurons) and neuroinflammation (glial activation and cytokine expression) in the spinal cord. Effective treatments are needed to block both neural plasticity (underlying the pain symptoms) and neuroinflammation (underlying the disease progression).

Serhan's group identified that several families of lipid mediators, such as resolvins and protectins, derived from omega-3 unsaturated fatty acids (DHA and EPA), possess potent anti-inflammatory and pro-resolution actions in animals (reviewed in Serhan et al., *Nature Reviews Immunology*, 2008). Our collaborative research uncovered that resolvins—namely, RvD1, RvD2, and RvE1, as well as protectin/neuroprotectin D1 (PD1/NPD1), potently inhibit inflammatory pain. The effective RvE1 dose in reducing inflammatory pain is 100 times lower than that of morphine (Xu et al., *Nature Medicine,* 2010). Notably, RvD2 also potently inhibits the function of

TRPV1 (IC_{50} = 0.1 nM) and TRPA1 (IC_{50} = 2 nM) ion channels in nociceptor neurons (Park et al., *The Journal of Neuroscience*, 2011).

However, neuropathic pain is often resistant to treatments that are effective for inflammatory pain, such as opioids and NSAIDS. In this study, we set out to test the actions of PD1 on neuropathic pain induced by chronic constriction injury (CCI) of the sciatic nerve in mice. Strikingly, our results demonstrated that peri-surgical PD1 delivery to the sciatic nerve prevented CCI-induced neuropathic pain (Xu et al., *Annals of Neurology*, 2013). PD1 also prevents nerve injury-induced neuroinflammation (microglial and astrocyte activation and expression of CCL2 and IL-1β) and long-term potentiation in the spinal cord. Like gabapentin, an extensively used drug for treating neuropathic pain, post-treatment of PD1 transiently reversed established neuropathic pain after CCI, but the effective dose of PD1 is 100 times lower than that of gabapentin. Given the well-known side effects of current analgesics (opioids, NSAIDs, and gabapentin) and the potency of these novel pro-resolution mediators, protectins and resolvins may offer new treatments for preventing and treating surgery, nerve trauma-induced postoperative pain, and neuropathic pain, in addition to treating inflammatory pain conditions as demonstrated in our recent publications.

EGFRvIII mCAR-Modified T Cell Therapy Cures Mice with Established Intracerebral Glioma and Generates Host Immunity against Tumor-Antigen Loss

Awardee: Laura A. Johnson
Award: New Innovator Award
Awardee Institution(s): Duke University, University of Pennsylvania

Co-authors: John H. Sampson, Bryan D. Choi, Luis Sanchez-Perez, David J. Snyder, Catherine T. Flores, Smita Nair, Elizabeth A. Reap, Pamela K. Norberg, James E. Herndon II, Chien-Tsun Kuan, Richard A. Morgan, and Steven A. Rosenberg
Co-authors' Institutions: Duke University, National Cancer Institute

Chimeric antigen receptor (CAR) transduced T cells represent a promising immune therapy that has been shown to successfully treat cancers in mice and humans. However, CARs targeting antigens expressed in both tumors and normal tissues have led to significant toxicity. Preclinical studies have been limited by the use of xenograft models that do not adequately recapitulate the immune system of a clinically relevant host. EGFRvIII is a constitutively activated mutant of the epidermal growth factor receptor that occurs naturally and is antigenically identical in both human and mouse gliomas, but is also completely absent from any normal tissues. We developed a murine, third generation, EGFRvIII-specific CAR (mCAR), and performed tests to determine its efficacy in a fully immune-competent mouse model of malignant glioma.

Our results indicated that at elevated doses, infusion with EGFRvIII mCAR T cells led to cures in all mice with brain tumors. Additionally, antitumor efficacy was found to be dependent on

lymphodepletive host conditioning. Selective blockade with EGFRvIII soluble peptide significantly abrogated the activity of mCAR-transduced T cells in vivo, and may offer a novel strategy to enhance the safety profile for CAR-based therapy. Lastly, mCAR-treated, cured mice were resistant to re-challenge with EGFRvIII-negative tumors, suggesting generation of host immunity against additional tumor antigens.

Together these data support that third generation EGFRvIII-specific CARs are effective against gliomas in the brain and highlight the importance of syngeneic, immune-competent models in the preclinical evaluation of tumor immunotherapies.

Combined RIP3 Necrosis and Caspase-8 Apoptosis Mediate Perinatal Lethality in RIP1-deficient Mice

Awardee: William J. Kaiser
Award: Early Independence Award
Awardee Institution: Emory University

Co-authors: Lisa P. Daley-Bauer, Roshan Thapa, Pratyusha Mandal, Aarthi Sundararajan, Hongyan Guo, Chunzi Huang, Linda Roback, Pete Gough, John Bertin, Samuel Speck, Sid Balachandran, Edward S. Mocarski
Co-authors' Institutions: Emory University, GlaxoSmithKline, and Fox Chase Cancer Center

Receptor interacting protein (RIP)1 kinase (RIPK1) collaborates with RIP3 kinase (RIPK3) to drive midgestational death of embryos in FADD or caspase-8 (Casp8)-deficient mice. Germ line RIP1-deficient mice exhibit a paradoxical perinatal lethality that contrasts the full viability of RIP3-deficient mice. Here, we define a vital role of RIP1 in preventing unleashed apoptosis and necrosis that underlies this phenotype. First, we show that RIP1 K45A kinase dead (KD) mutant mice are fully viable. Cells from these mice show an expected resistance to TNF-induced, RIP1 kinase-mediated necroptosis, eliminating the sensitivity of RIP1-deficient cells to TNF or interferon (IFN). This pattern exposes an unexpected cytoprotective kinase-independent role of RIP1. Second, even though neither $Rip1^{-/-}Rip3^{-/-}$ nor $Rip1^{-/-}Casp8^{-/-}$ mice survive, the combined elimination of $Casp8$ and $Rip3$ produced viable and fertile $Rip1^{-/-}Casp8^{-/-}Rip3^{-/-}$ (TKO) mice. Curiously, $Rip1^{-/-}Casp8^{-/-}Rip3^{+/-}$ (KKH) mice are also viable and fertile, indicating an apparent $Rip3$ gene dosage effect. Third, TKO mice are immunocompetent, developing myeloid and lymphoid lineages necessary to mount a robust immune response and control viral infection just like $Casp8^{-/-}Rip3^{-/-}$ (DKO) mice. Also like DKO mice, TKO mice exhibit lymphoid hyperplasia and autoimmune markers with age, a pattern that reinforces the role of $Casp8$ downstream of Fas death receptor signal transduction. Collectively, our results reveal a vital RIP1 adapter role in restricting both Casp8-dependent apoptosis as well as RIP3 kinase-MLKL-dependent necrosis through innate signals accompanying parturition; although, RIP1, RIP3 and Casp8 together are dispensable for mammalian development as well as the adaptive immune response to infection.

Motif-Directed Protein-Capture Reagents

Awardee: Shohei Koide
Award: Transformative Research Award
Awardee Institution: The University of Chicago

Protein-capture reagents are indispensable for delineating the molecular mechanisms of diseases, to detect and characterize cellular abnormalities, and to characterize biological effects of drugs. However, high-quality protein-capture reagents are still lacking for many areas of biomedical sciences. The overarching goal of this project has been to develop an innovative and powerful protein-capture technology with high levels of fidelity and predictability.

A major limitation of currently available technologies including antibodies is that specificity and epitopes are individually tested by laborious methods after generating protein-capture reagents. We have established a new concept, "affinity clamping," that enables us to direct reagents exclusively to a linear sequence motif with high specificity and high affinity. We have developed two classes of "affinity clamps," one directed to the C-terminal residues ("C-clamps") and the other directed to phosphotyrosine (pY)-containing motifs ("pY-clamps").

