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CC
Xu, Ziyue
Postdoctoral Fellow
Radiology/Imaging/PET and Neuroimaging
Computerized Inspection of Airways and Lungs (CIAL): Introducing a novel, robust, accurate, and fast software tool for analyzing CT scans
Pulmonary tuberculosis (TB) is a common infection with high mortality and morbidity. CT is often used for spatial information analysis, visualization, and volume quantification of airways and cavities. Characterization of airways and cavitation are typically assessed by radiologists via visual analysis, which can be time consuming and provide limited accuracy with high inter-observer variance. In this study, we designed and tested a novel computer aided detection and quantification tool to detect and quantify airways and cavities. Airway lumen and wall thickness are located and measured with a novel graph-based algorithm that worked quickly and reliably. Cavities were then detected and delineated with a proposed automatic algorithm. With our methodology, multiple advanced imaging markers like airway radius, wall thickness, cavity volume and shape dispersion, minimum distance from cavities to airway, and the evolution of the cavity over time were extracted in high accuracy and efficiency. This can provide an analytical tool for improving radiologic research and patient-care diagnosis in the clinical and preclinical settings. Airway segmentation is developed based on a novel hybrid multi-scale approach, wall thickness estimation is based on novel spatially constrained random walk segmentation, and cavity detection uses both intensity and shape information within an efficient fuzzy connectedness delineation framework. The integration of these methods for our algorithm can reliably identify both airway structure and cavitation from CT scans, enabling automatic extraction of features relative to clinical applications. We retrospectively tested our computer-aided system on 40 human CT scans and a longitudinal study of TB with 12 rabbits for 7 weeks. The airway identification algorithm was found to be among the best of state-of-the-art methods, in accuracy and time-cost. For airway wall estimation, dice similarity coefficients (DSC) were found to be 73.87% and 81.42%, with respect to the surrogate truths (defined by radiologist visual analysis); inter-observer agreement was found to be 77.61%. For cavity detection and delineation, DSC was found to be over 95%. Our experimental results confirm that cavities are formed within the vicinity of the airway structure, with a mean distance of 1.59 mm; volume change could be statistically integrated with clinical variables to improve mathematical modeling of the disease when identifying potentially important biomarkers.

FDA/CBER
Gupta, Charu
Postdoctoral Fellow
Metabolomics/Proteomics
Identification of biomarkers of Trypanosoma cruzi infection in blood using aptamer technology and proteomics
Most individuals infected with the blood borne protozoan parasite Trypanosoma cruzi (T. cruzi), causing Chagas disease, are not aware of their chronic infection. This causes a threat to blood supplies because T. cruzi can be transmitted by blood transfusion. Current antibody-based tests have issues of cross reactivity and sensitivity, suggesting a need for an alternative and sensitive non-antibody based assay for T. cruzi diagnostic and blood donors screening. We have developed an aptamer-based assay for the

detection of T. cruzi biomarkers in blood. Aptamers are small single stranded nucleic acid molecules which bind to their target based on shape recognition. We selected RNA aptamers against T. cruzi excreted/secreted antigens (TESA) from a library of 10 superscript 12 (10^12) sequences (170 nucleotide long) using a SELEX (Systematic Evolution of Ligands by Exponential Enrichment) strategy. Aptamers were used as T. cruzi specific ligands in an Enzyme Linked Aptamer (ELA) assay and were selected for their ability to bind TESA and react with T. cruzi infected mice plasma. Aptamer L44 showing high binding affinity and specificity to TESA also reacted specifically with T. cruzi infected mice plasma, in the ELA assay. We used a proteomics based approach to identify the target (biomarker) of aptamer L44. Whole cell extracts of T. cruzi (Tulahuen strain) trypomastigotes, were fractionated using chromatofocusing and the fractions were tested using Apt-L44 ELA assay. Positive and negative fractions were selected and digested with trypsin. The peptides generated were analyzed by 2D nano-LCMS/MS. A comparison of top hits, having zero false positive rates, from ELA positive and negative fractions, resulted in 13 proteins as possible targets of Apt-L44. Of those, 6 were reported to be secreted by some trypanosomes and other parasites. To identify the true target from these candidates, recombinant proteins will be made and assessed for their Apt-L44 binding activity. This biomarker will be used further to generate additional aptamers that will be used to modify the current ELA assay into a quantitative sandwich aptamer-based diagnostic test.

FDA/CBER
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Molecular Biology - Eukaryotic
Deletion of endonuclease G in Leishmania mexicana leads to disruption in mitochondrial homeostasis and abolition of immunopathology in Balb/C mice
Cutaneous Leishmaniasis is a disease caused by the blood borne human protozoan parasite Leishmania. There are no effective vaccines against cutaneous leishmaniasis which is a serious public health problem. Previous vaccination approaches using heat-killed parasites, recombinant protein(s) and DNA vaccines have not been successful in humans. In contrast, genetically attenuated parasites (GAP) allow for interaction between a broad repertoire of parasitic antigens and the host immune system that is essential for the development of protective immunity. An ideal GAP vaccine would allow limited replication of the parasites in the host thus facilitating development of robust immunity and importantly cause no pathology. Endonuclease G (endoG) is a mitochondrial nuclease implicated in apoptosis. Recent reports showed endoG function as a determinant of the mitochondrial functions in humans. Since mitochondrial activities are essential for amastigote (the stage of Leishmania parasites in humans) survival, we investigated if deletion of endoG from Leishmania mexicana results in impaired mitochondrial activities and thus strong attenuation in parasite virulence. Results showed that deletion of endoG by homologous recombination (endoG-/-) leads to a significant growth reduction of amastigotes in vitro. This reduction was accompanied by impaired mitochondrial functions including reduced mitochondrial respiration, impaired lipid metabolism and reduced ATP production. Furthermore, deletion of endoG led to altered karyotype, such as a high proportion of abnormal, multinucleated amastigotes indicating defects in the cell cycle progression. Importantly, EM studies revealed the presence of large lipid bodies in endoG-/- mutants indicating impairment in macro-autophagy which regulates lipid metabolism. On the other hand, infection experiments with sand flies, arthropod vectors of Leishmania parasites, showed poor differentiation of endoG-/- mutants into infective metacyclic parasites. Autophagy is known to be essential for metacyclogenesis and our sand fly infection results further supports that autophagy is impaired in endoG-/- mutants. Balb/C infection experiments showed a 6-log fold difference in the parasite burden (endoG-/- versus wild type) and no pathology 10 weeks after the infection. These results demonstrate that deletion of endoG leads to a

strong attenuation of virulence and endoG-/- mutants can be tested as a novel live attenuated Leishmania vaccine.

FDA/CBER
Dharmasena, Madushini
Postdoctoral Fellow
Molecular Biology - Prokaryotic
Chromosomal Super-recombineering in Development of a Combined Oral Vaccine for Simultaneous Protection Against Both Typhoid Fever and Shigellosis
Typhoid fever and shigellosis cause high morbidity and mortality worldwide, yet no anti-Shigella vaccine is currently available. A live, attenuated bacterial oral vaccine for typhoid fever, strain Ty21a, has been safely administered to > 200 million travelers/military worldwide and affords long-term protection (> 8 yrs) after 3 doses given over a 5-day period. We have utilized Ty21a as a safe, broad-based oral vaccine vector for expression of multiple foreign antigens to protect against shigellosis, anthrax, and plague. Shigella LPS is a potent vaccine antigen for serotype-specific protection against shigellosis. Previously, we separately cloned/expressed S. sonnei and S. dysenteriae 1 LPSâ€™s from a low copy plasmid in Ty21a. The pGB-2 plasmid carrying Shigella LPS genes was stable during nonselective growth, but inexplicably lost stability upon removal of a selectable antibiotic-resistance gene. In this study, we have manipulated exisitng recombineering methods to insert 10-15 kb blocks of Shigella O-antigen-encoding genes into a targeted site within the Ty21a chromosome. The chromosomally-inserted Shigella LPS genes are 100% stable during subsequent growth for > 75 generations. Ty21a containing chromosomally-inserted S. sonnei O-antigen genes (i.e. Ty21a-Ss) simultaneously expressed both S.Typhi and S. sonnei O-antigens, elicited high serum antibody titers against both LPSâ€™s in mice and protected mice against a virulent S. sonnei challenge. Separately, the S. dysenteriae 1 O-antigen genes were inserted into the Ty21a chromosome. To obtain a high level of heterologous LPS expression, the native operon promoter was replaced by the constitutive, highly expressed lpp promoter, which resulted in strain Ty21a-Sdl exhibiting enhanced LPS expression. Ty21a-Sd1 elicited significant serum antibody responses against both homologous and heterologous LPSâ€™s and protection against virulent S. dysenteriae challenge. These strains comprise two of five proposed multivalent candidates that are expected to provide protection against >85% of shigellosis worldwide as well as typhoid fever. In addition, we have already plasmid-cloned to expression in Ty21a the S. flexneri 2a and 3a serotype LPSâ€™s and are currently cloning S. flexneri 6 LPS genes, which will complete our pentavalent combination vaccine.

FDA/CBER
Suzuki, Akiko
Postdoctoral Fellow
Radiology/Imaging/PET and Neuroimaging
Development of Radiolabeled Interleukin-13 Pseudomonas exotoxin for Bio-imaging and Bio-distribution in Animal Model of Human Glioblastoma Multiforme
Previously, we have demonstrated that a variety of human cancers including glioblastoma multiforme (GBM) uniquely overexpress receptors for Th2 derived cytokine, interleukin-13 (IL-13). IL-13 receptor a2 (IL-13Ra2), one of the two chains of IL-13R, is shown to be involved in cancer metastasis and serves as an effective target for IL-13R directed immunotherapy of cancer. To target IL-13R, we have developed a chimeric fusion protein, which consists of human IL-13 and a truncated Pseudomonas exotoxin (IL-13PE). IL-13PE is a powerful immunotoxin for IL-13Ra2-positive tumors and is being tested for safety and effectiveness in clinical trials in GBM patients. However, no studies have been performed to evaluate distribution of IL-13PE after intra-cranial delivery. We developed a novel technology to radiolabel IL-

13PE with I-125 and demonstrated that I-125-IL-13PE maintained its specific binding activity to IL-13Ra2 positive U251 GBM cells but not to T98G GBM cells, as these cells did not express IL-13Ra2. I-125-IL-13PE was equally cytotoxic to glioblastoma cells as non-radiolabeled IL-13PE, which killed U251 cells in a concentration dependent manner but not T98G cells. Both binding and cytotoxic activities were blocked by 100-fold excess of IL-13 indicating IL-13PE binding was specific. Athymic nude mice with intracranially implanted U251 tumors were given stereotactic intratumoral injections of I-125-IL-13PE by either a bolus injection or a 3-day-convection-enhanced delivery (CED) for its bio-distribution with SPECT/CT imaging. The animals were imaged by micro-SPECT/CT techniques to study the retention and distribution of I-125-IL-13PE in U251 tumors at various time points. CED study showed significantly higher volumes of distribution and maintained detectable drug levels for longer periods of time compared to bolus injection. Our results demonstrate that IL-13PE can be successfully radiolabeled with I-125 without the loss of binding and cytotoxic activity and that the administration of I-125-IL-13PE by CED showed higher volumes of distribution than the bolus route. Additional studies are ongoing to determine the number of catheters needed for an effective volume of drug distribution in the entire brain as GBM tumor cells are spread in the entire brain. These studies will provide a better insight into the distribution of not only immunotoxin protein drug but also cell and gene therapy vectors, which are being tested in clinical trials.

NCI-CCR
Metzger, Meredith
Postdoctoral Fellow
Biochemistry - General and Lipids
Mechanistic insights into ubiquitylation: allosteric activation of a key component of the endoplasmic reticulum-associated degradation machinery
Efficient degradation of proteins from the endoplasmic reticulum (ER) is critical to cellular homeostasis and its dysregulation is associated with disorders ranging from neurodegeneration to cancer. Proteins are degraded from the ER by the conserved process of ER-associated degradation (ERAD). ERAD substrates are polyubiquitylated through the concerted action of ubiquitin-conjugating enzymes (E2s) and RING ubiquitin ligases (E3s) dedicated to ERAD. E2s covalently conjugated to ubiquitin (E2~Ub) bind to RING domains through canonical low affinity (>100 uM) interactions that stimulate transfer of ubiquitin from E2~Ub to substrate. A major conundrum is how multiple rounds of ubiquitin transfer, required for ubiquitin chain formation, are efficiently achieved with such weak E2-E3 interactions. To study this, we utilize the model organism Saccharomyces cerevisiae. The S. cerevisiae ERAD E2, Ubc7p, is recruited to the ER by Cue1p, an integral membrane protein essential to ERAD. Binding to Ubc7p occurs through the Ubc7p-binding region (U7BR) of Cue1p, which we previously identified. Interestingly, the U7BR is also required for ubiquitylation by Ubc7p. To examine this functionally unique interaction, we determined the structure of the U7BR-Ubc7p complex. The U7BR is a novel E2-binding domain consisting of three helices, each of which contacts Ubc7p extensively and is crucial for Ubc7p function in vitro and in vivo. The U7BR binds to Ubc7p on a face of the E2 removed from both its active site and the site of RING binding. Strikingly, we find that U7BR binding stimulates allosterically-induced conformational changes that â€œopen upâ€ the active site of Ubc7p. These changes significantly increase the rate of conjugation of Ubc7p with ubiquitin and the rate at which ubiquitin is transferred from Ubc7p to substrate. Further, the U7BR induces an ~8-fold increase in the affinity of Ubc7p for RING E3s. Thus, in addition to recruiting Ubc7p to the ER, Cue1p also has an indispensible function as an activator of Ubc7p, and the distinct allosteric effects imparted by the U7BR together provide a molecular basis for this activation. This work also provides a novel mechanism for how ubiquitin chain formation can be effected in vivo despite low affinity E2-E3 interactions. Our findings raise the intriguing possibility

that other â€˜third partyâ€™ proteins may also bind to and modulate the function of E2s in vivo, and thereby play a general role in facilitating ubiquitylation.

NCI-CCR
Tanaka, Naoki
Visiting Fellow
Biochemistry - General and Lipids
Dietary methionine deprivation reduces adipose tissue mass and improves insulin sensitivity in mice
The prevalence of obesity and metabolic syndrome is increasing worldwide due to sedentary lifestyle and overnutrition. Lifestyle modifications, such as increased physical activity and restriction of calorie intake, and pharmacological therapies, have been used to prevent/treat obesity. However, it is sometimes difficult to correct severe obesity by such interventions. Through studies of murine nonalcoholic steatohepatitis models, dietary methionine deprivation was found to cause significant body weight loss. Therefore, the effects of methionine deficiency on whole-body metabolism were investigated. Male C57/B6 mice (8-10 weeks of age) were treated with a control diet and methionine-deficient (MD) diet for 4 weeks and serum lipid profiles and glucose tolerance examined. The MD diet significantly reduced body weight, white adipose tissue mass, and hepatic triglyceride contents, without notable liver dysfunction. Serum lipid analysis revealed marked decreases in total cholesterol. Glucose tolerance and insulin sensitivity was significantly improved by the MD diet. These changes were likely associated with marked up-regulation of adiponectin in white adipose tissue, and fibroblast growth factor (FGF) 21, peroxisome proliferator-activated receptor (PPAR) gamma-coactivator 1 alpha, and pyruvate dehydrogenase kinase 4 in the liver that mimic the physiological response to fasting. Although PPARalpha is a master regulator of glucose/lipid metabolism in the fasting state and FGF21 expression in the liver, the increases in FGF21 mRNA levels by the MD diet were observed in a PPARalpha-independent manner in mice. FGF21 was reported to possess anti-obesity and anti-diabetic properties. Interestingly, levels of FGF21 mRNA were increased by MD diet in primary hepatocytes, suggesting that hepatocytes sense methionine deficiency in the external milieu and secrete FGF21 to adapt to alterations of nutrient/energy homeostasis. Collectively, dietary methionine deprivation could reduce adiposity and improve insulin sensitivity and lipid profiles, probably due to modulating the expression of FGF21 and adiponectin. These results provide a possible nutritional approach that could safely treat obesity/metabolic syndrome and ensuing diseases, such as diabetes, dyslipidemia, atherosclerosis, and nonalcoholic fatty liver disease.

NCI-CCR
HONG, JINGJUN
Visiting Fellow
Biochemistry - Proteins
Structural basis of recognition of histone variant H2A.Z by the nucleosome remodeler SWR1 subunit
Histone variant H2A.Z-containing nucleosomes are incorporated at most eukaryotic promoters. The conserved multi-subunit nucleosome-remodeling enzymes, SWR1 in budding yeast and p400/SRCAP in human, replaces histone H2A in canonical nucleosomes with H2A.Z in an ATP-dependent manner. The over-incorporation of H2A.Z in chromatin, however, is associated with breast cancer, and therefore H2A.Z and p400/SRCAP are targets for cancer therapy. Two subunits (Swc2 and Swr1) in the SWR1 complex have been identified that can recognize the H2AZ/H2B dimer in yeast. However, the structural basis of the recognition is unknown. Here, we have determined the crystal structure of the H2AZ/H2B in complex with the region N-terminal to the ATPase domain in the Swr1 subunit at a 1.95 Å resolution. We find that Swr1 binds to H2AZ/H2B using a short 3(10) helix and an irregular chain, which are separated by a nine-residue disordered loop. Swr1 forms hydrophobic interactions with the a3 and aC helices of

H2A.Z, and also with the a1 and a2 helices of H2B. Swr1 binding also leads to an extension of a helical turn of the aC helix in H2A.Z. Additionally, the Asp-rich sub-region in the histone-binding region (HBR) of Swr1 forms electrostatic interactions with Arg86 in the L2 loop, and with Arg90 at the N-terminus of the a3 helix in H2A.Z, and with Lys61 at the N-terminus of the a2 helix in H2B. Moreover, in vivo and in vitro mutation studies have confirmed that the above structural regions are bona fide recognition sites between H2A.Z and Swr1. Our results provide the first structural insights into how Swr1 recognizes H2AZ/H2B, and help identify similar binding regions between human H2AZ/H2B and p400/SRCAP, paving the road for structure-based cancer drug design.

NCI-CCR
Masaoka, Takashi
Postdoctoral Fellow
Biochemistry - Proteins
Multi-Enzyme Screening for Broad-Spectrum Thienopyrimidinone Inhibitors of HIV-1 Reverse Transcriptase-Associated Ribonuclease H
Introduction of combination antiretroviral therapy has resulted in dramatic improvements in human immunodeficiency virus type 1 (HIV-I)-related morbidity and mortality. However, the emergence of drug-resistant virus, which subsequently results in incomplete viral suppression, is a major obstacle to the effective long-term treatment of HIV-infected patients. Since none of the approved anti-HIV drugs target ribonuclease H (RNase H) activity of HIV-1 reverse transcriptase (RT), inhibitors that act against RNase H should be effective against all of the current drug-resistant viruses. We have identified the thienopyrimidinone, 5,6-dimethyl-2-(4-nitrophenyl)thieno[2,3-d]pyrimidin-4(3H)-one (DNTP), as a potent inhibitor for RNase H (IC50 = 0.85 μM). Site-directed mutagenesis has predicted that DNTP occupies the heterodimer interface between the C-terminal p66 RNase H domain and p51 thumb of HIV-1 RT. Thienopyrimidinones thus represent allosteric RNase H inhibitors that bind adjacent to the active site and induce a conformational change that is incompatible with catalysis. To further understand the inhibitory mechanism and to improve the potency of the thienopyrimidinone, we performed biochemical characterization of 39 novel derivatives of the thienopyrimidinone, DNTP, with antiviral testing of selected compounds. In addition to wild-type HIV-1 RT, thienopyrimidinones were evaluated with a panel of four rationally-designed, selectively-mutated p66/p51 heterodimers that display high-level sensitivity or resistance to DNTP. Using this strategy, we identified 3â€™,4â€™-dihydroxyphenyl (catechol)-substituted thienopyrimidinones with sub-micromolar in vitro activity against both wild type HIV-1 RT and engineered drug-resistant variants (IC50 = 0.26 â€“ 0.69 μM). Fluorescence-based thermal shift analysis indicates that, in contrast to active site RNase H inhibitors, these thienopyrimidinones destabilize the enzyme, in some instances reducing the Tm by 5oC. Importantly, catechol-containing thienopyrimidinones also inhibit HIV-1 replication in cells. Collectively, our data strengthens the case for allosteric inhibition of HIV RNase H activity, providing an important platform for designing improved antagonists for use in combination therapy with nucleoside and nonnucleoside RT inhibitors.

NCI-CCR
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Biochemistry - Proteins
Development of new HIV-1 Integrase inhibitors overcoming clinical resistance
Raltegravir (RAL) is the first HIV-1 integrase (IN) inhibitor approved by the FDA. The use of RAL in experienced and more recently in naïve patients has become part of mainstream AIDS therapy. However, resistance to RAL has emerged as a limitation. It involves IN mutations in three genetic pathways: N155H, G140S/A-Q148H/R/K and Y143H/R/C. We systematically investigated how these

mutations affect IN enzymatic activity and RAL resistance. We demonstrated that the clinically relevant IN mutations are sufficient to account for the phenotype of RAL-resistant viruses. We also assessed the resistance profile of elvitegravir (Gilead, approved in August 2012) and dolutegravir (ViiV Healthcare, phase III). Our studies show that both molecules have selective advantages over RAL but only dolutegravir presents an improved resistance profile. However, the Q148 pathway still induces around 6-fold resistance and new mutations are selected (G118R, R263K). Thus, we designed novel compounds with in vitro and cellular activities comparable to that of dolutegravir and performed structure-activity studies with wild-type and mutant recombinant IN, and solved the atomic structure of our new inhibitors in a model intasome (IN from the prototype foamy virus) by X-ray crystallography. We also observed a correlation between the drugsâ€™ resistance profile and their selectivity for IN activities. This finding led us to use RAL as a probe to evaluate the IN-DNA molecular interactions and we now show drastic differences between wild-type and mutant enzymes. Detailed analyses of IN mutants is critical to model the HIV IN active site and overcome resistance to the first generation IN inhibitors.

