FARE2016 WINNERS

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CC
Guilhen Faure
Postdoctoral Fellow
Protein Structure/Structural Biology
mRNA folding energy profile impacts on protein structure
Protein translation speed vary depending on species, environment and even at a translated protein level. It impacts on protein abundance and can affect substantially the protein folding which occurs both inside and on the ribosome surface during the translation (so called protein co-translational folding). Several synonymous mutations and mRNA specific structures have been shown to slow down the translation rate leading sometime to a non-functional translated protein. This suggests mRNA which codes the primary amino acid protein sequence could also contain protein folding information. In order to explore this new layer of information retained by the mRNA, using the available protein structures from two eukaryotes (H. sapiens and S. cerevisiae) and two bacteria (E. coli and B. subtilis), we explored the potential impact of mRNA structure, which was inferred from the mRNA predicted folding energy profile, on the structure of the encoded protein. We found that mRNA folding energy is positively correlated with protein solvent accessibility and protein compactness in all species. In other words, highly structured mRNAs, which slow down translation, typically code for compact proteins burying a large proportion of residue in the core (called large core protein). We further analyzed the relationship between protein structure and mRNA folding energy and found that the size of domains positively correlates with the local mRNA folding energy whereas the length of unstructured linkers showed no such correlation. Thus, there appears to be a robust local relationship between the mRNA folding energy profile and domain features. Most proteins appear to undergo dynamical, co-translational folding whereby the secondary structure forms in the ribosomal exit channel and the tertiary structure forms at the surface of the ribosome before the completion of translation. The mRNA structure might act as a protein folding controlling device, by reducing ribosome speed when the nascent peptide needs time to form and optimize the core structure. This conclusion is compatible with the results of previous studies which indicate that time is required by the mRNA helicase to unwind highly structured mRNA and increasing translation speed leads to protein miss-folding. mRNA structure could provide a protection to degradation and aggregation as well as information to fold the protein.

CC
Andrew Hedman
Postdoctoral Fellow
Molecular Biology - Eukaryotic
Absence of IQGAP1 impairs insulin signaling and leads to insulin resistance

Insulin binds to the insulin receptor (IR), inducing receptor autophosphorylation and phosphorylation of the receptor associated protein, insulin receptor substrate-1 (IRS-1). Activated IRS-1 directly interacts with and activates phosphatidylinositol-3-kinase (PI3K), promoting PKB/Akt signaling. This is an essential pathway for normal insulin action, and impaired insulin signaling is a key deficiency in diabetes. IQGAP1 is a scaffold protein that interacts with multiple binding partners to modulate and integrate cellular signaling cascades in response to diverse stimuli. Since IQGAP1 binds to several growth factor receptors and is a component of receptor signaling pathways, we hypothesized that IQGAP1 may participate in insulin signaling. We tested this hypothesis using several strategies, including binding assays with pure proteins, cultured cells and knockout mouse models. Here we demonstrate that IQGAP1 associates with both IR and IRS-1 and modulates insulin signaling. In vitro, IQGAP1 directly interacts with the intracellular domain of IR and with the phosphotyrosine binding domain (PTB) of IRS-1. A multiprotein complex comprising pure IR, IRS-1 and IQGAP1 was observed in vitro. Additionally, IR and IRS-1 co-immunoprecipitate with IQGAP1 from cells. Further, the function of this interaction in modulating insulin signaling was investigated using knockout models. Experiments performed in IQGAP1-null cells revealed that the absence of IQGAP1 significantly reduced the ability of insulin to stimulate phosphorylation of IRS-1 and Akt, indicating defects in IR activation and downstream PI3K signaling, respectively. Importantly, loss of IQGAP1 significantly impaired glucose homeostasis in vivo. Collectively, these data reveal that IQGAP1 functions as a scaffold for IR and IRS-1 and implicate IQGAP1 as a participant in insulin signaling.

CC

Holger Roth
Visiting Fellow
Radiology/Imaging/PET and Neuroimaging

Multi-level deep convolutional networks for improved computer-aided detection and organ segmentation

Computer-aided detection (CAD) and organ segmentation (OS) are important yet challenging problems for medical imaging research. Previous methods show high sensitivities but at the cost of high false-positives (FP) or fail at organs with high anatomical variability. We design “deep learning” methods for coarse-to-fine CAD and OS. In CAD, we generate candidates with high sensitivities but ~50 FP per patient. A volume of interest (VOI) at each candidate is then fed to a second classification tier using random local views. These views can be used to train deep Convolutional Neural Network (CNN) classifiers, inspired by the visual cortex of the brain. They learn image features automatically from examples. In testing, our CNNs assign probabilities at each VOI, aggregated over all views. This is a highly selective process of rejecting difficult FP while preserving high sensitivities in three different data sets: 59 cases for spine metastases, 179 cases for lymph nodes, and 1,186 cases for colonic polyps CAD. In all 3 datasets of different content and sizes, our CNN approach demonstrated improvement of CAD. We raise absolute CAD sensitivities by 13%, 34% and 17% at 3 FP per case for spinal metastases, lymph nodes and colonic polyps, respectively. This matches or outperforms previous work. For OS, we use a bottom-up approach for organ OS in CT. We design several variations of CNNs in the context of hierarchical, coarse-to-fine classification on image patches and regions (superpixels). First, we present a dense labeling of local image patches via P-CNN and nearest neighbor fusion. Then we apply a regional

CNN (R1-CNN) that samples a set of bounding boxes at superpixel in a “zoom-out” fashion. Our CNNs learn to assign probabilities for each superpixel. Lastly, we study a stacked R2-CNN leveraging the joint space of CT intensities and dense P-CNN probability maps with structured predictions as post-processing. Here, we evaluate on CT of the pancreas in 82 cases (62/20 for training/testing). We achieve the highest reported Dice Similarity Coefficients (OS accuracy) so far with 84.2%±3.6% in training and 75.8%±5.4% in testing. This raises the state-of-the-art from ~68% to ~76% at the computational cost of minutes, not hours as previously. Our deep learning approaches could be applied as multi-organ CAD/OS since CNNs naturally support this. CAD and OS problems with large variations and pathologies (such as in tumors) could be solved by similar deep learning methods.

CC

Veit Sandfort
Postdoctoral Fellow
Radiology/Imaging/PET and Neuroimaging

Rate of carotid disease progression in asymptomatic low to moderate risk individuals: the RIGHT study

Purpose: Cardiovascular disease is a leading cause of death in industrialized countries. Advances in imaging enable serial assessment of atherosclerosis and could provide insights into disease progression. The aim of this study was to examine the feasibility of serial carotid MRI in an asymptomatic low to moderate risk population and to analyze the association between the progression of carotid MRI findings and baseline clinical factors. Materials and Methods: High resolution 3 tesla carotid MRI was performed on 106 asymptomatic patients with hyperlipidemia at baseline and after 1 year follow up as part of the RIGHT study (Randomized Trial of Imaging Versus Risk Factor-Based Therapy for Plaque Regression). All patients received statin therapy. The average wall area per slice (defined as outer vessel area minus lumen area) was measured in five axial slices of the proximal internal carotid artery on both sides and averaged. Comparable slices in the follow up exam were selected and the images were then analyzed separately. The relationship of parameters of progression with clinical risk factors was evaluated using univariate and multivariable logistic regression analysis. Results: The mean subject age was 65 years (63 % male). The mean Framingham 10 year risk for CAD was 9%, the mean AHA 10 year risk was 12% and the mean BMI was 28. The mean carotid area change showed a slight regression (-0.3 square mm, not significantly different from zero). Overall regression occurred in 57 % of the subjects while in 43 % progression was noted. In a univariate analysis only obesity (defined as BMI>30) was significantly associated with progression of carotid artery disease. In the majority of obese subjects progression was seen (19 of 28 subjects showed progression, 68 %) while in the majority of non-obese subjects regression occurred (51 of 78 subjects showed regression, 65%, chi square p<0.005). In a multivariate model (including sex, age, BMI and AHA risk category) the only parameters associated with disease progression was obesity (p<0.005). Discussion: Obesity was associated with progression of carotid atherosclerosis in this study. There was no statistical correlation with other clinical factors (age, gender, LDL and AHA risk category). Obesity has been shown to cause higher levels of vascular inflammation and has an unfavorable effect on dyslipidemia. Both factors might offer an explanation for our findings.

NCATS

Ian Goldlust

Doctoral Candidate

Hematology/Oncology, Tumor Immunology, and Therapy

Modelling and targeting residual tumor cells in high grade serous ovarian cancer

Many types of cancer respond well to initial treatment, yet most patients develop and succumb to relapsed disease. This phenomenon is exemplified in ovarian cancers where first-line platinum-based chemotherapeutics and cytoreductive surgery leaves optimally debulked patients with no detectable disease, yet nearly 75% of these women relapse with disease that is clonogenically related to the primary cancer. Such a phenomenon indicates that residual tumor cells (RTCs) persist after treatment and that patients could benefit from consolidation therapies specifically targeting this population. RTCs cycle infrequently and have the capacity to repopulate a tumor from a low number of cells. This population is also the least sensitive to standard chemotherapy, is able to survive and metastasize to other vital organs, and leads to fatality in patients. Monolayer culture is a poor model for RTCs since monolayer cells cycle quickly and are sensitive to chemotherapeutics. We developed a model for RTCs by culturing tumor cells as three dimensional spheroids in specially defined media and low-attachment conditions. We performed differential expression analysis using RNA-sequencing on several tumor types grown in spheroid and monolayer and found that the cell cycle was impaired in the spheres relative to monolayer culture. Subsequently, we performed a metabolic profiling of the two culture conditions which indicated an increased reliance on oxidative phosphorylation in the spheroids whereas cells grown in monolayer relied more heavily on glycolysis. Taken together these results indicate that spheroids could be used as a model for RTCs. With this well-defined model, we then sought to screen for compounds that could specifically target RTCs. Our pharmacological profiling provided mechanistic insight into the baseline changes that these alternate culture conditions impart on cells of common origin. The small molecule elesclomol was identified as the most potent and reproducible compound with selective toxicity compared to cells grown in standard conditions. Leveraging a recently developed whole genome RNAi screening platform, we further screened for RNAi constructs that specifically target RTCs or increase elesclomol's activity. Finally, we tested elesclomol's efficacy in a genetically engineered mouse model for high grade serous ovarian cancer that closely resembles the human disease on histologic and molecular levels.

NCATS

Monica Kasbekar

Doctoral Candidate

Biochemistry - Proteins

Abstract removed by request of author

NCI-CCR

Fardokht Abulwerdi

Postdoctoral Fellow

Chemistry

A Microarray-Based Approach For The Development of RNA-Binding Small Molecules With Anti-HIV

Activity

RNA has emerged as a critical regulator of diverse biological processes, including viral infectivity, gene expression, signaling, and diseases. While RNA is an attractive therapeutic target for small molecule drugs, identifying small molecules that bind to RNA has lagged behind and novel approaches are in demand. Our laboratory has addressed this problem by developing a small-molecule microarray (SMM)-based screening platform. Here, small molecules are covalently linked to a glass surface and hybridized with an RNA of interest to screen for binding interactions. As an initial study, we previously screened a collection of 20,000 compounds for binders to the HIV-1 transactivation response element (TAR) RNA, a well- established target for inhibiting HIV replication. This work led to the identification of a thienopyridine compound that selectively binds to TAR, exhibits an EC50 of 28uM in a viral replication assay, and has no toxicity up to 1mM in uninfected cells. Herein we report a convergent 3-step synthetic route to generate a library of twenty analogs of this lead compound to improve potency and establish a structure-activity relationship (SAR). Analogs were evaluated in a Thermofluor assay to assess melting temperature (Tm) changes of TAR upon compound binding. A majority of analogs caused a 1-2o decrease in Tm indicating that binding of this class of compound causes destabilization of TAR. In addition, subtle changes in aliphatic substituents on the pyridine ring have pronounced effects on binding, while changes to a linker group are tolerated. Additionally, ligand-observed 19F NMR studies with 105FA, an analog that contains a trifluoromethyl group, showed concentration-dependent perturbation of the fluorine signal in the presence of TAR RNA. Finally, the analogs were evaluated in a viral replication assay, where two analogs showed 3-fold increase in potency compared to the initial lead with minimal toxicity. In summary, a novel class of small-molecule TAR binders has been identified. In this class, a clear SAR is observed with stringent structural requirements to recognize and bind TAR. This effort led to a more potent analog, 105FA, that has improved activity in a cell-based viral replication assay. Future work includes mapping the binding site of this class of compound to TAR by NMR and chemical probing techniques as well as using SMM technology more broadly in identifying small molecules for other RNAs important in diseases.

NCI-CCR

Ekaterina Allen

Postdoctoral Fellow

Vascular Disease and Biology

GPR124 functions as a WNT7-specific co-activator of beta-catenin signaling in brain endothelium

G-protein coupled receptor 124 (GPR124) is an orphan receptor in the adhesion family of GPCRs. Previous global or endothelial-specific disruption of Gpr124 in mice led to defective CNS angiogenesis and blood brain barriergenesis. Similar developmental defects were observed following dual deletion of Wnt7a/Wnt7b or deletion of beta-catenin in endothelial cells, suggesting a possible relationship between GPR124 and canonical WNT signaling. Canonical WNT/beta-catenin signaling is known to play important roles in many cellular processes such as body axis formation, cell proliferation, and angiogenesis. Wnt stabilized beta-catenin translocates to the nucleus where it interacts with TCF/LEF transcription factors to regulate gene expression. To study a possible role of GPR124 in canonical WNT/beta-catenin signaling, we created a reporter cell line by introducing a TCF/LEF-promoter driven luciferase vector into Gpr124-/- immortalized mouse embryonic brain endothelial cells (BECs). Co-

expression of GPR124 with WNT7 significantly enhanced beta-catenin signaling in BECs. GPR124 only potentiated WNT7A and WNT7B signaling among the 19 WNT family members, demonstrating the remarkable specificity of this effect. Next we identified structural determinants of GPR124 function by generating a series of N-terminal and C-terminal truncation mutants. The N-terminal leucine rich repeat region in the extracellular domain and the C-terminal four amino acids encoding an intracellular PDZ binding motif were both found to be important for GPR124 stimulation of beta-catenin signaling by WNT7. Finally, we addressed the role of GPR124 in WNT7 signaling in vivo. Lac-Z staining of BAT-GAL (beta-catenin driven beta-galactosidase) reporter mice revealed a significant reduction in beta-catenin signaling in brain endothelium of Gpr124-/- :BAT-GAL+ mice. Importantly, genetic interaction studies using Gpr124+/-:Wnt7a+/-:Wnt7b+/- compound mutant embryos revealed abnormal CNS vascularization and barriergenesis that was absent in Gpr124+/- or Wnt7a+/-:Wnt7b+/- embryos. This study reveals a vital role for GPR124 in potentiation of WNT7 induced canonical beta-catenin signaling with important implications for understanding and manipulating CNS-specific angiogenesis and blood brain barriergenesis. The fact that GPR124 is also upregulated in tumor vasculature suggests that manipulation of GPR124 also holds promise for the development of new anti-angiogenic agents to block tumor growth.

NCI-CCR
Heekyong Bae
Postdoctoral Fellow
Immunology - Autoimmune
Deletion of the IFN-gamma 3' UTR AU-rich element results in primary biliary cirrhosis in female C57/BL6 mice
Primary biliary cirrhosis (PBC) is an autoimmune liver disease and occurs primarily in women (> 90%). PBC is characterized by lymphocytic infiltrates near portal tracts, destruction of small bile ducts and presence of anti-mitochondrial Abs (AMAs), and eventually leads to liver failure. Although the incidence of PBC is increasing over time, there is a lack of effective treatment and current mouse models do not present the sex difference relevant to the clinical phenotype. IFN gamma is elevated in patients with PBC, but the functional role of IFN gamma on PBC is not known. Here, we characterized the distinctive pathological phenotype of PBC in a mouse model of chronic IFN gamma expression generated by deletion of the IFN-gamma 3' UTR AU-rich element. Histological assessment of the liver shows that female ARE-Del mice have moderate immune cell infiltration and bile duct destruction near portal tracts. Consistent with clinical features seen in PBC, female ARE-Del have over 30 fold upregulated total bile acids and spontaneous production of AMAs in serum. In liver function tests, female ARE-Del mice have significantly elevated levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum, indicating ongoing liver damage. To investigate the mechanisms involved in PBC development, we analyzed liver gene expression in ARE-Del mice using RNA-sequencing. By focusing on genes whose expression is highly specific to female ARE-Del, we determined that IFN gamma and interferon alpha receptor are top upstream regulators for this female specific disease progression based on pathway analysis. We also observed that TNF-alpha-induced mitochondrial dysfunction and nuclear factor of activated T-cell 5 (NFAT5) are critically involved in downstream signaling pathways. Remarkably, deletion of the interferon alpha receptor in ARE-Del mice significantly reversed the pathological

phenotypes of PBC. We are testing if IFN gamma neutralizing antibody can ameliorate disease progression in female ARE-Del, and we are also further characterizing the identified downstream signaling pathways for their role in disease progression. Taken together, the ARE-Del mice model mimics the features presented in human PBC and demonstrates that the IFN signature is critical in PBC progression. Importantly, this mouse model is the first to present the sex differences seen in PBC, and thus has a high potential for use in testing clinical therapeutic approaches for this disease.

NCI-CCR
Benjamin Blehm
Postdoctoral Fellow
Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology
Cells have feelings too: Dissecting in situ cell-ECM force interactions in 3D culture models and in Danio rerio

The physical properties of the microenvironment, including the stiffness, dimension, and topography have profound impacts on cell fate, tissue assembly and malignancy. However, delineating the complex interplay between cells and their physical microenvironment is challenging using existing techniques and remains elusive. What is needed is the ability to resolve and quantitate minute forces that cells sense in the local environment (microns) within thick tissue (mm). 3D culture models approximate in vivo architecture and signaling cues, allowing for real time characterization of cell-ECM dynamics. Using an optical tweezers we performed active-passive microrheology to measure material properties at length scales unobtainable by bulk rheology. Briefly, an optical tweezer employs a focused laser beam to apply forces to refractive sensors. Microspheres are used as these local sensors where we measure the local displacement both due to Brownian motion (passive) and force applied by the laser beam (active). These displacements are then converted into physical properties. We show for the first time the ability to generate stress-strain relationships in thick gels at frequencies relevant to cellular processes, allowing us to quantify minute forces. We spatially map the viscoelastic properties of the microenvironment in vitro and in vivo during tumorigenesis. First, we interrogated the cell-ECM interactions as cells proliferate and remodel the hydrogel. We determined that these hydrogels rapidly soften from stiffnesses of hundreds to tens of Pascals well before the first cell division. This was due to rapid swelling and reorganization of protein polymers. Modulating rate of expansion, we found a threshold where cells are unable to respond and undergo apoptosis. Pharmacological inhibition of myosin IIa ameliorates cell death implicating cell contractility for initial cell survival when cultured in a 3D hydrogels. We determined that the forces that cells sense or transmit are short-range, and are quickly dissipated within the hydrogel. We then applied our technique in live zebrafish, Danio rerio, mapping the viscoelastic properties of the fish tissue in regions where human tumor cells were actively proliferating. This is the first time in situ optical trap calibration and micron-scale mechanical characterization has been applied in a thick hydrogel and in vivo, accurately characterizing physical determinants of the local microenvironment.

NCI-CCR
Myriem Boufraqech
Postdoctoral Fellow
Tumor Biology and Metastasis

Lysyl Oxidase (LOX) regulates SNAI2 expression in thyroid cancer

Epithelial–mesenchymal-transition (EMT) is associated with cancer progression and metastasis. Patients with anaplastic thyroid cancer (ATC) uniformly succumb to locally advanced and metastatic disease, which is believed to be mediated by EMT. We previously reported that lysyl oxidase (LOX) is overexpressed in ATC and regulates cancer progression and metastasis, and is a prognostic marker in patients with differentiated thyroid cancer. Although LOX is thought to mediate EMT, the molecular mechanism involved remains unknown. Thus, in this study we investigated the effect of LOX on established mediators (vimentin, N-cadherin, E-cadherin, and transcription factors–TWIST1, SNAIL1, SNAI2 and ZEB1) of EMT. We found that LOX knockdown decreased SNAI2 mRNA and protein expression in ATC, HeLa, and breast cancer cell lines with high LOX expression. Furthermore, knockdown of LOX in a mouse model of metastatic ATC reduced SNAI2 expression and metastases. Because inhibition of LOX catalytic activity with BAPN did not affect SNAI2 expression, we next investigated if LOX directly regulates the transcription of SNAI2. We found that LOX binds the SNAI2 promoter in ATC and breast cancer cell lines and transactivates SNAI2 promoter as knocking down of LOX significantly reduced SNAI2 promoter-luciferase activity. Furthermore, we found a positive correlation between LOX and SNAI2 expression in thyroid cancer samples (p=0.009, r= 0.35) by RT-PCR and which was validated in a public genomic database (p<0.001, r=0.70). In addition, we showed a positive correlation between LOX and SNAI2 expression in a large publically available genomic dataset of breast cancer (p<0.0001, r=0.67). Similar to LOX, there was overexpression of SNAI2 in ATC compared to normal (p<0.02) and papillary thyroid cancer (p<0.01). Immunohistochemistry staining revealed co-localization of LOX and SNAI2 protein in aggressive thyroid cancer samples. Since we observed no change in Cadherins and vimentin with LOX knockdown, we hypothesized that a LOX-SNAI2 axis mediates EMT by targeting MMPs/TIMPs which regulate cancer cell invasion and metastasis. We found LOX or SNAI2 knockdown inhibited TIMP4 expression and secretion in vitro. These results provide the first evidence that LOX is a new transcriptional regulator of SNAI2 and that a LOX-SNAI2-TIMP4 axis is important in thyroid cancer progression and in other solid malignancies.

NCI-CCR
Chad Brocker
Research Fellow
Gene Expression

Peroxisome proliferator-activated receptor alpha-mediated regulation of long non-coding RNA and the potential influence on lipid homeostasis.

The advent of deep sequencing has revealed that the transcriptional landscape of the genome is overwhelming complex. One of the most striking observations is that the abundance of non-coding RNA (ncRNA) dwarfs protein-coding transcripts. In fact, only one-fifth of transcriptome is attributed to coding genes. Moreover, over 90% of genetic trait-associated SNPs occur within non-coding regions of the genome highlighting the functional importance of these sequences. Long ncRNAs (lncRNA) represent a relatively unstudied group of ncRNA with diverse functions within the cell including suppressing gene expression. Peroxisome proliferator-activated receptor alpha (PPARa) is a nuclear receptor and major regulator of lipid metabolism. PPARa is activated in response to fasting and promotes the use of fatty acids as an energy source. As such, PPARa and downstream genes represent important targets for

treating metabolic diseases including hyperlipidemia, type 2 diabetes and obesity. While PPARa activates expression of most target genes, it also suppresses expression of select genes. We hypothesized that this is due to transrepression possibly involving lncRNAs. To the regulation of lncRNA by PPARa, RNAseq was performed on livers from mice treated with the PPARa agonist WY14643. Analysis revealed 2557 putative lncRNA species, most of which represent novel transcripts. Of these, 523 were robustly regulated in response to WY. lncRNA levels did not correlate with neighboring genes indicating lncRNA are expressed as independent transcriptional units. PPARa-mediated changes in the eight most differentially regulated lncRNA were experimentally confirmed by qRT-PCR in WY-treated mice. Analysis of PPARa knockout mice showed that expression of these lncRNA were PPARa-dependent. Monitoring mRNA levels over 24 hours after activation revealed that lncRNA expression profiles paralleled known protein-coding target genes. PPARa ChIPseq datasets from agonist-treated mice were downloaded from the Gene Expression Omnibus and promoter regions were screened for PPARa binding. All lncRNA analyzed exhibited binding peaks within the 5' promoter region, which was further supported by those computationally predicted. Taken together this preliminary study provides evidence for the direct regulation of lncRNA by PPARa, suggesting that these RNA may play important roles during lipid homeostasis in vivo and possible function in transrepression of gene expression by PPARa activation.

NCI-CCR
Yanhui Cai
Visiting Fellow
Immunology - General
Long-lasting CTL responses can be efficiently boosted by a single pDNA vaccination in rhesus macaques
Background: To improve efficacy, DNA vaccines were developed to overcome HIV sequence diversity and to address potential competition with "decoy" epitopes. We have designed a vaccine focusing on the conserved elements (CE) of HIV gag. All CE DNA-vaccinated macaques developed robust CE–specific memory responses with a significant fraction of cytotoxic T cells. Macaques were vaccinated with CE DNA followed by boost with DNA expressing complete Gag, which significantly boosted the preexisting CE responses increasing both magnitude and breadth. This concept is currently developed for a clinical trial supported by NIAID/HVTN. Methods: The longevity of HIV-specific cellular immunity induced by plasmid DNA vaccination was monitored in macaques two years after immunization. PBMC samples were stimulated with peptide pools covering different protein regions and the antigen-specific responses were measured combining surface and intracellular cytokine staining followed by flow cytometry. Results: We found persistent levels of HIV-specific T cells in the blood of all 11 immunized animals even 2 years after the last vaccination. These responses were mediated by both CD4+ and CD8+ T cells characterized by a cytotoxic memory phenotype. The macaques received one single booster vaccination with plasmid CE DNA, which led to a rapid and significant (~6-22-fold) increase of the CE-specific responses reaching levels higher than those obtained during prior vaccinations. CE-specific CD4+ and CD8+ T cells comprised effector memory (EM; CD28+CD95+CCR7+) and transitional memory (Tm; CD28+CD95+CCR7-) T cells harboring Granzyme B indicative of their cytotoxic potential. Conclusions: Our data demonstrate that the HIV-1 CE DNA vaccine was able to induce long-lasting, potent cytotoxic

memory T responses that can be rapidly activated. This finding supports the use of DNA as a potent vaccine platform.

NCI-CCR
Smita Chandran
Research Fellow
Immunology - Lymphocyte Development and Activation
IL-7R and c-myc expression identify differentiated effector CD8+ T cells that can establish memory in humans after adoptive transfer
The optimal T cell attributes for the adoptive immunotherapy of cancer and viral diseases are currently unclear. Recent adoptive transfer clinical trials using ex vivo expanded tumor infiltrating lymphocytes has provided evidence that differentiated effector T cells can mediate durable anti-tumor responses in selected cancer patients. The capacity of these transferred cells to persist in the host was found to strongly correlate with their clinical activity. Thus, there is significant interest in identifying intrinsic markers that may predict whether antigen specific effector T cells develop into long-lived memory cells versus undergoing apoptosis after infusion in humans. We recently reported the long term persistence of ex vivo expanded tumor specific CD8+ T effector clones in refractory metastatic melanoma patients after adoptive T cell transfer. By utilizing these highly homogeneous clone populations, we sought to define the pre-infusion cellular and molecular attributes associated with their effector to memory transition. Comparative transcriptional profiling found the pre-infusion clone mRNA expression levels of the IL-7 receptor (IL-7R) and the proto-oncogene, c-myc, directly correlated with the level of clonal persistence after adoptive transfer in humans. The predictive value of these markers was further established by utilizing IL-7R protein, induced pSTAT5, and c-myc mRNA expression to prospectively identify human tumor specific effector clones that could engraft after controlled adoptive transfer into highly immunodeficient mice. These findings support that IL-7R and c-myc expression are valuable cell intrinsic markers that can predict the fate of effector CD8+ T cells after adoptive transfer.

NCI-CCR
Huimin Chen
Postdoctoral Fellow
Biophysics
Understanding the coupling of transcript elongation and splicing through CRISPR/dCas induced pausing
Splicing of pre-mRNA can occur concurrently with transcription, and studies have shown that the kinetics of splicing and transcription elongation are coupled. For example, co-transcriptional splicing occurs more efficiently when the pre-mRNA is synthesized by a slower elongating RNA Pol II mutant in vitro, however the evidence for this in vivo is inconclusive. In the ‘first in first out’ model, it is thought that pre-mRNA is processed in the approximate order of its synthesis with upstream introns getting a larger window of opportunity than downstream elements. In this scenario, the rate of elongation is expected to have an effect on the kinetics of splicing and also alternative splicing. Indeed, binding of protein factors on specific regions of the gene to slow down transcription elongation has been shown to favor inclusion of alternative exons. However, the mechanism by which splicing and elongation are coupled in vivo is still unknown. Here, we use live cell imaging of transcription of a reporter gene in real

time to observe the effects of stalling RNA Pol II at specific regions of the gene important for splicing to elucidate the mechanisms by which splicing and elongation are coupled. A cell line with a stably integrated inducible human betaglobin reporter gene and coding for PP7 and MS2 stem loops incorporated in an intron and the 3'UTR of the gene allow us to tag fluorescent proteins to the pre-mRNA as the stem loops are transcribed and to image the transcription sites in real time. To alter the rates of elongation in a site specific manner, we used the CRISPR technology to target catalytically inactive Caspase9 (dCas) molecules to three regions critical for proper splicing: the branch point, 3' splice acceptor and the 3'UTR. dCas has been shown to block elongation when targeted to the non-template strand. I obtained time traces showing that the movement of RNA Pol II through the gene is indeed blocked intermittently when dCas is bound to the non-template strand. Using correlation analysis on the fluorescence intensity fluctuations at the transcription site, we extracted meaningful parameters for transcript elongation and splicing. We also observed that stalling of RNA Pol II in the 3'UTR of the gene changed the kinetics of co-transcriptional splicing. Ongoing work comparing changes in splicing by CRISPR induced pauses all along the gene will provide a framework for elucidating the coupling between transcript elongation and splicing.

NCI-CCR
Jiandong Chen
Visiting Fellow
Molecular Biology - Prokaryotic
A Method to Efficiently Select mRNA Targets and Identify sRNA Regulators
Small regulatory RNAs are present in all three kingdoms of life, and their essential roles in developmental biology and cellular physiology have been well documented. Bacterial small regulatory RNAs (sRNAs) are key stress-response regulators that can shield bacteria against adverse conditions through fine-tuning a broad repertoire of stress-response genes. Mechanistically, bacterial sRNA regulation is achieved through controlling the translation and/or stability of target mRNAs by an antisense mechanism. The most critical protein factor involved in this post-transcriptional regulation is the global sRNA chaperone Hfq, and thus far ~30 Hfq-dependent sRNAs regulating ~100 mRNA targets have been found in Escherichia coli. However, an efficient and reliable method to select candidate mRNA targets of interest is not readily available yet. In this study, we present an effective and efficient combinatorial method to select mRNA targets and identify sRNA regulators. The candidate mRNA targets were selected based on physical association with Hfq protein plus transcript level change in the presence versus absence of Hfq. By analyzing genomic data (RNA microarray and RNA immunoprecipitation tiling-array), we predicted 94 candidate mRNA targets that highly associate with Hfq and largely change transcript levels in the hfq mutant. We further validated the selection method by experimentally identifying sRNA regulators for 3 selected candidate mRNA targets (recA, mutS and yhcN) using a reporter-based, sRNA overexpression library method. We report here that Spot42 sRNA strongly represses recA and yhcN, and ArcZ sRNA represses mutS gene expression, both by a post-transcriptional mechanism. More importantly, we showed that in all three cases ablation of physiological levels of the sRNA is sufficient to disturb target mRNA expression, indicating a bona fide regulation related to the bacterial physiology. This was further corroborated by studying sRNA regulation of MutS function where we showed sRNA-mediated dampening of mutS expression led to

elevated stress-induced bacterial mutagenesis. Taken together, this study builds a pipeline for efficiently identifying sRNA regulators involved in specific biological events of interest by providing guidelines for selecting candidate mRNA targets and experimentally identifying and validating sRNA regulators in action.

NCI-CCR
Christopher Chien
Postdoctoral Fellow
Tumor Biology and Metastasis
Chemotherapy induced signaling from bone marrow stromal cell niches aids survival of leukemia cells leading to disease recurrence
Despite high remission rates in pediatric acute lymphoblastic leukemia (ALL), ALL remains the number one cause of cancer-related deaths in children due to relapse of disease. Therefore therapies are greatly needed for the treatment of recurrent ALL. Patients with ALL that overexpress thymic stromal lymphopoietin receptor (TSLPR) have rates of relapse nearly double the rate of non-overexpressing patients. We hypothesize that TSLPR signaling in bone marrow stromal cell niches is driving relapse of ALL and sought to elucidate the mechanisms underlying the increased risk. We developed a syngeneic transplantable model of high TSLPR expressing leukemia (TSLPRhigh). We found an 8-fold difference in the percentage of TSLPRhigh cells in the bone marrow when compared to the parental cell line (TSLPRlow) 5 days after implantation into mice corresponding to an early stage of disease as leukemia burden was <2% of the BM and when ALL is likely in bone marrow niches. Interestingly, in vivo lethality endpoints and in vitro and in vivo cell growth were no different between the TSLPRhigh and TLSPRlow ALL indicating that TSLP does not alter ALL proliferation. From this data we can infer that TSLP/TSLPR signaling is likely to be most critical at early stages of leukemia development when bone marrow stromal niches are intact and not relevant once leukemia burden is so high that it has disrupted the normal bone marrow architecture. It has been reported that bone marrow stromal cells (BMSC) can produce TSLP after stimulation with inflammatory cytokine IL-1alpha and we found that TSLP mRNA and TSLP protein were elevated in mice injected with IL-1alpha in the bone marrow and serum respectively. Five days after injecting mice with ALL and IL-1alpha, we observed TSLPRhigh levels were enhanced relative to TLSPRlow ALL implying that IL-1alpha induced TSLP levels to aid early progression of TSLPRhigh ALL. Chemotherapy is known to induce inflammatory cytokines in patients and likewise we found cytarabine (commonly used to treat ALL) was able to dramatically induce IL-1alpha expression from both mouse and human ALL cells while dexamethasone, an anti-inflammatory agent did not. In total, these experiments suggest that chemotherapeutic treatment of ALL could potentially provide an unintended advantage to TSLPR overexpressing ALL at stages of low disease burden. Future studies are focused on developing TSLP blocking strategies to combine with chemotherapy to mitigate this effect.

NCI-CCR
Tobias Eggert
Postdoctoral Fellow
Tumor Biology and Metastasis
Senescent hepatocytes secrete CCL2 to accelerate liver cancer growth via accumulation of

immunosuppressive myeloid cells

Aim: Oncogene-induced senescence causes hepatocytes to secrete cytokines, which induce the immune-mediated clearance of these senescent cells, preventing malignant transformation and tumor initiation; a process termed 'senescence surveillance'. However, senescent hepatocytes can give rise to hepatocellular carcinomas (HCC), if additional oncogenic mutations, e.g. p53 mutation, abrogate the senescence program. We set out to investigate the effect of senescent cell secreted cytokines on growth of neighboring liver tumor cells. Experimental procedure: To induce senescence in mouse livers, we hydrodynamically injected a transposon system, which encodes for either oncogenic Nras (NrasG12V) or an effector loop mutant (NrasG12V/D38A) that is incapable of signaling to downstream pathways, into C57BL/6 or CCR2-/- mice. To achieve tumor development in senescent livers, hepatocellular carcinoma cells were intrasplenically injected into mice, which expressed either NrasG12V or NrasG12V/D38A in the liver. Hepatic immune cell infiltrates were analyzed using flow cytometry and immunohistochemistry. Immunosuppressive ability of CD11b+Gr1+ myeloid cells was analyzed in vitro after fluorescence-activated cell sorting. Furthermore, peritumor tissue of 226 HCC patients was hierarchical clustered based on the expression of 35 senescence-associated genes. Senescence-associated gene signature was then compared with CCL2 expression and survival. Results: Senescent hepatocytes promoted growth of hepatocellular carcinoma cells through accumulation of immunosuppressive CD11b+Gr1+ myeloid cells. CD11b+Gr1+ cells inhibited T cell proliferation via an iNOS-dependent mechanism. Immunosuppressive myeloid cell accumulation as well as tumor growth promotion was abrogated, when senescence was induced in livers of CCR2 knockout mice, indicating a pivotal role for CCL2 in creating a tumor promoting immune environment. Finally, gene expression analysis in patients with hepatocellular carcinoma confirmed the association of senescence-induced CCL2 expression in peritumor tissue and poor prognosis. Conclusion: Senescent hepatocytes not only induce an inflammatory response that leads to their own clearance, preventing tumor initiation, but surprisingly also induce the accumulation of immunosuppressive myeloid cells that promote growth of neighboring hepatocellular carcinomas.

NCI-CCR

Wei Gao

Postdoctoral Fellow

Biochemistry - Proteins

Regression of liver cancer by a novel immunotoxin targeting glypican-3 via inhibiting Wnt signaling and protein synthesis

Liver cancer is the fifth most common cancer worldwide. Hepatocellular carcinoma (HCC) accounts for 75% of all liver cancer cases. Despite the prevalence of HCC, surgery is still the most effective treatment to date but is only available for a limited number of patients identified at early stages. Sorafenib is the only-FDA-approved chemotherapeutic agent for HCC. It has only modest efficacy and most patients eventually develop resistance. Therefore, there is an urgent need to develop new strategies for the treatment of liver cancers. Glypican-3 (GPC3) is a glycosylphosphatidylinositol-anchored cell surface protein consisting of a core protein and two heparan sulfate chains. GPC3 is highly expressed in 70-100% of HCCs but not in normal adult tissues. The expression of GPC3 is correlated with poor clinical prognosis in HCC. GPC3 regulates major signaling pathways in HCC pathogenesis, including Wnt and Yap signaling.

Therefore, GPC3 is a potential target for HCC therapy. However, our work and others showed that the naked anti-GPC3 antibodies did not induce regression of liver tumors in mice, suggesting that the therapeutic effects of that type of naked antibody may not be potent enough for curative treatment of liver cancer. Our current study has demonstrated that GPC3 is efficiently internalized by tumor cells, suggesting that GPC3 can be established as a new target of antibody-toxin conjugates that act inside liver cancer cells. To this end, we develop two antibodies targeting GPC3, HN3 and YP7. The first antibody recognizes a functional epitope and inhibits Wnt signaling, whereas the second antibody recognizes a C-terminal epitope but does not inhibit Wnt signaling. Both are fused to a fragment of Pseudomonas exotoxin A (PE38) to create immunotoxins. Interestingly, the immunotoxin based on HN3 (HN3-PE38) has superior anti-tumor activity as compared to YP7 (YP7-PE38) both in vitro and in vivo. Intravenous administration of HN3-PE38 as a single agent or in combination with irinotecan induces regression of Hep3B and HepG2 tumors in mice. Our results demonstrate for the first time that GPC3 is a promising target for immunotoxin therapy. The HN3-PE38 immunotoxin induces tumor regression via dual mechanisms: inactivation of cancer signaling via the antibody and inhibition of protein synthesis via the toxin. Our future research aims to pursue clinical trials with this new anti-GPC3 immunotoxin in humans for the treatment of liver cancer.

NCI-CCR
Sudheer Kumar Gara
Postdoctoral Fellow
Endocrinology

HABP2, a novel tumor suppressor gene in familial papillary thyroid cancer

Familial nonmedullary thyroid cancer (FNMTC) accounts for 3–9% of all thyroid cancer cases and has an autosomal dominant pattern of inheritance. FNMTC may be syndromic (e.g. Cowden's, Carney complex), for which the susceptible genes are known but the majority of them are nonsyndromic, accounting for more than 95% of all cases. Unfortunately, the susceptibility gene(s) for nonsyndromic FNMTC are not known. In order to identify candidate FNMTC susceptible gene(s), we performed whole-exome sequencing on germline DNA of a large kindred with FNMTC without any known familial cancer syndrome. We identified a germ line heterovariant, G534E in the HABP2 gene that segregated with all affected members (7/7 cases) of the kindred. Further examination of this variant in 24 kindreds with FNMTC identified two additional kindreds who were carriers of the variant (4 of 4 affected members). In addition, analysis of The Cancer Genome Atlas (TCGA) of whole-exome sequencing data of germline DNA showed 20 of 423 (4.7%) patients with thyroid cancer had the HABP2 G534E heterovariant (4.7% vs. 0.7% in multiethnic population databases with unknown disease status, p<0.0001). HABP2 mRNA and protein expression in thyroid tumor samples from G534E variant carriers in the kindred was higher as compared to absent expression in adjacent normal thyroid tissue and sporadic thyroid cases. Functional knock down of wild type HABP2 increased colony formation, cellular migration and invasion in vitro in thyroid cancer cell lines and HEK293 cells suggesting a tumor suppressive function of HABP2. In order to further understand the role of the variant, we stably overexpressed both wild type and G534E variant of HABP2 in cell lines. We observed that the G534E variant expressing cells had increased colony formation, cell migration and invasion suggesting that the variant results in loss-of-function of HABP2. We then analyzed whether the heterovariant can initiate malignant transformation by transiently

overexpressing wild type HABP2 (+/+), G534E (-/-) variant and equal volumes of both wild type and G534E variant (+/-) in mouse fibroblast cell lines NIH-3T3 by preforming foci assay. We observed that the variant expressing cells (+/- and -/-) had higher number of foci and migration compared to the wild type (+/+) suggesting a dominant negative tumor suppressive effect. In conclusion, our data suggests that HABP2 is a susceptible gene for FNMTC and functions as a dominant negative tumor suppressor gene

NCI-CCR
Eileen Geoghegan
Postdoctoral Fellow
Virology - DNA
JC Polyomavirus Makes Facultative Use of Glycosaminoglycans For Infectious Entry Into Cells
JC polyomavirus (JCV) commonly infects the urinary tract of healthy adults. Although the infection generally does not cause overt disease in immunocompetent individuals, in patients with impaired immune function JCV can cause a lethal brain disease called progressive multifocal encephalopathy (PML). It is well established that wild-type JCV strains utilize glycans that bear sialic acid (SA) as receptors for infectious entry into cells. Surprisingly, the predominant JCV strains found in the brains of PML patients contain mutations that disrupt the SA binding site. Although mutant virions show minimal interaction with the known sialylated receptor glycans, the mutant virus readily infects primary brain cells, such as astrocytes. This suggests the existence of a SA-independent alternative pathway for infectious entry. Our findings document the existence of this alternative entry pathway and show that it requires a non-sialylated class of polysaccharides called glycosaminoglycans (GAGs). Heparin is a familiar example of a GAG. Both wild type (wt) and PML-mutant JCVs fail to bind a CHO-based cell line that lacks GAGs. Conversely, both wt and PML-mutant JCV virions readily attach to cell lines that lack SA. In an inhibition assay with exogenous heparin, the infectivity of the wt virus is inhibited by about 50%, while the infectivity of PML-mutant virus is completely abolished. This suggests that the wt virus is able to use either the known SA-dependent infectious entry pathway or the GAG-dependent pathway, while the mutant is restricted to only the GAG pathway. Treatment of cells with an inhibitor of sialyltransferase activity reduced the infectivity of wt JCV by about 50%, again suggesting that the GAG-dependent pathway remains operational even if the SA-dependent pathway is blocked. In contrast, treatment of cells with the sialyltransferase inhibitor slightly enhanced the infectivity of the SA mutant viruses, presumably reflecting a restriction of the mutant virus to the GAG-dependent entry pathway. Finally, we have preliminary evidence indicating that the region of the viral capsid responsible for attachment to GAGs is a highly conserved RYVD motif that is located at the base of deep canyons between the pentamers of the virion surface. This research defines a novel alternative entry pathway that mutant JCV strains use to enter cells, thus providing a target for treatment and prevention of PML.

NCI-CCR
Andrew Goey
Visiting Fellow
Pharmacology and Toxicology/Environmental Health
Towards personalizing belinostat therapy: dose adjustments based on UGT1A1 genotype in patients with advanced cancer

Belinostat (BEL), a histone deacetylase inhibitor recently approved for treatment of peripheral T-cell lymphoma, is metabolized by UDP glucuronosyltransferase 1 family, polypeptide A1 (UGT1A1). Polymorphisms of UGT1A1 associated with reduced function (e.g. UGT1A1*6, *28 and *60) could result in increased exposure of BEL, leading to increased risk of toxicities. Based on solely one preclinical study showing reduced BEL glucuronidation due to UGT1A1*28 alleles, the BEL package insert recommends a dose reduction in patients homozygous for UGT1A1*28 to minimize toxicities. Since a clinical basis for this recommendation is lacking, we carried out a pharmacogenomic (PG) analysis in which UGT1A1 genotype status was compared to systemic exposure of BEL, global protein lysine acetylation (AcK), and toxicities in patients with cancer receiving BEL-containing chemotherapy. Hereto, clinical data were evaluated from patients with advanced cancer participating in a Phase I (BPE) trial, in which BEL (400-800 mg/m2/24h) was administered by 48 h continuous infusion in combination with cisplatin and etoposide. Patients (n=25) were genotyped for UGT1A1*6, *28 and *60. Endpoints were associations between UGT1A1 genotype and BEL pharmacokinetics (PK), toxicities and AcK during cycle 1. UGT1A1*60 carriers had progressively higher BEL elimination half-lives than wild-types: P=0.0077. Other PK parameters were not significantly affected by UGT1A1 genotype (P>0.01). Examining the higher doses only, lead to additional progressive increases in area under the plasma concentration-time curve (P=0.0037) and decreases in clearance (P=0.020) in *60 carriers. Incidence of grade 3-4 thrombocytopenia, neutropenia and ?QTc prolongation was increased in patients with *28 (P<0.05), while *60 carriers had an even greater risk for grade 3-4 thrombocytopenia (P<0.01). UGT1A1*28 carriers tended to have increased AcK in peripheral blood mononuclear cells (P=0.075). We here show for the first time that UGT1A1 genotype was clinically associated with increased systemic BEL exposure (*60), increased AcK (*28) and increased incidence of toxicities (*28 and *60), particularly at doses >400 mg/m2/24h. Therefore, dose adjustments should be considered based on both UGT1A1*28 and *60 genotype to reduce the risk for toxicities. In addition, results of this PG analysis and a population PK/pharmacodynamic model led to a submitted amendment to incorporate genotype-based BEL dosing in the BPE trial.

NCI-CCR
Ido Goldstein
Visiting Fellow
Endocrinology

Massive chromatin landscape re-organization promotes a cascade of transcriptional regulation during fasting

During fasting glucose levels drop and the liver restores homeostasis through the secretion of alternative fuels. Fuel production during fasting is temporally organized whereby glucose serves as the major fuel produced in short-term fasting while ketones are produced in longer fasts. However, the mechanism enabling such temporal organization is undefined. A hyper-activated, dys-regulated response to fasting occurs when the liver becomes insulin resistant, eventually leading to type 2 diabetes. The response to fasting is heavily reliant on transcriptional regulation with many transcription factors enabling it, presumably with underlining alterations in chromatin structure. To determine how fuel production is temporally regulated during fasting at the transcriptional level, we assayed livers from fasted mice in three genome-wide experiments. We employed the DNase I hyper-sensitivity assay

(DNaseI-Seq) to globally map the accessible regions of chromatin; thus mapping active and inactive states for transcriptional regulatory elements (enhancers) in chromatin. We found ~4,000 sites in liver chromatin in which accessibility was altered following fasting, suggesting functional relevance. These altered regions were enriched in binding sites for transcription factors known to regulate the response to fasting. We obtained similar results by globally mapping active enhancers (by chromatin immunoprecipitation of the active enhancer mark H3K27ac followed by sequencing). Moreover, we determined the alterations in gene expression by profiling the liver transcriptome using RNA-seq. We found the transcriptional switch from a glucogenic to a ketogenic stage during fasting. Upon short-term fasting, glucagon is secreted, initiating a sequential cascade of transcription factor activation in liver cells, resulting in a coordinated response to nutrient scarcity. We report here the first genome-wide characterization of chromatin landscape following a physiological perturbation, showing that fasting leads to a massive re-organization of liver chromatin. Importantly, we describe here for the first time the mechanism behind the temporal organization of the response to fasting whereby fasting-enhanced chromatin alterations support the onset of a complex, temporally organized, mutli-stage transcriptional response. Understanding the transcriptional response and its underlining chromatin regulation following fasting is critical to elucidating the events leading to diabetes.

NCI-CCR
Kristen Greathouse
Research Fellow
Clinical and Translational Research

A LUNG MICROBIOME SIGNATURE IS CHARACTERIZED BY TP53 MUTATIONS IN SQUAMOUS CELL LUNG CARCINOMA

Lung cancer is the leading cancer diagnosis worldwide and a major health and financial burden to our healthcare system primarily due to late stage detection. Improvements in early stage detection tools would, therefore, significantly improve survival. Recent studies in colon cancer implicate the microbiome as a factor in inflammation and as a biomarker for early stage detection. We hypothesized therefore, similar to colon cancer; the lung microbiome may also be associated with inflammation and lung cancer etiology and may be useful in identification of novel biomarkers for diagnosis or treatment. Using the NCI-MD Case-Control Study we sequenced 16S rRNA in 398 lung tumor, non-tumor adjacent and healthy lung tissue. As validation, we used RNAseq unmapped reads from 1,112 lung tumor/non-tumor samples from The Cancer Genome Atlas (TCGA). In order to analyze these data we constructed custom 16S and metagenomic sequence pipelines. In comparison, these two separate sequence platforms and sample populations demonstrated significant overlap in abundance of microbial taxa, with 16S rRNA sequencing and metagenomic (RNA-seq) data (TCGA), sharing 224 Phyla in common. We also found a significantly lower alpha diversity (within sample) in normal lung as compared to non-tumor adjacent or tumor tissue, indicating a shift in the overall microbial community in lung cancer. Using three genera, Veillonella, Variovorax and Streptococcus we were able to classify normal healthy lung from lung tumor tissue with an AUC=0.8. More specifically, Variovorax abundance distinguish tumor from non-tumor adjacent paired lung tissue with a mean increase of 2.1%, which was also validated in the TCGA data set. Lung cancer subtype was also distinguished by a subset of species (7) in lung tumor tissue, which were significantly higher in abundance in squamous cell carcinoma (SCC) vs

adenocarcinoma (AD) and validated in the TCGA data set. Given that p53 mutations diminish the epithelial barrier, we analyzed these 7 SCC-associated bacteria to determine if somatic mutations were associated with abundance. Surprisingly, among SCC cases (TCGA dataset), those with p53 mutations had significantly higher abundance of the squamous-associated species, which was not seen in AD. Thus, the lung cancer microbiome not only has the ability to distinguish cancerous tissue and histological subtype, but also may be further altered in those tumors with p53 mutations.

NCI-CCR
Alena Gros
Research Fellow
Hematology/Oncology, Tumor Immunology, and Therapy

PD-1 expression on peripheral blood lymphocytes enables direct enrichment of tumor-reactive and mutation-specific lymphocytes

Accumulating evidence supports that T cells targeting unique somatic mutations expressed by the patients' tumors may play an important role in the anti-tumor responses observed following adoptive transfer of tumor-infiltrating lymphocytes. Tumors constitute a sink for tumor-reactive and mutation-specific T cells, and PD-1 expression in the tumor microenvironment can identify the CD8+ patient-specific tumor-reactive repertoire. However, mutation-specific cells are far less prevalent in peripheral blood and isolation of these cells from peripheral blood remains an elusive challenge. The identification of mutation-specific T cells from the blood would provide a novel strategy to harness naturally-occuring mutation-specific T cells present in peripheral blood for the development of personalized T-cell based therapies to treat cancer. In view of the significance of PD-1 expression in the tumor, we explored the utility of PD-1 expression on peripheral blood lymphocytes to detect and enrich for T cells targeting unique, patient-specific somatic mutations. In order to do this, we isolated bulk CD8+ lymphocytes from peripheral blood of melanoma patients and also separated them based on the expression of PD-1 into CD8+PD-1- and CD8+PD-1+. Following this isolation, we expanded these cells in vitro and screened these populations for the recognition of mutated antigens using an approach that enables the expression of all the potential tumor neoantigens in the autologous antigen-presenting cells. We found that selection of CD8+PD-1+ lymphocytes circulating in peripheral blood, but not the bulk or CD8+PD-1- cells, led to the direct enrichment of mutation-specific cells in three melanoma samples tested, with at least 1, 3 and 7 unique patient-specific neoantigens recognized, respectively. The PD-1+ derived cells also displayed autologous tumor recognition. Furthermore, CD8+PD-1+ and PD-1hi cells isolated and expanded from the blood of a patient with colorectal cancer led to the identification of mutation-specific cells targeting two unique mutations in the Caspase 8 and histone HIST1H3B genes. These mutation-specific cells were not detected in the bulk CD8+ or the CD8+PD-1- populations. Our findings support that CD8+PD-1+ cells circulating in peripheral blood of patients with cancer are enriched in mutation-specific cells and selection of this T cell subset can be used to identify mutation-specific cells and to isolate T-cell receptors that could be used to treat cancer

NCI-CCR
Berkley Gryder
Postdoctoral Fellow

Epigenetics

Genetic, Epigenetic and Molecular Sensitivity Landscape of Pediatric Rhabdomyosarcoma

Master transcription factors establish enhancers to regulate cell identity genes by recruiting epigenetic machinery, and are sequentially exchanged during changes in cell identity (ie, differentiation). Commonly, the fusion of transcription factors profoundly alters proper progression of cell identity, serving as the signature driving event in many malignancies. The most common soft tissue cancer of childhood, rhabdomyosarcoma (RMS), is characterized by an inability to exit the proliferative myoblast-like state, presumably by blocking myogenic transcription factors from advancing the active enhancer landscape. This is achieved by either chromosomal translocation resulting in the oncogenic fusion transcription factor PAX3/7-FOXO1 (alveolar subtype) or mutations in the tyrosine kinase/RAS/PIK3C axis (embryonal subtype). Patients who harbor a PAX fusion typically relapse despite aggressive therapy, and have very poor survival. To characterize the landscape of somatic alterations in this disease, we used a combination of whole-genome, exome and transcriptome sequencing along with high resolution SNP arrays in 147 tumor/normal pairs, the largest cohort to date of this pediatric cancer. PAX-fusion tumors are marked by a strikingly low occurrence of mutations. PAX-fusions typically partner with FOXO1, but the same transcriptome can be achieved by PAX partnering with chromatin remodelers NCOA1 or INO80D. Master transcription factors (MYCN, MYOD1, MYOG, POU4F1, RUNX2) expressed at unusually high levels collaborate with PAX3-FOXO1 almost exclusively at distal enhancers. Active enhancer marks (H3K27ac, H3K4me1/2) found at PAX3-FOXO1 bound enhancers decrease over myogenic differentiation, including the super enhancer regulating MYOD1. Utilizing both small molecule and RNAi screening, we identified certain epigenetic modulators as unique protein dependencies, especially in PAX-fusion RMS. RNA-seq after treatment with epigenetic inhibitors reveals inactivation of PAX-fusion target genes, including MYOD1 and MYCN. Mechanistic studies are underway to uncover the effects of epigenetic chemical probes on chromatin architecture and gene regulation. Collectively, our studies are pointing to epigenetic inhibition as a potential therapeutic option for children with PAX-fusion driven rhabdomyosarcoma.

NCI-CCR

Christine Happel

Postdoctoral Fellow

Virology - DNA

KSHV regulation of the microRNA biogenesis pathway

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression post-transcriptionally. Kaposi's sarcoma-associated herpesvirus (KSHV) encodes for 12 pre-miRNAs that give rise to at least 18 mature viral miRNAs. The processing of these viral miRNAs is Dicer-dependent and, in KSHV-associated cancers, a single KSHV miRNA can account for as much as 20% of all mature miRNAs within a cell. MCP-1-induced protein-1 (MCPIP1), is a critical regulator of immune homeostasis and we have recently identified MCPIP1 as a highly down-regulated gene after de novo KSHV infection. In addition to its known function as an RNase that destabilizes pro-inflammatory cytokine transcripts, MCPIP1 has also been shown to suppress miRNA biosynthesis via cleavage of the terminal loops of precursor miRNAs. We propose that KSHV prevents a host immune reaction to expression of foreign miRNAs through the reduction of MCPIP1 levels to ultimately allow expression of the KSHV viral miRNAs. Using KSHV miRNA

mimics we have identified three KSHV miRNAs that target the 3'UTR of MCPIP1 resulting in decreased expression. Since changes in MCPIP1 levels could in turn effect the expression of mature miRNAs, we used functional assays to demonstrate that MCPIP1 inhibits viral miRNA biogenesis. Over-expression of MCPIP1 indeed results in decreased expression of mature miRNAs without effecting primary miRNA transcript levels. In vitro cleavage assays demonstrate the MCPIP1 directly cleaves viral miRNA precursors. As opposed to MCPIP1, Dicer promotes miRNA biogenesis and we found an increase in Dicer expression following KSHV infection. In addition to two KSHV miRNAs that up-regulate Dicer expression, we have also observed increased Dicer expression following the down-regulation of MCPIP1. Expression of essential Dicer partners such as TRBP are also increased following de novo KSHV infection, indicating an increase in the core components of the RISC loading complex that is available for miRNA processing. Therefore, we see increased expression of a number of critical regulators of miRNA processing while expression of negative regulators of miRNA biogenesis is repressed. We propose that the expression of KSHV miRNAs inhibit MCPIP1 and up-regulate Dicer, to evade host mechanisms of inhibiting expression of foreign miRNAs. KSHV-mediated alterations in miRNA biogenesis represent a novel mechanism in which KSHV interacts with its host, and a novel mechanism for the regulation of miRNA expression.

NCI-CCR
Majda Haznadar
Research Fellow
Clinical and Translational Research

Lung Cancer Metabolomics Identifies Metabolites as Robust Risk Biomarkers

Lung cancer is a major health burden on the general population, as it will kill 160,000 people annually in the US, making it the number one cause of cancer related deaths. Fewer than 15% of patients survive 5 years after diagnosis, indicating the enormous necessity to search for biomarkers for early lung cancer detection when amenable to treatment. Currently accepted methods for early detection of lung cancer are limited to low-dose spiral CT (LDCT) scanning in high-risk populations. However, the specificity of LDCT is low, with a false positive rate of 96%. Our goal is to identify stable and reproducible biomarkers in non-invasively collected biofluids (i.e. urine) that may be powerful risk assessment screening tools. To that extent, we first conducted a first of its kind effort using mass spectrometry-based untargeted metabolic profiling of urine samples in the NCI-MD case-control study (N=469 cases, 536 controls). We identified four metabolites as independent and robust classifiers of lung cancer diagnosis and prognosis: novel metabolite creatine riboside, N-acetylneuraminic acid (NANA), cortisol sulfate and unidentified metabolite designated 561. Creatine riboside and NANA were also elevated in stage I lung tumor tissue, linking them directly to deregulated tumor metabolism. Next, we assessed whether these metabolites are elevated in subjects prior to lung cancer diagnosis in the prospective Southern Community Cohort Study (SCCS) (N=180 cases, 349 controls). Controls were individually matched to cases in a 2:1 ratio using incidence density sampling by age (+/-2 years), sex, race, recruitment site, menopausal status (women), and date of sample collection (+/-6 months). We confirmed that creatine riboside and NANA were significantly associated with lung cancer risk with smoking-adjusted Odds Ratios (OR) of 1.8 and 1.5, respectively. These metabolites, having been observed as deregulated in tumor metabolism, may become interesting targets for future studies regarding therapeutic interventions. The associations with risk were stronger among European than African Americans, and among whites were observed in

individuals having been diagnosed two or more years after cohort enrollment (OR=6.1 and 3.3, for creatine riboside and NANA respectively). These results provide strong preliminary data that identified metabolite biomarkers have a potential utility as clinical screening tools for early diagnosis of lung cancer, when prognosis is significantly better.

NCI-CCR
Carrie House
Postdoctoral Fellow
Stem Cells - General
Dissecting the role of NF-kappaB signaling ovarian cancer tumor-initiating cells
Ovarian cancer is the most lethal gynecological malignancy in the United States with the major cause of morbidity and mortality due to recurrence and chemoresistance. Our data suggest tumor-initiating cells (TICs), self-renewing cells capable of generating heterogeneous tumors, play an important role in disease biology. Clarifying the mechanisms by which TICs support tumor progression could lead to clinically favorable therapies. We previously showed that a subset of ovarian cancer cells are dependent on NF-kappaB signaling, and that expression of NF-kappaB proteins associates with poor patient survival. To investigate this pathway in TICs, we first designed a novel method to enrich for TICs from cell lines and patient samples by culturing non-adherent, floating cells in stem cell enriching conditions. These free-floating spheroids mimic malignant cells found in patient ascites, and exist in some cell lines. Preliminary data show that these cells have higher stem cell markers CD133, Nanog, TRA-1-60, and aldehyde dehydrogenase activity, are resistant to carboplatin, and are more tumorigenic in nude mice compared to their epithelial, adherent counterparts. Given that our previous studies demonstrated NF-kappaB expression correlated with a poor outcome for ovarian cancer patients, and that NF-kappaB activity supports drug resistance and tumorigenicity - two functional outputs associated with TICs - we hypothesize that NF-kappaB supports a TIC program responsible for ovarian cancer relapse. Supporting our hypothesis, preliminary data show that TIC-enriched culture conditions increased NF-kappaB expression and activity in TICs compared to adherent conditions. Stimulation of NF-kappaB signaling enhanced drug-resistance and stem cell marker expression. Current studies focus on dissecting the role of individual NF-kappaB proteins in TICs. First, we use specific inducible short-hairpin (shRNA) targeting RelA or RelB to measure changes in spheroid formation, drug resistance, tumorigenesis, and disease relapse following chemotherapy in a xenograft model. Second, we will measure system level changes induced by these shRNAs, and identify a gene signature specific to NF-kappaB in ovarian TICs. Clarifying the nuances of NF-kappaB signaling in TIC populations will fill an important gap in our understanding of ovarian cancer recurrence and may lead to improved therapeutic strategies for women in first remission, to prevent subsequent relapse to incurable disease.