C-clamps exploit the inherent specificity of PDZ and other protein domains to a peptide containing the free C-terminus. Virtually every protein has a unique C-terminal signature that could be recognized with high efficiency by C-clamps. The a priori knowledge of epitope location allows one to predict the level of specificity and to implement strategies to eliminate off-targets. Thus, C-clamps are particularly suited as a technology for generating a comprehensive set of protein capture reagents.

Similarly, pY-clamps exploit the inherent specificity of SH2 domains to pY-containing motifs. pY-motifs play critical roles in signal transduction and thus are "high-value" targets. We have developed pY-clamps to address a fundamental challenge in signal transduction, which is to define the roles of individual nodes and of individual protein-protein interactions embedded in large multi-protein networks that control cellular behaviour. We used pY-clamps directed to single pY-motifs to deconvolute a signaling network and elucidated the roles of single protein-protein interactions in embryonic stem cell differentiation.

On the Representation of Visual and Cognitive Information in the Human Brain

Awardee: Gabriel Kreiman
Award: Pioneer Award
Awardee Institution: Children's Hospital, Harvard Medical School

Our capacity to transform vast and complex sensory information into cognition depends on the computations performed by the neuronal circuitry in the cerebral cortex. In spite of rapid advances in creating ever more powerful sensors and computers, our brains outperform in silico algorithms in most cognitive problems. As a paradigmatic example of a challenging everyday task, we consider the question of visually identifying objects. We can recognize objects, faces, and patterns in a fraction of a second. Visual recognition depends on rapid, selective, and robust integration of incoming sensory information and relies on the cascade of linear and nonlinear processes along the ventral visual cortex. In collaboration with neurosurgeons and neurologists, we have taken advantage of a unique opportunity to interrogate the human visual cortex through implanted electrodes in patients while they perform diverse cognitive tasks. These recordings have demonstrated that the human ventral visual cortex combines strong selectivity to objects and faces with robustness to image transformations including changes in scale, position, rotation, clutter, and occlusion. The physiological responses underlying recognition occur within a few hundred milliseconds, are modulated by task demands, and are orchestrated by the integration of bottom-up and top-down pathways. These signals can be decoded in real time to read out the brain's internal representation of visual information. Taken together, these studies have begun to unravel the mechanisms and neural circuits involved in transforming sensory inputs into cognitive signals. Understanding the inner workings of neural circuits is helping inspire a new generation of biologically plausible computational algorithms that may in the future be able to perform complex cognitive operations. Furthermore, the processes of recording and decoding cortical signals in real time open the doors to build new devices to interact with the human brain at unprecedented resolution and hold the potential to help in devastating neurological conditions that affect cognition.

Development of *Saccharomyces Boulardii* as a Mucosal Vaccine Delivery System

Awardee: Tracey J. Lamb
Award: New Innovator Award
Awardee Institution: Emory University School of Medicine

Co-authors: Lauren Miller, Milo Fasken, Courtney McDermott, David Guiliano, Jan R. Mead, Anita H. Corbett
Co-authors' Institutions: Emory University School of Medicine, Emory University, University of East London

The majority of human pathogens initially enter the body through the mucosa. Vaccination is one of the most cost-effective health interventions that can protect people from infection in endemic areas, saving millions of lives every year. Although vaccines currently exist to protect against infection with rotavirus and cholera, uptake of these vaccines in developing countries is poor due to the financial cost. In addition, most current vaccination strategies rely upon needle-stick injection and the development of systemic immune responses; however, this approach only poorly correlates with protection at the mucosa where most human pathogens first encounter the immune system. Optimal protection against many of these pathogens may instead be achieved by specifically targeting the gut-associated lymphoid tissue (GALT). This proposal will develop a new affordable vaccine delivery platform that uses the probiotic yeast *Saccharomyce cerevisiae boulardii* to deliver vaccines directly to the gastrointestinal tract. Using genetic techniques already established for *Saccharomyces cerevisiae*, this project will genetically engineer *S. boulardii* to express antigen-adjuvant complexes that induce protective immune responses. Oral administration of genetically transformed *S. boulardii* will facilitate expression of these fusion proteins in situ at the mucosal surface. Co-administration of *S. boulardii* expressing polarizing cytokines will facilitate the arming of different immune effector mechanisms driven by Th1, Th2, or Th17 responses—an essential feature that will potentially allow this vaccine delivery platform the flexibility to be applied to both microscopic (bacterial/viral) and macroscopic (helminth) pathogens. During this project, the generation of immune responses delivered by genetically transformed *S. boulardii* via oral vaccination will be initially demonstrated in mice using the model antigen ova to describe the baseline immune responses generated by different dosing regimens. The protective nature of the immune responses elicited will subsequently be demonstrated using a mouse model of diarrheal infection, *Cryptosporidium parvum*, for which protective antigens have been described. Here we report our initial work to create *S. boulardii* auxotrophic mutants, a feature that will facilitate the manufacturing of the vaccine because no antibiotic selection will be required in the production process. We also show data that transfected *S. boulardii* is capable of expressing correctly folded protein. This project will radically change the way vaccines are administered both in developed and developing countries.

Host Microarray Molecular Signature and Serologic Evaluation of Stages of *Pseudomonas aeruginosa* Infection in Cystic Fibrosis

Awardee: Hara Levy
Award: New Innovator Award
Awardee Institution: Medical College of Wisconsin

Co-authors: Melissa Reske, Rachel Bersie, Joseph Barbieri, Shuang Jia, Mary Kaldunski, Pippa Simpson, Anita Laxova, Philip M. Farrell, Martin J. Hessner
Co-authors' Institutions: Medical College of Wisconsin, University of Wisconsin School of Medicine and Public Health

Cystic fibrosis (CF) is a genetic disorder caused by mutations in *CFTR*; lung disease is characterized by chronic infection by *Pseudomonas aeruginosa* (Pa)*, the major cause of morbidity and mortality in CF patients. However, oropharyngeal cultures for Pa in young children result in poor specificity for lower airway infection. We used a novel approach to generate genome-wide expression profiles to identify markers for Pa infection and CF disease severity. Transcription was induced in healthy unrelated peripheral blood mononuclear cells (PBMCs) through co-culture with autologous serum (self-baseline control), allogeneic serums (healthy unrelated controls), and CF patient serums.

All CF diagnoses included sweat chloride levels ≥60 mEq/L and/or two *CFTR* mutations. We carried out various ELISAs against 192 serum samples (43 individuals sampled over 15 years) and comparative cultures. Concurrently, we completed expression profiling of RNA (Affymetrix HGU133plus2.0 array) from co-culture of PBMCs with the serum of CF patients, who possessed increasing degrees of colonization with Pa. Differentially expressed probe sets were defined as those possessing a false discovery rate <10% and |log2 ratio| >0.5 among comparison groups.

Our ELISA data indicated that the host response to Pa infection increased over time, suggesting that serology to Pa may help determine initial airway pathology. Comparison of array signatures performed prior to and after seroconversion identified several hundred unique genes; the most significant differences in gene expression included host genes involved with airway defense, antigen presentation, and transcription.

Our methodology consistently identified a small number of genes that were unique to CF patients or to CF patients with initial Pa infection conversion. These genes merit investigation as active participants in the function of the lung microbiome and may serve as therapeutic targets for Pa infection in CF.

Dissecting Essential Signaling Pathways in Apicomplexan Parasites

Awardee: Sebastian Lourido
Award: Early Independence Award
Awardee Institution: Whitehead Institute

Apicomplexan parasites are important human pathogens and cause diseases ranging from lifelong asymptomatic infections with *Toxoplasma gondii* in about a quarter of the world's population to nearly a million deaths annually due to malaria. To decipher their biology and treat the diseases they cause, we must understand the signaling pathways unique to these successful pathogens. Calcium-dependent protein kinases (CDPKs) are attractive targets for intervention because they are conserved among apicomplexans, absent from the genomes of their animal hosts, and essential for the parasite life cycle. Prior work has shown that CDPKs regulate *T. gondii* entry and exit from host cells, in part through controlling calcium-regulated secretion of specialized organelles required for motility. Although we have identified key enzymes responsible for phosphorylation in *T. gondii*, we know little about the substrates and even less about the consequences of these modifications for the parasite life cycle. The proposed study will map essential signaling pathways regulated by apicomplexan CDPKs and inform their potential as therapeutic targets. We will address different aspects of CDPK biology by identifying the role of individual kinases, characterizing the substrates they regulate, and determining the function of these substrates. These goals will be achieved through a combination of chemical-genetic approaches and quantitative phospho-proteomics that will map essential signaling networks regulated by apicomplexan CDPKs and inform their potential as therapeutic targets. Newly identified substrates of individual kinases are likely novel components of these pathways since we don't know the function of nearly 40% of apicomplexan proteins or the pathways they participate in. Furthermore, this study provides the basis for comparing CDPK functions across the apicomplexan phylum to uncover how this kinase family regulates the behavior of different parasites.