NCI-CCR
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Biochemistry - Proteins
Isolation and characterization of a novel class of potent anti-HIV proteins from an Australian soft coral.
An estimated 34 million people worldwide are living with the human immunodeficiency virus (HIV). HIV infection requires fusion of cellular and viral membranes mediated by viral envelope glycoproteins (gp120 and gp41) and target cell surface receptors (CD4 and a chemokine receptor). The need for a female-controlled anti-HIV microbicide to prevent infection has been declared a critical world health priority by the WHO and U.S. government. Concerns about emergence of HIV strains resistant to current antiretrovirals (ARVs) remain especially when these agents are used in a low-dose prophylactic mode such as a microbicide. It is thus critical to identify novel non-ARV microbicide and post-exposure prophylaxis agents to prevent HIV infection. Here we report the isolation of a novel class of proteins (Cnidarins) from a soft coral Synthecium sp. (phylum Cnidaria). The proteins were purified by sequential ethanol and ammonium sulphate precipitation followed by hydrophobic interaction chromatography. The purified proteins, CNID-1, CNID-2 and CNID-3, were monomers of ~170 amino acids with molecular weights of ~18 kDa; and had picomolar to low-nanomolar activity against laboratory strains and primary isolates of HIV-1. They inhibited viral fusion in a concentration-dependant manner but not viral attachment indicating the proteinsâ€™ antiviral effects occur after initial virus-to-cell attachment but prior to viral entry. CNID-1 and CNID-3 were fully sequenced and showed no significant homology (>25%) to any known protein. CNID-1 being the most potent (EC50 of 85pM) was the focus of this study. CNID-1 bound equivalently to gp120 and gp41 in a concentration-dependant manner but not to other glycoproteins or soluble CD4. Pre-treatment with CNID-1 did not block subsequent binding of sCD4 to gp120 and vice versa and its gp120 binding was independent of glycosylation. Pre-treatment with other potent antiviral proteins cyanovirin and scytovirin, did not affect CNID-1 binding to gp120 and gp41 but promoted slightly better binding while griffithsin pre-treatment reduced CNID-1 binding but not completely. Recombinant production in E.coli resulted in C-terminal truncated forms of CNID-1 (14-16kDa, EC50 of 45-150nM). Ongoing work includes optimizing expression conditions to produce fully active, recombinant CNID-1. The Cnidarin proteins are a novel class of proteins that target viral fusion and inhibit HIV infection in a manner different from any currently identified anti-HIV agent.

NCI-CCR
Afonin, Kirill
Research Fellow

Biophysics

Development of therapeutic RNA switches selectively activating apoptosis in diseased cells

Over the past decade, the total number of RNA interference (RNAi)-based preclinical and clinical trials has increased rapidly with 22 different therapeutic small interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs) reaching clinical trials for the treatment of at least 16 diseases. The first clinical trial (in 2004) for siRNA-based therapy ran only three years after the discovery of siRNA function in mammalian cells; much faster than the usual six to seven years for small-molecule drug candidate preparations. Altogether, this illustrates a promising future in novel RNAi therapeutics. We have computationally designed novel therapeutic RNA switches with embedded siRNA functionalities that could represent an important step towards a selective cure of cancers. In the absence of a specific mRNA that triggers the switch (e.g. CTGF, VEGF or TWIST, which are over-expressed only in cancer cells) these switches containing the Dicer substrate (DS) siRNA are inactive. However, the switches are innovatively designed to possess sequence regions that can bind to the trigger mRNA and induce the conformational changes leading to the release of DS siRNAs selected against human apoptosis inhibitor genes (e.g. BCL-2, FLIP, STAT3 and XIAP). We comprehensively analyzed several switches in vitro and in human breast cancer cells as a model system. Native PAGE experiments confirmed the successful formation of switch construct and the release of DS siRNA during the incubation with complementary mRNA fragment (CTGF) at 37 C. Furthermore, the successful processing of DS siRNA by a human recombinant Dicer was shown. We determined that the affinities of switch activation by a trigger mRNA to be at least as low as 0.5nM. Several different cell culture experiments allowed tracking of the uptake of the fluorescently labeled switches by the breast cancer cells. The successful release of therapeutic moieties (siRNAs) was confirmed by the significant level of suppression (>60%) of the target gene expression when the target mRNA was over-expressed inside the cells. Interestingly, the extent of siRNA release is proportional to the level of expression of target mRNA and can be fine-tuned by altering the concentration of the switch. Overall, this novel approach opens a new route in development of conditionally activated nucleic-acids-based smart nanoparticles for a broad range of biomedical and nanotechnological applications.

NCI-CCR

Hu, Duosha

Postdoctoral Fellow

Carcinogenesis

Regulation of KSHV encoded viral interleukin 6 (vIL-6) by X-box binding protein 1 (XBP-1)

Kaposiâ€™s sarcoma-associated herpesvirus (KSHV) is the causative agent for Kaposiâ€™s sarcoma (KS), primary effusion lymphoma (PEL), and a subset of multicentric Castleman's disease (MCD). There is evidence that KSHV-encoded viral IL-6 (vIL-6) plays an important role in the pathogenesis of these disorders. vIL-6 mimics the activity of human IL-6 (hIL-6), although it can bind to a wider range of receptors. A large subset of KSHV-MCD plasmablastic B-cells produce KSHV vIL-6, and overexpression of vIL-6 is a major factor causing the severe inflammatory symptoms in this disorder. vIL-6 is produced during lytic replication of KSHV, but can also be produced by some cells during latency. The factors responsible for vIL-6 production in cells latently infected with KSHV are not known. Because high levels of spliced XBP-1 are expressed during plasmacytic differentiation of B cells in lymph nodes and XBP-1 has been shown to activate the KSHV lytic gene, RTA, we hypothesized that XBP-1 might also play a direct role in vIL-6 activation. Analysis of the promoter region of vIL-6 showed that it had several potential consensus XBP-1-response elements (XREs), which respond to the active spliced form of XBP-1. Luciferase reporter assays showed that spliced, but not unspliced, XBP-1 can activate the vIL-6 promoter. By deletion and mutation of the potential XRE sequence, 2 functional XREs were found. Using immunohistochemistry, we observed that XBP-1 and vIL-6 were co-expressed in KSHV-MCD

plasmablasts and also in PEL cells. Brefeldin A induction of XBP-1 in PEL cells led to upregulation of vIL-6 at the RNA and protein level by rt-PCR and Western blot respectively. In conclusion, this study provides evidence that vIL-6 can be directly up-regulated by spliced XBP-1 without the need for KSHV lytic activation. This suggests production of vIL-6 by KSHV-MCD plasmablasts can occur in an XBP-1 dependent fashion and independent of lytic activation. XBP-1-induced vIL-6 may contribute to the pathogenesis of KSHV-MCD, as well as PEL and other KSHV-related disorders.

NCI-CCR
Kuschal, Christiane
Visiting Fellow
Carcinogenesis
Effect of aminoglycosides and non-aminoglycosides on readthrough of premature termination codons in xeroderma pigmentosum group C cells
About 12% of human genetic disorders are caused by premature termination codon (PTC) mutations. Aminoglycoside antibiotics and non-aminoglycoside compounds have been proposed for restoring full-length proteins from genes with PTC in nonsense mutation causing human diseases including ataxia-telangiectasia, Hurler syndrome, and cystic fibrosis. They cause a conformational change of the ribosome, leading to increased mRNA translation. Many xeroderma pigmentosum (XP) patients have PTC in the XPC DNA repair gene, leading to defective nucleotide excision repair (NER) and a more than 10,000 fold increased skin cancer risk. There is no cure for xeroderma pigmentosum. We developed a sensitive method for assessing efficiency of readthrough by use of a panel of fibroblast cells from homozygous and compound heterozygous XP-C patients carrying different types of PTC. We investigated whether the aminoglycosides geneticin and gentamicin and the non-aminoglycoside compounds PTC124, BZ16 and RTC14 induce the production of an XPC protein and induce DNA repair. In 6 of 8 XP-C cell lines, treatment with aminoglycosides stabilized XPC-mRNA, which would otherwise have been degraded by nonsense-mediated decay. In addition, readthrough led to the expression of an XPC protein that is able to function in NER: immunofluorescence revealed that the expressed XPC protein localized to UV damaged sites and recruited downstream XPB and XPD helicases to UV DNA damages. Furthermore, readthrough induced the repair of UV-induced 6-4 photoproducts and cyclobutane pyrimidine dimers. None of these effects appeared in untreated cells or in cells harboring an XP-C initiation codon mutation. Site directed mutagenesis to mimic PTC in luciferase expression vectors revealed that readthrough depends on the sequence context. Treatment with non-aminoglycoside compounds resulted in similar increased XPC-mRNA expression and DNA damage removal with less toxicity than the aminoglycosides. This is the first study that shows readthrough of XPC. The readthrough efficiency depends on the specific PTC, its location in the XPC gene and type of compound. Our findings draw attention to the value of readthrough compounds as a new therapeutic approach for preventing skin cancer in XP-C patients harboring PTC by partially correcting their DNA repair defect.

NCI-CCR
Maachani, Uday Bhanu
Postdoctoral Fellow
Carcinogenesis
MPS1 kinase inhibition induces Radiosensitization in Glioblastoma (GBM) Cells by modulating DNA repair proteins.
During the cell cycle, genomic stability requires accurate chromosome segregation. Errors in this process can cause aneuploidy and lead to tumorigenesis. To ensure faithful chromosome segregation, cells develop a mechanism called the spindle assembly checkpoint (SAC). Cancer cells are addicted to the components of SAC machinery for a faithful entry of the cell into anaphase. Thus, targeting the

molecular mechanisms required for the growth of aneuploid cells may be a more cancer cell specific therapeutic approach applicable to broader tumor histologies. Previously, using a siRNA based RNAi screen we identified MPS1 kinase, as an important kinase for GBM cell survival. MPS1 is an essential SAC enzyme aberrantly overexpressed in a wide range of tumors and necessary for tumor cell proliferation. We observed inhibition of GBM cell growth when MPS1 was downregulated by number of MPS1 specific siRNAs. This was further validated using a selective and orally bioavailable MPS1 inhibitor NMS-P715 in various in-vitro cell assays. The induction of cell death was induced partly by apoptosis; however, the major mechanism was mitotic catastrophe. Cells treated with NMS-P715 showed an increase in cells in G2-M phase of cell cycle followed by mitotic catastrophe. Compared to control cells inhibition of MPS1 resulted in radiosensitization of GBM cells. We observed decrease in DNA damage repair and significant retention of ?H2AX foci after combination of radiation (RT) and with NMS-P715 compared to individual treatments. Radiation in combination with NMS-P715 was more potent in inhibiting viability of GBM cells as accessed by colony formation assay. Further, NMS-P715 could inhibit GBM tumor growth in an orthotopic brain tumor model. In order to determine MPS1 associated molecular pathways, we compared gene expression profile in MPS1 knockdown cells compared to the control by microarray analysis. Ingenuity pathway and Gene Set Enrichment Analysis were used to investigate the biological relevance of MPS1 modulated genes. We identified DNA Repair gene PRKDC (DNAPK) as a downstream modulator of MPS1 activity. Thus, inhibiting MPS1 kinase in combination with radiation could represent a promising new approach to GBM therapy.

NCI-CCR
Woditschka, Stephan
Postdoctoral Fellow
Carcinogenesis
Overexpression of RAD51 promotes brain metastases from breast cancer
Brain metastases are one of the most devastating consequences of breast cancer. They contribute increasingly to patient mortality and morbidity, as better chemotherapeutics â€“ nearly all of which are brain-impermeable â€“ target systemic disease and improve patient survival. We identified overexpression of the DNA double-strand break (DSB) repair gene RAD51 as a common molecular feature of brain metastases compared to both matched primary breast tumors from the same women (1.5-fold, p=0.0001) and systemic (bone/lung) metastases from breast cancer (1.4-fold, p=0.008). We hypothesized that RAD51 is a functional driver of brain metastasis development, independent of its recognized role in resistance to DNA-damaging chemotherapy. RAD51 was overexpressed in brain-metastatic variants of human MDA-MB-231 breast carcinoma cells (231-BR) and 4T1 murine mammary carcinoma cells (4T1-BR). In vitro, RAD51 overexpression results in a 35-40% (p<0.0001) reduction Gamma-H2AX foci during S-phase, a DNA DSB marker. Furthermore, using a Homologous Recombination (HR) assay, the frequency of HR was significantly increased (1.7-fold, p=0.002) in RAD51 overexpressers compared to vector controls. No effects on proliferation or invasion were observed with RAD51 overexpression. In intracardiac-injected mice, overexpression of RAD51 increased formation of large brain metastases, the equivalent of clinically detectable lesions, (3.9-fold, p=0.02; 1.8-fold, p=0.006) and micrometastases (3.8-fold, p=0.008; 1.5-fold, p=0.02), compared to vector controls in 231-BR and 4T1-BR models, respectively. ShRNA-mediated RAD51 knockdown in 4T1-BR cells had the opposite effect, reducing large brain metastases (2.5-fold, p=0.001) and micrometastases (2.5-fold, p=0.001). RAD51 modulation had no effect on lung metastasis development in tail-vein mouse models of 231-BR or 4T1-BR cells. To distinguish between roles for RAD51 in initiation vs. outgrowth of brain metastasis, we quantified iron-labeled 231-BR cells in the brain microenvironment at 3, 7 and 12 days post-injection. On day 12, we observed significantly more RAD51-overexpressing single cells compared to vector controls, with a clear trend discernable by day 7 (fold-changes over vector: 1.1-fold, p=0.8; 2.4-fold, p=0.07 and

2.8-fold, p=0.001 for days 3, 7 and 12, respectively). Together these data demonstrate that RAD51 drives initiation of brain metastases from breast cancer, which may be amendable to therapeutic development.

NCI-CCR
Roukos, Vassilis
Postdoctoral Fellow
Cell Biology - General
A spatiotemporal framework for the formation of chromosome translocations
Chromosomal translocations play an important role in tumorigenesis and can be causal in cancer. Despite their prevalence and functional importance, the sequence and timing of events leading to the formation of chromosome translocations in the context of the intact cell nucleus are largely unknown. We have developed a cell-based experimental system to visualize for the first time the formation of chromosomal translocations in living cells. In this system, we induce DNA breaks at defined genome sites in a controlled fashion to mimic endogenous double strand breaks (DSBs). The broken ends of the targeted chromosomes and the respective repair foci marked by the repair factor 53BP1 can be tracked in space and time using three different color fluorescent tags. We have used this system to characterize the spatial and temporal properties of chromosome translocations. We find that upon induction of DSBs repair factors are rapidly recruited to breaks sites and translocations form within several hours. We identify three distinct phases in translocation formation: DSB partner search, transient pairing and persistent pairing. While distinct pathways repair DSBs in different cell cycle stages we find that translocations form in a cell cycle-independent manner. Tracking of individual translocating double strand breaks by high-throughput microscopy shows that proximal breaks are prone to translocations and that they exhibit distinct dynamic motion compared to non translocating loci. Systematic perturbations of key factors of the DNA damage response by chemical inhibition and knock down approaches identified distinct effects on DSB pairing and the formation of translocations. While Mre11 knockdown decreases the pairing of DSBs and the formation of translocations, inhibition of the DNAPKcs kinase activity increases translocation frequency without affecting the pairing of breaks. These observations provide a spatial and temporal framework for the formation of chromosome translocation and they identify several molecular pathways as contributors to translocation etiology.

NCI-CCR
Kettenhofen, Christine
Postdoctoral Fellow
Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology
Membrane shaping proteins EHD1 and EHD3 function in primary cilium formation
Primary cilia are highly conserved microtubule-based organelles that project from the surface of most vertebrate cells. Numerous pathways including Hedgehog and Wnt depend on the cilium for optimal signaling. Consequently, perturbations of cilia function result in diseases referred to as ciliopathies. The molecular details governing the process of cilia formation remain poorly understood. The ciliary membrane is thought to develop from a ciliary vesicle that is progressively reshaped into a tubule structure around the developing axoneme. Previously, we demonstrated that a Rab11-Rab8 cascade functions in ciliary membrane formation. To investigate the mechanisms involved in membrane structural rearrangements occurring during ciliogenesis, we examined the potential role of membrane curving proteins known as EHDs (EHD1-4), proteins linked to Rab11 and Rab8 trafficking pathways. We find EHD1 and EHD3 but not EHD2 and EHD4 are present on the developing ciliary membrane. Structured illumination microscopy (SIM) super resolution imaging support a role for EHD1/EHD3 in regulating positive membrane curvature during ciliary membrane development and ablation of EHD1

impairs ciliogenesis in RPE and MEF cells. Interestingly, defects in ciliation could be rescued by EHD1 or EHD3, suggesting a redundancy in ciliary membrane assembly function. EHD1 -/- mice described by other groups do not display characteristic ciliopathy defects. Because our work suggests that EHD3 could compensate for EHD1 functioning we used zebrafish as model system to perform morpholino-based knock-downs of both ehd1 and ehd3 proteins and looked for developmental defects resulting from cilia abnormalities. Both ehd1 and ehd3 morphants featured overlapping organogenesis defects caused by cilia malfunction, in particular, smaller eyes (also reported in EHD1-/- mice). Histological and TEM analyses revealed that photoreceptor cells have proper centriolar structures but lack cilia. However, we also observed distinct ciliary phenotypes between the two morphants. ehd3 knock-down caused additional heart and kidney defects whereas analysis of ehd1a+b knock-down in a transgenic line with a marker for cilia (Scorpio-GFP) shows absence of immotile kinocilia in otic vesicles. Our study provides the first in vivo evidence that ehd1 and ehd3 are both required for vertebrate cilia formation and suggests some functional overlap, which could explain the lack of cilia phenotype observed in EHD1 -/- mice.

NCI-CCR
Hasegawa, Keisuke
Postdoctoral Fellow
Cell Cycle-General, Regulators and Checkpoints, Apoptotic Mechanisms
The role of RCC1 and Ran in the regulation of mitosis and oncogenic reprogramming
The role of the mitotic spindle in chromosomes segregation was shown to depend on mitotic factors activated by small GTPase Ran. RanGTP activates such factors by releasing them from complexes with importins, supporting normal assembly and function of the spindle. Moreover, due to the chromatin-binding of the single Ran-specific exchange factor RCC1 and cytoplasmic localization of RanGAP1, mitotic chromosomes are surrounded by a steep RanGTP gradient which could accelerate spindle assembly by activating mitotic factors at the chromosomes. However, while many Ran-regulated mitotic factors are highly expressed in proliferative cells and tumors, their levels are low in normal tissues and cells which also contain lower Ran levels. We therefore examined whether the mitotic role of Ran is universal or an adaptation in some cells, including cancer cells. Using FRET biosensors, we performed live cell measurements of mitotic RanGTP gradients in ~20 different human cell types, including normal primary cells, immortalized, cancer-derived and tumorigenic cells. We found that steep mitotic RanGTP gradients and high RanGTP levels were present in rapidly growing cells and that the gradients were significantly reduced in slow growing normal cells. We showed that increased RCC1 expression was sufficient to drive the steep RanGTP gradients in primary cells and the steep gradients were required for rapid spindle assembly and cell cycle rate in HeLa cells. Moreover, we demonstrated that polyploidization of primary cells resulted in cells displaying steep mitotic RanGTP gradients, showing that large chromosomal gain is a key driver of steep mitotic RanGTP gradients. Such chromosomal gain-driven activation of Ran could promote mitosis in cells resulting from cytokinesis failure, supporting their survival and oncogenic transformation. We are currently testing the hypothesis that the activation of Ran supports oncogenic reprogramming by examining the role of Ran in in vitro induced transformation of primary cells into cancer stem cells (CSCs). Our results so far show that that RCC1 levels and RanGTP gradients are strongly increased during oncogenic reprogramming and also during the induction of pluripotency in normal cells by the Yamanaka factors. These results are consistent with the idea that increased RCC1 expression is critical for the de-differentiated and highly proliferative state of CSCs.

NCI-CCR
Kim, Tae Sung
Visiting Fellow

Cell Cycle-General, Regulators and Checkpoints, Apoptotic Mechanisms

Regulation of genomic stability by Plk1-dependent stepwise phosphorylation of the PBIP1-CENP-Q complex

Mammalian polo-like kinase 1 (Plk1) is critically required for proper M-phase progression. Plk1 regulates diverse biochemical and cellular processes, such as G2/M transition, centrosome maturation, metaphase/anaphase transition, mitotic exit and cytokinesis. Whether and, if so, how Plk1 promotes proper chromosome segregation remains largely elusive. Plk1 localizes to centrosomes, centromeres/kinetochores, and midbody through the targeting activity of its C-terminal non-catalytic region, called polo-box domain (PBD). Disruption of the PBD function results in various mitotic defects, including chromosome missegregation. A failure in this process ultimately leads to aneuploidy, a hallmark of cancer. We have shown that Plk1 recruits itself to interphase centromeres by phosphorylating the T78 residue of a centromere scaffold, PBIP1 (also called MLF1IP and CENP-U/50) and binding to the self-generated phospho-T78 (p-T78) motif through its PBD. PBIP1 also tightly interacts with another centromere component, CENP-Q, and forms a stable complex all throughout the cell cycle. Here, we demonstrated that, at the late stages of the cell cycle, Plk1 bound to the p-T78 motif of PBIP1 directly phosphorylates the CENP-Q subunit of the PBIP1-CENP-Q complex at multiple Ser/Thr residues. Mutation of Plk1-dependent 9 phospho-Ser/Thr residues on CENP-Q to Ala (CENP-Q-9A) greatly delayed the delocalization of the PBIP1-CENP-Q complex from mitotic kinetochores, suggesting that timely phosphorylation of CENP-Q by rising PBIP1-p-T78-bound Plk1 activity is critical for triggering dissociation of the PBIP1-CENP-Q complex from mitotic kinetochores. On the other hand, mutation of the 9 Ser/Thr residues to Asp/Glu, respectively, (CENP-Q-9D/E) crippled the capacity of CENP-Q to dynamically localize to interphase centromeres and delocalize from mitotic kinetochores. At a cellular level, CENP-Q RNAi cells expressing the CENP-Q-9A mutant, but not the corresponding CENP-Q wild-type, exhibited significantly increased mitotic index, lagging chromosomes, and micronucleation. CENP-Q RNAi cells expressing CENP-Q-9D/E also displayed the defects but at reduced levels. Thus, we propose that proper phosphorylation/dephosphorylation processes constitute the molecular basis of how the PBIP1-CENP-Q complex localizes to and delocalizes from centromeres/kinetochores, and that Plk1 promotes genomic stability by sequentially phosphorylating PBIP1 and CENP-Q.

NCI-CCR
Nelson, Christopher
Postdoctoral Fellow
Chemistry

Design and synthesis of robust targeting drug conjugates: Selenocysteine antibody-drug conjugates

The highly specific cellular targeting and pharmacological stability of antibodies, combined with the diverse structural and cytotoxic properties of small-molecule drugs, make antibody-drug conjugates (ADCâ€™s) a premier strategy for targeted drug delivery in cancer therapy. A prototypical ADC is generated by covalently linking electron-rich amino acid sidechain functionalities (ie. OH, NH or SH) of a monoclonal antibody (mAb) to an electrophillic â€œlinkerâ€ of the drug molecule. However, controlling the degree of drug loading (number of drugs/mAb), retaining the native binding of the mAb portion of the ADC and designing linkers for target-specific drug liberation remain problematic to practitioners of the art. Our approach to forming ADCâ€™s address these issues with a combination of design elements. The degree of drug loading is controlled by utilizing a selenocysteine residue (Sec, U), engineered into the C-terminal Fc domain of a mAb, which allows chemoselective drug conjugation. The distinct reactivity of the selenol sidechain (relative to thiols or amines), combined with a judiciously chosen drug-linker, facilitates the formation of ADCâ€™s with excellent selectivity at low pH. Paramount to the success of this strategy is the choice of a highly cytotoxic drug payload because the conjugation is limited to one drug molecule per mAb. Auristatin F, a tubulin targeting cytotoxic pentapeptide, has

served as the scaffold to create a small library of synthetic derivatives with IC50 values ranging from micromolar to sub-picomolar! Such high potency has required the design of a robust chemical linker for mAb conjugation to avoid premature drug liberation, which could lead to off-target cytotoxicity. The linking agent of choice incorporates two distinct chemical moieties to prevent premature drug loss. First, irreversible mAb-drug conjugation is achieved using a very stable selenoacetimide linkage. Second, a specific dipeptide sequence is included within the linker that functions as an â€œenzyme triggerâ€ for drug release by being susceptible to proteolysis only by enzymes within the targeted cells. Efforts are currently underway to assess both the cellular cytotoxicity (in vivo) and specificity (in vitro) of these highly engineered ADCâ€™s in a variety of cancer cell lines.