NCI-CCR
Elad Jacoby
Clinical Fellow
Hematology/Oncology, Tumor Immunology, and Therapy
B cell acute lymphoblastic leukemia relapse following CD19 CAR immune-pressure demonstrates global alterations suggesting significant plasticity and de-differentiation

Novel treatments for acute lymphoblastic leukemia (ALL), the most common childhood cancer, and an aggressive cancer in adults, are utilizing the patient's T cells targeting surface molecules on the leukemic blasts. CD19 is a surface marker restricted to B cells and highly expressed on B cell ALL. CD19 has been targeted through genetically engineered chimeric-antigen receptor (CAR) T cells, and via bispecific CD3-CD19 antibodies, that bring into close proximity CD19 on leukemia and CD3 on T cells. Though these targeted therapies show response rates up to 90% of patients, reports have been emerging of relapsed leukemia in about 20% of patients. Interestingly, relapsed leukemic cells have lost expression of CD19, thought to be essential for ALL, and making them inherently resistant to this therapy. To study CD19 negative ALL we established a murine ALL model expressing CD19. Mock treated ALL recipients died of disease in 21 days. Recipients of CAR T cells went into remission, with late relapses despite long-term persistence of CAR T cells. Compatible with preliminary reports from patients, all late relapse samples have lost CD19 expression. This was accompanied by down regulation of CD19 and its regulator PAX5 in the mRNA level. Overall, the later the post-CD19-CAR relapse was, the greater phenotypic alterations it had, with loss of B cell markers and gain of myeloid markers (Mac1, Gr-1) and c-kit. Of note, no myeloid markers were expressed when examining multiple single cell clones of the original cell line. In-vivo passage of some but not all of the CD19-negative leukemia in secondary and tertiary recipients with no additional CAR treatment resulted in reemergence of CD19 expression in leukemia blasts (mostly in tertiary recipients). However, these were inherently resistant to CAR treatment and relapsed rapidly following CD19 CAR. Expression microarray analysis of primary negative escape samples compared with CD19+ samples showed downregulation of B cell markers (CD19, AICDA, CD79a, RAG1 and EBF1) and HOXA clusters, and upregulation of MYC, TET2 and DR1, similar to expression profiles reported in leukemia stem cells. Altogether, these data demonstrate plasticity of leukemic blasts under novel antigen-specific immune pressure. Current efforts are focused on RNA sequencing of the antigen negative escapes in both human and murine samples, along with prospective study of patients treated with CD22 CAR for potential antigen alterations of leukemia.

NCI-CCR
Smita Kakar
Visiting Fellow
Protein Structure/Structural Biology

Allosteric Activation of Bacterial Swi2/Snf2 Protein RapA by RNA Polymerase: Biochemical and Structural Studies

Members of the evolutionary conserved Swi2/Snf2 (switch/sucrose non-fermentable) family depend on their ATPase activity to mobilize nucleic acid-protein complexes for gene expression. In bacteria, RapA is an RNA polymerase (RNAP)-associated Swi2/Snf2 protein that mediates RNAP recycling during transcription. It is known that the ATPase activity of RapA is stimulated by its interaction with RNAP. However, how the RapA-RNAP interaction activates RNAP is not known. Previously, we determined the crystal structure of RapA. The structure revealed the dynamic nature of its N-terminal domain (Ntd), which prompted us to elucidate the solution structure and activity of both the full-length protein and its Ntd-truncated mutant (RapAdN). Here, we report the solution structures of RapA and RapAdN, either ligand-free or in complex with RNAP determined by small-angle X-ray scattering. The solution structures reveal a new conformation of RapA, define the binding mode and binding site of RapA on RNAP, and

show that the binding sites of RapA and sigma70 on the surface of RNAP largely overlap. We also report the ATPase activity of RapA and RapAdN, in the absence or presence of RNAP. We conclude that the ATPase activity of RapA is inhibited by its Ntd but stimulated by RNAP in an allosteric fashion and that the conformational changes of RapA and its interaction with RNAP are essential for RNAP recycling. These findings outline the functional cycle of RapA, which increases our understanding of the mechanism and regulation of Swi2/Snf2 proteins in general and of RapA in particular. The new structural information also leads to a hypothetical model of RapA in complex with RNAP immobilized during transcription.

NCI-CCR
Edward Kim
Postdoctoral Fellow
Molecular Biology - Prokaryotic
SpoVM localizes by sensing convex membrane surfaces
Understanding the mechanisms that determine how proteins localize within a cell has broad impacts, from biotechnology to human health. The small peptide SpoVM, expressed in Bacillus species during sporulation, localizes via a mechanism previously unobserved in prokaryotes: recognition of membrane curvature. Fluorescence microscopy of Bacillus expressing SpoVM-GFP shows almost exclusive localization to the convex surface of the forespore during sporulation, while a single amino acid mutation, SpoVM(P9A), results in promiscuity to all membrane surfaces. This mislocalization mutant results in a loss of function, and the sporulation efficiency drops by six orders of magnitude compared to the wild type. We have developed an in-vitro system to reconstitute the convex surface of the forespore by coating silica microbeads with a lipid bilayer. In a competition between 2um beads and 8um beads, microscopy shows that purified SpoVM-GFP preferentially binds to 2um beads, as the radius of curvature is closest to that of the Bacillus forespore. In contrast, SpoVM(P9A)-GFP binds equally well to both 2um and 8um beads, in agreement with what we observe in vivo. To quantify the binding of SpoVM-GFP to the beads, we use flow cytometry to analyze the fluorescence from the 2um and 8um beads separately from the mixed population. When normalized per surface area, binding of SpoVM-GFP at low concentrations is up to eight fold greater in the 2um beads compared to the 8um beads. Near saturation concentrations, about 4uM, fluorescence per surface area is nearly equal. In contrast, SpoVM(P9A)-GFP has equal fluorescence per surface area even at low concentrations, due to the promiscuity of the mutant protein. We then use this flow cytometry assay to investigate how the kinetics of binding determine the preference toward different radii of curvature. We find that the rate of dissociation is higher in 8um beads than 2um beads. These data support a model in which SpoVM can sample all membranes, but the higher rate of dissociation in incorrect surfaces leads to preferential binding to surfaces with the correct radii of curvature. These findings reveal detail in what may be a general mechanism for localizing proteins to the surfaces of organelles.

NCI-CCR
Nard Kubben
Visiting Fellow
Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology

Repression of the longevitiy-promoting NRF2 pathway in Hutchinson-Gilford Progeria Syndrome
Aging is a fundamental biological process and directly linked to many common diseases. The naturally occurring rare human premature aging disorder Hutchinson-Gilford Progeria Syndrome (HGPS) is a powerful tool to study human aging. HGPS is caused by constitutive production of progerin, a mutant form of the nuclear architectural protein lamin A, which causes extensive nuclear defects, including loss of various nuclear proteins, decreased amounts of heterochromatin and elevated levels of DNA damage. The mechanisms leading to these defects are unknown. Identification of disease mechanisms would benefit our understanding of the molecular defects underlying aging as well as help the development of novel therapeutic strategies for HGPS and other age-related diseases. To identify cellular factors involved in bringing about progerin-induced aging defects, we developed a high-throughput siRNA screen. A human siRNA library targeting 320 human ubiquitin-ligases was screened for proteins which prevent formation of progeria-associated morphological, epigenetic and DNA damage phenotypes in inducible progerin-expressing cells. We identified CAND1 as a cellular factors whose knockdown prevented the formation of aging defects in progerin expressing cells, as well as reversed established aging defects in HGPS patient cells. CAND1 is a regulator of the antioxidant and longevity promoting NRF2 transcription factor. We find NRF2 transcriptional activity is severely reduced by progerin, through an aberrant interaction between progerin and NRF2, which causes nuclear mislocalization of NRF2. Progerin-mediated impaired activation of NRF2 transcriptional targets, including many antioxidants, increases the cellular levels of reactive oxygen species. Knock-down of NRF2, as well as induction of oxidative stress in wildtype cells was sufficient to recapitulate HGPS aging defects. Correcting impaired NRF2 activity in HGPS patient cells by NRF2 overexpression lowered the oxidative burden, as well as decreased progerin levels by two-fold and thereby reversed progerin-associated aging defects. These findings demonstrate a key role for NRF2 in HGPS etiology and an anti-aging role of NRF2. They point to potential preventative and therapeutic applications of NRF2-activating drugs in HGPS and in major aging-related diseased, including cardiovascular disease, cancer and Alzheimer disease.

NCI-CCR
Rhushikesh Kulkarni
Visiting Fellow
Chemistry
Profiling the role of non-enzymatic acetylation in cancer
Protein acetylation is a posttranslational modification critical for regulation of diverse cellular processes such as gene expression, metabolism and cell death. Altered cellular acetylation, found in many cancers, can modulate key tumor suppression and oncogenic signaling pathways. While these findings implicate acetylation as a major driver of cell transformation and proliferation, the enzymes regulating many cellular acetylation events remain unknown. It has been hypothesized that the acetylation of many proteins may be caused by their spontaneous enzyme-independent reaction with the electrophilic thioester of acetyl-CoA; however, current methods to study this phenomenon experimentally are limited. Here we report the development of an unbiased chemical proteomic method to investigate the non-enzymatic reactivity of acetyl-CoA in cellular environments. We have developed a chemical probe, p-NAC, that specifically identifies proteins susceptible to non-enzymatic acetylation. This probe incorporates the intrinsically reactive thioester of acetyl-CoA, but lacks the adenine base and

phosphopantetheine arm required for enzymatic acetylation. Following reaction with cancer cell proteomes, p-NAC can be ligated to a chemical reporter using “click chemistry” facilitating identification of thioester-reactive proteins. Our initial results show that p-NAC is cell permeable and non-toxic. Furthermore it reports exclusively on non-enzymatic acetylation events, as it is not a substrate for acetyltransferases. Preliminary studies indicate that p-NAC labeling is sensitive to conditions that alter cellular acetyl-CoA levels, suggesting that p-NAC targets tightly overlap with targets of non-enzymatic acetyl-CoA reactivity. Pilot proteomic experiments identified several candidate metabolic enzymes as p-NAC targets implicating non-enzymatic acetylation in metabolic regulation of cancer cells. Our current efforts are aimed at studying how non-enzymatic acetylation drive cell signaling in vivo, and harnessing insights into intrinsic thioester reactivity for the development of novel therapeutic approaches.

NCI-CCR
Brandon Kwong
Postdoctoral Fellow
Immunology - Autoimmune
T-bet expression in Th17 cells regulates the recruitment of inflammatory cells to the central nervous system during experimental autoimmune encephalomyelitis (EAE)
Experimental autoimmune encephalomyelitis (EAE) is a murine model for multiple sclerosis in which autoreactive CD4+ T helper (Th) cells infiltrate the central nervous system (CNS) and induce neuroinflammation. We have previously demonstrated that expression of the transcription factor T-bet is required in adoptively transferred CNS antigen-specific (2D2-transgenic) Th17 cells for the induction of EAE. Indeed, histological examination of the CNS following the adoptive transfer of T-bet-deficient (T-bet-/-) 2D2 Th17 cells revealed that in the absence of T-bet expression, 2D2 cells were unable to breach the blood-brain-barrier and induce CNS parenchymal inflammation. However, the transcriptional targets of T-bet that are required for the autoimmune pathogenicity of Th17 cells have not been identified. We first analyzed global gene expression profiles of wild-type or T-bet-/- Th17 cells, in conjunction with ChIP-sequencing, to identify differentially expressed target genes that could be directly regulated by T-bet binding. Notably, we found that T-bet regulates the expression of several chemokines in Th17 cells, including known chemoattractants for monocytes, dendritic cells (DCs), and other inflammatory effectors, and these results were confirmed by quantitative PCR. Thus, a critical pathogenic role of T-bet in 2D2 Th17 cells may be to trigger the recruitment of inflammatory effectors into the CNS via chemokine production, thus propagating neuroinflammation. When T-bet was conditionally deleted in T cells, we observed that the loss of T-bet impaired the ability of CD4+ T cells to recruit DCs, monocytes, and neutrophils to the CNS during EAE, although the infiltration of natural killer (NK) cells was unaffected. To identify specific T-bet-regulated chemokines (and the responding inflammatory cells) that are essential for EAE induction, we constructed retroviral vectors for the ectopic expression of candidate target genes in T-bet-deficient cells. Current studies are focused on testing whether the adoptive transfer of T-bet-deficient 2D2 Th17 cells, transduced with these candidate retroviral vectors, can recapitulate the level of EAE disease induced by T-bet-expressing 2D2 cells. Ultimately, the identification of pathogenic T-bet target genes could lead to novel therapies that inhibit autoreactive T cell pathogenicity without requiring the global suppression of T-bet, which remains critical to mounting effective immune responses against many foreign pathogens.

NCI-CCR

Tineke Lenstra

Other

Gene Expression

Single-molecule imaging reveals switch between spurious and functional ncRNA transcription

Genome-wide transcriptome analyses have revealed that eukaryotic transcription is pervasive, with up to 98% of all transcripts being non-coding. Non-coding transcripts are emerging as important regulators of coding genes, including tumor-suppressor genes. However, given that transcription is also known to be stochastic, it has also been proposed that ncRNA represents transcriptional noise that arises from leakiness of the transcription machinery. A general understanding of when ncRNA transcription is functional or spurious is still lacking. One major difficulty in addressing the functional role of individual ncRNAs lies in the fact that these transcripts often originate from genomic regions, which overlap coding genes. Therefore two primary tools for assessing function – evolutionary conservation and mutagenesis – are largely ineffective. In this study, we developed a new approach for dissecting the role of ncRNA. We use time-lapse single-molecule imaging in living cells in tandem with strand-specific transcriptional blocking of the ncRNA by CRISPR/Cas9d to interrogate the role of antisense ncRNA transcription at the GAL locus of S. cerevisiae. Both coding and non-coding RNA syntheses were visualized in single cells in real-time using the PP7 and MS2 RNA labeling technique. We observed ncRNA synthesis before, and even during sense transcription, suggesting that the template is highly permissive for transcription. By targeting an enzymatically-dead mutant of Cas9, we were able to selectively block transcription of one strand without affecting transcription of the opposite strand. Blocking ncRNA in this manner under repressive conditions results in increased transcriptional leakage of two genes at the locus, one of which encodes a signaling molecule. Leakage of this signaling molecule alters the activation threshold, response time, and hysteresis of the network. However, blocking ncRNA had no detectable effects on the actively transcribed gene. Using a computational model we were able to quantitatively determine that the ncRNA controls the initial conditions of signaling molecules, but does not alter the topology or rate constants of the network. We thus experimentally uncouple the appearance of ncRNA from the function of the ncRNA. In conclusion, we find that transcription of the same ncRNA is functional under repressive conditions but spurious and pervasive under activating conditions, highlighting the nuanced roles that ncRNA can play in gene regulation.

NCI-CCR

Doreen Matthies

Visiting Fellow

Biophysics

High-resolution single-particle cryo-EM of a 200 kDa membrane ion channel

Magnesium ions are the most abundant divalent intracellular cations and are essential for life, as they play key roles in signaling, nucleic acid action and metabolism. Mg2+-deficiency is associated with diseases affecting the heart, muscle, bone, immune and nervous system, so it is very important to fully understand this ion uptake system. The ~200 kDa pentameric membrane channel CorA is the major Mg2+ uptake system in bacteria and a homolog of the eukaryotic mitochondrial Mrs2 proteins which it can complement. CorA contributes to Mg2+ homeostasis through a negative feedback loop, where

Mg2+ binding at the subunit interface leads to channel closure and low Mg2+ concentrations stabilize the open conformation. Electron paramagnetic resonance (EPR) spectroscopic studies of purified CorA revealed large quaternary conformational changes associated with magnesium binding/unbinding. Using single-particle cryo-EM, we have determined the structure for the "closed" magnesium-bound state of CorA at a resolution of 3.9 Å. At this resolution, we were able to trace the entire peptide chains and build an atomic model of the 200 kDa protein complex. Side-chain densities as well as densities for two magnesium ions per subunit can be clearly discerned. We are actively pursuing efforts to determine the extent and nature of conformational changes induced by Mg2+ binding. Initial observations suggest that channel opening are likely to involve significant changes in quaternary structure including changes in symmetry.

NCI-CCR
Manasi Mayekar
Visiting Fellow
Stress, Aging, and Oxidative Stress/Free Radical Research
A critical role for C/EBPG in regulating the integrated stress response through heterodimerization with Atf4

Stress signals such as amino acid deprivation and redox imbalances activate the integrated stress response (ISR), which allows cells to recover or to undergo apoptosis if the stress is unresolved. ISR triggers up-regulation of the transcription factor ATF4, which subsequently activates stress response genes through recruitment to cis-regulatory sites known as C/EBP:ATF response elements (CAREs). Although the role of ATF4 in regulating stress response is well studied, the identity of the C/EBP partner that heterodimerizes with ATF4 to execute this crucial function remains obscure. Here we show that the transcription factor C/EBP gamma is a critical partner of ATF4 and that C/EBPG:ATF4 heterodimers are the predominant CARE-binding species in stressed cells. We found many similarities in the phenotypes of MEFs lacking C/EBPG and ATF4. Similar to ATF4, C/EBPG is also necessary for resistance of MEFs to oxidative stress. MEFs lacking C/EBPG show increased levels of reactive oxygen species (ROS) as a consequence of impaired glutathione biosynthesis, as was also seen in ATF4-deficient MEFs. C/EBPG is required for stress-induced association of ATF4 with CAREs and the subsequent activation of stress-responsive genes. Mice lacking C/EBPG are smaller in size and show defective eye lens formation, similar to ATF4-deficient mice. The absence of C/EBPG also causes perinatal mortality due to pulmonary atelectasis and respiratory failure. This mortality can be rescued by supplementation of the pregnant mothers with the anti-oxidant, N-acetyl cysteine. Accordingly, our gene expression analysis suggests the presence of increased oxidative stress and impaired expression of stress-responsive genes in the lungs of newborn mice lacking C/EBPG. Furthermore, our molecular analysis of pulmonary surfactants indicates modest but significant reductions in the pulmonary surfactant lipids which may be a consequence of elevated oxidative stress. Increased expression of C/EBPG in tumors has also been shown to be associated with poor patient prognosis in several clinical studies. Hence, activation of antioxidant pathways and other stress genes through up-regulation of C/EBPG could be a mechanism deployed by cancer cells to mitigate the high levels of ROS and metabolic stresses that they experience.

NCI-CCR
David Montgomery
Postdoctoral Fellow
Metabolomics/Proteomics
Chemoproteomic profiling of lysine acetyltransferases
Lysine acetyltransferases (KATs) play a critical role in the regulation of gene expression, metabolism, and other key cellular functions. The disruption of acetylation-dependent signaling cascades is an emerging paradigm in oncology, but methods to discover, characterize, and inhibit KATs are limited. One shortcoming of traditional KAT assays is their inability to study KATs in their native contexts, where a complex network of binding partners and posttranslational modifications are often required for proper enzyme function. To address this challenge, we have developed the first chemoproteomic platform capable of profiling KAT activity directly in biological contexts. Conversion of KAT bisubstrate inhibitors to affinity probes enables the selective labeling and active site–dependent enrichment of over 50% of cellular KAT enzymes in a single step from cell lysates. These cofactor-based affinity probes report on KAT activity in cell lysates, allowing the determination of ligand active site occupancy in competition studies for inhibitor screening efforts or global selectivity profiling. Chemical affinity purification from crude cell lysates and unbiased LC-MS/MS profiling identified two non-canonical KAT enzymes with high levels of enrichment, highlighting an expanded landscape of orphan lysine acetyltransferases present in the human genome. Chemical affinity profiling provides a powerful method to study the molecular interactions of KATs in native contexts, and will aid investigations into the role of KATs in cell state and disease.

NCI-CCR
Aizea Morales Kastresana
Visiting Fellow
Metabolomics/Proteomics
Extracellular vesicle subset analyses by nanoFACS
Extracellular vesicles (EV) are a heterogeneous population of nano-sized vesicles with important regulatory roles. The targets and functions of EVs are determined by their constituent protein, lipid and RNA components. It has been hypothesized that EV subsets produced by different cells (and under different conditions) have distinct compositions, leading to functional differences. We have developed nanoFACS as an ultra high resolution flow cytometric method for analyzing and sorting EV subsets, based on features of single EV particles. Effective separation of relevant EV subsets requires identification of relevant subset-specific markers. We used mass spectrometry (LTQ XL, Thermo) and nanoFACS (AstriosEQ, Beckman Coulter) analysis to identify and characterize subset-specific surface markers. EVs isolated by differential ultracentrifugation from irradiated and non-irradiated 4T1 mouse mammary tumor cell lines were used as a model for EV subset identification. EV concentration and size distribution was determined by nanoparticle tracking analysis (Nanosight, LM10) and EV light scattering and fluorescent labeling was evaluated with nanoFACS. To identify subset-specific proteins, we compared proteomic profiles of EVs released by irradiated and unirradiated 4T1 tumor cells. Among 549 proteins identified, 197 were uniquely present or significantly enriched in EVs isolated after irradiation. Among the 197 radiation-associated EV proteins, transmembrane proteins were ranked according to the

number of repetitive peptides identified, and 5 proteins (Mcam, Icam1, Ly75, Vcam1 and endoglin) were selected as candidate surface markers of radiation-specific EV subsets. 4T1 tumor-specific EV markers were similarly identified by comparing EVs isolated from serum of tumor-bearing and tumor-free mice. NanoFACS offers a unique platform for the identification and isolation of relevant tumor- and treatment- associated EV subsets. In these studies, we used a well-established preclinical tumor model system to identify markers for tumor- and radiation-associated EVs. We are using those markers with nanoFACS, to investigate the functions of EV subsets. Further testing will determine whether nanoFACS sorting can be used to purify EV subsets that modulate anti-tumor immune responses, and whether nanoFACS analysis of peripheral blood can be used to detect tumor-derived EVs.

NCI-CCR
Meera Murgai
Postdoctoral Fellow
Vascular Disease and Biology
KLF4 dependent smooth muscle cell plasticity promotes metastasis
Metastasis is the major cause of cancer death, for which developing new targeted therapies are required in order to improve patient survival. In addition to their impact on the local microenvironment, tumors elicit changes at distant sites that promote metastasis, including resident stromal cells activation and bone marrow derived cell (BMDC) recruitment. An expansion of PDGFRa-expressing stromal cells in pre-metastatic sites is associated with increased extracellular matrix (ECM) proteins that recruit integrin-expressing BMDCs. One potential component of this stromal cell population is comprised of vascular smooth muscle cells (SMCs) and pericytes, a plastic cell type capable of switching from a contractile to an activated, inflammatory phenotype characterized by increased proliferation, migration and ECM secretion. SMC phenotypic switching is KLF4-dependent and can be regulated by the microRNAs miR143/145 in development and vascular injury. Although SMC plasticity plays crucial roles in many disease states, whether SMC plasticity occurs in the metastatic microenvironment is unknown. We hypothesized that KLF4-mediated SMC/pericyte plasticity contributes to a metastasis-promoting microenvironment. We found that SMCs are activated in the pre-metastatic setting, where they proliferate and express KLF4 and PDGFRa in tumor-bearing mice, and in non-tumor-bearing mice exposed to tumor-derived factors. SMCs treated with tumor-derived factors in vitro undergo phenotypic switching, and produce ECM that enhances tumor cell adhesion, proliferation, and migration. Disruption of KLF4-dependent SMC activation decreases metastasis in a SMC-specific, inducible KLF4 knock-down murine model. Consistent with these pre-metastatic changes, miR143/145 levels are elevated in pre-metastatic tumor-bearing mouse blood, in metastatic patient blood, and in tumor conditioned media. shRNA-mediated disruption of tumor-derived miR143/145 delays metastasis and improves survival in vivo. Together these findings demonstrate that KLF4- and miR143/145-dependent SMC plasticity promotes metastasis in part by altering the extracellular mileu at pre-metastatic sites. SMCs represent a novel and previously unappreciated stromal cell target in metastasis, for which identifying mechanisms that mediate SMC plasticity may inform our ability to identify patients with a heightened metastatic risk, and find potential therapeutic strategies to modulate stromal cell plasticity at pre-metastatic sites.

NCI-CCR
Thomas Musich
Postdoctoral Fellow
HIV and AIDS Research
Applying Flow Cytometric Principles to Virions
Flow cytometry is utilized extensively for the analysis and characterization of various cell types, but due to technological limitations, it has not been applicable to individual virions. Nanoscale fluorescence activated cell sorting (nanoFACs) technology is now allowing the analysis and sorting of virus in a way that was never before possible. While flow cytometric analysis of virus has been demonstrated previously, it relied upon magnetic nanoparticles for separating the desired virus populations. Here we demonstrate that we can fluorescently label and sort infectious pseudovirions. R5-tropic HIVBaL and X4-tropic HIVNL4.3 Env-expressing pseudoviruses were generated and labeled utilizing different carbocyanine fluorescent membrane stains. Fluorescent antibodies specific for cellular molecules found on budding virions were also used to label Bal and NL4.3 Env-expressing pseudovirus made in THP-1 cells (monocyte/macrophage) and H9 cells (T-cells) respectively. After mixing the two virus populations together, they were sorted from one another using a high-speed jet-in-air system. The sorted virus was then re-analyzed to demonstrate sort purity. In addition to re-analysis, the sorted virus was titrated onto CCR5 (R5) and CXCR4 (X4) restrictive cell lines, which were stained by X-gal and quantified. Bal was able to infect R5-expressing cells and not the X4-expressing cells post sorting, while NL4.3 was able to infect the X4-expressing cells but not those expressing R5. Electron microscopy was used to further confirm that virus was sorted, in addition to RT-PCR, and p24 and P27 ELISAs. We were also able to sort SIV from three-year old frozen plasma, and demonstrate that it remained infectious. Thus we have demonstrated that we can label and sort infectious virus, and maintain its infectivity downstream of sorting. This technique can potentially be expanded to the labeling and sorting of infectious virus directly from HIV-infected patients, allowing for the analysis of both the antigenic and genomic makeup of the virus.

NCI-CCR
Masao Nakagawa
Postdoctoral Fellow
Tumor Biology and Metastasis
Frequent gain-of-function CCR4 mutations in Adult T-cell leukemia/lymphoma (ATLL)
High expression of CC chemokine receptor 4 (CCR4) has been identified as a hallmark gene in ATLL, an aggressive peripheral T-cell neoplasm. CCR4 is a chemokine receptor, which has a critical role in immune cell trafficking. CCR4 ligands, CCL17 and CCL22, were produced in lymph nodes and skin from dendritic cells, macrophages and Langerhans cells. Most ATLL cases express surface CCR4 (90%) and infiltrate to lymph nodes and skin. These observations suggest that CCR4 could have a role in ATLL biology, but it is still unclear whether dysregulation of CCR4 function contributes to ATLL pathogenesis. Human T-cell lymphotropic virus type-I (HTLV-I) is believed to be the causative agent for ATLL. However, only a small proportion of HTLV-I carriers (2-7%) develop ATLL with a long latency (40-50 years). Thus, acquisition of somatic mutations in cellular genes is likely to be crucial for the development of ATLL. But our knowledge of genetic aberrations in this malignancy is incomplete. We performed RNA-Seq for two primary ATLL cases and discovered recurrent non-sense mutations in CCR4. Though an extended analysis

using Sanger sequencing, CCR4 mutations were detected in 14/53 ATLL samples (26%) and consisted exclusively of nonsense or frameshift mutations that truncated the coding region at C329, Q330 or Y331 in the carboxy-terminus. In 5 cases for which paired normal DNA was available, three different CCR4 mutations were detected only in the ATLL cells (Q330*; Q330 frameshift; Y331*), demonstrating that they were acquired somatically during malignant transformation or progression. Chemotaxic assay using an ATLL cell line clarified that the ectopic expression of CCR4-Q330* enhanced the chemotactic ability of the transduced cells toward CCL17 and CCL22 rather than CCR4-WT transduced cells. Through the analysis of the change in surface CCR4 levels by flow cytometry, we found that ligand-induced internalization of CCR4-Q330* was significantly impaired compared with CCR4-WT, which likely contributes to the enhanced chemotaxis of cells bearing these mutants. Furthermore, we demonstrated that CCR4-Q330* mutant enhanced PI(3) kinase/AKT activation following receptor engagement by CCL22 in ATLL cells, and conferred a growth advantage in long term in vitro cultures. Our findings implicate somatic gain-of-function CCR4 mutations in the pathogenesis of ATLL and suggest that inhibition of CCR4 signaling might have therapeutic potential in this refractory malignancy.

NCI-CCR
Hidetaka Ohnuki
Visiting Fellow
Vascular Disease and Biology

EphrinB2 controls vessel pruning through STAT1-JNK3 signaling

Angiogenesis produces primitive vascular networks that need pruning to yield hierarchically organized and functional vessels. Although the signals that orchestrate endothelial cell (EC) sprouting have been investigated, little is known about the mechanisms controlling blood vessel pruning despite the importance of this process to the patterning, density and function of blood vessels. EphrinB2, a transmembrane ligand for Eph receptors, plays pivotal roles in angiogenesis. However, the function of EphrinB2 cytoplasmic tyrosines has not been previously investigated in post-angiogenic vessel pruning. We now found that tyrosine phosphorylation of EphrinB2 is critical for regulation of vessel survival and pruning. Knock-in mice with a targeted mutation of tyrosine residues in EphrinB2 display significantly increased EC death and reduced branching in hyaloid vessels compared to EphrinB2 wild-type mice at postnatal days 3, 4 and 5. To understand the mechanisms of phosphorylated EphrinB2 signaling, we performed immunoprecipitation experiments, and identified the tyrosine phosphatase SHP2, the tyrosine kinase JAK2 and the signal transducer/activator of transcription STAT1 as EphrinB2-interacting proteins that are modulated by EphrinB2 tyrosine phosphorylation. Through biochemical and genetic experiments we established that phospho-tyrosine EphrinB2 associates with SHP2 better than non-phosphorylated EphrinB2 in vitro and in hyaloid vessels of mice, and through this association promotes JAK-STAT1 de-phosphorylation and inactivation. Non-phosphorylated EphrinB2 lacking associated SHP2, activates STAT1 in the endothelium. These data indicated that phosphorylated EphrinB2 interacts with SHP2 and represses JAK2-STAT1 activation. We further identified the pro-apoptotic kinase JNK3 as a target of activated STAT1 by chromatin immunoprecipitation and JNK3 promoter reporter assays, and found that EphrinB2-5Y promotes JNK3 expression and EC death. In hyaloid vessels, JNK3 is expressed in degenerating endothelium that lacks phosphorylated EphrinB2. Since we found that JNK3-null mice show persistence of hyaloid vessels in the adult, we conclude that JNK3 is a mediator of cell death

modulated by EphrinB2. Taken together, we have identified a novel pathway, EphrinB2-STAT1-JNK3 controlled by phosphorylation of EphrinB2 that is critical for regulation of vessel survival and pruning in the vasculature of the eye.

NCI-CCR
Mary Olanich
Postdoctoral Fellow
Cell Cycle-General, Regulators and Checkpoints, Apoptotic Mechanisms
CDK4 amplification reduces sensitivity to CDK4/6 inhibition in fusion-positive rhabdomyosarcoma
Rhabdomyosarcoma (RMS) is the most common pediatric soft tissue sarcoma and includes a PAX3- or PAX7-FOXO1 fusion-positive subtype. Amplification of chromosomal region 12q13-q14, which contains the CDK4 proto-oncogene, was identified in an aggressive subset of fusion-positive RMS. Other tumor types with CDK4 amplification, including liposarcoma and neuroblastoma, are sensitive to CDK4/6 inhibition, suggesting that CDK4/6-targeted therapies may provide a new treatment strategy in fusion-positive RMS. We first confirmed CDK4 overexpression in RMS cell lines and diagnostic patient tumors harboring CDK4 amplification. To evaluate the role of CDK4 amplification in fusion-positive RMS and the potential clinical utility of CDK4/6 inhibition in this disease setting, we examined the biological consequences of CDK4 knockdown, CDK4 overexpression, and pharmacologic CDK4/6 inhibition in fusion-positive RMS in vitro and in vivo. Knockdown of CDK4 abrogated proliferation, transformation, and xenograft tumor growth of 12q13-14-amplified and non-amplified fusion-positive RMS via G1-phase cell cycle arrest. This arrest was mediated by reduced RB phosphorylation and E2F-responsive gene expression. Significant differences in E2F target expression, cell cycle distribution, proliferation, or transformation were not observed in RMS cells overexpressing CDK4. Treatment with LEE011, a highly selective CDK4/6 inhibitor, phenocopied CDK4 knockdown, decreasing viability, RB phosphorylation, and E2F-responsive gene expression and inducing G1-phase cell cycle arrest. Though all fusion-positive cell lines showed susceptibility to CDK4/6 inhibition, diminished responsiveness was associated with CDK4 amplification and/or overexpression. The inverse relationship between CDK4 expression and LEE011 sensitivity was recapitulated in xenograft models of CDK4-amplified and non-amplified fusion-positive RMS. Our data demonstrate that CDK4 is necessary but overexpression is not sufficient for RB-E2F-mediated G1-phase cell cycle progression, proliferation, and transformation in fusion-positive RMS. Moreover, our studies indicate that LEE011 is active in the setting of fusion-positive RMS and suggest that low CDK4-expressing tumors may be particularly susceptible to CDK4/6 inhibition. Thus, we propose a novel model of tumor sensitivity to LEE011 in which CDK4 amplification and resultant overexpression confer reduced rather than enhanced vulnerability to CDK4/6-targeted therapies.

NCI-CCR
Puspa Pandey
Postdoctoral Fellow
Carcinogenesis
PAX3-FOXO1 is essential for initiation but not for recurrence during rhabdomyosarcoma tumorigenesis
The PAX3-FOXO1 (P3F) fusion gene is generated by a 2;13 chromosomal translocation in rhabdomyosarcoma (RMS), and is associated with aggressive behavior and poor prognosis. This study

utilizes a novel inducible expression system in human myoblasts to dissect the molecular mechanism and contribution of P3F in RMS tumorigenesis. A human myoblast cell line (immortalized by BMI1 and TERT expression) was transduced with a retroviral construct that constitutively expresses MYCN and/or a lentiviral based-doxycycline inducible construct that variably and reversibly expresses P3F. Transformation in vitro and tumorigenesis in vivo were assessed by cell culture focus formation and animal xenograft experiments, respectively. Myogenic differentiation was assessed by light microscopy and by western blot and immunohistochemical assays of muscle-specific protein expression. P3F-transduced myoblasts treated with doxycycline demonstrated a time- and dose-dependent increase in expression of P3F mRNA and protein and its downstream targets genes; doxycycline withdrawal led to cessation of fusion protein expression. Though myoblasts expressing P3F or MYCN alone did not show evidence of transformation in culture, combined P3F and MYCN expression resulted in myoblast transformation. Under differentiation-promoting culture conditions, combined P3F and MYCN expression inhibited myogenic differentiation. Intramuscular injection of myoblasts with MYCN and P3F resulted in rapid RMS tumor formation in NOD-SCID mice when fusion protein expression was induced by feeding mice a doxycycline-containing diet. Myoblasts with MYCN expression alone did not form any tumors while P3F induction without MYCN expression resulted in RMS tumors after a much longer latency period. After tumors formed from myoblasts expressing P3F with or without MYCN, down-regulation of P3F expression by doxycycline withdrawal resulted in tumor regression associated with cell death and myogenic differentiation. The regressed tumors slowly grew back in the absence of doxycycline demonstrating a P3F-independent oncogenic mechanism for recurrence. In summary, the P3F fusion protein collaborated with MYCN in the initial stage of RMS tumorigenesis to promote dysregulated cell proliferation and inhibit myogenic differentiation. Though most cells in the initial tumor were dependent on the fusion protein, recurrent tumors formed in which the fusion protein was not required to maintain the tumorigenic phenotype.

NCI-CCR
jeongwon park
Visiting Fellow
Endocrinology
Abstract removed by request of author

NCI-CCR
Sunmi Park
Postdoctoral Fellow
Endocrinology
Abstract removed by request of author

NCI-CCR
Adam Parks
Research Fellow
Microbiology and Antimicrobials
Engineering Bacteriophage To Target KPC+ K. pneumoniae
KPC+ Klebsiella pneumoniae are extensively resistant to commonly used antibiotics. I have developed a

system for rapidly and efficiently modifying bacteriophages for use as therapeutic agents. The overall goal is to produce a bacteriophage-based treatment that can be used to specifically reduce the abundance of Klebsiella in the GI-tract, thus preventing the development of bacteremia without harming other bacterial residents of the microbiome. By creating a library of modified Klebsiella phage, bacterial resistance to phage therapy can be circumvented. The Red-Recombinase system was used to modify phage T7 as a model system for determining the conditions necessary to generally modify lytic phage genomes. These experiments focus on exchanging tail fiber genes, since they are the primary players in bacterial cell recognition. Genomic DNA from bacteriophage T7 was co-eletroporated into cells, along with synthetically produced oligonucleotides that encode a desired genetic change. Cells that expressed Red-Recombinase showed a 1000-fold increase in phage transfection frequency over controls that do not express the recombinase. Phage particles that infect Klebsiella can also be recovered from Red-expressing E. coli cells that have been transfected with Klebsiella specific phage genomic DNA, indicating that E. coli may be used as a general-purpose recombination host. Oligonucleotide recombination frequencies up to ~10% have been achieved to make simple point mutational changes, and deletions of phage genes are made with 1-5% efficiency within the targeted phage genome. In addition to direct recombination within the phage genome, I have recombined tail fiber genes onto the E. coli chromosome; construction of recombinant tail fiber genes is less complicated and more efficient when carried out on the E. coli chromosome than when attempted on the phage genome itself. I have shown that such reconstructed genes on the bacterial chromosome are efficiently recombined into the T7 genome, when sufficient DNA homology is present. In other experiments when tail fiber genes are expressed from the E. coli chromosome the fibers made can assemble onto T7 phage defective for their own tail fiber production. The series of bacterial and phage strains that I have generated will be useful in generating diverse phages that lyse and kill antibiotic resistant Klebsiella, and the tools developed in this study can be broadly applied to phages of other intractable pathogens.

NCI-CCR
SHEIKH RAHMAN
Visiting Fellow
Intracellular Trafficking

HIV-1 RNA partner selection: when, where, and how?

Infectious HIV-1 contain two copies of the viral RNA genome that forms a dimer. RNA dimerization occurs prior to packaging into particles. Biochemical studies showed that HIV-1 Gag protein, the main driver of particle assembly, can interact with HIV-1 RNA in the cytoplasm. Thus, it is generally assumed that the two co-packaged RNAs dimerize in the cytoplasm and form a complex with few Gag molecules, then travel to the main assembly site, the plasma membrane, to form infectious particles. However, the sub-cellular location(s) in which HIV-1 RNA dimerization occurs is currently undefined. To investigate this question, we tagged RNAs derived from two viruses, one with yellow fluorescent protein (YFP) and the other with a red fluorescent protein mKate. In this system, each virus was engineered to contain RNA sequences recognized by either MS2 bacteriophage coat protein or E.coli antitermination protein BglG; by tagging these two RNA-binding proteins with different fluorescent proteins, we can distinguish RNAs derived from the two viruses. We also tagged the viral protein Gag with a cerulean fluorescent protein (CeFP). We then studied HIV-1 RNA dimerization using live-cell imaging and analyzed the behavior of

viral RNAs near the plasma membrane by total internal reflection fluorescence (TIRF) microscopy. We envisioned two possible mechanisms for RNA dimerization: 1) HIV-1 RNAs dimerize in cytoplasm and travel to assembly site together, or 2) HIV-1 RNAs dimerize not in cytoplasm, but at the assembly site on the plasma membrane. Using single-molecule tracking, we followed >30 events in which two RNA colors co-localized near the plasma membrane. In most of these events, the two RNA signals appeared sequentially on the plasma membrane. One RNA signal was detected first and a second RNA with a different color joined the first signal at a later time point, after which these co-localized signals migrated together, and followed by the increase of Gag-CeFP signal indicating active assembly. These results reveal that most of the viral RNA dimerization occurs at the plasma membrane and the RNA dimer promotes Gag assembly. To our knowledge, this is the first detection of RNA dimerization in living cells, and the first direct evidence on the dimer RNAs promote HIV-1 assembly. These results provide novel insights into how RNA genomes are packaged and how infectious HIV-1 viruses are produced from cells.

NCI-CCR
Arnab Ray Chaudhuri
Visiting Fellow
DNA-binding Proteins/Receptors and DNA Repair

PTIP recruitment of MRE11 nuclease drives replication fork instability in BRCA-deficient cells

BRCA1 and BRCA2 (BRCA1/2) are tumor suppressor genes essential for cellular proliferation whose inactivation markedly increases the risk of developing breast and ovarian cancers. BRCA-deficiency triggers genomic instability and growth arrest by compromising DNA double strand break (DSB) repair by the homologous recombination (HR) and also by promoting DNA degradation at sites of stalled replication forks. HR defects associated with BRCA1 deficiencies can be rescued by loss of 53BP1 (a factor involved in error-prone DSB repair). To date, no mutations have been described that bypass the growth arrest in BRCA2-deficient cells. In this study, using mouse genetics and single molecule DNA fiber assays for replication fork analysis, we show that replicative stress induced fork degradation and chromosomal instability observed in BRCA1/2 deleted cells can be rescued by a loss of PTIP, an effector of 53BP1. Surprisingly, this rescue of genomic stability is not dependent on functional HR, as Rad51 foci a hallmark of HR, is absent at sites of DSBs in these cells. Loss of PTIP confers protection to replication forks from nuclease mediated degradation in the absence of BRCA1/2 and remarkably rescues the viability of BRCA2-deficient embryonic stem cells. Biochemical analysis of stalled replication forks by iPOND (isolation of Proteins On Nascent DNA) revealed that PTIP was involved in the recruitment of MRE11 nuclease to stalled replication forks. In the absence of PTIP, nascent DNA strands were protected from degradation. This function of PTIP is independent of its interaction with 53BP1 but mediated via the MLL3/4 histone methyltransferase complex. Our data suggest that increased methylation of H3K4me1/3 by MLL3/4 complex at stalled forks lead to enhanced loading of MRE11 and results in degradation of nascent DNA at these sites. Our data provides the first evidence of a genetic alteration that overcomes growth arrest and platinum sensitivity of BRCA-deficient cells by counteracting the HR-independent functions of BRCA1/2. The clinical relevance of PTIP deficiencies in chemo-resistance has been validated using public databases, where low levels of PTIP in BRCA1/2-deficient ovarian cancers correlated with poorer survival in patients treated with replication stress inducing platinum drugs. This

suggests that DNA repair proteins like PTIP can be used as predictive biomarkers of chemo-resistance and will contribute to the design of more efficient chemotherapeutic regimens.

NCI-CCR
David Sanchez Martin
Visiting Fellow
Tumor Biology and Metastasis
Deleted in Liver Cancer 1 (DLC1) regulates cell survival in primary endothelial cells through TNFAIP3
DLC1 is a tumor suppressor gene very frequently down-regulated in cancer. Current understanding of DLC1 mostly derives from over-expression studies in malignant cells that have lost DLC1 expression during the tumorigenic process. These studies have shown the importance of DLC1 in metastasis, clonogenic growth and migration. However, this approach provides limited information on the physiological role of DLC1. To address this limitation, we explored the physiological role of DLC1 in primary endothelial cells (EC): Human Umbilical Vein Cells (HUVEC) and Human Dermal Microvascular Endothelial Cells (HMVEC-D). We found that DLC1 expression is dependent on endothelial cell confluence: DLC1 is barely detectable in cells growing at low density, whereas DLC1 expression is easily detected in confluent cell cultures. When reaching confluence, primary EC first maintain a monolayer by equilibrium between cell division and cell death by anoikis, but after several days at confluence undergo a “crisis” characterized by abundant cell death. By contrast, we found that DLC1-silenced primary EC are significantly more resistant to confluence-associated death; maintain extended cell growth, show moderate stratification and form some dense foci. Consistent with this, DLC1-silenced, but not control cells, formed small subcutaneous tumors in immunodeficient mice that subsequently regressed. Microarray analysis of control and DLC1-silenced HUVEC revealed the presence of a group of differentially expressed genes; this difference was confirmed at the protein level by western blotting. Network integration using Ingenuity Pathway Analysis uncovered a previously unreported link between DLC1 and A20 (also known as tumor necrosis alpha-induced protein 3, TNFAIP3), a zinc finger protein that inhibits TNFa-induced cell death by blocking NFkB activation. Silencing TNFAIP3 reversed, in part, the death resistance phenotype in confluent cell culture of HUVEC and HMVEC-D induced by the loss of DLC1. These observations provide evidence for a previously unrecognized role of DLC1 as a physiological regulator of contact-inhibited cell growth and survival, suggesting an important role of DLC1 in early stages of tumorigenesis.

NCI-CCR
Kazuhide Sato
Postdoctoral Fellow
Radiology/Imaging/PET and Neuroimaging
Selective cell elimination in vitro and in vivo from tissues and tumors using near infrared photoimmunotherapy
Cell cultures and tissues often contain cellular subpopulations that potentially interfere with or contaminate other cells of interest. However, it is difficult to eliminate unwanted cells without damaging the very cell population, which one is seeking to protect, especially in established tissue. Near infrared photoimmunotherapy (NIR-PIT) is a new treatment that combines the specificity of intravenously

injected antibodies that target tumors with the toxicity induced by photosensitizers activated with NIR-light. Here, we present a method of eliminating a specific subpopulation of cells from a mixed 2D or 3D cell culture and a mixed-population in vivo tumor model by using NIR-PIT without damage to non-targeted cells. Using RFP, GFP and luciferase as optical reporters, we could demonstrate selective cell elimination. In vitro and in vivo experiments were conducted with an EGFR, luciferase and GFP expressing cell line (A431-luc-GFP) as a target, and no-EGFR, RFP expressing cell line (Balb/3T3-RFP) as a non-target. An antibody-photosensitizer conjugate consisting of panitumumab and a phthalocyanine dye, IRDye-700DX, was synthesized. In vitro NIR-PIT cytotoxicity was confirmed with dead staining, luciferase activity, and GFP fluorescence intensity. In vivo NIR-PIT effect for A431-luc-GFP xenografted tumor was demonstrated with bioluminescence image (BLI), and fluorescence image (FLI). Specific cell elimination from almost confluent in vitro 2D mixed cell culture and in vitro mixed 3D (spheroid) cell culture were evaluated with optical reporters. Specific cell elimination from in vivo mixed tumor was assessed with BLI and FLI. As a result, elimination of targeted cells from mixed 2D and mixed 3D cell cultures were done without damaging non-target cells. Repeated NIR-PIT also led to complete elimination of target-expressing cells from mixed tumors in vivo. BLI and FLI of ex vivo tumor confirmed the results. All these data were demonstrated with BLI and FLI, and quantified by luciferase activity and fluorescence intensity. With these demonstrations, we propose that NIR-PIT is a practical method for eliminating a selective set of cells from cell culture or tissue in vitro or local environment in vivo without damaging the remaining cells. Locally specific cell elimination by NIR-PIT has potential application in many fields, for instance, regenerative medicine, immunomodulation, and tumor immunity.

NCI-CCR
Iain Sawyer
Visiting Fellow
Chromatin and Chromosomes

Cajal bodies shape genome configuration

The nucleus is highly organized and gene expression is regulated by both spatial genome organization and nuclear architecture. Thus, a variety of domains and nuclear bodies (NBs) exist within the nucleus. These regions are often observed at actively transcribing genes and perform a number of essential, but thermodynamically unfavorable, processes. Despite their prominence in the nucleus, NB formation and contribution to genomic function is unclear. A prime example is the Cajal body (CB), which is present in aneuploid transformed cells, such as HeLa cells. The CB catalyzes small nuclear RNA (snRNA) biogenesis and spliceosomal snRNP recycling after each round of RNA splicing. We studied the reciprocity between gene activity and CB function to determine whether CBs are passive by-products of specific gene activity or if they actively influence functional genome configuration. Using a novel 6-color DNA FISH imaging method it was established that CBs are non-randomly positioned in HeLa cells and are often situated at a multi-chromosome interface. In this region CBs are in contact with snRNA genes from several chromosomes. By performing genome-wide chromatin conformation capture analysis (4C-seq) using several CB-proximal loci as references we defined all gene pairing events which occur in close proximity to CBs. Notably, these data suggest that a single CB was responsible for the restructuring of chromosome 1 into a rosette structure which enveloped the CB. Furthermore, CBs simultaneously interact with multiple chromosomes and gene loci to form specific gene clusters. These pairing events

are not detectable in primary diploid cells lacking CBs. RNAi-mediated disassembly of CBs led to a reorganization of global chromatin structure and reduced the number of CB-proximal gene pairing events. As CBs contain factors necessary for the processing of snRNA genes, this list of CB-proximal genomic loci was integrated with RNA-seq datasets after CB depletion. CBs specifically promote the expression of spliceosomal RNA genes and all categories of small U RNA genes but not proximal genomic regions. Transcriptional analysis revealed that CB disassembly induces a subtle global gene expression change and a substantial increase in splicing noise. These observations suggest that CBs support the 3D organization of the human genome and will help to reveal the mechanism by which a specialized nuclear structure directly contributes to the regulation of genome topology and function.

NCI-CCR
Sigal Shachar
Visiting Fellow
Chromatin and Chromosomes

Systematic identification of genome positioning factors by high-throughput screening

The DNA in a mammalian nucleus measures more than 2 meters in length, yet it has to fit into a 10um nucleus in a manner that will enable proper function of all cellular activities. It has been known for decades that the genome is not randomly arranged in the 3D space of the cell nucleus. The specific location and environment of a genomic locus affects numerous essential functions, including transcription, replication timing and repair. Despite the undisputed importance of genome organization, little is known regarding the identity of the cellular factors that establish and maintain the 3D location of genomic loci. In this study we have developed a method to identify genome organization factors in a high-throughput and unbiased manner. Previous studies that investigated genome organization used traditional fluorescent in situ hybridization (FISH) which is often qualitative in nature and requires prior knowledge of candidate proteins. Our method relies on a high-precision, high-throughput, automated FISH imaging pipeline followed by novel image analysis tools that enabled us to conduct an unbiased siRNA screen to identify factors involved in genome organization in human cells. Using this novel approach we identified 50 cellular factors required for accurate positioning of a set of endogenous genome regions. Most of the factors identified were not previously implicated to function in genome organization. Prominent among the identified factors were components of the replication and post-replication chromatin re-assembly machinery. We were able to show that replication per se is required for proper gene positioning and that when replication is perturbed genomic regions are mis-organized and occupy a different region in the nucleus. Additionally, we showed that replication, but not mitosis, is required for correct genome positioning. These results establish for the first time a method for the unbiased identification of molecular mechanisms involved in genome positioning and they provide a compendium of genome organization factors. The identification of candidate genome positioning factors now opens the door to the investigation of their precise mechanism of action. The method we developed is applicable to various biological systems and will be a useful tool to study the molecular basis of numerous aspects of genome architecture.

NCI-CCR
Jonathan Shrimp

Postdoctoral Fellow
Chemistry
A signal-amplifiable, ligand-displacement assay for small molecule epigenetic modulator discovery
Lysine acetyltransferase (KAT) enzymes are key regulators of gene expression programs in many diseases, including cancer. For example, the acetyltransferases Gcn5 and p300 function as coactivators of the oncogenic transcription factor c-Myc, facilitating changes in chromatin accessibility and protein-protein interactions necessary for c-Myc-mediated gene expression. While such examples provide a strong rationale for targeting acetylation-dependent signaling cascades in cancer, few KAT inhibitors are currently known. Current in vitro assays for KATs are insensitive and prone to the identification of false-positives, both of which limit high-throughput screening efforts. To address this challenge, we have developed a highly sensitive, ligand-displacement assay for KAT enzymes. This strategy uses a proximity immunoassay approach known as AlphaScreen to detect the binding interaction of a biotin-labeled KAT cofactor analogue (biotin-H3K14-CoA) and a His-tagged KAT (Gcn5). This KAT-cofactor interaction brings a singlet-oxygen producing streptavidin "donor" bead into close proximity to an anti-His "acceptor" bead that upon reaction with singlet oxygen, produces an intense fluorescence signal. Since each KAT-cofactor interaction causes multiple singlet oxygen release events, the fluorescence signal is greatly amplified compared to traditional binding assays. This signal amplification permits highly sensitive detection of Gcn5 (signal: noise ratio >400:1), which is reduced in the presence of competitive inhibitors, thus providing a powerful platform for small-molecule inhibitor discovery. Using Gcn5 as a prototypical KAT we have demonstrated the assay is effective in a miniaturized (384-well) format and enables the rapid, quantitative determination of dissociation constants for known Gcn5-interacting ligands. Current efforts are focused on applying this approach in screening efforts to identify small molecules able to probe the targetable role of Gcn5 in c-Myc regulated gene expression. More broadly, we envision this approach may provide a general strategy for the development of highly sensitive activity assays for "orphan" chromatin modifiers that utilize a cofactor (i.e. acetyl-CoA, ATP, NAD+) but whose substrate is not known. Thus, signal-amplifiable ligand-displacement assays have the potential to greatly accelerate efforts to define the function of chromatin modifiers in cancer biology.

NCI-CCR
Nathan Simon
Postdoctoral Fellow
Clinical and Translational Research
Targeting Triple Negative Breast Cancers with an Immunotoxin Directed to Tumor-Associated EGFR
Triple negative breast cancers (TNBC) are typically more aggressive and result in poorer outcomes than other breast cancers because treatment options are limited due to lack of hormone receptors or amplified HER2. Many TNBCs overexpress the epidermal growth factor receptor (EGFR) or manifest amplification of the EGFR gene, supporting EGFR as a therapeutic target. Using the single chain variable fragment (scFv) of the 806 monoclonal antibody that binds only to cells with overexpressed, misfolded, or mutant-variants of the EGFR, a recombinant immunotoxin was engineered through a gene fusion with truncated Pseudomonas aeruginosa Exotoxin A (806-PE38). 806-PE38 reduced the viability of multiple TNBC lines through inhibition of protein synthesis in a concentration-dependent manner, while not affecting cells with wild-type EGFR. Deletion of a catalytic residue, E553, from the toxin resulted in loss

of cytotoxic activity, confirming the toxin's ADP-ribosyltransferase activity as the mediator of cell death. Systemic treatments with 806-PE38 resulted in reduced tumor burdens and increased survival in two TNBC mouse xenograft models. These data support the development of the 806-PE38 immunotoxin as a therapeutic agent for the treatment of patients with EGFR-positive TNBC.

NCI-CCR
Eric Sterner
Postdoctoral Fellow
Biochemistry - General and Lipids
Profiling Mutational Significance in Germline-to-Affinity Mature 3F8 Variants
Antibody immunotherapy is rapidly becoming the standard-of-care practice in the treatment of many cancers. One such instance is the treatment of the childhood extra-cranial cancer neuroblastoma. In this case, the up-regulation of the disialoganglioside GD2 on the cellular surface is a hallmark of tumor cells and as such has become a clinically critical target. Two clinically successful anti-GD2 candidates, 14.18 and 3F8, demonstrate remarkable affinity and have become integral to neuroblastoma treatment. Considering the difficulty of developing high affinity antibodies against carbohydrate antigens, we wanted to better understand how the immune system evolved these antibodies. Our work explores the immunological evolution of 3F8, including the synthesis of germline, mutational intermediates, and affinity mature antibodies. Our long term goal is to apply lessons learned from this work in the development of better anti-glycan antibodies. One of the challenges of comparing germline and affinity mature antibodies is the breadth of reactivity. Given that the germline antibody is expected to have broader specificity than the affinity matured antibody and that the binding partners of the germline may be difficult to predict, tools that adequately probe the interactome are needed to fully characterize germline specificity. Within our laboratory, we have developed a high-throughput, glycan array platform that allows for the screening of a single carbohydrate binding antibody against hundreds of antigens in a single assay. We profiled the binding of anti-GD2 monoclonal antibodies, including 3F8. Our results not only validated the high affinity of these anti-GD2 antibodies, but also demonstrated their remarkable specificity. These antibodies bound almost exclusively GD2 with >100-fold selectivity over closely related gangliosides. An IgBLAST of the 3F8 nucleotide sequence yielded a putative germline sequence with two light chain and four heavy chain complementary determining region mutations. DNA for the germline, affinity mature, and six single-mutant knockouts were synthesized, transfected into HEK293 cells, and then product antibodies were screened using our glycan array. Interestingly, preliminary data from these experiments indicate that the germline 3F8 antibody is already highly specific for GD2, an interesting revelation counter to the typical view of a highly non-specific germline structure.

NCI-CCR
Lei Sun
Visiting Fellow
Stem Cells - General
Loss of Folliculin disrupts hematopoietic stem cell quiescence and homeostasis resulting in bone marrow failure
Folliculin (Flcn) modulates many different pathways that regulate growth, proliferation, metabolism,

survival, motility and adhesion. It is an essential protein required for murine embryonic stem cell commitment and Drosophila germline stem cell maintenance. However, the function of Flcn in adult stem cells has not been investigated. Hematopoietic stem cells (HSCs) sustain multi lineage blood cell development over the life of the animal by their unique ability to proliferate, self-renew and differentiate. Normally, HSCs are protected from proliferative exhaustion by remaining in a quiescent or dormant state, but the mechanisms that regulate these processes are largely unknown. To determine if Flcn is required for adult stem cell development, we conditionally inactivated Flcn in HSCs. This drove HSCs into proliferative exhaustion resulting in the rapid depletion of HSCs, loss of all hematopoietic cell lineages, acute bone marrow failure, and mice became moribund after 40 days. HSCs that lack Flcn failed to reconstitute the hematopoietic compartment in recipient mice, demonstrating a cell-autonomous requirement for Flcn in HSC maintenance. Flcn null bone marrow cells also showed increased phosphorylation of AKT and mTOR. Treatment with rapamycin significantly reduced hyperplasia, suggesting that the mTOR pathway was activated by loss of Flcn expression in hematopoietic cells in vivo. Furthermore, Tfe3 was activated and preferentially localized to the nucleus of Flcn knockout HSCs. Tfe3 overexpression in HSCs impaired long term hematopoietic reconstitution in vivo, recapitulating the Flcn knockout phenotype, supporting the notion that abnormal activation of Tfe3 contributes to the Flcn knockout phenotype. Flcn knockout mice develop an acute histiocytic hyperplasia in multiple organs, and an expansion of abnormally activated macrophages, which engulf hematopoietic cells resembling human hemophagocytic lymphohistiocytosis (HLH). These data demonstrate that Flcn is required to maintain HSC quiescence, and Flcn loss ultimately results in bone marrow failure. Therefore, Flcn loss in human patients may also contribute to bone marrow failure, which threatens patient survival. We believe our results can help understand the development of bone marrow failure, and result in the identification of novel biomarkers that can be used for prognosis and potential treatment of this disease.