Developing a Reduced-Complexity Model Gut Microbiome in the Behavior Model, *Drosophila melanogaster*

Awardee: Will Ludington
Award: Early Independence Award
Awardee Institution: University of California, Berkeley

The gut is a major site of interaction between microbes and the host immune system. In the fruit fly *Drosophila melanogaster*, microbial products such as lipopolysaccharides and peptidoglycan stimulate host production of antimicrobial peptides (such as Diptericin). Changes in the microbial ecosystem can, in turn, modulate different aspects of host immunity, thereby inducing a feedback loop. We have begun to quantify how inter-microbial interactions affect the host by applying the principles of genetic epistasis to microbial ecosystems. Rather than

examining pairwise combinations of gene knockouts to map the gene interaction network, we are identifying phage and antimicrobial peptides that target and kill specific strains in the gut ecosystem. We are developing a gnotobiotic *D. melanogaster* model to simultaneously measure the microbial community, host immunity, and host behavior. Our goal is to reconcile the complex microbiome-host relationship with known principles of ecology.

Interrogating Diverse Epigenetic Roles of Protein Methyltransferases with Engineered Apparatus

Awardee: Minkui Luo
Award: New Innovator Award
Awardee Institution: Memorial Sloan-Kettering Cancer Center

Epigenetic regulations are involved in establishing cell-lineage diversity, and the errors in these processes have been linked to many diseases including developmental abnormalities, neurological disorders, and cancer. Among the key biochemical modifications in epigenetics is protein methylation, a process orchestrated by more than 60 human protein methyltransferases (PMTs) with *S*-adenosyl-*L*-methionine (SAM) as a cofactor. Defining the targets of the PMTs is pivotal toward elucidating their roles in normal physiology and disease states. Unfortunately, few prior tools were available for mapping proteome-wide and genome-wide methylation events in an unambiguous manner. To address this situation, the Luo laboratory recently developed BPPM (Bioorthogonal Profiling of Protein Methylation) technology for profiling the histone and nonhistone targets of multiple PMTs inside living cells. Here, human SAM synthetase was engineered to process metabolite mimics (terminal-alkyne-containing methionine analogs), thus allowing in situ production of the corresponding SAM analogues. Upon coupling with engineered PMTs, the SAM analogs will be processed to label the histone and nonhistone targets of the corresponding PMTs. The labeled substrates can then be readily enriched via alkyne-azide click chemistry for further analysis. Since only engineered PMTs recognize the SAM analogs, the resultant labeled targets can be assigned unambiguously to the designated (engineered) PMTs. We have successfully implemented the BPPM approach to > 10 human PMTs and showed that each of the PMTs can readily methylate 200 ~ 2,000 nonhistone targets, whose functions can associate with most essential biological pathways such as DNA replication, RNA processing, other posttranslational modulars, and metabolic enzymes. We will exemplify several findings about unprecedented roles of PMTs such as regulation of transcription factors and RNA splicing factors.

The Role of Pathogen-Environment Interactions in the Pandemic Potential of Influenza

Awardee: Linsey C. Marr
Award: New Innovator Award
Awardee Institution: Virginia Tech

Influenza is responsible for an estimated 36,000 deaths, 3.1 million hospitalization days, and 31 million outpatient visits per year in the United States, for a total economic burden of $90 billion. It is remarkable that we know so much about the infectivity and pathogenicity of influenza viruses and so little about transmission and the inter-host dynamics of the virus in the environment. Many critical questions remain unanswered surrounding the dominant mode of transmission, seasonality, and factors that enable a certain strain to "go airborne." This project will investigate the hypothesis that pathogen-environment interactions may play a key role in the transmissibility of the virus. Specifically, we hypothesize that evaporation-induced changes in the chemical composition of aerosols, such as lowered pH, increased salt and protein concentrations, crystallization, and/or phase separations, affect the structure and/or function of the virus inside the aerosols. The overall goal of the project is to elucidate the mechanisms by which humidity affects influenza virus transmissibility. Our specific objectives are (1) to determine the relationship between virus viability and solute concentrations in droplets/aerosols; (2) to characterize respiratory droplet/aerosol composition in terms of solute concentrations, pH, crystallization, and phase separation as a function of relative humidity (RH); (3) to pinpoint the location of viruses within droplets/aerosols; (4) to identify the mechanism(s) by which the virus is inactivated in droplets/aerosols; and (5) to confirm findings by exposing aerosolized viruses at known concentrations to a guinea pig model under various levels of RH. This research is innovative for five significant reasons. First, it aims to redefine the current paradigm used to describe airborne transmission of diseases, which relies on outdated terminology and concepts that have been eclipsed by major advances in aerosol science. Second, this research introduces pathogen-environment interactions as playing a significant role in the transmission of infectious diseases. Third, we have developed an innovative hypothesis that addresses a question that has stymied researchers for decades: "How can humidity affect a virus that is encased in an aerosol?" The fourth innovative aspect of this research is the use of an interdisciplinary approach, which is essential to making a large leap in understanding airborne transmission of infectious disease. Fifth, our innovative technical approach brings modern aerosol, biological, and nanoscience methods to the problem. Results of this research have the potential to promote major advances in predicting the pandemic potential of influenza virus strains, forecasting of disease dynamics, and development of infection control strategies.

Tolerance during CRISPR-Cas Immunity: Domesticating the Virus

Awardee: Luciano A. Marraffini
Award: New Innovator Award
Awardee Institution: The Rockefeller University

CRISPR-Cas loci of bacteria and archaea provide adaptive immunity against viruses that infect these organisms (phages). CRISPR loci consist in an array of short (~ 30 bp) repetitive sequences separated by equally short "spacer" sequences of viral origin that are acquired upon viral infection. Spacer sequences are transcribed into small antisense CRISPR RNAs that guide CRISPR-associated (Cas) nucleases to the phage genome for its destruction. Temperate phages usually carry beneficial genes for the host (even essential in certain conditions) that are incorporated into the bacterial genome after the integration of the viral DNA, or lysogeny. Therefore the destruction of these viruses by CRISPR immunity can present a problem for the cell. Here we investigated whether this immune system can tolerate commensal viruses. We found that, at least in staphylococci, Cas-mediated cleavage requires transcription of the target DNA. As most of the viral genes are silenced upon lysogeny, this requirement facilitates the tolerance of the lysogen. Tolerance, however, is contingent on the "good behavior" of the phage. When the lysogen initiates a lytic cycle, of lethal consequences for the host, its genome is transcribed and thus becomes a target for Cas nucleases. Therefore the staphylococcal CRISPR-Cas immune system possesses a tolerance mechanism that allows the cell to "domesticate" its viruses: maintaining them when they provide beneficial traits, but destroying them if they become harmful.

Optical Imaging of Newly Synthesized Proteins in Living Cells, Tissues, and Animals

Awardee: Wei Min
Award: New Innovator Award
Awardee Institution: Columbia University

Co-authors: Lu Wei and Yihui Shen
Co-authors' Institution: Columbia University

Synthesis of new proteins, a key step in the central dogma of molecular biology, has been a major biological process by which cells respond rapidly to environmental cues in both physiological and pathological conditions. For instance, long-lasting forms of synaptic plasticity, such as those underlying long-term memory, require new protein synthesis in a space- and time- dependent manner. However, visualization of newly synthesized proteome in live systems with subcellular resolution has been proven to be highly challenging despite the extensive efforts along the lines of fluorescence staining, autoradiography, and mass spectrometry. Herein, we report a novel live-cell imaging technique to visualize nascent proteins by harnessing the emerging stimulated Raman scattering (SRS) microscopy coupled with metabolic incorporation of deuterium-labeled amino acids.