NCI-CCR
Burman, Bharat
Doctoral Candidate
Chromatin and Chromosomes
Epigenetics of Chromosomal Breakage Sites and Translocations
Chromosomal translocations are genetic hallmarks of most cancer cells. Translocations require the formation of DNA double-strand breaks (DSBs) at two or more genomic loci, followed by the illegitimate joining of broken chromosomal ends through DNA repair. There is increasing evidence that translocations occur at non-random sites in the genome, suggesting that certain regions of the genome are more susceptible to DNA breakage than others. We hypothesize that altered chromatin structure predisposes genomic sites to DNA breakage and translocations. To identify chromatin features that facilitate translocations, we have mapped histone modifications and DNase I hypersensitive sites (DHSs) at translocation-prone regions in anaplastic large cell lymphoma (ALCL) precursor cells. We find enrichment of active histone marks and a decrease in repressive marks near frequent translocation breakpoints. In addition, one of the two breakpoints is enriched in DHSs. In a complementary approach, we have identified altered chromatin features including specific histone marks at common leukemia and lymphoma breakpoints in CD34+ hematopoietic stem cells. In order to directly test the role of chromatin features in DNA breakage susceptibility, we have developed a protein-DNA tethering system that allows us to create local chromatin domains at pre-defined sites in the genome containing inducible DSB sites in vivo. By measuring the amount of DSBs using ligation-mediated PCR, we find that histone modifying enzymes that create active chromatin marks generally increase breakage susceptibility; however, repressive marks do not necessarily decrease breakage susceptibility. This suggests the involvement of other factors in breakage susceptibility. Taken together, these experiments are providing first insights into the role of chromatin structure in the formation of nonrandom chromosomal breaks and the mechanisms that lead to clinically relevant translocations.

NCI-CCR
Zhang, Shaofei
Postdoctoral Fellow
Chromatin and Chromosomes
The Nucleosome Binding Protein HMGN5 and the Lamina Associated Protein Lap2a Interact and Reciprocally Affect Their Genome Wide Chromatin Binding.
The interactions of nuclear lamins with the chromatin fiber play vital roles in diverse nuclear functions such as gene regulation, DNA repair and chromatin organization. A key challenge in understanding the full spectrum of these interactions is to identify all the components that establish the link between these two major networks in nucleus. Here we report a novel connection between chromatin and nuclear lamin formed by nucleosome-binding and lamin-binding proteins. HMGN5 binds to nucleosomes without any specificity for the underlying DNA sequence and de-compacts chromatin by competing with

linker histone H1. LAP2a is a soluble splice variant of the nuclear envelope protein LAP2, which interacts with A-type lamin. By fluorescent microscopy we show that LAP2a-Cherry and HMGN5-YFP extensively co-localize throughout the nucleus. Using HaloLink affinity chromatography, we found that LAP2a specifically bound to HMGN5. Co-immunoprecipitation experiments with extracts from cells co-expressing LAP2a-myc and Halo-HMGN5 verified that HMGN5 and LAP2a interact in living cells. Furthermore, in vitro co-immunoprecipitation with extracts from cells co-expressing deletion mutation of HMGN5 or LAP2a indicated that N-terminal region of HMGN5 interacts with C-terminal region of LAP2a. Significantly, fluorescence recovery after photobleaching analysis reveals that changes in the levels of either HMGN5 or LAP2a affects intranuclear mobility of its interacting partner, in living cells. ChIP-seq analysis indicates that HMGN5 and LAP2a are enriched and co-localize in the promoter regions of numerous genes. Strikingly, loss of either HMGN5 or LAP2a leads to significant redistribution of the other protein on chromatin. The altered organization of HMGN5 in LAP2a-deficient cells is not due to major changes in the nucleosomal organization since comparison of the micrococcal nuclease digestion patterns of nuclei isolated from wild type and LAP2a-deficient HeLa cells suggests that neither the kinetics of micrococcal nuclease digestion, nor the nucleosomal repeat is affected in the absence of LAP2a. We propose that loss of either HMGN5 or LAP2a alters the higher order chromatin structure, thereby affecting the chromatin binding of its interacting partner. Our studies identify a novel functional link between the chromatin and nuclear lamin network and provide insights into process that affect the organization and function of the nucleus.

NCI-CCR
Li, Ang
Research Fellow
Clinical and Translational Research
MicroRNAs profiling supports two different types of mucinous breast carcinoma
Background: Mucinous carcinoma (MC) is a rare subtype of ductal carcinoma of breast generally associated with good prognosis. Recently, two types have been recognized, type A or paucicellular, and type B cellular with neuroendocrine differentiation. MicroRNAs have been implicated in regulation of cellular growth and differentiation and found to dysregulate cell proliferation in human tumors including breast cancer. We evaluated the phenotypic and molecular characteristics of mucinous tumors and performed miRNA profiling searching for similarities/differences between the two types and the DCIS associated with type B. Design and methods: 30 cases of MC (age range: 36-86 years; tumor size: 0.2-4.5 cm; nodal metastasis: 5 cases) were examined in this study. Immunohistochemistry for ER, PR, and Her2 was performed to identify the molecular characteristics of these mucinous tumors. Tissues of 12 cases representing high and low cellularity tumors and associated DCIS were microdissected and RNA was extracted. MiRNAs expression was profiled with miRNA array (nCounter Human v2 miRNA assay, Nanospring) and the data was analyzed using nSolver and Partek. Results: Expression of ER, PR, and Her2 protein were observed in 100.0% (30/30), 86.7% (26/30), and 6.7% (2/30) of MC cases but one had FISH amplification, respectively. We identified 14 (9 up / 5 down) differentially expressed miRNAs associated with high but not low cellularity mucinous tumors (including upregulated miR-21-5p and miR-125b-5p and downregulated miR-494 and miR-720). 26 miRNAs (11 up and 15 down) were aberrantly expressed in the high cellular tumors relative to their associated DCIS (including upregulated miR-188-5p and miR-363-3p and downregulated miR-125a-5p and miR-1283). There were 9 commonly deregulated miRNAs (6 up and 3 down) in carcinomas with high cellularity compared to low cellularity tumors and DCIS (including upregulated miR-144-3p, miR-149-5p, miR-320e, miR-338-3p, miR-631, and miR-2682-5p and downregulated miR-200c-3p, miR-1260a, and miR-4454). Conclusions: Our study demonstrates that invasive highly cellular mucinous carcinoma has a different miRNA expression pattern from those with low cellularity and also with DCIS. This microRNA signature most likely reflects unique molecular

changes for each lesion of mucinous carcinoma, and it can not only prove to be useful in diagnosis and prognosis but also have great impacts in the development of new molecular targets and therapy.

NCI-CCR
Anderson, Matthew
Postdoctoral Fellow
Developmental Biology
FGF3 REGULATION OF BMP SIGNALING IS REQUIRED FOR NEURAL TUBE CLOSURE
Fibroblast growth factors (FGF) are well known for regulating early mesoderm and somitogenesis during axis extension. However, axis extension also requires a coordination of morphogenesis between all primary lineages, particularly the early mesoderm and neural tube (NT); defects in one often affect the other. However, little is known about the signals through which these two tissues communicate. Within this study we have uncovered a new role for FGFs in signaling from the mesoderm to the NT for the normal development of both tissues and normal axis extension. Adult Fgf3 null mice ("mutants") have axis extension defects with fewer and malformed caudal vertebrae, due to embryonic defects in somitogenesis and premature loss of the somite progenitor tissue. We observed a domain of cell death within this progenitor tissue that could explain the loss of tissue, however manipulations of the pro-cell death effectors Bax and Bak have shown that cell death is dispensable for the production of the mutant phenotype. These phenotypes however, are secondary to a NT closure defect (NTD), first evident at the 25-somite stage, preceding mesoderm malformation by ~ 16 hours. Furthermore, Bmp4, shown previously to inhibit NT closure, is upregulated and caudally expanded in the mutant dorsal NT, beginning at the 24-somite stage. Thus, we hypothesize that FGF3 repression of Bmp4 in the NT is required for NT closure and normal axis extension. To test this we performed three genetic manipulations. First, we increased BMP signaling by deleting a single allele of the BMP antagonist Noggin resulting in a greater severity in the NTD and a subsequent greater caudal truncation. Second, we attenuated BMP signaling via homozygous deletion of the receptor gene BmpRIb, partially rescuing the NTD and subsequent caudal malformations. Finally, we deleted NT Bmp4 expression in mutants, which rescued the NTD, confirming the role of BMP signaling in the etiology of the Fgf3 mutant. This work represents the first identification of a mouse FGF-ligand that signals to the NT thereby regulating both NT closure and normal axis extension. Through rescuing cell death with the Bax/Bak manipulations and failing to rescue mesoderm loss, we have additionally shown that the NTD caused loss of mesoderm is independent of cell death. This work represents a significant advance in our understanding of the etiology of NTDs, which are the second most frequent birth defect occurring in ~1% of human births.

NCI-CCR
Barlow, Jacqueline
Postdoctoral Fellow
DNA-binding Proteins/Receptors and DNA Repair
Genome-wide identification of early replicating fragile sites reveals a novel source of DNA rearrangement leading to B cell lymphomas
DNA double strand breaks (DSBs) in B lymphocytes arise spontaneously during replication (S phase) or as a result of targeted DNA damage during class switch recombination by the enzyme activation induced cytidine deaminase (AID) in G1. Lymphocytes are particularly susceptible to replication-stress-inducing agents such as hydroxyurea (HU) since they undergo several bursts of replications during their development. HU treatment generates replication stress by depleting dNTP pools to levels insufficient to complete replication, and cells arrest early in S phase. Here, we use chromatin immunoprecipitation followed by deep sequencing to identify genomic loci that are enriched for DNA repair proteins in response to replicative stress. By generating genome-wide profiles of DNA repair proteins RPA, SMC5, ?-

H2AX, and BRCA1 in primary murine lymphocytes treated with HU, we identified a novel class of recurrent DNA lesions at early replicating sites, termed Early Replication Fragile Sites (ERFS). ERFS colocalize preferentially with highly expressed gene clusters, particularly with regions of active divergent or convergent transcription. Genomic instability at ERFS was confirmed by fluorescence in situ hybridization (FISH), as cells exposed to HU exhibited elevated levels of DNA double strand breaks specifically at ERFS. Although distinct from common fragile sites (CFS), as shown by insensitivity of their stability to low doses of the polymerase inhibitor aphidicolin, the fragility of ERFS and CFS were similarly dependent on the replication-stress response kinase ATR. ERFSs also translocated to programmed AID-induced breaks occurring at the Immunoglobulin Heavy chain gene (IgH) during class switch recombination. Interestingly, genes rearranged frequently in B cell lymphoma such as IKZF1, BACH2 and BCL2 are located within the strongest identified ERFS. Further, human B cell lymphomas exhibit characteristic DNA rearrangements comprised of insertion and deletion events, termed copy number variations. Greater than 50% of the common copy number variations observed in human diffuse large B cell lymphoma map to ERFS. In summary, we have identified a new class of fragile sites in mammalian cells, which play a mechanistic role in recurrent DNA rearrangements during lymphomagenesis.

NCI-CCR
Ruzankina, Yaroslava
Research Fellow
Gene Expression
Analysis of molecular networks that drive development of malignant astrocytoma
High grade astrocytomas (HGA) are the most common tumors of the brain and include anaplastic astrocytoma and glioblastoma multiforme (GBM). These tumors remain incurable in spite of advanced aggressive treatments including surgery, radiation and chemotherapy. To identify disease-specific gene networks involved in astrocytoma initiation and progression, we utilized mouse models wherein key molecular pathways commonly perturbed in human GBMs were inactivated or induced specifically in adult astrocytes via tamoxifen-inducible Cre recombinase. Inhibition of Rb pathway via expression of T121(T; a dominant inactivating protein) initiated diffuse grade II astrocytoma formation by 2 months after induction. Additional activation of the KRas pathway (TR) facilitated progression to grade III anaplastic astrocytoma 4-5 months post induction. Additional PTEN loss or heterozygosity (TRPhet; TRPnull) led to rapid development of glioblastoma with characteristic features of angiogenesis and necrosis observed in human disease. Transcriptome analyses of GBMs in these mouse models showed concordance with highly aggressive mesenchymal and proneural subclasses of human GBMs. We analyzed gene and miRNA expression in more than 300 brain samples from T, TR, TRPhet and TRPnull mice and corresponding controls at different time points after induction. The genes that were induced early and gradually increased in expression with tumor grade belonged to several key networks: DNA replication and repair, cell cycle progression, metabolism, and pathways important for embryonic stem cell biology. Pathways significantly induced at later stages of disease (grades III-IV) included p53 and Notch signaling, inflammatory responses, and RNA processing. Significantly downregulated pathways were related to neuronal functions. We have confirmed expression of several pathways in mouse tumor samples and cell lines. For many confirmed genes (e.g. GBM stem-like factors: Notch1, Olig2, STAT3, Nestin; inflammatory: p65) similar upregulation has been reported in human HGAs. Novel findings included identification of several inflammatory cytokines and transcription factors (e.g. HoxA5) that have not been studied in the context of HGA development. However the upregulations of these factors in human HGAs are reported in TCGA database. Currently we are investigating potential roles of selected genes in HGA by in vitro and in vivo functional analysis.

NCI-CCR

Huang, Bau-Lin
Postdoctoral Fellow
Genetics
Gli3-5â€™Hoxd antagonism regulates digit joint formation by modulating BMP pathway activity
During limb development, the number of joints is one of the morphologic hallmarks of digit identity. 5â€™Hoxd (Hoxd11,12,13) genes and Gli3, a mediator of Hedgehog (HH) signaling, control limb pattern (digit numbers). However, their roles in regulating digit joint formation are unclear. The expression of 5â€™Hoxd and Gli3 overlap in interdigital mesenchyme (IDM) at E12.5 and in the interzone regions (IZ, presumptive joint region) by E13.5. Lineage-tracing analysis using a Gdf5Cre/RosaTomato reporter to specifically mark joint progenitors confirms the loss-of-joint phenotype in 5â€™Hoxd-/-, whereas proximal phalanges in Gli3-/- are transformed into an expanded joint-like structure filled with Gdf5+ cells. Previously our lab showed 5â€™HOXD proteins antagonize Gli3 repressor function via direct interaction. Ptch1 expression (a HH target) is reduced in 5â€™Hoxd-/- IZs but Gli3 RNA is not altered, indicating excessive Gli3 repressor activity. We generated 5â€™Hoxd;Gli3 compound mutants and found that normal digit joint formation was restored, indicating regulation by Gli3-5â€™Hoxd genetic interaction. It has been proposed that BMP signaling from the IDM controls digit identity at late stages (~E12.5). Msx1 RNA (a BMP target) is reduced in Gli3-/- and elevated in 5â€™Hoxd-/- IDMs. Noggin, a BMP antagonist, is expressed in cartilage and joints are absent in Noggin-/- embryos. To evaluate whether IZ BMP activity may be targeted by Gli3-5â€™Hoxd interaction, we generated Noggin+/-;Gli3-/- mutants and found that normal digit joints were restored. In contrast, in a 5â€™Hoxd hypomorph (5â€™HoxdFlox/-) that still forms normal joints, removing one Noggin allele (Noggin+/-) results in loss of digit joints. This suggests that IZ BMP activity is modulated by Gli3-5â€™Hoxd interaction. Moreover, conditional removal of Gli3 or 5â€™Hoxd genes only in cartilage precursors (with Sox9Cre) does not alter joint formation. Therefore, we hypothesize that Gli3-5â€™Hoxd interaction from the IDM regulates IZ BMP activity indirectly, and non-autonomously to control joint formation. We have generated an IDM-specific Cre line (Bmp2-Cre) and are currently testing if Gli3-5â€™Hoxd balance in IDM regulates digit joint formation, and which BMP ligands may be regulated. It has been shown conditional remove of the component of non-canonical BMP signaling, TAK, caused joint fusions. We are also testing if canonical (Smad) or non-canonical IZ BMP activity is altered by changing Gli3-5â€™Hoxd balance to regulate joint fate.

NCI-CCR
Parpart, Sonya
Doctoral Candidate
Genomics
Integrative Epigenomic and Genomic Profiling of HCC Patients to Identify Key Driver Signaling Pathways of HCC Progression and Poor Outcome.
Globally, hepatocellular carcinoma (HCC) accounts for 70-85% of primary liver cancers and is the second leading cause of male cancer death. Among HCC patients there is widespread heterogeneity, yet a single therapeutic, Sorafenib, which is only effective in patients with overactive MAP kinase signaling. Identifying homogeneous subgroups of patients will improve our ability to develop more effective treatments targeted to specific signaling pathways that lead to poor survival. Recently, many â€˜omics based studies have further described cancer heterogeneity. Genomics is the application of high-throughput methods to analyze the expression of genes on a global level, while epigenomics focuses on epigenetic modifications, such as DNA methylation, across the genome. Though genome-wide â€˜omics methods are popular, the integration of the data across platforms is uncommon. We hypothesize that the integration of patient data will reveal distinct epigenetic and genomic modifications in subsets of HCC patients that lead to poor outcome. In this study, we employed Illumina or Affymetrix array

platforms to analyze paired tumor and non-tumor tissue specimens from 82 HCC patients in an integrative approach incorporating DNA methylation, somatic DNA copy number alteration (SCNA) and expression of mRNA and microRNA genes. First we performed a class comparison analysis between tumor and non-tumor patients to identify 2,173 differentially methylated genes (Illumina 27k BeadChip). Of those genes, only 621 overlapped with tumor-specific genes differentially expressed in the same patients (Affymetrix array). We further narrowed the tumor-specific genelist by extracting only the subset of genes that correlated with methylation data. An epigenetic-driven gene signature of 46 genes was established using a correlation coefficient of -0.185 corresponding to the 95th percentile of the 1000-fold random distribution. In a similar manner, an SCNA-related gene signature made up of 2,722 tumor-specific genes was established using a correlation coefficient of 0.3 corresponding to the 99th percentile of the 1000-fold random distribution. We found that of 62 patients with poor outcome, epigenetic changes drove 1/3, SCNA-related changes drove 1/3 and a combination of the two drove the final 1/3. Moving forward, we plan to focus on key signaling pathways derived from integrated gene and miRNA expression data to elucidate the mechanisms of HCC progression and poor outcome in each HCC subgroup.

NCI-CCR
Kato, Shingo
Visiting Fellow
Hematology/Oncology, Tumor Immunology, and Therapy
Structure-function studies of agonists for subtypes of NKT cells
NKT cells are a unique population of T lymphocytes that recognize lipid antigens presented by a nonclassical MHC-like molecule CD1d. There are two types of NKT cells, type I and type II. Unlike conventional T cells, each subset of NKT cells recognizes distinct antigens. One of the defined antigens for type I NKT cells is alpha-galactosylceramide (aGC), and 3-o-sulfo-beta-D-C24:1-galactosylceramide (sulfatide) is one of the few defined antigens for type II NKT cells. Previous structure-function studies of NKT cell antigens focused solely on analogues of type I NKT cell agonist aGC. To our knowledge, there is no such study focusing on type II NKT cells. Compared to type I NKT cells and its agonist aGC, which are well characterized, type II NKT cells and its agonists are much less well characterized because of lack of widely available tools to identify them. Recently, our lab developed a new method to create CD1d tetramers loaded with sulfatide, which can specifically stain type II NKT cells. In this study we conducted a structure-function study of sulfatide analogues by taking advantage of this new method. First, we tested cytokine production of spleen cells or liver lymphocytes after stimulation with sulfatide. The production of IFN-gamma and IL-4 was observed in both populations. Compared to spleen cells, liver lymphocytes needed a higher concentration of sulfatide to induce the peak production of the cytokines. Sulfatide analogues induced lower levels of production of both cytokines compared to sulfatide. CD1d tetramers loaded with different sulfatide analogues reacted with different proportions of liver lymphocytes, where NKT cells are enriched, but none of them reacted with aGC-loaded CD1d tetramer. The frequency of T cells reacting with sulfatide analogues was lower than that of sulfatide reactive cells. Double staining of liver lymphocytes with CD1d tetramers loaded with two sulfatide species suggested that the majority of type II NKT cells react to multiple sulfatide analogues. In view of the facts that cancer cells naturally produce sulfatide analogues and the type II NKT cells have immunosuppressive effects on tumor immunity, detailed analysis of sulfatide analogues may reveal a mechanism by which cancer cells can escape from the immune system. This study may have great impact to develop new immunotherapies for cancer.

NCI-CCR
Long, Adrienne

Doctoral Candidate
Hematology/Oncology, Tumor Immunology, and Therapy
Enhanced glycolytic metabolism is associated with rapid senescence and poor anti-tumor efficacy in a xenograft model of chimeric antigen receptor T cell therapy for sarcoma
Chimeric antigen receptors (CARs) provide a promising new approach for generating T cell populations for the adoptive immunotherapy of cancer. CAR T cell (CART) therapies demonstrate activity against leukemias in preclinical and clinical studies, but CART targeting solid tumors have been less impressive. We hypothesized that the observed differences could be due to a more hostile microenvironment in solid tumors, and/or variable CAR potency. To address these issues, we created a CD19+GD2+ osteosarcoma (143B-CD19). This allowed us to compare the susceptibility of a solid tumor to both CD19-CART, which have potent anti-leukemic activity in vitro and in vivo, vs. GD2-CART, which show potent anti-tumor effects in vitro. 51Cr release demonstrated that CD19- and GD2-CART were equally active against 143B-CD19 in vitro. However, in vivo models showed a significant difference in anti-tumor efficacy. When NSG mice were injected with 143B-CD19 and treated with control, GD2- or CD19-CART 14d later, no difference in tumor growth was observed between GD2-CART and control treated animals. However, mice treated with CD19-CART showed complete eradication of their CD19+ disease. We also observed that CD19-CART expanded and persisted in vivo, whereas GD2-CART did not. Further comparison of GD2 vs. CD19 CARs demonstrated that GD2-CART depend more on glycolysis for metabolism, compared to CD19-CART or mock expanded controls. Using a Seahorse Extracellular Flux Analyzer, the ratio of the extracellular acidification rate (ECAR; measure of glycolysis) to oxygen consumption rate (OCR; measure of oxidative phosphorylation) of GD2-CART was found to be double that of CD19-CART or controls during in vitro expansion. This higher dependence on glycolytic metabolism was associated with a more senescent phenotype, as GD2-CART displayed lower CD127 and higher CD25, PD1 and TIM3 expression levels within 7 days of initial activation, compared to CD19-CART. GD2-CART also produced >10x lower levels of TNFa, IFNg, and IL2 upon incubation with 143B-CD19, compared to CD19-CART. We conclude that the solid tumor microenvironment is not a barrier to effective CART therapy, but hypothesize that the enhanced glycolysis induced in GD2-CART leads to early senescence and diminished anti-tumor effects in vivo. Future work will seek to define the basis for the differential metabolism observed in CD19- vs. GD2-CART and will address the contribution of metabolism to T cell fitness.