NCI-CCR
Erin Swinstead
Postdoctoral Fellow
Chromatin and Chromosomes

Steroid Receptors can facilitate the binding of the pioneer factor FoxA1 in breast cancer cell lines through a dynamic assisted loading (DynALoad) mechanism

The steroid receptors (SRs), estrogen receptor (ER) and glucocorticoid receptor (GR) in addition with forkhead box protein (FoxA1) all play an important role in breast cancer development. The ER, GR, and FoxA1 status in breast cancer is a significant factor for determining the outcome of the disease. However, the cellular interactions between ER, GR, and FoxA1 and the role these interactions play in the progression of breast cancer are not well understood. FoxA1 has been implicated in ER binding patterns serving as a pioneer factor; however, the effect of ER on the function of FoxA1 has been controversial. Furthermore, the molecular interplay between GR and FoxA1 is also poorly understood. Classically it has been proposed that FoxA1 has a slow residence time on DNA allowing chromatin to remain open permitting access of SRs to binding sites. Conversely, it has recently been identified that SRs can alter the binding landscape of other SRs facilitating selective access to the chromatin by a mechanism termed dynamic assisted loading (DynALoad). To determine if the DynALoad phenomena extends to FoxA1 we

characterised GR, ER, and FoxA1 crosstalk in three estrogenic breast cancer cell lines utilizing chromatin immunoprecipitation followed by high-throughput sequencing. Genome-wide analysis of FoxA1 binding upon hormone treatment shows that both activated ER and GR can recruit FoxA1 to specific binding sites. In addition, investigation of chromatin structure determined by genome-wide DNase hypersensitivity sequencing demonstrates an increase in chromatin accessibility at the newly established FoxA1 binding sites. This indicates chromatin reorganization upon activation of ER and GR and recruitment of FoxA1 binding through a DynALoad mechanism. Most importantly, there is a lack of a FoxA1 DNase footprint in these cells suggesting the binding pattern is highly dynamic with a short DNA residence time. These findings do not support a model wherein a specific set of pioneer factors which bind to closed chromatin and establish the binding landscape for other TFs. These results suggest rather that many TFs in a given cell have the potential to affect the binding landscape of other TFs, depending on the chromatin context. In addition, this study has shifted our classical understanding of pioneer factors in breast cancer, demonstrating that activated GR and ER have the capability to recruit and alter the response of FoxA1.

NCI-CCR
Gokhan Tolun
Postdoctoral Fellow
Protein Structure/Structural Biology
The oligomeric structure of human retinal protein retinoschisin provides insights into its molecular mechanisms underlying juvenile macular degeneration
Retinoschisin (RS1) is a retinal cell surface protein that is predicted to be an adhesion complex preserving the structure and visual function of the retina. Mutations in RS1 gene lead to macular degeneration in young males, characterized by splitting (schisis) of the inner retinal cell layers, disorganized synapses, reduced electrical response of the retina, and vision loss with age. Biochemical studies suggested that RS1 forms a homo-octameric ring, but in-depth structural studies have not been possible due to extremely low-yields of purified RS1, preventing x-ray crystallography studies. Hence, the molecular mechanisms underlying RS1 function remain unknown. We purified a sufficient quantity of full-length human RS1 for electron microscopy (EM) imaging. Using state of the art Cryo-EM technologies and single-particle 3D reconstruction, I determined the molecular structure of RS1 at an average resolution of 4.6 Å with local regions up to 3 Å. This structure shows that RS1 forms a stack of two octameric rings in a non-covalent sandwich arrangement. This novel observation allows me to propose a molecular mechanism that describes the adhesion function of RS1: two RS1 single-rings found on the surfaces of two opposing retinal layers bind to each other to hold the two retinal layers together. The structure also shows that RS1 has two domains. The N-terminal domain is in the center of RS1-rings and is unstructured and/or very flexible. The C-terminal residues form a well-known protein domain called discoidin that forms binding pockets for a diverse array of ligands in many proteins. Bioinformatics analysis I performed, supported by biochemical data, suggested that the discoidin domain in RS1 binds galactose. Glycan array data and EM experiments using galactose-conjugated gold beads confirmed this prediction. Accordingly, RS1 may be interacting with glycoproteins to either activate intracellular pathways, or to potentiate further adhesion through an unknown mechanism. In summary, the double-octameric oligomeric ring structure of RS1 shows greatly enhanced multivalency of discoidin domains,

presenting multiple binding surfaces for its ligand(s). This structure also enables us to propose a molecular mechanism for the adhesion function of RS1 in keeping the retinal layers together. Identification of its ligands and dissecting the structure and function of its N-terminal domain will provide us with further insights into RS1 function, facilitating future therapies.

NCI-CCR
Yusuke Tomita
Visiting Fellow
Gene Expression
Development of a third generation next-gen sequencing technique for the discovery of oncogenic androgen receptor splice variants driving castrate-resistant prostate cancer
Castrate-resistant prostate cancer (CRPC) is a lethal form of prostate cancer. Androgen receptor (AR) splice variants that excise the ligand binding domain (LBD) drive prostate cancer progression and contribute significantly to lethality by eliminating the molecular target for all approved antiandrogen therapies. It would be highly desirable to assess prostate tumor specimens for AR splice variants and monitor circulating tumor cells (CTCs) during patient therapy to analyze the evolution of splice variants, so that patients are not treated with ineffective therapy. The full-length AR mRNA is 10 kb. Twenty AR splice variants have been reported. Most next-generation sequencing (NGS) platforms including Illumina, with average read lengths of 150 bases are not well equipped to sequence the long and complex AR transcripts. The PacBio third generation NGS platform can generate markedly longer reads of greater than 10 kb. We hypothesized that we could combine nanoferrofluidic isolation of tumor cells from blood and multiparametric flow identification with imaging and sorting technology to isolate CTCs and sequence the entire AR mRNA in a single read. Our goal is to develop an assay that goes from a simple blood draw to sequencing of the full-length AR transcript and all variants, including novel variant discovery in a single read. We spiked 22Rv1 cells into peripheral blood mononuclear cells and enriched CTCs by more than three logs. Then, single cancer cells or hematopoietic cells were successfully sorted on the DEPArray platform. We confirmed valid calls by whole genome amplification, PCR of the AR LBD and Sanger sequencing. A 22Rv1 AR library was created by 3'RACE using an anchored oligo(dT) containing a universal priming site and PCR using a 5' gene specific primer and a universal 3' primer and sequenced on the PacBio RS II. Clustering algorithms enabled sequencing of full-length AR, multiple previously identified transcripts including AR-V7 and novel transcripts. Target-specific sequencing was confirmed on the Illumina HiSeq platform. Although this study is in an early phase, these results establish a pathway to CTC isolation and AR mRNA sequencing from blood facilitating a precision medicine approach to the molecular characterization of prostate cancer as it evolves during therapy, and the choice of therapy appropriate to the patient. We are applying this method to CRPC patients in a new clinical trial of neoadjuvant androgen deprivation and enzalutamide.

NCI-CCR
Carlos Tristan
Postdoctoral Fellow
Carcinogenesis
Hippo pathway effector TAZ is required for drug resistance and tumorigenesis of glioblastoma stem cells.

Glioblastoma Multiforme (GBM) is an extremely aggressive brain cancer characterized by rapid progression, high resistance to current therapeutic regimens, and survival rates of only 25% two-years post diagnosis. These characteristics of GBM are attributed to glioblastoma stem cells (GSCs), a highly tumorigenic subpopulation of cancer stem cells that have been identified as the true therapeutic targets for the treatment of GBM. Normal organ and tissue growth during development is regulated by the Hippo pathway, wherein cell-to-cell contact-induced activation of this kinase cascade inhibits cell growth and proliferation. In recent years, studies have suggested that dysregulation of the Hippo signaling pathway might underlie cancer progression, poor overall patient survival and treatment resistance in GSCs. Since high grade gliomas are positively correlated with the expression of TAZ; a key downstream effector of the Hippo pathway, and therapeutic resistant stem cells give rise to GBM, we hypothesized that dysregulation of the Hippo pathway in cancer stem cells may play a significant role in the propagation and sustainment of GBM. To address this question, we conducted loss-of-function studies using shRNAs against TAZ in GSCs and determined if TAZ plays a functional role in drug resistance and tumorigenesis in GBM. Here we demonstrate that TAZ knockdown sensitizes GSCs to chemotherapeutic tyrosine kinase inhibitors (TKIs) via a 10-fold increase in efficacy, when compare to controls. Furthermore, we demonstrate that knockdown of TAZ abrogates several tumorigenic characteristics of GSCs, including anchorage-independent cell growth, cell migration and invasiveness. In addition, gain-of-function studies using overexpression of TAZ in immortalized normal human astrocytes (iNHAs) confers anchorage-independent cell growth and augments cell migration in iNHAs. Our studies suggest that TAZ is functionally associated with resistance to TKIs and multiple tumorigenic characteristics of glioblastoma stem cells. Furthermore, the tumorigenic properties acquired by immortalized NHAs via overexpression of TAZ suggest that TAZ might drive the transformation of normal cells into cancerous cells. An increased understanding of the molecular interactions underlying the functional role of TAZ in drug resistance and tumorigenesis will help identify potential therapeutic targets for the treatment of GBM.

NCI-CCR
Casmir Turnquist
Doctoral Candidate
Cell Biology - General
p53 isoforms regulate astrocyte senescence in neurodegenerative disease
Aging is the main risk factor for neurodegenerative diseases. Similar to aging in other organs, brain aging is accompanied by increased cellular stress, accumulation of reactive oxygen species, and cellular senescence, all of which propagate neuronal dysfunction and loss. Many of these processes are mediated by astrocytes, which possess a senescence-associated secretory phenotype (SASP) that increases with aging and is further enhanced in neurodegenerative diseases. Previous studies have reported an increase in SASP astrocytes in amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (AD); however potential therapeutic strategies to moderate senescence remain unclear. Here we report that p53 isoforms, ?133p53 and p53ß, previously identified as regulators of cellular senescence in T cells and fibroblasts, are endogenous regulators of cellular senescence in the central nervous system (CNS). ?133p53 functions as a dominant-negative regulator of p53 and represses senescence, while p53ß as a co-activator of p53, promotes senescence. In neurodegenerative disease brain tissue, full-legth-53 and

p53ß are upregulated while ?133p53 is downregulated. We demonstrate that ?133p53 and p53ß directly regulate astrocyte senescence , including the release of key neurotoxic pro-inflammatory cytokines such as IL-6 and IL-1ß. Also, we show that the p53 isoform switch that occurs during aging and neurodegeneration promotes neuronal toxicity using co-culture experiments with human iPSC-derived motor neurons and astrocytes. We also demonstrate that astrocyte senescence can be reduced through overexpression of ?133p53, revealing a promising therapeutic approach to delay or inhibit the progression of neurodegeneration.

NCI-CCR
Oscar Vidal
Visiting Fellow
Carcinogenesis

A novel tumor suppressive role for the dopamine receptor DRD1 in lung cancer

We recently discovered an epidemiological association between genetic variation in the dopamine receptor DRD1 and risk of lung cancer. In addition, studies have shown that there is an inverse comorbidity between Parkinson's disease and lung cancer. Combined, these data suggest that the dopamine pathway (DAP) may play a role in lung cancer. First we comprehensively characterized the DAP in normal lung tissue from mice, and normal and cancer human cell lines, as this had not been done before. Our results showed that the DAP is present in the distal and peripheral areas of the normal lung, including the bronchus and alveoli. We also detected elements of the DAP in human cancer cells. However, there are differences between normal and cancer cells. For example, cancer cells secreted dopamine to the extracellular media and expressed tyrosine hydroxylase (TH), the enzyme responsible for synthesizing dopamine, while normal lung cells did not. In addition, DRD1 was significantly decreased in human lung cancer tissues compared with non-involved tissue, an observation that we replicated in both adenocarcinoma and squamous cell carcinoma using TCGA data. We also found that increased expression of miR-142-3p is linked to DRD1 down regulation in lung cancer. Specifically, exogenous expression of miR-142-3p decreases DRD1 mRNA and protein expression, there is a negative correlation between DRD1 and miR-142-3p in lung cancer and luciferase assays show direct binding between these two RNAs. Follow-up in vivo and in vitro experiments found that DRD1-positive lung cancer cells have decreased tumor growth in xenograft models, increased sensitivity to chemotherapeutic drugs, lower colony formation, and less migration and invasion capacity, as compared with DRD1-negative cells, consistent with a role as a tumor suppressor. We further assessed whether drugs that modulate dopamine in the peripheral nervous system are associated with lung cancer survival. Indeed, in an analysis of 1,097 lung cancer patients in the NCI-MD case control study, we observed that individuals taking norepinephrine/dopamine reuptake inhibitors had a significantly better outcome. Taken together our results suggest that the DAP may play a role as a tumor suppressor during lung cancer and further highlights the relationship between the DAP and cancer. These findings could impact both our understanding of the etiology of lung cancer, and also generate a new list of potential targets for lung cancer therapy.

NCI-CCR
Lizhen Wang

Postdoctoral Fellow
Cell Biology - General
Role of Vav1 in the regulation of age-associated endothelial dysfunction
A normal and healthy endothelium maintains vascular homeostasis by regulating the balance between vasoconstriction (constraction) and vasodilation (relaxation). Aging is associated with endothelial dysfunction that represents a pathological state as a decrease in the capacity of the endothelium to dilate blood vessels in response to physical and chemical stimuli due to increased stiffness. Here we report the role of Vav1 epxression in the regulation of vascular tone associated with aging. The study stems from the observation that mice deficient of vav1 expression exhibits dilated blood vessels. Force-tension myography analysis identifies that both young and aged vav1 deficient mice show greater relaxation in aorta than their correpsonding wild type littermates in an eNOS dependent manner. Moreover, aorta from Vav1 deficient mice is protected with aging by showing less stiffness in aged mice than the wild type littermates, suggessting that Vav1 is a negative regulator of eNOS dependent vascular tone in mouse arota. In addition, we found that aorta extracted from wild type mice show enhanced Vav1 protein levels in aged mice. Collectively, we hypothesize that the reduced eNOS activity and increased stiffness in aged mice aorta might be due to the accumulation of Vav1. Mechanistically, overexpression of Vav1 in human endothelial cells induces oxidative stress by accumulation of NADPH oxidase-dependent reactive oxygen species (ROS) production. The consequence of the pathological ROS production activates poly ADP ribose polymerase (PARP), the NAD+ consuming enzyme, and results in decline in the Sirt1 deacetylase level and finally attenuats eNOS activity via increased acetylation and subsequently reduces phosphorylation on S177. The inihibitory effect of Vav1 could be neutralized by the presence of small-interfering RNA against Vav1 or resveratrol to increase Sirt1 activity. Consistent to this clarification, aorta samples extracted from both young and old Vav1 deficient mice as well as wild type mice show similar pattern of expression. Since the age-associated alterations in endothelial relaxation is the characteristic changes that arises with age, independently of other known cardiovascular risk factors, our findings provide a molecular mechanism of age-associated endothelial dysfunction mediated by Vav1, which provides a target for future development of medical intervention strategies for endothleial dysfunction in elderly population.

NCI-CCR
I-Lin Wu
Visiting Fellow
Molecular Biology - Prokaryotic
A versatile nano-display platform from bacterial spore coat proteins
Bacterial spores are dormant cell types formed by some Gram-positive species in response to stressful conditions such as starvation. Spores protect the organism's genetic material from environmental insults such as high heat, caustic chemicals, and radiation. When favorable growth conditions resume, even after many centuries in some cases, spores germinate and the cell continues to grow normally. Spores are therefore one of the hardiest organisms on earth. Dormant bacterial spores are encased in a thick protein shell, the "coat", which contains ~70 different proteins and is among the most durable static structures in biology. Due to extensive cross-linking among coat proteins, this structure has been recalcitrant to detailed biochemical analysis, so molecular details of how it assembles are largely

unknown. Coat morphogenesis initiates with the assembly of a basement layer, a platform on top of which the other coat proteins deposit. The structural component of the basement layer is composed of SpoIVA, an exceptional cytoskeletal protein that hydrolyzes ATP to drive its irreversible polymerization. SpoIVA is anchored to the surface of the forespore by SpoVM, a small amphipathic a-helical protein that localizes properly by sensing the positive membrane curvature of the forespore surface. Here, we reconstitute the basement layer of the coat atop spherical membranes supported by silica beads to create artificial spore-like particles. We report that these synthetic spore husk-encased lipid bilayers (SSHELs) assemble and polymerize into a static structure, mimicking in vivo basement layer assembly during sporulation in Bacillus subtilis. This system may provide a robust in vitro assay for studying spore morphogenesis that helps to reveal the mystery for spore coat assembly and interactions. Additionally, we demonstrate that SSHELs may be easily covalently modified with small molecules and proteins. We propose that SSHELs may be useful display platforms for drugs and vaccines in clinical settings, or for enzymes that neutralize pollutants for environmental remediation.

NCI-CCR
YIBIN YANG
Research Fellow
Signal Transduction - General
Analysis and Therapeutic Targeting Non-proteolytic Protein Ubiquitination in Diffuse Large B Cell Lymphoma
The Inhibitor of Apoptosis (IAP) family E3 ubiquitin ligases, cIAP1 and cIAP2, regulate aspects of immune signaling pathways by catalyzing the synthesis of non-proteolytic K63-linked polyubiquitin chains. SMAC mimetic small molecules, which specifically target cIAP1/2 protein stability, have been developed for early clinical trials in diverse cancer types. However, the therapeutic potential of SMAC mimetics in human lymphoid malignancies has not yet been established, due to the lack of biological understanding of the oncogenic role of cIAP1/2 in this disease. Our current work revealed an essential oncogenic role of cIAP1/2 E3 ligases in Activated B Cell-like subtype of Diffuse Large B cell Lymphoma (ABC DLBCL). With cancer genomics SNP array, we identified cIAP1/2 loci as frequently gained/amplified chromosome regions in ABC DLBCL primary biopsies. Starting from this genomics evidence, we uncovered a required role for cIAP1/2 in maintaining constitutive NF-?B signaling and tumor survival in ABC DLBCL. Mechanistically, cIAP1/2 were associated with the constitutively assembled CARD11-BCAL10-MALT1 (CBM) complex downstream of B Cell Receptor (BCR) signaling, required for K63-specific ubiquitination of BCL10 in BCR signaling, and thus required for IKK? subunit recruitment to the CBM complex in ABC DLBCL lines. Importantly, SMAC mimetic birinapant displayed strong and specific anti-proliferation ability in ABC DLBCL cell line models and mice xenograft models in vivo. Moreover, a high-throughput small molecule screen was used to identify other drugs that exhibit synergistic toxicity for ABC DLBCL cells when combined with birinapant, to increase the response rates and durability. In summary, the biological function of cIAP1/2 in ABC DLBCL discovered in this study provides the rationality of the therapeutic potential of SMAC mimetic birinapant in this disease. Furthermore, this study also produces a new way to think about nonproteolytic protein ubiquitination in lymphoma that provides novel drug development targets that can be specific for a given disease process in cancer.

NCI-CCR

Weishi Yu

Postdoctoral Fellow

Chromatin and Chromosomes

DNA hypomethylation is associated with de novo enhancer formation and neuronal reprogramming

Regenerative medicine requires the generation of human tissues and organs for replacement of those damaged by injury, degenerative disease, aging or cancer. A promising strategy utilizes patient derived induced pluripotent stem cells (iPSc) which are then differentiated in vitro towards the desired cell type. The reprogramming of chromatin states, including the resetting of DNA methylation pattern, is key for successful generation of iPSc and differentiated tissues, however, the precise molecular mechanisms and co-factors of DNA methylation remain largely unknown. We examined the role of the chromatin remodeling factor Lsh in the generation of neural lineage cells from iPSc. Lsh belongs to SNF/ISWI family and we have previously shown that it alters nucleosome density and DNA methylation in vivo. Using whole genome-wide bisulfite sequencing, ChIPs and RNA-seq we assessed chromatin states and gene expression in Lsh+/+ and Lsh-/- murine embryonal fibroblasts (MEFs). We found de novo formation of putative enhancer at CG hypomethylated sites in Lsh-/- MEFs compared to Lsh+/+ MEFs. These H3K4me1 marked sites were not yet active and were found enriched at neuron lineage genes. Moreover, these sites were partially preserved during reprogramming to iPSc and became active upon neuron lineage differentiation. This led to increased acquisition of H3K27 acetylation (a mark of active enhancers) and augmented mRNA expression of those pre-marked neural lineage genes and accelerated neural lineage development of Lsh-/- iPS cells in vitro and in vivo. Finally, we could show that subcloning of pre-marked DNA sequences showed neuronal specific enhancer activity in a reporter system. Our data indicates a functional link of CG hypomethylation and enhancer formation that ultimately influences cellular plasticity. These results are relevant for regenerative medicine, underscoring the importance of complete reprogramming and resetting of DNA methylation patterns for successful tissue generation.

NCI-CPFP

Ronald Eldridge

Cancer Prevention Fellow

Epidemiology/Biostatistics - Prognosis and Response Predictions

A mediation analysis to investigate the mechanism underlying smoking and HPV re-acquisition

Smoking may be implicated at different stages throughout the natural history of human papillomavirus (HPV)-associated cancers. Smoking is an established risk factor for progression of HPV infection to cervical neoplasia, but its role in HPV re-acquisition is less clear. Also unclear is smoking's underlying mechanism for re-acquisition and progression. Our previous study found an inverse association between smoking and naturally acquired antibodies against HPV, suggesting an immune response mechanism for re-acquisition. In vitro studies suggest that tobacco condensate can induce DNA damage in HPV-infected cervical cells, suggesting a more direct mechanism. Recent methodological advances in mediation analysis allow for estimation of mechanisms in epidemiologic data – decomposing an overall effect into separate mechanistic effects. Using a two-year follow-up study of women (n=1,978), we investigated whether a lowered antibody response was smoking's underlying mechanism for increasing the risk of

HPV-16 re-acquisition. We first posit our causal model: smoking affects a woman's HPV serological antibody response; smoking and HPV antibodies affect HPV infection directly; sexual behavior can confound the stated effects. The model has two logistic equations, both adjusted for age, age at sexual initiation, lifetime number of sexual partners, and ever diagnosis of another sexually transmitted infection. From these equations, the natural indirect effect (antibody mechanism) and natural direct effect (alternative mechanism) are estimated. For the study population, baseline measurements of smoking and sexual behaviors were assessed by questionnaire, and HPV-16 antibodies by a Luminex GST ELISA assay; during follow-up, bi-annual cervical specimens were assayed for HPV-16 DNA infection (n=131). Compared to never smokers, current smokers had an increased odds of HPV infection by the antibody-mediated indirect effect (OR=1.24, 95% CI: 1.08, 1.60); the direct effect was not significant (OR=0.66, 95% CI: 0.34, 1.31). For women who smoke at least half a pack of cigarettes daily, the indirect effect was stronger (OR=1.62, 95% CI: 1.20, 2.31).This is the first analytic model to suggest that current smoking increases the risk of re-acquiring an HPV infection by reducing HPV antibody titers. The estimate appears dose-dependent, more harmful for women who smoke at least half a pack of cigarettes per day. Replication of the findings in an independent study is currently underway.

NCI-CPFP
Sarah Keadle
Cancer Prevention Fellow
Cultural Social and Behavioral Sciences
Impact of changes in television viewing on total mortality: A prospective cohort study
Background: Increasing physical activity later in life can improve health and longevity among older adults. However, older adults spend an average of 4.7 hours per day watching television, a discretionary sedentary behavior that consumes over half of available leisure-time. Prolonged television viewing is associated with greater mortality, but it is unknown whether changing television viewing habits subsequently alters mortality risk. We performed the first known analysis of changes in television viewing on mortality in a prospective study with repeated measures of television viewing habits. METHODS: The NIH-AARP Diet and Health Study enrolled participants in 1994-96 (Time 1) and administered a follow-up questionnaire in 2004-05 (Time 2). We evaluated 167,083 adults (50-71 yrs at Time 1) who provided exposure information at both time-points. Mortality follow-up began at Time 2 and continued through December 31, 2011. Cox proportional hazards regression was used to estimate risk associated with changes in television viewing and mortality from any-cause. All models were adjusted for age, sex, education, smoking, pre-existing disease and BMI. We also conducted analyses stratified by change in exercise participation. RESULTS: Over 6.6 years of follow-up, there were 20,387 deaths. Those who reduced viewing from >5 h/d (Time 1) to 3-4 h/d (Time 2) had a 15% lower mortality (HR[95%CI] 0.85 [0.80,0.91]) and to <3 h/d (Time 2) had an 11% lower risk (0.89[0.80,0.98]). Conversely, increasing from < 3h/d to >5 h/d was associated with a 45% increase in mortality (1.45[1.33,1.59]). Compared those who consistently exercised (> 1h/wk) and watched little television, exercisers who watched >3h/d of television had a 22% greater mortality (1.22[1.16,1.28]) and non-exercisers who increased TV viewing had the greatest risk (1.76[1.55,2.01]). CONCLUSIONS: Our data demonstrate for the first time that reductions in television viewing are associated with lower mortality in older adults. Exercise participation was also associated with lower risk, but there was some erosion of

mortality benefits from exercise for adults who watched more television. Older adults should exercise at recommended levels and limit their sedentary television viewing time to less than 3 h/d for optimal longevity. Given the high prevalence of television viewing among older adults, favorable changes in this leisure-time behavior could have substantial public health impact.

NCI-CPFP
Sarah Nash
Cancer Prevention Fellow
Epidemiology/Biostatistics - Prognosis and Response Predictions
Predictors of cigarette smoking-related mortality in adults over 70 years
Background: Despite the success of tobacco control efforts, tobacco use remains a leading modifiable cause of cancer incidence and premature mortality in the US. Approximately half of all smokers will die a cigarette-related death, and life expectancy is decreased by more than 10 years in lifetime smokers, relative to non-smokers. Associations of smoking with premature mortality in the elderly are of particular interest, given the aging nature of the US population. Several studies have examined the benefits of smoking cessation on mortality in mid-life (50-60 years); however, few studies have explicitly examined risks associated with smoking in adults older than 70 years. Methods: We used Cox proportional hazards models, adjusted for age, sex, education, and alcohol use, to examine associations of age at smoking initiation and age at smoking cessation with risk of all-cause and cause-specific mortality among 160,113 participants of the NIH-AARP study who completed a detailed lifestyle questionnaire in 2004-2005, who were aged 70-82 at baseline, and who were followed for mortality through December 31, 2011. Results: Substantial differences in mortality were observed by smoking status. During follow-up, 12.1% of never smokers died, while 27.9% of participants who quit between ages 60-69, and 33.1% of current smokers died. Relative to current smokers, the risk of all-cause mortality was lower in all categories of former smoking, with HRs (95% CI) of 0.41 (0.39-0.43), 0.51 (0.49 – 0.54), 0.64 (0.61 – 0.67), and 0.77 (0.73 – 0.81) for quitting between ages 30-39, 40-49, 50-59, and 60-69, respectively. Among current smokers, there was a strong inverse association of mortality with age at initiation. We observed similar findings for cause-specific mortality, including mortality from lung cancer, other smoking-related cancers, heart disease, stroke, diabetes, and respiratory disease. Conclusions: As with younger populations, age at smoking initiation and cessation are key predictors of mortality in US adults after age 70. In NIH-AARP participants, all former smokers, even those who quit in their 60s, were at substantially reduced risk of mortality after age 70, relative to current smokers. This finding highlights the need to emphasize smoking cessation to smokers of all ages. Furthermore, younger age at initiation was associated with increased risk of mortality, demonstrating the importance of youth and early-adult smoking behaviors on lifetime mortality risk.

NCI-DCEG
Bryan Bassig
Doctoral Candidate
Epidemiology/Biostatistics - Etiology, Risk, and Prevention
Soluble levels of CD27 and CD30 are associated with risk of non-Hodgkin lymphoma in a pooled study of three Chinese prospective cohorts

Background: Recent prospective epidemiological studies conducted in Western populations have indicated that higher plasma levels of biomarkers indicative of B-cell activation are associated with future risk of non-Hodgkin lymphoma (NHL). Two of these markers, CD27 and CD30, are members of the tumor necrosis factor superfamily and are expressed on specific subsets of activated lymphocytes and have broad immunoregulatory activities upon activation. No studies have prospectively evaluated the association between levels of B-cell stimulatory biomarkers and NHL risk in other ethnic populations, including in Chinese, who have different patterns of NHL incidence and subtype distributions. Given that the descriptive characteristics of NHL in East Asia differ from the West, it is of interest to evaluate mechanistic commonalities for NHL between these populations. Methods: We conducted a pooled nested case-control study from three prospective, population-based cohorts of Chinese men and women in Shanghai and Singapore, including 218 NHL cases and 218 individually matched controls. Plasma levels of soluble CD27 (sCD27) and CD30 (sCD30) were measured in duplicate by ELISA, with cases and their matched controls analyzed consecutively in the same batch. Conditional logistic regression was used to estimate odds ratios (OR) and calculate 95% confidence intervals (95% CI) evaluating analyte quartiles and NHL risk. Results: Compared to the lowest quartile, ORs (95% CIs) for the 2nd, 3rd, and 4th quartiles of sCD27 were 1.60 (0.83-3.09), 1.94 (0.98-3.83), and 4.45 (2.25-8.81), respectively (ptrend = <0.001). The corresponding ORs for sCD30 were 1.74 (0.85-3.58), 1.86 (0.94-3.67), and 5.15 (2.62-10.12) (ptrend = <0.001). The highest quartiles of both markers remained significantly associated with NHL in models mutually adjusted for the other. Notably, associations with NHL for both markers were significantly elevated in those diagnosed with NHL 10 or more years after blood draw. Conclusions: Higher levels of sCD27 and sCD30 were demonstrated for the first time to be associated with future risk of NHL in the Chinese population, and were demonstrated to precede lymphomagenesis >10 years before diagnosis. These data provide new insight into the mechanisms of NHL in Asians and are consistent with findings in Western populations, indicating common mechanistic characteristics for NHL in diverse populations with different patterns of NHL incidence and subtype distribution.

NCI-DCEG
Jiyeon Choi
Postdoctoral Fellow
Genomics

An intronic GGGCCC repeat variation of PARP1 confers melanoma risk via guanine quadruplex-mediated expression regulation

Recent genome wide association studies (GWAS) identified several new loci for melanoma susceptibility. While these results highlight potential pathways predisposing to melanoma, functional risk variants in these loci, as well as mechanisms by which they influence risk, have yet to be elucidated. Identifying functional variants from GWAS loci is particularly challenging because lead SNPs often tag linkage disequilibrium (LD) blocks harboring hundreds of SNPs and multiple genes. To nominate effecter gene(s) we performed expression quantitative trait loci (eQTL) analysis in 62 melanoma cell lines. Among 16 GWAS loci tested two chromosome 1 loci exhibited significant cis-eQTL. Subsequent validation using Taqman qPCR demonstrated that Poly [ADP-ribose] polymerase 1 (PARP1) expression is significantly associated with the lead SNP (p=0.03, copy number adjusted). Namely, the risk allele is correlated with

an increased PARP1 levels. We then interrogated the genotype-expression correlation by Taqman allele discrimination qPCR in 21 melanoma cell lines heterozygous for the GWAS lead SNP. The results demonstrated significantly higher proportion for the risk allele in PARP1 transcripts (p=0.0001). To identify functional risk variants mediating these effects we annotated the PARP1 locus using ENCODE database. Among 56 SNPs of high LD with the lead SNP (r2>0.5 using 1000 Genomes genotypes), six exhibited strong evidence as potential transcriptional enhancers in melanoma relevant cell types. One of them is a six-base pair indel (-/GGGCCC) in GC-rich region poorly covered by 1000 Genomes. Genotype reassessment of this indel using gel-based fragment analysis in 745 healthy Europeans from DCEG imputation reference set resulted in markedly improved LD with the lead SNP (r2=0.94 from 0.67) supporting direct link to melanoma susceptibility. Subsequent luciferase and Electro Mobility Shift Assays demonstrated that the indel exhibits allele-specific transcriptional activities and protein binding in melanoma cell lines. Finally, mass-spectrometry of allele-specific binding proteins identified a striking collection of Guanine-quadruplex (G4) binding proteins, which suggests that transcriptional inhibitory function of G4 in insertion allele leads to lower expression than deletion. These data demonstrate that increased PARP1 expression is correlated with melanoma risk, and suggest that an indel variant mediates differential PARP1 expression possibly through G4 binding proteins.

NCI-DCEG
Catherine Lerro
Doctoral Candidate
Cultural Social and Behavioral Sciences

Organophosphate insecticide use and cancer incidence among spouses of pesticide applicators in the Agricultural Health Study

Background: Organophosphates (OP) are among the most commonly used insecticide active ingredients in the US, comprising approximately 35% of insecticides used. They are registered for use in all market sectors, including agriculture, home and garden, industrial, commercial and government. OP use has been linked to cancer risk in some epidemiologic studies, which have been largely conducted in predominantly male populations. We evaluated personal use of specific OPs and cancer incidence among female spouses of private pesticide applicators (farmers) in the prospective Agricultural Health Study cohort. Methods: On a survey administered from 1993-1997, women provided information on lifetime use of specific pesticide active ingredients including 10 OPs, farming and pesticide application practices, demographic information, family, personal and reproductive health history, and other potential confounders. Incident cancer cases were obtained from the North Carolina and Iowa state registries through 2010 and 2011, respectively. We used Poisson regression to estimate relative risks (RR) and 95% confidence intervals (CI). We examined use of any OP and individual OPs (malathion, diazinon, chlorpyrifos, terbufos, dichlorvos, phorate, fonofos, coumaphos, parathion) where sample size permitted. We examined all cancer sites together, and individual sites with =10 exposed cases. Results: 30,003 women were included in our analysis, representing 437,198 person-years of follow-up. 25.9% of spouses reported OP use, and 718 OP-exposed women were diagnosed with cancer during the follow-up period. Any OP use was associated with an elevated risk of breast cancer (RR=1.20, 95%CI: 1.01, 1.43). Malathion, the most commonly reported OP, was associated with increased risk of thyroid cancer (RR=2.04, 95%CI: 1.14, 3.63) and decreased risk of non-Hodgkin lymphoma (RR=0.64, 95%CI: 0.41, 0.99).

Diazinon use was associated with ovarian cancer (RR=1.87, 95%CI: 1.02, 3.43). Conclusions: We observed increased risk with OP use for several hormonally-related cancers, including breast, thyroid, and ovary. Previous studies examining OP insecticide use and cancer focused primarily on men, making this a unique evaluation. The increased risks observed for hormonally-related cancers are consistent with the hypothesis that OPs might act as endocrine disruptors. This study represents the first comprehensive analysis of OP use and cancer risk among women, and thus a need for further evaluation.

NCI-DCEG
Orestis Panagiotou
Visiting Fellow
Epidemiology/Biostatistics - Etiology, Risk, and Prevention
Effect of bivalent human papillomavirus (HPV) vaccination on pregnancy outcomes: long-term observational follow-up in the Costa Rica HPV Vaccine Trial
Background: Millions of women receive prophylactic vaccination against the human papillomavirus virus (HPV) as part of the vaccination schedules worldwide. Previous studies could not rule out an effect on miscarriage of the bivalent HPV vaccine (HPV2) for pregnancies conceived <90 days from vaccination. Methods: We analyzed data from an NCI-sponsored randomized, double-blinded trial, the post-trial follow-up phase, and an unvaccinated cohort using pregnancy as the unit of analysis. We estimated the relative risk (RR) and 95% confidence intervals (CI) of miscarriage (i.e. fetal loss within 20 weeks of gestation) comparing the miscarriage rates in pregnancies conceived <90 days and any time since HPV2 vaccination, and in pregnancies conceived anytime from the control hepatitis A vaccine (HAV) and in the unvaccinated cohort. We evaluated the positive associations between HPV2 and miscarriage with the false-positive report probability (FPRP) using FPRP <0.1 as the noteworthiness threshold. Results: The miscarriage rate among the 3,394 pregnancies conceived any time since HPV2 vaccination was 13.29%. For pregnancies conceived <90 days of vaccination (n=381) the miscarriage rate was 13.12%. Unexposed were 3,227 pregnancies (2,507 after HAV vaccination and 720 in the unvaccinated cohort), of which 414 (12.83%) ended in miscarriage. There was no increased risk of miscarriage for pregnancies conceived <90 days from HPV2 vaccination (RR=1.02 [95% CI, 0.78-1.34], P1-sided =0.436) in unadjusted analyses. Results were similar when we adjusted or stratified for age at vaccination, age at conception, gestational age of miscarriage, and age at trial enrollment. When not restricting to time since vaccination, HPV2 was also not associated with increased miscarriage risk overall except for miscarriages at weeks 13-20 of gestation (RR=1.35 [95% CI, 1.02-1.77]; P1-sided =0.017); the corresponding FPRP values were >0.1. Conclusions: The data do not support an effect of HPV vaccination on miscarriage risk for pregnancies including those conceived <90 days from vaccination. The increased risk for miscarriages at weeks 13-20 of gestation is not noteworthy and constitutes a random finding.

NCI-DCEG
Elizabeth Yanik
Postdoctoral Fellow
Epidemiology/Biostatistics - Etiology, Risk, and Prevention

Elevated cancer risk among US pediatric solid organ transplant recipients

Solid organ transplant recipients have elevated cancer risk compared to the general population. Risk is particularly increased for infection-related cancers, largely attributable to immunosuppressant use. Among pediatric transplant recipients, the effects of transplantation and immunosuppression on cancer risk may differ from those observed in the adult transplant population, but no prior study has comprehensively described cancer risk in this population. The U.S. transplant registry was linked to 16 cancer registries to identify cancer diagnoses among solid organ recipients <18 years of age at transplant. Standardized incidence ratios (SIRs) were estimated for total cancer and for specific cancer types by dividing observed cancer counts in recipients by expected counts calculated based on general pediatric population cancer rates within strata of age, sex, race, calendar year, and registry. SIRs were calculated for all pediatric recipients, and for subgroups defined by age and transplanted organ type. We included 17,958 pediatric recipients transplanted during 1987-2011 (44% of the U.S. total). Among these recipients, 394 cancers were diagnosed, of which 279 (71%) were non-Hodgkin lymphomas (NHL) and 30 (8%) were Hodgkin lymphomas (HL). Compared to the general population, cancer incidence was 22 times higher overall (SIR=22, 95%CI=20-24). Specifically, incidence was higher for NHL (SIR=210), HL (SIR=19), leukemia (SIR=4), myeloma (SIR=230), and cancers of the kidney (SIR=16), thyroid (SIR=6), liver (SIR=28), testis (SIR=4), soft tissue (SIR=4), ovary (SIR=8), bladder (SIR=49), breast (SIR=9), and vulva (SIR=130). SIRs for lymphomas were highest for recipients <5 years old at transplant (NHL SIR=320, 95%CI=260-380; HL SIR=54, 95%CI=29-92). When evaluated by organ type, NHL risk was most elevated among intestine recipients (SIR=1300, 95%CI=530-2700) followed by heart/lung (SIR=320, 95%CI=250-390), liver (SIR=200, 95%CI=160-250) and kidney recipients (SIR=160, 95%CI=130-200). In conclusion, pediatric transplant recipients have markedly elevated risk for a number of cancer types, but the majority of the cancer burden is attributable to NHL. Increased NHL risk was exceedingly high among the youngest recipients, who are likely to develop primary Epstein-Barr virus infection after transplantation, and among intestine recipients, who receive intensive immunosuppression and are highly exposed to donor lymphoid tissue conveyed in the donor organ.

NEI

Aman George

Postdoctoral Fellow

Genetics

COlobomatous Microphthalmia, Macrocephaly, Albinism & Deafness (COMMAD) a novel syndrome caused by biallelic mutation of MITF gene

Mutations in MITF gene are associated with Waardenburg syndrome (WS). Here, we report a rare family with WS parents having disparate clinical manifestations due to heterozygous MITF mutations and a child with compound heterozygous mutations of MITF. This child exhibited extreme colobomatous microphthalmia, a complete lack of melanin pigment in the skin/hair/eyes, profound hearing loss, macrocephaly and low tone. DNA sequencing revealed a paternally inherited p.R318del mutation and maternally-inherited p.K307N mutation in MITF gene, the latter being previously unreported. The MITF gene encodes for microphthalmia-associated transcription factor, a basic helix–loop–helix protein critical for development of neural crest-derived melanocyte, neuro-ectoderm derived retinal pigmented epithelium and hematopoietic tissue derived osteoclasts and mast cells. Using live cell imaging and

western blotting of transfected HEK293 cells with GFP tagged wild type (WT) and mutant expression constructs, it was observed that WT-MITF translocate to the nucleus, whereas R318del was observed only in cytoplasm and K307N mutant localized in both nucleus and cytoplasm. As studied by electro-mobility shift assay, the R318del did not bind to DNA whereas the DNA binding ability of K307N mutant was significantly reduced compared to WT. Interestingly K307N mutant activated the TRP1, TRP2, TYR and Best1 gene promoters just like the WT-MITF while R318del mutant could not, as tested by dual luciferase assay. MITF is known to form homo-dimer inside the cytoplasm, then translocate into the nucleus and activate target gene promoters. By co-transfection and live cell imaging of RFP tagged WT-MITF and GFP tagged mutant MITF we show that R318del mutant acts in a dominant negative manner, whereby it binds the WT-MITF and retains it inside the cytoplasm. The K307N mutant had modest dominant negative effect on WT-MITF. Upon co-transfection of both the mutant isoforms stronger retention of MITF was observed in the cytoplasm, thus explaining the severe phenotype of the child with the compound heterozygous MITF mutations. We report for the first time the compound heterozygous MITF gene mutations causing coloboma in humans, its occurrence with microphthalmia, macrocephaly, albinism and deafness resulting in a novel syndrome termed COMMAD and its underlying molecular mechanism.

NEI
Jennifer Kielczewski
Postdoctoral Fellow
Immunology - Autoimmune
Tertiary lymphoid tissue with germinal centers and T follicular helper cells in immunologically privileged retinas of mice with chronic autoimmune uveitis
Although the eye is an immunologically privileged organ, it can be subjected to destructive immunity. This occurs in uveitis where autoreactive T cells enter into the eye attacking the retina, leading to irreversible vision loss. We developed a spontaneous uveitis mouse model (R161H), which expresses a retina-specific T cell receptor for Interphotoreceptor Binding Protein, a uveitic target antigen. These transgenic mice develop spontaneous uveitis that resembles human disease with distinct retinal lesions that resemble tertiary lymphoid tissue (TLT). TLT has been observed in a wide variety of autoimmune conditions, but has not been well characterized in inflamed retinal tissue. We determined whether the retinal lymphoid structures represent TLT. Mouse retinas underwent extensive immunohistochemical analysis. Laser capture microdissection was used to isolate the retinal lesions and gene expression arrays were performed. Immunohistochemistry revealed that the retinal lesions stained for CD4+ T cells and B cells in well-defined zonal areas. The retinal lesions also stained positively for germinal center markers, PNA and GL-7, which are typically expressed in TLT. Laser captured microdissected samples of the retinal lesions showed significant upregulation of T follicular helper cell markers, most notably CXCR5 and its ligand CXCL13, which are also typically expressed in TLT. In addition, immunohistochemical analysis of the retinal lymphoid aggregates revealed presence of T follicular helper cells (co-stained for CXCR5+/CD4+). We detected evidence for antigen presentation to retina-specific T cells within the TLT by co-labeling for CD11c+, pZap70+, and CD4+ IRBP specific T cells. Likewise, CD138+/B220+ plasma cells were detected in the retinal aggregates, suggesting the retinal lymphoid aggregates are a source of autoantibody production. Lastly, we found that mice with progressive late stage retinal aggregates had

significantly reduced visual activity compared to control mice lacking these retinal structures. Our findings suggest that the TLT are pathological sites where immune cells can accumulate to create an environment conducive to immune cell activation and autoantibody production, thereby contributing to chronic ocular inflammation, hence loss of visual function. Further studies are needed to determine how to inhibit immune cell aggregates from forming in the ocular environment resulting in pathological tissue damage.

NEI
Suddhasil Mookherjee
Visiting Fellow
Molecular Biology - Eukaryotic
Abstract removed by request of author

NEI
FNU RUCHI
Postdoctoral Fellow
Stem Cells - General
Developing Autologous Cell Therapy for Macular Degeneration Using Functional RPE Tissue Derived from Patient-specific iPS Cells
Purpose: Retinal pigment epithelium (RPE) is a monolayer of cells located adjacent to the retinal photoreceptors and is fundamentally important for photoreceptor health and function. RPE degeneration leads to photoreceptor cell death leading to blinding eye diseases like age-related macular degeneration (AMD). AMD is one of the leading causes of blindness worldwide and currently there is no cure for the “dry” form of this disease. Preliminary work suggests that replacing the damaged RPE with an autologous RPE monolayer can provide potential therapy for dry AMD. In this study we develop protocols to generate functionally mature RPE monolayers from patient-derived autologous induced pluripotent stem (iPS) cells. Methods: A reporter iPS cell line expressing GFP under RPE specific promoter was used to optimize the differentiation protocol to RPE. This tri-phasic developmentally guided protocol uses the dual SMAD inhibition combined with canonical WNT and FGF inhibition to generate RPE-primed neuroectoderm. Differentiation of neuroectoderm to committed RPE needs an activation of canonical WNT and TGF-signaling pathways. To generate mature and functional RPE monolayers, we induced primary cilium in committed RPE cells using known cilia inducers like aphidicolin and prostaglandin E2. The differentiation efficiency was analyzed using GFP expression and RPE-specific gene expression. Results: Generation of RPE-primed neuroectoderm is marked by increased expression of eye-field transcription factors PAX6, RAX and SIX3. Increase in expression of PAX6,MITF and OTX2 brings the RPE primed neuroectoderm to committed RPE fate. Maturation of RPE monolayers is marked by upregulation of RPE maturation makers like RPE65 and functional responses from iPSC-RPE cells that well mimic the native RPE. Expression of RPE-specific GFP reporter shows more than 98% RPE cells in these differentiation cultures. This differentiation protocol reproducibly makes RPE from healthy and AMD patient-derived iPS cell lines. Conclusion: With the help of a reporter line, we were able to optimize a highly efficient and reproducible differentiation protocol. Furthermore, we found out the

novel link between the primary cilium and RPE maturation. This protocol is currently being used to develop clinicial-grade RPE from AMD patient iPS cells for a phase I clinical trial.

NEI

Anthony St. Leger

Postdoctoral Fellow

Immunology - General

Ocular surface microflora modulates IL-17 and neutrophil recruitment within the conjunctiva associated lymphoid tissue

Dysregulation of immune homeostasis at the ocular surface is associated with inflammation, ocular discomfort, and potential blindness. In mucosal tissues, like the conjunctiva associated lymphoid tissue (CALT), cytokines and lymphoid follicles maintain immune homeostasis with commensal and potentially pathogenic bacteria. Data from studies show that potentially pathogenic microbes are routinely found in the ocular mucosa of healthy subjects as well as patients afflicted with Dry Eye Disease (DED), Sjogren's Syndrome, recurrent conjunctivitis, and various forms of keratitis. The exact mechanisms that regulate immune homeostasis in ocular mucosa remain largely unknown. While previous studies have suggested a critical role for the functionality of CALT in the maintenance of ocular surface health, no definitive conclusion has been made due to the absence of an adequate animal model. In this study, we establish a working animal model and study the functional properties of CALT. We found that the normal CALT of WT C57BL/6 mice harbored substantial numbers of neutrophils. Notably, the CALT contained innate lymphoid cells (ILCs), aß and ?d T cells that readily produced interleukin (IL)-17, a known recruiter of neutrophils, upon stimulation with PMA/ionomycin as well as IL-1ß, which suggested that these cells contribute to the neutrophil recruitment. Indeed, just 48 hours after a local injection of neutralizing IL-17A/F into one eye, we noted significantly reduced neutrophil numbers in the CALT compared to the PBS treated opposite eye, which supported the hypothesis that continual in situ production of IL-17 is required for neutrophil presence in the CALT. Additionally, IL-17A/F double knockout (DKO) mice failed to recruit neutrophils to the CALT, resulting in the outgrowth of Staphylococcus aureus and the development of severe conjunctivitis. Finally, we linked IL-17 production in CALT to presence of local ocular surface bacteria, as evidenced by a dramatic loss of IL-17 in CALT following antibiotic treatment. Furthermore, germfree mice had virtually no immune response within the CALT at steady state. Our data uncover an interdependence between the CALT and normal ocular microflora for regulating mucosal immune function at the ocular surface, and demonstrate its importance for maintaining normal ocular surface homeostasis.

NHGRI

Stephen Bond

Visiting Fellow

Informatics/Computational Biology

Evolution of the Pannexin Gap Junction Protein Family

Gap junctions (GJs) are a nearly ubiquitous feature of metazoan life, coupling the cytoplasm of adjacent cells into a partially selective syncytium. The range of physiological processes GJs partake in is extensive, a feature mirrored by an equally extensive diversity in the primary sequences of GJ-forming proteins.

Two protein families have independently evolved the ability to form GJs – connexins and pannexins (Panxs) – but to date there has not been a large systematic analysis of the Panxs. Here, we aim to formalize the nomenclature of Panxs, trace their evolutionary histories, and identify functional motifs that have been conserved or independently acquired. For this study, 974 Panx sequences were manually curated from public databases and non-public RNA-seq or whole-genome assemblies. Both Bayesian and maximum likelihood approaches were used to determine the phylogenetic relationships among these sequences. In place of conventional experimental controls, statistical approximation experiments rely on congruence among independent approaches. All methods used here unambiguously support the emergence of Panxs prior to separation of the earliest branching metazoans, but diversification of these proteins only occurred after the major contemporary superphyla were established, leading to at least five distinct classes. The current Panx nomenclature is based on order of discovery in model organisms, which implies orthologous relationships that do not actually exist. As such, we recommend adopting a naming scheme similar to that used for connexins, sub-classing the genes to better reflect evolutionary relationships. To identify orthologs within each newly identified Panx class, we have developed a novel refinement of established methods for ortholog assignment in whole genome analysis (i.e., Markov clustering based on BLASTP scores). Our approach uses Markov chain Monte Carlo simulation to predict optimal clustering parameters from training sets of manually assigned orthologs, and a second group of curated orthologs is included in test sets to control against over-fitting. We have currently placed about 30% of our Panxs into ortholog clusters. At the sequence level, the highest degree of Panx conservation exists in the transmembrane and extracellular loop domains, while the C-terminus and intracellular loop is highly variable. Putative functional motifs have been identified in both conserved and variable regions, which will be targets for future study.

NHGRI
Steven Boyden
Postdoctoral Fellow
Genetics

Vibration-induced urticaria due to aberrant mast cell degranulation caused by a mutation in ADGRE2

Vibratory urticaria (VU) is a rare condition in which sustained vibration against the skin induces both a localized hive and systemic manifestations such as facial flushing. We ascertained two large Lebanese kindreds in which VU segregates as an autosomal dominant trait. In affected family members, acute onset of symptoms and concurrent peripheral histamine release implicated mast cell degranulation in the pathogenesis. This hypothesis was supported by increased staining of tryptase, a component of mast cell granular contents, in post-vibration patient skin samples compared to controls. Through linkage analysis and exome sequencing we identified the missense mutation p.C492Y in ADGRE2 as the only rare nonsynonymous or splice variant co-segregating with VU in these kindreds. Gaps in exome coverage within the linkage interval were filled by Sanger sequencing to ensure no alternative candidate variants had been missed, and the p.C492Y mutation was absent from variant databases and 200 ancestry-matched controls, indicating it is not a common polymorphism. ADGRE2 encodes an adhesion G-protein coupled receptor that undergoes autocatalytic cleavage, producing an N-terminal extracellular alpha subunit that remains non-covalently bound to a C-terminal transmembrane beta subunit. ADGRE2 was found to be highly expressed in mast cells, and patient-derived but not control primary mast cells

degranulated when vibrated in culture. This activity was accentuated by both dermatan sulfate, the endogenous ligand of ADGRE2, and an anti-ADGRE2 antibody that ligates its alpha subunit. Likewise, human LAD2 mast cells expressing ADGRE2 with the p.C492Y mutation in the alpha subunit showed greater degranulation in response to vibration than control cells expressing wild type ADGRE2. This response was cleavage-dependent, suggesting the subunit interaction must be non-covalent to permit vibration-induced degranulation. Furthermore, LAD2 cells expressing an ADGRE2 truncation mutant encoding only the beta subunit showed constitutive degranulation, indicating the alpha subunit is auto-inhibitory. Our data suggest a pathogenic mechanism whereby the p.C492Y mutation destabilizes this inhibitory subunit interaction, sensitizing dermal mast cells to vibration-induced hyperactivation of beta subunit-mediated signaling. We describe vibration as a novel IgE-independent mechanism for mast cell degranulation and provide the first genetic basis for a mechanically induced urticaria.

NHGRI
Brennan Decker
Doctoral Candidate
Genomics

Under the Radar: Survival Strategies of an Ancient Clonally Transmissible Canine Tumor

Canine transmissible venereal tumor (CTVT) is a parasitic cancer clone that has propagated for thousands of years via direct sexual transfer of malignant cells from one canid to another. Little is understood about the genomic mechanisms that converted an ancient tumor into the world's oldest known continuously propagating somatic cell lineage. Since all worldwide cases of CTVT share a common origin in the single progenitor tumor, their genome sequences are comprised of 1) the genetic background of the founder canid, 2) the somatic mutations that initially enabled the tumor to escape immunosurveillance and be passed from one individual to another, and 3) lineage-specific somatic mutations that may be involved in clonal transmissibility. In order to distinguish between these possibilities, we created the largest existing catalog of canine genome-wide variation and compared it against two CTVT genome sequences, thereby separating alleles derived from the founder's genome from somatic mutations, some of which must drive clonal transmissibility. Variant metrics including transition to transversion ratio, nonsynonymous to synonymous ratio, and conservation across species at variant sites all support the hypothesis that novel variants are dramatically enriched for true somatic mutations. Gene Set Enrichment Analysis of the 1,341 protein-truncating somatic substitutions and indels, as well as the 2,329 protein-disrupting structural variants revealed the greatest enrichment for somatic mutations in the Reactome "Immune System" pathway, with p-values of 1.35E-12 and 1.48E-19, respectively. In this pathway, overlapping mutations disrupt every step of somatic cell participation immunosurveillance, especially self-antigen presentation and immune-mediated apoptosis. Unbalanced somatic structural rearrangements enabled identification of chronologically early somatic mutations in oncogenesis- and immune-related genes, including TP53, CDKN2A/B, TAP2, and CASP3, which could represent key initiators of clonal transmissibility. We also rebuilt surviving elements of the founder canid's germline genome, and our genome-wide maximum likelihood phylogeny supported CTVT origination in an ancient domesticated dog genetically similar to modern Siberian huskies. It is clear that CTVT is exquisitely adapted to its transmissible allograft niche, and we provide the first insights into the specific genomic aberrations that underlie CTVT's dogged perseverance in canids around the world.

NHGRI

Gustavo Sudre

Postdoctoral Fellow

Genomics

The connectome in the human brain: defining its heritability and association with ADHD

Introduction: While Attention Deficit Hyperactivity Disorder (ADHD) is highly heritable (h2=0.7), there has been limited progress in identifying the genes conferring risk. One strategy to accelerate progress is to use quantitative neural endophenotypes that both reflect genetic risk and may lie closer to the etiology of ADHD than the more distal clinical phenotype. Here we examine the connectome, defined as the interconnected brain regions or networks that support cognition, and focus on its structural basis provided by white matter tracts. We use multigenerational families with high prevalence rates of ADHD as this allows us to simultaneously estimate the heritability of the connectome and the strength of its association with ADHD. Methods: Participants were 165 members from 24 multi-generational, extended families (median size 8). In these families, the eleven major white matter tracts that constitute the structural connectome were defined using diffusion tensor imaging. Three microstructural variables were obtained for each tract: a) fractional anisotropy (FA), axial diffusivity (AD) and radial diffusivity (RD). The total additive genetic heritability (h2r) of each white matter metric was determined by modeling the covariance among family members as a function of genetic proximity using SOLAR. We used linear mixed model regressions to calculate the association between ADHD symptoms and the properties of white matter tracts, accounting for clustering of the observations within families. Results: Twenty-two of 33 white matter tract properties emerged as significantly heritable. Both the corpus callosum (FA: h2r =.41+-.16, p<10-2, AD: h2r =..43+-.17, p<10-2, RD: h2r =.60+-.15, p<10-4) and the right superior longitudinal fasciculi (FA: h2r =.32+-.15, p<.05, AD: h2r =..59+-.18, p<10-3, RD: h2r =.45+-.16, p<10-3) were among the most heritable tracts. However, only one of 33 white matter tract metrics (corpus callosum, AD) was also significantly associated with the symptoms of ADHD, specifically symptoms of inattention (t=2.2, p=0.03). Conclusions: These preliminary analyses include the first 165 participants who have been phenotyped. As a proof of concept study, it demonstrates how we can identify from a large number of candidate phenotypes within the connectome a small number that are both heritable and associated with ADHD. Such endophenotypes may prove rich targets for understanding how genes implicated in ADHD act on the connectome.

NHGRI

Tuoqi Wu

Visiting Fellow

Immunology - Lymphocyte Development and Activation

The transcription factor TCF1 is required for viral-specific T follicular helper (Tfh) cell responses

Early after viral or intracellular bacterial infections, effector CD4 T cells differentiate into two distinct lineages: Th1 cells and T follicular helper cells (Tfh cells). Tfh cells provide critical help for the initiation and maintenance of germinal centers (GCs), which are indispensible for developing high affinity antibodies and generating long-lived humoral immunity. Both of these two lineages are essential for controlling infection and developing immunological memory. However, the molecular mechanisms that govern this division of labor are still unclear. In this study, using lymphocytic choriomeningitis virus

infection in mice as a model, we demonstrate that TCF1, a transcription factor critical for thymocyte development and CD8 T cell memory, is also essential for Tfh-cell responses against viral infection. RNA-sequencing revealed that Tcf7, which encodes TCF1, was highly transcribed in naïve and Tfh cells but strongly repressed in Th1 cells. Reciprocal expression of TCF1 and Blimp1, an inhibitor of Tfh-cell differentiation, between viral-specific Tfh and Th1 cells occurred early after infection. We further found that TCF1 expression in Th1 cells was suppressed by IL-2 and Blimp1, which bound to enhancers upstream of Tcf7. Strikingly, conditionally deleting TCF1 in T cells severely compromised Tfh-cell and GC responses, without evidently affecting the Th1 cells. By deleting TCF1 in adoptively transferred viral-specific T cell receptor transgenic Tcf7 inducible knockout cells immediately prior to infection, we further showed that viral-specific Tfh responses relied on high levels of TCF1 in a cell autonomous manner independent of the role of TCF1 in T cell development. Transcriptome analyses demonstrated that the few remaining Tcf7-deficient Tfh cells had lost Tfh gene expression signatures and gained Th1 gene expression signatures. Moreover, Tcf7-deficient Tfh cells adopted a metabolism pattern closer to Th1 cells involving less oxidation in mitochondria. Among the genes we found most affected by Tcf7-deletion were Il2ra and Prdm1 (encoding Blimp1), which were bound and repressed by TCF1 as determined by chromatin immunoprecipitation assays. Finally, over-expression of Bcl6, an antagonist of Blimp1, rescued Tfh defects caused by Tcf7 deficiency, suggesting that TCF1 acts upstream of the Bcl6-Blimp1 axis. Thus, TCF1 functions through forming a negative feedback loop with IL-2 and Blimp1, which separates the Tfh-cell fate from the Th1-cell fate.

NHLBI
Katarzyna Placek
Visiting Fellow
Epigenetics

MLL4 regulate Treg cell differentiation by remote methylation of chromatin

Gene activation and silencing is associated with distinct chromatin modifications. T cell differentiation is accompanied by changes in chromatin modification patterns at specific loci encoding genes important for immune functions. While histone modification patterns are well established in different T cell subsets, little is known about the function of histone modifying enzymes in T cell specification. In this study we address the role of histone H3 lysine (K)4 methltransferase, Mll4, in CD4+ T cell development using conditional Mll4 knockout (Mll4KO) mice in CD4+ T cells. Our data show that Mll4 deficiency results in reduced number of T cells in periphery. Although Mll4 deletion does not change a global level of H3K4methylations in CD4+ T cells it leads to a striking redistribution of H3K4me1 and H3K4me2 modifications genome-wide with globally decreased methylation at enhancers but increased methylation at promoters. We further found that Mll4 deficiency not only results in decreased H3K4me1 and H3K4me2 levels at enhancers directly bound by Mll4 but more importantly decreases these modifications at enhancers not bound by Mll4, suggesting that Mll4 control the chromatin modifications remotely through chromatin looping formation. Indeed, we identify direct contacts between the MLL4-bound enhancers and the enhancers not bound by MLL4 but exhibiting decreases in H3K4 methylations in the knockout cells. We show that Mll4 remotely regulates H3K4me via chromatin looping at Foxp3 locus that encodes the master transcription factor of regulatory T (Treg) cells. Moreover differentiation of Treg cells is compromised in the absence of Mll4, indicating that the remote regulation of Foxp3

methylation pattern by Mll4 is functionally relevant. Furthermore we find that MLL4 -bound regions are enriched in a sequence motif recognized by the ETS family of transcription factors and show that the MLL4 complex physically interacts with ETS1 and co-localizes with the ETS1 on chromatin in CD4+ T cells. Deletion of MLL4 severely compromised chromatin accessibility and the binding of ETS1 in the cells, suggesting a critical role of MLL4 in allowing targeting of transcription factors. In summary our results indicate a critical role of histone H3 lysine methylation in T cell development. We propose a new mechanism by which MLL4 regulates remotely H3K4 methylation through chromatin looping.