When hydrogen is replaced by deuterium, the biochemical activities of amino acids remain almost unmodified. When added into the media for culturing cells, these deuterium-labeled amino acids will be taken up by the natural cell machineries as the necessary building blocks for new protein production. Hence, the newly synthesized proteins will carry the special deuterium atoms connected to carbon atoms. Intriguingly, the carbon-deuterium bonds (C–D) vibrate at a distinct frequency different from almost all the natural chemical bonds existing inside cells, which can be sensitively imaged by SRS microscopy. Technically, incorporation of deuterium-labeled amino acids is minimally perturbative to live cells, whereas SRS imaging of exogenous C–D in the cell-silent Raman region is highly sensitive, specific, and compatible with living systems.

As a demonstration of proof of principle, we imaged newly synthesized proteins in live mammalian cancer cell lines with high spatial-temporal resolution without fixation or staining. Subcellular compartments with fast protein turnover in HeLa and HEK293T cells, and newly grown neurites in differentiating neuron-like N2A cells, are clearly identified via this imaging technique. Moreover, coupled with label-free SRS imaging of the total proteome, our method can readily generate spatial maps of the quantitative ratio between new and total proteomes. We further optimized both the C–D labeling efficiency and the SRS imaging system, and achieved imaging newly synthesized proteins in primary hippocampal neurons, acute brain tissue slices and animals including mice. Thus, SRS imaging of deuterium incorporation will be a valuable tool for studying the complex spatial and temporal dynamics of newly synthesized proteome in vivo.

ATP-Independent Diffusion of Double-Stranded RNA Binding Proteins

Awardee: Sua Myong
Award: New Innovator Award
Awardee Institution: University of Illinois at Urbana-Champaign

The proteins harboring double-stranded RNA binding domains (dsRBDs) play diverse functional roles such as RNA localization, splicing, editing, export, and translation, yet mechanistic basis and functional significance of dsRBDs remain unclear. To unravel this enigma, we investigated transactivation response RNA binding protein (TRBP) consisting of three dsRBDs, which functions in HIV replication, protein kinase R (PKR)-mediated immune response, and RNA silencing. Here we report an ATP-independent diffusion activity of TRBP exclusively on dsRNA in a length-dependent manner. The first two dsRBDs of TRBP are essential for diffusion, whereas the third dsRBD is dispensable. Two homologs of TRBP, PKR activator and R3D1-L, displayed the same diffusion, implying a universality of the diffusion activity among this protein family. Furthermore, a Dicer-TRBP complex on dsRNA exhibited dynamic diffusion, which was correlated with Dicer's catalytic activity. These results implicate the dsRNA-specific diffusion activity of TRBP that contributes to enhancing siRNA and miRNA processing by Dicer.

Metabolic Approaches to Treat Severe Viral and Inflammatory Diseases

Awardee: August Ochoa
Award: Transformative Research Award
Awardee Institution: Louisiana State University Health Sciences Center

Co-authors: Timothy Foster, Paulo Rodriguez, James Hill, Maria Dulfary Sanchez, Stephanie Cormier, David Munn
Co-authors' Institutions: Stanley Scott Cancer Center, Louisiana State University Health Sciences Center, University of Tennessee, Georgia Regents University

Severe viral diseases are caused by a combination of virus-mediated cytopathic effects and an acutely overactive or a chronic inflammatory response. Diseases, such as keratoconjunctivitis by herpes simplex virus 1 (HSV1) or adenovirus, encephalitis by HSV2 (in neonates) or West Nile virus, and emerging pandemic diseases, such as SARS and influenza, frequently cause severe complications and disabilities including blindness, severe mental retardation, pneumonia, acute respiratory distress, and even death. Current therapies, when available, rely almost entirely on virus-specific antiviral drugs. However, elimination of the virus alone does not prevent the inflammatory complications. Corticosteroids are frequently used when inflammatory complications ensue. However, steroid usage leads to increased viral replication or reactivation of latent viruses, resulting in a vicious cycle that is difficult to interrupt. No single drug currently exists that can both inhibit viral replication and control the deleterious inflammation. Our data, however, support _the hypothesis_ that simple and achievable metabolic changes can

concurrently inhibit viral replication, modulate the inflammatory and angiogenic responses, and promote tissue healing, while allowing the development of a protective immune response. Our team will test this hypothesis through the use of virally induced inflammatory disease models. In addition to demonstrating the therapeutic efficacy of the proposed metabolic manipulations, we plan to identify the cellular and molecular pathways by which metabolic changes can control viral replication, and inflammation, while allowing for the development of a protective immune response. The proposed research could provide a new approach to treat or prevent severe viral illnesses, and create a new platform for screening metabolic therapies for infectious and inflammatory diseases.

Utilizing TALEN Technology to Regulate Human MicroRNAs

Awardee: Ryan O'Connell
Award: New Innovator Award
Awardee Institution: University of Utah

MicroRNAs (miRNAs) have quickly emerged as important regulators of mammalian physiology owing to their precise regulation of critical protein coding genes. Importantly, perturbations in expression or function of specific miRNAs have been linked to a plethora of human pathological conditions including cancer, autoimmunity, cardiovascular disease and neurodegeneration. Although miRNA gene deletion in mice via homologous recombination has led to an improved understanding of how they function during times of health and disease, there remains a fundamental need to manipulate miRNA genes and their target binding sites in the human germ line. This would allow for the study of their biology in man, and may be a strategic means by which miRNAs can be targeted therapeutically to combat human disease. We propose to develop a novel approach to activating, repressing or disrupting human miRNA genes in vivo by engineering TALE proteins that will function as transcription factors or nucleases that specifically target miRNA genes or their binding sites in the 3'UTRs of key target mRNAs. We will focus on targeting human miRNA-155, an "oncomiR" involved in regulating immune responses, and miR-146a, a tumor suppressor miRNA that inhibits inflammatory responses. Following proper targeting and disruption of these miRNAs or their binding sites in the 3' UTRs of relevant target mRNAs, a series of functional assays will be carried out to assess the roles of human miR-155 and miR-146a in regulating immune responses and cancer phenotypes. Beyond these two miRNAs, this approach will be compatible with any human miRNA making it a powerful, specific, and versatile technology that has not yet been used to study miRNAs.

Altered Regulatory Circuitry of Follicular Lymphoma Revealed
by Tumor Epigenomes

Awardees: Jacqueline Payton and Eugene Oltz
Award: Transformative Research Award
Awardees' Institution: Washington University

Co-authors: Olivia I. Koues, Rodney Kowalewski, Li-Wei Chang, Jennifer A. Schmidt, Amanda Cashen
Co-authors' Institution: Washington University

An understanding of epigenetic mechanisms that coordinate gene expression will provide unprecedented opportunity for therapeutic intervention because, unlike genetic lesions, pathogenic changes in the epigenome are reversible. Epigenomic alterations disrupt the normal transcriptional circuitry of cells (ref), including cis-acting regulatory elements (REs), perhaps engaging oncogenic or other disease-associated expression programs. In this regard, gene expression changes that occur in non-Hodgkin lymphoma (NHL), a broad category of B cell tumors, have been characterized extensively (ref). However, perturbations in the underlying transcriptional circuitry that lead to pathogenic changes in gene expression remain elusive. Here, we bioinformatically integrate new epigenomic, transcriptomic, and genomic data for follicular lymphoma (FL), a common form of NHL, to identify tumor-specific REs. Importantly, molecular profiles were obtained from primary B cells purified directly from FL biopsies, thus avoiding cell culturing and long-term propagation, both of which significantly alter the epigenomic landscapes. In addition, direct comparisons were made between the epigenetic landscapes of an FL and patient-matched, normal B cells. Many of the FL-altered REs (FLAREs) are predicted to be transcriptional enhancers. A subset of these serve as rheostats for genes involved in cellular transformation, proliferation, and survival. Although some of the FLAREs are employed in normal B cell counterparts, we find that FL also usurps regulatory circuitry from other cell types to promote viability and expansion, while blocking their terminal differentiation. As such, our study provides a rich resource for deciphering intrinsic aberrations in the transcriptional circuitry of FL that foster pathogenesis, with an emphasis on the underlying transcription factors, chromatin modifiers, and their RE targets. Our epigenome-centric approach for analysis of primary tumors also revealed two previously unappreciated sub-classes of low-grade FL, which resemble categories reserved for more aggressive types of NHL (ref). These discoveries, at the interface of genetics and epigenetics, provide new opportunities for prognostics and therapeutics, as well as a framework for the dissection of faulty gene regulation pathways that drive human diseases.