NCI-CCR
Yan, Hannah
Other
Hematology/Oncology, Tumor Immunology, and Therapy
CCL9 is a Critical Mediator in Tumor Cell Survival and Metastasis
Cancer metastasis accounts for over 90% of cancer-associated deaths. One rate-limiting step is tumor cell survival in the hostile distant organ. We previously reported a significant infiltration of Gr-1+CD11b+ cells or myeloid derived suppressor cells (MDSCs) in lungs of mice bearing mammary adenocarcinomas prior to tumor cell arrival. Here we observed increased tumor cell survival when co-cultured with MDSCs. Using cytokine protein arrays, we found that CCL9 (macrophage inflammatory protein-1 gamma) was highly produced only in the co-culture supernatant. Consistently, CCL9 neutralization increased whereas recombinant CCL9 decreased the expression level of cleaved caspase 3. We found CCR1, the only receptor for CCL9, was expressed by tumor cells. Importantly, the expression level correlates with metastatic capability. This data was obtained by using cell line series of 4T1 mammary carcinoma that model different stages of metastasis. 4T1 and 4T07 cells (highly metastatic) had significantly higher CCR1 expression than 67NR and 168FARN (low metastatic). We are in the process to knockdown or over express CCR1 to investigate how tumor cell survival and metastasis will be affected.

We recently discovered that deletion of transforming growth factor beta (TGF-ß) receptor II in myeloid cells, including these MDSCs (Tgfbr2MyeKO), resulted in a significantly decreased metastasis and CCL9 expression. In addition, unlike wild type MDSCs, when Tgfbr2 deficient MDSCs were co-injected, tumor cells do not increase survival nor decrease apoptosis in vivo. Our data suggest CCL9 as a critical mediator in myeloid TGF-ß regulation of tumor metastasis. Further experiments showed that inhibition of P38, a down stream mediator of TGF-ß signaling, decreased CCL9 expression in wild type but not Tgfbr2 deficient MDSCs. We plan to over express CCL9 in Tgfbr2 deficient MDSCs and test whether it rescues tumor metastasis defect in Tgfbr2MyeKO mice. Furthermore, using a public available database, we found that the expression level of CCL23, the human homologue for CCL9, in peripheral blood mononuclear cells correlates with the progression of lung cancer. In summary, we hypothesize that CCL9 is a critical mediator of TGF-ß function in myeloid cells that promotes tumor cell survival and colonization in the distant metastatic organ. CCL9 is specifically and highly produced due to interaction of MDSCs and tumor cells. Our work may identify a unique target for metastasis treatment.

NCI-CCR
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Postdoctoral Fellow
HIV and AIDS Research

Highly active antiviral therapy (HAART) increases frequency and function of NKG2A+NK cell subsets but failed to increase NKp44+NK subset in the rectal mucosal tissues of Rhesus macaques infected with Simian Immunodeficiency Virus (SIV)

The gastrointestinal tract is one of the main target tissues of HIV/SIV entry and pathogenesis. Gut associated mucosal immune system that comprised of lymphoid tissues and lymphoid cells may play a major role in controlling and preventing HIV/SIV infection. Recent studies have shown two types of NK cell subsets present in the Rhesus macaqueâ€™s mucosal tissue. NKG2A+ NK cell subset is more cytotoxic as NKp44+NK cell subset is more regulatory. NKp44+ NK cells resemble the NK22 cells reported in humans and produce LIF, BAFF, and cytokines including IL22, IL17, and may be important for the maintaining of gut mucosal integrity. Growing evidence suggests that NK cells play a significant role in controlling HIV/SIV infections. However, little is known about mucosal NK cell subsets and their functions during HIV infection and particularly during anti retroviral treatments. Here, in this study, we characterized the phenotypic and functional alterations of rectal mucosal NK cell subsets during SIV infection in Rhesus macaques by multiparametric flow cytometry. Furthermore, we studied the mucosal NK cell responses to HAART in chronically infected macaques. Our results showed that a rapid increase of cytotoxic NK cell subset (NKG2A+) (p=0.009) during the acute phase of the infection. However, it decreased during progression to the chronic phase. Meanwhile, we observed that the NKG2A+ cells increased cytotoxicity (CD107a+) (p<0.001) and IFN gama (P<0.0001) production during the infection. Nevertheless, TNF alpha production (p<0.001) significantly reduced in the acute phase. The regulatory mucosal NK cell subset (NKp44+) was significantly reduced (P=0.0072) during early phase and further diminished in the chronic stage. Furthermore, IL17 (p=0.005) and TNF alpha production (p<0.001) was drastically reduced in NKp44+ subset. Surprisingly, NKp44+ NK cells acquired cytotoxic capability during SIV infection. Eleven weeks post HAART treatment, SIV DNA copy number dramatically reduced in the rectal mucosa tissues and concurrently NKG2A+ NK cell subset (p=0.003) significantly increased. Nevertheless, regulatory subset (NKp44+) continues to decline even after the HAART treatment. These results suggest that the mucosal NK cell subsets may play significant role during the SIV pathogenesis and the HAART can at least reconstitute NKG2A+ NK cell subset in the mucosal tissue of chronically infected Rhesus macaques.

NCI-CCR

Ramsuran, Veron
Visiting Fellow
HIV and AIDS Research
Allele specific variation in HLA-A gene expression and its effect on HIV Viral Load control
Host genetic variation is estimated to account for about a quarter of the observed differences in control of HIV across infected individuals. Several genome-wide association studies have confirmed that polymorphisms within the human leukocyte antigen (HLA) class I locus is the primary host genetic contributor to determining outcome after infection. To date there are numerous studies demonstrating the effects of HLA-B and HLA-C genes on HIV disease, however not much attention has been placed on HLA-A. In this study we aim to first determine the impact of HLA-A alleles on HIV viral load (VL); second, examine the effect of differential expression of HLA-A on HIV VL; third, identify factors contributing to differential HLA-A gene expression levels. We determined the association of individual HLA-A, -B, -C alleles with HIV VL control in a large cohort of 2527 HIV infected Caucasians. HIV controllers (VL <2,000 copies/ml of plasma, n=834) were compared to HIV non-controllers (VL >10,000 copies/ml of plasma, n=1690). Results of a logistic regression analysis with stepwise addition of each allele with frequency of =1% revealed eight HLA-A alleles that associate significantly with higher VL. We next determined mRNA expression levels of all HLA-A alleles using a real time quantitative PCR assay. HLA-A mRNA expression was then used as a continuous variable in a logistic regression analysis with stepwise selection in order to determine if there is an independent effect of HLA-A expression on VL control. A comparison of HIV controllers to non-controllers showed that higher HLA-A expression was significantly associated with poor HIV control in both Caucasians (OR=2.98, p=6E-05, n=2461) and African Americans (OR=5.34, p=1E-06, n=862). Subsequently we have identified differential levels of methylation in the promoter region of the HLA-A gene that contributes to differential HLA-A gene expression levels. HLA-A alleles with low expression have increased DNA methylation levels. This is the first report of novel associations between HLA-A alleles and HIV viral load control. We also show that increased mRNA expression of HLA-A contributes to poor viral control. Furthermore we identified DNA methylation as a factor contributing to differential HLA-A gene expression levels. Characterization of this regulation pathway could potentially identify drug targets that modulate HLA expression and thus modulate the host response in viral infections, autoimmune diseases and tumors.

NCI-CCR
Kwong, Brandon
Visiting Fellow
Immunology - Autoimmune
T-bet expression is required in both donor and host mice for the passive induction of 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 initiate autoimmune inflammation, resulting in axon demyelination and neurodegeneration. Although Th1 and Th17 cells are both capable of inducing EAE, it has been established that expression of the Th1-associated master transcription factor T-bet is required for disease induction. Indeed, the â€œpassiveâ€ adoptive transfer of CNS antigen-specific wild-type (WT) Th17 cells into WT recipients causes severe EAE, while the transfer of CNS antigen-specific T-bet-deficient (T-bet-/-) Th17 cells results in minimal disease. However, whether or not T-bet expression in immune cells other than CD4+ Th cells is involved in the induction of EAE remains largely unexplored. To assess the potential pathogenic contribution of T-bet expression in non-Th cells, we transferred transgenic 2D2 T-bet-/- or 2D2 WT Th17 cells (expressing T cell receptor specific for a CNS antigen) into T-bet-/- or WT hosts. Consistent with previous reports, 2D2 T-bet-/- Th17 cells were unable to cause significant disease in either recipient,

despite the substantial infiltration of 2D2 T-bet-/- cells into the CNS. In contrast, 2D2 WT Th17 cells induced severe EAE in WT mice but not in T-bet-/- hosts. Surprisingly, the infiltration of 2D2 WT cells into the CNS of T-bet-/- recipients was almost completely abolished. These results suggest that T-bet expression in Th17 cells is necessary, but not sufficient, to induce EAE via adoptive transfer. T-bet expression in inflammatory monocytes and dendritic cells has also been previously reported. We observed that the infiltration of these innate cells was highly elevated in the CNS of WT mice that received 2D2 WT Th17 cells, but absent in T-bet-/- recipients, correlating with the disease severity. Interestingly, while the germline deletion of T-bet protected mice from developing EAE after â€œactiveâ€ immunization with CNS-derived antigens, the cre/lox-mediated CD4-specific deletion of T-bet failed to protect mice from EAE in this model. Collectively, these results suggest that T-bet expression in cells other than CD4+ Th cells plays a pathogenic role in the induction of EAE, and ongoing studies using additional cell-specific deletions of T-bet expression will further elucidate this previously unknown role for T-bet in other cells of the immune system.

NCI-CCR
Wang, Yichuan
Postdoctoral Fellow
Immunology - General
Vaginal type-II mucosa acts as an inductive site during the generation of primary CD8+ T cell mucosal immune responses
It is widely believed that primary immune induction in type-II mucosa (vagina, foreskin & cornea) occurs in the draining lymph nodes (LNs) due to a lack of mucosa-associated lymphoid tissue (MALTs). In this process, naïve T cells located in the draining LNs are primed by antigen (Ag)-bearing dendritic cells migrating from the Ag-exposed mucosa. Primed T cells then travel to the mucosal site through the bloodstream. In contrast to this paradigm, we show that vaginal mucosa itself can act as an immune inductive site during the generation of primary CD8+ T cell mucosal immunity. As evidence, we found that naïve CD8+ T cells routinely migrated to the female reproductive tract and that Ag-specific CD8+ T cells could be generated in the LN-deficient mice after intravaginal (Ivag) immunization. Further, the adoptively transferred naïve OT-1 CD8+ T cells were activated in the vaginal mucosa but not in the draining LNs at 24 hours after Ivag immunization, even in the presence of FTY720, a drug blocking the egress of T cells from LNs. In addition, the Ag-bearing cells isolated from immunized vaginal mucosa were able to stimulate naïve OT-1 CD8+ T cells to secret IFN-gamma and undergo proliferation. Finally, vaginal mucosa largely supported the expansion of Ag-specific CD8+ T cells. In conclusion, we present evidence for a new paradigm for primary CD8+ T cell immune induction in type-II mucosa of the vagina, one that occurs locally without the help of draining LNs or MALTs.

NCI-CCR
Izhak, Liat
Postdoctoral Fellow
Immunology - Innate and Cell-mediated Host Defenses
The balance between three types of T cells in regulation of tumor immunity
Many studies have demonstrated the importance of regulatory cells such as Tregs and type II NKT cells in the immune regulation of cancer. However, it is still not clear why different suppressive cells play a dominant role in different tumor models. Here by using syngeneic models of colorectal and renal cancer , we examined the relative role of the two suppressors, Tregs and type II NKT cells, in three strains of mice: wild-type, NKT cell-deficient CD1dKO mice and Ja18KO mice, which lack type I NKT cells but still retain type II NKT cells. Treg blockade led to tumor rejection in WT and CD1dKO, but surprisingly not in Ja18KO mice, suggesting that Tregs are not necessary for the suppression of tumor immunity in Ja18KO

mice. Based on our previous findings that type I and type II NKT cells can counter-balance each other, we hypothesized that cross regulation between type I and type II NKT cells in WT mice leaves Treg cells as primary suppressors, whereas in mice lacking type I NKT cells (Ja18KO mice), unopposed type II NKT cells can suppress tumor immunity even when Tregs are blocked. We confirmed this by blocking both suppressors, Tregs and type II NKT cells using antibodies, as well as by reconstituting type I NKT cells in Treg-depleted Ja18KO mice. These results support our hypothesis that it is necessary to block both suppressors, type II NKT cells and Tregs, in order to achieve protection. Moreover, shifting the balance between the two types of NKT cells by specifically stimulating type II NKT cells with an agonist, sulfatide, suppressed type I NKT cells and abrogated the protective effect of Treg blockade. These results indicate that shifting the balance toward immunosuppressive type II NKT cells suppresses tumor immunity even in the absence of Tregs. We conclude that in the absence of type I NKT cells, blockade of both type II NKT cells and Tregs is necessary to abrogate the suppression of tumor immunity and that a third cell, the type I NKT cell, therefore determines the relative roles of these two regulatory cells, regulating the regulators. Finally, as cancer patients often have defects in type I NKT cell functions like Ja18KO mice, controlling both suppressors may be critical for the success of immunotherapy of human cancer.

NCI-CCR
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Research Fellow
Immunology - Innate and Cell-mediated Host Defenses
Effect of intratracheal CpG immunotherapy with biodegradable polyketal nanoparticles on murine lung cancer
Lung cancer is the leading cause of cancer deaths in the US, with a 5 year survival rate of only 15%. Our work examines whether immune elimination of established lung tumors can be achieved by localized activation of the innate immune system. This strategy builds on recent findings that the intra-tumoral delivery of immunostimulatory CpG oligodeoxynucleotides (ODN) can overcome tumor-induced immunosuppression and lead to tumor regression. Building on those findings, we examined whether the intra-pulmonary delivery of CpG ODN could be effective against lung tumors. To maximize the local delivery of CpG ODN, we conjugated them to biodegradable polyketal nanoparticles (MW; 4000, particle size; 200 - 600 nm, endotoxin contamination < 0.1 EU/mL determined by LAL test) at an ODN : particle ratio of 1:27-30). In vitro studies showed that the polyketal-ODN nanoparticles induced TLR9 dependent cell proliferation and IL-12 production by murine splenocytes. In vivo studies showed that the intratracheal administration of polyketal-ODN particles improved pulmonary macrophage accumulation by 2-fold, lymphocyte accumulation by 10-fold and IL-12 production by 5-fold vs free ODN (determined by analyzing bronchoalveolar lavage fluid collected 2 days after instillation). To evaluate the effect of intratracheal polyketal-ODN nanoparticles on lung tumors, we developed a model that mimics bronchogenic lung cancer. In this model, 10e6 Lewis lung carcinoma cells were instilled intratracheally into 5 - 6 wk old C57/BL6 mice. In untreated animals these tumor cells infiltrate peribronchially, form tumor nodules, and cause terminal cancer by 3 wk. The intratracheal delivery of ODN (7 days post tumor challenge) significantly improved survival. The survival of polyketal-CpG treated recipients was 100% after 7 wk while those treated with free CpG ODN showed only 40% survival (all untreated animals were dead). The efficacy of polyketal-CpG therapy was site and dose dependent. Mechanistic analysis of the effect of polyketal-ODN is underway. These findings suggest that the intratracheal administration of CpG nanoparticles may provide a novel and effective strategy for the immunotherapy for lung cancer.

NCI-CCR
Bowen, Steven
Postdoctoral Fellow

Immunology - Lymphocyte Development and Activation

Simultaneous VDJ recombination at multiple TCR loci results in trans-rearrangement encoding functional hybrid TCR chains

During the CD4-CD8- (DN) stage of T cell development, RAG-dependent DNA breaks and V(D)J recombination occur at three T cell receptor (TCR) loci: TCRb, TCRg and TCRa/d During this stage, abnormal trans-rearrangements also take place between TCR loci, occurring at increased frequency in absence of the DNA damage response mediator ATM . Here we use this model of physiologic trans-rearrangement to study factors that predispose to rearrangement and the role of ATM in preventing chromosomal translocations. We combine fluorescent in situ hybridization (FISH) at the TCR loci with immunofluorescent staining for the DNA damage marker 53BP1 in DN thymocytes to examine the temporal coincidence of rearrangement at more than one TCR locus. The frequency of DN thymocytes with DNA damage foci at multiple TCR loci simultaneously is increased 2-3 fold in the absence of ATM. However, measurement of trans-rearrangement frequency by real-time PCR reveals a 10,000-100,000 fold increase in the absence of ATM, indicating that ATM function extends beyond timely resolution of DNA breaks. RAG-mediated synaptic complex formation occurs between recombination signal sequences (RSS) with unequal 12 and 23 base spacer sequences (12/23 rule). TCR trans-rearrangements violate this rule as we observed similar frequencies of 12/23 and aberrant 12/12 or 23/23 recombination products by real-time PCR and sequencing. This suggests that trans-rearrangements are not the result of trans-synaptic complex formation, but are instead due to unstable cis synaptic complexes that form simultaneously at distinct TCR loci. ATM thus suppresses trans-rearrangement primarily through stabilization of DNA breaks at TCR loci. Furthermore we identify peripheral T cells in WT and ATM-/- mice that express a hybrid Vg-Cb TCR chain on the cell surface encoded by a trans-rearranged chromosome. The Vg-Cb hybrid chain is capable of mediating allelic exclusion at the TCRb locus, as Vg+Cb+ lack an intact TCRb chain. Cells expressing the Vg-Cb hybrid receptor are developmentally dependent on TCRa expression. These cells are CD4 or CD8 single positive and MHCII and MHCI restricted, respectively, and are capable of differentiating into memory T cells. This work provides insight into the molecular mechanism of TCR trans-rearrangement, as well as the selection and function of a novel population of cells that express TCR chains encoded by trans rearrangement in mouse T cells.

NCI-CCR

Park, Joo Young

Visiting Fellow

Immunology - Lymphocyte Development and Activation

A dual requirement for IL-7 and IL-15 in iNKT cell development and survival

Invariant natural killer T (iNKT) cells are thymus-generated T lineage cells with shared phenotypic and functional characteristics of innate NK cells. iNKT cells can mount rapid and vigorous cytokine responses upon antigen challenge, and are deemed critical for raising an early immune response during microbial infection, inflammation, and in tumors. While generation and maintenance of all T lineage cells require signaling by interleukin-7 (IL-7), iNKT cells have been presumed to be IL-7-independent. Instead, iNKT cells were thought to be dependent on IL-15, which is a non-redundant cytokine for NK cells. Thus, the cytokine requirement for iNKT cell was proposed to be distinct from that of T lineage cells and proposed to be rather in line with NK cells. Here we report that this is not the case. In contrast to the prevailing view, we found that iNKT cells survival and homeostasis were dependent on IL-7 and not IL-15. Specifically, we found that IL-15-deficient mice had normal numbers of iNKT cells in peripheral tissues despite impaired iNKT cell generation in the thymus. On the other hand, IL-7 was critical for both the development and survival of iNKT cells as IL-7-deficient mice showed dramatically reduced iNKT cell numbers both in the thymus and spleen. Moreover, using a new in vivo model of peripheral IL-7 deficiency, which we generated by expressing a thymocyte-specific IL-7 transgene into IL-7-deficient

mice (K7 mice), we further confirmed that IL-7 is a survival factor for iNKT cells. In agreement with this idea, IL-7 transgene dramatically increased peripheral iNKT cell numbers, whereas IL-15 overexpression failed to do so. These data suggest that IL-7, and not IL-15, sets the size of the iNKT cell pool. Notably, IL-7 was not only important for iNKT cell survival but also for their function, as in vivo iNKT activation by alphaGalCer injection into K7 mice resulted in blunted IL-4/IFNgamma production compared to WT mice. Mechanistically, we identified STAT5 as the transcriptional effector molecule downstream of IL-7 signaling that drives iNKT cell development because STAT5-deficient mice failed to generate and maintain iNKT cells. Collectively, these data provide an entirely new perspective on cytokine requirements for iNKT cell survival and function, and proposes a new role for IL-7 in iNKT cell biology.

NCI-CCR
Roychoudhuri, Rahul
Visiting Fellow
Immunology - Lymphocyte Development and Activation
Bach2 is required for the generation of CD8+ T-cell memory
Following acute infection or vaccination, naïve antigen-specific CD8+ T cells undergo rapid clonal expansion to generate an effector response. While these cells undergo contraction upon clearance of antigen, a small number survive to form long-lived memory cells. These cells play an important role in protective immunity against subsequent infection and, thus, their formation is a desired consequence of vaccination. Despite this, mechanisms by which CD8+ T cells differentiate into memory cells are poorly elucidated. In this study, we found that generation of memory CD8+ T cells is critically dependent upon a transcription factor, Bach2. Bach2 was highly expressed in naïve and memory CD8+ T cells, but lost upon effector differentiation in vivo. Strikingly, overexpression of Bach2 prevented differentiation into effector cells, suppressed cytokine production and resulted acquisition of memory-cell characteristics. Consistent with its known function as a transcriptional repressor, whole transcriptome analysis of these cells revealed down-regulation of a majority of differentially-expressed genes including Prdm1, which encodes Blimp-1 and antagonizes memory formation by inducing sensecence in CD8+ T cells. ChIP-Seq analysis revealed binding of Bach2 at intronic regions within the Prdm1 gene and its mRNA was exuberantly induced upon stimulation of naïve CD8+ T cells in the absence of Bach2. Consequently, while naïve Bach2-deficient CD8+ T cells formed effector responses following viral infection in vivo, there was a near-complete cell-autonomous defect in their ability to generate memory cells (CD44+ CD62L+) and form long-lived memory. This could be attributed to decreased proliferation. Instead, Bach2-deficient CD8+ T cells expressed high levels of KLRG-1, a marker of senscence in CD8+ T cells and selectively failed to migrate to central lymphatic structures. Accordingly, priming of adoptively transferred naïve Bach2-deficient OT-1 transgenic CD8+ T cells in wildtype hosts resulted in impaired protective immunity against challenge with recombinant Listeria monocytogenes-Ova. Bach2-deficient cells also exhibited impaired long-term anti-tumor functionality. Thus, Bach2 is required for the generation of CD8+ T cell memory. These findings provide insight into transcriptional events that lead to the formation of CD8+ T cell memory and a basis for the design of targeted approaches aimed at augmenting memory responses.