NHLBI
Martin Skarzynski
Doctoral Candidate
Hematology/Oncology, Tumor Immunology, and Therapy

Targeting deposited complement component C3d to potentiate monoclonal antibody cancer therapy

Anti-CD20 monoclonal antibodies (mAbs) are a key component of treatment regimens for B-cell malignancies. During administration of anti-CD20 mAbs, tumor cells lose CD20 expression and thus evade mAb therapy. These cells are nevertheless labelled by complement proteins deposited on their cell surface. In particular, complement component C3d is covalently bound to these cells and constitutes a neoantigen. Here, we generated a chimeric anti-C3d mAb and sought to determine their potential therapeutic value. We first confirmed that tumor cells remaining in circulation 24 hours after administration of the CD20-targeting mAb ofatumumab had lost CD20 antigen and carried abundant C3d deposited on their cell surface. Next, we demonstrated that the anti-C3d mAb mediate complement-dependent cytotoxicity, NK cellular cytotoxicity and phagocytosis of CLL cells. Importantly, the anti-C3d mAb specifically bound only B cells but no other blood cells, indicating that the deposition of C3d is highly targeted to cells initially bound by ofatumumab. Given that CD20 antigen is lost during treatment with anti-CD20 mAbs we sought to determine whether C3d would be equally lost during targeting with our anti-C3d mAb. On the contrary, repeat targeting of C3d opsonized cells in the presence of human serum in vitro resulted in increased deposition of C3d leading to a 5-fold increase of binding sites per cell and killing of > 95% of cells through complement. Finally, we tested the activity of the anti-C3d mAb in vivo in a xenograft mouse model. Leukemic cells obtained from patients 24 hours after administration of ofatumumab were injected into mice. The C3d-targeting mAb reduced CLL cell counts in blood and spleen by 80% compared to mice treated with trastuzumab, a non-targeting mouse/human chimeric mAb used as a control. We provide proof of concept that targeting cell deposited C3d can enhance the potency of mAb therapy. Given that our anti-C3d mAb preserves the specificity of the initial mAb, it could augment the potency of many mAbs currently in clinical use by delivering a “one-two punch”. Specifically, the anti-C3d mAb could eliminate antigen loss variants that arise during treatment with other mAbs. Further, by depositing additional C3d the number of available target sites is amplified, which could enhance to potency of mAbs directed against low abundance targets. Further studies are necessary to study the safety of such an approach before clinical testing could begin.

NHLBI
Andrea Stoehr

Visiting Fellow
Biochemistry - Proteins
Discovering new pathways in the heart during hypoxia: Proteomic approaches to identify the cardiac prolyl hydroxalome and to analyze protein stability during hypoxia-mimicking conditions
Hypoxia activates signaling pathways that allow the cell or organism to adapt to a reduced oxygen level. These mechanisms are important in a cardiac cell which relies heavily on oxygen to supply energy to pump blood to the organs. Hypoxic signaling as an adaptive response is important to preserve heart function during impaired conditions such as in myocardial infarction. This research aims to understand hypoxic mechanisms in cardiac diseases which might be useful to develop therapies. One well characterized class of oxygen-dependent enzymes is prolyl hydroxylases (PHD). In the presence of oxygen, PHD enzymes add OH groups to prolines on HIF-1 that targets it for degradation. So far, only a few proteins have been shown to undergo prolyl hydroxylation. The goal of this study was to (I) identify new targets of PHDs in the heart, (II) test whether inhibition of prolyl hydroxylation alters protein stability and (III) investigate potential biological consequences. A proteomic approach (Orbitrap LC-MS/MS) was used to identify prolyl hydroxylated proteins in rat heart extracts. 45 prolyl hydroxylated peptides from 18 unique proteins were identified. Examples were collagen-binding protein serpin H1, sarcomeric protein troponin I or mitochondrial cytochrome c oxidase. To address whether prolyl hydroxylation influences protein stability and to transfer the results to human, we used a cell culture model of iPS cell-derived cardiomyocytes and Stable Isotopic Labeling with Amino Acids (SILAC). Medium was switched to heavy amino acids (13C6 L-Lysine-2HCL, 13C615N4 L-Arginine-HCl) and cells were divided into two groups: vehicle or dimethyloxalylglycine (DMOG, 1 mM), a PDH inhibitor. After 2, 6 and 18 hours samples underwent mass spectroscopy (Orbitrap Fusion). 96 proteins with significant differences in light peptides between DMOG and vehicle were identified 18 hours after media switch (p<0.05). To measure protein turnover, the rate of decay of the light peptides was measured. Slopes calculated by a multi-point first-order fit equation confirmed targets such as serpin H1, polyadenylate-binding protein 1 and SRSF1 as significantly stabilized by DMOG. Panther analysis revealed that mRNA processing factors were the largest group of stabilized proteins, suggesting a potential effect on alternative splicing in the presence of DMOG. In summary, stabilization of additional proteins besides HIF-1 appears to play important roles during hypoxic conditions in cardiomyocytes.

NHLBI
Adam Trexler
Research Fellow
Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology
Temporally resolving protein and lipid colocalization at exocytic sites
Regulated exocytosis, the controlled release of vesicle-enclosed molecules from cells, is essential for eukaryotic life and human health. Release of vesicle cargo requires that the vesicle membrane fuse with the plasma membrane of the cell, and the SNARE proteins driving this process form the core of the exocytic machinery. However, there are over a dozen other molecular factors that are absolutely critical to exocytosis. It is largely unknown how all these components come together in living cells to achieve exocytosis. To address this outstanding question, we used fluorescence microscopy to visualize individual exocytic events in live cells which enabled us to observe proteins present at exocytic sites

before, during, and after vesicle fusion. Understanding the temporal dynamics of exocytic proteins in living cells provides direct mechanistic insight into how these proteins work together to achieve exocytosis. We observed stimulated exocytosis in insulinoma cells using two-color total internal reflection fluorescence microscopy. We labeled a vesicle cargo called neuropeptide Y with GFP (NPY-GFP) and exocytic proteins of interest with a red fluorescent protein. Visualizing NPY-GFP allowed us to identify the location of exocytic events. We could then use these coordinates to examine the exocytic protein of interest relative to when and where exocytosis occurred. To quantitatively analyze these data we temporally aligned many exocytic events to extract average fluorescence intensities over time. We visualized the behavior of cytosolic and membrane bound mCherry as non-specific fluorescent controls and did not observe meaningful changes in these upon exocytosis. In this project we examined over 20 proteins involved in exocytosis, and one of our most surprising findings was that tomosyn, a protein believed to negatively regulate exocytosis, was present at exocytic sites until the moment of fusion after which it diffused away rapidly. Tomosyn is thought to bind and physically block proper SNARE complex formation so we did not expect to observe it where exocytosis occurs. We hypothesize that tomosyn may be responsible for fine-tuning the number of properly assembled SNARE complexes at the exocytic site. Collectively our data begin to build a better cellular context into which the vast biochemical knowledge of exocytosis can be better understood at a functional level.

NHLBI
Lingdi Wang
Visiting Fellow
Stress, Aging, and Oxidative Stress/Free Radical Research
Gcn5-like Protein 1 (Gcn5L1) regulates gluconeogenesis via the integrated regulation of ER-mitochondrial biology.
The nutrient sensing Gcn5-like 1 (Gcn5L1) protein is enriched in mitochondria and in the endoplasmic reticulum (ER), although its function in these distinct compartments has not been fully characterized. Cellular Gcn5L1 knockdown results in accelerated mitophagy whereas the genetic knockout of Gcn5L1 is embryonic lethal in mice. To further characterize the role of Gcn5L1, protein interactions studies were performed, we identified ER enriches chaperone Grp78 and mitochondrial shuttle enzyme GPD (glycerolphosphate dehydrogenase) are interacting proteins with Gcn5L1. To investigate the physiological role of Gcn5L1, we employed Cre-loxP technology to generated hepatic specific Gcn5L1 knockout (Gcn5L1 LKO) mice. We found that Gcn5L1 LKO mice were viable, had lower fasting blood glucose and displayed an exaggerated unfolded protein response (UPR) signature. Moreover using primary hepatocytes, glucose productions under basal conditions and in response to glucagon stimulation were dramatically decreased in Gcn5L1 LKO. We also found that the hepatic expression levels of the key gluconeogenic enzymes, PEPCK and G6Pase, were significantly down regulated in Gcn5L1 LKO hepatocytes and liver tissues. Meanwhile, deletion attenuated glucose production by using glycerol or lactate as substrate, but not pyruvate, which indicates the functional role of Gcn5L1 in gluconeogenesis via regulation mitochondrial redox state. Our findings provide novel insights into a potential in vivo ER-mitochondrial role of Gcn5L1 in regulating hepatic gluconeogenesis.

NIA
Murat Bilgel
Doctoral Candidate
Epidemiology/Biostatistics - Etiology, Risk, and Prevention
The APOE e4 allele is associated with an earlier onset of amyloid accumulation
Cerebral amyloid deposition is the defining characteristic of preclinical stages of Alzheimer's disease (AD) and begins years before cognitive symptoms are evident. The APOE e4 allele is the most influential known genetic risk factor for late-onset AD and is associated with higher cortical amyloid burden in cognitively normal individuals. Age at onset of AD is a phenotype of great clinical interest, as delaying onset will decrease population prevalence and disease burden. Furthermore, finding characteristics associated with onset age might provide clues about disease mechanisms. Among AD risk genes, APOE exhibits the strongest relationship with the age at onset of AD, with the presence of one or more e4 alleles shifting the onset to earlier ages. However, the effect of APOE genotype on the age at onset of amyloid accumulation at the individual level has not been investigated. Using data for 132 participants with longitudinal amyloid measures obtained from Pittsburgh compound B (PiB) positron emission tomography scans in the Baltimore Longitudinal Study of Aging, we estimated the age at which each PiB+ individual began accumulating amyloid using a nonlinear mixed effects model. To account for the possibility that individuals who remained PiB- during the study might have begun accumulating amyloid after their last visit, we used survival analysis methods to investigate the associations with the age at onset of amyloid accumulation. We used the Cox model to quantify the risk of accumulating amyloid, and the accelerated failure time model to assess differences in the age at onset of amyloid accumulation in relation to APOE e4 status and sex. APOE e4 positivity conferred a 3-fold risk of accumulating amyloid (chi-squared test p=0.0006) after adjusting for sex and education. On average, e4+ individuals began accumulating amyloid 12.3 years (16.9%, 95% CI 7.4–26.4) earlier than e4- individuals. The average age at onset of amyloid accumulation for the APOE e4- and e4+ groups was 73.1 and 60.7, respectively. In conclusion, APOE e4 positivity was associated with a higher risk as well as an earlier onset of amyloid accumulation. Our method for estimating the onset age of amyloid accumulation allows for novel analyses using longitudinal amyloid data. Determination of onset age of amyloid accumulation is of critical importance for evaluation of treatments to prevent or delay AD and can inform the optimal time window for anti-amyloid interventions.

NIA
Martine EL BEJJANI
Postdoctoral Fellow
Radiology/Imaging/PET and Neuroimaging
Sex-hormones and brain volumes in middle-age men
Animal and human research suggests that sex-hormones play important roles in brain organization and function and that testosterone has neuro-protective actions in the brain. Parallel to these findings, declining testosterone levels in older men have been linked to the risk of dementia and brain alterations. However, little is known on the links between sex-hormones and brain outcomes in middle age. This study examines the relationships of testosterone (T) and sex hormone-binding globulin (SHBG) levels with global and regional brain volumes in 267 middle-age men participating in the Coronary Artery

Risk Development in Young Adults CARDIA-brain magnetic resonance imaging (MRI) sub-study. We used the mean of 3 sex-hormones measures collected at the Year 2, Year 7, and Year 10 study exams (at the ages of 24 to 41) to approximate the average total T, bioavailable T, and SHBG levels over 8 years. Brain volumes were collected at the Year 25 exam (at the ages of 42 to 56). Multivariable linear regression model analyses, adjusted for potential confounders, revealed that higher levels of SHBG were associated with larger WM volumes, with a 4 cm3 (95% confidence interval (CI)=0.69, 7.56)) increase in WM volume for each z-score increase in SHBG level. There was a pattern of associations between higher total T and SHBG levels and smaller total GM volume; these associations were marginally non-statistically significant. Some associations were more pronounced with certain regions: higher SHBG levels were associated with smaller GM volume in the parietal lobe (-1 cm3 (95%CI=-1.75, -0.24)) and with larger WM in the frontal and temporal lobes (+2 cm3 (95%CI=0.22, 3.79) and +1 cm3 (95%CI=0.08, 1.99)). There was no interaction between the sex-hormones; fractional polynomial analyses showed no indication of non-linear relations between hormones levels and brain volumes. SHBG levels were associated with global WM volumes; regionally, they were associated with GM volume in the parietal lobe -a brain region reported to have distinct characteristics and development in males- and with WM in the frontal and temporal lobes -two regions linked to neurocognitive disorders. Results support the hypothesized links between sex-hormones and brain measures and highlight a relationship in middle-age men between SHBG levels, which might reflect differential regulation of sex-hormones, and brain volumes that have been previously linked to behavioral and neurocognitive outcomes.

NIA

Evandro Fang
Postdoctoral Fellow

Cell Biology - General

Restoration of the NAD+/SIRT1 pathway prevents mitochondrial dysfunction in Ataxia telangiectasia neurons

A major problem in the elderly is neurodegeneration which brings a heavy socioeconomic burden and calls for urgent mechanistic and therapeutic investigations. Ataxia telangiectasia (A-T) is an ideal model disease to study brain aging since it shows premature aging and neurodegeneration. A-T is caused by mutations in the gene encoding ATM, a master regulator of the DNA damage response. We recently reported mitochondrial dysfunction via defective mitophagy in some DNA repair-deficient diseases due to PARP-1 hyperactivation and NAD+/SIRT1 pathway reduction. A role for mitochondrial dysfunction in ATM-deficient neurons, however, has not been explored. We hypothesize that mitochondrial dysfunction contributes to neurodegeneration in A-T, and that recovery of NAD+/SIRT1 signaling may offer neuroprotection in this incurable disorder. The aim of our study was to investigate various methods of increasing endogenous NAD+ levels and thereby improve signaling via the NAD+/SIRT1 pathway. First, we documented that in ATM-depleted primary rat neurons, relative to controls, there was lower levels of NAD+, impaired SIRT1 activity, accumulation of damaged mitochondria, and mitochondrial dysfunction as judged by higher oxygen consumption rates (OCR) and increased mitochondrial ROS levels. We then tested three methods to improve signaling via the NAD+/SIRT1 pathway: a) supplementation with NAD+ precursors, b) SIRT1 activators, or c) PARP inhibition. Using primary rat neurons, we showed that each method was able to palliate the mitochondrial dysfunction

and raise signaling by SIRT1. These treatments were also neuroprotective as demonstrated by increased neuronal dendritic spine production and improved synapse formation in ATM-depleted neurons, relative to no treatment. We also independently tested these findings in another model system, C. elegans. Using the same three strategies as aforementioned, we observed that raising the NAD+/SIRT1 signaling restores the impaired short- and long-term associated learning in an atm-1 C. elegans model. Interestingly, the learning profiles of the N2 control worms also benefitted from increased NAD+ levels. Thus, our study highlights the importance of the NAD+/SIRT1-dependent nuclear-mitochondrial signaling in maintaining mitochondrial health in neurons, and may provide a basis for therapeutic interventions for a broad range of neuronal disorders originating from the dysregulation of DNA damage processing.

NIA
GITA KUMARI
Postdoctoral Fellow
Epigenetics

ACCESSIBILITY/CONFORMATIONAL CONTROL OF IgH GENE IN THYMOCYTES

T and B lymphocytes produce diverse antigen receptors through recombination of variable (V), diversity (D) and joining (J) gene segments at T cell receptor and immunoglobulin loci. Rag1/Rag2 endonuclease initiates VDJ recombination by introducing double strand breaks. This reaction is tightly regulated to minimize genomic damage by permitting RAG access only to the appropriate antigen receptor locus depending on cell type and developmental stage. In one exception to this rule immunoglobulin heavy chain (IgH) gene rearrangements have been reported in 30-50% of developing T cells. To understand the basis for this unexpected activity, we examined IgH chromatin structure, transcription and recombination in CD4+CD8+ (DP) thymocytes generated by expression of a rearranged TCRß transgene. Using chromatin immunoprecipitation experiments we showed that several histone modifications that characterize a fully active locus such as H3K9 acetylation or H3K4 trimethylation were reduced in CD4+CD8+ thymocytes compared to pro-B cells. In addition, specific noncoding RNAs and DNAseI hypersensitive sites within the locus were reduced in CD4+CD8+ thymocytes in comparison to pro-B cells. We suggest that IgH alleles are trapped in a partially activated state in CD4+CD8+ thymocytes. To test the hypothesis that this partially active state may be generated by partial Eμ enhancer activity in the wrong lineage, we assessed interaction of Eμ binding proteins. We observed reduced binding of enhancer binding proteins to Eμ enhancer and this may be one of the factors leading to reduced enhancer activity. Locus contraction and looping have been demonstrated to play an essential role in VDJ recombination and is regulated directly or indirectly by key transcription factor such as CTCF, Pax5 and YY1. Using DNA-FISH we showed that large scale locus construction of IgH locus was absent in CD4+CD8+ thymocytes. DNA-FISH experiments clearly indicate potential involvement of CTCF in insulating VH segments from the DJH part of the locus which in turn blocks VH to DJH recombination in CD4+CD8+ thymocytes. Our studies provide the mechanistic basis for incomplete assembly of functional immunoglobulin heavy chains in wrong cell types and also support the hypothesis that cell type specificity of VDJ recombination is regulated by enhancer.

NIA

HUIMING LU

Visiting Fellow

DNA-binding Proteins/Receptors and DNA Repair

A Physical and Functional Interaction between RECQL4 and DNA2

RecQ helicase RECQL4 deficiency is associated with three human diseases, Rothmund-Thomson Syndrome (RTS), RAPADILINO and Baller-Gerold Syndrome. Our previous study showed that dysfunction of RECQL4 results in persistent DNA damage and induced senescence and contributes to clinical features in a RTS mouse model. However, how RECQL4 deficiency leads to genome instability is not clear. We are interested in determining its protein interaction partners to clarify its role in DNA metabolic pathways including DNA repair. Helicase/nuclease DNA2 is essential in Okazaki fragment maturation, stalled replication fork processing, long patch base excision repair and DNA end resection in double-stranded DNA break repair. Both RECQL4 and DNA2 localize to mitochondria and nucleus, and we hypothesize that the two proteins work together in DNA repair. Here, we detected a physical interaction between RECQL4 and DNA2 in HeLa cells which increased after treatment with H2O2. In 293T cells after ? radiation, more RECQL4 was pulled down with Flag-DNA2 than that from untreated cells. A direct interaction between RECQL4 and DNA2 was then confirmed by co-immunoprecipitation with the purified proteins RECQL4 and DNA2. Interestingly, we found that only about 25% of DNA2 remains on chromatin in RECQL4 depleted HeLa cells with treated ? radiation or camptothecin (CPT), indicating that RECQL4 is required for DNA2 binding to chromatin after DNA damage. The nuclease activity of DNA2 is important in DNA metabolism. To understand if RECQL4 promotes the nuclease activity of DNA2, multiple substrates were tested. Interestingly, at a protein concentration ratio of 1: 1, RECQL4 stimulated DNA2 nuclease activity about 2 fold in the degradation of single-stranded DNA in the 5' flap and the chicken foot substrate. Moreover, the stimulation was independent of RECQL4's helicase activity. To determine the biological significance of the RECQL4-DNA2 complex, cell survival after DNA damage with CPT, mitomycin C, hydroxyurea and H2O2 were conducted. Depletion of either protein causes cell sensitivity to each of these agents, but there were no additive effects in DNA2 and RECQL4 double knockdown cells, suggesting that RECQL4 and DNA2 work in the same pathways. In conclusion, RECQL4 interacts with DNA2 and recruits it to chromatin after DNA damage and promotes DNA2 nuclease activity. This novel observation provides important clues to the mechanism of both proteins in maintaining genome stability.

NIA

Hachi Manzur

Visiting Fellow

Neuroscience - Integrative, Functional, and Cognitive

Temporal backpropagation of reward prediction error signals in the basal forebrain underlies new associative learning

Animals learn to use environmental cues to predict relevant outcomes such as rewards. When these cues no longer predict the anticipated consequences, the internal models of the environment need to be updated. Central to this process is the representation of the difference between what is expected and what is actually obtained as a reward-prediction-error (RPE) signal. During the acquisition of the

association of a reward with a reward-predicting cue, RPE signals, which have been widely observed in several brain regions, are triggered by the initially unpredicted reward, and are then transferred to the predictive cue late in the learning process. However, little is known about how the transference of RPE signals occurs. Here, for the first time we provide direct electrophysiological evidence that the transference of RPE signals from reward to the reward-predicting cue occurs by a temporal backpropagation of neural signals during learning. We trained rats in a discrimination task, and then we performed in-vivo extracellular single-unit neural recordings in the basal forebrain (BF) while they learned a new stimulus-reward association. We found that, unexpected reward following the new stimulus led to an abrupt increase of BF responses early during learning. This neural response gradually decreased over successive training sessions and was inversely correlated with behavioral indicators of accuracy, suggesting that it was a true RPE signal. Before being transferred to the new reward-predicting stimulus, this RPE signal quickly backpropagated to events occurring immediately before reward (i.e. approach to the reward port). If this intermediate state acquired value by itself, as required by the beackpropagation hypothesis, then we should observe RPE signals at this state regardless of the action or stimulus that led to it. Our data shows RPE signals time locked at this state even in the absence of stimulus, which confirms our hypothesis. Finally, the RPE signal was transferred to the new reward-predicting stimulus and was correlated with outcomes of learning such as reaction times. Overall, these results provide direct experimental support for the key prediction of reinforcement learning theories that RPE signal temporally backpropagates during learning and provide a framework on how animals update internal models of their environments.

NIA
Magdalena Misiak
Postdoctoral Fellow
DNA-binding Proteins/Receptors and DNA Repair
Modulation of Alzheimer's disease Phenotypes in DNA Repair Deficient Mice by Intermittent Fasting
Age-related cognitive impairment and Alzheimer's disease (AD) are major health problems without effective interventions. Previous findings showed that the essential protein DNA polymerase beta (PolB) was reduced in brain during normal aging, and to a greater extent in patients with AD. Here, we used an AD mouse model, 3xTgAD, heterozygous for PolB, in order to better mimic human AD. We assessed the impact of reduced PolB levels on several aspects of neurodegeneration. We found that reducing PolB levels in the 3xTgAD background was sufficient to accelerate synaptic and cognitive deficits, elicit an AD-like molecular fingerprint and increase neuronal degeneration relative to the parental 3xTgAD strain. Thus the findings confirmed our hypothesis that a deficiency of PolB would sensitize hippocampal neurons to age- and AD-related synaptic dysfunction. Moving forward, we wanted to test if we could ameliorate some effects of PolB loss. Intermittent fasting (IF) improves hippocampal synaptic plasticity and memory. We hypothesize that this may occur by increased DNA repair, or by improved mitochondrial function invoked via nuclear to mitochondrial DNA damage signaling. We predicted that IF would ameliorate deficits of hippocampal synaptic plasticity and memory resulting from PolB deficiency. Our results show that IF rescued the enhanced anxiety in 3xTgAD/PolB+/- mice. In addition, 3xTgAD/PolB+/- mice consumed significantly more food than 3xTgAD mice, without weight increase. This was reversed by IF. PolB mice have a high energy demand consistent with recent findings that

persistent DNA damage can increase energy consumption. We found that 3xTgAD/PolB+/- mice showed a decline in oxidative phosphorylation gene expression compared to 3xTgAD mice. We believe that the decline of metabolic homeostasis caused by the increased DNA damage-associated energy burden is a key attribute that promotes more rapid neurodegeneration in the 3xTg/PolB+/- mice relative to the 3xTgAD mice. Presently, we are quantifying the degree of neurodegeneration seen in the mouse cohorts. In summary, we have created a mouse model, the 3xTg/PolB+/- strain that more faithfully recapitulates neurodegeneration as seen in AD patients and we are investigating molecular mechanisms that are responsible for synaptic dysfunction and memory impairment. Our findings may provide insight into how diet might counteract neurodegeneration in AD and other neuropathological conditions.

NIA
Wei Peng
Visiting Fellow
Biochemistry - General and Lipids
The RNA topoisomerase Top3b is required for normal synapse structure and function in mice
Top3b is the first RNA topoisomerase identified in eukaryotes. It biochemically and genetically interacts with FMRP, a protein that is deficient in fragile X syndrome (FXS). Increasing evidence suggests that Top3b regulates RNA metabolism and promotes the formation of neuromuscular junctions. A recent study also shows that individuals carrying a homozygous deletion of Top3b exhibit intellectual disability and mental disorders such as schizophrenia and autism, suggesting a critical role of Top3b during brain development. Here we show that Top3b-deficient mice have abnormal synapse formation, a phenotype similar to FMRP-knockout mice. Specifically, cultured primary neurons from Top3b mutant mice exhibit abnormal presynaptic vesicles and synapses represented by reduced immunostaining of the presynaptic and postsynaptic markers, synaptophysin and PDS95. Notably, two forms of protein synthesis-dependent synaptic plasticity, long-term depression (LTD) and long-term potentiation (LTP) that involve activation of metabotropic glutamate receptors (mGluRs), are impaired in the hippocampus of Top3b-deficient mice. This is in contrast to FMRP-knockout mice, which show normal LTP but enhanced LTD in the hippocampus. Importantly, we found that an mGluR5 antagonist can largely rescue the abnormal LTP evoked by synaptic stimulation in hippocampus of Top3b-knockout mice. This resembles previous findings that the same type of antagonists can largely rescue the abnormal synaptic transmission in FMRP-deficient mice. Furthermore, compared with wildtype mice, Top3b-deficient mice show increased freezing time and frequency in a fear conditioning test, suggesting that these mice are more sensitive to fear shock, a similar phenotype of schizophrenia and autism patients. Mechanistically, Top3b binds to a group of synaptic mRNAs, which are crucial for synaptogenesis and synaptic activation, such as Syt11, Notch2, and Camk2a, and may regulate their stability and translation. These findings indicate that Top3b plays an important role in regulating synaptic formation and activity-dependent synaptic plasticity in the brain. Moreover, the data also raise a possibility that mGluR antigonists may be used to treat not only patients with Fragile X syndrome, but also those with schizophrenia and intellectual disability caused by Top3b mutations.

NIA
Jaya Sarkar

Postdoctoral Fellow
DNA-binding Proteins/Receptors and DNA Repair
SLX4 contributes to telomere metabolism via regulated processing of telomeric joint molecule intermediates
Mutations in SLX4 have been linked to the genetic disease Fanconi Anemia (FA) characterized by congenital abnormalities, increased susceptibility to cancer, and sensitivity to DNA interstrand crosslinking agents. Human SLX4 assembles an endonuclease toolkit to function in DNA inter strand cross link (ICL) repair, DNA replication, and telomere maintenance. Telomeres are chromosome end protective nucleoprotein structures. Because of their high propensity to form branched DNA intermediates during normal metabolism, telomeres pose an inherent challenge to DNA replication. Resolution of these intrinsic unusual DNA forms at telomeres is critical for timely progression of telomere metabolism and hence genome stability. Thus, telomere maintenance requires extra attention from genome DNA repair proteins such as the SLX4-assembled nuclease complex, and the helicase BLM. The SLX4-nuclease complex is recruited to telomeres via direct interaction of SLX4 with TRF2, an inherent telomeric DNA-binding protein. Here we provide mechanistic and regulatory insight into how SLX4 functions in telomere maintenance. SLX4 associates with telomeres, peaking in the late S phase and also under replication stress. Disruption of the interaction of SLX4 with TRF2 or SLX1 independently causes telomere fragility (a manifestation of defective telomere replication), suggesting a requirement of the SLX4 complex at telomeres for nuclease-dependent resolution of branched intermediates during telomere replication. Indeed, we find that in vitro the SLX1-SLX4 complex processes a variety of telomeric joint molecules, with kinetic preference for telomeric D-loops and Holliday Junctions (HJs), the most frequently occurring intermediates in DNA metabolism. The nucleolytic activity of the SLX4-nuclease complex is negatively regulated by telomeric proteins TRF1 and TRF2 and by the helicase BLM in vitro. In vivo, we find that SLX4-complex sponsored nucleolytic processing of telomeric homologous recombination (HR) is negatively regulated by BLM, thus resulting in suppressed extrachromosomal telomeric circle (TC) formation and telomere sister chromatid exchange (T-SCE) events. We propose that the SLX4-nuclease toolkit is a bona fide telomere accessory complex that in conjunction with other telomere maintenance proteins ensures unhindered, but regulated progression of telomere maintenance. We believe these findings will help advance our understanding of human genetic disorders, such as FA.

NIA
jian sima
Visiting Fellow
Stress, Aging, and Oxidative Stress/Free Radical Research
EDA and its targets DKK4 and LRP6 regulate Wnt action during Meibomian Gland development
Meibomian glands (MGs) are large sebaceous glands in the eyelids. They produce lipids that protect tears from evaporation. MG dysfunction is thought to be one leading cause of “dry eye” condition that affects over 10 million people in the United States. Previous data has demonstrated the mutations in EDA gene cause MG ablation both in “EDA” patients and in the Tabby mouse. Besides EDA, however, other possible signaling pathways controlling MG development and physiology remain unknown. To study the development and function of MG, we utilize RNA microarray, knockout (KO)/transgenic (TG)

mouse model and CRISPR technology to clarify signaling crosstalk during MG development. Firstly, we performed microarray expression profile of MG tissues from Tabby mice and wide type controls. Data strongly suggested that the Wnt pathway may be regulated by EDA, and especially DKK4 and LRP6, which were markedly down-regulated in Tabby. Further experiments including CHIP, luciferase assays and cell-based functional studies all confirmed that both DKK4 and LRP6 are direct transcriptional targets of EDA. These findings suggested canonic Wnt signaling as a key player and led us to analyze the MG growth phenotypes using KO/TG mice. Interestingly, a MG ablation phenotype was observed in DKK4Tg mice and further histological analysis indicated that phenotype is due to “germ” early phase growth arrest that is very similar to Tabby. We also analyzed the phenotype of ?-catenin KO mice and found a more severe defect in which early germ induction was totally blocked. This evidence confirmed the importance of the wnt pathway, but also suggested possible Wnt function as an upstream signal of turning on EDA. To study the function of LRP6, a second direct target of EDA, we are planning to analyze the phenotype of LRP6 KO mice. Meanwhile, we set up MG tissue culture as an ex vivo assay with CRSIPR KO and activation of LRP6 to analyze phenotypes of MG growth. Besides phenotypic analysis, we also performed in vitro biochemical study and found that 1) DKK4 protein can bind to LRP6 and inhibit Wnt/?-cat activity; and 2) DKK4 can be cleaved at its N-terminal and loss the Wnt inhibitory function. Thus, our data suggest that EDA turns on further MG development and also turns on DKK4 as a feedback inhibitor to Wnt pathway. Overall, this study focuses on a previously unknown signaling crosstalk during MG development, and may provide potential targets to alleviate related eye conditions in the elderly.

NIAAA
Jessica Chancey
Postdoctoral Fellow
Neuroscience - Integrative, Functional, and Cognitive
Removal of D2 receptors on striatal cholinergic interneurons impairs sequence learning
The dorsal striatum (dStr) encodes action initiation and action sequences. Medium spiny neurons (MSNs) in dStr change their firing rate at the start and stop of learned action sequences. MSNs also encode learned action sequences as a single action by increasing or decreasing firing throughout the sequence. Recently, it has become increasingly evident that acetylcholine (ACh) released by striatal cholinergic interneurons (CINs) plays an important role in striatal function. ACh, acting through a diverse group of receptors, drives the release of dopamine and possibly co-release of other neurotransmitters, governs MSN excitability, modulates glutamate release, regulates GABAergic interneuron activity, and regulates plasticity in the striatum. CINs are spontaneously active, establishing a cholinergic tone, and pause their firing during action sequences, but the significance of this physiology has not been explored. We hypothesize that CIN pausing is critical for regulating neurotransmitter release and MSN excitability, allowing for the plasticity necessary for learning. Slice physiology studies suggest that pausing occurs via activation of D2 dopamine receptors (D2Rs) on CINs. Here we test how removing D2Rs from CINs affects learning a sequence of actions to obtain sucrose reward. We adapted a fixed-ratio lever-pressing paradigm that produces reliable performance of action sequences in mice, and determined that eliminating D2Rs from CINs by crossing D2flox/flox mice with Chat-Cre mice (D2F-ChatCre) caused deficits in sequence learning. Specifically, D2F-ChatCre mice learned the sequence task at a significantly slower rate than D2flox/flox littermate controls. Trained D2F-ChatCre mice also demonstrated less lever

pressing and received fewer reinforcers per session than littermate controls. The behavioral deficits were not due to deficits in motor skill learning or general motivation, as D2F-ChatCre mice performed similar to controls in accelerated rotarod, home cage sucrose intake, and progressive ratio breakpoint tasks. Thus, the deficits in sequence learning may be specific to action chunking or behavioral flexibility necessary for this sequence task. We are currently using behavioral paradigms to further investigate the learning deficits and performing in vivo recordings of unit activity in dStr throughout training to gain a better understanding of the role of CIN activity in striatal encoding of learned action sequences.

NIAAA
Resat CINAR
Research Fellow
Pharmacology and Toxicology/Environmental Health
Peripherally restricted, Dual Target cannabinoid CB1R/ inducible NO synthase (iNOS) inhibitors with improved safety and anti-fibrotic Efficacy in liver fibrosis
Liver fibrosis, a major contributor to liver-related mortality, currently has no effective treatment. Liver fibrosis is associated with increased activity of the endocannabinoid/CB1R system, and the CB1R antagonist/inverse agonist rimonabant mitigates fibrosis in animal models. However, the antifibrotic efficacy of rimonabant is low, and neuropsychiatric side effects halted its therapeutic development. Liver fibrosis is also associated with increased activity of inducible nitric oxide synthase (iNOS) and decreased activity of adenosine monophosphate kinase (AMPK), and iNOS inhibitors or AMPK activators have been shown to mitigate liver fibrosis. In order to improve the safety and antifibrotic efficacy of CB1R antagonism, we have developed highly potent, orally bioavailable hybrid CB1R antagonists that are behaviorally inactive due to low brain penetrance, and have additional activity either as iNOS inhibitors or AMPK activators. We have tested the antifibrotic efficacy of the two lead compounds in a murine model of liver fibrosis induced by bile-duct ligation (BDL). The compounds, (-)MRI1867 (CB1R antagonist/iNOS inhibitor) and (-)MRI1891 (CB1R antagonist /AMPK activator) selectively block peripheral CB1R (Ki 2.5 nM and 0.5 nM, respectively) due to their limited brain penetrance (plasma:brain ratio of 0.03 and 0.05, respectively). MRI1867 directly inhibits iNOS activity in vitro by 37% and MRI-1891 activates AMPK in vitro by 20%, whereas rimonabant affects neither iNOS nor AMPK activities, when tested at 1 uM, which is below their concentration in the liver after chronic in vivo administration. Mice were subjected BDL and were treated simultaneously with equipotent daily oral doses of rimonabant (3 mg/kg), (-)MRI1867 (3 mg/kg), (-)MRI1891 (1mg/kg) or vehicle. Both (-)MRI1867 and (-)MRI1891 were more efficacious than rimonabant in mitigating liver fibrosis as quantified by Sirius red staining and TGFß1, Collagen 1A, TIMP1 and aSMA gene expression. Importantly, both hybrid compounds were also able to reduce fibrosis in CB1R-/- mice subjected to BDL. Unlike rimonabant, neither dual-target compound elicited CNS-mediated effects such as hyperambulatory activity or anxiogenic behavior. We conclude that dual-target CB1R/iNOS or CB1R/AMPK compounds can provide a novel type of pharmacotherapy for liver fibrosis with improved efficacy and safety

NIAAA
Joshua Gowin
Postdoctoral Fellow

Neuropharmacology and Neurochemistry

Varenicline administration diminishes amygdala response to fearful faces in heavy drinkers

Over one in ten Americans will develop an alcohol use disorder during their lifetime, underscoring the need for effective treatment. A number of studies have shown that pharmacotherapies can reduce drinking in alcoholics and improve their overall health. The effect sizes for the effectiveness of these medications, however, are small, making it important to expand the range of therapeutics and develop personalized treatment approaches. Neuroimaging offers a potentially valuable tool to predict which treatments may be successful for specific groups of people. Recent studies have suggested that varenicline, an a4ß2-nicotinic partial agonist widely used for smoking cessation, can help alcoholics reduce drinking, but the neurocognitive underpinnings of its effectiveness remain untested. In this double-blind study, 32 heavy drinkers were randomized to receive varenicline (2 mg/day) or placebo. After 2 weeks of dosing, participants underwent functional MRI scans, during which they viewed 90 images of faces with either a neutral or a fearful expression—a well-validated task to elicit amygdala activation and anxiety. Blood-oxygen-level dependent response was analyzed with Analysis of Functional Neuroimaging software. A linear mixed-effects model was used to examine the effects of facial expression (fearful versus neutral) and medication (placebo versus varenicline). Participants also completed the Obsessive Compulsive Drinking Scale (OCDS), a measure of anxiety-driven alcohol consumption, and the 90 day Timeline Follow Back, which quantifies drinking for the three months prior to the study. Results indicated a significant facial expression-by-medication interaction in the left amygdala (p<0.05, corrected). The groups showed equivalent activation to neutral faces, but, whereas the placebo group showed increased activation to a fearful face, the varenicline group showed no change in activation. Amygdala activation to fearful faces correlated with number of drinks in the previous 90 days and the total score on the OCDS. Our results suggest that varenicline may disrupt amygdala response to fearful faces in heavy drinkers. Further, amygdala activation correlated with alcohol consumption, suggesting that varenicline's effects may be related to aspects of drinking behavior. These results suggest that altered amygdala activation may be developed as a biomarker of the effectiveness of medications being developed for the treatment of alcohol use disorder.

NIAAA

Lindsay Halladay

Postdoctoral Fellow

Neuroscience - Integrative, Functional, and Cognitive

Brain mechanisms mediating the suppression of alcohol-seeking in the face of punishment

Seventeen million people in the US are afflicted by alcohol use disorders (AUDs). AUDs are characterized by continued alcohol abuse despite harmful consequences, suggesting that the brain processes that normally serve to regulate punished behaviors are impaired. Prior work has shown that the amygdala is a key brain region encoding the emotional valence of environmental stimuli signifying punishment. However, though alcohol is known to have strong effects on neurotransmission in the amygdala, its role in mediating effects of punishment on alcohol-seeking are unknown. To address this issue, we chronically implanted microelectrodes in the amygdala to record in vivo neuronal activity in freely-moving mice performing a punished-suppression of alcohol-seeking task. Mice were first trained to lever press in order to obtain an alcohol reward, and then during subsequent probe-tests, subjected to a mild

footshock when the lever was pressed. Neuronal recordings revealed sub-populations of cells in the basolateral and central regions of the amygdala that significantly altered their firing rate either during alcohol consumption or during the interval just prior to a punished lever press. Moreover, mice that displayed sustained lever pressing during punishment showed a greater degree of neuronal inhibition during punished lever presses, as compared to mice that largely ceased responding during punishment. Retrospective analysis found that in sessions prior to punishment, the 'punishment-resistant' mice also displayed a higher number of cells that fired in response to alcohol consumption. Together, these data demonstrate neuronal encoding of punished alcohol-seeking in distinct subregions of the amygdala and suggest that the development of resistance to the suppression of alcohol-seeking in the face of harmful consequences may be related to aberrant amygdala function.

NIAAA

Yong He

Visiting Fellow

Immunology - Innate and Cell-mediated Host Defenses

Interleukin-20 promotes T cell-mediated hepatitis in mice: a novel therapeutic target for the treatment of hepatitis

Background and Aims: T cell-mediated fulminant hepatitis is a life-threatening event for which the underlying mechanism is not fully understood, and no effective therapies exist at this time. Injection of Concanavalin A (Con A) into mice recapitulates the histological and pathological sequelae of T cell-mediated hepatitis. We have found interleukin (IL)-20, a member of the IL-10 family of cytokines, is markedly elevated in the serum from Con A-induced T cell hepatitis; but the role of IL-20 remains unknown. Results: To explore the potential function of IL-20 in Con A-induced hepatitis, we generated for the first time IL-20 knockout mice (IL-20KO) and generated adenovirus expressing IL-20. Our data revealed that IL-20KO mice were completely resistant to Con A–induced liver injury as evidenced by lower serum levels of alanine transaminase (an enzyme produced by hepatocytes and a biomarker for liver injury) and liver necrosis compared with wild-type mice. Administration of IL-20 adenovirus markedly accelerated Con A-induced hepatocellular damage. Compared with wild-type mice, Con A-treated IL-20KO mice had much higher levels of hepatoprotective cytokine IL-6 in the serum and liver. In agreement with this, hepatic activation of the IL-6 downstream signal transducer and activator of transcription 3 (STAT3) was elevated, while activation of the IFN-gamma downstream signal STAT1 was decreased in Con A-treated IL-20KO mice compared with wild-type mice. In vitro treatment with recombinant IL-20 inhibited the LPS-mediated stimulation of IL-6 production in macrophages. In addition, IL-20 receptors including IL-20R1, IL-20R2, and IL-22R1 messenger RNA, were detected in the liver and further elevated after Con A injection. These receptors are differentially expressed on hepatocytes, liver resident macrophages, and T cells. By targeting these receptors, IL-20 activated several different signals in these cells. Conclusions: IL-20KO mice are resistant to Con A-induced liver injury, suggesting that IL-20 promotes T cell hepatitis. The detrimental effect of IL-20 is partly mediated via the inhibition of hepatoprotective cytokine IL-6 and its downstream cell survival signal STAT3. Blocking IL-20 could be a novel and effective therapy for T cell-mediated hepatitis.

NIAAA

Teresa Ramirez

Postdoctoral Fellow

Stress, Aging, and Oxidative Stress/Free Radical Research

Aging down-regulates hepatic SIRT1 and exacerbates chronic-plus binge ethanol-induced liver injury and fibrosis

Alcoholic liver disease (ALD), one of the most prevalent forms of liver disease worldwide, is often caused by excessive alcohol consumption. This contributes to oxidative stress, inflammation and down-regulation of enzymes necessary for cell survival. The spectrum of ALD includes fatty liver, steatohepatitis, fibrosis/cirrhosis and hepatocellular carcinoma. SIRT1 protein is essential for cellular metabolism and cell survival. The mechanisms by which chronic ethanol consumption and aging play a role in ALD remain unclear. We hypothesized that aging may have an interaction effect with ethanol exposure that may play a role in down-regulating hepatic SIRT1 protein expression, inducing liver injury and fibrosis. For our studies, female and male C57BL/6 mice age 2, 4, 12 or 16-19-months were used. These mice were first acclimatized to control liquid diets for 5 days. Once acclimatized, mice were chronically fed with control or liquid diets containing 5% (v/v) ethanol for 10 days, followed by a single ethanol binge (5g/kg body weight [b.w.]) or fed up to 8 weeks, which included multiple binges of ethanol (5g/kg [b.w.]). Liver injury and fibrosis were measured using histology, protein and gene-expression levels. The results from liver histological analysis from a single binge revealed that there was a greater degree of steatosis (fatty deposits) and fibrosis in the livers from ethanol-fed old mice when compared to young mice. Results showed that the chronic binge ethanol fed mice showed reduced hepatic protein levels of SIRT1 in old mice. Chronic multiple binge ethanol exposed old mice demonstrated more steatosis, neutrophil infiltration and fibrosis when compared to young mice, correlating to the characteristics of ALD. The protein levels of SIRT1 in stellate cells (which are a cell type involved in liver fibrosis and mostly due to aging) were also down-regulated in 12month old mice. Deletion of SIRT1 in stellate cells accelerated stellate cell activation in vitro; while overexpression of SIRT1 attenuated HSC activation. The current results suggest that aging plays a role in down-regulating hepatic SIRT1 protein expression in hepatocytes and HSCs in old mice, consequently inducing alcoholic liver injury and fibrosis. These novel findings will help us to better understand the importance of how aging and alcohol induced liver injury greatly affects the elderly population, and develop ways to help prevent further injury.

NIAAA

Ming-Jiang Xu

Postdoctoral Fellow

Physiology

Hepatocyte-derived lipocalin-2 plays an important role in inhibiting bacterial infection and promoting liver regeneration

Background and Aims: Lipocalin-2 (LCN2), which is the mouse homologue of human neutrophil gelatinase-associated lipocalin (NGAL), is a 24-kDa secreted glycoprotein and is found in many cell types and various organs, including the bronchus, stomach, small intestine, pancreas, kidney, prostate gland, thymus, and liver. However, the functions of LCN2 and the cell types that are primarily responsible for

serum LCN2 production remain unclear. Results: We generated for the first time Lcn2floxed mice and hepatocyte-specific knockout mice to determine whether hepatocytes are responsible for LCN2 production under the condition of infection and partial hepatectomy (PHx). Hepatocyte-specific Lcn2 knockout mice were subjected to bacterial infection (with Klesbsiella pneumoniae or Escherichia coli) or PHx. Studies of hepatocyte-specific LCN2 knockout (LCN2Hep-/-) mice revealed that hepatocytes contributed to 25% of the low basal serum level of LCN2 protein (~62 ng/ml) but were responsible for more than 90% of the highly elevated serum LCN2 protein level (~6,000 ng/ml) post-infection and more than 60% post-PHx (~700 ng/ml). Both LCN2Hep-/- and global LCN2 knockout (LCN2 KO) mice demonstrated comparable increases in susceptibility to infection with K. pneumoniae or E. coli. These mice also had increased enteric bacterial translocation from the gut to the mesenteric lymph nodes and exhibited reduced liver regeneration after PHx. Treatment with IL-6 stimulated hepatocytes to produce LCN2 in vitro and in vivo, and hepatocyte-specific ablation of the IL-6 receptor or STAT3, a major downstream effector of IL-6, markedly abrogated LCN2 elevation in vivo. Furthermore, chromatin immunoprecipitation (ChIP) revealed that STAT3 was recruited to the promoter region of the LCN2 gene upon STAT3 activation by IL-6. Conclusion: Hepatocytes are the major cell type responsible for LCN2 production after bacterial infection or PHx, and this response is dependent on IL-6 activation of the STAT3 signaling pathway. Thus, hepatocyte-derived LCN2 plays an important role in inhibiting bacterial infection and promoting liver regeneration.

NIAAA
Jia Yan
Postdoctoral Fellow
Psychiatry

Evidence for Influence of the Interaction between CHRNA5 and Childhood Adversity on Alcohol Self-administration and Related Traits in a Sample of Nonsmoking Drinkers

Nicotinic acetylcholine receptors have been reported to play roles in reward and cognitive processes, and in response to drugs of abuse and risk for substance use disorders (SUDs). In particular, a single-nucleotide polymorphism (SNP) in the alpha5 subunit of the nicotinic acetylcholine receptor (CHRNA5), rs16969968, has been identified as a risk variant for nicotine and other SUDs across several samples. Additionally, childhood adversity, which is a significant risk factor for SUDs, has been shown to interact with rs16969968 to influence nicotine dependence. Here, we aimed to characterize the impact of rs16969968 and its interaction with childhood trauma on alcohol-related traits in a sample of nonsmokers. The sample in this study consisted of 350 participants, a subset of whom participated in an intravenous alcohol self-administration (IV-ASA) study (n=70), wherein participants press a button to self-administer alcohol via IV-infusion, providing greater control over brain exposure to alcohol than oral intake. The association between rs16969968 and its interaction with childhood trauma and alcohol traits was assessed using linear and logistic models with sex, age, and ancestry as covariates. Variation in rs16969968 was characterized using a dominant model (AA/AG, n=202 vs. GG, n=148). Childhood adversity was measured using the Childhood Trauma Questionnaire (CTQ), which assesses history of emotional, sexual, and physical abuse and emotional and physical neglect. Alcohol-related traits included alcohol consumption and problems, subjective response to alcohol, and peak and average breath alcohol concentration (BrAC) reached during the IV-ASA. Main effects for rs16969968 were found

for alcohol self-administration in the IV-ASA sample, including the peak and average BrAC reached during the session (p<0.05). An interaction between A-allele status and CTQ total score was found to be associated with peak BrAC reached and whether or not the participant had a binge exposure during the session (p<0.05). No main effects were observed for other phenotypes; however, an interaction was found for decreased subjective response to the impairing effects of alcohol (p<0.05). Individuals with the A allele showed a stronger association between childhood trauma scores and alcohol self-administration compared with GG homozygotes. These findings provide independent support for the impact of the interaction between childhood trauma and rs16969968 on substance use.

NIAAA
Zhou Zhou
Visiting Fellow
Physiology
High-fat diet and acute alcohol binge are a deadly combination that induces liver damage through hepatocyte CXCL1 production and neutrophil recruitment
Background and Aims: Alcohol drinking has long been known as a source of liver injury. Moreover, obesity and excess energy consumption are a growing problem of public health in the modern western world. Several recent epidemic publications suggest a synergistic effect of obesity and alcohol uptake in hepatic damage. However, no studies from animal models confirmed this notion or discussed the mechanisms to pinpoint the potential therapeutic targets. Results: In the present study we investigated into this question by developing a high fat diet (HFD) feeding plus acute alcohol binge model. Our results showed that both short term (3 days) and long term (3 months) HFD feeding exacerbated liver damage by the following acute alcohol binge. The serum alanine transaminase (a marker for liver damage) level reached 700 to 1000 IU/L in the 3-month HFD plus binge group. As inflammation is well related to the deteriorating effects of obesity and alcoholism, we examined the cell number and functions of immune cells in the livers. Flow cytometry analysis showed that HFD plus ethanol resulted in a significant change in the functional groups of the adaptive and innate immune cells. Kupffer cells were drastically dwindled and more importantly, the neutrophils greatly accumulated after HFD. Since neutrophils were found responsible for liver damage in many animal models and epidemic studies, we further studied the mechanisms of their infiltration in the liver. We found CXCL1, an important neutrophil specific chemokine, was dramatically induced by HFD plus binge synergistically, up to 1600 pg/ml in the serum. Surprisingly, hepatocytes themselves, but not other inflammation related cells in the liver or any other organs, were dominant in expressing CXCL1. Instead of commonly known inflammatory factors, CXCL1 was induced by free fatty acid (FFA) accumulation and the downstream signals, JNK, ERK1/2 and NF-kappaB. Blocking CXCL1 with a neutralizing antibody or genetic deletion of the Cxcl1 gene abrogated the hepatic damage of HFD plus binge treatment, supporting the HFD-FFA-CXCL1-neutrophil-liver damage pathway. Conclusions: Our present work suggests a deteriorating effect of HFD and acute alcohol on liver injury through a novel mechanism of FFA deposition, CXCL1 expression in hepatocytes and neutrophil accumulation in the liver. CXCL1 could be a novel therapeutic target for the treatment of HFD plus acute ethanol-induced liver damage.

NIAID
Yaw Adomako-Ankomah
Visiting Fellow
Microbiology and Antimicrobials
Seasonal dynamics of P. falciparum gametocyte carriage
P. falciparum malaria transmission is initiated by the passage of the gametocyte stage of the parasite from infected individuals to mosquitoes. For current transmission control programs to be successful it is critical to clearly define the dynamics of gametocyte carriage in malaria endemic areas. However, there are limited data on the presence and stability of a primary gametocyte reservoir throughout the year and under different transmission settings. In this study we sought to investigate changes in P. falciparum prevalence and gametocyte reservoir over the course of a full year in an area of highly seasonal transmission. From May, 2013 we conducted a yearlong longitudinal cohort study involving 500 individuals aged 1 to 63 years in Kenieroba, Mali. In order to assess parasite carriage at a high temporal resolution we collected peripheral blood samples from subjects at biweekly intervals and screened them for general P. falciparum parasite prevalence as well as gametocyte carriage using the highly sensitive 18S rDNA-targeted PCR and Pfs25 RNA-targeted RT-PCR methods, respectively. As expected, peak P. falciparum prevalence coincided with the peak of the wet season in November, while the lowest prevalence was observed in May. Longitudinal prevalence was found to be age dependent and increased with increasing age, peaking in the 9-12 age group. Interestingly, we also observed a significant gender-based difference in P. falciparum prevalence throughout the year, with males having a significantly higher longitudinal prevalence compared to females. More importantly, gametocyte carriage ranged from a high of 41% in the wet season to a low of 27% in the dry season, with the 9-12 age group contributing the highest proportionate prevalence in both periods. Future work will examine the direct contribution of different age groups to transmission by assessing their relative infectiousness in mosquitoes throughout the year. This work will help define the temporal dynamics of the P. falciparum transmission reservoir which will be important for developing more targeted and efficient malaria transmission control programs.

NIAID
Jesse Arbuckle
Postdoctoral Fellow
Virology - DNA
Abstract removed by request of author

NIAID
Gaspar Canepa
Visiting Fellow
Molecular Biology - Eukaryotic
Anopheline vectors drive selection of Plasmodium falciparum Pfs47
Plasmodium falciparum Malaria is a life-threatening disease transmitted to people through the bites of infected anopheline mosquitoes. P. falciparum originated in Africa and dispersed as a result of human migration thus having to adapt to several different anopheline species in other continents. The mosquito

immune system can greatly limit infection by Plasmodium but this parasite evolved a strategy to evade these responses mediated by the surface-expressed Pfs47 protein. Pfs47 is a polymorphic gene with signatures of diversifying selection and a strong geographic genetic structure around the world. The greatest haplotype diversity of Pfs47 was detected in Africa, while haplotype diversity was low in Asia and the lowest in America. We hypothesize that selection of Pfs47 is driven by adaptation of P. falciparum to different anopheline species around the world. To test our hypothesis we generated a P. falciparum Pfs47 Knock Out line by single cross over recombination with a plasmid bearing an attB integration adaptor sequence to facilitate stable complementations. We generated different parasites lines expressing representative haplotypes of Pfs47 from Africa, Asia and America within the same genetic background. The Pfs47 Knock Out line generated has a decreased capacity to infect the African vector Anopheles gambiae, the Asian vector Anopheles dirus X and the American vector Anopheles albimanus. When complemented with the most representative haplotypes of Pfs47 from Africa, Asia or America these parasite presented higher compatibility (i.e. higher infection intensity and prevalence) with the anopheline species of the same region. The role of the mosquito's immune system on the different Pfs47 lines was also investigated. After silencing components required for immune response in the mosquito by RNAi we were able to rescue the infection of the Pfs47 Knock Out line but no change in infection was observed between the haplotype-vector compatibility pairs. These results showed that within the same genetic background the presence of a specific allele of Pfs47 determines the compatibility of the parasite, suggesting that selection of compatible Pfs47 haplotypes may be required for P. falciparum to evade the mosquito immune system of a new vector, influencing parasite's population structure and malaria epidemiology.

NIAID
David Cantu
Postdoctoral Fellow
Immunology - Lymphocyte Development and Activation
Polycystic liver disease is suppressed by IL-4/IL-13 and TGF-beta signaling
Polycystic liver disease (PLD) is a relatively rare but devastating condition that causes cysts – fluid filled sacs – to grow throughout the liver. The immunological mechanisms controlling liver cyst formation, however, remain unclear. In this study, we show that during infection with the helminth parasite Schistosoma mansoni, severe PLD-like disease develops when infected mice are deficient in both IL-4 and IL-13, suggesting that Stat6-mediated signaling is critically required to prevent the development of PLD. We hypothesized that the parasite eggs might be contributing to cyst formation by promoting damage to microvessels in the liver, which are not efficiently repaired when mice are deficient in both IL-4 and IL-13. Because IL-4 and IL-13 are linked with wound repair and are potent inducers of transforming growth factor-beta (TGF-b) expression, we examined whether cyst formation in IL-4/IL-13-deficient mice was linked with uncontrolled type 1-associated inflammation and/or maladaptive repair. Although IL-17A was up-regulated in IL-4/IL-13 double knockout (dKO) mice, supporting the emergence of a Th17-driven inflammatory response, IL-17A KO and IL-4/IL-13/IL-17A triple KO (tKO) were equally susceptible to the development of PLD, although the tKO displayed slightly improved survival. Histological analysis of liver tissues from IL-4/IL-13 dKO mice revealed an elevated infiltration of F4/80+ macrophages that co-localized with TGF-b-inducible protein along the cyst border, suggesting TGF-b

signaling was elevated in the dKO mice. To elucidate the role of TGF-b, infected IL-4/IL-13 dKO mice were treated with a pan neutralizing TGF-b monoclonal antibody (mAb). Strikingly, the number and size of cysts was dramatically increased when type-2 cytokines and TGF-b signaling were simultaneously blocked, suggesting that IL-4, IL-13, and TGF-b collaborate to prevent PLD during chronic S. mansoni infection. These studies suggest that therapeutic strategies that increase IL-4/IL-13 and TGF-b signaling might provide a rational approach to treat patients with progressive PLD.

NIAID
Julio Castillo
Visiting Fellow
Immunology - Innate and Cell-mediated Host Defenses
Cellular mechanisms of hemocyte-mediated parasite killing in Anopheles gambiae mosquitoes infected with Plasmodium berghei

The mosquito midgut epithelium constitutes a major barrier against Plasmodium parasite infection in disease vector mosquitoes. Hemocytes -the insect's equivalent of lymphocytes- are essential for parasite killing. Previous work have shown that hemocyte differentiation factor (HDF), produced by mosquitoes infected with Plasmodium is essential for immune priming and immune memory. Moreover, HDF induces hemocyte differentiation into granulocytes which are required for the establishment of immune memory. However, the direct role that these cells play during parasite midgut invasion remains unknown. Also, there is evidence of the presence of hemocyte-specific transcripts found in Plasmodium-infected midguts, yet no hemocytes have been found physically associated with the midgut epithelia. To try to address this problem, we develop a protocol to specifically label hemocytes using a cell membrane-specific lipophilic dye. First, granulocytes are the only circulating hemocyte type found labeled in the mosquito hemolymph. Confocal microscopy analysis of midguts from Anopheles gambiae mosquitoes (carrying fluorescently-labeled hemocytes) infected with P. berghei parasites show the presence of labeled microvesicles (presumably of granulocyte origin) inside Plasmodium-infected midgut cells. When we pretreat mosquitoes with HDF (inducer of hemocyte differentiation) we found a dramatic increase in dye co-localization inside infected cells presumably due to an increase in granulocytes. Similarly, when we manipulate hemocyte numbers by depleting the pool of granulocytes available by injecting fluorescent beads (granulocytes are phagocytic), we found a dramatic decrease in microvesicles inside infected cells and an increase in infection. Furthermore, when we silenced two hemocyte-specific genes using RNAi, we observed that the gene AGAP000095 increased the intensity of labeling of infected cells, whereas silencing the gene AGAP002243 caused a decrease in vesicle formation. To summarize, our hemocyte manipulation data further support our hypothesis that hemocytes physically interact with Plasmodium-infected midguts, and microvesicles might be involved as a component of this interaction. Currently, we are investigating the cellular mechanism of parasite killing used by hemocytes, as well as trying to identify the nature of the cargo they shuttle.