Adaptive Translation Facilitates Proteome Adaptation to Varying Environmental Conditions

Awardee: Tao Pan
Award: Pioneer Award
Awardee Institution: University of Chicago
Presenter: Mike Schwartz

Co-authors: Michael Schwartz, Chloe Weisberg, Molly Evans
Co-authors' Institution: University of Chicago

Genetic diversification of species is a fundamental requirement for adaptation to changing environments. This process is generally regarded to be time-consuming and beyond the scope of the biochemistry within individual cells, which cannot deliberately alter their genetic code. However, recent evidence has revealed that cells can circumvent their genetic confines by making diversified proteins which do not precisely adhere to the amino acid sequence specified in DNA. This process is mediated by the incorporation of nongenetically encoded methionine residues in specific amino acid positions, which is accomplished by misacylation of nonmethionyl-tRNAs with methionine. This process is highly regulated and cells are capable of charging 0.01–10% of their Met to non-Met-tRNAs in response to environmental changes. We find that Met-misacylation occurs in all three domains of life. In *E. coli* and the hyperthermophile *Aeropyrum pernix,* this adaptive translation does not occur under the optimal conditions for laboratory cultivation, but it can be activated within seconds or minutes in response to anaerobiosis, temperature fluctuations, or steady-state growth on certain carbon sources depending on the organism. We are testing the hypothesis that adaptive translation can adapt the proteome of an organism for optimal function in varying environments and may be advantageous in conditions for which there is no effective transcriptional response. Our investigation aims to establish that deviation from the central dogma has evolved to facilitate adaptation to fluctuating growth conditions known to be encountered in natural habitats.

Successful Development of a Nonhuman Primate Model of Congenital Cytomegalovirus Transmission

Awardee: Sallie Permar
Award: New Innovator Award
Awardee Institution: Duke University

Co-authors: Amitinder Kaur, Peter Barry, Kristy Bialas, Dollnovan Tran, Valerie Varner, Erika Kunz, Joshua Amos, Jennifer Kirchherr, Lisa Kattenhorn, Lynn Wachtman, Takayuki Tanaka
Co-authors' Institutions: New England Primate Research Center, University of California at Davis, Duke University

Congenital cytomegalovirus (CMV) infection is the leading infectious cause of brain damage and hearing loss in infants, resulting in permanent disabilities in 5,500 U.S. infants annually. Pre-existing maternal immunity to CMV provides protection against intrauterine virus transmission, as the transmission rate falls from 40% with no pre-conception immunity to less than 1% in mothers previously-exposed to CMV. Defining protective maternal immune responses will require a highly relevant animal model that recapitulates the anatomic, virologic, and immunologic aspects of congenital human CMV transmission. Intrauterine inoculation of fetal rhesus monkeys with rhesus CMV (rhCMV) results in neuropathogenesis similar to that of human congenital CMV, yet placental transmission of rhCMV has not been reported. Thus, a rhesus monkey model of rhCMV placental transmission is highly desirable for evaluating immunologic interventions that will eliminate congenital CMV transmission.

We aimed to develop a rhesus monkey model of congenital CMV transmission in rhCMV-seronegative pregnant monkeys, utilizing a herpes virus-free rhesus monkey colony. To increase placental virus exposure, we first depleted CD4+ T lymphocytes from pregnant, rhCMV-seronegative monkeys at 7 weeks of gestation via infusion of a CD4-depleting monoclonal antibody. The CD4+ T cell-depleted pregnant monkeys were inoculated intravenously 1 week later with a mixture of three rhCMV viral stocks, including 180.92, UCD52 and UCD59, and rhCMV viral load was assessed in maternal plasma and amniotic fluid. RhCMV immunohistochemistry was performed on placental and fetal tissues.

Complete depletion of peripheral CD4+ T cells was achieved in all three pregnant monkeys for 2 to 3 weeks post depletion, with a slow rise in peripheral CD4+ T cells thereafter. Maternal rhCMV viremia peaked at 2 to 3 weeks post-infection ($1.6–3.9 \times 10^6$ CMV DNA copies/ml) and was detectable through at least 10 weeks post-infection in surviving mothers. Two of the three CMV-exposed fetuses were spontaneously aborted at 3 weeks following inoculation, with foci of rhCMV-infected cells in placenta by immunohistochemistry. Concurrent with the fetal loss, one pregnant monkey sustained a gastrointestinal intussusception and was euthanized. RhCMV was detected in the amniotic fluid of all infected mothers ($2.1–5.8 \times 10^2$ copies/ml), with the peak amniotic fluid viral load occurring three weeks post inoculation in the nonaborted fetus.

We have developed a nonhuman primate model of congenital CMV transmission that can be utilized to define the virologic and immunologic factors associated with placental CMV transmission and assess immunologic interventions to block intrauterine CMV transmission.

RNA-Guided Genome Engineering Using CRISPR/Cas

Awardee: Lei Qi
Award: Early Independence Award
Awardee Institution: University of California, San Francisco

Targeted genome engineering is a key enabler for studying and programming cells, yet available tools for efficient and specific genome manipulation are limited. The bacterial CRISPR system offers a simple and powerful platform for RNA-guided DNA targeting. Here we show that the CRISPR system can be used to efficiently control gene expression, with the site of delivery determined solely by a small guide RNA (sgRNA). The regulation is specific for endogenous genes and works in diverse organisms including bacteria, yeast, and human cells. We show that the CRISPR system can be used as a modular and flexible platform for targeted regulation of gene expression, which opens new doors for the systematic perturbation and interrogation of gene function in both prokaryotic and eukaryotic cells.

Engineered Proteins Detect Spontaneous DNA Breakage in Human and Bacterial Cells

Awardee: Susan Rosenberg
Award: Pioneer Award
Awardee Institution: Baylor College of Medicine

Co-authors: Chandan Shee, Ben Cox, Franklin Gu, Elizabeth Luengas, Mohan Joshi, Li-Ya Chiu, David Magnan, Jennifer Halliday, Ryan Frisch, Janet Gibson, Ralf Nehring, Huong Do, Marcos Herandez, Lei Li, Christophe Herman, P.J. Hastings, David Bates, Reuben Harris, Kyle Miller
Co-authors' Institutions: Baylor College of Medicine, University of Texas at Austin, University of Minnesota, University of Texas MD Anderson Cancer Center

Spontaneous DNA breaks instigate genomic changes that fuel cancer and evolution, yet direct quantification of double-stranded ends (DSEs) has been limited. Predominant sources of spontaneous DSEs remain elusive. We report synthetic technology for quantifying DSEs using fluorescent-protein fusions of DSE-binding protein, Gam of bacteriophage Mu. In *Escherichia coli* GamGFP forms foci at chromosomal DSEs, and pinpoints their subgenomic locations. Spontaneous DSEs occur mostly one per cell, and correspond with generations, supporting replicative models for spontaneous breakage, and providing the first true DNA-breakage rates. In mammalian cells GamGFP—labels laser-induced DSBs antagonized by end-binding protein

Ku; co-localizes incompletely with DSE marker 53BP1 suggesting superior DSE-specificity; blocks exonucleolytic resection; demonstrates DNA breakage via APOBEC3A cytosine deaminase. We demonstrate directly that some spontaneous DSEs occur outside of S phase, when most DNA replication occurs. The data illuminate spontaneous DNA breakage in *E. coli* and human cells and illustrate the versatility of fluorescent-Gam for interrogation of DSEs in living cells.