NCI-CCR
Daily, Kenneth
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Informatics/Computational Biology
The UISO cell line is not representative of Merkel cell carcinoma tumors
When using a cell line to study cancer, phenotypic similarity to the original tumor is paramount. Cell lines are particularly important for rare cancers such as Merkel cell carcinoma (MCC) where tumor tissue

is scarce. MCC is an aggressive cutaneous neuroendocrine tumor with an annual incidence of ~1,500 in the United States, and ~80% of MCC tumors are infected with the recently discovered Merkel cell polyomavirus (MCV). Like most tumors types, little has been done to characterize how closely cell lines model native MCC tumors. The cell line UISO has been used extensively in the research of MCC since it was first characterized in 1993. To investigate its suitability as an experimental model, we characterized the UISO cell line and two other commonly used MCC cell lines (Mkl-1 and WaGa) with gene expression microarrays. Using a computational bioinformatic approach, we identified significant differences between the whole transcriptome gene expression signatures of the UISO cell line and the other MCC cell lines. We also identified striking differences between the UISO cell line and fresh frozen MCC tumors, irrespective of the MCV status in the tumors. In comparison, the Mkl-1 and WaGa cell lines more closely approximated the global transcriptome of MCC tumors. Gene set analysis identified similar differences at the pathway level between the UISO cell line and the other MCC cell lines, and between UISO and MCC tumors. Machine learning algorithms were then applied to the expression data to classify the UISO cell line among multiple tumor types. In contrast to the Mkl-1 and WaGa cell lines, UISO cells were consistently classified as non-MCC cells, indicating their global gene expression profile more closely resembles other tumor types than MCC. For validation of these findings in vivo, we characterized UISO cells grown as xenografts in immunocompromised mice. Histologically and immunophenotypically, UISO tumors did not resemble typical MCC tissue. In contrast, WaGa xenograft tumors showed immunohistological features diagnostic for MCC. Spectral karyotyping and short tandem repeat analysis of the UISO cell line matched that of the originally described UISO cells, ruling out contamination by another cancer cell line. Our results indicate that the UISO cell line is not representative of MCC tumors and should be used as an experimental model with caution, whereas the Mkl-1 and WaGa cell lines more closely model MCC tumors.

NCI-CCR
Shiba, Yoko
Visiting Fellow
Intracellular Trafficking
ArfGAP3 regulates the Transport of Mannose 6-phosphate Receptor in the post-Golgi compartment
ArfGAP family proteins catalyze the hydrolysis of GTP on Arf, the small GTP-binding protein that regulates membrane traffic. In pre-Golgi traffic, GTP hydrolysis on Arf is known to be involved in cargo sorting. However, the role of ArfGAPs in post-Golgi traffic has not been defined. Post-Golgi traffic is crucial for normal cell physiology and is disrupted in a number of human genetic disorders. Mannose 6-phosphate receptor (MPR) is an essential receptor required for proper targeting of many lysosomal enzymes with a known role in lysosomal storage disorders. MPR cycles between post-Golgi compartments, such as the trans-Golgi network (TGN) and endosomes. To determine the function of ArfGAPs in post-Golgi traffic, we performed an siRNA screen for ArfGAPs that affect MPR localization and found that down-regulation of ArfGAP3 resulted in the peripheral localization of MPR. The phenotype of cells transfected with ArfGAP3 siRNA was rescued by ArfGAP3 but not by homologous ArfGAP1, ArfGAP2 or the GAP domain mutants of ArfGAP3, suggesting the effect on MPR is specific for ArfGAP3 and dependent on its GAP activity. The transport of a classic secretary cargo, vesicular stomatitis virus G-protein, was not affected by the loss of ArfGAP3 protein. We found that ArfGAP3 localized to the TGN. In cells with reduced expression of ArfGAP3, maturation of Cathepsin D, a ligand of MPR, was slowed and its secretion was accelerated, suggesting MPR transport is perturbed. Retrograde transport of endogenous MPR, but not truncated MPR lacking the luminal and transmembrane domain, from the early endosomes to the TGN was perturbed in cells with reduced expression of ArfGAP3. Furthermore we found that the exit of epidermal growth factor receptor (EGFR) from the early endosomes was slowed in cells with reduced expression of ArfGAP3. We suggest that ArfGAP3 regulates

the transport of full-length MPR and EGFR specifically from the early endosomes to the late endosomes en route to the TGN and lysosome, respectively. The role of ArfGAP3 in post-Golgi traffic is unexpected given its known role in pre-Golgi traffic and when considering the specificity of the effect of ArfGAP3, our study raises the possibility that each ArfGAP functions nonredundantly in a specific set of cargos for specialized itineraries in the post-Golgi compartment.

NCI-CCR
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Clinical Fellow
Metabolomics/Proteomics
Phenformin induces metabolic shift of prostate cancer cells towards utilization of fatty acid metabolism
~Introduction: Biguanide drugs (most notably metformin) have generated significant interest due to their unanticipated anticancer properties seen in prostate cancer (PCa). The mechanism(s) responsible for this activity remain unclear although preliminary data point to involvement of energy production pathways, either through complex 1 in the mitochondria or AMPK. We sought to further characterize the metabolic changes incurred in prostate cancer cells upon exposure to phenformin (Pf), a more potent analogue of metformin. ~Methods: LNCaP, PC3, DU145, and LaPC4 PCa cell lines as well as primary explant PCa and normal prostate epithelium were examined in this study. Pf exposures were for 2-24 hours at 0.1-100uM. Western blot analysis was used to assess for changes in protein expression. The Seahorse XF96 analyzer was used to measure oxygen consumption rate (OCR) following injection of Palmitic acid bound to BSA to measure fatty acid oxidation (FAO) and glutamate + malate to measure complex I activity. Cells were also exposed to 5 uM AICAR (a direct AMPK activator) and 10 uM compound C (CC, an AMPK inhibitor) to assess the influence of AMPK on Pf induced changes. ~Results: PCa cells exposed to Pf demonstrated a 390% to 1240% increase in OCR in response to fatty acid injection compared to cells not exposed to Pf (p<0.0001). No such induction of FAO response was seen in normal prostate epithelium. No changes in FAO were observed after treatment of cells with the AMPK activator AICAR or AMPK inhibitor CC, suggesting that Pf effect is at least not solely due to AMPK activation. Co-treatment with AICAR and Pf demonstrated no significant change in FAO compared to Pf alone, however co-treatment with Pf and CC demonstrated a statistically significant blunting of the effect of Pf (50%; p<0.0001). Western blot densitometry demonstrated a significant induction of fatty acid synthetase and acetyl-CoA carboxylase in Pf-treated cells (p<0.001). Pf blunted OCR response to glutamate + malate injection (p<0.0001) suggesting mechanism of action on complex I of the mitochondria. ~Conclusion: Pf seems to shift the metabolic balance of PCa cells but not normal cells towards FAO. Although not directly induced by AMPK, this phenomenon seems to be modulated by AMPK activity state. Future studies are ongoing to investigate whether targeting fatty acid metabolism in combination with Pf represents a novel therapeutic strategy to treat prostate cancer.

NCI-CCR
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Postdoctoral Fellow
Molecular Biology - Prokaryotic
Change in membrane curvature drives differential gene expression in bacteria
Upon nutrient limitation Bacillus subtilis cells undergo sporulation, a simple developmental process, which results in the formation of spores that can withstand harsh environmental conditions. The hallmark of sporulation is asymmetric septum formation in the progenitor cells, which is followed by the activation of a sigma factor cascade that activates different set of genes in the smaller forespore compartment and the larger mother cell compartment. The molecular mechanism behind the shifting of cell division from medial site to asymmetric site and how the activation of first compartment-specific

sigma factor in the cascade takes place remains unclear. SpoIIE, a transmembrane protein, has been linked to the asymmetric shift in the septum formation site and also in activating the very first transcription factor, SigF, specifically in the forespore compartment. But how SpoIIE performs these roles with temporal and spatial precision is unknown. Here, we studied DivIVA, a negative curvature sensing peripheral membrane protein that resembles eukaryotic tropomyosins, in sporulating cells. We report that: (i) DivIVA localizes at the asymmetric septum, presumably by sensing negative curvature at the onset of septation; (ii) using a co-immunoprecipitation assay we found that DivIVA physically interacts with SpoIIE; (iii) deletion of divIVA or the genes encoding two other cell division proteins recruited by DivIVA, results in the mislocalization of SpoIIE and uncompartmentalized activation of SigF; (iv) timed-degradation of DivIVA after sporulation initiation also results in the mislocalization of SpoIIE and uncompartmentalized activation of SigF; (v) sporulating cells devoid of divIVA display severe inability to elaborate asymmetric septa; (vi) using structured illumination microscopy - a super resolution microscopic technique, we observed that after the completion of septum formation DivIVA and SpoIIE persist preferentially on the forespore side of the asymmetric septal membrane. We conclude that we have identified a factor, DivIVA, which mediates the asymmetric shift of the septation and likely sequesters SpoIIE at the asymmetric septum on the forespore side so that SpoIIE is now aptly placed to perform its second function in activating SigF in a compartment-specific manner. Thus, the membrane curvature sensing protein, DivIVA, through its interaction with SpoIIE controls asymmetric division and differential gene expression during development in B. subtilis.

NCI-CCR
Bakhsheshian, Joshua
Research Fellow
Neuroscience - Cellular and Molecular
Bioluminescent Imaging of ABCG2 Function at the Blood-Brain Barrier Using the Specific Substrate D-luciferin
ATP binding cassette (ABC) transporters are a group of transmembrane proteins that maintain chemical homeostasis by effluxing compounds from the apical cell membrane to the extracellular space, blocking cell entry. ABC transporters play a key role in protecting the brain parenchyma by exerting their action at the blood-brain barrier (BBB). However, these transporters also block the entry of therapeutic drugs, including cancer chemotherapeutics, limiting their efficacy against primary malignancies and metastases. One of the key transporters playing this role is ABCG2. While other ABC transporters can be studied through PET and SPECT, no probe exists for directly imaging ABCG2 function at the BBB. As such, there is little current knowledge on the absolute contribution of ABCG2 to chemodefense at the BBB. This has taken on particular interest given the finding that individuals of the Jr(a-) blood type carry two null alleles of ABCG2. D-luciferin, the endogenous substrate of fLuc, has been shown to demonstrate decreased bioluminescence in ABCG2-expressing cells. Biodistribution and cell uptake kinetic studies have confirmed low brain distribution of D-luciferin. Given that the BBB probably restricts entry of D-luciferin, we hypothesized that we can image ABCG2 function at the blood-brain barrier using bioluminescent imaging in transgenic mice expressing fLuc in the brain. The three most prevalent ABC efflux transporters at the blood-brain barrier are P-glycoprotein, multidrug resistance protein 1, and ABCG2. We sought to directly measure the selectivity of D-luciferin among these three transporters. The selectivity of D-luciferin was measured in vitro by the accumulation of fluorescence in human and mouse cells that overexpress each transporter and, in future research, it will be measured in vivo as the production of bioluminescence in mouse brains. Accumulation of D-luciferin was lowest in cells overexpressing ABCG2, and the accumulation increased when co-administered Ko143, a potent and selective ABCG2 inhibitor. Among the three most prevalent efflux transporters at the blood-brain barrier, D-luciferin is a specific substrate for ABCG2. These findings suggest that bioluminescence

imaging, using D-luciferin as the probe substrate, can be used for in vivo evaluation of ABCG2 activity for the first time. Furthermore, ABCG2â€™s role in the efflux of D-luciferin at the blood-brain barrier should be considered for future bioluminescence neuroimaging protocols.

NCI-CCR
Qu, Aijuan
Visiting Fellow
Physiology
Hypoxia-inducible factor 2 alpha activation disrupts liver cholesterol metabolism and accelerates atherosclerosis in apoE-null mice
Patients with obstructive sleep apnea (OSA) have an increased incidence of hypercholesterolemia and atherosclerosis, which has been attributed to liver dysfunction induced by chronic intermittent hypoxia (CIH). However, the mechanism is still elusive. Cellular adaptive response to hypoxia is mainly mediated by hypoxia-inducible factor (HIF), which consists of a ubiquitously expressed beta subunit and two related oxygen sensitive alpha subunits, HIF1alpha and HIF1beta. Under normoxia, HIFalpha subunits are rapidly degraded via the von Hippel-Lindau tumor suppressor protein (VHL) E3 ubiquitin ligase complex. In the present study, CIH resulted in increased hepatic HIF2alpha expression and elevated cholesterol in liver and serum. Hepatocyte-specific disruption of VHL resulted in constitutive activation of HIF signaling, and the resultant increased HIF expression augmented liver cholesterol accumulation, disturbed serum lipoprotein profiles and hypercholesterolemia. These effects were completely abolished in mice with compound knockout of VHL and HIF2alpha, thus demonstrating that HIF2alpha plays an essential role in control of cholesterol metabolism in liver and serum hypercholesterolemia. Mechanistically, HIF2alpha activation repressed the expression of Cyp7a1 and Cyp27a1 to block the conversion of free cholesterol to bile acids, downregulated acyl-CoA:cholesterolacyltransferase 2 (Acat2) to suppress the synthesis of cholesterol ester from free cholesterol, and inhibited ATP-binding cassette g5 (Abcg5) and Abcg8 to decrease the transport of cholesterol from liver to small intestine, thus leading to the accumulation of cholesterol in liver. In vivo luciferase assays via the Xenogen imaging system revealed that HIF2alpha activation could inhibit the transcriptional activity of the farnesoid X receptor (FXR), which is a critical transcription factor controlling liver cholesterol homeostasis. Moreover, disruption of HIF2alpha in apoE-null mice could decrease serum cholesterol levels and alleviate the development of atherosclerosis as revealed by reduced oil red O staining in aortic sinus sections and entire aortas. Together, these findings demonstrate that HIF2alpha activation in liver is critical for liver cholesterol dysregulation, hypercholesterolemia and atherosclerosis, thus providing a potential target for the therapeutics for hypercholesterolemia and atherosclerosis.

NCI-CCR
ZHOU, YANG
Postdoctoral Fellow
Radiology/Imaging/PET and Neuroimaging
Optical molecular imaging of HER1-positive cancer using validated near-infrared indocyanine green-labeled panitumumab
Optical molecular imaging has attracted remarkable attention in recent years for cancer visualization and localization due to its characteristics of real-time, high sensitivity and specificity, cost-effectiveness and non-ionization. Indocyanine green (ICG), the only clinically approved near-infrared fluorophore, is attractive to researchers in the development of targeted optical imaging agents by conjugation with antibodies or their fragments. However, ICG is hydrophilic and forms aggregates which cannot be removed by dialysis after antibody conjugation because of their high molecular weight. This condition is also frequently overlooked by many researchers. To characterize and purify ICG-conjugated antibody, in

this study, size-exclusion HPLC (SE-HPLC) was used after conjugation with an anti-epidermal growth factor receptor (anti-HER1) antibody, panitumumab. From the SE-HPLC profile, the percentages of the formed bioconjugates are ~55%, 30%, 15% for reactions performed with a molar ratio of dye to antibody of 5, 10, or 20, respectively (5×, 10×, 20×). After purification, the purity of ICG-panitumumab is >98% for all three bioconjugate products, with the molar ratios of linked ICG to panitumumab 1, 2 and 6 for 5×, 10×, and 20×, respectively. Competitive radioimmunoassay demonstrates that the targeting moiety of these bioconjugates was conserved. Fluorescence microscopy also demonstrates strong cellular binding and excellent fluorescent intensity of the bioconjugates in both fixed and live HER1-expressing A431 and SKOV3 cells. Importantly, visualization and quantification of HER1 in overexpressing LS-174T xenografts was successful. In contrast, the negative control antibody, ICG-HuM195 which targets CD33, showed negative tumor imaging; excess panitumumab antibody also blocked tumor uptake further demonstrated the specificity of ICG-panitumumab. Taken together, our results for the first time show in vivo optical imaging of HER1-expressing tumors using validated ICG-conjugated panitumumab. This should facilitate developing clinically applicable optical imaging strategy with ICG-conjugated antibodies.

NCI-CCR
Wakano, Clay
Postdoctoral Fellow
Signal Transduction - General
â€œInside-Out Signalingâ€: A Novel Non-Transcriptional Regulatory Mechanism of Receptor Signaling by a RNA Polymerase II Elongation Factor.
Cellular homeostasis is orchestrated by a complex and tightly regulated system of signal transduction pathways, cascading from receptors on the cell surface into the nucleus to activate transcription. Here we describe a novel â€œinside-outâ€ signaling mechanism that flows in the opposite direction from the nucleolus to the cell membrane. The Eleven Nineteen Lysine-Rich Leukemia protein (ELL), an RNA pol II elongation factor, forms regulated interaction with nucleophosmin (NPM1), a nuclear chaperone predominantly localized to the nucleolus. Our results suggest that the NPM1-ELL interaction sequesters ELL within the nucleolus where its subsequent release into the nucleoplasm and cytoplasm occurs in response to receptor activation. Remarkably, while in the cytoplasm ELL forms interactions with the endosomal sorting complexes (ESCRT) required for regulating the steady-state levels of important signaling receptors. This release of ELL from the nucleolus renders ELL available to modulate the ESCRT complexes and their subsequent recruitment into multivesicular endosomes (MVEs) to regulate the availability of active mitogenic membrane receptors like EGFR. This NPM1-ELL-ESCRT cycling therefore represents a novel, non-genetic, â€œreverse transductionâ€ mechanism for regulating cell-surface receptor internalization, degradation and signal attenuation through â€œinside-outâ€ signaling.

NCI-CCR
Walia, Vijay
Research Fellow
Signal Transduction - General
LPA-PI3K-Akt Signaling Network Regulates Primary Cilium Assembly
Defects in primary cilium formation and signaling are associated with a growing list of genetic diseases and has been linked to certain cancers. Cultured cells, rapidly cycling in the presence of serum suppress primary cilia assembly whereas G0/G1-arrest, deprivation of serum factors or cell-confluency stimulates primary cilia assembly. To identify the serum factors and most relevant cell-signaling cascade that regulate ciliogenesis, we screened 30 ubiquitous growth factors regulating 12 major signaling pathways in humans. We identified lysophosphatidic acid (LPA) and TGF-beta as two novel regulators of ciliogenesis in retinal pigment epithelial cells. We show that LPA but not TGF-beta blocks Rab11-

dependent preciliary membrane transport required for building cilium membrane by live microscopy. Using RNAi and chemical-inhibitor studies, we show that G-protein coupled LPA receptor 1 (LPAR1), but not LPAR2-5, activation by LPA inhibits ciliogenesis. Furthermore, to identify the downstream mediators activated by LPA-LPAR1, we used kinase inhibitors and found that inhibition of PI3K-Akt signaling strongly induces cilia growth. Ciliation was accelerated by Akt knockdown in serum and suppressed by overexpressing wild-type Akt upon starvation. GFP-LPAR1 is detected on the plasma membrane and Rab11-positive intracellular vesicles in serum fed cells, but relocalizes completely to the plasma membrane within minutes of serum deprivation. We found that blockage of LPAR1/PI3K endocytosis by Dynasore promotes Rab11-dependent preciliary membrane transport and ciliation indicating that inhibition of LPAR1 endocytosis is sufficient to engage cellâ€™s machinery for ciliogenesis. To further understand how the serum-switch regulates Rab11 preciliary transport and ciliogenesis, we investigated Akt phosphorylation of Rab11 associated proteins linked to endosome transport. To determine if this pathway is a global regulator of ciliogenesis, we tested 57 adherent cell lines from NCI-60 panel. We found that nearly 40% (23/57) of the cancer cell lines ciliate and 60% (34/57) do not ciliate. Among ciliators, 61% (14/23) cell lines significantly showed induction in ciliation upon LPAR1/PI3K/Akt inhibition. Together these findings demonstrate that a novel growth factor signaling network mediated by LPA-PI3K-Akt regulates ciliogenesis.

NCI-CCR
Kennedy, Mark
Visiting Fellow
Stem Cells - General
Sp1-like transcription factors are essential effectors of Wnt signaling during mammalian embryogenesis
Neuro-mesodermal (NM) stem cells generate the cellular precursors of the central nervous and musculoskeletal (MS) systems during mammalian embryogenesis. The Wnt3a/beta-catenin signalling pathway is essential for NM cell development. Loss of Wnt/beta-catenin signalling results in MS cells adopting neural fates and the depletion of NM cells. Yet, the mechanisms by which Wnt3a/beta-catenin signalling regulates NM development remain unclear. We sought to identify Wnt3a target genes to obtain insight into Wnt-mediated NM cell fate decisions. We transcriptionally profiled Wnt3a-/- mouse embryos to identify the Wnt3a-mediated gene regulatory network. This approach identified the Sp1-like family transcription factors Sp5 and Sp8 as putative Wnt3a target genes. Analysis of Sp5/Sp8 mRNA expression in Wnt3a-/- embryos and conditional beta-catenin loss- and gain-of-function embryos confirmed Sp5 and Sp8 as Wnt3a/beta-catenin regulated genes in vivo. In addition, treatment of mouse embryonic stem cells with recombinant Wnt3a protein showed that Sp5 and Sp8 were the only Wnt-responsive Sp1-like family members. Interestingly, whereas Sp5-/- embryos have no overt phenotypic defects, Sp8-/- embryos fail to develop caudal vertebrae, implicating Sp8 in caudal MS progenitor cell development. Direct comparisons of Sp5 and Sp8 gene expression in vivo determined that both genes are co-expressed in MS progenitors, thereby raising the possibility of redundant functions. Hence we established conditional, MS progenitor cell-specific Sp5/Sp8 double knockout (dko) mice. Dko embryos display a body truncation that is more severe than that observed in Sp8 single mutants. Analysis of the stem cell markers T and Msgn1 indicated a total loss of MS progenitor cells before E9.5 demonstrating a critical requirement for Sp5 and Sp8 in NM cell development. Furthermore, dko embryos display Sox2+ ectopic neural tubes which is a cell fate transformation also observed in Wnt3a-/-, and Tcf1-/-;Lef1-/- mutants suggesting that Sp5/Sp8 play an important role in Wnt signalling. In fact, overexpression of either Sp5 or Sp8 in ES cells is sufficient to activate the expression of many known Wnt target genes. Together these results provide tantalizing evidence that Sp1-like genes are novel components of the Wnt/beta-catenin signalling pathway.

NCI-CCR
KIM, WONIL
Postdoctoral Fellow
Stem Cells - General
Impaired Erythropoiesis of Gfi-1 Null Hematopoietic Progenitor Cells Is Rescued by Reducing Id2 Levels
The survival, self-renewal and differentiation of hematopoietic stem and progenitor cells (HSPC) are regulated by extrinsic signals from the microenvironment, and intrinsically by transcription factors and transcriptional networks. Previously, we found that inhibitor of DNA binding/differentiation 2 (Id2) is a physiological regulator of B cell development, and that growth factor independence-1 (Gfi-1) is part of the B cell transcription factor network, where it negatively regulates the expression of Id2. Gfi-1 is required for the development of multiple cell lineages including HSPC, however, the mechanisms by which Gfi-1 mediates these effects are currently unknown. Since Id2 expression is elevated in HSPC and Id2 can promote cell proliferation, we hypothesized that lowering Id2 expression could rescue the HSPC defects observed in Gfi-1 knockout (KO) mice. By transplanting Gfi-1 KO mouse bone marrow, we found that short-term (ST) reconstituting activity is rescued by heterozygosity at the Id2 locus, while the long-term reconstitution defect of HSC was not. Furthermore, we found that reduced levels of Id2 expression in Gfi-1 KO mice restore erythroid cell development by rescuing ST-HSC, common myeloid progenitor and megakaryocyte-erythrocyte progenitor in Gfi-1 KO mice. Pro-erythroblasts are expanded in Gfi-1 KO mice and their differentiation is rescued by lowering Id2 levels, suggesting that erythroid development is blocked at an early stage in Gfi-1 KO mice. Using Id2 promoter-driven YFP reporter mice, we found that Id2 expression is decreased as pro-erythroblasts mature, confirming that Id2 expression is repressed in later stages of erythroid differentiation. The dramatic changes of Id2 expression during erythroid development support our findings that the overexpression of Id2 which occurs in the absence of Gfi-1-mediated transcriptional repression causes impaired erythropoiesis at the pro-erythroblast stage. To identify the molecular mechanisms that restore erythropoiesis in Gfi-1 KO mice, we examined the expression of genes and proteins required for erythroid proliferation and differentiation. We found that reduction of Id2 expression in Gfi-1 KO mice results in increased expression of Gata1, Eklf, and EpoR genes, which are essential for erythroid differentiation. Consequently, we concluded that reducing Id2 levels rescues impaired erythropoiesis of Gfi-1 null hematopoietic progenitor cells by increasing erythropoietic gene expressions.