NIAID
Abhilash Chiramel
Visiting Fellow

Immunology - Innate and Cell-mediated Host Defenses
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NIAID
Rebecca Drummond
Visiting Fellow
Immunology - Infectious Disease
Impaired Chemokine Production and Neutrophil Recruitment in CARD9-Deficient Mice and Humans with Brain Fungal Infection
Candida albicans is the most common human fungal pathogen and causes systemic infections that are associated with an unacceptably high mortality rate, despite the availability of antifungal therapy. C-type lectin receptors (CLRs), expressed on myeloid cells, are crucial for innate Candida recognition and initiation of antifungal immune responses. Many CLRs utilize a common signaling pathway, which uses the adaptor protein CARD9. Humans with autosomal recessive CARD9 deficiency are susceptible to fungal infections that have a unique tropism for the brain and meninges. However, the mechanisms of CARD9-dependent brain antifungal immunity are poorly defined. Here, we report a novel CARD9 missense mutation (c. 170G>A; p. R57H) in a 10 year-old girl with Candida meningoencephalitis, whose monocytes exhibited profound defects in production of proinflammatory cytokines to fungal stimuli ex vivo. Notably, the patient had a distinct absence of neutrophils in the cerebrospinal fluid (CSF) despite uncontrolled fungal disease (0-7% of total cells), which is strikingly suboptimal compared to the robust neutrophil response observed in CARD9-sufficient patients with Candida meningoencephalitis (>75% of total cells). Absence of neutrophils in the CSF was not due to peripheral neutropenia, impaired neutrophil survival or a chemotaxis defect. Instead, we found that neutrophil-recruiting chemokines CXCL1, CXCL2, CXCL5 and IL-8 were absent in the patient CSF, which was not chemotactic for CARD9+ or CARD9-null neutrophils ex vivo. We phenocopied the human susceptibility in Card9 knockout (KO) mice, which develop uncontrolled brain candidiasis with profound tissue fungal invasion, and have severely impaired neutrophil accumulation in the infected brain. This defect was not caused by decreased neutrophil production in the bone marrow or egress into the blood, nor due to impaired neutrophil survival or induction of neutrophil-targeted adhesion molecules in the KO brain. While KO neutrophils showed no intrinsic chemotaxis defects in mixed bone marrow chimeras, the levels of CXCL1, CXCL2 and CXCL5 were significantly decreased in infected KO brains. Using qRT-PCR in FACS-sorted brain cells and transgenic reporter animals, we found that the major cellular source for these chemokines was neutrophils. Thus, CARD9 is critical for neutrophil recruitment and control of fungal invasion in the brain, acting to promote neutrophil-targeted chemokine production.

NIAID
John Gallagher
Postdoctoral Fellow
Protein Structure/Structural Biology
Cryo-electron microscopy and image analyses of influenza vaccine nanoparticles suggest conformational and orientational design constraints correlated with multivalent binding and increased vaccine response.
Vaccines based upon recombinantly engineered proteins are revolutionizing the process of vaccine

design. Presentation of multiple copies of antigens on nanoparticles substantially increases the immune response to vaccination when compared to unitary monovalent counterparts, in part due to multivalent binding sites on immunogens facilitating B-cell receptor activation. To determine structural and geometrical parameters for multivalent nanoparticle design, we determined the structure of a ferritin-based nanoparticle designed to present a conserved influenza hemaglutinnin (HA) stem epitope on its surface. We used the methods of cryo-electron microscopy, image processing and molecular modeling to determine the molecular organization and conserved epitope disposition of the nanoparticle. Plain ferritin particles, which have well characterized properties, were used as a positive control for the imaging and analysis. Our results indicated that the ferritin-HA was rigid and homogeneous, which would present a well-defined structure to the immune system. Molecular modeling indicated twenty-four HA protomers assembled into eight trimers on the ferritin particle surface with overall octahedral symmetry. Using the cryo-EM density map, we resolved the angular orientation of protruding HA trimers relative to the threefold symmetry axis of HA. The angular orientation of the HA trimers were offset from each other, permitting us to model Fab fragments binding to all 24 epitopes on the ferritin-HA particle. Fabs passed next to each without overlap, while protruding outward from the particle where sufficient space existed to accommodate a full antibody. Thus, the structure indicated that a full complement of 24 antibodies may bind to the ferritin-HA nanoparticle. In support of our conclusions from the cryo-EM structure, animal studies indicated a robust immune response to the ferritin-HA nanoparticle in ferrets. Guidelines for nanoparticle based vaccine design based upon validated structural data such as ferritin-HA promise to lead to improved immune responses to nanoparticle based vaccine candidates and a rapid development process for more efficacious influenza vaccines.

NIAID
Richard Gieseck
Doctoral Candidate
Immunology - Infectious Disease

Type II IL-13 Signaling on Hepatic Progenitor Cells Initiates Ductular Reaction

Schistosomiasis, a chronic helminth infection notorious for the severe IL-13 driven fibrosis it causes, affects over 200 million individuals worldwide. Chronic Schistosoma infection often leads to a destructive complication known as ductular reaction (DR), characterized by neoplastic ductal structures which cannot drain properly, causing local necrosis and increasing risk of cancers such as cholangiocarcinoma (CC). Presence of DR is used widely as a prognostic marker and is highly correlated with severity of fibrosis; however, whether DR develops as a direct result of chronic fibrogenesis or by a distinct mechanism remains unknown. To investigate whether IL-13 plays a direct or indirect role in DR, we utilized IL4RaLiverKO (knockout specific to hepatocytes, cholangiocytes, and hepatic progenitor cells (HPCs)) and WT mice to deplete IL-13 signaling in ductal cells. After 18 weeks infection with Schistosoma mansoni, no differences in fibrosis were observed between the IL4RaLiverKO and WT groups; however, the WT group exhibited extensive DR, whereas the IL4RaLiverKO group had normal ductal architecture. Additionally, the use of an IL13Ra1 knockout yielded no DR, suggesting that the type II IL4Ra:IL13Ra1 signaling complex is necessary for DR, and importantly, that fibrosis is not sufficient to cause DR in the absence of IL-13 signaling on ductal cells and HPCs. To determine if IL-13 is sufficient to drive DR in the absence of a helminth infection, we constructed an IL-13 overexpression vector and hydrodynamically

delivered the plasmid to restrict expression to the liver. IL4RaLiverKO and WT groups of mice developed levels of fibrosis normally seen in 12-week schistosoma infection in just 9 days. WT mice developed florid DR whereas IL4RaLiverKO mice had none. Microarray analysis of HPCs isolated from these mice and restimulated in vitro with IL-13 demonstrated that IL-13 acts as both a mitogen and differentiation factor on HPCs. Overall, these data have shown that fibrosis is not sufficient to cause DR, but rather that IL-13 signaling drives DR, by directly stimulating the expansion and differentiation of HPCs, and fibrosis concurrently, thus explaining the high correlation between the two. It is our hope that this previously unknown function of IL-13 in DR will help further the development of novel therapeutic interventions to help prevent this serious complication and reduce the need for liver transplantation in advanced cirrhosis.

NIAID
Anderson Guimaraes Baptista Costa
Postdoctoral Fellow
Immunology - Infectious Disease
Neutrophil recruitment during Leishmania infection: The role of sand fly salivary proteins
Leishmaniasis comprises a group of diseases endemic in 98 countries, affecting mostly tropical and subtropical areas, caused by parasites of Leishmania genus. Leishmania infection begins when an infected sand fly bites a host inoculating metacyclics parasites into the skin. Neutrophils are the first cells recruited to the site of an infected sand fly bite and interact with Leishmania parasites, playing a pivotal role during infection. It has been proposed that the recruitment of neutrophils to the site of the bite increases the ability of Leishmania to establish an infection. Here we investigate the effect of sand fly salivary proteins on neutrophil recruitment. We purified bone marrow neutrophils from C57BL/6 mice or from peripheral blood of healthy human donors. Chemotaxis driven by sand fly saliva of vectors of visceral (Lutzomyia longipalpis) or cutaneous (Phlebotomus duboscqi) disease was measured by modified Boyden chamber assay. Human neutrophils migrated towards saliva glands of the vectors P. duboscqi and L. longipalpis, in a dose-dependent manner. Moreover, L. longipalpis saliva recruited more human neutrophils than P. duboscqi. Inversely, P. duboscqi recruited mouse neutrophils in a dose-dependent manner, but L. longipalpis had no impact on mouse neutrophils migration. Proteinase K treatment of P. duboscqi saliva completely abrogated neutrophil recruitment, suggesting a protein as the chemotactic factor. To unearth the identity of the chemotactic factor from the saliva, we have generated plasmids coding for the 20 most abundant salivary proteins from P. duboscqi and injected them in the ears of C57BL/6 mice. Neutrophil recruitment was analyzed by flow cytometry staining at 2, 6, 12 and 24 hours post injection. We have identified 3 different salivary protein-coding plasmids with neutrophil recruitment activity. Our next step is to perform the in vitro and in vivo chemotaxis assays with the recombinant proteins. We plan to validate the role of the salivary chemotactic factor in vivo and test if its neutralization can alter Leishmania infection. Our results demonstrate for the first time that saliva from both sand fly vectors possesses a protein chemotactic factor for neutrophils recruitment. We put forward the hypothesis that blockage of this protein activity will disrupt neutrophil migration early in the Leishmania infection possibly altering the disease outcome.

NIAID

Seong-Ji Han

Postdoctoral Fellow

Immunology - General

Immunity in adipose tissue

Each abdominal organ is associated with visceral adipose tissue (VAT), which contains a variety of immune cells that have been shown to exacerbate metabolic disorders. While these cells have been extensively studied in the context of obesity and diabetes, little is known about their contribution to tissue-specific immunity. Furthermore, it remains unclear whether the VAT associated with each organ differs in its cellularity and function. The immunological function of the mesenteric adipose tissue (MAT), a component of the VAT that connects the intestine to the mesenteric lymph node with lymphatics and blood vessels, has been unappreciated so far. Our results from various model of infection revealed that the MAT can be profoundly remodeled, in some cases permanently. These observations led us to hypothesize that the MAT may be more than a connective tissue and, together with the MLN, may function as first line of defense to oral infections and against the translocation of commensals. Our analysis of the MAT under steady state conditions has revealed a significant accumulation of lymphocytes associated with the control of bacterial or parasitic infection, CD4+ T helper 1 (Th1) cells and unique antigen presenting cell subsets, including BATF3-dependent dendritic cells (DCs). To establish whether these DCs are required for the accumulation of Th1 cells in the MAT, we examined mice deficient in BATF3 and found that the CD4+ T cells no longer express makers associated with Th1 cell and switched to a Th2 phenotype. These results suggest that the BATF3-dependent DCs present antigens to CD4+ T cells and constitutively initiate a Th1 response in the MAT. Further, in response to oral antigen, we observed the accumulation and clustering of antigen-specific CD4+ T cells in the adipose tissue, further suggesting that antigen presentation occurs in this tissue. Transcriptional analysis of the MAT revealed a high level of antimicrobial peptides, indicating that this tissue may be poised to sense and control the microbiota and pathogens. On going studies are aimed at further understanding the cross talk between this compartment and defined pathogens and members of the microbiota. Together, our results uncover a novel role of the adipose tissue and support the idea that the mesenteric adipose tissue can act as a shield by inducing early immune responses against intestinal microbes that breach the intestinal wall, preventing pathogen spread and translocation of commensals.

NIAID

Kevin Hart

Postdoctoral Fellow

Immunology - General

Immune Modulation of NAFLD progression to NASH and Hepatic Fibrosis in Obesity

Worldwide incidence of obesity has attained epidemic proportions and continues to rise. Globally, an estimated 300-500 million people are obese, with an increased risk for the myriad of health concerns now termed the metabolic syndrome. This includes Non-alcoholic fatty liver disease (NAFLD), and its pathophysiologic complications including non-alcoholic steatohepatitis (NASH) and cirrhosis, which have become the most common forms of progressive liver disease in developed countries. Evidence suggests

that progression of NAFLD is intricately tied to both chronic low-level inflammation and the resulting insulin resistance present in the context of obesity. Disease progression is marked by abnormal fat accumulation in hepatocytes (steatosis), immune cell infiltration, and eventual scarring, or fibrosis, of the liver. The specific immunological mechanisms underlying progression to NASH and fibrosis are still poorly understood, in part due to limited animal models and reports that mice are resistant to NASH progression. Contrary to these reports, we found that mice on a chronic high fat diet (HFD) for 40 weeks do in fact recapitulate markers of human NASH, including increased steatosis, inflammation, and characteristic hepatic fibrosis. To determine how some of the proinflammatory cytokines increased in obesity regulate NASH progression, we hypothesized that elimination of interferon-gamma (IFN-g) or IL-12 would reduce the progression and severity of liver disease. However, knockout mice on HFD lacking these proinflammatory cytokines were more susceptible to steatosis, and IFN-g knockout animals displayed rapid progression to NASH with evidence of fibrosis at 15 weeks. This rapid progression was linked to altered regulation of the profibrotic molecule transforming growth factor-beta (TGF-b), and antibody mediated blockade of TGF-beta markedly reduced expression of fibrotic markers in mice fed a chronic HFD. Thus, instead of driving disease, our data revealed a protective role for some inflammatory cytokines in the fibrotic response of NAFLD. As such, these findings suggest a more complex paradigm between inflammation, obesity and metabolism in the liver than has been previously proposed. Moreover, our studies indicate that IFN-g deficient animals represent a rapid and novel model to study downstream mechanisms propagating NASH. Finally, they also illustrate the therapeutic potential of targeting TGF-b signaling in NASH-associated fibrosis.

NIAID
Amy Kullas
Postdoctoral Fellow
Immunology - Infectious Disease

Role of Monocyte Autophagy in Host Susceptibility to Cryptococcus gattii infection

Cryptococcus gattii is an emerging fungal pathogen, causing an outbreak of meningitis in the U.S. Pacific Northwest among previously healthy individuals. The fungus is related to C. neoformans, responsible for the deaths of an estimated 600,000 AIDS patients annually. However, components contributing to the susceptibility to C. gattii infection remain largely unknown. Recently, granulocyte-macrophage colony-stimulating factor, GM-CSF, neutralizing antibody has been identified as a predisposing factor to C. gattii in healthy individuals. We thus sought to characterize how antibody to GM-CSF may be affecting the immune response to C. gattii. We incubated healthy donor monocytes with healthy donor or patient sera, and found that sera containing anti-GM-CSF autoantibody accelerated p62 degradation, a marker of increased autophagic flux. Autophagy is a highly conserved, homeostatic pathway in eukaryotes that degrades cytosolic components to promote survival. Autophagy proteins also modulate aspects of innate and adaptive immunity including the inflammasome response to pathogens. Thus, to understand the relationship of antibodies to GM-CSF and autophagy in C. gattii infections, we used an established myeloid-specific ATG5 Cre-Lox knockout mouse model to examine if monocyte autophagy plays a role in C. gattii infection, as ATG5 plays a central role in autophagy. Atg5flox/flox-Lyz-Cre littermates and wild-type littermates were infected by intrapharyngeal aspiration of 50,000 CFU of C. gattii. At various times post-infection, lungs and brain were harvested to determine fungal burden and characterize the

immune response. The data showed that ATG5 deficiency, presumably an autophagy deficiency, in macrophages resulted in better survival of the host: decreased fungal burden in lungs and brains, increased concentration of IL-1beta in lungs, and less macrophage recruitment to the lungs. These data thus identify Atg5 as the first mammalian host gene shown to alter susceptibility to C. gattii. In summary, these data implicate a role for the modulation of autophagy by autoantibodies to GM-CSF that may alter susceptibility of the host to C. gattii infections. This data does not follow previous paradigms where myeloid cells utilize autophagy for protective microcidal actions, but rather, autophagy serves to increase susceptibility to infection and suggests novel mechanisms for the role of autophagy in microbial infections.

NIAID
Bo Liang
Research Fellow
Virology - RNA and Retroviruses

Enhanced immunogenicity of respiratory syncytial virus F protein expressed by a parainfluenza virus: effects of pre-fusion stabilization and packaging in the vector particle

Human respiratory syncytial virus (RSV) and parainfluenza virus type 3 (HPIV3) are the most common viral pathogens causing severe lower respiratory infection in infants and young children. Vaccines are not available, and an RSV vaccine is particularly needed. A live-attenuated chimeric bovine/human PIV3 vector (B/HPIV3) expressing RSV fusion (F) glycoprotein from an added gene has been under development as a bivalent intranasal vaccine against RSV and HPIV3. In a previous clinical study by another group, the induction of RSV neutralizing serum antibodies (NAb) by this vaccine in seronegative children was less than expected. In the present study, we explored whether the RSV NAb response could be improved by (i) enhancing the packaging of RSV F into the vector virion and (ii) using a highly immunogenic pre-fusion form of RSV F involving two serine to cysteine mutations designed by others, called DS, that stabilize RSV F in the pre-fusion conformation. Analysis of RSV F present in purified vector virions by electron microscopy with immunogold labeling and Western blot analysis indicated that the packaging efficiencies of wild-type (wt) and pre-fusion (DS) forms of RSV F were enhanced 20 times (to levels resembling RSV virions) by substituting the cytoplasmic tail alone (CT) or together with the transmembrane domain (TMCT) with their counterparts from the vector F protein. In cell culture, packaging of RSV F did not impair vector replication. The CT, but not TMCT, dramatically increased the RSV F induced cell-to-cell fusion; however, neither CT/DS nor TMCT/DS caused fusion, likely due to their stabilized pre-fusion conformation. In hamsters, vectors with TMCT and DS, but not CT, independently resulted in dramatically increased levels of high-quality RSV NAbs (i.e., neutralizing in vitro without added complement). In a rhesus macaque model, the vector with TMCT/DS was 100-1000 times more attenuated than vectors expressing the wt and DS forms of RSV F, but induced 100 and 30 times higher titers of serum RSV NAb than vectors expressing the wt and DS forms of RSV F, respectively. Thus, TMCT-mediated packaging and DS contributed independently to the enhanced NAb response of TMCT/DS. The packaged wt and pre-fusion forms of RSV F were generally stable during vaccine virus replication in vitro and in vivo. The B/HPIV3 vector expressing TMCT/DS is under development for clinical evaluation.

NIAID
Eugene Liu
Clinical Fellow
Metabolomics/Proteomics
Proteomic predictors of Plasmodium falciparum specific IgG antibody responses in an area of intense seasonal malaria transmission
During an infection some antigens of the infecting pathogen elicit higher antibody responses than others, but the factors underlying this heterogeneity are unclear. In this study we sought to understand the factors underlying differential antibody reactivity to natural Plasmodium falciparum infections. Using a protein microarray containing 1087 P. falciparum proteins, we profiled P. falciparum-specific IgG responses in plasma samples collected from 267 Malian subjects aged 3 months to 25 years who are exposed to intense seasonal malaria transmission every year. We examined the relationship between the level of P. falciparum antigen-specific IgG levels and a number of features of the antigens on the array including their subcellular location, presence of MHC class II epitopes, protein abundance, molecular weight, presence of human orthologs, and degree of polymorphism. We found that IgG reactivity was significantly higher to extracellular and plasma membrane proteins, proteins with MHC class II epitopes, highly abundant proteins and highly polymorphic proteins; whereas IgG reactivity was significantly lower to proteins with human orthologs. Multivariate analysis revealed that subcellular location (extracellular and plasma membrane proteins) independently predicted higher IgG reactivity, a finding we observed in the same cohort the following malaria season. These results provide insights into the proteomic features of antigens that underlie the variation in antibody responses during a natural infection, information that could inform vaccine strategies.

NIAID
Carrie Lucas
Research Fellow
Immunology - Lymphocyte Development and Activation
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NIAID
joseph mudd
Postdoctoral Fellow
HIV and AIDS Research
Loss of NKp44+ type 3 innate lymphoid cells in mesenteric lymph nodes during chronic SIV infection is linked to disease progression.
Chronic immune activation is a key determinant of HIV pathogenesis. Early in disease course, the integrity of the gut mucosal barrier is compromised and this allows microbial products to translocate into systemic circulation. There they stimulate the immune system and exacerbate immune activation. Under healthy conditions, gut epithelial integrity is maintained, in part, by IL-17. CD4+ Th17 cells are important sources of IL-17 and these are lost early in disease course. Yet, these cell types are not the only subsets responsible for maintaining gut integrity. A recently described type 3 innate lymphoid cell (ILC3s) that express NKp44 reside in gut tissues and also secrete IL-17. In mice, loss of these cells induces

bacterial dissemination to peripheral tissues. SIV infection in Asian macaques closely mimics the natural course of HIV infection, and it was our goal to study whether SIV infection is associated with dysfunction of NKp44+ ILC3s that secrete IL-17. For the first time in non-human primates, we have characterized Nkp44+ ILC3s by flow cytometry in infected and uninfected rhesus macaques. In chronically infected rhesus macaques (N=5), we find that frequencies of NKp44+ ILC3s are decreased in mesenteric nodes (mean ILC3= 13.5%) when compared to NKp44+ ILC3s of uninfected animals (N= 5)(mean ILC3= 30.8%)(p= 0.008). NKp44+ ILC3 in the mesenteric lymph node are significantly more activated, as chronically infected animals display higher surface expression of the activation marker HLA-DR (mean HLA-DR= 51.7%) when compared to HLA-DR expression on NKp44+ ILC3s of uninfected animals (mean HLA-DR= 16.8). Loss of CD4 T cells (which ultimately results in susceptibility to opportunistic infections) correlates directly with low frequencies of ILC3 cells (N=8, R^2= 0.8, p= 0.001). Moreover, NKp44+ ILC3s are lost in parallel with CD4 T cells that secrete IL-17 in SIV+ mesenteric lymph nodes (N= 8, R^2= 0.7, p= 0.003). Given the important role of NKp44+ ILC3s in maintaining gut integrity and promoting anatomical containment of commensal bacteria, the dysfunction of this subset in SIV infection may be important in contributing to pathogenesis.

NIAID
Sethu Nair
Visiting Fellow
Genetics
Abstract removed by request of author

NIAID
Alexandra Ortiz
Postdoctoral Fellow
HIV and AIDS Research
Contribution of IL21 Treatment and Probiotic Supplementation to Th17 Reconstitution in ARV-Treated Nonhuman Primates.
Human Immunodeficiency Virus (HIV) infection results in damage to the gastrointestinal (GI) immune system, which is only partially restored upon the initiation of antiretroviral (ARV) therapy. In particular, the specific depletion of antibacterial Th17 CD4+ T-cells within the GI tract correlates inversely with disease progression and persists despite prolonged ARV therapy. Recent findings have implicated that the competency of the GI tract immune system is dependent upon signaling and metabolites originating from commensal flora and that the composition of the microbiome is altered under certain diseased states (dysbiosis). We have considered that incomplete GI tract immune reconstitution in ARV-treated individuals might result from persistent dysbiosis. We previously evaluated the effect of an oral regimen of probiotics in a non-human primate model of progressive HIV infection. We found that probiotic supplementation resulted in decreased lymphoid fibrosis, increased antigen presenting cell frequency and function, and increased GI tract CD4+ T-cell reconstitution concurrent with ARV-treatment. However, we did not observe an increase in the frequency of Th17 cells. We next considered that IL21 administration might promote Th17 recovery. IL21 has been shown to promote Th17 cell differentiation and function in vitro and the administration of IL21 to simian immunodeficiency virus (SIV)-infected

nonhuman primates in vivo has been previously demonstrated to preserve Th17 cells. Here, we infected 11 Pigtailed Macaques (PTM) with SIVmac239 and at day 98 post-infection, treated all PTM with ARVs L'812, PMPA and FTC and with or without probiotic (VSL#3®) and IL21. We evaluated treatment efficacy by tracking SIV viremia by RT-PCR and immune function by multi-parameter flow cytometry, ELISA and immunohistochemistry. Probiotic and IL21-supplementation of ARVs in SIV-infected PTMs promoted intestinal B-cell expansion, CD4+ T-cell reconstitution, and increased Th17 frequency and polyfunctionality. Our treatment was not associated with increased viral load or with increased immune activation. Importantly, treatment resulted in significantly fewer subclinical opportunistic infections and ARV-associated complications as compared to controls. Our results are the first to demonstrate in vivo Th17 recovery in a non-human primate model of progressive HIV infection.

NIAID
Gabriel Parra
Research Fellow
Virology - RNA and Retroviruses

A Population Dynamics Approach for Charting the Evolution of Norovirus Strains Associated with Acute Gastroenteritis

Noroviruses (NoV) are important pathogens of acute gastroenteritis. An effective vaccine could save thousands of lives each year, but the number of antigenic components needed for broad coverage is not known. Like many RNA viruses, NoV are genetically diverse with two major genogroups (GI and GII) containing over 30 different genotypes. To understand the rules that govern NoV evolution and antigenic diversity, we: (i) assembled a large database that included over 800 sequences of the complete ORF2 (encoding the major capsid protein VP1) representing all known GI and GII NoV genotypes; and (ii) developed an efficient platform to analyze complete NoV genomes by Next-Generation Sequencing (NGS) to improve the depth of the database. Phylogenetic analyses of all available sequences revealed the presence of intragenotypic clusters within most of the genotypes, with GII.4 (the predominant NoV genotype) presenting the largest number of clusters. The other genotypes formed clusters with strains that either differed by only a few residues over several decades or that formed distinct clusters within the same year with marked variation, while the GII.4 followed a temporal pattern of evolution. To gain insight into the apparent varying mechanisms of evolution, we analyzed by NGS differences in the population dynamics of NoV infecting healthy individuals. The first set of samples was collected from patients with GII.3, GII.4, or GII.6 infection that, despite resolving symptoms within days, shed NoV for up to 4 weeks. The GII.6 and GII.3 strains were stable and did not show evidence of adaptive changes during the prolonged shedding phase, while the GII.4 viruses showed a number of nucleotide changes as infection progressed. A second set of samples was obtained from cases of person-to-person transmission of non-GII.4 strains, which showed that although minor changes could be detected during transmission (acute phase), the virus would often revert to the original virus sequence during the recovery phase of the infection. Taken together, our data suggest different patterns of evolution among NoV strains. The GII.4 viruses are most prone to change and follow a linear evolution of clusters, similar to influenza A virus, which allows the emergence of new epidemic strains. Other NoV genotypes explore only a few clusters that remain static over time, which limits their antigenic diversity and prevalence. Population dynamics offers a new tool in the development of NoV vaccines.

NIAID
Vanessa Ridaura
Visiting Fellow
Carcinogenesis
The effect of skin resident commensals in treatment and progression of melanoma
The skin serves as an important biological barrier. It is home to a microbiota with complex and varying biogeographical features. Resident members of the microbiota, such as Staphylococcus epidermidis, can confer immunity against cutaneous pathogens by stimulating the host's immune system or by occupying empty niches that would otherwise be colonized by more pathogenic organisms. Preliminary data from our laboratory has suggested that specific strains of commensal bacteria can modulate CD8+ T cells in the skin. Furthermore, tumor bearing, germ-free animals have slower tumor progression and are less responsive to immunotherapy than their wild-type specific pathogen free counterparts. Interestingly, malignant melanoma is a common cancer in the US and prognosis has been associated with tumor infiltrating CD8+ lymphocytes. We hypothesize that resident bacteria strains on the skin occupy particular niches eliciting specific immune responses that can modify the progression of malignant melanoma. We have isolated novel bacterial species from the skin and lymph nodes of wild-type and immune-deficient mice and identified isolates using amplicon sequencing. Microbes promoting specific immune responses in healthy mice raised without specific pathogens were sequenced, and draft genomes were assembled. To test the effect of these isolates on tumor progression, we injected mice ears with B16 melanoma 14 days after colonizing their skin with immunogenic microorganisms. Remarkably, we observed that each microbe had a different effect on tumor progression demonstrating that skin microbiota can impact tumorigenesis and should potentially be considered as a therapeutic target during cancer treatment. Tumor size was associated with an increase of myeloid cells at the tumor site and with commensals that conferred a Th17 response in the tissue. Going forward we will seek to understand the mechanisms by which these microbes and their effect on the immune system impact tumor progression. Furthermore, we will test if the associated skin microbiota can modify treatment efficacy by treating tumor-bearing animals with a characterized immunotherapy and monitoring its effectiveness in mice associated with different immunogenic isolates. Together, these studies will provide an innovative and generalizable approach for dissecting the mechanisms by which the skin microbiota influences host immunity and impact tumor progression and immunotherapy.

NIAID
Nadeene Riddick
Postdoctoral Fellow
Virology - RNA and Retroviruses
SIVagm from African green monkeys can use non-CCR5 entry pathways in vitro and ex vivo
Background: African nonhuman primates (i.e. African green monkeys [AGM] and sooty mangabeys [SM]) are natural hosts of SIV and infection is non-pathogenic in these animals, whereas SIV-infected non-natural hosts, (i.e. rhesus macaques [RM]) exhibit a pathogenic infection. CCR5 has been described as the primary entry coreceptor for SIV in vivo. However, natural hosts exhibit extremely low levels of CCR5 on their CD4 T cells while maintaining robust virus replication. A recent study identified SIV-infected SM that were genetically CCR5-deficient, indicating that some SIV strains can use non-CCR5 pathways in

vivo. We sought to examine CCR5-dependence by SIV derived from another natural host species, the AGM. We also examined the ability of AGM-derived alternative coreceptors, GPR15 and CXCR6, to serve as potential entry coreceptors. Methods: PBMC were infected with SIVagm in the absence or presence of CCR5 antagonist, maraviroc. Viral replication kinetics were determined by measuring reverse transcriptase activity in culture supernatant. In vitro pseudotype infections were conducted in target cells expressing CD4 and various coreceptors. PBMC were stained and sorted using a FACSAria cell sorter, and cDNA synthesized from extracted RNA was used in qPCR assays to determine coreceptor mRNA levels. Results: SIVagm efficiently replicated in AGM and RM PBMC in the absence and presence of maraviroc, suggesting this virus can use non-CCR5 entry pathways in cells isolated from a natural host and a non-natural host. Pseudotype infections revealed that viruses carrying SIVagm envelopes (envs), including transmitted/founder (T/F) envs, used AGM-CXCR6 more efficiently than AGM-CCR5. All of the envs tested modestly used AGM-GPR15. We also found that in addition to CCR5 mRNA, GPR15 and CXCR6 mRNA were detected in AGM CD4 T cells. Overall, coreceptor mRNA levels were higher in the memory CD4 T cell subset than the naïve CD4 T cell subset. Conclusions: These results indicate that SIVagm can use non-CCR5 entry pathways ex vivo, and SIVagm envs, including T/F envs, can use CXCR6 and GPR15 for entry into target cells. Detection of GPR15 and CXCR6 mRNA in AGM CD4 T cells supports the notion that these alternative coreceptors may serve as potential SIV entry coreceptors in vivo. These data suggest that the use of non-CCR5 entry pathways may be a common feature attributed to SIV derived from natural hosts, and may contribute to non-pathogenicity in these animals.

NIAID
Eri Saijo
Visiting Fellow
Neuroscience - Cellular and Molecular

Abstract removed by request of author

NIAID
Karlie Sharma
Postdoctoral Fellow
Immunology - Autoimmune

Graft Versus Host Disease (GvHD) is Attenuated by Administration of Pregnancy Specific Protein 1 (PSG1) Through Induction of Immune Tolerance

Hematopoietic stem cell transplantation is curative for many disorders; however, it can be associated with significant morbidity and mortality, often as a result of graft versus host disease (GvHD). GvHD is an immune mediated reaction in which donor T cells recognize the host antigens as foreign, causing donor T cells to proliferate and attack host tissues. Establishment of a tolerogenic immune environment while preserving immune response to infectious agents is required for successful bone marrow transplantation and GvHD presents a significant obstacle to this. Pregnancy specific glycoprotein 1 (PSG1) is synthesized by the placenta at the onset of pregnancy. PSG1 is essential to maintaining a tolerogenic immune environment to prevent rejection of the fetus by the maternal immune system and serves to maintain the immune system to protect both the mother and fetus from infections. PSG1 has

been shown to increase secretion of transforming growth factor-ß (TGFß), a cytokine essential to suppression of inflammatory T-cells and important for differentiation of tolerance inducing CD4+CD25+FoxP3+ regulatory T cells (Tregs), a cell population important in the prevention of GvHD. We hypothesized that PSG1 could be used to treat or prevent GvHD by inducing immune tolerance. In vitro data using naïve T cells isolated from wildtype mice showed that upon treatment with PSG1, 18% of CD4+CD25+ cells expressed FoxP3 compared to only 2% in untreated controls. When a TGFß inhibitor was added, FoxP3 expression was effectively blocked, further supporting the hypothesis that PSG1 induces expression of FoxP3+ Tregs through regulation of TGFß. Since IL-2, like TGFß, is important for the stability of Tregs in vivo, we removed IL-2 from the cellular media and found that the absence of IL-2 prevented expression of FoxP3 in PSG1 treated cells in vitro, suggesting that PSG1 is dependent on IL-2 as well as TGFß signaling pathways for FoxP3 expression. Using an in vivo murine model of GvHD, we further showed that mice receiving PSG1 had reduced numbers of infiltrating inflammatory CD3+ T cells in the colon and showed a marked improvement histologically over untreated controls. These same PSG1 treated mice also showed expression of FoxP3 in 30% of CD4+CD25+ splenic cells when compared to approximately 7% expression in untreated controls. Combined, this data strongly suggests that PSG1 induces immune tolerance in GvHD and may be a future treatment option for these patients.

NIAID
Troy Sutton
Visiting Fellow
Virology - RNA and Retroviruses

Prophylactic administration of broadly neutralizing monoclonal antibodies CR6261 and CR9114 protect against lethal H2 influenza challenge in mice.

Influenza strains of the H1N1, H2N2, and H3N2 subtypes have caused pandemics in the last century. H2N2 initiated a pandemic in 1957 and circulated until 1968. Given that H2 viruses continue to circulate in wild birds, and that an increasing proportion of the population has no pre-existing immunity, H2 influenza represents an on-going pandemic threat. In the event of a pandemic, anti-viral agents are the primary treatment, however, broadly neutralizing anti-HA stem monoclonal antibodies may also represent an effective prophylactic strategy. The monoclonal antibodies, CR6261 and CR9114, have been shown to protect mice from infection with H1N1 and H5N1, and H1N1, H3N2, and influenza B viruses, respectively. Thus, we evaluated if CR6261 and CR9114 were protective against lethal recombinant A/Ann Arbor/6/1960 (H2N2) [rA/AA] and A/swine/MO/4296424/06 (H2N3) [Sw06] challenge. In vitro neutralization assays showed that CR6261 neutralized both H2 isolates, while CR9114 was only capable of neutralizing Sw06. In mouse studies, Balb/c mice were treated with CR6261 or CR9114 (0, 1.7, 5, or 15 mg/kg via i.p injection), and challenged 24 hours later with 25 MLD50 of rA/AA or Sw06. Both antibodies provided complete protection from lethality at doses of 5 mg/kg or higher, and at the 1.7 mg/kg dose, CR6261 protected against lethality while CR9114 treatment protected 7 of 8 mice. Following challenge, all mice lost weight, with more substantial weight loss observed at lower antibody doses. We hypothesize the in vivo protection of CR9114 despite a lack of in vitro neutralizing activity is the result of complement-mediated activity. Experiments utilizing KA and LALA variants of CR9114 with reduced complement and Fc receptor binding affinity are underway to define the in vivo mechanism of action. Collectively, these studies demonstrate that HA stem antibodies, CR6261 and

CR9114, are effective against H2 influenza strains and are capable of limiting severe H2 influenza infection in mice.

NIAID
Kevin Tosh
Doctoral Candidate
Immunology - Innate and Cell-mediated Host Defenses
Proinflammatory cytokine production by human CD16+ monocytes and CD1c+ dendritic cells in response to Toxoplasma gondii requires the phagocytosis of live parasites
Toxoplasma gondii is a protozoan parasite that infects one-third of the world's population. In the murine model of infection, host resistance to this pathogen requires the induction of a Th1 response that is dependent on IL-12 production by dendritic cells (DC). This innate response in turn is initiated by DC recognition of soluble parasite profilin by Toll-like receptors 11 and 12 (TLR11/12). Although well defined in mice, humans do not express TLR11/12 and no study has systematically evaluated which human innate myeloid cells are capable of detecting and responding to T. gondii. To this end, we exposed primary human CD14+ elutriated monocytes from 42 healthy donors to live T. gondii tachyzoites and found that cells from each individual secreted significant amounts of p40 IL-12 at varying levels. Similarly, DC purified directly from donor blood were also found to be highly responsive. Analysis of 18 different cytokines and chemokines by multiplex assay demonstrated that the cytokine response of primary monocytes and blood DC to T. gondii is highly similar yet distinct from that elicited by LPS. In the murine model, different studies have shown that that the CCR2+ Ly6Chi inflammatory monocyte and CD8alpha+ DC subsets are important sources of IL-12 during infection. In contrast, when purified by FACS, the CD16neg human equivalent of inflammatory monocytes and the CD141+ equivalent of murine CD8alpha+ DCs failed to respond to tachyzoite stimulation while human CD16+ monocytes and CD1c+ DC produced high levels of the cytokine. Also, in direct contrast to the murine system, none of the human cells tested produced IL-12 in response to heat killed parasites, soluble parasite extract (STAg) or live parasites separated by a transwell, indicating that the cytokine response is dependent on physical contact of the cells with live parasites. By pretreating the host cells or parasites with an irreversible inhibitor of actin-polymerization to block phagocytosis or parasite invasion respectively, we demonstrated that parasite invasion is dispensable, while phagocytosis is necessary, for cytokine induction. Together these findings indicate that innate IL-12 induction by T. gondii in humans involves a different pathogen recognition mechanism and distinct myeloid subsets from those utilized in mice.

NIAID
Neeltje van Doremalen
Visiting Fellow
Virology - RNA and Retroviruses
Development of a small animal model for MERS-CoV
The emergence of Middle East respiratory syndrome coronavirus (MERS-CoV) marked the second time a betacoronavirus emerged in the human population, merely a decade after the global pandemic caused by severe acute respiratory syndrome coronavirus (SARS-CoV) infected ~8000 people. Both

coronaviruses are associated with high mortality rates and cause acute respiratory distress. Currently, no clinically approved coronavirus-specific vaccines or antivirals are available and thus the development of either is crucial. Likewise, research on this subject will improve our ability to respond to the next novel emerging coronavirus. Although rhesus macaques and marmosets can be used to study MERS-CoV, inoculation of smaller animals such as mice, hamsters and guinea pigs has not resulted in viral replication. Meanwhile, experiments performed with non-human primates are hampered by costs. Furthermore, studies are conducted only at a small scale. Combined, these facts explain the relatively slow progression within the field. Previously we discovered that differences in the receptor of MERS-CoV, dipeptidyl peptidase 4 (DPP4), between species were responsible for this observed host tropism. We hypothesized that expression of human DPP4 (hDPP4) in non-susceptible hosts, such as mice, would generate a MERS-CoV small animal model. To obtain a transgenic mouse, hDPP4 was cloned into the ROSA26 locus, and hDPP4 expression was confirmed via quantitative polymerase chain reaction (qPCR) as well as flow cytometry. Groups of four hDPP4-expressing mice were inoculated intranasally or intratracheally with MERS-CoV. Three days post inoculation (DPI), all mice had lost 15-25% of body weight accompanied by hunched posture, ruffled fur and increased respiratory rate. Infectious virus was found in the respiratory tract of all mice at 3 DPI, but not in any other examined tissues including brain, liver, spleen, kidney and cervical lymph nodes. Furthermore, viral RNA could be detected in nasal and oral swabs at 1, 2 and 3 DPI. The severe disease observed in this mouse model is a substantial contribution to the MERS-CoV field, and will be instrumental in the assessment of vaccine and antiviral efficacy as well as detailed description of disease mechanisms. The possibility to study MERS-CoV replication and disease in a small animal model ameliorates the need for non-human primates, thereby significantly increasing experimental opportunities.

NIAID
Oliver Voss
Postdoctoral Fellow
Signal Transduction - General
Cd300lb functions as a novel pattern-recognition receptor and amplifies TLR4-lipopolysaccharide-induced lethal endotoxemia.
Sepsis, which commonly originates from a severe gram-negative bacteria infection, initiates a systemic immune response causing a pro-inflammatory cytokine storm that results in multi-organ dysfunction and, subsequently, death. Immune response initiation requires pattern-recognition receptors (PRRs) on immune cells, such as TLR4, which recognize lipopolysaccharide (LPS), a component found on the outer membrane of bacteria. Recently, additional immune receptors have been implicated in the initiation of the innate immune response to bacterial infection, including Cd300lb (CLM5). Yet, the precise mechanism by which Cd300lb regulates the immune response remains unknown. The goal of our study is to define the Cd300lb ligand involved in bacterial recognition and the mechanism(s) responsible for the Cd300lb-mediated immune response. Here, we identify LPS as a ligand for Cd300lb, and show that LPS treatment promotes the binding of Cd300lb to TLR4. Consistently, LPS-treated bone marrow-derived macrophages (BMDM) from wild-type (WT) mice produce higher levels of pro-inflammatory cytokines (TNFalpha, IL-6 and INFgamma) when compared with Cd300lb-/- BMDM. We show that Cd300lb-/- mice are less susceptible to LPS-induced lethal inflammation, and demonstrate an increased serum level of

the anti-inflammatory cytokine, IL-10. Furthermore, neutralization of endogenous IL-10 in Cd300lb-/- mice, prior to LPS treatment, leads to a substantial upregulation of TNFalpha, IL-6 and INFgamma serum levels, and diminishes their survival advantage over WT mice. Depletion of macrophages, but not neutrophils or NK cells, decreases the survival rate of Cd300lb-/- mice. Moreover, adoptive transfer of Cd300lb-/- BMDM, but not those from WT mice, improves the survival of WT and Cd300lb-/- mice, suggesting that macrophage-derived IL-10 promotes the survival of Cd300lb-/- mice. Indeed, compared to WT BMDM, Cd300lb-/- BMDM produce higher levels of IL-10 after LPS treatment. We further demonstrate that the increase in IL-10 production in Cd300lb-/- BMDM requires the Syk-MEK1/2-ERK1/2 signaling axis and is independent of p38-MAPK activation. Collectively, these findings describe a previously unappreciated dynamic regulatory network by which Cd300lb acts as a potential PRR to synergistically amplify TLR4-LPS-induced lethal endotoxemia, and identifies key nodes for a future clinical intervention in inflammatory diseases like sepsis.

NIAID
Chao Zhong
Postdoctoral Fellow
Immunology - General
GATA-3 regulates the development of ILC3 at multiple stages
Innate lymphoid cells (ILCs), mainly enriched in mucosal tissues, are emerging lymphocytes that maintain the homeostasis between the host and commensals, and play essential roles during secondary lymphoid organogenesis and innate immune responses to various infections. Mature ILCs, similar to their adaptive counterparts of CD4 T helper cells, can be divided into three major subsets based on their signature cytokine production and selective expression of master transcriptional regulators, T-bet, GATA-3 and ROR?t. However, how the development and functions of these subsets are precisely regulated, especially by transcription factors including GATA3 that are expressed at various levels, is still elusive. We found that in addition to its critical role in the development and maintenance of ILC2s, GATA-3 is also required for the development of other IL-7Ra+ ILCs including ILC3s. Our results also showed that GATA-3 is continuously expressed by all ILC3 subsets and has crucial regulatory functions at multiple stages during the development of ILC3 subsets. Deletion of Gata3 gene after ROR?t expression by Rorc-Cre resulted in a significant reduction of ILC3s, especially for the NKR+ ILC3 subset. Genome wide analysis of gene expression in the wild type and Gata3-deficient ILC3s revealed that the expression of Il7r and a number of genes associated with the functions of ILC3s was dramatically reduced in the absence of GATA-3. Thus, our results shed light on the regulatory function of GATA-3 at multiple developmental stages of ILC3 subsets.

NIAID-VRC
Nami Iwamoto
Visiting Fellow
HIV and AIDS Research
Development of simian immunodeficiency (SIV)-specific chimeric antigen receptor (CAR) for evaluation in non-human primate AIDS model
HIV is a chronic infectious disease, and elimination of viral reservoir is required for cure from HIV

infection. Cytotoxic T lymphocytes (CTL) have a potential to eliminate viral reservoir, but virus prevents antigen presentation to CTL by down-regulating human leucocyte antigen (HLA). In addition, epitope recognition by CTL is determined by HLA genotype and inducing CTL effective for viral control by vaccination has not succeeded. We are applying CAR (Chimeric Antibody Receptor) technology developed for cancer therapy, to HIV therapy. The advantages of CAR in HIV therapy are direct recognition of target molecules on cell surface with no restriction by genetic background. The CAR contains a membrane bound chain of a variable antibody fragment (scFv) coupled to intracellular domains of CD137 and CD3 zeta chain to transduce signals necessary for target killing and in vivo persistence after target recognition. We have cloned SIV-specific neutralizing antibodies (NAb) with different specificity to SIV envelope (Env). We selected scFv specific to CD4 binding site (CD4bs) and variable region (V)1/2 for introduction into CARs.. After SIV-CAR transduction to isolated rhesus PBMC, CAR+ cells were sorted and in vitro viral suppressive efficacy was evaluated by co-culture with SIV-infected cells. Tier 1 and 2 SIVsmE660 viruses were used as targets in this assay. In neutralization assay, tier 1 virus infection is completely blocked, but tier 2 virus is only partially neutralized at most by our NAb. However, SIV-CAR+ cells showed 100% suppression on both tier 1 and 2 virus replication. This result shows that binding to (but not neutralization of) Env is important for viral suppression by CAR; thus CAR has the possibility to eliminate various HIV regardless of neutralization sensitivity. We also found that V1/V2-specific CAR showed more potent viral suppression than CD4bs-specific CAR. We recently succeeded making dual CAR transduced cells that express two SIV-CAR with different Env specificities. Dual expression did not have any negative effect and showed as potent in vitro viral suppression as single CARs. in vivo, dual CAR transfected CAR clones might detect infected cells more sensitively, and escape from dual CAR control should be harder. Initial in vivo experiments show persistence and expansion of CAR+ cells in NHP. Our result indicates potential for elimination of virus-infected cells by CAR as HIV therapy and binding to Env is important for CAR design.

NIAID-VRC
April Killikelly
Postdoctoral Fellow
Biochemistry - Proteins
Pre-fusion F glycoprotein is not displayed on the surface of formalin-inactivated respiratory syncytial virus
Respiratory syncytial virus (RSV) infects all children by 2-3 years of age, and is the leading cause of hospitalization in children under 5. RSV infection is a primary cause of mortality in children <1 year in developing countries and can also cause serious disease with excess mortality in the elderly. Passive administration of a commercial monoclonal antibody (mAb) reduces hospitalization rates, but is only available to high-risk infants. A safe and effective vaccine that could be made widely available would make a large impact on public health, however many vaccine candidates have been unsuccessful to date. In a vaccine trial done in 1969 using formalin-inactivated RSV (FI-RSV) to immunize children aged 2-7 months, more frequent and severe illness was induced in vaccinees, leading to two deaths. Further analysis shows immunization with FI-RSV induces high titers of antibodies with low neutralization activity. A major antibody target is the RSV surface glycoprotein (F) that triggers fusion between the viral and host membranes. Atomic level resolution structures have been solved of F in two distinct

conformations: pre- and post-triggered. Despite RSV virions presenting a combination of pre- and post-F conformations on their surface, antibodies targeting pre-F account for >90% of neutralization activity. To determine the composition of pre- and post-F on FI-RSV relative to live virus, we used a non-denaturing and non-reducing dot blot. To detect different F conformations we used a series of monoclonal antibodies targeting pre-F, post-F or both conformations. We found that antibodies targeting epitopes only present on pre-F do not react with FI-RSV. FI-RSV is produced by incubating RSV with formalin at 37C. RSV incubated with formalin for 24hrs is still reactive with pre-F specific antibodies; however, RSV incubated with formalin for 72hrs looses its ability to react with pre-F specific antibodies. As a control we also demonstrated that pre-F retained the ability to react with pre-F specific antibodies after incubation with formalin for 96hrs. These data indicate that pre-F present on the surface of RSV transforms into a post-F conformation by incubation with formalin at 37C for 72hrs. This novel finding suggests that a pre-F immunogen would induce a distinct immune response from the vaccine-enhanced illness observed in the 1969 FI-RSV vaccine trials.

NIAID-VRC
Syed Moin
Visiting Fellow
Signal Transduction - General
Wnt/ Beta-catenin pathway negatively regulates RSV replication
The Wnt/beta-catenin pathway is involved in diverse physiologic cellular functions governing development and various human diseases. However, there are few reports on its potential role in viral replication and propagation. We have studied the Wnt pathway in context of human respiratory syncytial virus (RSV), an important respiratory pathogen that causes significant lower respiratory tract disease worldwide. We found that the Wnt pathway regulates RSV at multiple levels. Screening a panel of 59 human cell lines (NCI-60) for RSV permissivity revealed that non-permissive cells (34%) had significantly higher levels of beta-catenin than permissive cells (66%). This was confirmed in beta-catenin knockout cells H28 that showed over 8-fold increase in infection over its parent H661. No significant difference was observed between viral binding and entry in permissive and non-permissive cells as revealed by their respective assays. We attributed this to intracellular signaling events that increased full-length genomic RNA synthesis and replication. The GSK-3beta independent beta-catenin inhibitor, cardamonin, reversed the infectivity in non-permissible cells resulting in over 12-fold increase in viral replication while having a more subtle effect on its permissive counterparts (2-fold). This increase was dose dependent and negatively correlated with beta-catenin expression levels. In immunofluorescence assays, a higher number of replication complexes were seen in response to cardamonin except in beta-catenin deleted H28 cells. A qPCR based genome quantitation also showed significantly higher replication activity (>5 fold) with cardamonin that could be reversed by beta-catenin stabilization via the proteasomal inhibitor MG132 (>3 fold). Similar inhibitory effects were observed in a luciferase reporter based RSV minigenome assay. An siRNA mediated beta-catenin knockdown in a non-permissive lung cell line, H226 resulted in significantly increased RSV infectivity. Lithium chloride, a GSK-3beta inhibitor that mediates beta-catenin accumulation diminished RSV infection in H661 cells but had very limited effect on H28 cells. Here, we demonstrate that Wnt pathway negatively regulates RSV replication at multiple levels without affecting virus attachment or entry. Finding that elements of the Wnt/beta-catenin

pathway negatively regulate RSV replication identifies novel therapeutic targets, and may provide new insights into the biological properties and pathogenesis of RSV.

NIAMS
Carolyne Smith
Doctoral Candidate
Immunology - Autoimmune
Lupus oxidized high density lipoprotein promotes macrophage inflammation by blocking ATF3 activity
Systemic lupus erythematosus (SLE) is an autoimmune disease of unclear etiology that primarily affects women. While the life expectancy in lupus has significantly improved over the last several decades, cardiovascular disease (CVD) is still a leading cause of death in these patients. Indeed, SLE patients are fifty times more likely to experience a heart attack or stroke compared to age- and sex-matched controls. High density lipoprotein (HDL) cholesterol is believed to be beneficial for cardiovascular health and has been associated with anti-inflammatory responses within macrophages. When HDL is oxidatively modified (oxHDL) however, it is linked to pronounced CVD risk. We previously demonstrated that SLE patients have higher levels of plasma oxHDL than healthy donors, but we now sought to examine whether SLE oxHDL mediates aberrant inflammatory responses in macrophages. To do so, we exposed healthy macrophages to no HDL, SLE oxHDL, or healthy HDL. Affymetrix gene expression analysis was performed and demonstrated that, compared to macrophages exposed to healthy HDL, SLE oxHDL increased the levels of mRNA for the inflammatory cytokines IL-6, IL-12B and TNF-alpha. This induction was confirmed by RT-qPCR and ELISA analysis. As the transcription factor ATF3 is a known negative regulator of these cytokines, we assess whether ATF3 expression or nuclear localization was effected by SLE oxHDL. RT-qPCR and confocal microscopy revealed that while healthy HDL induces ATF3 expression and translocation to the nucleus, SLE oxHDL impeded ATF3 expression and nuclear translocation. This activity was restored if a receptor known to bind oxidized lipoproteins, LOX-1, was inhibited with blocking antibodies. Together, these findings show that when SLE oxHDL binds to macrophage LOX-1 receptor, it prevents ATF3 activation. Without ATF3 activity in the nucleus, the macrophages become extremely inflammatory and may become detrimental to cardiovascular health. As such, therapies which target HDL oxidation in SLE may prove beneficial for the prognosis of SLE patients.

NIAMS
Vivek Thumbigere-Math
Research Fellow
Clinical and Translational Research
A Novel Interferon Regulatory Factor-8 (IRF8) Mutation Associated With Idiopathic Tooth Root Resorption
Periodontal disease, an immune-mediated inflammatory response to oral microbiome, affects nearly 50% of adults worldwide, resulting in a significant socioeconomic burden and public health concern. Currently, molecular mechanisms driving the inflammatory-mediated tissue destruction in periodontal disease are unclear including a pathology termed multiple idiopathic root resorption, thereby affecting development of targeted therapeutic interventions. Following IRB approval from University of Detroit

Mercy and NIH, dental/medical histories, x-rays, and saliva samples were collected from a kindred (3 affected and 3 unaffected members) presenting with idiopathic root resorption. The proband, affected son, and daughter exhibited severe root resorption affecting multiple teeth, and subsequent tooth loss, but no other significant medical history. Micro-CT on exfoliated teeth revealed a unique pattern of severe cervical root resorption distinct from tooth decay and other diseases. Whole exome sequencing revealed an autosomal dominant missense mutation in interferon regulatory factor-8 (IRF8). IRF8, a transcription factor expressed in immune cells including monocytes/macrophages, B/T-lymphocytes, is a key regulator of myeloid cell differentiation, inflammatory response, and osteoclast activation, thereby negatively influencing bone resorption. The identified amino acid change (G388S) in IRF8 was localized to a highly conserved C-terminal motif, leading to altered serine phosphorylation motifs and phosphoserine binding domains, and is predicted to cause a large shift in 3D protein folding. These data strongly support that G388S mutation impairs heterodimerization with other transcription factors, altering osteoclast regulation, effectively producing overactive osteoclasts that target root structures. Irf8-/- mice exhibited increased numbers of osteoclasts and alveolar bone loss, suggesting that IRF8 deficiency has a broader impact on the periodontia. To further understand IRF8 functions, we are using WT and mutated IRF8(G388S) vectors to define protein-protein/DNA-protein interactions, osteoclast differentiation/activity, and inflammatory cytokine production in vitro, which will provide valuable information in generating IRF8(G388S) mice, and delineating the role of IRF8(G388S) mutation in vivo. In conclusion, our results indicate that IRF8 plays a critical role in modulating periodontal tissue destruction, specifically multiple idiopathic root resorption.

NIBIB
PANAGIOTIS CHANDRIS
Research Fellow
Biochemistry - General and Lipids
Dissecting localization and dynamics of key players in the de novo sphingolipid synthesis pathway using bimolecular fluorescence complementation system and super resolution microscopy
Sphingolipids constitute an essential family of membrane and bioactive lipids that have roles in diverse cellular functions such as cell proliferation, growth arrest, apoptosis and migration. Their metabolism is comprised of two major axes: the de novo pathway and the salvage/recycling pathway. Serine palmitoyltransferase (SPT) is a key enzyme in the de novo pathway of sphingolipid synthesis, as it catalyzes the initiation or commitment step. Mammalian SPT consists of at least three subunits: LCB1, LCB2 (a or b), and a small subunit (Sptssa or Sptssb) that has catalytic activity. Regulation of the holoenzyme especially in mammalian systems remains an unsolved puzzle, although recent data point to a family of modulatory proteins, the ORMDLs, as negative regulators of SPT. Although it is known that de novo sphingolipid synthesis takes place in the ER there is no information regarding dynamics and live monitoring of the key players. We dissected the dynamics of these key players individually but also as a cohort by live cell imaging using fluorescent chimeric proteins and fluorescence recovery after photobleaching (FRAP). Furthermore, by setting up a novel bimolecular fluorescent complementation system, we found evidence for specific bimolecular interaction between SPT isoforms and ORMDLs. We also monitored the sensitivity of ORMDL isoforms to distinguish between active and catalytically dead SPT mutants, by flow cytometry and and co-immunoprecipitation experiments. Finally, we employed

super resolution microscopy (both structured illumination microscopy and single-molecule imaging) both in fixed and live specimens to map the distribution and organization of ORMDLs and SPT molecules and to follow their nanoscale dynamics. By visualizing the spatiotemporal orchestration of SPT subunits and modulators in the endoplasmic reticulum milieu employing super resolution microscopy we present evidence for clustered organization of SPT and ORMDLs into the ER with a possible sequestration of SPT-ORMDL complexes juxtaposed to ER exit sites.

NIBIB
Ryan Christensen
Postdoctoral Fellow
Informatics/Computational Biology
A four dimensional analysis suite for examining Caenorhabditis elegans embryonic neurodevelopment in toto.
Despite identification of many key neurodevelopmental mechanisms acting on the level of individual cells or simple, defined circuits, a systems-level understanding of the dynamics of nervous system assembly remains limited. Examining development of simple nervous systems, like that of Caenorhabditis elegans, offers one way of bridging the gap between identified mechanisms and larger-scale, systems-level development. The small number of neurons in the nematode and its optical transparency raises the possibility that the development of each of these cells could be followed in intact animals, enabling understanding of the morphological events underpinning the development of an entire nervous system. The vast majority of C. elegans neurons (222) are born during embryogenesis, but study of embryonic neurodevelopmental events has proven difficult due to changes in embryo morphology and muscular activity which cause rapid twisting and movement, interfering with imaging and analysis of developmental events during this time period. We have developed a computer algorithm and image visualization software which allows a user to computationally straighten images of twisted-up embryos, obviating problems associated with embryo movement and greatly simplifying the analysis of developmental events in moving embryos. We have also added the capability to track the position of user-defined cells or structures over time, allowing for quantitative analysis of cell movement, growth and shape change. We have used this annotation capability to track overall morphological change in five embryos, and have found that changes in overall morphology are remarkably stereotyped, with variance in the position of epidermal cells limited to a couple of cell diameters throughout development. We have also tracked the movements of three pairs of neurons, and noted a clear difference in positional variability between actively-migrating and non-actively-migrating neurons. Non-migrating neurons showed low positional variability, similar in magnitude to epidermal cells, while actively migrating neurons showed high variability along the anterior-posterior axis of migration. We plan to extend our tracking of neuronal cell position and neurite outgrowth to all 222 neurons developing during embryogenesis, providing the first comprehensive morphological look at how an entire nervous system develops.

NIBIB
Peng Huang
Postdoctoral Fellow

Radiology/Imaging/PET and Neuroimaging

Magnetic melanin nanoparticles as activatable theranostics for PET/MRI/PA tri-modal imaging guided photothermal therapy

Background: The separation of tumor diagnosis and treatment is one of major issues in the clinic. Use of activatable theranostics for the combination of diagnosis and treatment of cancer can provide the direct evidence to early diagnosis, occurrence and development progresses of cancer, and also enables online imaging of drug for the detection of cancer, image-guided drug delivery and treatments, guidance of surgical resection, and monitoring of treatment response. Herein, we report a radionuclide 64Cu labeled magnetic melanin nanopaticles (64Cu-MMNPs) for positron emission tomography (PET), magnetic resonance imaging (MRI), and photoacoustic (PA) tri-modal imaging guided photothermal therapy (PTT) of U87MG glioblastoma cancer. Methods: MMNPs were synthesized by biomimetic synthesis method using bioploymer melanin as the biotemplate. The radionuclide 64Cu was labeled with MMNPs by the high affinity of metal ions of melanin. PET, MRI, and PA imaging were carried out on U87MG tumor-bearing mice. PTT was conducted both in vitro and in vivo. Results: MMNPs were successfully synthesized by biomimetic synthesis method. The size of MMNPs is ~15 nm. The r2 value of MMNPs is 163 mM (superscript-1) s (superscript-1), which is much higher than spherical magnetic nanoparticles (16 nm, r2=125.86 +/- 9 mM (superscript-1) s (superscript-1); and 10 nm, r2=59.91 +/- 6 mM (superscript-1) s (superscript-1)). MMNPs were successfully radiolabeled with 64Cu and purified to give radiochemical yield ~100%. 64Cu-MMNPs were stable in PBS and mouse serum up to 24 h. In vivo PET imaging showed high tumor uptake of MMNPs after intravenous injection (150 muCi, 9.5%ID/g, 24 h). MRI and PA imaging also showed high tumor uptake of MMNPs. Afterwards, upon localized laser irradiation (808 nm, 0.5 W/cm (superscript2), 5 min), 100% tumor elimination was achieved in MMNP treatment group (10 mg/kg of MMNPs). Conclusion: MMNPs showed great potential as cancer photoactivatable theranostics with PET/MRI/PA multi-modality imaging capability and potent PTT effect.

NICHD

Anush Arakelyan

Research Fellow

HIV and AIDS Research

Characterization of Envs on individual HIV particles by flow virometry.