Proteome Multitarget Drug Discovery

Awardee: Ram Samudrala
Award: Pioneer Award
Awardee Institution: University of Washington
Presenter: Jeremy Horst

Co-authors: Gaurav Chopra, Jeremy Horst, George White, Ambrish Roy
Co-authors' Institution: University of Washington

We have developed a shotgun system-based multitarget drug discovery pipeline to find new therapeutics for underserved diseases with higher efficiency, lowered cost, and increased success rates compared to current approaches. Computational structural biology and bioinformatics algorithms are applied to evaluate how all human-approved drugs interact with human and pathogen proteomes, and thus predict new therapeutic applications. The top predictions for herpes and dental caries are verified in the laboratory and predictions for thalassemia and tuberculosis are underway for experimental and clinical verification. I will present an integration of applied research on therapeutic discovery, building upon basic protein structure, function, and interaction prediction research that exploits evolutionary information using state of the art informatics techniques and using machine learning to build highly predictable drug-target relationship networks. Two foundational tenets underlie our approach: (1) the efficacy of a putative drug depends on its interaction with full proteomes and not just one target protein, and thus understanding relationships between signatures of compound-proteome binding will help identify new drugs more accurately; (2) a focus on drugs already approved for human use solves the toxicity problem and leads to faster turnaround between drug discovery and clinical verification.

Strong Inter-population Cooperation Leads to Partner Intermixing in Microbial Communities

Awardee: Wenying Shou
Award: New Innovator Award
Awardee Institution: Fred Hutchinson Cancer Research Center

Patterns of spatial positioning of individuals within microbial communities are often critical to community function. However, understanding patterning in natural communities is hampered by the multitude of cell-cell and cell-environment interactions as well as environmental variability. Here, through simulations and experiments on communities in defined environments, we examined how ecological interactions between two distinct partners impacted community patterning. We found that in strong cooperation with spatially localized large fitness benefits to both partners, a unique pattern is generated: partners spatially intermixed by appearing successively on top of each other, insensitive to initial conditions and interaction dynamics. Intermixing was experimentally observed in two obligatory cooperative systems: an engineered yeast community cooperating through metabolite-exchanges and a methane-producing community cooperating through redox-coupling. Even in simulated communities consisting of several species, most of the strongly-cooperating pairs appeared intermixed. Thus, when ecological interactions are the major patterning force, strong cooperation leads to partner intermixing.

Stochastic Regulation of Cellular Mechanotransduction:
Implications for Mitochondrial ATP Production, Extracellular
Matrix Remodeling and Contractility

Awardee: Béla Suki
Award: Transformative Research Award
Awardee Institution: Boston University

Co-authors: Harikrishnan Parameswaran, Jasmin Imsirovic, Nuria Martinez,
Erzsébet Bartolák-Suki
Co-authors' Institution: Boston University

Cells are active material that use ATP to sense and respond to external cues. ATP is generated in the mitochondria that can serve as mechanotransducers during regular cyclic stretch. However, cells in the body are exposed to variability in their mechanical microenvironment due to fluctuations such as blood pressure. Here we report that ATP production is downregulated in vascular smooth muscle cells stretched with monotonous sinusoids compared to cells stretched with physiological level of cycle-by-cycle stochastic variability in strain. Variable stretch enhances ATP production by increasing the expression of ATP-synthase's catalytic domain, cytochrome c oxidase and its tyrosine phosphorylation. These phenomena are mediated by the ability of variable stretch to increase the microtubule network's fractal dimension and enhance its association with mitochondria, which are destroyed during monotonous stretch that typifies current laboratory experimentation. Variable stretch also influences mitochondrial structure and function in stem cells. Furthermore, stochastic regulation of mechanotransduction appears to be a general theme of biology: we also show that cell proliferation, post-translations modification, calcium uptake, secretion of extracellular matrix molecules and cellular contraction are also influenced by the body's natural rhythms. Since such stochastic regulation represents normal physiology in vivo built into cell function by a billion years of evolution, our results have implications for all ATP-dependent and mechanosensitive intracellular processes.

Induction of Cancer Cell Death by Selective DNA Misincorporation

Awardee: Derek Taylor
Award: New Innovator Award
Awardee Institution: Case Western Reserve University

Telomeres cap and protect the ends of all human chromosomes. In healthy adult tissue, telomeres shorten with each round of cell division as part of the natural aging process. By limiting human cells to a finite number of divisions before induction of programmed cell death, telomere erosion functions as a tumor suppressor. Conversely, in cancer cells, an enzyme called telomerase is upregulated to synthesize telomere DNA and, thus, nullify the limited number of cell divisions. The upregulation of telomerase in ~90% of metastatic tumors is a primary contributor to the cancer cell's unlimited proliferative properties. Due to this unique and critical role in cancer biology, telomerase provides a novel target for innovative therapeutics. As such, direct telomerase inhibitors are currently being developed, with several compounds showing promise in treating a wide range of human cancers. However, the primary shortcoming with this methodology is that even after telomerase inhibition, the cancer cells must go through multiple rounds of division before telomere attrition results in replicative senescence. This delay allows cancer cells to develop other mechanisms of survival, such as alternative lengthening of telomere mechanisms, to overcome the effects of telomere shortening caused by telomerase inhibition.

This project is designed to explore a novel mechanism to use telomerase to deliver small molecule drugs to cancer cells specifically. Telomere DNA is bound and protected by specialized proteins including telomere repeat binding factors 1 and 2 (TRF1 and TRF2) and protection of telomeres 1 (POT1) proteins. TRF1/2 and POT1 bind telomere DNA with high specificity, such that a single change in telomere DNA sequence drastically reduces the binding efficiency. Abrogation of POT1 or TRF1/2 binding to telomeres induces an immediate DNA damage response. We predict that the misincorporation of non-native nucleotide analogs by telomerase into telomeric DNA will abrogate POT1/TRF1/TRF2 binding. The inability of telomere proteins to bind and protect the telomeres should elicit an immediate DNA damage response and initiate cell death specifically within cancer cells. The cell-killing potential of these non-native nucleotide compounds will be validated by measuring their potency and selectivity against telomerase-positive breast cancer cells and xenograft mouse models. If successful, our strategy will provide a selective mechanism to potentially treat a wide range of human cancers.

High-Throughput Identification of *Mycobacterium tuberculosis* Persistence Mechanisms

Awardee: Anna Tischler
Award: New Innovator Award
Awardee Institution: University of Minnesota

Mycobacterium tuberculosis (*Mtb*), the causative agent of the pulmonary infection tuberculosis, has evaded eradication from the human population due to its extraordinary ability to both persist in latently infected individuals and to generate antibiotic-tolerant persister cells. We hypothesize that different molecular mechanisms account for these two types of *Mtb* persistence and propose novel high-throughput genetic screens to identify and characterize these mechanisms. We will use a new technology, Tn-seq, which simultaneously identifies and quantifies transposon (Tn) insertion mutants within large random Tn mutant pools by massively parallel sequencing of Tn-genome junctions. Tn-seq has previously been used to analyze fitness of bacterial Tn mutants in various culture conditions in vitro and in some animal infection models *in vivo*. But Tn-seq has limited utility for studying growth conditions or infection models in which there are narrow colonization bottlenecks. We propose to adapt the Tn-seq method using bar-code sequence tags and multi-plexing to enable cost-effective analysis of smaller Tn mutant pools. We will use Tn-seq combined with infection of genetically modified mice to define on a genome-wide scale the factors that *Mtb* requires for persistence in the face of specific host adaptive immune defenses. We will additionally use an in vitro antibiotic selection strategy combined with Tn-seq to define factors that *Mtb* requires for optimal formation of antibiotic-tolerant persister cells. Ultimately, we will expand these results to relevant animal infection models, to demonstrate that the *Mtb* persistence factors we identify are viable drug targets. Therapeutics targeting these persistence factors would represent novel approaches to tuberculosis control that would sensitize *Mtb* either to natural host immune defenses or to existing antibiotics.