NCI-CCR
Xiao, Ying
Postdoctoral Fellow
Stem Cells - General
Hair Follicle Stem Cells Reside in a Venule Vascular Niche
Maintenance of stemness is especially important for hair follicle stem cells because follicles are highly proliferative and continually cycle through phases of regression and stem cell mediated regeneration. In addition to trophic support, vasculature is involved in regulating many processes including the creation of microenvironments that maintain stem cells in some tissues. We sought to investigate if vasculature also provides a hair follicle stem cell niche. We found that dermal blood vessels form a loop around Keratin 15 negative (K15-) hair follicle stem cells in the upper bulge. This vascular annulus consistently remains associated with the K15- region despite extensive angiogenesis around the regenerating follicle and its subsequent regression during hair follicle cycling. After each regression the follicle enters a resting phase where the vascular annulus is spatially associated with two adjacent yet distinct stem cell populations: label-retaining cells residing in the lower isthmus, and the K15- upper bulge stem cells. Using the venous marker EphB4 and arterial marker ephrinB2, we discovered the vascular annulus is comprised of post-capillary venules, suggesting the possibility that relative hypoxia and other venous

traits might contribute to the stem cell niche. Intriguingly, the K15- upper bulge specifically expresses Egfl6, a signaling molecule with angiogenic properties that can influence endothelial cell migration, suggesting a mechanism for maintaining its association with the vascular annulus. Developmentally, we found that nascent hair placodes hosting primitive K15- stem cells begin recruiting blood vessels by embryonic day E14.5, and a vascular annulus forms by birth when K15- upper bulge cells are specified. In a transplant model of folliculogenesis, a stereotypical vascular annulus is recapitulated around the upper bulge of de novo reconstituted hair follicles. The consistent nature of this association in different contexts strongly suggests a necessary regulatory relationship between the perivascular microenvironment and K15- upper bulge stem cells. We propose that specialized hair follicle stem cells are associated with a venule vascular niche that may be instrumental in establishing and maintaining tissue specific stem cell. Identifying niche components for normal hair follicle stem cells should help in understanding the niche for basal cell carcinoma cancer stem cell, which might originate from hair follicle stem cells.

NCI-CCR
Maeda, Daisuke
Visiting Fellow
Stress, Aging, and Oxidative Stress/Free Radical Research
Activated macrophages are a major source for genomic instability.
It is estimated that 20% of worldwide cancer deaths may be due to chronic inflammation. One chronic inflammatory response shown to increase cancer risk is silicosis due to repeated inhalation of fine silica dust. Macrophages resident in the lung attempt to phagocytize and destroy the silica particles, a process involving the generation of reactive oxygen species (ROS). ROS react with cellular components including DNA to produce an array of oxidative lesions. While most of these DNA lesions are single-stranded in nature, double-strand breaks (DSBs) also occur. Among the most serious of DNA lesions, DSBs increase the risk of genomic instability and cancer. Yet, to what extent DSB levels are elevated under chronic inflammatory conditions is unclear. We previously showed that the implantation of subcutaneous tumors in mice led to increased DNA damage including DSBs in tissues distant from the tumor in a macrophage-dependent manner. Similarly, we hypothesized that in lung tissues silica particles may activate lung macrophages, leading to ROS generation and DSB induction in susceptible bystander cells. In order to study this process in a system more amenable to experimental manipulation than whole animals, we adapted a model where permeable inserts containing macrophages are placed in fibroblast cultures about one mm above the cell layer. When the macrophages are stimulated by lipopolysaccharide (LPS) or silica, the incidence of gamma-H2AX foci, markers of DSBs, is significantly elevated in the fibroblasts. Removing macrophages, LPS, or silica nearly eliminates gamma-H2AX formation. Compared to controls, numbers of gamma-H2AX foci are increased about 3 fold and 2.6 fold in fibroblasts co-cultured with macrophages activated by LPS and silica respectively. Ongoing studies indicate that the mechanisms involved in DSB formation may differ between LPS and silica. We are now examining the DSB-containing fibroblasts for other signs of genomic instability. In addition, we will compare these in vitro results with those obtained in vivo. Such studies are significant because mechanistic insights into inflammation-induced genomic instability in normal tissues will be instrumental in assessing its medical importance as well as in developing methods to mitigate its effects in patients suffering from chronic inflammatory diseases.

NCI-CCR
WEYEMI, Sossou
Visiting Fellow
Stress, Aging, and Oxidative Stress/Free Radical Research

NADPH Oxidase 4 is a critical mediator in A-T disease

Ataxia telangiectasia (A-T) is a rare, autosomal recessive disorder characterized by radiosensitivity, progressive cerebellar degeneration, oculocutaneous telangiectasia, immunodeficiency and predisposition to lymphoid malignancies. To date A-T remains incurable. Symptoms generally appear in early childhood with most individuals dying in early adulthood from cancer or severe neurologic problems. It has been established that a protein kinase named ataxia telangiectasia mutated (ATM) is defective or missing in A-T patients. While ATM is best known for its function in DNA repair, recent findings have revealed a role for ATM in the control of reactive oxygen species (ROS) levels in mammalian cells. One feature of A-T disease is continuous oxidative stress. The NADPH oxidase 4, NOX4, has been described as a constitutive producer of ROS in a large range of human cell types. In addition, because NOX4 is in close proximity of the nucleus, changes in its expression may lead to altered levels of ROS-induced DNA damage. Therefore, we hypothesized that accrued ROS production in A-T cells may result from NOX4 dysregulation. We provide here the first evidence that pharmacological inhibition of ATM or its inactivation by small interference RNA (siRNA) leads to increased expression of NOX4 in primary fibroblasts, consistent with our findings that relative NOX4 expression is higher in A-T fibroblasts. Furthermore, we show that when NOX4 is silenced by siRNA in A-T primary fibroblasts, ROS levels are reduced, accompanied by reduced levels of oxidative DNA damage, DNA double-strand break formation, and replicative senescence, coupled with increased rates of proliferation. Altogether, these results suggest a pivotal role for NOX4 in regulating oxidative stress in A-T cells. A-T patients suffer from cerebellar degeneration. We show that analyses of brain tissues from A-T patients reveal elevated levels of NOX4 in the cerebellum that also correlate with increased levels of DNA damage and apoptosis. To substantiate this correlation, we examined mouse models of A-T disease which do not display cerebellar degeneration and failed to detect abnormal NOX4 cerebellar expression. We conclude that in A-T disease, NOX4 may be critical mediator and targeting it may open up new avenues for therapeutic intervention.

NCI-CCR
El Touny, Lara
Postdoctoral Fellow
Tumor Biology and Metastasis

Identification of a novel combination therapy that prevents the metastatic outgrowth and reduces the viability of dormant breast cancer cells: implications for clinical translation

Recurrent metastatic disease many years after initial therapy is a major cause of breast cancer (BC) mortality and strongly suggests that disseminated cells survive long periods in a growth-arrested state that is refractory to chemotherapy, known as â€œcellular dormancyâ€. Alterations in the components of the extracellular matrix (ECM), such as fibrotic foci and collagen-1 (Col-1) deposition, are associated with more aggressive breast cancer, increased metastasis and poor prognosis for BC patients. This suggests that ECM alterations may have a role in influencing the behavior of dormant BC cells. In fact, we have previously shown both in vitro (using 3-dimensional culture) and in vivo (preclinical model of fibrosis at the lung metastatic site) that alterations in the ECM surrounding disseminated, dormant BC cells can induce signaling pathways requiring Src and ERK1/2 activation that switch dormant cells into a proliferative state. We now demonstrate using the Src inhibitor AD0530 or Src shRNA, that the proliferative outbreak of dormant BC cells in Col-1-enriched microenvironments is prevented through the induction of a reversible G1-phase arrest and the upregulation of nuclear cyclin dependent kinase inhibitor p27Kip1, without affecting the viability of the dormant cells. Src inhibition, however, is not efficacious in regressing already established metastatic lesions, suggesting that Src signaling is crucial to engage the dormant-to-proliferative switch but is not necessary for survival once the cells have begun to proliferate. Since ERK1/2 activation is also required for the dormant-to-proliferative switch, we

determined the effect of the MEK1/2 inhibitor AZD6244 that prevents ERK1/2 activation on the proliferative switch alone and in combination with the Src inhibitor. Importantly, we show that combination treatment with AZD0530 and the MEK1/2 inhibitor AZD6244 of dormant cells undergoing the dormant-to-proliferative switch induces apoptotic cell death in a significant fraction of the dormant cells, which is not seen with either inhibitor alone, and significantly delays metastatic outgrowth in the lung metastatic site. In conclusion, inhibition of Src alone prevents the proliferative response of dormant cells to external stimuli without affecting survival, but the addition of a MEK1/2 inhibitor suppresses survival of dormant cells, indicating that this combination may have clinical value in preventing breast cancer recurrence.

NCI-CCR
Faraji, Farhoud
Doctoral Candidate
Tumor Biology and Metastasis
Network analysis reveals Cnot2 and miR-3470 transcriptional circuitry regulates breast cancer metastasis in vivo
Metastasis is responsible for the majority of cancer related mortality and is a result of stochastic genomic and epigenetic events leading to gene expression profiles that drive tumor dissemination. Prognostic gene signatures to predict patient risk for metastatic disease are in clinical trials; however, these minimal gene sets appear to be merely correlated to metastatic disease and have provided little insight into the mechanisms of metastasis. Here we exploit the notion that metastatic propensity is modified by the genetic background to link prognostic gene signatures with molecular mechanisms driving metastasis. To accomplish this we employ global gene expression profiling of tumors of the AKXD recombinant inbred panel followed by network analysis to identify a network of co-expressed genes centered on Cnot2 that predicts metastasis free survival in breast cancer patients. Modulating Cnot2 expression, results in changes in tumor cell metastatic potential in vivo: down-regulation of Cnot2 enhances metastasis, while up-regulation of Cnot2 suppresses metastasis, demonstrating functional involvement of Cnot2 expression in metastasis. miR-seq analysis in the same tumor panel revealed miRNA-3470b as a potential upstream regulator of the Cnot2 network. As such, overexpression of miR-3470b in 6DT1 breast cancer cells down-regulated expression of 4 network hub genes including Cnot2 and enhanced metastasis in vivo, phenocopying Cnot2 knock-down. Cnot2 is a core component of the CCR4-NOT complex, the primary deadenylase in mammalian cells which carries out the rate limiting step of mRNA degradation. Cnot2 also functions as a transcriptional regulator that recruits chromatin modifying complexes. We demonstrate by two independent methods that Cnot2 physically interacts specifically with the pro-metastatic short isoform of Brd4, a chromatin reader, but not the anti-metastatic long isoform of Brd4. We further show that it binds the metastasis driver genes Rrp1b and Sipa1, further supporting the critical role of Cnot2 in both expression and protein interaction networks causally involved in metastasis. Here, we have identified a gene expression signature regulated by miR-3470b, whose central node Cnot2 functionally regulates breast cancer metastasis. This not only reveals an unexpected potential role for mRNA deadenylation in breast cancer metastasis, but also links miR-3470b and Cnot2 to several metastasis modifier genes involved in chromatin regulation.

NCI-CCR
Giles, Amber
Postdoctoral Fellow
Tumor Biology and Metastasis
Circulating bone marrow-derived progenitor cells predict metastatic risk and develop into myeloid-derived suppressor cells at metastatic sites

The ability of tumors to metastasize to distant tissues is the most lethal aspect of cancer. Primary tumors impact the local microenvironment and elicit changes in distal tissues, such as bone marrow and spleen, which permit their growth and survival. The tumor can also condition tissues to create a specialized microenvironment, termed the pre-metastatic niche, which promotes the growth and survival of disseminated tumor cells. We have found in multiple murine cancer models that prior to metastasis, a primary tumor enhances production and mobilization of progenitor cells from the bone marrow. We find this phenomenon is recapitulated in breast and rhabdomyosarcoma cancer patients when compared to healthy controls. The significance of these cells is underscored by our finding in these patients that circulating progenitors predict metastatic risk. In patients and mice, clusters of bone marrow-derived cells are found at metastatic sites before tumor cells and are essential for the development of metastases. We sought to characterize the fate of these progenitors and determine how they contribute to the metastatic process. To demonstrate that bone marrow-derived cells accumulate at the primary tumor and metastatic lesions, we utilize bone marrow transplant models and an ex vivo lung culture system. As our cancer models approach detectable metastases, we observe increased myeloid cells in pre-metastatic sites. To determine if circulating progenitors contribute to the myeloid population in the pre-metastatic lung, we traced adoptively transferred progenitors in tumor-bearing mice. These progenitors home to the pre-metastatic lung and develop into myeloid cells within 24 hours of adoptive transfer. Myeloid cells isolated from the pre-metastatic lung, but not normal lung, are potent suppressors of T cells activated ex vivo with anti-CD3/anti-CD28 stimulatory beads. Together, these data suggest that circulating progenitor cells contribute to metastatic progression by developing into myeloid-derived suppressor cells at pre-metastatic sites and may protect newly disseminated tumor cells from immune attack. Furthermore, these cells provide an early prognostic tool to predict metastatic dissemination and a potential target of the metastatic process. Monitoring these cells at time of diagnosis, throughout treatment, and after treatment may help the identify patients at highest risk of metastatic progression and who may benefit from adjuvant immunotherapies.

NCI-CCR
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Tumor Biology and Metastasis

An adaptive unfolded protein response is required for metastatic tumor cell growth in the lung microenvironment

Background: Cure rates for pediatric osteosarcoma patients with lung metastases have remained low over the past 30 years. This clinical problem underscores the need to understand how metastatic tumor cells survive and grow within the lung. Once within the lung, tumor cells face a gauntlet of micro-environmental â€œstressorsâ€ that negatively affect tumor cell survival. In particular, metastatic tumor cells experience nitrosative stress, which results from exposure to high levels of nitric oxide released by host lung cells. Nitrosative stress has been shown to cause endoplasmic reticulum (ER) stress, and induce apoptosis in tumor cells. Despite this fact, a small subset of metastatic tumor cells are able to adapt, survive, and grow within the lung. By using a novel ex vivo lung tissue explant model, the following research investigates the ER and the adaptive unfolded protein response (UPR) as a mechanism by which highly metastatic tumor cells survive in the lung. Hypothesis: Highly metastatic osteosarcoma cells can mount an adaptive UPR in order to survive and grow in the lung. In contrast, low metastatic osteosarcoma cells are unable to mount an adaptive UPR resulting in a diminished ability to colonize the lung. Methods & Results: To dynamically image how osteosarcoma cells mount an adaptive UPR in the lung micro-environment, high and low metastatic murine osteosarcoma cells were transduced with a fluorescent-protein reporter construct that consists of spliced X-box binding protein-1 (sXBP-1) fused to a red fluorescent protein (RFP). This reporter construct is only expressed during ER

stress (ie. accumulation of unfolded proteins in the ER). Highly metastatic cells exhibited robust and sustained expression of sXBP-1/RFP from the time of initial arrest in the lung, to the formation of large metastatic deposits. In contrast, low metastatic cells had little to no expression of sXBP-1/RFP, and formed smaller lesions. Since the expression of sXBP-1 upregulates ER chaperone proteins, we examined the expression of GRP78 (a major ER luminal chaperone protein) in high and low metastatic cells in lung tissue by quantitative immunofluorescence microscopy. Highly metastatic cells had a larger ER compartment, whereas low metastatic cells had a smaller ER compartment. Conclusion: These results suggest that the robust expression of sXBP-1 and expansion of the ER compartment are important cellular adaptations for metastatic tumor cell growth in the lung micro-environment.

NCI-CCR
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Tumor Biology and Metastasis
The Nm23 metastasis suppressor binds Gelsolin and inhibits its actin severing activity.
Nm23 is the first metastasis suppressor gene to be identified and validated in several cancer cell lines by in vitro and in vivo assays. The molecular mechanism(s) leading to the anti-metastatic property of Nm23 is incompletely defined. After overexpressing flagâ€“tagged Nm23 in the murine mammary carcinoma cell line 4T1, flag immunoprecipitation was performed to isolate protein complexes associated with Nm23. Using mass spectrometry, we identified a new Nm23 interaction partner, the actin-binding protein Gelsolin. Two-way co-immunoprecipitations were performed in 4T1 cells and several human breast carcinoma cell lines to confirm the association. In the highly metastatic 4T1, MDA-MB-435 and MDA-MB-231 cell lines, the interaction of Nm23 and Gelsolin was observed only after Nm23 overexpression suggesting that an Nm23-Gelsolin interaction may only occur when relatively high levels of Nm23 are expressed. Co-immunoprecipitation of the two proteins at endogenous expression levels was observed in MCF-7 breast carcinoma cells. The functional interaction of Nm23 and Gelsolin was investigated by overexpressing each protein, or the combination, in both murine (4T1) and human (MDA-MB-231) breast carcinoma cell lines. Cell motility and F-actin-severing activity were measured. Gelsolin overexpression was associated with an increase in both cell motility (58% and 64% in 4T1 and MDA-MB-231 cells, respectively) and F-actin-severing (22- and 7-fold increase in 4T1 and MDA-MB-231, respectively) while Nm23 overexpression reduced cell migration (46% and 37% in 4T1 and MDA-MB-231, respectively), without affecting the actin-depolymerization, compared to the empty vector-expressing cells. Both Gelsolinâ€™s stimulation of motility and depolymerization of actin were abrogated by co-overexpression of Gelsolin and Nm23 suggesting an inhibitory effect of Nm23 on Gelsolinâ€™s pro-motility properties. A spontaneous metastasis model was used to confirm those observations.. Gelsolin overexpressing 4T1-injected mice developed 2-fold greater lung metastases (p=0.001) and diffuse liver metastases compared to the vector-expressing group. Nm23 overexpression resulted in 48% reduction in lung (p=0.0002) and no diffuse liver metastasis. Interestingly, the Nm23 and Gelsolin co-overexpression abrogated the pro-metastatic effect of Gelsolin. The in vitro and in vivo data suggest a potential role of Nm23 on inhibiting the pro-motility and pro-metastatic effect attributed to Gelsolin.

NCI-CCR
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Tumor Biology and Metastasis
Mammary tumorigenesis in haploinsufficient Cripto-1 mice
The embryonic gene Cripto-1 (Cr-1) is expressed at very low levels in normal adult tissues and detected at high levels in several human tumors. Transgenic mice overexpressing the Notch4 intracellular domain

(Int3) in the mammary gland under the control of the whey acidic protein (WAP) promoter rapidly develop mammary hyperplasias and undifferentiated mammary adenocarcinomas in aged nulliparous females and after multiple cycles of pregnancy. Considering that Cr-1 facilitates the proteolytic intracellular processing of Notch receptors, we sought to assess the contribution of Cr-1 in the hyperplasias and tumors that arise in WAP-Int3 mice. Therefore, we developed a bi-transgenic mouse model lacking one allele of Cr-1, by crossing the knock-in Cr-1/LacZ mice (containing a promoter-less bacterial ß-galactosidase gene which disrupts one of the Cr-1 alleles) to WAP-Int3 transgenic mice. We submitted these Wap-Int3/Cr-1/LacZ mice to multiple cycles of pregnancy, and observed that in the absence of one allele of Cr-1, Int3-induced transformation of mouse mammary epithelial cells was delayed, and additional cycles of pregnancy were required to observe tumor growth. Notably, only 20% of the Wap-Int3/Cr-1/LacZ mice developed mammary tumors by the second pregnancy, as compared to 80% of Wap-Int3 mice. Additionally, we found a 90% reduction in the frequency of mammary hyperplasias in these Wap-Int3/Cr-1/LacZ. Immunohistochemistry and Western blot analysis revealed that the few tumors derived from these Cr-1 haploinsufficient mice significantly expressed more milk proteins, such as Caseins, when compared to Wap-Int3 tumors. In fact, histological analysis showed that Wap-Int3/Cr-1/LacZ tumors showed a significantly more differentiated histology, preserving glandular-like mammary structures. Fluorescence-activated cell sorting (FACS) analysis revealed that Wap-Int3 tumors expressed Integrin beta-3 (CD61), a luminal progenitor marker, while Wap-Int3/Cr-1/LacZ tumors did not, indicating that Cr-1 might be important to keep a mammary luminal progenitor population. This study suggests that Cr-1 contributes to the development of Int3-induced mammary tumorigenesis and might be important in reprogramming differentiated tumor cells into cancer stem cells. We are currently generating a mouse model conditionally lacking the two alleles of Cr-1 in the mammary gland to better address our question regarding the functional significance of Cr-1 in mammary tumorigenesis.

NCI-CCR
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Virology - DNA

KSHV microRNAs repress breakpoint cluster region protein expression to enhance Rac1 activity and in vitro angiogenesis

MicroRNAs (miRNAs) are small ~22-nt long RNAs that regulate gene expression post-transcriptionally. Kaposi's sarcoma-associated herpesvirus (KSHV) encodes 12 pre-miRNAs during latency and the cellular targets of these miRNAs largely remain unknown. Using a previously reported microarray profiling analysis, we identified breakpoint cluster region protein (Bcr) as a cellular target of the KSHV-encoded miRNA, miR-K12-6-5p (miR-K6-5). Bcr protein levels were repressed in human umbilical vein endothelial cells (HUVECs) upon transfection with miR-K6-5 and during KSHV infection. Luciferase assays, wherein, the Bcr 3â€™-UTR was cloned downstream of a luciferase reporter showed repression in the presence of miR-K6-5 and mutation of one of the two predicted miR-K6-5 binding sites relieved this repression. Further, inhibiting miR-K6-5 in KSHV-infected BCBL-1 cells showed an increase in Bcr protein levels. Together, these results show that Bcr is a direct target of the KSHV miRNA, miR-K6-5. To understand the functional significance of Bcr knockdown in the context of KSHV-associated disease, we hypothesized that the knockdown of Bcr, a negative regulator of Rac1, might enhance Rac1-mediated angiogenesis. We found that HUVECs transfected with miR-K6-5 had increases in Rac1-GTP levels and tube formation, compared to control miRNAs. Furthermore, an siRNA against Bcr was able to recapitulate a similar effect suggesting that the effect of miR-K6-5 on tube formation was likely mediated via repression of Bcr. Together, our results reveal a new function for both KSHV miRNAs and Bcr in angiogenesis and suggest that KSHV miRNAs repress expression of Bcr to promote an angiogenic environment.