HIV remains one of the most variable viruses due to its high mutation rate, in particular in the envelope encoded by env. As a result, these quick changes allow the virus to evade the host immune responses that target predominantly viral surface proteins. HIV envelope plays a critical role in HIV infection as only in the right conformation of Env HIV is able to bind to cell receptors/coreceptors and to fuse with the plasma membrane. Functional HIV envelope proteins are organized in spikes consisted of trimers of three external gp120 subunits and three trans-membrane gp41 subunits that are linked to each other via non covalent bonds. This functional conformation of spikes may be spoiled in a number of ways rendering them dysfunctional: in particular Env can remain uncleaved, form dimeres or monomeres or lost leaving behind the “gp120 stumps”. Since on each virion there are 13 or 14 spikes, it was assumed that the majority of virions is mosaic and carry both functional and non-functional spikes. Understanding the extend of this mosaicism is important both for understanding of the basic mechanisms of HIV infection and for development of new therapeutic and prevention strategies, in particular vaccines. To

answer this question it is necessary to analyze the conformation of individual Env proteins on individual virions. We developed a new nanoparticle-based technique, “flow virometry”, that permits the analysis of antigenicity of individual viral particles. We used flow virometry and a panel of antibodies that discriminate between various gp120 conformations to investigate the conformation of Envs on individual virions. In contrast to what was generally accepted regarding the mosaicism of HIV virions our results demonstrate that on the majority of virions in a HIV preparation there are either only functional trimeric Envs or predominantly defective Envs. Only a small subfraction of the virions that carry defective spikes was mosaic with virions carrying both functional and nonfunctional Envs. Contribution of this minor fraction in HIV infection of human tissue ex vivo seems to be small. The results of our study suggest that spikes may not be formed independently and our approach can be used to describe mosaicicsm of HIV-1 in plasma of individual patients thus determining individual treatment.

NICHD
Brad Busse
Postdoctoral Fellow
Biophysics

Introducing Resin Embedded Multicycle Imaging (REMI) of the Plasma Membrane

Although the plasma membrane and its associated protein complexes are involved in critical cellular functions, including orchestrating cell-cell interactions and cellular information transduction, current imaging techniques are not able to image multiple rounds of protein identification on the same sample. This shortcoming limits the ability to multiplex protein labels. To resolve this limitation, we utilize resin-embedded multicycle imaging (REMI), which stabilizes protein structure and antigenicity in acrylic resin, allowing labels to be removed and replaced. This enables imaging an arbitrarily large number of proteins of interest on the same specimen. My first implementation of this technology, array tomography, has proven of great benefit to uncovering the heterogeneity of synapses in the brain. Studies involving large, distributed organelles or spatially restricted volumes of interest such as the plasma membrane, however, rapidly encounter increased complexity in imaging and analysis. We have now developed a series of novel preparative methods to address the problem of imaging multiple proteins in larger areas of the plasma membrane of individual cells. After affixing cells to glass-bottomed dishes, we can embed whole cells, or isolate their basal membranes by applying intracellular media with sufficient shear to remove the bulk of the protoplasm. We then fix and embed these cells and membranes in a hydrophilic resin directly on the cover slip in an oxygen-free environment, forming an ultra-thin resin sheet similar to a cut section of a resin block prepared for electron microscopy. These techniques result in a stable, thin layer in which the plasma membrane's epitopes are trapped in-situ. Any barriers to the free diffusion of antibodies appears to be minimal, allowing for immuno-histochemical labeling of proteins. Furthermore, we have identified conditions for removing antibodies after imaging, followed by relabeling - multiplexed imaging. With straightforward computational techniques for aligning sequential images of the same membrane sheet, we can repeat this process as many times as needed. Moreover, we can utilize super-resolution imaging techniques to determine colocalization of proteins. We are using our new technology of REMI on human b-cells, mouse fibroblasts, and human adipocytes, where a novel 'signalosome' appears to mediate response to insulin.

NICHD

Yu Chen

Research Fellow

Intracellular Trafficking

Identification of EARP (Endosome-Associated Recycling Protein), a novel multisubunit tethering complex involved in endocytic recycling

Continuous transfer of material between organelles of the endomembrane system is crucial to maintain the overall metabolic balance of the cell. This transfer is mediated by transport vesicles that bud from a donor compartment and fuse with an acceptor compartment. The first physical link between a transport vesicle and its acceptor compartment is established by tethering factors. Previous work showed that the multisubunit complex GARP (Golgi-Associated Retrograde Protein), composed of Ang2, Vps52, Vps53 and Vps54 subunits, functions as a tethering factor in retrograde transport from endosomes to the trans-Golgi network (TGN). A mutation in Vps54 is the cause of spinal muscular atrophy in the "wobbler" mouse, whereas mutations in Vps53 cause progressive cerebello-cerebral atrophy type 2 in humans. In the current study, we found that a previously uncharacterized protein named "Syndetin" assembles with Ang2, Vps52 and Vps53 – but not Vps54 – to form another complex named "EARP" (Endosome-Associated Recycling Protein). Syndetin was identified as a top hit by mass spectrometry following tandem-affinity purification using Ang2, Vps52 and Vps53 as baits. Reciprocally, Ang2, Vps52 and Vps53 - but not Vps54 - were identified when Syndetin was used as the bait. Co-immunoprecipitation confirmed interactions of Syndetin with all the GARP subunits except for Vps54. The molecular mass of EARP calculated from sedimentation velocity and gel filtration analysis indicated this complex is a 1:1:1:1 heterotetramer. In contrast to the TGN localization of Vps54 (i.e., GARP), co-localization analysis placed Syndetin (i.e., EARP) at Rab4-positive recycling endosomes. To establish the function of EARP, we depleted Syndetin in HeLa cells using siRNA and examined endocytic recycling and retrograde transport. Fluorescent transferrin pulse-chase analysis revealed a dramatic delay in the recycling of endocytosed transferrin receptor back to the cell surface in Syndetin-knockdown cells as compared to control cells transfected with scrambled siRNA. In contrast, Syndetin depletion had no effect on retrograde trafficking of fluorescently-labeled Shiga toxin B subunit to the Golgi complex, for which GARP was shown to be essential. These studies thus identified EARP as a novel multisubunit tethering complex specifically involved in endocytic recycling. Moreover, our findings shed light on the pathogenesis of neurodegenerative disorders caused by mutations in GARP and/or EARP subunits.

NICHD

Yong Cheng

Postdoctoral Fellow

Neuroscience - Neurodegeneration and Neurological disorders

A Mutation in the NF-a1 Gene Linked to Neurodegeneration and Alzheimer's Disease

Neurotrophic Factor-a1 (NF-a1), a newly discovered neurotrophic factor, previously known as Carboxypeptidase E, has been reported to be involved in neuroprotection, neurodegeneration and depression. The neuroprotective role of NF-a1 prompted us to search for possible mutations in the human NF-a1 gene that might be linked to human neurodegenerative diseases. We searched GeneBank EST database and identified an EST sequence entry from Alzheimer cortex tissue that had three

adenosine inserts. This introduces 9 amino acids in the first beta-pleated sheet after the pro-domain of the mutant protein, herein called QQ NF-a1 due to the presence of two glutamine residues in the new sequence. Two other ESTs were also identified that are predicted to give rise to insertion of the same amino acids in the NF-a1 protein. Expression studies of QQ NF-a1 demonstrated that it was made but failed to be secreted in neuro2a cells. It accumulated in the ER where it was targeted for degradation by the proteasome and lysosome in neuro2a cells. Co-expression of WT (wide type) and QQ NF-a1 in neuro2a cells resulted in the degradation of both forms of the protein and reduction in the secretion of WT NF-a1, indicating that the mutant was acting in a dominant recessive manner. Overexpression studies of QQ NF-a1 by adenovirus transduction in rat primary cultured hippocampal and cortical neurons resulted in increased levels of ER stress marker CHOP (n=4, t-test, p<0.05) and decreased levels of the pro-survival protein, Bcl-2 (n=4, t-test, p<0.01) in addition to increased neuronal cell death as measured by LDH release (n=10, t-test, p<0.001) and MTT (n=10, t-test, p<0.001) assays. Moreover, over-expression of Bcl-2 rescued the cell death induced by QQ NF-a1 in the hippocampal neurons (n=5, p<0.05, t-test). We then made QQ NF-a1 transgenic mice and found that QQ NF-a1 transgenic mice had memory deficits compared to WT mice as tested by Morris water maze (n=8, Two-way ANOVA test, p<0.05). Furthermore, the mutant mice showed more active Caspase-3 expression in the CA2 and CA3 regions of hippocampus as measured by immunofluorescence (n=4, t-test, p<0.05), indicative of neurodegeneration. We also found amyloid beta deposition in hippocampus of QQ NF-a1 transgenic mice (n=4), suggesting that this mutation of NF-a1 could lead to Alzheimer's disease. Taken together, we demonstrated that a mutation in the NF-a1 gene can cause neurodegeneration linked to Alzheimer's disease.

NICHD
Sarah Cohen
Visiting Fellow
Intracellular Trafficking
Mitochondrial fusion promotes fatty acid oxidation during starvation
In order to survive, cells must have the ability to shift their metabolism in response to changing conditions. Cells store energy as triglycerides, within organelles called lipid droplets (LDs). In response to nutrient deprivation, fatty acids (FAs) are released from triglycerides by lipases, and oxidized within mitochondria in order to provide the cell with energy. It has previously been shown that mitochondria fuse into a highly tubulated continuous network in response to starvation, but whether this tubulation plays a role in the metabolism of FAs is unclear. To address this question we directly visualized FA trafficking in live cells using a fluorescent FA analogue (Red C12). Spinning-disk microscopy and thin-layer chromatography revealed that Red C12 accumulated in triglycerides within LDs in well-fed cells, but moved from LDs into mitochondria and was metabolized when cells were starved. LDs in starved cells were closely apposed to mitochondria, suggesting that FA transfer may occur at interorganellar contact sites. In addition, we found that in starved wild type (WT) mouse embryonic fibroblast cells, Red C12 was homogeneously distributed throughout the mitochondrial network. In contrast, in Mfn1 knockout (Mfn1KO) or Opa1 knockout (Opa1KO) cells – which have impaired mitochondrial fusion – FAs did not become homogenously distributed throughout mitochondria, resulting in either very low or exceedingly high FA concentrations within individual mitochondrial elements. Together, these data

suggest that in response to starvation, FAs are transferred from LDs to mitochondria at sites of close contact, and that mitochondrial fusion functions to equilibrate FAs throughout the network and maximize FA availability for beta-oxidation reactions. Indeed, mitochondrial oxygen consumption measurements revealed that Mfn1KO and Opa1KO cells were unable to sustain increased beta-oxidation in response to starvation. This inability to efficiently metabolize FAs had drastic consequences: Mfn1KO and Opa1KO cells both stored more FAs within LDs, and effluxed FAs from the cell. A co-culture assay in which "donor" cells pulsed with Red C12 were incubated with green-labeled "acceptor" cells revealed that acceptor cells cultured with Mfn1KO or Opa1KO donor cells were exposed to higher levels of FAs than acceptor cells cultured with WT donor cells. Thus, disregulation of mitochondrial morphology has metabolic consequences not only for the cell itself, but also for its neighbors.

NICHD
Marie Reine Haddad
Visiting Fellow
Neuroscience - Neurodegeneration and Neurological disorders
AAV-mediated gene therapy rescues a mouse model of Menkes disease
Menkes disease is a lethal infantile neurodegenerative disorder of copper metabolism caused by mutations in a P-type ATPase, ATP7A. Untreated patients die by 3 years of age. Currently, the only available treatment is daily subcutaneous copper injections. This treatment, however, is not effective in the majority (75%) of affected individuals, who would benefit from a gene therapy regimen. The mottled-brindled (mo-br) mouse recapitulates the Menkes phenotype and the mutants die by 14 days (D) of age. To develop a treatment, we designed Adeno-Associated Virus (AAV) vectors carrying human ATP7A cDNA. We previously demonstrated that a gene therapy approach using AAV serotype 5 (AAV5) in combination with copper both given into the brain's lateral ventricles (ICV) was able to significantly prolong the survival of the mutant mice, compared to untreated mutants. However, subtle abnormalities in growth and neuromotor function persisted in long-term surviving mice. In this study, we report far superior outcomes using AAV9 given ICV at D2 in combination with subcutaneous injections of clinical grade copper histidine (n=40 mice) given at D4 to 6. We employed AAV9 and –rh10 vectors given their broad tropism (neurons and glial cells) compared to AAV5. We assessed single treatments with either AAV alone or copper alone and none were able to significantly enhance the survival. We used 3 different AAV doses: high (1.6e10 vg), intermediate (5.0e9 vg) and low (1.6e9 vg) in combination with Cu; our intermediate and high AAV9-ATP7A doses were most effective. In correlation with the best survival benefit, cerebrospinal fluid (CSF)-directed AAV9 with subcutaneous Cu resulted in superior murine neurobehavioral outcomes and growth compared to AAVrh10 and to our previous results with AAV5 serotype. In addition, this synergistic treatment effect with high dose AAV9 and Cu showed markedly improved brain neurochemical and brain copper levels in comparison to untreated mutants and correlated with viral genome copies. Copper measurements in combination-treated mutants (d12) were not statistically different in comparison to wild type littermates suggesting a restored copper transport to the brain. Our findings provide definitive evidence that gene therapy may have clinical utility in the treatment of Menkes disease. Based on our combined data, we propose recombinant AAV9 as a serotype of choice for first-in-human intrathecal administration in Menkes patients.

NICHD

Joo Yun Jun

Postdoctoral Fellow

Physiology

Role for the Melanocortin 3 Receptor in determining mesenchymal stem cell fate

The human melanocortin 3 receptor (MC3R) is known to play an important role in regulating appetite, food intake, and energy expenditure, possibly playing a pivotal role in human metabolic function. However, the cellular mechanisms by which insufficient MC3R signaling causes childhood obesity phenotypes are still unclear. To characterize these mutations in association with metabolic function, we generated homozygous knock-in mouse models replacing murine Mc3r with wild type human MC3R (MC3RhWT/hWT) and a double mutant (C17A + G241A) hypofunctioning human MC3R (MC3RhDM/hDM) that we found was associated with pediatric obesity. On both chow and high-fat diets, MC3RhDM/hDM had significantly greater body weight and fat mass but less fat-free mass compared to MC3RhWT/hWT or C57BL/6 mice even at an early age (by 7 weeks). Dual-energy x-ray absorptiometry showed MC3RhDM/hDM mice had significantly lower bone area and bone mineral contents compared to MC3RhWT/hWT. Therefore, we hypothesized that multipotent mesenchymal stem cells (MSCs) from MC3RhDM/hDM mice are predisposed towards adipocyte differentiation and away from osteoblast differentiation. Bone-derived MSCs were cultured and differentiated into either adipocytes or osteoblasts, and the accumulation of triglycerides for adipocytes and calcium for osteoblasts were measured by specific staining (Oil Red O and Alizarin Red S, respectively). Oil red O staining showed MC3RhDM/hDM MSCs had ~40% increased capacity to differentiate into triglyceride-storing adipocytes than MC3RhWT/hWT MSCs, and mRNA of adipocyte markers were correspondingly increased. On the other hand, Alizarin red S staining showed that MC3RhDM/hDM MSCs have ~50% less capacity to differentiate into osteoblasts than MC3RhWT/hWT MSCs, and mRNA of osteoblast markers were correspondingly reduced. Similar data were found for mice with Mc3r knockout. These data suggest that MC3R activity may regulate MSC fate, with low activity facilitating differentiation into lipid storing cells. These findings are the first to identify a role for MC3R in differentiation and suggest a novel mechanism through which MC3R signaling regulates energy homeostasis and metabolism.

NICHD

Byoungkwan Kim

Visiting Fellow

Microbiology and Antimicrobials

Identifying virulence-critical Legionella pneumophila effectors using CRISPR/dCas9-mediated gene interrogation

The bacterium L. pneumophila is the causative agent of a severe pneumonia called Legionnaires' disease. During infection, L. pneumophila modifies host cell processes by injecting almost 300 effector proteins into the host cytosol. One question that has continued to puzzle the field is which of the 300 effectors critically contribute to the infection of human cells as opposed to effectors that only play a role in amoeba, the natural host of Legionella. Randomized screening approaches such as transposon or chemical mutagenesis were mostly unsuccessful in addressing this issue due to a high level of functional redundancy among Legionella effectors. We now established a novel genetic tool for the simultaneous

deactivation of several effector-encoding genes by adapting the Streptococcus pyogenes CRISPR/dCas9 gene silencing (CRISPRi) technology in L. pneumophila. We successfully introduced a copy of dcas9 under a tetracycline-inducible promoter into the L. pneumophila chromosome, while the genes encoding the trans-activating (tracr) RNA and the target gene-specific CRISPR (cr) RNA(s) are provided on a plasmid. We showed that the trimeric complex of dCas9-tracrRNA-crRNA can specifically silence individual L. pneumophila genes without off-target effects, validating that the CRISPRi system is an effective gene editing tool in L. pneumophila. We also confirmed simultaneous silencing of two genes or even up to ten effector-encoding genes in L. pneumophila by CRISPRi. To our knowledge, this is the first time that such a large number of genes has been simultaneously silenced in any laboratory organism using the CRISPRi system, and it paves the way for a systematic interrogation of Legionella's effector-encoding genes. Importantly, the ability of CRISPRi-mediated gene silencing to attenuate L. pneumophila infection was validated by silencing dotA, a gene known to be essential for virulence. In summary, the CRISPR/dCas9 system presented here is destined to decipher the network of L. pneumophila effectors that critically contribute to the infection of human cells, and will pave the way for a more streamlined analysis of virulence mechanisms that contribute to Legionnaires' disease and related illnesses.

NICHD
Pei-Chung Lee
Visiting Fellow
Microbiology and Antimicrobials
Identifying human targets for Legionella pneumophila effector kinases by combining human protein arrays with a non-radioactive ATP labeling technique
Protein phosphorylation is the most abundant post-translational modification within living cells. Hundreds of kinases and thousands of kinase substrates have been predicted in human cells, highlighting the importance and complexity of phosphorylation networks. During infection, many bacterial pathogens interfere with cellular signaling pathways in order to avoid clearance by the host. Legionella pneumophila causes a form of bacterial pneumonia called Legionnaires' disease. Upon phagocytosis by alveolar macrophages, L. pneumophila uses a type IV secretion system (T4SS) to translocate more than 300 effector proteins into the host. How these effectors alter cellular functions in order to create conditions favorable for L. pneumophila replication has remained largely unclear. Using a bioinformatics approach, we discovered five novel L. pneumophila effectors with putative protein kinase domains, three of which we already validated using a non-radioactive ATP-gamma-S labeling technique. In order to identify host targets phosphorylated by these effector kinases, we combined the ATP-gamma-S labeling technique with a protein array containing more than 9,300 human proteins. Once transferred from ATP-gamma-S onto the target protein, the thiophosphate group is chemically alkylated, and the resulting thiophosphate ester epitope can be visually detected on the array by a fluorophore-conjugated antibody. Using this approach, we discovered that the L. pneumophila effector kinase, LegK6, phosphorylates human PKCbeta1, MAPK activated protein kinase 5 (MK5), vaccinia related kinase 1, and cortactin. Interestingly, both cortactin and MK5 are involved in F-actin rearrangement , implying that manipulation of the host cell cytoskeleton is critical for infection by L. pneumophila, probably by affecting endocytosis/phagocytosis or by altering actin-dependent cargo transport. Since L. pneumophila alters host endocytosis/phagocytosis pathway to create a unique legionella-containing

vesicle for its intracellular replication, our future studies are aimed at investigating, in a cellular context, how the downstream signaling events that are manipulated by L. pneumophila effector kinases promote bacterial infectivity.

NICHD
Shanshan Li
Postdoctoral Fellow
Epidemiology/Biostatistics - Etiology, Risk, and Prevention
Trans-generational root of obesity: maternal diabetes in pregnancy is associated with higher obesity risk in the offspring during childhood, adolescence and early adulthood
Background: Accumulating data from experimental studies suggest that hyperglycemia in utero environment as exemplified in pregnancies complicated by gestational diabetes (GDM) might increase obesity risk in offspring. However, data from epidemiological studies have been conflicting and the long-term trans-generational impacts of GDM through adolescence and adulthood are unknown. Furthermore, it is unclear whether the trans-generational impacts of GDM differs by offspring sex. Methods: We conducted a prospective cohort study of 19,956 participants (10,410 female and 9,546 male) in the US from the Growing-Up Today Study and followed up them from age 9-14 years through 23-28 years. Obesity was defined using International Obesity Task Force criteria for children under age 18 years and World Health Organization definition for age 18 or older. History of physician diagnosis of GDM during the index pregnancy was reported by participants' mother and has high validity in a previous validation study. The association between GDM and offspring obesity risk was examined using generalized linear models and generalized estimating equations adjusted for pre-pregnancy body mass index (BMI), age, and major pre-pregnancy maternal factors. Results: During 14 years of follow-up, 8.8% female and 9.2% male offspring became obese. In general, male offspring born to a GDM pregnancy had significantly higher BMI (p=0.001) and greater risk of obesity from childhood through early adulthood than those born to a non-GDM pregnancy. Adjusted relative risks (RRs) (95% Confidence Intervals (CIs)) of obesity associated with maternal GDM among male offspring were 1.64 (1.14-2.37) during late childhood (9-12 years), 1.58 (95% CI: 1.16-2.16) during adolescence (12 -18 years), and 1.42 (95% CI: 1.04-1.95) for early adulthood (age >18 years). There was a significant interaction between pre-pregnancy BMI and GDM in relation to obesity risk in the male offspring, with the highest risk being observed among offspring born to obese GDM women (p for interaction=0.03). Among female offspring, however, no significant association was observed. Conclusion: Hyperglycemia in utero environment may have a gender specific effect on obesity risk in the offspring; it is related to higher risk of obesity from childhood through early adulthood among male offspring, but not among female offspring.

NICHD
Uri Manor
Postdoctoral Fellow
Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology
Spire1C is a novel mitochondrial protein that regulates actin- and ER-dependent mitochondrial fission.
Improper regulation of mitochondria fission results in multiple disorders. Regulated fission is essential for damage control and apoptosis, both of which are implicated in neurodegeneration. The dynamin-like

protein Drp1 mediates fission, but mitochondria must first be constricted before Drp1 can act. Recently, it was reported that the endoplasmic reticulum (ER) wraps around and constricts mitochondria prior to division. A role for actin in this process was established when the ER-anchored formin protein, INF2, was found to promote constriction at ER-mitochondria intersections via actin polymerization. How INF2 activity is triggered at these intersections is unknown. We hypothesized that a mitochondrial protein might regulate fission by binding and activating INF2. Spire proteins are actin-nucleators that cooperate with formin proteins to promote actin assembly, but they have never been found on mitochondria. We identified a novel splice isoform of Spire1, Spire1C, which specifically localizes to mitochondria in all tissues. To test whether Spire1C localizes to the mitochondrial outer membrane (MOM), where it would have access to INF2 and actin, we utilized the super-resolution imaging technique, structured illumination microscopy (SIM), which allows us to resolve the inner and outer mitochondrial compartments. Spire1C was consistently on the MOM and could be distinguished from mitoRFP, a control protein localized to the mitochondrial matrix. In order to test whether Spire1C plays a role in INF2- and actin-dependent mitochondrial fission, we used SIM to image mitochondria, ER, and actin in Spire1C/INF2 knockout (KO) vs. control cells. We observed that Spire1C assembles actin on MOMs, and that Spire1C actin-nucleating and formin-binding activities promote ER-mediated mitochondrial constriction and division in an INF2-dependent fashion. To further test the hypothesis that Spire1C acts through INF2, we confirmed that Spire1C and INF2 directly interact in vitro. Finally, by imaging GFP-cytochrome C (cytoC), a protein that exits mitochondria during apoptosis, we found that in Spire1C KO cells, cytoC failed to exit mitochondria after 18 hours of treatment with the apoptosis-inducing drug staurosporine, whereas control cells released cytoC after just 3 hours of treatment. Our results reveal Spire1C as a novel MOM protein that interacts with both actin and the ER to regulate fission and apoptosis, which may have important implications for many diseases.

NICHD
Sarine Markossian
Visiting Fellow
Cell Biology - General
Quantitative Assessment of Chromosome Instability Promoted by Chemotherapeutic Agents
Most solid tumors are aneuploid, carrying an abnormal number of chromosomes, and they missegregate whole chromosomes in a phenomenon termed chromosome instability (CIN). CIN is associated with poor prognosis in many cancer types, and targeting of CIN is an attractive strategy for anti-cancer therapeutics. The mechanisms causing CIN and its contributions to tumor initiation and growth are not well defined, partly because there is no straightforward, quantitative assays for CIN in human cells. To address this problem, we have developed the first Human Artificial Chromosome (HAC)-based quantitative live-cell assay for mitotic chromosome segregation in mammalian cells, with which we can easily score the rates of CIN within one cell division under different experimental conditions. We have constructed a HAC encoding copies of enhanced green fluorescent protein (eGFP) fused to the destruction box (DB) of hSecurin, a substrate of the anaphase promoting complex/cyclosome (APC/C) ubiquitin ligase, which becomes active during anaphase to catalyze the proteolysis of critical mitotic target proteins. This HAC also contains tet operator (tetO) arrays and sequences encoding the tetracycline repressor fused to monomeric cherry fluorescent protein (tetR-mCherry). We have

produced human U2OS cells carrying this HAC, in which we monitor HAC segregation in two ways: First, APC/C degrades the DB-eGFP fusion expressed from the HAC at anaphase onset, and DB-eGFP re-accumulates in the daughter cells after G1 phase, when APC/C becomes inactive. Daughter cells that do not obtain a copy of the HAC will thus be GFP negative in the subsequent interphase. Second, because tetR-mCherry binds to the tetO arrays, the HAC itself could be followed by live imaging. Using this system, we can not only detect HAC mis-segregation within a single cell division but also extrapolate the rate of mis-segregation after a defined number of cell divisions from the proportion of GFP positive cells. Following the HAC by live cell imaging experiments, we show that HAC-bearing U2OS cell lines have low inherent levels of CIN, but HAC mis-segregation is markedly increased by treatment with chemotherapeutic drugs such as Reversine, an inhibitor of Mps1, and microtubule agents Nocodazole and Taxol. In summary, we have developed new assays to score CIN levels in human cells and have shown that CIN levels increase upon treatment with chemotherapeutic agents, which makes our assays ideal for chemical screens.

NICHD
Robert Mitchell
Postdoctoral Fellow
Biochemistry - Proteins
Elucidation of the Interneuron Glutamatergic Postsynaptic Proteome via an Endogenous Cre-dependent Affinity Tag
In higher organisms, networks of excitatory and inhibitory neurons sculpt patterns of neurotransmission to orchestrate complex behaviors. Of growing interest are the inhibitory GABAergic interneurons, as although greatly outnumbered by their excitatory counterparts, are instrumental in regulating neuronal network activity. This is evidenced by the fact that dysfunction of GABAergic neurons is linked to neuropsychiatric and neurodegenerative disorders such as schizophrenia, autism and epilepsy. GABAergic neurons are highly diverse and specialized in their function. For example, the fast-spiking, parvalbumin expressing (PV) neurons are required for specific patterns of electrical oscillatory activity disrupted in many neurological disorders. Moreover, whereas excitatory neurons have well characterized synaptic compartments called spines- that allow for spatial and temporal resolution of incoming electrical activity- these are absent on the majority of GABAergic neurons. Therefore, a major question in neuroscience is to determine how GABAergic neurons function at the synaptic level. However, this poses an extreme challenge at the protein biochemical level, as major synaptic targets are common between both excitatory and inhibitory neurons. To circumvent this challenge, I utilized the FLEx recombination strategy, in combination with CRISPR/cas9 genomic engineering of mouse embryonic stem (ES) cells, to generate a Cre recombinase-inducible, genomic, V5 affinity tag on the ubiquitously expressed synaptic protein Gria1. The ES line was differentiated into neurons and infected with an aav cre-gfp. Infected neurons expressed the Gria1-V5 tag, validating the construct at the genomic level. The ES cell line has since been injected into blastocysts to generate a Gria1-FLEx-V5 tag mouse strain. These mice will be crossed with a PV-cre driver strain to generate V5 tagged synapses exclusively on PV neurons. Large-scale purification of V5-taged Gria1 from PV neurons will then be subjected to LC/MS/MS-based proteomics. In addition, the Gria1-FLEx-V5 mouse will be crossed with a CamKIIalpa cre driver mouse (CamKIIalpa is a protein exclusively expressed in excitatory neurons).

Analysis of the proteome of these two types of synapses will enhance our general understanding of how GABAergic neurons function at the synaptic level and may lead to PV synapse-specific therapeutic targets for neurological disorders.

NICHD
Apratim Mitra
Postdoctoral Fellow
Epigenetics
Enhancer-dependent genes regulated by a shared muscle enhancer at the H19/Igf2 locus
Mutations of regulatory regions controlling the expression of Insulin-like growth factor 2 (Igf2) and H19 are associated with various human diseases, such as the overgrowth disease Beckwith-Wiedemann syndrome and rhabdomyosarcoma, a cancer of the connective tissues. Igf2 and H19 are linked genes believed to have opposing functions in development. Igf2 plays a crucial role in promoting growth, while the highly expressed non-coding H19 RNA acts in opposition to Igf2 and limits growth. In mice, the expression of these genes in skeletal muscle is controlled by an enhancer (muscle enhancer, ME) located downstream of H19. A mutation deleting the ME (called DME) results in complete loss of both Igf2 and H19. However, several questions remain: What is the effect of this deletion on the chromatin structure of the H19/Igf2 locus? How does the enhancer deletion affect local and global transcriptional status? To answer the first question, we conducted genome-wide chromatin immunoprecipitation sequencing (ChIP-Seq) experiments to study the histone marks associated with enhancers, promoters and active transcription in wild-type and DME/DME cells. We found that the patterns of histone marks were largely unchanged in the mutant cells suggesting that the deposition of these marks was unaffected even while transcription levels were reduced by > 300-fold. Thus an enhancer is not required to establish an active chromatin but instead controls the efficiency with which locus can generate transcription. To answer the second question, we performed genome-wide mRNA-sequencing.. As expected, the enhancer deletion ablated the expression of H19 and Igf2, but in addition, there were 186 other genes that were significantly changed in mutant cells. To disentangle the effects of the enhancer from that of Igf2 and H19, we utilized heterozygous deletions of the enhancer – maternal inheritance of the deletion (DME/+) results specifically in the loss of H19, while paternal inheritance (+/DME) causes Igf2 loss with normal H19 levels. Global comparisons of the transcriptomes of the mutant cells with wild-type revealed a set of 131 putative enhancer-dependent genes that were changed only in the DME/DME cells, but not in the heterozygous deletions. These results suggest that the muscle enhancer at the H19/Igf2 locus regulates distal genomic loci, and work is ongoing to further understand this phenomenon and especially to distinguish primary and secondary effects.

NICHD
Fearghal O'Brien
Visiting Fellow
Cultural Social and Behavioral Sciences
The Influence of Previous Parental Supervision on Adolescent Alcohol Use after Moving Out
Early alcohol use is associated with heavy drinking later in life. Parental supervision has been found to reduce alcohol use during the high school years. After high school, parents cannot offer direct

supervision if their child moves out of the family home. Is it the case that good parental supervision ceases to matter when a child moves away? We used data from the NEXT Generation Health Study to address this question. This 7-year, nationally-representative longitudinal study of health behaviors among emerging adults began in 2009. There were 1406 participants (818 females) who completed the survey in the first year after high school (wave 4). The outcome measure was alcohol use at wave 4 and the predictors were accommodation at wave 4 (Living at Home vs. Not Living at Home) and parental supervision during the last year of high school (wave 3). Parental supervision was the mean of a responses to a number of questions about how much a participant's parents knows about the participant's life (e.g. what they do after school). Multiple regression modelling tested the relationship between the predictors and alcohol use while controlling for age, gender, race, family affluence, school status, and direct parental advice about alcohol. Mothers' and fathers' supervision were separate variables in the model, along with their interaction with accommodation. A significant interaction was found between accommodation and both fathers' supervision (Beta = .1, p < .05) and mothers' (Beta = .07, p < .05). For those living away from home at wave 4, increased supervision from their father at wave 3 predicted less drinking. There was no effect of mothers' supervision for this group. For those still living at home at wave 4, increased supervision from their mother at wave 3 predicted less drinking. There was no effect of fathers' supervision for this group. Collectively, these findings are novel as they are the first to address longitudinal questions of parental supervision. The results suggest that parental supervision can be predictive of alcohol use even after a child has moved out. The differential effect of mothers' and fathers' supervision was not expected and may reflect the different parenting roles that each parent plays with late adolescent children. The positive effects of parental supervision emerged even when controlling for parents giving direct advice about alcohol use which suggests that it may be supervision in general that confers a protective benefit.

NICHD
Morihiro Okada
Postdoctoral Fellow
Metabolomics/Proteomics
Roles of histidine metabolism gene during adult intestinal stem cell development.
Research on stem cells has gained considerable recognition because of the promise of stem cell-based therapies for diseases. On the other hand, to apply stem cells toward treatment of organ-specific disease, it is critical to understand how the organ-specific adult stem cells are formed during vertebrate development. This area is, however, poorly studied due to the difficulty in utilizing mammalian models for the postembryonic period when most adult stem cells are formed. The thyroid hormone (T3)-dependent frog metamorphosis resembles mammalian postembryonic development and offers a unique opportunity to study how adult stem cells are developed, largely due the ability to manipulate the externally developing frog embryo. In particular, the remodeling of the intestine during metamorphosis mimics neonatal maturation of mammalian intestine, the well-characterized organ for studying adult stem cells due to the life-long epithelial self-renewing system. Our earlier studies have shown that the formation of the adult intestine during Xenopus metamorphosis involves complete degeneration of the larval epithelium and de novo formation of adult stem cells. A tissue-specific microarray analysis of intestinal gene expression during metamorphosis has identified a number of novel candidate stem cell

genes. Among them is the amidohydrolase domain containing 1 (AMDHD1) gene, which encodes an enzyme involved in the catabolism of histidine, leading to the formation of glutamic acid. In this study, we show that AMDHD1 is exclusively expressed in the proliferating adult intestinal epithelial stem cells during metamorphosis with little expression in other intestinal tissues. We further provide evidence that T3 activates AMDHD1 gene expression directly at the transcription level through T3 receptor binding to the AMDHD1 gene in the intestine. These together with our earlier finding that histidine ammonia-lyase (HAL) gene in histidine catabolic pathway is similarly regulated by T3 in the intestine suggest that histidine catabolism plays a critical role in the formation of adult intestinal stem cells during metamorphosis. Consistently, our preliminary transgenic studies suggest that altering AMDHD1 and HAL expression level indeed influence cell proliferation in the tadpole intestine. Thus, our findings provide the first evidence for the involvement of metabolic pathways in intestinal adult stem cell formation and likely metabolic disorders in intestinal diseases as well.

NICHD
Amy Palin
Postdoctoral Fellow
Immunology - Lymphocyte Development and Activation
Reducing T cell receptor signaling capacity during thymocyte development supports an instructive model of regulatory T cell lineage commitment
T cell receptor (TCR) signal strength is a critical determinant of T cell fate. During T cell development in the thymus, TCR signaling can initiate development and survival of moderately self-reactive cells (positive selection), or apoptosis of strongly self-reactive cells (negative selection). In mature T cells, TCR signaling can initiate activation or a functionally non-responsive state known as anergy, depending on co-receptor and cytokine signaling. Our lab has generated a knock-in mouse with tyrosine-to-phenylalanine mutations in the 6 Immunoreceptor Tyrosine Activation Motif (ITAM) tyrosines of the CD3? chain of the TCR/CD3 complex, which is the major intracellular signaling component. T cells from mice with germline expression of the mutant 6F CD3? chain have a 60% reduction in TCR signaling potential. The lab previously found that there were surprisingly few defects in T cell composition and effector function between mice with germline expression of 6Y CD3z and 6F CD3z, with the notable exception of an increase in the number and percentage of regulatory T cells (Tregs) in mice with germline 6F CD3z expression. Tregs are vitally important in preventing autoimmunity by suppressing damaging immune responses. Our results indicate that highly self-reactive thymocytes receive a lower TCR signal when expressing 6F CD3z and become Tregs, instead of undergoing negative selection and apoptosis. Because the 6Y to 6F switch is inducible by expression of Cre recombinase, we have manipulated the timing of 6F CD3z expression in thymic development. These experiments address two questions: the timing of Treg lineage commitment in the thymus, and whether commitment is instructive. We have found that 6F CD3z expression before the double positive stage and positive selection results in an increase in Treg generation, similar to that seen in mice with germline 6F CD3z expression. Using two Cres active beginning at the late double positive stage and through the single positive stage, we have found that 6F CD3z expression at the time of selection does not result in increased thymic or peripheral Tregs. Our data support an instructive model of Treg generation in the thymus, whereby the strength of TCR signaling in response to self induces development into a Treg. Our

data also indicate that Treg commitment occurs at the time of positive selection. We are investigating the function of Tregs from these mice, and the role of TCR signal strength in maintenance of Tregs.

NICHD
Solji Park
Visiting Fellow
Stem Cells - General
COMPARISON OF THE ABILITY OF IPSCs INTO GERM CELLS
According to the World Health Organization, infertility is considered a disease and it affects 48.5 million couples worldwide. Historically, primordial germ cells (PGC) develop only into sperm and oocytes in vivo during fetal development. However, oocytes were recently derived from induced pluripotent stem cells (iPSCs) via PGC intermediaries in mice, which introduced the possibility of making patient specific gametes in an ethically favorable method. In the iPSC literature, some studies suggest that the starting somatic cell type for iPSCs reprogramming affects the downstream differentiated potential to the cell or tissue type of interest. Previous studies have reported that iPSCs exhibit a propensity for differentiation into their original cell lineages, while others did not. In this study, we compared the ability of iPSCs derived from either rhesus ovarian stem cells (OSC) or rhesus fibroblasts (FB) to differentiate into PGCs. We established the first serum-free and defined differentiation protocol for inducing PGC-like cells (PGCLC) from rhesus iPSCs. Efficient reprogramming of both OSC and FB-derived iPSCs was confirmed by the presence of teratoma formation, immunofluorescence staining of pluripotency markers as well as RT-PCR. Using surface marker SSEA1 to enrich for PGCLCs by flow cytometry, we found that FB-derived iPSCs exhibited higher efficiency in differentiation to PGCLCs compared to OSC-derived iPSCs (18.33+/-4.03% vs 3.07+/-1.34%, respectively; P<0.05), despite OSC-derived iPSCs originating more closely to PGC than FB. FB-derived iPSC also produced PGCLCs with higher expression of the germ cell marker DDX4 compared to OSC-derived iPSCs (5.88+/-1.09 vs 2.86+/-1.12, respectively; P<0.05), while no differences were detected in STELLA and PRDM1. Although some studies have shown that iPSCs created from a similar cell origin are more efficiently reprogrammed back towards a similar cell type, OSC-derived iPSCs are not significantly more efficient in differentiating to PGC than FB-derived iPSCs. Taken together, these data suggest that patients who have lost their ovaries may still be amenable to iPSCs approaches using skin fibroblasts for generating PGC and subsequent oocytes.

NICHD
Jing Pu
Visiting Fellow
Cell Biology - General
BORC, a Multisubunit Complex that Regulates Lysosome Positioning
Lysosomes are membrane-enclosed cellular organelles whose main function is the degradation of biomacromolecules. In addition, lysosomes participate in various other cellular processes, including microbial killing, detoxification, cholesterol homeostasis, apoptosis, metabolic signaling, plasma membrane repair, cell migration, and cancer invasion and metastasis. Recent evidence indicates that at least some of these processes are influenced by the positioning of lysosomes within the cytoplasm. In the course of studies on the BLOC-1 complex that is defective in some types of Hermansky-Pudlak

syndrome, we discovered a related complex named BORC (for BLOC-one-related complex) that regulates lysosome positioning. Using affinity-purification/mass-spectrometry, co-immunoprecipitation, size-exclusion chromatography and sedimentation velocity analyses, we found that BORC comprises eight subunits, of which some are shared with BLOC-1 and the others are previously uncharacterized proteins. BORC peripherally associates with the lysosomal membrane, where it functions to recruit the small GTPase Arl8. This initiates a chain of interactions that promotes Kinesin-1-dependent movement of lysosomes towards the plus ends of microtubules in the cell periphery. CRISPR/Cas9 knockout (KO) or siRNA knockdown (KD) of BORC subunits in HeLa cells results in dissociation of Arl8b from lysosomes and consequent collapse of the peripheral lysosome population into the pericentriolar area. Relative to WT cells, BORC-KO cells exhibit a significant (30%) reduction of the footprint area in adherent culture but a similar diameter in suspension culture, consistent with a defect in cell spreading. Two-dimensional cell migration analysis using a circular gap closure assay showed that the migration velocity of BORC-KO cells was also reduced ~30% relative to WT cells. These experiments thus identified BORC as a critical effector of lysosome positioning and motility, and uncovered a physiological requirement of BORC-dependent lysosome transport in the regulation of cell spreading and migration.

NICHD
Cathy Ramos
Postdoctoral Fellow
Developmental Biology

Neto-mediated interactions shape postsynaptic composition at the Drosophila neuromuscular junction

In mammals, learning and memory processes require synaptic plasticity that is sustained by ionotropic glutamate receptors (iGluRs). The iGluRs are localized to the postsynaptic densities (PSDs) and their properties are regulated by membrane auxiliary subunits such as Neto-1 and -2 (Neuropillin and Tolloid-like). At the Drosophila neuromuscular junction (NMJ), our lab found that the iGluRs clustering at the PSDs also requires an auxiliary subunit, Neto, which is highly conserved. Since the Drosophila NMJ is similar in composition and physiology to mammalian glutamatergic synapses, it is a powerful genetic model to study the developmental mechanisms of the proper PSDs assembly that is hardly feasible with mammalian models. In this study, we found Drosophila neto encodes two isoforms generated by alternative splicing, Neto-a and -ß, which only differ in their intracellular domain. By qPCR/RNAi experiments, we found that Neto-ß is the predominant isoform at the NMJ (80%). We generated two different neto-ß alleles: a neto-ß genetic null and a neto-ß truncation. Both mutants show partial lethality, small NMJs and physiological defects. At the fly NMJ, the iGluRs are heterotetrameric complexes noted type-A or -B, conferring by the GluRIIA or -IIB subunit. The type-A iGluRs arrive first at the PSDs, forming the cluster core and then, type-B accumulate surrounding the type-A core. Thus, the GluRIIA/IIB ratio is a read out for proper synapse strength and synaptic plasticity. When Neto-ß is missing/truncated, the NMJs show drastic changes in the iGluR subtype ratio. This is due to reduced accumulation of GluRIIA at the PSDs that is normally sustained by the postsynaptic component p21-activated kinase (PAK). By epistasis experiments and netonull rescue experiments, we showed that only the intracellular part of Neto-ß, and not Neto-a, recruits PAK at the PSDs, then enabling PAK to stabilize type-A iGluRs and leading to normal synapse development. Our findings show that Neto-ß is required for the proper iGluRs recruitment at the PSDs during the synapse development. Also, these results strongly

suggest that Neto isoforms use their different cytoplasmic domains to recruit specific proteins to sculpt the PSD composition. To further study the roles of Neto proteins, we generated neto-a KO flies using the CRISPR system and we are currently analyzing the mutants.

NICHD
Neelam Dabas Sen
Research Fellow
Molecular Biology - Eukaryotic
Tale of two DEAD-box RNA helicases: Ded1 and eIF4A have distinct but overlapping functions in regulating eukaryotic translation initiation in vivo
Initiation is the most regulated and rate limiting step of translation. In eukaryotic translation initiation, 43S pre-initiation complex (PIC) containing the small ribosomal subunit and methionyl initiator tRNA typically attaches to the 5'end of the mRNA and scans the 5' UTR for the start codon. Resolving mRNA secondary structures in 5' UTR is thus a key regulatory step in both 43S PIC attachment and subsequent scanning of the mRNA. DEAD-box RNA helicases eIF4A and Ded1 are believed to promote translation initiation by resolving mRNA secondary structures that impede 43S PIC attachment or scanning, but whether they perform distinct functions or act redundantly in vivo is poorly understood. We compared effects of mutations in Ded1 or eIF4A on global translational efficiencies (TEs) in yeast by ribosome profiling. Ribosome profiling involves deep-sequencing of mRNA fragments protected from nuclease digestion by translating 80S ribosomes. Quantification of ribosome footprints together with mRNA abundance measurements from the same cultures (RNA-seq), allows estimation of the TE of each mRNA in the transcriptome. Despite similar reductions in bulk translation, inactivation of a cold-sensitive Ded1 mutant substantially reduced the TEs of >600 mRNAs, whereas inactivation of a temperature-sensitive eIF4A mutant yielded <40 similarly impaired mRNAs. The broader requirement for Ded1 did not reflect more pervasive secondary structures at low temperature, as inactivation of temperature-sensitive and cold-sensitive ded1 mutants gave highly correlated results. Interestingly, Ded1-dependent mRNAs exhibit greater than average 5'UTR length and propensity for secondary structure, implicating Ded1 in scanning though structured 5' UTRs. Reporter assays confirmed that cap-distal stem-loop insertions increase dependence on Ded1 but not on eIF4A for efficient translation. While only a small fraction of mRNAs is strongly dependent on eIF4A, this dependence is significantly correlated with requirements for Ded1 and 5'UTR features characteristic of Ded1-dependent mRNAs. These findings suggest that Ded1 is critically required to promote scanning through secondary structures within 5'UTRs; and while eIF4A cooperates with Ded1 in this function, it also promotes a step of initiation common to virtually all yeast mRNAs. Our study unveils, for the first time, a division of labor among these two essential DEAD box helicases in the critical process of eukaryotic translation initiation.

NICHD
Giampaolo Trivellin
Postdoctoral Fellow
Genetics
X-linked acro-gigantism (X-LAG): a new form of infant-onset pituitary gigantism
Introduction: High growth hormone (GH) secretion is a rare condition that leads to gigantism in

childhood and acromegaly in adults. Aim: We studied gigantism and acromegaly for genetic defects. Methods: We performed genome-wide analyses in an international cohort of 46 patients with gigantism and 248 patients with acromegaly. The expression of a candidate gene was investigated in different species. Results: We detected a novel microduplication at chromosome Xq26.3 in 2 unrelated kindreds and 13 sporadic cases with infantile gigantism. All patients had disease onset before 5 years of age and presented with mixed GH/prolactin-secreting tumors and/or hyperplasia. All sporadic cases harbored non-recurrent duplications, whereas familial cases inherited the duplications from their mothers. Breakpoint junctions revealed microhomology, suggesting a replicative mechanism for their formation. Patients shared a common duplicated region of approximately 500 Kb containing 4 protein-coding genes, of which only GPR101, a G-protein coupled receptor (GPCR) that activates cAMP signaling, was consistently overexpressed in patients' pituitary lesions. We also identified a recurrent GPR101 variant in 11 out of 248 patients with acromegaly, mostly in tumors. When transfected into somatotrope cells the mutation led to increased GH secretion. Low GPR101 expression was seen in non-duplicated GH-secreting tumors and in most normal adult human tissues, including the pituitary. On the contrary, high expression was observed in human fetal pituitary. Adult pituitaries of both rhesus monkey and rat expressed GPR101 but in different cell types. In the developing zebrafish embryo a strong and brain-specific GPR101 staining (including the hypothalamus and pituitary) was seen. Conclusions: We describe a new genomic disorder characterized by early-onset gigantism and caused by Xq26 microduplications (X-LAG for X-linked acrogigantism). This syndrome is likely caused by overexpression of GPR101, a dosage-sensitive GPCR that activates the cAMP pathway, whose mitogenic effects in pituitary somatotropes are well established. GPR101 may also be mutated in adult patients with acromegaly. The brain is the major site of GPR101 expression across different species, although divergent species- and developmental stage-specific expression patterns are evident, especially concerning the pituitary. These differences might reflect the very different growth, development and maturation patterns among species.

NICHD
Alex Valm
Postdoctoral Fellow
Intracellular Trafficking

Novel spectral imaging and analysis to unravel the organelle interactome

The organization of the eukaryotic cell into membrane-bound compartments allows for regulation of cellular processes. However, the activities of spatially distinct organelles must be coordinated to enable proper cell function. Membrane contacts have emerged as key sites for exchange of metabolites and signaling molecules, as well as playing roles in organelle division and maturation. Much progress has been made recently in elucidating the functions and molecular components of pairwise organelle interactions. However, a systems-level understanding of the dynamic organelle interactions within eukaryotic cells, i.e., the organelle interactome, remains unattained due to the inability to label and distinguish more than a few different fluorescent proteins in a single sample. Here, we present a novel cell labeling, image acquisition and analysis approach to study the spatial distribution of multiple organelles within single eukaryotic cells. We adapted a lattice light sheet imaging system for spectral imaging by introducing multiple laser lines coupled with fast acousto-optical beam splitting to record

spectral images in the excitation regime. We further developed a computational image analysis procedure that we used to track the observed inter-organelle contacts through time in our live cell time lapse images. We imaged cells that simultaneously express fluorescent fusion protein markers for peroxisomes, lysosomes, mitochondria, the endoplasmic reticulum (ER) and the Golgi apparatus, as well as a vital chemical stain to label lipid droplets (LDs). As a negative control, we generated computer models of cells with their organelles randomly distributed. We applied our organelle interactome analysis to test the hypothesis that there are subpopulations of LDs – the organelles responsible for storing and redistributing lipids – within mammalian cells that differ in their organelle interactions. We found that LDs are heterogenous, but that all LDs interact extensively with ER, which is the site of many lipid synthesis enzymes. We found that at any given time 58% of LDs are associated with ER and that the frequency of LD associations with peroxisomes, a site of fatty acid metabolism, was approximately 3-fold higher than in our random models. We have demonstrated the first live cell imaging of 6 different fluorescently labeled organelles in single cells and our results demonstrate the prevalence and significance of the ER contact sites in the eukaryotic cell.

NICHD
Jyothsna Visweswaraiah
Visiting Fellow
Molecular Biology - Eukaryotic

The ß-hairpin of 40S ribosomal protein Rps5/u7 is a critical determinant of efficient and accurate translation initiation in vivo

In eukaryotic translation, the 43S pre-initiation complex (PIC), containing initiator tRNAMet bound to the 40S ribosomal subunit, scans the mRNA leader for an AUG triplet in favorable sequence context. eIF1 and various other initiation factors enhance the efficiency and fidelity of this process, but contributions of the 40S subunit itself are poorly understood. Recent structural analyses revealed that the ß-hairpin of yeast Rps5 protrudes into the mRNA exit channel of the 40S subunit and contacts both the eIF2·GTP·Met-tRNAi ternary complex (TC) and context nucleotides of the mRNA in the E site. To explore the role of 40S exit channel proteins in AUG selection, we mutated residues in the ß-hairpin of yeast Rps5 and screened the mutants for altered fidelity of AUG selection: either increased initiation at near-cognate UUG codons (Sui- phenotype) or decreased UUG initiation in cells harboring a Sui- mutation in another factor. We identified substitutions in ß-strand 1 and the nearby C-terminal residue of Rps5 that confer slow growth, reduce bulk translation initiation and increase initiation at near-cognate UUG codons (Sui- phenotype) as judged by HIS4 reporters. However, unlike other Sui- mutations that increase eIF1 expression by suppressing poor context at the AUG codon of SUI1 (encodes eIF1), these mutations actually decrease eIF1 levels by exacerbating the effect of poor sequence context of the eIF1 AUG start codon and thereby reducing eIF1 abundance. Restoring eIF1 levels in these rps5 mutants with plasmid borne SUI1 eliminated their Sui- phenotype, indicating that the Sui- phenotype was due to low eIF1 levels. In fact, the rps5 mutants with restored eIF1 levels suppress the Sui- phenotype of a mutation in eIF5, indicating that they increase the stringency of selecting AUG over near-cognate start codons (Ssu- phenotype) in addition to discriminating against poor context. Consistent with these genetic findings, these mutations greatly destabilize the binding of tRNAiMet to AUG in reconstituted 43S·mRNA complexes in vitro. We further identified substitutions in loop residues of the ß-hairpin that selectively

discriminate against UUG start codons in vivo and likewise destabilize tRNAiMet binding at UUG, but not at AUG start codons, in vitro. These results indicate that the Rps5 ß-hairpin is on par with well-studied soluble initiation factors in insuring efficient and accurate recognition of start codons in eukaryotic cells.

NICHD
Maeve Wallace
Postdoctoral Fellow
Cultural Social and Behavioral Sciences
Pregnancy-associated homicide and suicide in US states with enhanced pregnancy surveillance on the standard certificate of death: 2005-2010
Improvements in access to and quality of maternal health care in the US since the mid-1950s have drastically reduced mortality from obstetric causes. Less attention has been paid to pregnancy-associated violent death – homicide or suicide of a woman while pregnant or within 1 year postpartum – despite reports from city- and state-level data that consistently find homicide ranks among the leading causes of death in the peripartum period. Insufficient sample sizes and inadequacy of pregnancy information from mortality data sources have limited previous attempts to compare risk of violent death during pregnancy/postpartum to risk among non-pregnant/non-postpartum women and national-level estimates have not been reported. We analyzed newly available data from death records in 37 states that included items identifying female decedents age 10-54 as pregnant, early postpartum (pregnant within 42 days of death), late postpartum (pregnant within 43 days to 1 year of death) or not pregnant/postpartum from 2005-2010, inclusive. Given documented underreporting of pregnancy status on death records, we adjusted our number of pregnant/postpartum decedents based on two published estimates of misclassification before computing mortality ratios for comparison to the non-pregnant/non-postpartum population. Young, unmarried, undereducated, and non-Hispanic Black women were at greater risk for homicide, while suicide was more likely to occur in older, unmarried, and non-Hispanic White women. Pregnancy-associated homicide ranged from 2.9-6.2 per 100,000 live births, depending on degree of misclassification, compared to 2.6 per 100,000 non-pregnant/non-postpartum population. Pregnancy-associated suicide ranged from 2.1-3.7 per 100,000 live births compared to 5.7 per 100,000 non-pregnant/non-postpartum population. Adjustment for the most conservative estimate of misclassification indicated pregnant/postpartum women were at 1.77 times greater risk of homicide compared to non-pregnant women (95% confidence interval[CI]: 1.64, 1.90) while the risk of suicide was decreased (relative risk=0.62, 95% CI: 0.57, 0.67). In fact, the direction of these findings was robust to misclassification of less than half the most conservative reported rate. Our results add to the small body of literature aiming to shed light on violent causes of maternal death. These losses of life merit the same vigilance paid to incidents of obstetric-related maternal mortality in their surveillance and prevention.

NICHD
Gernot Wolf
Visiting Fellow
Epigenetics
KRAB zinc finger proteins are evolutionarily adaptive repressors of parasitic DNA elements
Retroviruses have been invading mammalian germ lines for millions of years, accumulating in the form

of endogenous retroviruses (ERVs) that account for nearly one-tenth of the human genome. Several ERV families have been shown to alter gene expression profiles by introducing retroviral enhancer and promoter elements into the host genome. While this mechanism may lead to the evolution of new regulatory networks and gene function, ERV-associated transcription has also been linked to several human diseases including cancer. To protect their genomic integrity, mammals have developed a number of anti-retroviral defense mechanisms that include epigenetic repression of ERVs during development. However, the adaptive cellular factors that recognize and repress ERVs and other parasitic DNA elements during development have remained elusive. Here we demonstrate that ZFP809, a member of the highly diversified and rapidly evolving Krüppel-associated box zinc finger protein (KRAB-ZFP) family, initiates silencing of a defined subset of ERVs in a sequence-specific manner via recruitment of heterochromatin inducing complexes. ZFP809 knock-out mice develop normally but display up to several hundred-fold increased expression of ZFP809-targeted ERVs in the majority of somatic tissues and organs. ERV reactivation is accompanied by an epigenetic shift from repressive to active histone modifications and partial loss of DNA methylation. Importantly, using conditional alleles and rescue experiments, we demonstrate that ZFP809 is required to initiate ERV silencing during embryonic development, but becomes largely dispensable in somatic tissues. Furthermore, we show that ZFP809 silences a handful of non-viral host genes that have acquired ZFP809 binding sites via ancient ERV insertions. Finally, we determined the genome-wide binding profiles of several previously uncharacterized murine KRAB-ZFPs using ChIP-seq technology and show that the majority of these proteins bind to distinct ERV families. In sum, our data strongly support the hypothesis that KRAB-ZFPs evolved in response to germ-line colonization events by ERVs and other transposable elements, which repeatedly re-wired transcriptional networks during mammalian evolution.

NICHD
Megan Wyeth
Postdoctoral Fellow
Neurotransmission and Ion Channels
Neto regulation of kainate receptors on inhibitory interneurons
Kainate receptors have been shrouded in controversy, but they are particularly interesting because they serve a modulatory role, influencing both excitatory and inhibitory transmission. Neto proteins were recently discovered to be auxiliary proteins to postsynaptic kainate receptors on hippocampal CA3 pyramidal cells, influencing synaptic targeting and conferring proper function. The effect of kainate receptor activation on inhibitory interneurons is contentious, with increased or decreased GABA release possibly varying by interneuron type, and pre- versus postsynaptic localization. Therefore we investigated kainate receptors, and the question of their regulation by Neto proteins, by interneuron class. We used immunohistochemistry to demonstrate that among interneuron types cholecystokinin-containing (CCK) interneurons and especially somatostatin-containing (Som) interneurons express Neto1. In situ hybridization indicated that both CCK and Som interneurons also express the Neto2 isoform. We localized the kainate receptor subunits GluK1 and GluK2 to Som and CCK interneurons by immunohistochemistry and in situ hybridization. Immunocytochemistry suggested that GluK2 is targeted to the somatodendritic membrane in Som cells, avoiding the axonal domain. Electron microscopy revealed immunogold for GluK2 in CCK-labeled terminals, along with the high affinity GluK5 subunit.

Electrophysiology recordings targeting interneurons from Som and CCK populations confirmed that kainate enhanced their holding current, consistent with kainate receptor-mediated depolarization. The effect of kainate was stifled in Neto1 but not Neto2 knockout mice. In recordings from CA3 pyramidal cells spontaneous IPSCs were enhanced by kainate, an effect again lost in Neto1 but not Neto2 knockout mice. Pharmacologically silencing CCK interneurons suggested that Som interneurons are the primary contributors to Neto1-dependent kainate enhancement of inhibition. On the other hand, paired recordings between CCK interneurons and CA1 pyramidal cells demonstrated that kainate receptor suppression of GABAergic transmission was reduced in Neto2 knockout mice, and further reduced in Neto1+2 knockout mice. Altogether these data clarify the variation of particular kainate receptor subunits and their subcellular localization by interneuron type in addition to their functional regulation by Neto1 and Neto2.

NICHD
Yeyi Zhu
Postdoctoral Fellow
Epidemiology/Biostatistics - Prognosis and Response Predictions
Maternal ambient air pollution exposure preconception and during early gestation and offspring congenital orofacial defects
The ubiquitous nature of air pollution provides an opportunity for significant public health impact since the adverse effects on birth outcomes could potentially be prevented. Maternal air pollution exposure and orofacial defect risk has been studied but findings are inconsistent with no evaluation of potential chronic preconception effects. Further, most previous studies have small geographic coverage. We are the first to study criteria air pollutant exposures during three months preconception and we also examined risk for gestational weeks 3-8 (overall and by individual week) in relation to orofacial defects in a large, contemporary multi-region US cohort. Among 188,102 births from the Consortium on Safe Labor (2002–2008), 63 had isolated cleft palate (CP) and 159 had isolated cleft lip with/without cleft palate (CL+/-CP). Exposures were estimated using a modified Community Multiscale Air Quality model. Logistic regression with generalized estimating equations, adjusted for site/region and maternal demographic, lifestyle and clinical factors, calculated the odds ratio (OR) and 95% CI per interquartile range (IQR) of pollutant increase. Preconception, carbon monoxide (CO; OR=2.24; CI: 1.21-4.16) and particulate matter (PM) =10 µm (OR=1.72; CI: 1.12-2.66) were significantly associated with CP, while sulfur dioxide (SO2) was associated with CL+/-CP (OR=1.93; CI: 1.16-3.21). During gestational weeks 3-8, CO (OR=2.74; CI: 1.62-4.62), nitrogen oxides (NOx; OR=3.64; CI: 1.73-7.66) and PM =2.5 µm (PM2.5; OR=1.74; CI: 1.15-2.64) increased the risk of CP. Analyses by individual week revealed that CP risks associated with NOx and PM2.5 were most prominent from weeks 3-6 and 3-5, respectively. If our findings are confirmed, more than 6,000 orofacial cleft cases per year in the US could be prevented with a 0.3 ppm decrease in CO levels and 3.5 ppb decrease in SO2 levels three months before conception, with an additional 6,000 CP cases with a 30 ppb decrease in NOx levels in early pregnancy. Greater benefits might be seen in areas of the world where the levels of exposure are higher. Our novel data on preconception exposure risk suggests the need for future research to understand the potential adverse effects of chronic preconception exposure to air pollution on embryo-fetal development. Further, the

observed week-specific associations demonstrate temporal variability in the relation between air pollution and time-dependent outcomes such as birth defects.