T Follicular Helper Cell Dynamics in Germinal Centers

Awardee: Gabriel Victora
Award: Early Independence Award
Awardee Institution: Whitehead Institute

Co-authors: Ziv Shulman, Sasha Targ, Alexander D. Gitlin, Mila Jankovic, Giulia Pasqual, Michel C. Nussenzweig
Co-Authors' Institution: The Rockefeller University

T follicular helper cells (Tfh) are a specialized subset of effector T cells that provide help to and thereby select high-affinity B cells in germinal centers (GCs). To examine the dynamic behavior of Tfh cells in GCs in mice we combined two-photon microscopy and optical highlighting using a photoactivatable fluorescent reporter. Unlike GC B cells, which are clonally restricted, Tfh are distributed among all GCs in lymph nodes and continually emigrated into the follicle and neighboring GCs. Moreover, newly activated Tfh cells invaded pre-existing GCs, where they contributed to B cell selection and plasmablast differentiation. Our data suggest that dynamic exchange of Tfh between GCs ensures maximal diversification of T cell help, and their ability to enter ongoing GCs accommodates antigenic variation during the immune response.

Formation and Regulation of the Translating mRNP

Awardee: David E. Weinberg
Award: Early Independence Award
Awardee Institution: University of California, San Francisco

Translational control is a major mode of gene regulation in eukaryotes that is often perturbed in human diseases. Despite extensive mechanistic studies of translation, it has not been possible to monitor in vivo most steps of translation initiation, which is the primary point of translational control. As a result, how endogenous translating messenger ribonucleoprotein particles (mRNPs) are formed and regulated in the competitive cellular environment is not completely understood. The goal of the proposed research is to develop and apply in vivo methods to study the complex process of translation initiation. The premise of our approach is that the translating mRNP contains many mRNA-binding proteins, so individual steps of initiation can be studied by identifying and characterizing the underlying mRNA-protein interactions. This novel approach to the translating mRNP leverages in vivo crosslinking to capture RNA-protein interactions as they exist in the cell, and high-throughput sequencing to identify these interactions across the transcriptome. We are now applying this approach to investigate three aspects of the translating mRNP: how mRNAs adopt the closed-loop structure, which is thought to enhance translation and mRNA stability; what determines the efficiency with which mRNAs are translated, which varies widely among endogenous genes for largely unknown reasons; and what role helicases play in scanning by the 40S ribosomal subunit, a

process that has not previously been observed or directly assayed. Collectively, the proposed research will improve our knowledge of cellular translation mechanisms and our understanding of how these mechanisms go awry in disease.

An Endogenous "Accelerator" for Viral Transcription Confers a Fitness Advantage

Awardee: Leor Weinberger
Award: Pioneer Award
Awardee Institution: University of California, San Francisco

Co-authors: Cynthia Bolovan-Fritts, Melissa Teng, Roy D. Dar, Brian Linhares
Co-authors' Institution: University of California, San Francisco

Biological signaling circuits, like electrical circuits, face a fundamental tradeoff between speed and amplitude: faster rates of initial increase are typically obtained at the cost of a higher steady-state level. This creates an evolutionary tradeoff when rapid signaling is essential but the signaling molecule is cytotoxic at high levels (e.g., for fever response, inflammatory cytokines, and many viruses). We describe a transcriptional circuit in a human herpes virus (CMV) that overcomes this tradeoff—and confers significant fitness to the virus—by converting signaling inputs into faster expression rates without amplifying final equilibrium levels in individual cells (Teng et al., *Cell*, 2012). Strikingly, the accelerator circuit maps to a transcriptional negative-feedback loop encoding an exceptionally high self-cooperativity (Hill coefficient ≈ 7). Binding of the virus's essential transactivator protein, IE2, to a single 14-bp sequence in its own promoter generates negative auto-regulation. In general, such accelerator circuits may provide a mechanism for signal-transduction circuits to respond quickly to external signals without increasing steady-state levels of potentially cytotoxic molecules.

Evolutionary Dynamics of Melanoma Metastasis

Awardee: Richard White
Award: New Innovator Award
Awardee Institution: Memorial Sloan-Kettering Cancer Center

Metastatic disease remains the defining feature of advanced malignancy, and is responsible for the vast majority of cancer deaths. Metastasis can be conceptualized as an evolutionary landscape, composed of key elements of Darwinian evolution: heritable (epi)genotypes, geographic dispersal, and novel microenvironmental selection pressures. I hypothesize that altering these evolutionary landscapes would provide a strikingly new method for treating cancer, in which the cancer cells can co-exist with the host over long periods of time. To achieve this requires a deep mechanistic understanding of the ways in which tumors generate novel genotypes, and how natural selection in the microenvironment amplifies these mutations. My work utilizes the zebrafish, a small vertebrate organism that has only recently come to light as an important cancer model. The zebrafish offers several unique capacities for studying metastasis: high-throughput transgenesis, unbiased genetic screens, and single cell imaging in the optically transparent casper adult fish. For the period of this proposal, I plan to address three primary questions in metastatic melanoma: (1) Can we identify the incipient genetic changes that allow for metastatic progression, whether they arise in the primary tumor or after dissemination?, (2) Is adaptive mutation required for metastasis, and can this be modified?, and (3) Can we identify host microenvironments that disfavor metastatic progression? To do this, I will build upon a zebrafish model of melanoma in which the BRAFV600E allele is expressed in melanocyte progenitors, in the context of p53 loss of function (the BRAFV600E;p53-/- model). By combining the "brainbow" fate mapping system with the BRAFV600E;p53-/- fish, I will use exome sequencing to identify genomic lesions associated with metastasis based on lineage, space, and time. These candidate changes can be functionalized using a metastasis assay I have developed in the transparent casper strain. I hypothesize that selection stressors during metastasis lead to a state of adaptive mutation, in which the error rate of DNA replication is temporarily increased to find an evolutionary solution to that stress. To test this, I will generate zebrafish with mutation reporters, and then use this system to probe whether adaptive mutation mediated by error-prone Y family DNA polymerases promotes metastatic progression. Finally, since the host microenvironment provides the ultimate selection pressure, I will perform an unbiased genetic screen to identify novel stromal regulators of metastatic progression. Together, these studies provide a comprehensive framework that considers both tumor cell-intrinsic and microenvironmental dynamics of metastatic disease. The long-term goal of my laboratory is to utilize this information to identify therapies which can convert disseminated disease into a stable state, unable to further progress, leading to large improvements in long-term survival in patients with established metastases.