NCI-CCR
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Visiting Fellow
Virology - DNA
Antibody-Mediated Neutralization of Neurovirulent JC Polyomaviruses
Progressive multifocal leukoencephalopathy (PML) is an AIDS-defining fatal brain disease. PML is also a dangerous side effect of recently developed immunosuppressive agents, including monoclonal antibody (mAb) therapeutics used in the treatment of multiple sclerosis, rheumatoid arthritis, and certain types of B cell cancers. PML is caused by uncontrolled replication of JC polyomavirus (JCV) in various brain cell types. A great majority of adults, including patients at risk of PML, have serum antibodies capable of neutralizing lab-adapted JCV strains. Its unclear why these antibodies fail to protect immunosuppressed patients against PML. Intriguingly, several recent reports indicate that JCV isolates from the cerebrospinal fluid (CSF) of PML patients often contain distinctive mutations in the viral major capsid protein VP1. This observation raises the possibility that JCV variants found in the CSF of PML patients can evade recognition by neutralizing antibodies. To explore this idea, we engineered a set of seven JCV-based reporter vectors (pseudoviruses) and performed neutralization serology on hundred individuals. Results showed that while most healthy individuals have serum antibodies capable of neutralizing a broad range of wildtype and PML-derived JCV variants, some individuals fail to neutralize a subset of PML-variant JCV strains. The results suggest that rare individuals have a â€œblind spotâ€ in their JCV-neutralizing antibody repertoire, resulting in poor humoral protection against JCV variants associated with PML. We speculate that such individuals might be at increased risk of developing PML under immunosuppression. Our model suggests two potential approaches towards clinical management of PML. First, it might be possible to prevent/treat PML by administering humanized monoclonal antibody capable of neutralizing a broad range of JCV variants. Second, administration of virus-like particle vaccine based on the capsid protein VP1 might help broaden the neutralizing antibody repertoire in patients at risk of PML. Also, in course of performing the serology studies, we discovered a non-antibody protein innately present in all human sera that potently neutralizes PML-variant JCV isolates. Interestingly, the innate neutralizing activity is absent from healthy human CSF samples. In principle, the innate neutralizing factor might serve as a therapeutic agent against neurovirulent forms of JCV and could be delivered to the CSF of PML patients.

NCI-CCR
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Virology - DNA
Kaposi Sarcoma Herpes Virus (KSHV) ORF57 is a molecular switch to facilitate translation during stressful lytic infection
RNA granules are non-membranous compartments containing mRNAs and associated proteins playing a major role in translational repression during physiological stress and viral infections through TIA-positive stress granules (SGs) and during routine mRNA regulation though GW182-positive P bodies. In this study, we observed that B cells with lytic KSHV infection do not exhibit visible SGs, but the B cells without lytic KSHV infection displayed many SGs in the presence of oxidative stress of arsenite. We found that KSHV lytic ORF57 protein, also named mRNA transcript accumulator or MTA, prevents the formation of SGs during KSHV lytic infection. ORF57 in HeLa and HEK293 cells not only inhibited the formation of SGs during arsenite or Poly I:C treatment, but also restricted the formation of P bodies. ORF57 mutant and ORF59 failed to exhibit such inhibitory effects on SGs and P bodies. By western blotting, we confirmed the strong inhibition of EIF2 alpha phoshorylation by ORF57 in arsenite-treated

cells. Phosphorylation of EIF2 alpha is required for the formation of SGs and is increased remarkably by protein kinase R (PKR) during arsenite treatment. We found that KSHV ORF57 blocks PKR activity and inhibits PKR expression in arsenite-treated cells. Coimmunoprecipitation experiments showed that ORF57 could interact with PKR and this binding is important for both downregulation of PKR and its activity. However, ORF57 couldnâ€™t inhibit heatâ€“induced, PKR-independent EIF2 alpha phosphorylation and thus, the formation of SGs during heat stress, implying that ORF57 doesnâ€™t inhibit EIF2 alpha phosphorylation catalyzed by other EIF2 alpha kinases. Moreover, ORF57 showed interaction with several components of P bodies, including AGO2 and XRN2, and thus inhibits formation of the P bodies. Altogether, our study clearly shows that ORF57 facilitates translation during antiviral response in stressful lytic KSHV infection. Our study demonstrates the novel mechanism employed by a DNA tumor virus to inhibit antiviral response through a nuclear viral protein ORF57 and helps understand the stress involved and its viral modulation during lytic phase of a tumor virus infection.

NCI-CCR
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Virology - RNA and Retroviruses
The contribution of monocytes/macrophages to HTLV-1 infection and persistence
Proviral load (PVL) has been considered a strong risk factor for the development of HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP). However, PVL alone is not enough to differentiate symptomatic patients from healthy carriers, suggesting the participation of other factors. Early studies showed that monocyte/macrophage cells expressed HTLV-1 antigens when cultured short-term. In humans and non-human primates, peripheral blood monocytes can be classified into three main subsets based on expression levels of CD14 and CD16 molecules: CD14++CD16- (classical), CD14+CD16++ (non-classical), and CD14++CD16+ (intermediate). The last two subsets of monocytes (CD16+) are expanded in a variety of inflammatory and infectious states. In here, we investigated whether the different monocyte subpopulations are potential carriers of HTLV-1, contributing to viral infection and persistence. In order to investigate the distribution of monocytes subsets during HTLV-1 infection, PBMCs of 17 HTLV-1-infected patients (HTLV-1-IP) and 11 normal donors (ND) were phenotypically analyzed. The classical monocyte frequency was lower in HTLV-1-IPs compared to the NDs (p=0.048). Moreover, when we compared the monocyte frequencies with the PVL, we found a positive correlation between PVL and intermediate monocytes (r=0.6735, p=0.0042). Focusing on the presence of HTLV-1 provirus DNA in the different monocyte subsets, cell populations were isolated from PBMCs of 16 HTLV-1-IPs. Genomic DNA was isolated from sorted cells and a fragment of the HTLV-1 gag gene was amplified by nested PCR. All cell populations isolated from PBMCs of the infected patients (CD4+, CD8+, CD14++CD16-, CD14+CD16++, and CD14++CD16+) were positive for HTLV-1 gag in patients with high PVL. In contrast, one or more monocyte subsets were negative or very weakly positive for gag in patients with low PVL. To test whether natural STLV-1 infection recapitulates what we find in HTLV-1 infected individuals, we analyzed the monocyte distribution in 8 STLV-1 infected Rhesus macaques and 16 naïve animals. In the macaques, the frequency of intermediate monocytes was higher in infected compared to naïve animals (p=0.0001). As seen in HTLV-1 infection, there is a positive correlation between PVL and intermediate monocyte frequencies (r=0.6530, p=0.03). In conclusion, our results suggest that monocytes play an important role in viral dissemination and persistence and are potential viral reservoirs.

NCI-CCR
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Virology - RNA and Retroviruses

A unique â€œAâ€ shape topology of the HIV-1 Rev response element explains the specificity and cooperativity of Rev binding

The (+) RNA genome of the human immunodeficiency virus, type 1 (HIV-1) contains multiple splicing sites. Since unspliced RNAs normally are not exported from the nucleus, this problem is circumvented via the virus-coded Rev protein, which binds cooperatively and specifically to the Rev response element (RRE), an RNA structural element located in the env gene and contained in all partially and unspliced viral mRNA transcript. The Revâ€“RRE complex then sequesters Crm1 (Xpol1), a host exporting complex, to export partially spliced or unspliced RNA into the cytoplasm as a source of the viral genome and a template for protein synthesis. The topology of RRE that enables such a specific interaction, however, is not known. We report here the RRE three-dimensional (3D) topology using small-angle X-ray scattering (SAXS), showing it assumes a unique, extended â€˜Aâ€™-like shape. Analysis of RRE sub-fragments suggests that the specific Rev binding sites,IIB and IA, are located at the two â€˜legsâ€™ of the â€˜Aâ€™, facing each other by a separation of ~60 Å. These dual binding sites form a topological constraint, and together with its unique extended shape, may constitute the structural basis for the specificity, cooperativity and oligomerization of the Revâ€“RRE complex. The RRE topology provides important insights into the possible mechanism of how the seemingly promiscuous Rev and its cognate RNA partner select each other for nuclear export as well as a structural basis for designing drugs to target the nucleation sites of the export complex.

NCI-CPFP
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U.S. Childrenâ€™s Physical Activity by Time of Day: Results from the National Health and Nutrition Examination Survey (NHANES)

Purpose: Evidence is needed to understand physical activity patterns of youth in order to develop more appropriate and better targeted intervention strategies. To date, no population-based studies have evaluated U.S. youth physical activity by time of day. This study used objective physical activity measurement to investigate differences in youth activity patterns related to gender, age, race/ethnicity, body mass index (BMI), day, and time of day. Methods: Cross-sectional data are from the 2003â€“2004 National Health and Nutrition Examination Survey and based on participants (6-19 years) that had any days with = 10 hours of detected physical activity monitor wear-time (N = 2520). Mean counts per minute were summed by day. Weekday time was subdivided to reflect a typical US school day: before (6-9 AM), during (9-3 PM), after (3-6 PM) school, and evenings (6-9 PM). Descriptive, unweighted statistics for the rate of activity calculated as the total counts for each time period divided by the total hours of the time period were analyzed overall and by subgroup. Weighted, multivariate analyses will be conducted. Results: Preliminary data suggest that the lowest activity rate (mean counts/hr (SE)) occurred before school (10333 (127)) and highest rate after school (28822 (253)) vs. during school (20482 (161)). Boys had a higher rate of activity. This sex difference was largest after school. Obese youth had the lowest rate of activity overall vs. healthy weight youth with the largest difference after school (24532 (476) vs. 30715 (350)). Non-Hispanic Blacks had a higher rate of activity during weekday evenings compared to non-Hispanic Whites and Mexican-Americans (26527 (452) vs. 21471 (415) and 22508 (393), respectively). Children (6-11 yrs) had the highest activity rate, except before school when adolescents (12-15 yrs) were more active (12032 (222) vs. 10586 (202)). All findings are significant (p<0.001). Conclusions: There are significant differences in youth physical activity by time of day and for specific subgroups. These results provide much needed insight into the temporal patterns of physical activity by U.S. youth. Once additional weighted analyses have been performed and extended with the

2005-2006 NHANES data, findings from this study may help guide and better target population-level prevention intervention strategies for youth most at risk for physical inactivity.

NCI-CPFP
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Epidemiology/Biostatistics - Etiology, Risk, and Prevention
Pre-diagnostic circulating adipokines levels and endometrial cancer risk in the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial
BACKGROUND:Obesity, as measured by body mass index (BMI), is one of the strongest risk factors for endometrial cancer in postmenopausal women. While some of this association may be related to higher levels of circulating estradiol (E2), other potentially important mechanisms are not well understood. Adipokines, such as adiponectin, leptin and visfatin are produced and secreted by adipose tissue and have roles in such carcinogenic processes as cell proliferation, angiogenesis and metastasis. Few studies have prospectively evaluated the relationship of pre-diagnostic levels of adiponectin and leptin, and none have examined visfatin, with endometrial cancer risk. METHODS:We conducted a nested case-control study of postmenopausal women in the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial (n=78,216), including all 167 incident endometrial cancer cases with available pre-diagnostic serum and randomly selected 327 controls that were matched to cases 2:1 on age, study center, race, year of diagnosis, year of blood draw, time of day of blood draw and menopausal hormone therapy (MHT) use. Adipokine levels were categorized into tertiles (T) and odds ratios (ORs) and 95% confidence intervals (CIs) were estimated by conditional logistic regression, adjusting for known endometrial cancer risk factors, including BMI and E2. Because BMI shows the strongest relationship with endometrial cancer risk in women not using hormones, we also restricted our analyses to non-MHT users (86 cases; 176 controls). RESULTS:Adiponectin levels were inversely associated with endometrial cancer risk [OR T3vsT1: 0.48 (95%CI: 0.29-0.80); p-trend<0.01], whereas elevated leptin levels showed a positive association [2.77 (1.60-4.79); p-trend<0.01]. These results remained significant after adjustment for E2, but not after further adjustment for BMI. When analyses were restricted to non-MHT users, associations for adiponectin and leptin were stronger and remained significant after adjustment for E2 and BMI [0.25 (0.08-0.75), p-trend=0.01 and 4.72 (1.15-19.38), p-trend=0.02, respectively]. Although not statistically significant, estimates suggested a positive association of visfatin with risk. CONCLUSION:Our data suggest that adipokines may influence postmenopausal endometrial cancer risk through pathways other than E2-mediated cell growth, especially in non-hormone users. Continued evaluation of adipokines may help elucidate mechanisms underlying obesity-cancer associations.

NCI-DCCPS
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Carcinogenesis
Blockade of CD47 induces radiation tumor growth delay through regulation of autophagy and bioenergetics of the tumor microenvironment
CD47 is a widely expressed transmembrane receptor for members of the signal regulatory protein (SIRP/SHPS) family and the matricellular protein thrombospondin-1. We previously demonstrated that a lack or blockade of CD47 results in profound protection of cells and normal tissue from death induced by ionizing radiation. WT or CD47-/- mice were injected with B16 melanoma cells to form subcutaneous tumors and exposed to local irradiation. WT mice bearing B16 tumors were also treated with an antisense CD47 morpholino prior to irradiation. At the end of the study tumors were excised and analyzed. Tumors on a CD47 deficient microenvironment in combination with radiation treatment show

remarkable reduction in tumor growth. Tissue sections showed an increase in macrophage infiltrate as measured by CD68 positive staining. Moreover, targeting of CD47 enhanced macrophage mediated killing of melanoma. In vitro viability studies demonstrate that blockade of CD47 protects macrophages from death from IR indicating that blockade of CD47 may selectively protect cytotoxic macrophages that in turn reduce tumor growth. The radioprotection observed with CD47 inactivation in macrophages is associated with a reduction in cell death and increased in autophagy related genes ATG5, ATG7 and LC3. Moreover, cell mediated cytotoxicity is mediated also by the activation of autophagy by CD47 blockade in macrophages. Blockade of CD47 does not protect cancer cells from IR however blockade of CD47 caused a remarkable reduction of autophagy gene expression in tumors as measured by decreased Beclin-1 and reduction in LC3 puncta. Since autophagy is a homeostatic process dependent of cell metabolism we measured the oxygen consumption rate and acidification rate of cells using a seahorse XF24 system. Our results show a reduction in mitochondrial oxygen consumption rate and extracellular acidification rate in cancer cells which is associated with sensitization to death. This indicates that CD47 blockade selectively activates protective autophagy in macrophages but reduces this pathway in cancer cells. These differences are associated with the metabolic demand between transformed and non-transformed cells. Together our results demonstrate that targeting CD47 causes a dual effect in the tumor microenvironment by regulating autophagy and cell metabolism indicating that agents targeting CD47 may allow for more aggressive application of radiation to increase the percentage of curative responses

NCI-DCEG
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Clinical and Translational Research
Undetectable Levels of Anti-Müllerian Hormone in Women with Fanconi Anemia
Fanconi anemia (FA) is a rare genetic disorder caused by mutations in the Fanconi/BRCA DNA repair pathway and is characterized by birth defects, bone marrow failure, increased frequency of malignancy, and impaired fertility. To further examine fertility in FA, we measured serum levels of anti-müllerian hormone (AMH), a marker of ovarian reserve, in our cohort of female FA patients less than or equal to 40 years of age (n=20; median age=23; range: 7-37 years old). Unaffected female relatives of patients with FA (n=19; median age=34.5; range: 3-40 years old) and unrelated healthy females (n=21; median age=27; range: 12-40 years old) were used as controls. FA females had very low or undetectable AMH levels (median=0.0185 ng/ml; range: 0-2.316 ng/ml; p<0.0001) compared to both, unaffected FA relatives (median=2.062 ng/ml; range: 0.044-4.726 ng/ml) and unrelated healthy females (median=1.916ng/ml; range: 0.311-6.643 ng/ml). Normal levels of AMH for post-pubertal women of reproductive age typically range from 1-8 ng/ml. Post-pubertal females with FA had negligible levels of AMH (n=11; median=0 ng/ml; range: 0-1.179 ng/ml). Ovarian senescence before the age of 40, defined by amenorrhea and serially elevated levels of follicle stimulating hormone (FSH), is known as primary ovarian insufficiency (POI). In FA females, impaired fertility is often a result of POI before the age of 30. All five FA patients over 25 years of age were previously diagnosed with POI and were found to have undetectable AMH levels in our study. Because AMH levels do not significantly fluctuate during the menstrual cycle or with androgen use, AMH may be a better marker than FSH for diagnosis of POI in female FA patients known to have irregular cycles and in those who are treated with hormone therapy. This study is the first to demonstrate AMH deficiency in FA patients. Low or absent levels of AMH appear to be a shared phenotypic trait across females in our FA cohort, which is comprised of multiple FA genotypes and phenotypes. AMH deficiency in FA females suggests that ovarian reserve defects may be a common factor in an otherwise heterogeneic clinical disease. Identification of genetic alterations

affecting ovarian reserve in the FA population may elucidate mechanisms of infertility and, potentially, other determinants of FA-related complications.

NCI-DCEG
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Cultural Social and Behavioral Sciences
Case-case comparison of smoking and alcohol risk associations with Epstein-Barr virus-positive gastric cancer
BACKGROUND: Although the bacterium Helicobacter pylori is the primary cause of gastric cancer, monoclonal Epstein-Barr virus (EBV) nucleic acid is present in 8-10% of these tumors worldwide. Viral prevalence is increased with male sex, non-antral subsite localization and surgically disrupted anatomy. To further examine associations of EBV with sufficient statistical power, we have organized an international consortium of multiple gastric cancer studies with EBV assessment by in situ hybridization. Here we compare data on EBV-positive and EBV-negative gastric cancers to identify behavioral risk factors. METHODS: From eight studies (four from Asia, two from Europe and two from Latin America), we pooled individual-level data for aggregated analysis of 1,545 gastric cancer cases, including 152 (9.8%) that were EBV-positive. We assessed associations with cigarette (61% smokers) and alcohol (69% drinkers) use in case-case comparisons --rather than the original case-control approach-- to account for possible interactions with tumor-specific characteristics. Odds ratios (OR) and 95% confidence intervals (CI) were estimated by multi-level logistic regression to control for within-population clustering. ORs were adjusted (aOR) for distributions of sex (67% male), age (mean 60 years), educational attainment (44% secondary school or greater), body-mass index (49% obese, WHO Classification), tumor histology (63% Lauren intestinal-type) and anatomic subsite (85% noncardia). RESULTS: In unadjusted analyses, the OR for tumor EBV positivity among smokers was 2.4 (95% CI, 1.6-3.6) overall without substantial difference between current and former smokers (respective ORs, 2.4 and 2.5). The association was attenuated by adjustment for the possible confounders but remained statistically significant (aOR, 1.6; 95% CI, 1.01-2.5). There was a crude association of tumor EBV positivity with drinking (OR, 1.8; 95% CI, 1.02-3.3) that was explained by smoking and the other confounders (aOR, 1.1; 95% CI, 0.6-2.1). CONCLUSIONS: The association of cigarette smoking with gastric cancer may be stronger for EBV-positive than EBV-negative tumors. Conversely, the null association of gastric cancer with alcohol drinking does not apparently vary by EBV status. These epidemiologic characteristics further implicate EBV as an etiologic co-factor in gastric carcinogenesis. Risk of EBV-positive tumors, and gastric cancer in general, could potentially be decreased by smoking cessation.

NCI-DCEG
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Cultural Social and Behavioral Sciences
Home kitchen ventilation, cooking fuels and oils, and lung cancer risk in a prospective cohort of non-smoking Chinese women
Lung cancer incidence has been on the rise in less-developed countries. Indoor air pollution caused by poor ventilation, burning of solid fuels, and cooking oils has been associated with lung cancer risk in developing poor and rural countries in retrospective case-control studies. However, few studies have been conducted in urban and economically developed areas. To assess the relationship of cooking conditions, fuel use, oil use and risk of lung cancer in a modern population, an analysis was conducted in a prospective cohort of women, the Shanghai Womenâ€™s Health Study. 71,320 never smoking women were followed through December 2009 and 429 incident lung cancer cases were identified.

Standardized and structured questionnaires were used to collect information on household living and cooking practices for the three most recent residences lived, cooking fuel utilized, cooking oil utilized, ventilation conditions, smoking patterns of family members at home, and coworker smoking at work. Effect estimates for kitchen ventilation conditions, cooking fuels, and cooking oils use for the risk of lung cancer are presented as hazard ratios (HR) with 95% confidence intervals (95% CI) from Cox proportional hazards regression. Ever use of coal was not significantly associated with lung cancer risk compared to never use of coal, but ever having poor kitchen ventilation was associated with a 44% increase in lung cancer risk (HR: 1.44; 95% CI: 1.11-1.85) compared to never having poor ventilation. Ever having poor ventilation and being a coal user was associated with a 50% increase in lung cancer risk compared to never coal and poor ventilation users (HR: 1.50; 95% CI: 1.07-2.09). Coal (r: 0.19; p-value: <0.0001) use and poor ventilation (r: 0.12; p-value: <0.0001) were correlated with less education. This analysis of non-smoking Chinese women in Shanghai, China suggests that exposure to cooking coal with poor ventilation increases the risk of lung cancer. These results suggest that coal use can still be a public health issue in modern cities which may disproportionately affect women of the lowest socioeconomic status.

NCI-DCEG
Koutros, Stella
Research Fellow
Epidemiology/Biostatistics - Etiology, Risk, and Prevention
Differential urinary specific gravity as a molecular phenotype of the bladder cancer genetic association in the urea transporter gene, SLC14A1
Genome-wide association studies (GWAS) identified associations between markers within the solute carrier family 14 (urea transporter), member 1 (SLC14A1) gene and risk of bladder cancer. SLC14A1 defines the Kidd blood groups in erythrocytes and is also involved in concentration of the urine in the kidney. We evaluated the association between a representative genetic variant identified from GWAS (rs10775480) of SLC14A1 and urine concentration, as measured by urinary specific gravity (USG), in a subset of 275 population-based controls enrolled in the New England Bladder Cancer Study. Overnight urine samples were collected and USG was measured using refractometry. Analysis of covariance was used to estimate adjusted least square means for USG in relation to rs10775480. Expression of SLC14A1 and SLC14A2 genes in a panel of human tissues was evaluated by RNA-sequencing. USG was decreased with each copy of the rs10775480 risk T allele (p-trend= 0.011) with a significant difference observed for CC vs. TT genotypes (p-value tukey=0.024). RNA-sequencing in the bladder tissue showed high expression of SLC14A1 and the absence of SLC14A2, while both transporters were expressed in the kidney. We suggest that the molecular phenotype of this GWAS finding is the genotype-specific biological activity of SLC14A1 in the bladder tissue. The composition of urine in the bladder is thought to reflect that produced by the kidney however, our data suggest that SLC14A1 could be a unique urea transporter in the bladder that has the ability to influence urine concentration and that this mechanism might explain the increased bladder cancer susceptibility associated with rs10775480. Factors affecting urine volume and concentration, including urination frequency and fluid intake, have been evaluated as risk factors for the disease in epidemiologic studies, however, no study has specifically evaluated urine concentration and risk of bladder cancer. The regulation of USG by SLC14A1, and other potential mechanisms, needs further evaluation in future experimental studies.