NIDA
David Barker
Postdoctoral Fellow
Neuroscience - Cellular and Molecular
Lateral Habenula-Mediated Aversion is Regulated by an Unexpected Glutamatergic Input from the Lateral Preoptic Area
The Lateral Habenula (LHb) has been identified as a brain region that plays important roles in drug abuse, psychiatric illnesses and motivated behavior. Convergent evidence has established that the LHb receives a major input from the Lateral Preoptic Area (LPO). Although the nature of this LPO-LHb pathway hasn't bee determined, it has been prosed to be inhibitory, presumably from LPO GABAergic neurons. Here, we characterized the anatomical and functional network between the LPO and the LHb. To identify specific LPO neurons targeting the LHb, the retrograde tracer fluorogold was injected into the LHb (1% via iontophoresis). Next, we established the phenotype of the fluorogold-tagged neurons by in situ hybridization detection of transcripts encoding either glutamic acid decarboxylase 65/67 mRNA (GAD, a marker of GABAergic neurons) or vesicular glutamate transporter 2 mRNA (VGluT2, a marker of glutamate neurons). Surprisingly, we found that within the total population of fluorogold-positive neurons only 15.9 ± 3.2% expressed GAD mRNA, and as many as 74.7 ± 3.2% expressed VGluT2 mRNA. These neuroanatomical findings indicate that the major projection from the LPO to the LHb is from excitatory glutamatergic neurons rather that from inhibitory GABAergic neurons, as has been previously proposed. We next used an optogenetic approach to determine the participation of the LPO-LHb glutamatergic pathway in behavior. The light activated channel Channelrhodopsin2 (ChR2) was selectively expressed in LPO glutamatergic neurons by injecting a viral vector encoding a Cre-dependent ChR2 in the LPO of VGluT2::Cre mice. These infected mice were implanted with an optical fiber (dorsal to LHb) for the selective LHb stimulation of glutamatergic fibers from the LPO, Optogenetic activation of the LPO-LHb glutamatergic pathway resulted in escape behaviors and the formation of a conditioned place aversion to the compartment associated with optical stimulation. We conclude that LHb activity is regulated by a major excitatory (glutamatergic) pathway from the LPO, which activation results in a powerful aversive response. Thus far, hypotheses on the role of the LPO in LHb regulation have been based on the incorrect assumption that this connection is inhibitory, rather than excitatory, as shown here. The discovery of this LPO-LHb excitatory pathway opens a new avenue for investigating its role in depression, drug abuse, and other psychopathologies associated with the LHb.

NIDA
Comfort Boateng
Postdoctoral Fellow
Chemistry
Novel and high affinity Dopamine D3 receptor-selective ligands attenuate heroin self administration in WT but not D3KO mice
Dopamine receptors (D1-like (D1R, D5R) and D2-like (D2R, D3R, D4R)) are G-protein coupled receptor

proteins that regulate physiological functions such as movement, emotion and cognition. Dopamine D3 receptors (D3Rs) are enriched in mesolimbic circuits of the brain that are involved with drug addiction. Chronic administration of drugs of abuse leads to an overexpression of D3R in the ventral striatum, further supporting a role for D3R in addiction and as a potential target for medication development. D3R-selective antagonists have been studied in both animal models of drug abuse and in human substance abusers; however poor bioavailability, metabolic instability, and/or predicted toxicity have preempted success with translating promising drug candidates to clinical use. One of our most selective and high affinity D3R antagonists, PG648 (N-(4-(4-(2,3-Dichloro-phenyl)piperazin-1-yl)-3-hydroxybutyl)-1H-indole-2-carboxamide), has recently been developed as a lead drug candidate. Although pharmacokinetic and behavioral data in rats looked promising, it was not active in nonhuman primates and was metabolically unstable in this species. Thus we explored structural modifications on the PG648 backbone to discover novel and more metabolically stable ligands that retained high D3R binding affinity and subtype selectivity. Bioisosteric replacement of the 2,3-diCl-phenylpiperazine with a 2,3-naphthylpiperazine or 2-OMe,3-Cl-substituted-phenylpiperazine and replacing the terminal indole amide with various heteroaryl ring systems led to a series of compounds. Binding affinities were determined using [3H]N-methylspiperone displacement in HEK293 cells expressing hD2R or hD3R. We discovered several high affinity D3R antagonists, e.g. BAK4-54 (Ki=0.1 nM) and CAB2-015 (Ki=0.3 nM) compared to PG648 (Ki=1.9 nM). These analogues were potent antagonists (IC50=8 nM) in a mitogenesis functional assay (hD3 transfected CHO cells) and improved metabolic stability in mouse microsomes was demonstrated. Mice were trained to self-administer heroin (0.025 mg/kg/infusion) and both BAK4-54 and CAB2-015 effectively reduced heroin self-administration in WT but not D3KO mice, suggesting that they reduced the rewarding effects of heroin in a D3R-dependent manner. These are the first studies to show the effectiveness of D3R antagonists in heroin self-administration and suggest an alternate medication strategy to methadone maintenance or buprenorphine, the current treatments for heroin addicts.

NIDA
Lindsay De Biase
Postdoctoral Fellow
Neuroscience - Cellular and Molecular
Microglia establish region specific phenotypes within the basal ganglia and exhibit diverse responses to normal aging
Microglia possess highly motile processes that contact and sometimes phagocytose synapses. Microglia also release inflammatory and trophic signaling factors that can influence synaptic signaling and neuronal activity. We recently showed that microglia in the basal ganglia (BG) of young adult mice differ dramatically in their density, morphology, electrophysiological properties, and gene expression, indicating that these cells are not equivalent throughout the CNS as previously assumed. Such differences could profoundly impact the degree to which BG neurons are exposed to microglial signaling factors and whether BG synapses are contacted by microglial processes. To extend this analysis, we quantified microglial density and morphology in 2-week-old and 18-month-old mice and found that region-specific microglial phenotypes emerge by the second week of life and are maintained into late middle age. In addition, when transgenic approaches were used to transiently ablate microglia from the

CNS of young adult mice, microglia that repopulated the BG over the next 3 weeks re-established the same regional differences in density observed in naïve mice. Together these observations argue that throughout life, local regulatory signals within distinct BG nuclei orchestrate the density and process branching of resident microglia. During aging, inflammatory factors become elevated in the CNS and recent studies show that microglia increase phagocytosis of synapses during presymptomatic stages of Alzheimer's disease. Select BG nuclei are susceptible to neurodegeneration and changes in BG circuit function contribute to decline in cognitive and motor abilities in old age. To explore whether alterations in BG microglia may precede age-induced changes in circuit function, we performed high-resolution analysis of microglial morphology in late middle age (18-month-old) mice. Although region-specific differences in microglial branching complexity are largely maintained, abnormal soma shape and bulbous swellings along cell processes were more prominent in midbrain as compared to forebrain BG nuclei. Similarly, aging-related increases in microglial density were more prominent in midbrain BG nuclei containing dopamine neurons. This is the first report of regional differences in microglial responses to normal aging and these findings raise important questions about whether microglial heterogeneity contributes to cognitive decline and susceptibility to neurodegeneration.

NIDA
Yi He
Visiting Fellow
Neuropharmacology and Neurochemistry
Abstract removed by request of author

NIDA
Xuan Li
Research Fellow
Neuroscience - Neurodegeneration and Neurological disorders
Characterization of cue-activated dorsal striatal neurons during incubation of methamphetamine craving
A key challenge to treat methamphetamine addiction is relapse after abstinence. In rats, cue-induced methamphetamine seeking progressively increases after prolonged withdrawal (30 day), but not short withdrawal (2 days) from extended-access intravenous methamphetamine self-administration. We recently demonstrated that this "incubation of methamphetamine craving" was associated with Fos induction (a neuronal activity marker) in dorsal striatum. Furthermore, blocking Fos expression in dorsal striatum by a D1-family antagonist (SCH23390) decreased incubation of methamphetamine craving. Here, we characterized these cue-activated (Fos-positive) dorsal striatal neurons during incubation of methamphetamine craving. At anatomical and cellular levels, we examined Fos expression in three sub-regions of dorsal striatum (dorsomedial, dorsal center, and dorsolateral striatum) and cell type of the Fos-positive neurons (dopamine D1 versus D2 receptor expressing neurons). We performed RNAscope® in situ hybridization, a highly sensitive multiplex fluorescent assay, and triple-labeled Fos, Drd1 and Drd2 mRNA after cue-induced methamphetamine seeking tests during prolonged withdrawal. We observed that three sub-regions of dorsal striatum expressed similar numbers of Fos-positive neurons, and these neurons were comprised of both D1- and D2-type neurons. At molecular level, we measured mRNA expression of brain-derived neurotrophic factor (Bdnf) and its receptor (Trkb), glutamate receptors and

epigenetic enzymes in Fos-positive and Fos-negative dorsal striatal neurons after cue-induced methamphetamine seeking tests during prolonged withdrawal. We used fluorescent-activated cell sorting (FACS) to isolate Fos-positive and Fos-negative neurons, and assessed mRNA levels by gene targeted pre-amplification and qPCR. Surprisingly, we observed a selective increase of Bdnf and Trkb, glutamate receptors (Gria1, Gria3, Grm1 and Grin2a) and epigenetic enzymes (Hdac3, Hdac4, Hdac5 and Dnmt3a) in Fos-positive neurons compared with Fos-negative neurons. In summary, cue-activated Fos-positive neurons in dorsal striatum are neither sub-region specific nor cell-type specific, but exhibit a distinct molecular profile from non-activated Fos-negative neurons during incubation of methamphetamine craving. Ongoing studies are underway to examine the causal role of persistent changes of candidate gene expression in cue-activated dorsal striatal neurons in incubation of methamphetamine craving.

NIDA
Nathan Marchant
Postdoctoral Fellow
Neuroscience - Integrative, Functional, and Cognitive
In the wrong place at the wrong time: the neurobiology of how context promotes relapse to alcohol seeking after punishment-imposed abstinence
A major issue of alcoholism treatment is relapse rates during abstinence. In humans, environments previously associated with alcohol use often provoke relapse. This phenomenon has been modeled in laboratory animals using the context-induced reinstatement procedure where reinstatement of alcohol seeking is seen when the animal is tested in the original training context (A) after extinction of alcohol seeking in a different context (B). One limitation of this approach is that extinction training does not adequately capture the motivation for abstinence in human alcoholics who typically self-initiate abstinence, despite drug availability, due to the negative consequences of excessive use. We recently adapted the context-induced reinstatement procedure to study context-induced relapse in laboratory rats after abstinence is imposed by negative consequences (footshock punishment). Alcohol preferring P rats were first trained to lever press for alcohol in context A. We then continue alcohol self-administration in a physically distinct context (B) however lever pressing now causes response-contingent footshock. We increase the shock intensity until the rats have completely suppressed alcohol seeking. We tested the rats for context-induced relapse by returning them to context A (without alcohol or shock) and we observed increased alcohol seeking in context A compared to context B. We have taken a multi-disciplinary approach to study the neurobiology of this relapse. Using intracranial micro-injections, we have found that Lateral Hypothalamus (LH), Nucleus Accumbens shell (NAc shell), and Ventral Subiculum (vSub) are all critical neural substrates of this relapse. Using immunohistochemical detection of the neural activity marker Fos in combination with retrograde tracing, we have shown that context-induced relapse is associated with increased activity in NAc shell projections to LH, and vSub projections to NAc shell. Together, these data describe the critical neural circuitry by which alcohol-associated contexts promote relapse to alcohol seeking during abstinence. These results will pave the way for future clinical studies for relapse prevention using pharmaco-therapeutics, and/or repetitive transcranial magnetic stimulation to prevent relapse to alcohol use.

NIDA

Rachel Slack

Postdoctoral Fellow

Neuropharmacology and Neurochemistry

Using Click Chemistry Toward Novel 1,2,3-Triazole-Linked Dopamine D3 Receptor Ligands

Drug overdose death rates have risen steadily, becoming the leading cause of injury-related deaths in 2012. While medications exist for opioid and alcohol addiction, there remains no FDA-approved medication to treat psychostimulant addiction. Psychostimulant-induced behavioral activation and euphoria is caused by enhanced dopamine signaling, making dopamine receptors attractive targets for addiction pharmacotherapy. Drug-dependent subjects show an elevated expression of the D3 receptor (D3R) in the nucleus accumbens, a dopamine-rich brain region responsible for mediating reward and motivation. Furthermore, as D3Rs are minimally expressed in motor regions of the brain, extrapyramidal side effects that decrease compliance and complicate usefulness of the clinically available nonselective dopamine receptor antagonists (e.g. haloperidol) could be avoided. Thus, the development of D3R-selective ligands provides valuable tools for the study of mechanisms underlying addiction and may offer leads toward therapeutics. Although D3R-selective ligands with promising anti-addictive actions in animal models have been developed, metabolic instability has halted their clinical investigation. Many share the established molecular template that connects a 4-phenylpiperazine with an extended aryl ring system via a butylamide linker. This amide bond has proven to be both synthetically convenient and biologically critical for high D3R affinity and selectivity; however, its susceptibility to metabolism may be a limitation. Therefore, we designed a series of compounds where the amide was bioisosterically replaced with a 1,2,3-triazole; this moiety maintains many of the desirable physicochemical properties of the amide but should add metabolic stability. A copper-catalyzed azide-alkyne click reaction allowed for the facile synthesis of 30 novel triazoles. Several had D3R binding affinities in the low nanomolar range and compared favorably to the amide compounds. Indole-containing triazole RDS-02-28, in particular, had high D3R affinity (Ki = 5.85 nM) and excellent D3R selectivity (D2/D3=165). Furthermore, when tested for P450-mediated phase I metabolism in mouse liver microsomes, RDS-02-28 and other triazoles were metabolized slower than related amides. We showed that click chemistry is a convenient synthetic strategy that offers diversity to the traditional D3R ligand template, thereby increasing the armamentarium of tools to study the role of this receptor subtype in addiction.

NIDA

Wendy Xin

Doctoral Candidate

Neuroscience - Cellular and Molecular

Functional Characterization of Astrocytes within Midbrain Dopaminergic Nuclei

Astrocytes are the most abundant cell type in the brain. Through their release of neurotrophic factors and their inflammatory response to injury, they can be both critical and detrimental to neuron survival. Most reports of astrocyte properties have focused on the cortex or hippocampus. However, given the extensive evidence for astrocyte heterogeneity between brain regions, it is clear that a complete understanding of astrocyte biology will require regional characterization. Almost nothing is known about astrocytes in the substantia nigra (SN) and ventral tegmental area (VTA) - two midbrain areas that house

the majority of dopamine (DA) neurons in the brain. Impairments in DA signaling have been implicated in multiple psychiatric and neurological disorders, including substance use disorders and Parkinson's disease (PD). Previous studies provide indirect evidence of DA receptor expression on astrocytes, but this has yet to be directly demonstrated. Thus, we set out to define the basal characteristics of ventral midbrain astrocytes and determine whether they are affected by DA signaling. Immunohistochemistry with Aldh1L1 eGFP mice (in which eGFP is selectively expressed in astrocytes) revealed that VTA and SN astrocytes are much sparser than their hippocampal counterparts and express low levels of traditional astrocyte markers (e.g. GFAP, glutamine synthetase). Whole cell patch clamp recordings from eGFP+ cells revealed significant differences in membrane resistance and potassium conductance between hippocampal and midbrain astrocytes. Since changes in astrocytic calcium levels have been linked to the release of various signaling molecules, we injected GFAP-cre animals with a cre-dependent virus to get preferential expression of the calcium indicator GCaMP6m in astrocytes and imaged calcium activity in acute slices using two-photon microscopy. Bath application of DA receptor agonists altered the pattern of calcium activity, and cocaine (a blocker of DA uptake) produced a transient increase in calcium within these cells. These effects were abolished in the presence of blockers of synaptic transmission. Collectively, these results demonstrate that ventral midbrain astrocytes have unique molecular and electrophysiological properties and can be indirectly influenced by DA signaling. This work provides new insights into midbrain astrocyte function as well as the necessary foundation for future studies looking at the role of astrocytes in addiction and PD.

NIDA
Wen Zhang
Research Fellow
Neuroscience - Neurodegeneration and Neurological disorders
Schizophrenia Susceptibility Gene Dysbindin Maintains Prefrontal Function via BDNF
Schizophrenia is a chronic brain disorder that affects ~ 1% of general population. Current pharmacological treatments offered limited success, especially for improving negative and cognitive symptoms. Unraveling pathological mechanisms of schizophrenia is critical for developing effective therapeutic strategies. Although the pathophysiology of schizophrenia is still unclear, genetic studies have identified substantial number of susceptibility genes. Dysbindin (Dys) is one of the earliest identified susceptibility genes. Genetic variations of Dys have been well replicated as genetic risks associated with schizophrenia, and reduced dysbindin expression has been found common in brain areas of schizophrenia patients. To illustrate the mechanism of Dys on prefrontal function, we injected AAV1-CaMKII-Cre-eGFP virus into the prefrontal cortex (PFC) of Dys conditional knockout mice, a well-established brain region for its involvement in cognition and schizophrenia. We analyzed synaptic transmissions and behavior of virally injected mice. We found that loss of Dys in prefrontal pyramidal neurons significantly reduced GABAergic inhibitory synapses in PFC and led to deficits in pre-pulse inhibition (PPI), a schizophrenia-relevant behavior test. Excitatory synaptic transmission remained undisturbed. Dysbindin is a protein involved in protein trafficking. Using super-resolution optical imaging technique, we found that dysbindin molecules localized in proximity of brain-derived neurotrophic factor (BDNF) well within the size of single vesicles. Then we used total internal reflection fluorescence imaging technique to directly visualize BDNF release from cultured neurons, and found deletion of Dys

significantly reduced activity-dependent BDNF release while the trafficking of synaptic receptors was normal. Utilizing the same viral method we found the same synaptic dysfunction in Bdnf conditional knockout mice as that of Dys mice. We further found that viral overexpression of BDNF in PFC failed to rescue the deficits after Dys deletion, while chronic infusion of BDNF into PFC with osmotic minipumps rescued these deficits and PPI. In summary, our data showed dysbindin played an essential role in maintaining prefrontal cortex function via facilitating activity-dependent secretion of BDNF. Our results identify dysbindin dependent BDNF secretion is a novel pathogenic mechanism for schizophrenia, and suggest BDNF restoration as a novel therapeutic strategy.

NIDCR
Tomoko Ikeuchi
Visiting Fellow
Vascular Disease and Biology
A New Potential Therapy for Age-Related Macular Degeneration (AMD): Anti-Angiogenic Activity of Fibulin-7 and Its Fragments In Vivo
AMD is the leading cause of blindness in patients older than 50 years. The blindness is caused by abnormal angiogenesis of the choroidal vasculature via Brunch's membrane. Fibulin-7 (Fbln7) is a newly discovered member of the extracellular fibulin family proteins, functioning as a cell adhesion molecule and interacting with other ECM proteins and receptors. Fbln7 is expressed in retinal pigment epithelium cells as well as choroidal capillary walls., Using a recombinant protein approach, we previously showed that the C-terminal Fbln7 fragment (Fbln7-C) inhibits tube formation of human umbilical vein endothelial cells (HUVEC) in cultures and blocks vessel sprouting in aortic ring assays in vitro. In this study, we hypothesize that Fbln7-C has anti-angiogenic properties in vivo and could be a potential therapeutic compound for angiogenesis. We studied anti-angiogenic activity of full-length Fbln7 (Fbln7-FL) and Fbln7-C in vivo using a rat corneal model. In the assays, we prepared three types of biodegradable implants, each of which contained a combination of a pro-angiogenic lipid and 7-ketocholesterol (7KCh); the control implant contained nothing more, while the remaining two implant types had an addition of either Fbln7-FL or Fbln7-C. Implants were placed within the anterior chamber of the eye. Fluorescein was injected intraperitoneally 7 and 10 days after implantation, and implants were imaged for angiography. The implant with 7KCh alone started to induce neovascularization from the limbus vasculature in the eye 4 days after implantation, peaking at day 10. The implants with added Fbln7-FL or Fbln7-C inhibited neovascularization in a dose-dependent manner, with stronger inhibition displayed by Fbln7-C. We identified active peptides within the Fbln7-C protein for HUVEC attachment in cell cultures using a synthetic peptide approach. We are now testing these peptides for anti-angiogenic activities in the rat corneal model; our preliminary data show promising results. We are also studying the mechanism of the anti-angiogenic activity of Fbln7-C. Our preliminary data show that Fbln7-C binds to the vascular endothelial growth factor receptor (VEGFR), suggesting that inhibition of VEGF signaling is involved in Fbln7-mediated anti-angiogenic activity. We will further study how the interaction affects endothelial cell, migration, proliferation, and differentation.

NIDCR
Huanyu Xu

Postdoctoral Fellow
Endocrinology
Small cell lung cancer growth is inhibited by miR-342 and its target gene IA-2
Background: Small cell lung cancers (SCLC) are neuroendocrine tumors. Earlier studies from our laboratory showed that SCLC express high levels of IA-2 as compared to normal lung cells. The secretion of hormones from these cells is thought to have an autocrine effect on lung tumor growth. IA-2 is known to be involved in the secretion of hormones and neurotransmitters. Recently, we showed that one of the targets of the microRNA-342 was IA-2 and that miR-342 mimics suppressed the expression of IA-2. Aim: To evaluate SCLC tumor growth by knocking down IA-2 with RNAi or by overexpressing miR-342 with a mimic. Results: Electron microscopic studies confirmed the presence of IA-2 on secretory granules distributed throughout the cytoplasm of the SCLC lines NCI-H82 and NCI-345. Knockdown of IA-2 by RNAi reduced tumor growth within 4 days by 40% or more. Similar results were obtained when these cell lines were transfected with a miR-342 mimic. The knockdown of IA-2 by RNAi or the miR-342 mimic also resulted in a decrease in the secretion of acetylcholine, one of the autocrine hormones secreted by SCLC. Further studies revealed that the growth of SCLC cell lines that had been treated with the miR-342 mimic was restored to nearly normal levels by treatment with acetylcholine. Conclusion: Our studies show for the first time that both miR-342 and its target gene IA-2 are involved in the growth of SCLC tumors and act by their effect on autocrine secretion. These findings point to possible new therapeutic approaches for the treatment of autocrine-induced tumor proliferation.

NIDDK
Peng Chen
Visiting Fellow
Genomics
Identification of Differentially Methylated Genes in Children at High Risk for Type 2 Diabetes due to Intrauterine Exposure
It was first reported in studies of Pima Indians that the offspring of a diabetic pregnancy (children whose mother had type 2 diabetes [T2D] while they were in utero) have extremely high rates of T2D. Studies comparing sibling pairs showed that siblings born after the mother's development of T2D had much higher risk for developing this disease as compared to siblings born prior to the mother's development of T2D, suggesting this effect is not due solely to transmission of T2D genes. Therefore the in utero environment may be responsible for this increase in risk. In the current study, we explore whether DNA methylation is a mechanism whereby epigenetic signals are passed from mother to offspring. Peripheral blood leukocytes from 420 non-diabetic Pima Indians were epigenotyped at 485,577 CpG sites using the Illumina Human Methylation 450K Array. Subjects were selected as being either the offspring of a T2D mother (mother had documented hyperglycemia defined as 2-hr post-load plasma glucose =11.1 mmol/l or fasting plasma glucose =7.0 mmol/l at an exam during the 9 months preceding the child's birth) or the offspring of a non-T2D mother (mother had documented normoglycemia defined as 2-hr post-load plasma glucose <7.8 mmol/l and fasting plasma glucose <5.5 mmol/l during the 9 months prior to the child's birth and had a non-diabetic exam >1 year after the child's birth). Data from 423,343 CpG sites in 388 individuals (N= 187 with exposure and 201 without exposure to intrauterine diabetes) passed all quality control measures. A Logistic Regression model with appropriate adjustments was used

to estimate the association between exposure and methylation extent. Thirty-nine differentially methylated genes achieved epigenome-wide significance after correction for false discovery rate (FDR). A Cox Proportional Hazards model in 303 individuals with follow-up data on diabetes status determined that an intergenic CpG site (maps between FLJ42875 and PRDM16) significantly increased T2D risk (P value = 9.7E-5). This study is the first to report differentially methylated genes in response to intrauterine diabetes exposure at the epigenome-wide significance. Detailed studies of the biologic pathways affected by these differentially methylated genes are ongoing, which will lead to knowledge of the metabolic changes that underlie the epidemiologic observation that maternal diabetes affects diabetes risk in offspring.

NIDDK
Vineet Choudhary
Other
Biochemistry - General and Lipids
A conserved family of proteins facilitates nascent lipid droplet budding from the ER into the cytosol.
Lipid droplets (LDs) are found in almost all cells and play central roles in energy and lipid metabolism; it is also becoming clear that LDs have additional functions, including important roles in protein degradation, ER stress, and viral replication. Unlike all other membrane-bound organelles, LDs are surrounded by a phospholipid monolayer rather than a bilayer. How this unique structure is formed has remained an important unsolved mystery for many years. Quite a number of models of LD biogenesis have been proposed but the simplest and arguably most popular is one that has sometimes been termed the “lens” model. Neutral lipids, triacylglycerols (TAG) and steryl esters (SE) which form the core of all LDs, are synthesized in the ER and it is thought that as these lipids accumulate in the ER bilayer they form a lens (or blister) in the ER that can grow and bud. We visualized nascent LD formation in yeast using electron microscopy (EM)-tomography and found that the lens model is correct. Lens of neutral lipid approximately 30-60 nm wide were found between the two leaflets of the ER bilayer. This is the first time the beginnings of LD biogenesis have been visualized and resolves a long-standing question in LD biogenesis. We next asked whether proteins are necessary to facilitate nascent LD budding from the ER and focused on a conserved family of proteins, called fat storage-inducing transmembrane (FIT) proteins. Yeast has two FITs (called Fit2a and Fit2b) and we found that in cells missing both most LDs are wrapped by the ER membrane and that this is caused by LD budding into the ER lumen rather than the cytosol. The wrapping membrane is not derived from the phagophore or related to autophagy since we found that wrapping still occurred in mutants lacking the FIT proteins and various proteins required for autophagy. The role of FIT proteins in LD budding is conserved; knock down of FIT2 in 3T3 fibroblasts and in C.elegans caused LDs to become wrapped in the ER. Our findings indicated that nascent LDs form lenses in the ER and that FIT proteins are necessary to promote proper budding of LDs from the ER. The mechanism of how FIT proteins control the directionality of LD budding remains to be investigated.

NIDDK
David Libich
Visiting Fellow
Biophysics

The Dark State of GroEL: Direct Demonstration of Intrinsic Foldase/Unfoldase Activity by Solution NMR
Chaperonins, a sub-class of molecular chaperones, are ubiquitous to all three kingdoms of life and are absolutely essential for cellular functions such as signaling, protein transport and immune and stress responses. Despite intensive study, the details of exactly how these machines fold proteins to their correct native state remain elusive. Further confounding the problem are the observations that encapsulation of the substrate protein is not a strict requirement for successful refolding. Thus the question becomes: Exactly how do chaperonins refold proteins? Previous investigations of the prototypical group I chaperonin GroEL, a double ring 780 kDa complex, have theorized at the presence of intrinsic unfoldase activity although the conclusions were drawn from indirect evidence and on poorly defined, heterogeneous systems. Here, a system comprised of apo-GroEL and a monomeric, kinetically well-defined triple-mutant of the Fyn SH3 domain that exists in dynamic equilibrium between the natively folded (major) and a sparsely populated on-pathway intermediate (minor) state was employed to directly demonstrate of the ability of GroEL to accelerate the interconversion between these two states by three orders of magnitude. Simultaneous analysis of three complimentary relaxation-based NMR experiments permit the determination of the catalytic rate constants and ascertain the location of the GroEL binding site on the folding intermediate under conditions where the populations of GroEL-bound native and intermediate states are less than 1%. Further, deuteration of the SH3 domain modulates GroEL foldase/unfoldase activity indicating that catalysis of the exchange between states occurs as a result of the interaction of the substrate with the surface of GroEL. These data unequivocally demonstrate the foldase/unfoldase activity of GroEL and indicate that this function is distinct from the encapsulation cycle. Additionally, these results provide a detailed kinetic description of the 'work' GroEL preforms on a substrate polypeptide to enable it to reach the correctly folded native state. The research described here offers a detailed molecular description of the ability of chaperones to passively fold proteins and thus provide a protective mechanism against protein misfolding - a process at the heart of numerous human diseases.

NIDDK
Paolo Piaggi
Postdoctoral Fellow
Genomics
Genome-wide association study using a custom genotyping array identifies variants in GPR158 associated with reduced energy expenditure and increased Body Mass Index in American Indians
We have previously shown that a lower energy expenditure (EE) predicts long-term body weight and fat mass gain, implying that energy metabolism contributes to the pathogenesis of human obesity. Heritable factors are estimated to explain 40-70% and 10% of the inter-individual variance in body weight and EE, respectively. The aim of this study is to identify genetic variants that affect EE and thereby influence Body Mass Index (BMI) in American Indians, an ethnic group with extremely high rates of obesity and type 2 diabetes. 548206 tag SNPs (minor allele frequency=5%, r2=0.85) derived from whole-genome sequence data of 335 Pima Indians were genotyped using an Affymetrix Axiom Custom Array in a population-based sample of 3494 full-heritage Pima Indians. Data were analyzed for associations with maximum lifetime BMI (n=2762) and 2 separate measures of EE: resting metabolic rate (RMR) after overnight fasting measured by a ventilated hood system (n=498) and 24h EE measured by a

whole room calorimeter (n=407) when subjects were free from diabetes. Results were adjusted for age, sex, body composition and family membership. Rs11014566 (A/G), which maps to an intron in GPR158, demonstrated one of strongest associations for both BMI and EE, where the G allele (frequency=0.60) associated with an increased maximum lifetime BMI (b=1.6% per risk allele,p=6x10-3) and a lower EE as assessed by both RMR (b=-39 kcal/day,p=5x10-4) and 24h EE (b=-37 kcal/day,p=3x10-4) in 2 independent experiments. To assess replication of the BMI association, rs11014566 was further genotyped in 3950 mixed-heritage American Indians, where it associated with BMI in this replication sample (b=2.2%, p=8x10-4) and in the combined data (p=2x10-5). GPR158 encodes the G protein-coupled receptor 158 that binds to N-type voltage-gated calcium channel (CACNA1B) in the brain. CACNA1B-deficient mice are protected from obesity. Rs11014566 tags 3 other SNPs rs144895904, rs34673593, and rs16925884 (r2=0.86-0.99) in intron 4, any of which could play a role in transcriptional regulation. Therefore, an in vitro luciferase assay is currently being conducted to functionally characterize these SNPs. Our results indicate that common variation in GPR158 influences EE and obesity in American Indians. Identification of novel genes/gene pathways that influence EE and BMI in humans will lead to a better understanding of the complex pathophysiology of body weight regulation and could potentially lead to new drug targets

NIDDK
Jessica Pierce
Postdoctoral Fellow
Microbiology and Antimicrobials
Abstract removed by request of author

NIDDK
Mohammad Rahman
Postdoctoral Fellow
Cell Cycle-General, Regulators and Checkpoints, Apoptotic Mechanisms
Plk1 is required for nuclear envelope breakdown and single nucleus formation in C. elegans embryonic cells
During mitosis, nuclear envelope breakdown (NEBD) allows faithful segregation of the duplicated chromosomes. The nuclear envelope (NE) reforms at the end of mitosis, generating a single nucleus in each daughter cell. A special case of NEBD occurs during fertilization, when the NEs of the maternal and paternal pronuclei disassemble in order to allow mixing of the two genomes and the formation of a single nucleus. Polo-like kinase 1 (Plk1) is a conserved kinase involved in multiple steps of mitosis. Plk1 has not been shown to directly regulate NEBD. Moreover, while Plk1 function is studied extensively in somatic cells, little is known about its role in embryogenesis. The C. elegans embryo is an excellent model system for cell cycle studies due to its invariant cell division cycles. In C. elegans, complete inactivation of Plk1 causes failure in meiotic progression. However, we discovered that in embryonic cells of a temperature-sensitive (ts) plk1 mutant grown at a semi-permissive temperature, each cell contained two nuclei. This defect arose from a failure in NEBD as determined by live cell imaging of embryos expressing the nuclear lamina protein, LMN-1:GFP, or a component of the nuclear pore complex, NPP-1:GFP. Our analyses further showed that in the plk1(ts) cells, the initial defect was in the

NEBD of the parental pronuclei. Consequently, the two genomes failed to mix and underwent independent, albeit synchronized, mitoses, resulting in a paired-nuclei that was maintained through numerous cell divisions. These data show that in C. elegans, Plk1 is required for the mixing of parental genomes and for NEBD in subsequent cell divisions. If NEBD defect in plk-1(ts) cells was due to a defect in disassembly of one or more NE components, then a reduction in NE components by RNAi would facilitate NEBD and re-establish a single nucleus in plk1(ts) cells. Indeed, our data show that reducing certain NPC components, e.g., Nup98 or Nup107, rescues the paired-nuclei phenotype in plk1(ts) cells. Moreover, we found that during the first mitosis NEBD is linked to metaphase chromosome alignment, which fails to occur in the plk1(ts) mutant and is rescued by the aforementioned RNAi treatment. Thus, Plk1 may contribute to the first embryonic NEBD by facilitating metaphase chromosome alignment and/or by directly promoting NEBD. In summary, our study uncovered a novel involvement of Plk1 in NEBD and single nucleus formation following fertilization in an intact organism.

NIDDK
Simona Rosu
Postdoctoral Fellow
Cell Cycle-General, Regulators and Checkpoints, Apoptotic Mechanisms
Germ cell proliferation and quality control in the C.elegans germline
In animals, germlines are the tissue that give rise to sperm and egg cells and ensure the continuity of the species. I am using the C.elegans germline as a system to study the mechanisms regulating germline proliferation, as well as the mechanisms of quality control that operate in the production of oocytes. Many questions remain about the operation of the germline stem cell compartment and the control of germline proliferation: where and how large is the stem cell pool? Is there a transit-amplifying population of germ cells? How is the number and rate of cell divisions regulated during germline proliferation? In addition, during the oogenesis program, apoptosis occurs such that only a subset of germ cells become oocytes. How this quality control mechanism operates is not well understood. To gain insight into these questions, I have recently developed a new tool that enables lineage tracing in the C.elegans germline. I have constructed a C.elegans strain containing germline-expressed histone H2B::Dendra. Dendra is a green-fluorescing protein, which, when stimulated by laser light at 405 nm, photo-converts to red fluorescence. This tool allows me to photo-convert chromatin in selected nuclei of germ cells in vivo and follow their fate over long periods of time, as well as the fate of daughter cells arising from cell division. I have shown that I can successfully photoconvert germ cells in the germline, and that the daughters of the photoconverted cells can be followed for many divisions. I am currently determining the lineage of germ cells and the parameters that govern germ cell proliferation and the transition from germ cell to oocyte in wild-type situations. I am also using altered conditions to probe the regulation of these processes. One example of altered conditions occurs in the atl-1 (homolog of ATR, a DNA damage sensing kinase) mutant background. I have found that in the alt-1 mutant: 1) germline expansion is reduced, suggesting an unknown mechanism that reduces proliferation (possibly in response to DNA damage) and 2) germ cells exhibit micronuclei, however, oocytes do not, suggesting a mechanism to either eliminate or remodel these nuclear abnormalities. These studies will advance our knowledge of germline stem cell niches, proliferation and quality control, which are important both in

the context of reproductive health and in informing our understanding of cancer, where misregulation of proliferation and apoptosis are common.

NIDDK
Seung-Wook Shin
Visiting Fellow
Developmental Biology
Dppa3, a maternally derived epigenetic reprogramming factor, is ubiquitinated and partially cleaved in early mouse embryos
After mammalian fertilization, maternal control of gene expression is coordinately transferred to the newly established embryonic program in totipotent 1-cell zygotes. This maternal-to-zygotic transition (MZT) is accompanied by degradation of maternal proteins by the ubiquitin-proteasome system (UPS) and activation of the embryonic genome. However, mechanisms underlying this changeover remain largely unknown. Here, we identify maternal proteins that are regulated by ubiquitin and investigate the effect of UPS cleavage on their localization and function following fertilization. To detect candidate maternal proteins, we isolated and parthenogenetically activated 4000 eggs. Half were treated with epoxomicin, a proteasome specific inhibitor, and half were untreated controls. Ubiquitinated proteins were isolated from each sample with agarose-TUBEs (Tandem ubiquitin binding entities) and differentially labeled with stable-isotope prior to microscale tandem mass spectrometry. A total of 627 proteins were identified including maternal and UPS-associated proteins. In initial studies, we focused on Dppa3 (Developmental pluripotency associated 3), a maternal-effect protein also known as PGC7 or Stella. Dppa3 has been implicated in protecting embryonic DNA from TET3-mediated demethylation and maternal genetic ablation of Dppa3 results in cleavage-stage embryonic lethality. The Dppa3 protein is highly expressed during the MZT, but its abundance dramatically decreases after the 4-cell stage of embryogenesis. We document that Dppa3 is partially cleaved in 2-cell embryos and determine the cleavage sites by Edman degradation after 20S proteasome digestion of purified recombinant Dppa3 protein. Using mutant RNA constructs microinjected into 1-cell zygotes, we demonstrate that the normal export of Dppa3 from pronuclei to cytoplasm is prevented by point mutations that preclude cleavage and result in Dppa3 persistence in the nuclei of 2-cell embryos. These results suggest that after proteasome cleavage, maternal Dppa3 is exported from pronuclei to the cytoplasm, and this mechanism may be essential for loss of DNA methylation and epigenetic chromatin remodeling during cleavage-stage mouse embryogenesis. Currently, we are focusing on the whether the embryonic arrest at 4-cells can be rescued by normal and mutant Dppa3 isoforms and plan to determine epigenetic effects by comparing global DNA methylation patterns of transgenic embryos expressing control and mutant maternal Dppa3.

NIDDK
Jonathan Street
Visiting Fellow
Physiology
Sepsis reduces kidney function in mice before clinical symptoms become apparent, independent of tubuloglomerular feedback

Introduction: Acute kidney injury (AKI) increases the mortality and morbidity of sepsis, a systemic inflammatory response to infection. A rise in serum creatinine is used to detect AKI clinically and experimentally, but the slow kinetics prevents early detection of injury, when novel therapies are most likely to succeed and pathophysiological insights are most likely to be gained. We used a novel transcutaneous measurement of a fluorescent marker (FITC-sinistrin) to detect early decreases in kidney function by continuously monitoring the glomerular filtration rate (GFR). To prevent renal salt wasting during sepsis, renal tubular injury is hypothesized to reduce GFR by activation of tubuloglomerular feedback ("acute renal success"). Because adenosine 1a receptor (A1aR) knockout mice lack tubuloglomerular feedback (TgF), we tested whether TgF contributes to sepsis AKI. Methods: Sepsis was induced in male A1aR knockout mice and littermate controls by cecal ligation and puncture. FITC-sinistrin was injected intravenously at 0 and 90 minutes after surgery, and GFR was monitored for 5 hours; transcutaneous fluorescence was measured in conscious mice by a miniaturized fluorimeter attached to the shaved mouse flank. Results: The baseline GFR was the same in A1aR WT and KO mice. During the first hour following the induction of sepsis the GFR was stable in WT mice. Following a gradual decline over the next hour, the GFR fell rapidly 2 hours after the induction of sepsis, decreasing by 5-fold and remaining low for the duration of the study. In contrast, immediately following the induction of sepsis in KO mice the GFR was lower than in WT mice following sepsis ($p=0.0226$), and lower than at baseline ($p=0.0067$). The GFR then fell gradually, without the sudden decrease observed in WT mice ($p=0.0286$). Conclusions: Transcutaneous fluorescence enables greater temporal resolution in GFR measurement than prior approaches, revealing novel pathophysiology during early acute kidney injury. GFR falls significantly 2 hours after the induction of sepsis, much earlier than when clinical symptoms appear at 6 hours. In mice lacking tubuloglomerular feedback GFR begins to decrease earlier suggesting that tubuloglomerular feedback may initially stabilize GFR following sepsis. Our data does not support the "acute renal success" model, where TgF is supposed to dramatically lower GFR to prevent salt wasting.

NIDDK
Hina Sultana
Visiting Fellow
Developmental Biology

Genes expressed in early oogenesis are enriched in potential Doublesex targets and are important for ovary development

Sex determination is regulated by Doublesex-related transcription factors (DMRTs) in many species including Drosophila melanogaster. Though DSX is a master regulator of somatic sex determination, it does not control sex determination of germ cells directly because it is not expressed there. It is well understood that germline sex determination requires non-autonomous signals from the soma, but these signals have not been identified for female germline. We hypothesize these signals might be downstream of DSX because DSX is expressed in the somatic cells that are adjacent to the germ cells. In most sexually reproducing organisms' germ cells reside in the gonads. In Drosophila melanogaster female gonad consists of a pair of ovaries, each of which consist of 15-30 strings of developing egg chambers known as ovarioles. At the most anterior region of the ovariole is a structure known as germarium that contains germline stem cells (GSCs), which are surrounded by somatic cells forming the

GSC niche. To characterize transcription in the niche, we performed expression analyses using Poly-A RNA-Seq on 12 biological replicates of the anterior ovariole (consisting of germaria and early stage egg chambers). In order to identify the genes enriched in the anterior ovariole, we compared the expression profile of the anterior ovariole with that of the whole ovary and discovered 1317 genes with anterior ovariole biased expression. Next to test if DSX targets might be enriched in this region, we overlaid the list of potential DSX targets in ovary (identified earlier in our lab using DamId-seq) with that of the genes with anterior ovariole biased expression and remarkably found 58 percent of genes with anterior ovariole biased expression occupied by DSX (Fisher's Exact Test, p < 2.2x10-16). We selected 38 putative DSX targets with significantly higher anterior ovariole expression compared to the whole ovary (DESeq adjusted p-value < 0.01) for functional characterization. We performed loss-of-function study using RNAi-mediated knockdown of these genes in somatic cells of the gonad with traffic-jam gal4 and found three genes broad, misshapen and skuld that are specifically required for female but not male gonad development.

NIDDK
Jaira Vasconcellos
Visiting Fellow
Hematology/Oncology, Tumor Immunology, and Therapy
Targeted reduction of let-7a miRNA increases fetal hemoglobin in human adult erythroblasts
Induction of fetal hemoglobin (HbF) has therapeutic importance for patients with sickle cell disease and beta-thalassemia; HbF at levels of 20% or greater is an effective approach toward therapy. MicroRNAs (miRNAs) are a class of small, noncoding RNAs that regulate target messenger RNAs. The let-7 family of miRNAs consists of twelve genes encoding nine highly conserved mature miRNAs. Previous studies showed that let-7 miRNAs levels are regulated during the fetal-to-adult transition in the erythroid lineage with significant increases in let-7 miRNAs levels in adult compared to umbilical cord blood reticulocytes. Further studies indicated that reduced expression of let-7 in adult CD34+ cells by targeting the miRNA family seed region caused increased HbF; however these studies did not address the potential for targeting an individual let-7 miRNA family member to regulate HbF levels. We initially determined the expression levels of the let-7 miRNAs in purified cell populations sorted from human peripheral blood and let-7a was identified as a predominantly expressed let-7 family member in reticulocytes. Therefore, we hypothesized that specifically targeting let-7a may be sufficient to regulate HbF levels. For this purpose, a lentiviral construct that incorporated the tough decoy (TuD) design to target let-7a was compared with empty vector controls. Transductions were performed in human CD34+ cells from five adult healthy volunteers cultivated ex vivo in erythropoietin-supplemented serum-free media for 21 days. Down-regulation of let-7a was confirmed by Q-RT-PCR at day 14 (control: 1.4E+07 +/- 2.4E+06 copies/ng; let-7a-TuD: 1.6E+06 +/- 4.6E+05 copies/ng; p=0.0003). Expression levels of globin genes were evaluated upon let-7a-TuD by Q-RT-PCR with significant increases in gamma-globin mRNA expression levels after let-7a-TuD transduction (control: 1.2E+06 +/- 6.8E+05 copies/ng; let-7a-TuD: 1.1E+07 +/- 4.5E+06 copies/ng; p=0.004). Remarkably, HPLC analyses at day 21 demonstrated robust increases in HbF levels after let-7a-TuD transduction (control: 4.7 +/- 0.6%; let-7a-TuD: 38.2 +/- 3.8%; p=0.00003). Our findings identify the let-7a miRNA as a predominantly expressed let-7 family member in human reticulocytes and that targeted reduction of let-7a in erythroblasts is sufficient to cause robust

increases in gamma-globin mRNA expression and HbF levels, which may be useful for therapeutic induction of HbF in patients with hemoglobinopathies.

NIDDK
Yuchen Xia
Visiting Fellow
Virology - DNA
Hepatitis B virus infection of human stem cells-derived hepatocyte-like cells
Background and aim: Hepatitis B virus (HBV) remains a major public health threat with more than 350 million people chronically infected worldwide. The major obstacle of HBV research is the lack of efficient cell culture system or readily available small animal model, permissive for viral infection and replication. Primary human hepatocytes (PHHs) have been used for many years as an in vitro model, but the availability and donor variability restrict its application. Other cell culture models like HepaRG and NTCP-expressing hepatoma cell lines are not optimal due to the transformed nature of those cells. The aim of our study was to establish a novel HBV infection model by using human stem cells-derived hepatocyte-like cells (HLCs). Methods: HLCs were differentiated from human embryonic stem cells. Maturation of hepatocyte functions was evaluated by the expression of albumin, alpha-fetoprotein (AFP) and hepatocyte nuclear factor 4a(HNF4a). Differentiated HLCs and PHHs were infected with infectious HBV produced and concentrated from HepG2.2.15 cell culture supernatant. Three antivirals, entecavir, interferon-a and Myrcludex-B were tested. HBV total DNA, cccDNA, total RNA, pgRNA HBeAg, HBsAg were measured at different time points. Host innate immune response were determined by qPCR of different cytokines and interferon-stimulated genes (ISG). Results: Differentiated HLCs presented key features of PHHs: increased albumin and HNF4a production and decreased AFP expression. Similar to PHHs, all HBV replication markers were elevated during the first 14 days of infection in HLCs. HBsAg staining revealed about 30% of the cells were infected. Entecavir, interferon-a or Myrcludex-B treatment significantly reduced HBV infection. In contrast to hepatitis C virus, HBV infection induced little type I or type III interferon and their downstream genes in HLCs and PHHs, which favors the concept of HBV being a “stealth virus”. Conclusion: Stem cells-derived HLCs fully support HBV infection. Their close resemblance to PHHs makes them suitable for many applications including drug development and virus-host interaction studies.

NIDDK
XIAONAN ZHAO
Postdoctoral Fellow
DNA-binding Proteins/Receptors and DNA Repair
DNA repair gone wrong? The roles of the mismatch repair complexes MutSbeta and MutSalpha in repeat expansion in a mouse model of the Fragile X-related disorders
The Repeat Expansion Diseases are a group of more than 20 human genetic disorders that arise from expansion of a single tandem repeat tract. The Fragile X (FX)-related disorders (FXDs) are members of this disease group in which the repeat unit is CGG/CCG and the repeat tract is located in the 5’ UTR of the FMR1 gene. The individual strands of most of the disease-associated repeats, including the FX repeats, form hairpins that are thought to trigger expansion. The mismatch repair complexes MutSbeta

and MutSalpha normally protect the genome against microsatellite instability. In this study, we investigated the role these proteins play in repeat expansion, a specialized form of microsatellite instability. We first carried out in vitro studies of the interactions of the individual strands of FX repeats with purified human MutSbeta and MutSalpha. Using electrophoretic mobility shift assays we showed that both MutS complexes bind to both CGG-and CCG-hairpins. These data raise the possibility that both complexes could potentially interact with the expansion substrates in vivo and thus affect FX repeat instability. A thermal denaturation assay of hairpins containing 10 FX repeats showed that both MutS complexes stabilize the hairpins formed by the CCG-rich strand thus providing the first biochemical evidence that MutS complexes can facilitate the CCG-hairpin formation. No effect of either protein was seen on the stability of the CGG-hairpins since these hairpins are stable well above the denaturation temperature of MutS complexes. We then carried out the genetic studies by crossinga FXD mouse model with mice containing mutations in these proteins. We found that MutSbeta is actually required for 98% of germ line expansions and all somatic expansions. It also affects the average size but not frequency of repeat contractions. In contrast, MutSalpha protects the genome against both expansions and contractions. These findings demonstrate that both two MutS complexes are involved in the repeat instability, however they have very different effects. We are now examining other aspects of the binding of these proteins to the repeats and to other protein interacting partners in order to understand why these complexes behave so differently and why MutSbeta which normally protects the genome against microsatellite instability, is actually responsible for repeat expansion.

NIDDK
LU ZHU
Visiting Fellow
Physiology

Beta-arrestin2 is essential for the proper function of pancreatic beta cells

Beta-arrestins (barr1 and barr2) are G protein-coupled receptor (GPCR)-associated proteins that function to dampen GPCR signaling but can also act as scaffolding proteins to facilitate G protein-independent signaling. Studies with whole body barr2 KO mice have shown that barr2 plays a critical role in maintaining euglycemia. However, it remains unclear which specific cell types and signaling pathways are involved in this phenomenon. To gain insight into the function of barr2 in pancreatic beta cells, we generated beta cell-specific barr2 knockout mice (beta-barr2 KO mice). In vitro studies with barr2-deficient islets showed that glucose- and KCl-stimulated insulin release and increases in intracellular calcium levels were greatly reduced in the mutant islets. However, the lack of barr2 had no significant effect on islet morphology, total pancreatic insulin content, and beta cell mass. In vivo studies demonstrated that beta-barr2 KO mice maintained on a high-fat diet showed greatly reduced glucose tolerance, associated with a dramatic reduction in insulin secretion. Interestingly, a recent study showed that inhibition of CaMKII in beta cells resulted in phenotypes that closely mimicked those displayed by the beta-barr2 KO mice. We therefore examined whether barr2 is required for CAMKII function in beta cells. Treatment of WT islets with a CaMKII inhibitor (AIP2) led to similar deficits in insulin secretion and calcium influx as observed with beta-barr2 KO mice. Furthermore, expression of a constitutively active version of CaMKII in MIN6 beta cells fully rescued the deficits in insulin secretion observed after barr2 knockdown, while expression of a dominant negative version of CaMKII mimicked the impairment in

insulin release displayed by barr2-deficient MIN6 cells. We also found that the activity of L-type calcium channels was impaired in barr-2 deficient islets, consistent with the known role of CAMKII to promote the activity of these channels. Taken together, these observations strongly suggest that barr2 is required for CAMKII activity in beta cells, probably by acting as a scaffolding protein for CAMKII. Our findings convincingly demonstrate that barr2 is essential for the proper function of pancreatic beta cells. Strategies aimed at enhancing barr2 activity in pancreatic beta cells may prove useful to improve beta cell function for therapeutic purposes (e.g. in type 2 diabetes).

NIEHS
Amanda Conway
Postdoctoral Fellow
Stem Cells - General
GABPa positively regulates transcriptional circuitry controlling ESC identity by direct hindrance of a repressive chromatin-modifying complex
Embryonic stem cells (ESCs) can self-renew indefinitely and differentiate into all derivatives of the three germ layers, making them an attractive model for regenerative medicine and disease modeling. While many transcription factors and molecules involved in chromatin and epigenetic modifications have been found to be critical for ESC identity, their interplay is less understood. Here, we find that the Ets transcription factor GABPa is essential for ESC maintenance, and GABPa associates with and negatively regulates a repressive chromatin-modifying complex. Using RNAi, we determined that depletion of GABPa causes ESC differentiation characterized by flattened cellular morphology, down-regulation of pluripotency markers Oct4 and Nanog, and up-regulation of lineage-specific markers Gata3 and Cdx2. ChIP-seq studies revealed GABPa binding at the promoters of transcriptionally active genes, which positively correlates with RNA Pol II occupancy and active H3K4me3 histone mark. Importantly, we found that RNAi-mediated loss of GABPa leads to reduced H3K4me3 levels and down-regulated gene expression, suggesting that GABPa directly activates its target genes. To gain insights into the mechanism by which GABPa controls gene expression in ESCs, we performed immunoprecipitation followed by mass spectrometry (IP/MS) to identify GABPa-interacting proteins. We discovered that GABPa interacts with subunits of the Sin3b/HDAC complex, the H3K4me3-specific demethylase Jarid1a, the H3K4me3 reader GataD1, and the transcriptional repressor Emsy; all of which together form a previously identified chromatin-modifying complex. Consistent with our IP/MS results, ChIP-Seq analyses revealed co-localization of GABPa with Jarid1a and Sin3b at active promoters. Interestingly, in contrast to GABPa depletion, RNAi-mediated knockdown of Sin3b, Jarid1a, GataD1, or Emsy does not cause ESC differentiation and instead leads to elevated target gene expression. These results suggest that GABPa and its binding partners have opposing functions on chromatin and that GABPa may directly hinder the repressive complex. Indeed, combined RNAi-mediated depletion of GABPa and Emsy partially restored the differentiation phenotype and target gene expression. Altogether, our findings establish GABPa as a regulator of ESC identity by direct hindrance of a repressive chromatin-modifying complex.

NIEHS
Shannon Farris
Postdoctoral Fellow

Gene Expression

Plasticity in Hippocampal Area CA2: Lost in Translation?

In eukaryotic cells, the localization of mRNA to subcellular compartments is highly regulated to afford tight spatial and temporal control over gene expression. In neurons, a subset of mRNA is localized to the neuronal dendrites, specialized compartments extending for up to hundreds of microns away from the nucleus. In response to local cues such as growth factors, proteins can be synthesized locally to modify connections between neurons, a process termed synaptic plasticity. Local protein synthesis is thought to play an essential role in the brain throughout development and during learning. This process is disrupted in neurodevelopmental disorders such those in the autism spectrum. In fact, drugs targeting protein synthesis pathways have improved neurological symptoms in mouse models of autism and autism patients. Our lab studies the function of hippocampal area CA2, a region that does not undergo typical forms of synaptic plasticity thought to underlie learning. Currently, nothing is known about the transcripts localizing to CA2 dendrites. We hypothesize that CA2 neurons express unique transcripts that are trafficked into dendrites to locally regulate the lack of plasticity there. In order to isolate CA2 neuronal cell bodies from their dendrites, as well as from surrounding hippocampal subregions, we used fluorescence-assisted laser capture microdissection and transgenic mice that express green fluorescent protein (GFP) selectively in CA2 neurons. We captured and pooled 100- 8 micron-thick sections of the cell body or dendritic layers from each mouse (N=3, male, 6 weeks old) and extracted the RNA for RNAseq and whole transcriptome microarray. Transcript localization was verified using multiplexed single molecule fluorescent in situ hybiridization. Interestingly, we found a number of transcripts present at high levels in CA2 dendrites involved in calcium handling, such as Pcp4 and S100b, both of which are known to regulate calcium signaling, a critical step in plasticity. CA2 dendrites were also enriched for transcripts that regulate translation. For example, the small noncoding RNA, Bc1, which represses translation, was found at very high levels in CA2 dendrites. Future studies will compare CA2 dendritic expression to the surrounding hippocampal subregions, CA1 and CA3. Determining the transcripts present in CA2 dendrites will provide vital insight into which molecules might play a role in limiting CA2 synaptic plasticity and its role in learning.

NIEHS

Kristin Gabor

Postdoctoral Fellow

Clinical and Translational Research

Smith-Lemli-Opitz Syndrome Reveals Requirement for Sterol Biosynthesis in the Innate Immune Response

Smith-Lemli-Opitz Syndrome (SLOS) is a rare developmental disorder caused by mutation of 7-dehydrocholesterol (7DHC) reductase (DHCR7), a terminal enzyme in the cholesterol biosynthesis pathway. SLOS is associated with cholesterol deficit and 7DHC excess. SLOS patients have increased incidence of infections, but no mechanism of immunosuppression has been identified. Toll-like Receptors (TLRs), key pathogen-recognition receptors of the innate immune system, localize to cholesterol-rich lipid raft membrane microdomains. We predicted that SLOS cells would have attenuated TLR signaling due to deficient raft cholesterol. To address this, a panel of primary dermal fibroblasts from SLOS patients with varying degrees of deficit in DHCR7 activity was used to assess the TLR4 response to lipopolysaccharide (LPS). Upon stimulation, significantly lower NF-kB activation and

interleukin (IL)-6 and IL-8 production were observed in SLOS cells than in wild-type controls. Across patients, a direct relation between residual DHCR7 enzyme activity and IL-6 production was observed, together with an inverse relation between clinical severity scores and IL-8 production. Microarray analysis revealed that several pro-inflammatory chemokines associated with activated TLR4 signaling are less robustly induced by LPS in SLOS fibroblasts. The signaling deficiency in SLOS cells upon LPS stimulation is selective, as normal cytokines are produced in response to both TNFa and the TLR3 ligand poly(I:C). Deficient LPS-induced cytokines were also observed in both macrophages from Dhcr7 mutant mice and a pharmacological model of SLOS that we generated by using RAW264.7 macrophages treated with DHCR7 inhibitors. Supporting cholesterol deficit and not 7DHC excess as the cause of the TLR4 impairment, fibroblasts from a lathosterolosis patient (mutant for the enzyme one step upstream in the cholesterol pathway that produces 7DHC substrate for DHCR7) had defective LPS responses that were partially rescued by cholesterol loading. Mechanistically, confocal microscopy revealed that TLR4 colocalization with lipid rafts is decreased in SLOS macrophages. Taken together, these findings indicate that the SLOS mutation confers an abnormal TLR4 response that may arise from deficient raft cholesterol. We propose that deficient innate immunity may be a clinically relevant contributor to SLOS pathogenesis, and that SLOS highlights the importance of cholesterol biosynthesis to innate immunity.

NIEHS
Bo He
Postdoctoral Fellow
Signal Transduction - General
Human Glucocorticoid Receptor beta (hGRbeta) Has Unique Transcriptional Activity in Mouse Liver
Glucocorticoids govern diverse physiological processes and exert their effects through the classic glucocorticoid receptor (GR) alpha in many tissues and organs, such as the liver. The human GR exists as two alternative splice variants, hGRalpha and hGRbeta. Hepatic GRalpha has been shown in vivo to be critically involved in the regulation of liver metabolism and inflammatory response. The in vivo function of hGRbeta, however, is poorly understood. In vitro studies indicate that hGRbeta, unlike hGRalpha, is unable to bind glucocorticoids yet resides exclusively in the nucleus, where it harbors intrinsic gene regulatory activity. Moreover, when co-expressed with GRalpha, GRbeta acts as a dominant-negative inhibitor of GRalpha and elicits glucocorticoids resistance. These in vitro observations suggest that GRbeta serves as a homeostatic mechanism to modulate cellular responses to glucocorticoids. To investigate the role of hGRbeta in vivo, we created an Adeno-Associated Virus (AAV) to express hGRbeta under the control of the hepatocyte-specific alpha1-antitrypsin promoter to achieve hepatocyte-specific hGRbeta expression (AAV-GFP was used as a control). Our approach successfully achieved hGRbeta specific expression in the livers of 3-month-old C57BL/6 mice. The AAV-hGRbeta-injected liver displayed normal function. Immunofluorescence staining revealed that hGRbeta resides in the nucleus of hepatocytes, consistent with in vitro studies. Genome wide expression analysis comparing gene expression profiles between AAV-hGRbeta-injected and AAV-GFP-injected mice livers showed that hGRbeta significantly altered the expression of numerous genes, many of which are involved in the inflammatory response and infectious diseases. RT-PCR confirmed hGRbeta significantly up-regulated the expression of several immunomodulatory genes, such as signal transducer & activator of transcription 1 (STAT1), suppressor of cytokine signaling1 and some interferon-stimulated genes.

Strikingly, injection of AAV-hGRbeta into GR liver knockout mice also significantly increased the expression of these genes, including STAT1, which was reflected by the binding of hGRbeta to a glucocorticoid response element downstream of the STAT1 gene. Furthermore, treatment with RU486, previously shown to bind to hGRbeta, inhibited the up-regulation of STAT1 mediated by hGRbeta. In summary, our data indicate for the first time that hGRbeta can regulate gene expression in vivo in a GRalpha-independent fashion.