Modulating Innate Immune Responses in the Genital Mucosa to Facilitate Transport and Biodistribution of Nanoparticle Vaccines

Awardee: Kim Woodrow
Award: New Innovator Award
Awardee Institution: University of Washington

Co-authors: Renuka Ramanathan and Jaehyung Park
Co-authors' Institution: University of Washington

Nanoparticle (NP)-based vaccine protocols for modulating immunity in the genital mucosa still remains severely understudied. Transport of intravaginally delivered nanoparticles faces several challenges including penetrating the mucus layer and crossing the epithelium. Our research focuses on the role of mucosal antigen presenting cells in mediating the transport of particulate vaccines across these mucosal barriers and to draining lymph nodes of the vaginal tract. We first mapped the transport and biodistribution of differentially sized nanoparticles delivered intravaginally to mice. We show that 20 nm but not 100 nm diameter nanoparticles have shorter residence times in the local vaginal tissue and are transported to lumbar lymph nodes within 24 hours. These results indicate that size affects both the rate of NP transport distally from the site of delivery as well as NP accumulation in select organs. We also evaluated the role of exogenously delivered or endogenously produced chemokines on the recruitment of phagocytic immune cells to facilitate NP transport across the vaginal epithelium. We examined DC chemokines (MIP-3α and β-defensin) and growth factors (GM-CSF), as well as natural and synthetic adjuvants for their ability to modulate the composition of the local APC population and facilitate NP transport. Our data show that administration of the DC growth factor GM-CSF alone or in combination with the MIP-3α chemokine enhances recruitment and expansion of MHC II+ CD11c+ CD11b+ myeloid-derived dendritic cells. Enrichment of this specific DC subset was >2-fold higher when using GM-CSF alone or in combination with MIP-3α than when using only CpG. These results demonstrate that peptide chemokines may be administered topically to recruit dendritic cell subsets to the vaginal tract. We leveraged this method as a strategy to expand the functional immune population relevant for uptake and trafficking of mucosally delivered NP and found that pre-treatment with chemokines and CpG, or other mucosal adjuvants, influenced significantly NP distribution and transport from the vaginal tract. It is expected that these studies provide insight to control of the host immune response at the site of the vaccine delivery and infection, and will inform the design of nanoparticle-based mucosal vaccines.

A Chemical Rescue Screen for Identification of Apicoplast Inhibitors as Anti-Malarials with Novel Mechanisms of Action

Awardee: Ellen Yeh
Award: Early Independence Award
Awardee Institution: Stanford University

The apicoplast is an essential plastid organelle unique to *Plasmodium* spp. and a promising anti-malarial drug target. Previously we showed that the essential function of the apicoplast in *P. falciparum* blood-stage parasites is the production of isoprenoid precursor, isopentenyl pyrophosphate (IPP). Herein, we report (1) a chemical rescue screen using IPP that specifically identifies small-molecule inhibitors of the apicoplast and (2) a pipeline of functional assays for detailed interrogation of their activity on specific apicoplast pathways. Using the "Malaria Box," a library of prioritized growth-inhibitory anti-malarial compounds, we identify inhibitors with novel modes of action against the apicoplast. Two apicoplast inhibitors are identified that can serve as drug candidate leads and/or chemical probes for studying and discovering essential apicoplast targets. This simple screen can be applied to large libraries of uncharacterized growth-inhibitory anti-malarials generated by several high-profile screening efforts and facilitate identification of specific inhibitors against this key anti-malarial target.

A Microfluidic Bone Marrow Niche for the Study of Hematopoiesis

Awardee: Ying Zheng
Award: New Innovator Award
Awardee Institution: University of Washington

The bone marrow produces nearly 500 billion blood cells per day in an adult human. Each type of blood cell is required for life: red cells deliver oxygen, white cells provide immunity, and platelets prevent bleeding, among other functions of these cells. Dysregulation of blood cell production leads to severe anemia, leukopenia, and thrombocytopenia, and produces substantial morbidity and mortality. Blood cell transfusion remains the mainstay of therapy, with concurrent concerns related to variations in quality and quantity of these products, and the risks of infectious contamination. New strategies to stimulate hematopoiesis in vivo or to produce sufficient numbers of blood cells in vitro would revolutionize the management of anemia and cytopenias. Here, we aim to develop an in vitro microfluidic bone marrow niche that recapitulates the bone marrow in its cellular and matrix components, but which can also be manipulated to determine the roles of each component in normal bone marrow function, and allow us to elucidate and control hematopoiesis. In this presentation, I will show our preliminary efforts towards building a microvascular environment, followed by recapitulating the specific structure and function of the marrow. Our microvascular system exhibits in vivo-like properties that they (1) have strong barrier function, (2) grow with pro-angiogenic stimuli, (3) mature with perivascular cell coating, and (4) are non-thrombotic but can become thrombotic

with inflammatory stimuli. The microvasculature undergoes dynamic remodeling when surrounded by hematopoietic stem cells (HSCs) or progenitor cells. For example, megakaryocytes embedded in the matrix migrate towards the vessel wall, penetrate across the endothelium, and shed platelets into the circulation. Marrow stromal cells build the extracellular scaffolds in the matrix that support HSC proliferation and differentiation. HSCs in the circulation home into the marrow stroma and the ones in the matrix mobilize into the circulation, which all depend on the chemokines, blood flow and stromal microenvironment in our niche. I will further indicate our plan to optimize the niche architecture for both microvessels and bone osteoblast surfaces to fulfill the complex structure and functions within the marrow and to generate blood cells. The success of this system would allow us to study hematopoiesis through the stepwise addition or removal of individual components of the marrow. This could also revolutionize preclinical testing for therapies to increase blood cell count in diseases where they are low, and holds the potential to generate blood cells in quantities sufficient for transfusion.

High-Contrast Visualization of Endogenous PSD-95 in Live Tissue

Awardee: Haining Zhong
Award: New Innovator Award
Awardee Institution: Oregon Health & Science University

Despite recent developments in fluorescence microscopy that have pushed imaging resolution beyond the diffraction limit, it remains challenging to investigate the dynamics of individual protein species in living cells under physiological conditions. A current bottleneck is the lack of an approach to mark endogenous protein species stoichiometrically with molecular precision and high contrast in living cells.

We have developed a mouse genetic strategy, named endogenous labeling via exon duplication (ENABLED), for systematic fluorescence labeling of endogenous proteins at their C-termini in either all or a subset of cells. We have used this method to label the critical postsynaptic protein PSD-95 with the yellow fluorescence protein mVenus at endogenous levels *in vivo*. Biochemical, electrophysiological, and morphological analyses provided the first demonstration that PSD-95^{mVenus} can functionally replace endogenous PSD-95. Notably, synaptic transmission and plasticity, which are sensitive to traditional overexpression approaches for visualizing PSD-95, were not altered. Using this mouse, we have visualized PSD-95 in live brain tissue with high contrast. We are examining the subcellular dynamics of PSD-95 in the context of neuronal plasticity. The ENABLED strategy can be used to systematically study the endogenous spatiotemporal dynamics of a large number of protein species that are compatible with C-terminal tagging by fluorescence proteins.

Balancing Hematopoietic Stem Cell Fate and Endothelial Identity in Hemogenic Endothelium

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There has been increased interest in the developmental phenomenon of hemogenic endothelium (HE), where hematopoietic stem cells (HSC) are generated during embryogenesis from specialized vascular beds; the process is transient and its precise regulation remains unknown. The concept of endothelial derived HSCs has broad clinical implications as it may open new avenues for in vitro blood production. We aim to dissect the signaling pathways that regulate hematopoietic versus endothelial fate in the context of hemogenic endothelium for the eventual application of reprogramming mature endothelium for blood production. One important obstacle is to identify possible activators and silencers of the hemogenic program. A prime candidate for HE activation is the Notch1 pathway, as Notch1 loss-of-function studies demonstrate impaired artery-vein identity as well as defective hematopoiesis from HE sites. However as hemogenic endothelium occurs in arterial vascular beds, arterial identity is a likely prerequisite for hemogenic endothelial generation. Thus, the true role of Notch1 in initial HSC generation is unclear, and the signaling that controls notch receptor and ligand gene expression is unknown. Sox17, an important transcription factor in early endodermal development, has been noted to exhibit arterial vascular expression later in development. Loss of Sox17 effects hemogenic endothelial development and fetal HSC survival. We have noted a lack of Sox17 expression in the hematopoietic cell clusters that emerge from aortic HE, as opposed to high expression in the endothelium. Our bioinformatics analyses and in vitro Sox17 loss of function studies suggest that Sox17 is able to regulate the notch signaling pathway through receptor and ligand expression. We demonstrate through timed loss and gain of function studies that Sox17 maintains arterial identity and silences the hemogenic program through a Notch1 mediated mechanism. Thus, Sox17 and Notch1, initially perceived as activators of HE, act as repressors of the HE program during HSC emergence. The continued expression of Notch1 and Sox17 prevents endothelial to hematopoietic transition, and Sox-Notch endothelial regulation may be a key target for re-initiation of endothelial hematopoiesis in mature vascular beds.