NIEHS
Ashutosh Kumar
Visiting Fellow
Pharmacology and Toxicology/Environmental Health
Formation and Implications of Alpha-synuclein Radical Formed in Maneb- and Paraquat-induced Models of Parkinson's Disease

Parkinson's disease (PD) is a progressive neurodegenerative disease characterized by the selective and progressive loss of dopaminergic neurons. Though PD is multifactorial, several epidemiological reports show an increased incidence of PD with co-exposure to pesticides such as maneb and paraquat. Free radicals have been implicated in pesticide-induced PD, but how they affect the process of neurodegeneration is poorly understood. Various reports suggest free radical-mediated formation of alpha-synuclein aggregates as a central mechanism of PD pathogenesis; however, the mechanism that initiates and promotes intraneuronal alpha-synuclein aggregation, remains unknown. We hypothesized protein radical formation as an initiating mechanism for alpha-synuclein aggregation. Therefore, we used the highly sensitive immuno-spin-trapping technique to investigate protein radical formation as a possible initiating mechanism of alpha-synuclein aggregation as well as to investigate the underlying causes of protein radical formation in the midbrains of maneb and paraquat co-exposed mice. Co-exposure to maneb (30 mg/kg bw, ip) and paraquat (10 mg/kg bw, ip) for 6 weeks resulted in active microgliosis, NADPH oxidase activation, and iNOS induction. Concurrent NADPH oxidase activation and iNOS induction leads to formation of peroxynitrite. Peroxynitrite decomposes to the reactive free radicals •OH, CO3•- and •NO2,which induced radical formation on alpha-synuclein. Results obtained with immuno-spin-trapping and immunoprecipitation experiments confirmed formation of alpha-synuclein radicals in dopaminergic neurons, which preferentially die during PD pathogenesis. Alpha-synuclein radical formation requires NADPH oxidase and iNOS, as indicated by decreased alpha-synuclein radical formation in mice treated with inhibitors such as FeTPPS (a peroxynitrite decomposition catalyst), 1400W (an iNOS inhibitor), or apocynin (NADPH oxidase inhibitor) and in knockout mice (P47phox-/- and iNOS-/-). A concurrence of alpha-synuclein radical formation with dopaminergic neuronal death in maneb and paraquat co-exposed mice indicated a link between protein radicals and PD pathogenesis. Taken together, these results show for the first time the formation and detection of the alpha-synuclein radical and suggest that NADPH oxidase and iNOS play roles in peroxynitrite-mediated alpha-synuclein radical formation and subsequent neuronal death in the midbrains of maneb and paraquat coexposed mice.

NIEHS
Rui Liu

Research Fellow
Epidemiology/Biostatistics - Prognosis and Response Predictions
Ambient Air Pollution Exposure and Risk of Parkinson Disease
Air pollution is a complex and dynamic mixture consisting of particulate matter (PM), gases, organic components, and metals. Numerous studies have shown deleterious effects of air pollution on human health. Ambient PM10 (<10 microns in diameter), PM2.5 (<2.5 microns in diameter) and nitrogen oxides are among the top air pollutants that have been associated with increased risk of pulmonary and cardiovascular diseases. Limited evidence now suggests that chronic exposure to high levels of ambient air pollution may also be linked to neurodegenerative diseases. However, few studies have systematically evaluated the effects of major air pollutants on risk of Parkinson's disease (PD) with the state-of-art exposure assessment. We investigated the association between ambient PM10, PM2.5, and nitrogen dioxide (NO2) exposures and PD risk in the Parkinson's, Genes and Environment study, a nested case-control study in a large prospective cohort of U.S. older adults. We geocoded baseline (1995-1996) home addresses for 1,556 self-identified PD cases and 3,313 age-, sex-, and race-matched controls. All diagnoses occurred in or after 1995. Average ambient PM10, PM2.5 and NO2 concentrations were estimated for year 1990, 2000, and 2006, respectively, using a national land-use/kriging model incorporating roadway information. Air pollutant exposures were categorized into quintiles and multivariate odds ratios (OR) and 95% confidence intervals (CI) were estimated by logistic regression adjusting for age, sex, race, education, smoking status, caffeine intake, and physical activity. Overall, we observed no statistically significant association between PM or NO2 exposures and PD risk. However, in pre-planned subgroup analyses among women or never smokers, PM10 concentration was positively associated with the risk for PD. Compared to the lowest quintile (Q1), the OR for the highest quintile (Q5) was 1.65 (95%CI:1.11-2.46, p-trend=0.02) among women and 1.38 (95%CI:1.01-1.89; p-trend=0.04) among never smokers. In post-hoc analyses among female never smokers, both PM2.5 (OR for Q5vs.Q1=1.78; 95%CI:1.00-3.15; p-trend=0.05) and PM10 (OR for Q5vs.Q1=2.32; 95%CI:1.27-4.21; p-trend=0.01) showed positive associations with PD risk. Our findings suggest that high exposures to PM2.5 and PM10 may be associated with higher risk for PD in female never smokers. Further research is needed to investigate potential neurodegenerative effects of air pollution and possible underlying mechanisms.

NIEHS
Julie Lowe
Postdoctoral Fellow
Cell Cycle-General, Regulators and Checkpoints, Apoptotic Mechanisms
The novel p53 target Tumor Necrosis Factor-a-Induced Protein 8 variant 2 is increased in human cancers and can offset p53-dependent tumor suppression
Tumor Necrosis Factor-a-Induced Protein 8 (TNFAIP8) is a stress-response gene that has been associated with cancer, but no studies have differentiated among nor defined the regulation or function of any of its six recently described expression variants. Here, we specifically identify TNFAIP8 variant 2 (v2) as a gene product that promotes cancer through complex, reciprocal regulatory interactions with p53. Using variant-specific primers and RNA-seq analysis, we determined that v1 and v2 are the predominantly expressed variants of TNFAIP8 and that they are differentially expressed in many types of human

cancers. Specifically, v2 was overexpressed in seven of ten human tumor types that we surveyed in The Cancer Genome Atlas as well as in 5 different cancer cell lines, whereas v1 was typically downregulated. Silencing of v2 in cancer cells induced a dramatic reduction in DNA replication, as indicated by BrdU incorporation. This was p53-independent as it was also observed in p53-deficient cells. Consistently, PCNA expression was significantly lower in v2-depleted p53-sufficient and –deficient cells compared to controls. v2 depletion also resulted in widespread binding of p53 to target genes, p53 target gene (p21, Gadd45A) induction, cell cycle arrest, senescence, and senescence-associated secretory phenotype (IL-6 induction). p53 was required for v2 depletion-induced cell cycle arrest and senescence, as neither event occurred upon v2 silencing in p53-deficient cells, nor were the p53 targets p21, Gadd45A, or IL-6 induced. Moreover, cell cycle arrest induced by v2 depletion was identified to operate through the p53 target p21, as it was averted in p21-silenced cells. We also found that v2-depleted cells were highly sensitive to chemotherapy-induced DNA damage (higher annexin V staining and caspase-3/7 activation in response to staurosporine and the chemotherapeutic doxorubicin) in a p53-dependent manner. In response to doxorubicin, v2 was induced in cancer cells in a p53-dependent manner that involved p53 binding to an intragenic enhancer region brought into proximity of the v2 promoter via chromatin looping. Taken together, we propose that TNFAIP8 v2 and p53 function in reciprocal feedback loops, and that TNFAIP8 v2 may contribute to both carcinogenesis and chemotherapeutic resistance by broadly repressing p53 activation and tumor suppression. Our studies suggest potential for TNFAIP8 v2 as a novel molecular target in cancer therapy.

NIEHS
VIJAY MORE
Visiting Fellow
Pharmacology and Toxicology/Environmental Health

A lipid-sensing transcription factor, peroxisome proliferator activated receptor alpha, regulates blood-brain barrier efflux transporter expression and transport activity

The blood-brain barrier (BBB) is a network of specialized microvessels with tight intercellular junctions and high expression of membrane drug efflux transporters including P-glycoprotein (Pgp/Abcb1), multidrug resistance associated protein 2 (Mrp2/Abcc2) and breast cancer resistance protein (Bcrp/Abcg2). These transporters are major obstacles to the delivery of therapeutic drugs to the brain. Peroxisome proliferator activated receptor alpha (Ppar-a) is a lipid sensor and a master regulator of lipid metabolism. The fibrate class of drugs, endogenous free fatty acids and certain persistent environmental pollutants, e.g., perfluoroalkyl flame-retardants, are Ppar-a ligands. Here, we define a role for Ppar-a in regulating the expression of P-glycoprotein, Bcrp and Mrp2 at the BBB. Exposing isolated rat brain capillaries to 25 and 50uM clofibrate significantly increased protein expression and transport activity of all three transporters. Exposing rat brain capillaries to 10 uM linoleic acid, 1nM perfluorooctane sulfonate (PFOS) and 0.1nM perfluorononanoic acid (PFNA) also increased transport activity by two fold. The Ppar-a antagonist, GW6471, blocked the effects of clofibrate, linoleic acid, PFOS and PFNA. Rats treated with 200mg/kg of clofibrate significantly decreased brain accumulation of the P-glycoprotein substrate, verapamil by 50%. Moreover, fasting C57Bl/6 mice for 24 hours significantly increased serum lipids and BBB P-glycoprotein transport activity measured ex vivo. Fasting did not alter BBB P-glycoprotein activity in Ppar-a knockout mice. These results, implicating Ppar-a in the regulation of P-

glycoprotein, Bcrp, and Mrp2 at the BBB lead to three important conclusions. First, as fibrates are highly prescribed in metabolic syndrome, our results suggest reduced delivery to the brain of drugs that are substrates for these efflux transporters in patients taking fibrates. Second, we show increased transporter activity in response to nanomolar and subnanomolar concentrations of PFOS and PFNA respectively; indicating that occupational exposure to these flame-retardants could alter BBB properties. Finally, we show for the first time that Ppar-a activation is a mechanistic link between fasting-induced rise in serum fatty acids and altered BBB function. Thus, pharmacological, toxicological and physiological activation of Ppar-a increases drug efflux transport activity at the BBB, reducing drug delivery to the brain.

NIEHS
Barbara Nicol
Visiting Fellow
Developmental Biology
New Insights into the Maintenance of Somatic Cell Identity in the Mouse Fetal Ovary
Identity of the gonads depends upon the fate of somatic cell precursors to differentiate into ovarian granulosa cells or their male counterpart Sertoli cells. In the ovary, establishment and maintenance of granulosa cell identity is a coordinate event driven by multiple factors. In the mouse, inactivation of Foxl2 leads to transdifferentiation of ovarian granulosa cells into testis-specific Sertoli cells. Surprisingly, this sex reversal only occurs postnatally. We hypothesized that in the fetal ovary, granulosa cell identity is acquired and maintained by the complementary action of other pro-ovarian genes in addition to Foxl2. Runx1 gene was a good candidate because it is an ortholog of runt, a gene essential for ovarian determination in Drosophila. RUNX1 belongs to a family of transcription factors involved in cell lineage determination and is expressed in the fetal granulosa cells. Such characteristics led us to speculate that RUNX1 is involved in granulosa cell differentiation. To address this hypothesis, Runx1 was specifically ablated from the somatic cell lineage of the fetal ovaries using the Sf1-Cre mouse model. We compared ovarian differentiation in wild type, Runx1 and Foxl2 single knockout, and Runx1/Foxl2 double knockout ovaries. While granulosa cell identity was maintained during fetal life in Runx1 or Foxl2 single knockout ovaries, the somatic cells in the Runx1/Foxl2 double knockout ovaries were sex-reversed to male specific Sertoli cells. This observation demonstrates that RUNX1 and FOXL2 play redundant roles in maintaining fetal granulosa cell identity, possibly through the repression of Sertoli cell program. If this is the case, a forced expression of FOXL2 or RUNX1 in Sertoli cells in the fetal testis should transform Sertoli cells into granulosa cells. We therefore created a Rosa26-STOP-Foxl2 mouse model that allows us to induce ectopic FOXL2 expression in the somatic cell lineage of the fetal testis (the RUNX1 mouse model is under development). Upon ectopic induction of FOXL2 expression in the fetal testis, Sertoli cells lost their identity and transdifferentiated into granulosa cells, resulting in sex reversal. Altogether, these results led to the identification of a new factor, Runx1, in the maintenance of granulosa cells identity and bring to light the ability of Foxl2 to drive and maintain fetal granulosa cell identity by suppressing the program for Sertoli cell differentiation.

NIEHS
Clinton Orebaugh

Postdoctoral Fellow
Genetics
Repair of Ribonucleotides Incorporated into the Nascent Lagging Strand
Ribonucleotides are the most abundant lesion incorporated into the genome during nuclear DNA replication. The vast majority of these ribonucleotides are repaired via the RNase H2-dependent Ribonucleotide Excision Repair (RER) pathway. Deletion of the RNase H2 gene in Saccharomyces cerevisiae results in persistent ribonucleotides in genomic DNA. Mutations have been introduced into the DNA polymerase alpha (Pol a), delta (Pol d) and epsilon (Pol e) genes in RNase H2-deficient strains that promote increased ribonucleotide insertion. Ribonucleotides inserted by a variant DNA replicase in these strains serve as biomarkers for the activity of that polymerase. By fragmenting genomic DNA at ribonucleotides with alkali in these strains and subsequently mapping the DNA ends by next generation sequencing we have developed a technique named Hydrolytic End-sequencing, or HydEn-seq. With this new technology in hand we have mapped Pol a, Pol d and Pol e DNA polymerase activity across the nuclear genome in a strand-specific manner. We identified origins of replication by polymerase strand transitions, confirming several hundred origins that were previously demonstrated and revealing dozens of origins that were previously undiscovered. By combining these analyses we have confirmed our previous finding that Pol e predominately replicates the leading strand and Pol a and Pol d replicate the lagging strand across the entire yeast genome. In concurrent work, we have evidence suggesting that topoisomerase I (Top1) repairs ribonucleotides in an error-prone backup pathway to RER in a leading strand specific manner. Several hypotheses for this specificity include recruitment of Top1 to positive supercoils in the leading strand that do not form in the lagging strand. Another possibility is that the lagging strand mutator polymerase alleles do not incorporate enough ribonucleotides to illicit Top1 activity. In order to test the latter hypothesis, new Pol a and Pol d polymerase mutator alleles that incorporate even more ribonucleotides than those tested previously were cloned into RNase H2-deficient yeast strains in the absence and presence of Top1. Rates of mutation and specificity were determined in a CAN1 forward mutation assay and HydEn-seq libraries analyzed for each of the genotypes toward the goal of determining the role of Top1 in repairing misincorporated ribonucleotides in the lagging strand.

NIEHS
Sonika Patial
Research Fellow
Signal Transduction - General
Genetic deletion of an instability motif in the 3'-untranslated region of tristetraprolin (TTP) mRNA increases TTP mRNA stability and protein expression and protects against immune-mediated inflammatory diseases
Tristetraprolin (TTP) is an mRNA binding protein that binds to AU-rich elements (AREs) in 3'-untranslated regions (3'UTR) of specific mRNAs, such as that encoding tumor necrosis factor (TNF), and increases their rate of turnover. TTP deficiency in mice leads to a systemic inflammatory syndrome characterized by polyarticular arthritis, dermatitis, and myeloid hyperplasia, due in part to increased stability of Tnf mRNA, resulting in an unregulated increase in TNF production. In addition to TNF, other inflammatory mediators including IL23, IL10, GM-CSF, IL3, IL12, IFN?, CXCL1, and CXCL2 have been identified recently

as TTP targets, suggesting a complex endogenous anti-inflammatory role for TTP. Therefore, we hypothesized that increasing endogenous levels of TTP in an intact animal might protect against immune and inflammatory diseases. However, early attempts to generate transgenic mice expressing TTP from strong general promoters inevitably led to prenatal mortality. In an alternative approach, we deleted a 136 base instability motif in the 3'UTR of the TTP mRNA in the endogenous genetic locus, using knock-in technology, anticipitating that this would result in increased stability of the otherwise highly labile TTP mRNA, and result in modest increases in TTP mRNA and protein levels. These mice, termed TTP?ARE mice, appeared normal, had equivalent growth rates to WT mice, and had no apparent clinical, anatomical or histopathological abnormalities. Bone marrow-derived macrophages and mouse embryonic fibroblasts isolated from TTP?ARE mice exhibited increased TTP mRNA stability and increased levels of TTP protein. TTP protein expression was moderately increased in selected tissues. LPS-induced expression of TTP target mRNAs, particularly Tnf, Il-1ß, and Cxcl2 mRNA, was significantly decreased in TTP?ARE macrophages. Most importantly, TTP?ARE mice were essentially completely protected from collagen antibody-induced arthritis, and exhibited reduced inflammation in an imiquimod-induced psoriasis model. These data have translational implications, raising the possibility that increasing TTP levels in humans might be an attractive therapeutic approach in some immune-mediated inflammatory conditions. Preliminary screens have uncovered a set of small molecules that can induce TTP expression without increasing TNF levels, suggesting that such compounds might represent therapeutic options in the treatment of inflammatory diseases.

NIEHS
Matthew Schellenberg
Visiting Fellow
Protein Structure/Structural Biology
Abstract removed by request of author

NIEHS
Alisa Suen
Doctoral Candidate
Carcinogenesis
Aberrant uterine SIX1 expression may promote uterine adenocarcinoma following neonatal xenoestrogen exposure
Human exposure to estrogenic chemicals during sensitive time points in development can result in reproductive diseases. However, the mechanisms underlying these disease processes are not understood. Here we use a mouse model of developmental estrogen exposure to understand the mechanisms underlying uterine adenocarcinoma development. Exposing mice on days 1-5 of life to the synthetic estrogen, diethylstilbestrol (DES), or the phytoestrogen, genistein, results in 90% and 35% incidences of uterine adenocarcinoma, respectively, by 18 months of age. Prepubertal ovariectomy abrogates this phenotype suggesting that a second hit of estrogen is necessary to drive cancer development. Sine oculis-related homeobox 1 (SIX1) is a cancer-associated transcription factor that becomes permanently upregulated in the uterus after exposure. Aberrant SIX1 expression is specific to a population of abnormal uterine epithelial cells that develop following exposure and proliferate with age.

We hypothesize that neonatal xenoestrogen exposure directs permanent changes in uterine Six1 expression, resulting in aberrant cellular reprogramming that is perpetuated by endogenous estrogen exposure and leads to cancer. In this study we compared uterine Six1 mRNA expression, SIX1 expression patterns, and histopathology in control and neonatal DES- or genistein-treated mice at 6, 12, and 18 months of age and tested whether prepubertal ovariectomy impacted aberrant Six1 mRNA expression. We found that Six1 mRNA expression was increased in DES and genistein-treated mice at all ages and correlated with increasing incidence of uterine adenocarcinoma development. Furthermore, SIX1 protein localized to all cells in hyperplastic and neoplastic lesions in DES and genistein-treated animals and was absent in control uteri. DES treatment resulted in increased Six1 mRNA expression regardless of ovariectomy. However, prepubertal ovariectomy of neonatal DES-treated mice resulted in significantly lower Six1 mRNA expression than in intact or adult ovariectomized mice. These findings indicate that uterine SIX1 expression is a biomarker for exposure and disease, and suggests that SIX1 could play a role in carcinogenesis. To determine if SIX1 plays a causative role in uterine adenocarcinoma development, we are investigating if uterine specific overexpression of SIX1 induces cancer development and if uterine specific deletion of Six1 prevents cancer development following neonatal DES exposure.

NIEHS
Seddon Thomas
Postdoctoral Fellow
Immunology - Innate and Cell-mediated Host Defenses
Dendritic and epithelial cell crosstalk in the lung: the impact of cell-specific Myd88 expression on chromatin accessibility in dendritic cells and consequent immune responses to allergens
Allergic asthma is an inflammatory disease of the airway stemming from inappropriate immune responses to inhaled environmental allergens. Although asthma was previously regarded as a single disease, it is now seen as a heterogeneous set of diseases. Some forms of asthma are predominantly eosinophilic and steroid-responsive, whereas others are neutrophilic and steroid-resistant. Thus, there is an urgent need for new therapies that target specific types of asthma. MYD88, the adaptor molecule for TLR and IL-1 family member signaling, is required for allergic sensitization through the airway. However, it is unclear which cell types in the lung must express Myd88 for allergic sensitization and how this cell-specific expression affects immune signaling in the airway. It has been proposed that airway epithelial cells (AECs) communicate with lung dendritic cells (DCs), but the molecular signals involved remain poorly understood. To address these questions, we used mice bearing a ‘floxed’ version of the Myd88 gene to generate animals lacking Myd88 in either AECs (AEC-KO), or in DCs (DC-KO), and studied the impact of these genetic changes on responses to various allergens. Following allergic sensitization and challenge, AEC-KO mice had significant reductions in eosinophils compared to WT mice, but retained large numbers of airway neutrophils. Conversely, DC-KO mice had marked reductions in Th17-associated cytokines and airway neutrophils, but retained eosinophilic inflammation. These surprising findings reveal that airway eosinophilia and neutrophilia are separable allergic events and suggest that new treatments can be developed to target specific forms of asthma. To better understand how MyD88 signaling in AECs and DCs affect immune responses in the airway, we analyzed RNA prepared from the whole lung of AEC-KO and DC-KO mice at various times post-sensitization. MyD88 signaling in AECs triggered immune response gene activation at early time points, whereas MyD88 signaling in DCs

directed delayed responses. Analysis of RNA from purified DCs and AECs revealed several genes (and gene families) whose expression in DCs depended on Myd88 signaling in AECs. Finally, ATAC-seq was used to study how DC chromatin structure is modulated by signals from AECs. Collectively, these observations identify specific molecular pathways that mediate crosstalk between AECs and DCs to promote allergic sensitization to inhaled allergens.

NIEHS
Pengyi Yang
Research Fellow
Informatics/Computational Biology
Reconstruction of signaling networks from time-series phosphoproteomics data
Cell signaling controls various aspects of basic cellular processes including homeostasis, proliferation, and cell fate decisions, with defects in mechanisms underlying these processes associated with a wide range of diseases. Protein post-translational modifications (PTMs), which can activate/inhibit protein function, have emerged as key regulators of various signaling pathways. Protein phosphorylation is a common type of PTM that increases the functional diversity of the proteome by altering target proteins between activate and inactive forms for signal transduction and integration. It is characterized by the addition of a phosphate group by a protein kinase to a serine, threonine, or tyrosine residue on a substrate protein. Recent advances in mass spectrometry-based technology make it possible to profile proteome-wide phosphorylation events in vivo (at a single amino acid resolution) for investigating signal transduction cascades. However, development of algorithms to analyze and identify signaling events from high-throughput phosphoproteomics data is still in its infancy. The first step toward reconstruction of signaling networks is to identify key kinases involved in signaling cascades. To this end, we have developed a knowledge-based CLUster Evaluation (CLUE) approach that utilizes known kinase-substrate annotations to not only identify enriched active kinases and signaling cascades but also their order of occurrence. We demonstrate the utility of the approach on two time-series phosphoproteomics datasets and identify key signaling cascades associated with embryonic stem cell differentiation and insulin stimulation. Another critical step for reconstructing signaling networks involves de novo substrate prediction for key kinases of interest. Much of the past efforts were based on approaches that relied solely on kinase recognition motifs, with the dynamic information from phosphoproteomics profiling largely ignored. We have developed an approach (based on ensemble of positive unlabelled classifiers and cascade learning) that integrates dynamic quantitation of each phosphorylation site with known kinase recognition motifs to predict novel substrates for a given kinase. We find that the integrated approach is more effective compared to using sequencing information alone. Together, these approaches will serve as a valuable resource for dissecting signaling cascades and making biological inferences from phosphoproteomics data.

NIEHS
Xiaofeng Zheng
Visiting Fellow
Developmental Biology
Cnot3 maintains the pluripotent state in early embryos and embryonic stem cells

In the blastocyst stage embryos, the epiblast cells in the inner cell mass (ICM) possess a unique developmental plasticity, termed pluripotency. As development progresses, they differentiate to form all cell lineages. Upon culture, the epiblast cells give rise to embryonic stem cells (ESCs), which perpetuate in vitro the broad developmental potential. The pluripotent state of the epiblast cells and ESCs is governed by signal transduction pathways, transcription factors, chromatin and epigenetic regulators. However, how it is regulated by post-transcriptional mechanisms has only begun to be revealed in recent years. We have previously shown that three subunits of the Ccr4-Not complex, Cnot1, Cnot2, and Cnot3, are required for the maintenance of ESC pluripotency. The Ccr4-Not complex is the main deadenylase complex in eukaryotic cells. Here, we show that Cnot3 also plays an essential role in the maintenance of the epiblast cells in vivo. Cnot3 is up-regulated in the blastocysts, and is enriched in the epiblast. Its deletion leads to embryonic lethality before E7.5. Further, during diapause, a model commonly used to test the maintenance of the epiblast cells, Cnot3 null embryos appear to be smaller, defective in morphology, and have significantly decreased number of epiblast cells based on immunofluorescence staining of pluripotency markers Oct4 and Nanog. They are quickly lost over prolonged diapause. Finally, the epiblast cells from the Cnot3 null blastocysts fail to expand and grow in an ICM outgrowth assay in vitro. Thus, these results strongly suggest that Cnot3 is required for epiblast maintenance during embryonic development. To dissect the mechanism through which Cnot3 regulates the pluripotent state in epiblast and ESCs, we determined the mRNAs bound by the Ccr4-Not complex using the photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation method. In addition, we also derived conditional Cnot3 knockout ESCs and determined gene expression changes caused by Cnot3 deletion. We found that Ccr4-Not preferentially interacts with pluripotency gene mRNAs, such as those of Oct4, Nanog, Esrrb, Klf4, and Nr5a2. Moreover, Ccr4-Not-bound pluripotent mRNAs are down-regulated immediately after Cnot3 deletion. Together, we propose the model that Cnot3 controls the pluripotent state by regulating pluripotency mRNA stability via the Ccr4-Not complex. Our findings uncover a novel post-transcriptional mechanism in the regulation of pluripotency.

NIMH
Elizabeth Ballard
Research Fellow
Psychiatry

Nocturnal Wakefulness is Associated with Next-Day Suicidal Ideation in Major Depression and Bipolar Disorder: A Proposed Warning Sign and Acute Indicator of Risk

Over 40,000 Americans killed themselves in 2013. Despite advances in the treatment of depression and anxiety, the suicide rate has remained relatively stable over the last few decades. Research on suicide has focused on risk factors both stable (i.e. race or gender) and modifiable (i.e. depression, anxiety or sleep). One promising modifiable risk factor for suicide is sleep. Self-reported sleep disturbance has been linked to suicide, even when controlling for the effects of depression. As a modifiable risk factor, it is possible that treating sleep problems in individuals at risk for suicide will lead to reductions in suicidal thoughts, attempts and deaths. This literature has been limited by a reliance on self-report measures of sleep rather than gold standard measures such as polysomnography (PSG) (also known as a sleep study) to objectively measure and define sleep disturbances. In the current analysis, participants with major depressive disorder or bipolar depression (n = 65) underwent overnight PSG monitoring. Participants

were medication-free with the exception of mood stabilizers in individuals with bipolar disorder. The Hamilton Depression Rating Scale was administered the morning after PSG recording to assess next-day suicidal ideation and depression symptom severity; 39 participants (60%) reported some level of suicidal thoughts. It was hypothesized that nocturnal wakefulness would be associated with suicidal thoughts the next morning, representing an acute warning sign for suicide risk. Using a generalized linear mixed model, a significant time-by-ideation interaction was found, indicating greater nocturnal wakefulness at 4 AM among participants with suicidal ideation. Increased time awake during the 4 AM hour (4:00 to 4:59) was significantly associated with elevated suicidal thoughts the next day. This relationship persisted after controlling for age, gender, diagnosis, and severity of depressive symptoms. Results suggest that greater nocturnal wakefulness, particularly in the early morning hours, is significantly associated with next-day suicidal thoughts. Strengths of this approach include an objective measure of sleep disturbance and adjustment for depressive symptom severity. In conclusion, PSG-documented sleep disruption at specific times of night may represent an acute warning sign of suicidal ideation that warrants additional research and signals the importance of sleep in suicide prevention.

NIMH
Michael Gregory
Clinical Fellow
Neuroscience - General

Individual Cognitive Ability is Associated with Regional Variations in Cortical Gyrification

Background: It is widely hypothesized that increased folding of the brain's surface, or gyrification, supports superior cognition in humans compared to other species. This is supported in other species as those thought to have greater cognitive ability, such as cetaceans, also show a higher level of brain gyrification. Few studies, however, have examined associations between cortical gyrification and cognitive abilities in humans. Here, we tested for this brain-behavior relationship in a large sample of healthy adults using structural neuroimaging. Methods: 440 healthy adults (31.3+/-9.4 years, 250 females) completed T1-weighted MRI scanning on a 3T scanner as well as a neuropsychological battery. MRI images were processed using Freesurfer software, including computation of local gyrification index (LGI) forat each of 141,000 nodes on the cortical surface nodes. A composite of scores from 25 neuropsychological variables was computed, representinged general cognitive ability (g). The Weschler Adult Intelligence Scale, the Culture-Fair Intelligence Test and the Wide-Range Achievement Test provided additional indexes of broad cognitive ability. MRI images were processed using Freesurfer software, including computation of local gyrification index (LGI) for each of 141,000 cortical surface nodes. Correlations were computed between LGI at each node and each measures of cognitive ability, while controlling for effects due to age and sex. Reported findings are significant at p<0.05, FDR-corrected for multiple comparisons. Results: All measures of cognitive ability, including g, produced similar results, showing significant associations with increasing LGI in nearly the entire frontal lobe, insula, temporoparietal junction, posterior cingulate and parahippocampal gyrus. No regions showed a negative association between cognitive ability and LGI. These findings did not significantly differ between the sexes. Conclusions: Our results demonstrate that regional variation in brain gyrification is significantly associated with cognitive ability. Interestingly, all regions where increased gyrification was significantly associated with increased cognitive abilities are part of the neocortex, without significant

results in allocortical regions. These findings are consistent with comparative evolutionary research, showing rapid expansion of the neocortex in humans, relative to other species, perhaps providing a neuroanatomical basis for human cognitive ability.

NIMH
Angela Ianni
Doctoral Candidate
Neuroscience - Integrative, Functional, and Cognitive
Dopamine Synthesis and Receptor Profile Vary as a Function of Body Mass Index in Humans
Obesity is a worldwide epidemic with significant adverse health consequences. While behavior clearly plays an important role, the neurobiological underpinnings of weight control require further delineation in humans. Dysfunction of the mesolimbic dopamine system, which mediates the rewarding effects of appetitive stimuli such as food, has been implicated in the development of obesity. Furthermore, striatal D2 receptor levels are decreased in obese individuals and inversely correlated with future weight gain and food seeking-behavior in rats. However, no studies to date have assessed the roles of both dopamine synthesis and receptor density in healthy humans in the normal to obese range. Characterizing the dopaminergic signature related to higher BMI could provide insight into an important neural mechanism underlying the obesity epidemic. Seventy-three healthy volunteers (mean age 36.36, range 18.75-57.90; mean BMI 25.92, range 17.91-35.93; 34 females) were recruited from the local community and completed three PET scans on separate days to assess dopamine presynaptic synthesis capacity (with [18F]DOPA), and D1 and D2 receptor binding potential (with [11C]NNC112 and [18F]Fallypride, respectively). Subjects also completed a T1-weighted MRI scan that was segmented using Freesurfer and manual adjustments to generate ROIs of the basal ganglia including the putamen, caudate nucleus, ventral striatum, and midbrain. MRI images were registered to native space PET images and values were extracted for each of the ROIs using the Patlak method (FDOPA) and MRTM method (NNC112 and Fallypride), both using a cerebellar reference region. Correlations between BMI and PET measures were calculated in SPSS and significance was assessed using a threshold of p<0.05, uncorrected. We found that BMI correlated positively with presynaptic dopamine synthesis capacity (FDOPA) in the midbrain (r=0.271, p=0.022) and the left ventral striatum (r=0.300, p=0.011). In addition, BMI correlated negatively with D2 receptor binding potential (Fallypride) in the midbrain (r=-0.232, p=0.049) and the caudate nucleus (bilateral: r=-0.239, p=0.046; left: r=-0.272, p=0.022). BMI did not correlate with D1 receptor binding potential (NNC) in any of the ROIs. Taken together, these data suggest that a neural signature related to BMI across the normal to obese range includes an overactive tonic mesolimbic dopamine reward system with impaired regulation by D2 receptors.

NIMH
Rachel Scheinert
Postdoctoral Fellow
Neuroscience - General
Reversing Depression with Immune Cells
Recent research from our group has promoted the concept of a bidirectional relationship between brain and the immune system by showing that psychological stress can modify adaptive immune cells in a

manner that may boost resilience to stress. Here, we hypothesize that adaptive immune cells from stressed mice confer anti-depressive effects when transferred into mice lacking an adaptive immune system. First, we demonstrated that like wild type C57BL/6 mice, immune-deficient Rag2-/- mice, which lack mature lymphocytes, expressed a depressive-like behavioral phenotype and decreased hippocampal cell proliferation following chronic (14 days) restraint stress. This depressive-like phenotype was then "rescued" by an adoptive transfer of lymphocytes from donor mice that express green fluorescent protein in all cells. We tracked the green donor lymphocytes to the spleen and lymph nodes of the recipient Rag2-/- mice to confirm that transfer was successful 14 days later. Restraint-stressed mice that received adoptive transfer from naive donor mice demonstrated anti-depressive/anxiety like behaviors on three behavioral tasks: open field, light:dark and tail suspension. Interestingly, mice that received adoptive transfer from donor mice that had previously been exposed to chronic (14 days) of social defeat stress expressed a more pronounced anti-depressive like phenotype. These findings suggest that the adaptive immune system may play a key role in promoting recovery from chronic stress and depression. Furthermore, this research supports using lymphocytes as a novel therapeutic target for anti-depressant strategies.

NIMH
Saurav Seshadri
Postdoctoral Fellow
Neuroscience - Neurodegeneration and Neurological disorders

NMDAR-mediated alterations in critical dynamics are associated with cognitive impairment in a rat schizophrenia model

Brain networks at rest exhibit spontaneous activity, even in the absence of a stimulus. In diverse experimental systems, from organotypic slice cultures to the human brain, this activity forms scale-invariant, spatiotemporally ordered cascades. These cascades are termed neuronal avalanches, and their emergence from local, weakly interacting network elements is a hallmark of systems at criticality. Criticality in brain dynamics optimizes several aspects of information processing, and is impaired by pharmacological disruption of the excitatory-inhibitory balance. We therefore hypothesized that deviations from criticality would be associated with impaired cortical functioning, such as that seen in psychiatric disorders. To test this hypothesis, we used a neurodevelopmental rat model of schizophrenia. Sprague-Dawley rats were administered phencyclidine (PCP, 10 mg/kg s.c.) at postnatal day 7, 9, and 11. PCP is an antagonist to the excitatory N-methyl-D-aspartate receptor (NMDAR), and this model has been shown to reproduce key neuroanatomical and behavioral phenotypes associated with schizophrenia. Neuronal activity was recorded in superficial layers of cortex by in vivo 2-photon imaging of pyramidal neurons expressing the genetically encoded calcium indicator YC 2.60. At the individual neuron level, we found that spontaneous firing was increased in PCP treated rats, while variability in spiking was unchanged. At the group level, avalanche size distributions deviated significantly from the power law distributions predicted at criticality. To test the behavioral consequences of this deviation, we used the novel object recognition test of visual working memory. PCP treated rats showed significant impairment in maintenance of visual information in working memory across a short delay period. Finally, we attempted to rescue PCP-induced deficits by treatment with an NMDAR co-agonist, D-serine (800 mg/kg, i.p.). When tested or imaged 30 minutes post

injection, D-serine treated rats showed improved working memory performance and normalized avalanche size distributions. These results provide mechanistic insight at the group level (with single cell resolution) into NMDAR hypofunction in schizophrenia. They also suggest that altered critical brain dynamics may underlie cognitive deficits in mental disorders. We are currently using optogenetic modulation of fast-spiking interneurons to determine their role in mediating NMDAR related pathophysiology.

NIMH
Salvatore Torrisi
Postdoctoral Fellow
Psychiatry
Resting State Connectivity of the Bed Nucleus of the Stria Terminalis at Ultra-high Field
The bed nucleus of the stria terminalis (BNST), a portion of the 'extended amygdala', is a neural structure highly implicated in the pathophysiology of anxiety and addiction disorders. Its small size and connection to other small structures, however, prevents standard MRI imaging techniques from capturing it and its connectivity with confidence so is therefore little studied in humans. Seed-based resting state functional connectivity is an established method for mapping functional connections across the brain from an anatomical mask. For maximal accuracy we drew subject-specific BNST masks on 27 healthy adult subjects' high resolution structural scans (0.7mm isotropic voxels). We then mapped the BNST resting state network with high spatial resolution (1.3mm isotropic) using 10-minute functional runs collected with an ultra-high field (7 Tesla) MRI scanner. Methodologically, we used nonlinear registration in conjunction with noise reduction methods using subject-specific anatomical parcellations, cardiac and respiratory recordings, and local white matter signal removal to ensure accurate functional mapping with the BNST, a small structure adjacent to known sources of noise. Our results identified very strong BNST functional connectivity (p<1.0e-10) with a number of other regions, including: the central nucleus of the amygdala, anterior hippocampus, midline thalamus, medial caudate, periaqueductal gray, hypothalamus and cortical areas such as the medial PFC and precuneus. This specifically reveals the in vivo reproduction of many BNST connections previously described only in rodent and primate tract tracing studies and more generally demonstrates the power of ultra-high field MRI for mapping highly precise functional connections in the human. This work represents an important first step towards future task-based activation and connectivity studies that will probe the BNST network in healthy and pathological populations.

NIMH
Wan-Ling Tseng
Postdoctoral Fellow
Psychiatry
Neural Correlates of Irritability: A Novel Cross-Diagnosis Dimensional Approach
Severe, chronic irritability affects approximately 10 million youth in the U.S., and predicts adult depressive and anxiety disorders and long-term impairment. Despite the high prevalence and public health impact, the pathophysiology of irritability remains unclear. Moreover, irritability represents a stable trait that is distributed continuously in the population and is common in many pediatric disorders

including anxiety disorders (ANX), disruptive mood dysregulation disorder (DMDD), and attention-deficit hyperactivity disorder (ADHD). Thus, irritability is an ideal construct to be studied under the NIMH Research Domain Criteria (RDoC) initiative, focuses on dimensions of observed behaviors and brain functions across categorically-defined diagnoses. Here, we used a frustrating fMRI task to explore the neural correlates of irritability within the RDoC framework. Specifically, we examined associations between brain activation and a dimensional measure of parent-reported irritability across four diagnostic groups: DMDD (n=22), ADHD (n=19), ANX (n=26), and healthy volunteers (HV; n=36). Participants (M=12.8 years; 54.4% girls) completed an attention-reward task. We modeled irritability by eliciting frustration through providing participants rigged feedback and withholding an expected reward. Dimensional analyses revealed a cubic trend in bilateral lentiform nucleus (extending to insula), left fusiform gyrus, and right middle frontal gyrus (BA 9; corrected ps<.05). Highest levels of irritability were related to greater activation during rigged vs. positive feedback. Analyses using a traditional categorical approach demonstrated consistent patterns with the dimensional approach, i.e., DMDD youth, who had the highest level of irritability, displayed greater activation during rigged vs. positive feedback compared to other groups. These findings suggest that abnormal neural activation in regions implicated in reward processing, early visual processing, and emotion regulation may mediate irritability in youth. This study supports the RDoC dimensional approach for uncovering underlying pathophysiology that cuts across psychiatric disorders. Ultimately, research under RDoC framework, as the current one, may identify potential biomarkers to index risk for multiple mental illnesses and provide targets for neurobiologically-informed treatments.

NINDS
Xiutang Cheng
Visiting Fellow
Neuroscience - Cellular and Molecular

Axonal autophagosomes recruit dynein for retrograde transport through fusion with late endosomes

During autophagy, an essential catabolic cellular pathway, double-membrane autophagosomes are formed and enclose bulk cytosol and/or organelles. They then fuse with late endosomes (LEs), generating a hybrid organelle called the amphisome, or with lysosomes for final degradation. Impairment of this pathway is associated with several neurodegenerative disorders. Thus, efficient degradation of those autophagic vacuoles (AVs) via lysosomes is critical for cellular homeostasis. This is particularly challenging for polarized neurons with long processes where mature acidic lysosomes are relatively enriched in the soma. Although dynein motor was suggested for retrograde transport of AVs, a fundamental mechanistic question still remains how nascent autophagosomes generated at distal axons acquire dynein motors for retrograde transport toward the soma. To observe axonal transport of AVs, we used dorsal root ganglion (DRG) neurons as cell model. By time-lapse imaging of autophagy marker GFP-LC3 and LE marker mRFP-Rab7, we found 97% of AVs were amphisomes and displayed a predominant retrograde motility similar to LEs moving from distal axon to soma. Our previous study reveals that snapin acts as an adaptor protein recruiting dynein intermediate chain (DIC) to LEs. We then applied a snapin dominant-negative mutant defective in DIC-binding to disrupt DIC-snapin coupling and found impaired retrograde transport of both amphisomes and LEs, along with an accumulation of AVs in the axons. Consistently, we observed an elevated number of double-membrane AVs within the neurites

and presynaptic terminals from neurons of snapin KO mice by transmission electron microscopy (TEM). These results indicated that LE-loaded dynein-snapin complexes drive axonal retrograde transport of AVs. To address if autophagosomes share dynein with LEs upon fusion or directly recruit it, we knocked down syntaxin 17, a snare protein specifically mediating fusion between nascent autophagosomes and LEs. Live-cell imaging showed a robust increase in the number of nascent autophagosomes (LC3 only) and a decrease in amphisomes (LC3 with Rab7). Surprisingly, the majority of autophagosomes remained stationary, suggesting fusion is a prerequisite step for nascent autophagosomes to acquire retrograde motility. Thus, we demonstrate a novel motor-adaptor sharing mechanism for retrograde transport of AVs in distal axons and highlight the importance of LE transport upon autophagic clearance in neurons.

NINDS
Angel de la Cruz Landrau
Doctoral Candidate
Neurotransmission and Ion Channels
Locking the open state of a voltage-dependent concatemer potassium channel with metal bridges
Voltage-gated potassium (Kv) channels are involved in many channelopathies, diseases that are caused by a malfunction of an ion channel. In excitable cells, Kv channels regulate the duration of action potentials as well as the neuron's excitability status. They are homotetramers and each subunit is formed by six transmembrane segments, S1 to S6. The permeation pathway for K+ ions is formed by the assembly of S5, the P-region, and S6 from each subunit to create a central canal that crosses the membrane. Mutation of a valine to a cysteine at position 476 in S6 (near the intracellular gate) traps the mutant channel in the open state when Cd2+ is added intracellularly because metal bridges form between the cysteine at position 476 of one subunit and a native histidine at position 486 in an adjacent subunit. Since Kv channels are homotetramers, four metal bridges are present in a mutated V476C channel. Are all four metal bridges needed to lock the channels in the open state? What is the contribution of each subunit to the opening or closing of a Kv channel? To answer these questions, we developed a concatemer Kv channel with all subunits linked at the DNA level. This construct provides the flexibility of introducing mutation V476C in one, two, three and/or all subunits. After introducing the mutation V476C, ionic currents were measured using voltage clamp inside-out technique, which allows access to the intracellular part of the channel to perfuse Cd2+. Irrespectively of the number of V476C mutations in a concatemer Kv construct, the channels opened and closed normally in the absence of Cd2+. In the presence of Cd2+, concatemer KV channels with mutation V476C in only one subunit closed slower than in control conditions. In a concatemer with mutation V476C in two subunits, intracellular Cd2+ further slowed the closure of the channel. The successive addition of the mutation V476C to each subunit of the concatemer increasingly slowed down the closing of the channel, until it was unable to close and it was left in a locked open state when all four subunits have the mutation V476C. Therefore, all subunits of a Kv channel work together to open and close the channel.

NINDS
Rebekah Evans
Postdoctoral Fellow
Neuroscience - General

T-type calcium channels trigger a hyperpolarization induced afterdepolarization in vulnerable subpopulation of substantia nigra dopamine neurons

The dopamine neurons of the substantia nigra pars compacta (SNc) are essential for the initiation of movement. The degeneration of these neurons is the hallmark of Parkinson's Disease patients, and damage to these neurons in animal models causes Parkinson's like symptoms. One prominent hypothesis is that calcium through voltage gated calcium channels causes or exacerbates the degeneration of the SNc neurons in Parkinson's Disease. Most work on this calcium toxicity hypothesis has focused on the L-type calcium channel. However, we have recently found that under hyperpolarized conditions, the T-type calcium channel can trigger a large after-depolarization and a corresponding influx of calcium into the dendrites of these neurons. Specifically we have used computational modeling, patch clamp electrophysiology, and two-photon calcium imaging in mouse midbrain slices to show that this hyperpolarization-induced afterdepolarization (HI-ADP) and the corresponding dendritic calcium transient depends on T-type, but not L-type calcium channels. Interestingly, we observed that not all the SNc dopamine neurons displayed this HI-ADP. The cells that showed very little HI-ADP also showed very small dendritic calcium transients. Other studies have shown that there are two subpopulations of dopamine neurons within the SNc, and that these two populations differentially degenerate in Parkinson's Disease. During the course of human Parkinsonism and in Parkinson's model animals, the ventral SNc neurons appear to be more vulnerable and die more quickly while the dorsal SNc neurons are relatively resilient. We used immunohistochemistry to stain for calbindin, a marker for the resilient SNc cells, in slices previously used for electrophysiology. In this way we could compare the size of the HI-ADP in the resilient and vulnerable populations. We found that the HI-ADP was much stronger in the vulnerable, calbindin-negative, SNc neurons than in the resilient neurons. These results suggest that the T-type calcium channel expression density or characteristics may be different in the dendrites of the vulnerable and resilient population of SNc dopamine neurons. Because these channels can cause huge, possibly toxic, calcium transients when activated from hyperpolarized potentials, they may contribute to the vulnerability of specific SNc dopamine neurons in Parkinson's Disease.

NINDS

Travis Hage

Postdoctoral Fellow

Neurotransmission and Ion Channels

Tonic firing rate controls dendritic Ca2+ signaling and synaptic gain in substantia nigra dopamine neurons

Dopamine neurons of the substantia nigra (SN) are critical for voluntary movement and reward signaling. The malfunctioning of dopamine neurons contributes to a variety of disorders including Parkinson's disease, resulting from their neurodegeneration. Despite their importance, little is known about how dopamine neurons process synaptic information to drive motivated behaviors. Most studies of dendritic function have focused on pyramidal neurons in hippocampus and cortex. Firing in midbrain dopamine neurons differs from pyramidal neurons in an important way. Unlike pyramidal neurons that require synaptic input to generate action potentials (APs), dopamine neurons fire spontaneously as a result of intrinsic conductances. Therefore, synaptic inputs that drive reward-relevant bursts of APs occur on a background of tonic firing and are more frequently influenced by AP-evoked conductances. I

demonstrate a novel role of tonic firing rate in modulating the gain of synaptic responses in SN dopamine neurons. By combing whole cell recording, two-photon glutamate uncaging and calcium (Ca) imaging, I show that somatic depolarization enhances NMDA receptor (NMDAR)-mediated Ca signals over 400 um distal to the soma, due to unusually tight electrotonic coupling of the soma to distal dendrites. Consequently, increases in tonic firing rate (1-6 Hz) intensify synaptically-driven burst firing. Blockade of NMDA receptors eliminates the enhancement of burst output at high tonic firing rates. My results show that modulation of tonic firing rate provides a simple yet powerful mechanism to dynamically regulate the gain of synaptic input. After finding that small somatic depolarizations have far-reaching and robust control of synaptic integration, I asked how tonic firing rate influences dendritic Ca influx resulting from backpropagating APs. I find that small increases in tonic firing rate generated dendritic Ca signals up to five-fold greater than predicted by linear summation of single AP-evoked Ca-transients. This amplification was present throughout the dendrites and required depolarization of the interspike voltage and activation of L-type Ca channels. It is suggested that ongoing Ca influx promotes neurodegeneration in SN dopamine neurons due to increased metabolic load. I show that small, fractional increases in the tonic firing rate of SN dopamine neurons produce striking, multiplicative increases in Ca influx – perhaps predisposing these cells to neurodegeneration.

NINDS
Jermaine Ross
Postdoctoral Fellow
Gene Expression

The identification and characterization of cis-regulatory DNA within the Drosophila pdm-1 and pdm-2 gene locus

One of the major goals in human disease studies is to understand the mechanisms that control gene expression. One approach is to decipher the regulatory logic embedded in conserved non-coding DNA, which accounts for ~98% of the human genome. Linkage and genome-wide association studies have shown that DNA enhancer mutations are a significant cause of human diseases. Our focus is to study the cis-regulation of the transcription factor (TF) genes pdm-1 & pdm-2, denoted collectively pdm, which are required for survival during Drosophila neurogenesis. These genes are also related to the human TF genes Oct-1 & Oct-2, which have been implicated in glioma, the most common malignant brain tumor. During embryogenesis, cascade expression of TFs Hunchback->Krüppel->Pdm->Castor (Cas) coordinates specification of neuronal identity. We showed that Pdm activates cas expression in neural stem cells (NSCs). In turn, Cas represses pdm. To examine whether Cas directly regulates pdm-1, we scanned the pdm-1 locus for Cas DNA binding sites and confirmed that Cas binds repeatedly within a 5 kb genomic region. To delimit the enhancer boundaries, we used our program EvoPrinter, which reveals ultra conserved DNA. Our analysis identified a 1 kb cluster of conserved sequences (CSC) containing Cas sites. Enhancer-reporter analysis showed that this genomic region recapitulates pdm-1 expression in NSCs. We next showed that the loss of Cas or removal of Cas sites from the pdm-1 enhancer triggers ectopic expression during neurogenesis, validating Cas as a direct regulator of pdm. Given that the identified enhancer does not recapitulate the full pdm expression, we predicted that multiple enhancers regulate pdm. EvoPrinter revealed 116 CSCs within the 125 kb pdm loci. Thus, we generated over 100 transgenic fly lines and tested each CSC for enhancer activity during embryonic, larval and adult neurogenesis.

Surprisingly, our screen revealed over 70 unique pdm enhancers. To catalogue these enhancers, we created an online database called cisPatterns (cispatterns.ninds.nih.gov). From these studies, we also developed a novel machine learning algorithm that accurately (>90%) classifies enhancer function using conserved DNA sequence and image data from ~1000 characterized enhancers, including pdm enhancers. These results argue that enhancer function can be predicted from unique combinations of conserved DNA sequences. Together, we have formed a new systematic approach for studying gene regulation.

NINDS
Thom Santisakultarm
Postdoctoral Fellow
Neuroscience - General
Longitudinal Two-Photon Imaging of Neurovascular Coupling in Awake and Anesthetized Marmoset Monkeys
Cerebral blood flow is precisely controlled to ensure that homeostasis is maintained during neural activation when metabolic demand is heightened. This neurovascular coupling at the cellular level can be visualized with two-photon microscopy (2PM). Often, this optical imaging technique is performed in anesthetized animals albeit the unclear influence of the anesthetic agents. Here, we aim to quantify the effects of a commonly used anesthesia, isoflurane, on cortical vessels and neural activation in marmosets with 2PM. Marmosets were acclimated to 2 h of awake imaging in the sphinx position. A cranial chamber was then implanted over the somatosensory cortex, along with a headpost to stabilize imaging. To visualize functional activity, AAV1-GCaMP5G was injected for optical detection of calcium influx during neural firing. After recovery, 2PM revealed vascular topology in the cortex and enabled measurement of red blood cell motion in individual vessels. The vascular characteristics, along with neural responsiveness to peripheral stimulation, were quantified in the marmosets in both awake and anesthetized states. To evaluate the impacts of the surgical procedures and the implants, behavioral assessments were performed before surgery, and throughout the following 6 months. In addition, a healthy marmoset was euthanized for ex vivo 2PM to evaluate possible cerebrovascular reorganization. Under isoflurane anesthesia, arterioles of the same marmosets dilated by 16%, and had a 24% decrease in flow speed, compared to while awake. 19% of GCaMP5G-expressing neurons were responsive to stimulation, compared to 0% while anesthetized. Capillary density was 6695 capillaries/mm3 with a median diameter of 6.4 um, segment length of 67 um, and tortuosity of 1.2 arc-chord ratio. The median number of capillaries connecting penetrating arterioles to ascending venules was 8 branches. These vascular structures were similar to that of the control. Behavioral assessment demonstrated no change on cognitive and motor functions following surgical procedures. These results suggest significant alterations in hemodynamics and neural activity under isoflurane, and highlight the importance of studying brain function without anesthesia's confounding effects. This work provides an insightful imaging technique to assess neurovascular coupling at the cellular level in awake non-human primates, and allows for longitudinal investigation of critical mechanisms in neurological disorders.

NINDS
Shahriar Sheikhbahaei

Doctoral Candidate
Neuroscience - General
Oxygen Sensitivity of the Brainstem Astrocytes
In mammals, highly specialized oxygen sensing cells have evolved to monitor and ensure adequate oxygenation of the brain. Strategically located in the carotid bifurcation, the main peripheral respiratory chemoreceptors (carotid body, CB) detect decreases in arterial partial pressure of oxygen (PO2) and trigger adaptive changes in breathing. However, PO2 at the level of this peripheral chemoreceptor is not sensitive to regional differences in brain O2 demands. In the brain, astrocytes, the ubiquitous electrically non-excitable cells, enwrap all penetrating and intracerebral arterioles and capillaries and directly sense physiological changes in cerebral blood flow and brain oxygenation. In this study, we examined the role of astroglial signaling in regulating activity of neural circuits in the pre-Bötzinger complex (preBötC), a functionally specialized brainstem region controlling homeostatic breathing including respiratory responses to hypoxia. To specifically block astroglial vesicular release involved in this signaling, we generated an adenoviral vector for astrocyte-specific expression of the light chain of tetanus toxin (TeLC), which cleaves certain SNARE proteins required for vesicular docking and fusion. Whole-body plethysmography was used to measure the augmentation of lung ventilation in response to hypoxia (10% O2) in CB intact and CB ablated (for 8 weeks) conscious freely moving adult rats expressing GFP (control) or TeLC in astrocytes within the preBötC. Bilateral expression of TeLC in preBötC astrocytes reduced the augmentation of lung ventilation during hypoxia both in the CB intact (by 24%; n=6, p=0.011) and CB ablated animals (by 35%; n=5, p=0.006). Also, bilateral astrocytic TeLC expression in the CB intact animals (n=6) and bilateral CB ablation alone (in rats expressing control GFP transgene, n=5) resulted in quantitatively similar reductions in the magnitude of the hypoxic response. These latter observations suggest that the relative contribution of the preBötC astroglial O2 sensitive mechanism to the overall hypoxic ventilatory response is comparable to that of the specialized CB peripheral respiratory O2 chemoreceptors. Oxygen sensing by astrocytes may be important for local control of cerebral microcirculation and neuronal excitability when PO2 in a particular brain microdomain decreases. Failure of this mechanism may have adverse effects on neuronal function and contribute to the development of neurological disease(s).

NINDS
Phillip Swanson II
Postdoctoral Fellow
Immunology - Infectious Disease
CD8 T cells induce cerebral malaria through antigen-dependent interactions with brain vascular endothelial cells
Malaria, a devastating illness caused by the protozoan parasite Plasmodium falciparum, affects up to 500 million people worldwide. A subset of these individuals, mostly children, develops a fatal neurological condition called cerebral malaria characterized by seizures, coma, and intense vascular pathology. Because the mechanisms that lead to cerebral malaria are unknown, we investigated the cause of this disease using a murine model of experimental cerebral malaria (ECM). B6 mice infected with Plasmodium berghei (Pb) ANKA develop a neurological disease that mirrors human cerebral malaria. This disease is characterized by severe vascular pathology and death at 6 days post-infection.

We observed that CD8+ T cells, CD4+ T cells, and innate myelomonocytic cells are significantly increased in the brain during ECM. To identify the immune cells involved in ECM, we selectively depleted individual immune cell populations in infected mice and found that only depletion of CD8+ T cells prevented breakdown of CNS vasculature and rescued mice from death. To understand how CD8+ T cells induced this disease, we adoptively transferred fluorescent protein tagged Pb-specific CD8+ T cells into mice and used two-photon microscopy (TPM) to track their location and motility in the brain. Interestingly, Pb-specific CD8 T cells were primarily observed crawling along or arresting on the lumen of cerebral blood vessels during the development of ECM - a behavior not observed in a tissue without pathology (ear). Antigen presentation and adhesion molecules were expressed more highly on cerebral endothelial cells when compared to those extracted from the ear, which could explain the differential CD8+ T cell adherence observed by TPM. Because cerebral endothelial cells can acquire parasite antigen, we hypothesized that the interactions between CD8+ T cells and endothelial cells were antigen-specific. To address this theory, we demonstrated by TPM that Pb-specific CD8+ T cells spent significantly more time arrested along brain vasculature than activated non-specific CD8+ T cells. In addition, we generated bone marrow chimeras lacking antigen-presenting machinery on endothelial cells. The absence of antigen presentation by endothelial cells reduced Pb-specific CD8+ T cell arrest and completely prevented ECM. These data indicate CD8+ T cells drive ECM through antigen-dependent interactions with cerebral blood vessels.

NLM
Yuqing Mao
Postdoctoral Fellow
Informatics/Computational Biology
Large-scale Literature Indexing using Machine Learning
The rapid growth of scholarly publications in PubMed makes the search of relevant information in literature increasingly more difficult. To facilitate PubMed search, new articles are manually indexed with a set of relevant and controlled keywords known as Medical Subject Headings (MeSH) terms. The process (known as MeSH Indexing) is highly important for improving literature retrieval and many other scientific investigations in biomedical research such as citation analysis. Unfortunately, given its manual nature, the process of MeSH indexing is highly time-consuming, costly and challenging (the inter-annotator agreement between two human indexers is only about 40%). As a result, it takes on average three months for a new article to be indexed upon entering PubMed. To improve productivity and assist human indexers, automated MeSH indexing been attempted but several key issues remain including both method accuracy and scalability. To this end, we propose a novel approach for automatic MeSH indexing in real time. More specifically, given a new article, our method first obtains a list of candidate MeSH terms from multiple sources (e.g. previously indexed MeSH terms from related articles). Next it combines these different inputs systematically using a novel machine-learning framework to rank the candidates based on their relevance to the target article. Finally, it selects and returns the highest-ranked MeSH terms for the target article. Unlike previous studies, our method has a robust framework that allows systematic integration of multiple inputs for improved performance. To our best knowledge, it is also the first approach that integrates and ranks candidate terms using machine learning (as opposed to heuristic rules in other studies). Our method achieved an accuracy of 61.4% in F1-score using

a public benchmarking dataset, showing a 12.7% increase in performance over the state of the art. As a matter of fact, our method competed in a 2014 global science challenge on automatic MeSH indexing and obtained the highest performance among 62 participating systems. Finally, to demonstrate its utility in practical applications, our method has been streamlined to process articles at PubMed scale through parallel computing. As a result, our method is able to return predicted MeSH terms in a real-time fashion. Given its performance and scalability, we plan to explore its use in practical applications in future work.

NLM
Chih-Hsuan Wei
Research Fellow
Informatics/Computational Biology
A Hybrid Approach for Simplifying Composite Named Entities in Biomedical Literature
The biomedical literature is a critical foundation for future knowledge discovery. However, with its current size (over 24 million article in PubMed) and annual growth rate (more than a million each year), it is increasingly challenging for individual researchers to make the effective use of this content-rich knowledge resource. Automatic information retrieval/extraction are greatly needed. To start, many past studies have focused on the critical issue of automatically recognizing the bio-entities (e.g., genes, diseases) in the biomedical literature. However, automatic entity recognition is not always straightforward but rather difficult due to the ambiguity and variation in natural language. One particular challenge is to identify and resolve composite named entities, where a single span refers to more than one concept (e.g., colorectal adenomas and carcinoma). Most previous studies have either completely ignored this issue, used simple ad-hoc rules, or only handled coordination ellipsis, which is only one of the many types of composite mentions (e.g. range mentions like SMAD1-3). Here we propose a hybrid approach by integrating a machine learning model with a pattern identification strategy to identify individual mentions from a composite named entity. More specifically, we first trained and built a Conditional Random Fields model to detect the composite mentions and subsequently identify the antecedent (e.g., colorectal) and conjuncts regions (e.g., adenomas and carcinoma) of a composite mention. Next, we manually developed four patterns to model the six different types of composite mentions in our study. Finally, by applying our patterns to those previously identified regions in the composite mention, individual mentions are generated in our final output (e.g. colorectal adenomas and colorectal carcinoma). Our method achieved high performance in benchmarking experiments: identifying and resolving composite mentions in biomedical literature with an accuracy of 90.4% in F1-score compared to 37.9% using a rule-based method (baseline). More importantly, our method can subsequently result in improved performance of bio-entity concept recognition. To our best knowledge, this is the first systematic method to handle multiple types of composite mentions with high performance (as opposed to coordination ellipsis only). As a demonstration of its practical utility, our method has been successfully integrated into multiple bio-entity taggers.