NCI-DCEG
DellaValle, Curt
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Epidemiology/Biostatistics - Prognosis and Response Predictions

Risk of colorectal cancer associated with dietary nitrate and nitrite intake may be reduced by vitamin C: a prospective investigation in the Shanghai Womenâ€™s Health Study

Dietary intake of nitrate and nitrite can lead to the endogenous formation of N-nitroso compounds (NOCs), which are known animal carcinogens and potential human carcinogens. Associations between consumption of processed meats, which contain nitrate and nitrite as well as other necessary precursors for NOC formation, and increased risk of colorectal cancer have been observed in Western populations. However, the nitrosation reactions that form NOCs in the body can be inhibited in the presence of vitamin C and other antioxidants. We prospectively investigated the association between dietary nitrate and nitrite intake and risk of colon, rectum, and colorectal cancer combined in the Shanghai Womenâ€™s Health Study. We also evaluated potential effect modification by factors that affect the endogenous formation of NOCs; vitamin C (at or above/below median) and red meat intake (at or above/below median). Nitrate, nitrite and other dietary intakes were estimated from a 77-item food frequency questionnaire administered at baseline. Over a mean of 11 years of follow-up, we identified 619 colorectal cancer cases (n=383, colon; n=236, rectum) among 73,118 women. Cox proportional hazard regression was used to estimate hazard ratios (HR) and 95% confidence intervals (CI) for the association between colon, rectum and colorectal cancers combined and quintiles of nitrate and nitrite intake. Overall nitrate intake was not associated with colorectal cancer risk (HR = 1.08; 95% CI: 0.73-1.59). However, among women with vitamin C intake below the median (83.9 mg/day), risk of colorectal cancer was increased in the highest quintile of nitrate intake compared to the lowest quintile (HR = 2.45; 95% CI: 1.15-5.18; p-trend = 0.02). We found no association between nitrite intake and risk of colorectal cancer. This was the first study to evaluate dietary nitrate and nitrite intake and risk of colorectal cancer in an Asian population, which has much higher dietary nitrate intakes than those typically observed in Western populations. Our findings suggest that high levels of nitrate intake among subgroups expected to have high exposure to endogenous N-nitroso compounds, specifically women with low vitamin C intake, are associated with an increased risk of colorectal cancer. Incorporating vitamin C and antioxidant rich foods into the diet may reduce this risk.

NCI-DCEG
Gu, Fangyi
Visiting Fellow
Epidemiology/Biostatistics - Prognosis and Response Predictions

Time to first cigarette (TTFC) after morning wake-up and lung cancer risk

Lung cancer risk varies widely among smokers, making it challenging to select the best target population for lung cancer screening due to imprecise risk prediction. Different degrees of nicotine dependency impact smoking behavior and tobacco metabolism, therefore influencing exposure to biologically effective doses of tobacco carcinogens. Time to first cigarettes (TTFC) after wake-up (minutes: =5, 6-30, 31-60, >60), a phenotype capturing nicotine dependence, is a strong predictor of plasma cotinine (the major nicotine metabolite) and was associated with lung cancer risk in a hospital-based case control study. However none of the few studies that collected TTFC information has followed-up on this finding. To verify and extend this finding, we examined TTFC in relation to lung cancer in a unique population-based case control study designed to capture smoking behavior factors. We included 1437 cases and 1812 controls who were ever-smokers. First, we examined the association between TTFC and lung cancer with logistic regression, adjusting for cigarettes per day (cig/day), packyears, smoke duration, current/former, years since quit, age at smoke initiation and other lung cancer risk factors. Second, we calculated population absolute lung cancer rates for joint categories of TTFC and other smoking phenotypes by a novel blm R package, using sampling fractions to recover the underlying cohort. Compared to smokers with TTFC>60 (lowest dependency), the lung cancer odds ratio and 95% confidence intervals were 2.57(2.03-3.26), 2.27(1.79-2.88), and 3.5(2.64-4.64) for smokers with TTFC 31-

60, 6-30 and =5, respectively (p-trend<0.0001). We also confirmed the association in an independent cohort. Joint effects of TTFC and cig/day showed the lung cancer rates/100,000 person years (PY) for those smoking 1-10, 11-20, 21-30, >30 cig/day were 15.5, 36.1, 43.8, 109.1, respectively among lowest dependency smokers (TTFC>60); while the corresponding rates were 140.9, 157.1, 207.3, 281.8 per 100,000 PY among smokers with TTFC=60. The increase in lung cancer rates by duration and packyears was greater among those with shorter TTFC (higher dependency). In conclusion, our results suggested shorter TTFC is an independent lung cancer risk factor, and TTFC explains at least part of the heterogeneity of lung cancer risk among smokers. We are planning to next examine the impact of adding TTFC to lung cancer prediction models in order to improve risk stratification.

NCI-DCEG
Guertin, Kristin
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Epidemiology/Biostatistics - Prognosis and Response Predictions
The Human Metabolome: Mechanistic Insights into Diet-Cancer Associations
Dietary factors are associated with many health outcomes, including cancer; however, assessment of usual diet as captured by self-report is subject to measurement error. Metabolomics may improve upon self-reported dietary data, which do not reflect differences at the molecular level. Additionally, metabolomics may offer a substitute measure of exposure when self-report is unavailable. Furthermore, metabolites are intermediate measures in the causal pathway between diet and cancer and may provide information about the mechanisms of such associations, some of which may not have been previously considered. For example, in a recent analysis the association with reduced colorectal cancer was stronger for coffee metabolites than for self-reported coffee intake. We investigated partial Pearson correlations between self-reported diet and ~500 serum metabolites, measured by HPLC and GC-MS, in 254 adults in the Prostate, Lung, Colorectal, and Ovarian (PLCO) cancer screening trial, a large U.S. cohort. After Bonferroni correction and adjustment for age, gender, smoking, and energy intake, >43 metabolites were correlated with food items (p<6.0 x 10 superscript -7), including citrus fruits, seafood, peanuts, coffee, alcohol, calcium, or multivitamins. Serum proline betaine, a marker of citrus consumption previously identified by human feeding studies, was correlated with citrus intake (r=0.39). As expected, multivitamins were associated with higher serum a-tocopherol (r=0.37) and lower ?-tocopherol (r=-0.31). 3-Carboxy-4-methyl-5-propyl-2-furan propionic acid (CMPF), a uremic toxin which induces oxidative stress in vitro and impedes drug metabolism, was associated with fish intake (r=0.32); CMPF is of unknown origin but has been detected in fish. A further 17 unknown metabolites were associated with diet (r=0.23-0.38); identifying these metabolites may provide insight into downstream effects and diet-disease associations. Metabolomics is a useful technology to identify novel dietary biomarkers that may be more accurate measures of exposure given variability in metabolism. We demonstrated that dietary metabolites are detectable in human serum and identified metabolites of potential epidemiologic use; future studies should investigate correlations reported herein, associations of these metabolites with disease, and determine whether dietary and metabolite data can be combined to better characterize associations with disease.

NCI-DCEG
Mondul, Alison
Research Fellow
Epidemiology/Biostatistics - Prognosis and Response Predictions
Genetic Variation in the Vitamin D Pathway in Relation to Risk of Prostate Cancer â€“ Results from The Breast and Prostate Cancer Cohort Consortium (BPC3)

Background: There is experimental evidence that vitamin D compounds promote prostate cell differentiation and inhibit proliferation and invasion. In contrast to this basic research, a meta-analysis of epidemiologic studies concluded there was no evidence that higher vitamin D status assessed by circulating 25-hydroxyvitamin D (25(OH)D) levels is associated with a reduced risk of prostate cancer. Furthermore, men with higher circulating 25(OH)D were recently reported to have a statistically significantly elevated prostate cancer risk in a nested case-control analysis of 1,000 cases and 1,000 controls. Two recent genome-wide association studies have identified genetic variants in or near four genes that predict circulating 25(OH)D levels. In order to further elucidate the vitamin D-prostate cancer association, we examined risk in relation to SNPs in these four genes in a large pooled analysis within the NCI Breast and Prostate Cancer Cohort Consortium (BPC3). Methods: SNP markers localized to each of four genes (GC, CYP24A1, CYP2R1, and DHCR7) previously associated with 25(OH)D were genotyped in 10,018 cases and 11,052 controls from the BPC3. Logistic regression models were used to estimate the individual and cumulative association between genetic variants and risk of overall, aggressive, and non-aggressive prostate cancer. Results: We observed a decreased risk of aggressive prostate cancer among men with the allele in rs6013897 near CYP24A1 associated with lower serum 25(OH)D (per A allele, OR=0.86, 95%CI=0.80â€“0.93, p-trend=0.0002), but an increased risk for non-aggressive disease (per A allele: OR=1.10, 95%CI=1.04â€“1.17, p-trend=0.002). Examination of a polygenic score of the four SNPs revealed a statistically significantly lower risk of aggressive prostate cancer among men with a greater number of low vitamin D alleles (OR for 6-8 vs. 0-1 alleles = 0.66, 95% CI = 0.44 â€“ 0.98; p-trend=0.003). Conclusions: In this large, pooled analysis of men of European ancestry, we found that genetic variants near CYP24A1 related to lower vitamin D status could be associated with a decreased risk of aggressive prostate cancer, and a polygenic vitamin D score was similarly related to both overall and aggressive prostate cancer. Our findings do not support a protective association between higher vitamin D status and lower risk of prostate cancer, and point to the possibility of a positive association.

NCI-DCEG
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Visiting Fellow
Epidemiology/Biostatistics - Prognosis and Response Predictions
Detection for non-additive effects of SNPs at extremes of disease-risks
Background: Genome-wide association studies (GWAS) have now led to the discoveries of thousands of susceptibility SNPs across many complex diseases. A crucial next step for post-GWAS investigation of disease etiology is to characterize risk associated with multiple SNPs simultaneously. The starting point for such investigation is often to assume SNPs affect disease risk in an additive fashion under some chosen scale and test the adequacy of such model based on goodness-of-fit statistics. All of these statistical methods for this purpose, including the popular Hosmer-Lemeshow (HL) statistic, face the common limitation that they may not be very sensitive at the extremes of risk distributions where the departure of joint risk from the underlying additive models may be actually more prominent in practice. Methods: We develop a new method for testing adequacy of an underlying assumed model for the joint risk of a disease associated with multiple risk factors. To make the test more sensitive for detection of departures near the tails of risk distributions, we propose forming a test statistic based on squared Pearson residuals summed over only those individuals who achieve certain high- or/and low risk-threshold and then maximizing such test statistics over different risk-thresholds. We derive the asymptotic distribution for the test statistic and use such theory for evaluating p-values. Results: Through extensive simulations, we show the proposed procedure is valid and much more powerful than HL test and other goodness-of-fit tests under realistic models for describing joint effects of SNP markers emerging from GWAS. We apply the proposed method to a large study of breast cancer involving 4168 cases and 4930 controls from Breast and Prostate Cancer Cohort Consortium to explore the adequacy of

some standard and non-standard models for describing polygenic risk associated with 19 SNPs that have been previously shown to be associated with the disease. Our proposed test concludes there is not much evidence for departure from multiplicative model but strong evidence for departure from additive model. Conclusions: The proposed method is promising for testing adequacy of commonly used models for joint effects of SNPs. Significance: As subjects with extreme high- or low- risk may be impacted most from knowledge of their risk estimates, checking adequacy of risk models at the extremes of risk is very important for clinical applications of such models.

NCI-DCEG
Choi, Jiyeon
Postdoctoral Fellow
Genomics
Functional characterization of melanoma-associated common variants in PARP1
Recent genome wide association studies (GWAS) identified several new loci for melanoma susceptibility. While these results highlight potential pathways predisposing to melanoma, the functional risk variants in these regions, as well as the mechanism 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 potential functional variants we assessed whether SNPs in melanoma loci affect flanking gene levels by expression quantitative trait loci (eQTL) analysis. Affymetrix U133Plus2 expression microarray measured transcript levels in 62 melanoma cell lines. SNPs (>700K) were then typed on Illumina OmniExpress arrays and the ones in GWAS loci were further imputed using 1000 genomes data. Among 16 loci only one encompassing Poly [ADP-ribose] polymerase 1 (PARP1) exhibited significant eQTL, which was validated by meta-analysis adding an independent set of 50 melanoma cell lines (p=0.008) as well as Taqman quantitative PCR (qPCR). Namely, the risk allele is correlated with an increased PARP1 transcript levels after adjusting for genomic copy number (p=0.03). We then further interrogated the genotype-expression correlation by Taqman allele discrimination qPCR in 21 melanoma cell lines heterozygous for the GWAS lead SNP out of initial 62. 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 43 SNPs of strong LD with the GWAS lead SNP (r2>0.8), five exhibited strong evidence as potential transcriptional enhancers in melanoma relevant cell types. Subsequent Electro Mobility Shift Assays indicated allele-specific protein binding for at least two SNPs. Among them, rs208426 displayed preferential protein binding for the protective allele, which is consistent with allelic motif change prediction for Forkhead box proteins. Protein identification using antibody super-shift and mass-spectrometry as well as luciferase reporter assays will provide further evidence for the functionality of this SNP. Our data suggest that increased PARP1 levels are correlated with melanoma risk. Further analyses will better elucidate PARP1 function in melanoma susceptibility.

NEI
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Postdoctoral Fellow
Cell Biology - General
Macf1 is required for microtubule and actin interactions during ciliated sensory cell maturation and function.
Microtubule-actin cross linking factor 1 (Macf1) is one of two vertebrate spectraplakins that associate with F-actin and microtubules, thus integrating cytoskeletal networks. Linkage between the two cytoskeletons has a major role in intracellular trafficking and migration. As Macf1 is required to remove components from actin-rich focal adhesions a defect in Macf1 impedes cell migration. Macf1 was also

identified as the third most abundant protein in the mouse photoreceptor connecting cilium, and therefore may underlie certain forms of retinal degeneration and ciliopathies. To further characterize Macf1 function, we generated conditional knock out mice, in which Macf1 had been deleted in developing sensory tissues (retina and cochlea) and Macf1 antibodies against its actin-binding domain. We found that Macf1 localized to apical junctions of the developing neural retina. These structures require microtubule-actin interactions for their dynamic assembly and disassembly, and are critical for the maturation of late-born neurons including photoreceptors and bipolar cells. In developing cochlea sensory neurons, Macf1 is highly enriched at the base of the kinocilium and decorated the ciliary axoneme. Loss of Macf1 in the developing retina results in a complete loss of visual function due to severely disrupted retinal lamination affecting primarily photoreceptors and bipolar cells. Development is disrupted in mutant cochlea sensory neurons, which display apical-basal polarity and stereociliary bundle defects. Disturbed microtubule arrangements are observed in both these systems, likely due to their decoupling from actin networks. These findings suggest a critical role for Macf1 in developing sensory neurons, and highlight possible novel functions for microtubule and actin interactions in ciliary related processes. These insights provide the cellular basis for clinical phenotypes associated with ciliopathies. We are currently investigating whether Macf1 also plays a separate role in mature photoreceptors at the point of actin-microtubule network connection for proteins en route to the outer segments via the connecting cilia.

NEI
Yang, Hyun-Jin
Visiting Fellow
Epigenetics

Epigenetic Regulation of Photoreceptor-Specific Gene Expression

Rod and cone photoreceptors in the retina are light-detecting neurons specialized for night and day vision, respectively. Retinal degenerative diseases resulting from dysfunction or loss of rod photoreceptors are the most prevalent cause of blindness. Given the multifactorial nature of rod degeneration, understanding of rod-specific gene regulatory networks (GRNs) would greatly facilitate the development of therapies for retinal degenerative diseases. Recent progress in photoreceptor development has identified a combinatorial regulatory program mediated by key transcription factors that control development and maintenance of rods. However, an epigenetic contribution to rod-specific GRNs has not been elucidated. To investigate epigenetic regulation of rod gene expression, we performed genome-wide profiling of histone modifications using purified rods from distinct stages of retinal development. Developing and mature rod photoreceptors were isolated by flow-sorting from Nrl-GFP transgenic mice, in which GFP is expressed only in rods under the control of the promoter of Nrl that determines rod cell fate. As the number of flow-sorted rods is limited for a standard ChIP-seq assay, we have established a new ChIP-seq protocol for small numbers cells (as few as 10,000). We show that H3K4me3, an active histone mark, is acquired late during rod development, in contrast to general photoreceptor genes. This is consistent with the late onset of expression of rod-specific phototransduction genes in developing rods. We also demonstrate that genes specific to S cones, the proposed â€œdefaultâ€ subtype of photoreceptors, are initially free of but later become enriched with H3K27me3, a repressive histone mark, in mature rods, consistent with the hypothesis that the fate of immature rods is plastic until fixed irreversibly at later stages. Notably, rod-specific transcriptional activators (such as Nrl, Crx and Nr2e3) are present in newborn rods yet the phototransduction genes are not expressed. Our results therefore suggest that changes in epigenetic state are critical for rod maturation. To decipher the trigger of change in histone modification profiles, we are investigating the interplay between transcription factors and epigenetic marks by comparing histone profiles of rods in

the presence or absence of Nrl. Our study provides novel and direct evidence of epigenetic control of neuronal differentiation in mammalian retina.

NEI
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Postdoctoral Fellow
Immunology - General
Commensal microbiota trigger autoimmune inflammation of the retina in a spontaneous model of uveitis
Autoimmune uveitis (AU) has an incidence similar to multiple sclerosis and is a major cause of blindness. Animal models implicated a T cell mediated process, but in contrast to spontaneous development of the disease in humans, disease in animals must be induced by immunization with retinal antigen in adjuvant. In view of anecdotal evidence linking AU to systemic infections as well as accumulating data on importance of commensal microflora as a regulator of the host immune system, we hypothesized that AU could be triggered by a stimulus from endogenous microbiota. We tested this hypothesis in a new mouse model of AU expressing a transgenic (Tg) T cell receptor (TCR) specific to peptide 161-180 of the retinal protein IRBP (R161H). The TCR-Tg T cells are detectable with a specific peptide-MHC-class II dimer reagent. R161H mice develop spontaneous uveitis that starts at 3-4 weeks of age and peaks around 9 weeks. R161H mice treated with an antibiotic cocktail had drastically reduced intestinal flora in terms of biomass and species complexity and experienced a significant delay in onset and reduction in intensity of disease compared to untreated controls. The same was true of R161H mice reared under germ-free conditions, indicating an involvement of the endogenous microbiome in induction of retinal autoimmunity. In untreated mice, analysis of gut lamina propria (LP) lymphocytes showed increased IL-17-producing cells in R161H mice compared to wild-type littermates, with a high proportion of the cells expressing the IRBP-specific TCR. Notably, R161H mice that were made IRBP deficient still demonstrated an increase in Th17 cells in the LP, suggesting that activation of R161H cells occurred independently of the cognate antigen IRBP. To examine whether the activation involved a TCR-mediated signal, we crossed R161H with the TCR signaling reporter Nur77-GFP mice. Presence of GFP-bright 161-180 TCR specific cells in the intestinal LP of R161H-Nur77-GFP mice was consistent with the interpretation that IRBP-specific cells receive a signal through their TCR in the gut. Taken together, these results suggest that gut microbiota can provide an activation signal for autoreactive T cells. These data may have major mechanistic implications not only for uveitis, but also for other autoimmune diseases.

NHGRI
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Postdoctoral Fellow
Genetics
Mapping Genetic Contributors to Canine Hind Limb Conformation
Domestic dogs have been selectively bred to perform a variety of tasks, from herding to hunting to hauling. This has lead to limbs that are specialized for endurance, agility, speed, or strength. Physical variation in the hind leg is seen in the stifle (knee), pelvic, femoral, and hock (ankle) angles, as well as the relative lengths of the bones that comprise the main limb components. We seek to identify genes that impact the overall structure of the hind limb in dogs, and draw connections between physical shape, genetic markers, and contribution to the intended working or sporting function of the dog. A preliminary genomewide association study (GWAS) was conducted on 148 dogs of 13 breeds visually determined to have straight legs and 185 dogs of 16 breeds visually determined to have angled legs. Significance was found for a SNP on chromosome 9, near the T-Box Transcription Factor 4 (TBX4) gene; a gene that is responsible for hind limb bud initiation and soft tissue patterning in mice, and Small Patella Syndrome in humans. Sequencing of select coding and regulatory regions of TBX4 identified five

polymorphisms: four regulatory SNPs and one non-synonymous SNP in exon 8. Subsequently, breed average stifle angles were calculated for 47 dog breeds utilizing professional award portraits of 535 dogs competing in conformation events. The TBX4 SNPs were genotyped in 71 dogs of 13 of these breeds and significant association of breed average stifle angle was found with two of the regulatory region SNPs. To account for breed-specific stance variation present in the award portraits, we developed a standardized method to pose dogs in a balanced neutral position. This allows for the collection of 8 key hind limb measurements that can then be compared within and across breeds. Principle component analysis (PCA) was performed using these measurements from 68 dogs of 26 breeds. PC1 accounts for 33.9% of the variation in hind limb conformation, which comprises the matching changes in the angle of the stifle and hock. PC2 comes from the length of the hock and tibia relative to the femur length, and accounts for 28.3% of the variation. In order to better understand the impact of TBX4 on hind limb conformation, the dogs included in the PCA will be genotyped for the five TBX4 SNPs. In addition, a second GWAS will be completed on dogs with standardized measurements to identify additional genes involved in canine hind limb structure.

NHGRI
Rimbault, Maud
Postdoctoral Fellow
Genetics
Polymorphisms at Six Genes Determine Eighty-Five Percent of Size Variation in Small and Medium-Sized Dogs
Selective breeding of dogs by humans has generated extraordinary phenotypic diversity. The diversity in canine body size is illustrated by breeds such as Mastiffs, which can be 50 times heavier than Chihuahuas, and Great Danes, which can be five times taller than Pekingese. Codified breed standards and closed breeding populations have resulted in a simplified genetic architecture that is particularly amenable to multi-breed genome wide association studies. Several studies have identified regions of the genome associated with canine body size, but the intervals were too large to assign causative roles to genes. We analyzed four loci discovered using the CanMap data set, in which 60,968 single-nucleotide polymorphisms were genotyped on DNA from 915 dogs representing 80 domestic breeds. We performed fine mapping to define critical intervals that included the candidate genes GHR, HMGA2, SMAD2 and STC2, identifying five highly associated markers at the four loci. These markers, together with previously reported variants in the IGF1 and IGF1R genes, were genotyped on a panel of 500 domestic dogs from 93 breeds. Ancestral and derived alleles for all markers were defined by additional genotyping on a panel of gray wolves, the species from which dogs originated. We observed that the derived alleles at all markers correlated with reduced body size and smaller dogs are more likely to carry derived alleles at multiple markers. However, breeds are not typically fixed at all markers; multiple combinations of genotypes are found within most breeds. Finally, we show that 85% of the variance in body size of dogs < 90 lbs can be explained by these six genes. For dogs = 90 lbs, the six genes account for only 5% of size variation, suggesting that additional genes contributing to gigantism remain to be found. The determination of body size in dogs contrasts starkly with studies of human height, which paint a more complicated picture. Human height appears to be governed by hundreds of small effect loci, which taken together account for only ~10% of the adult human height variation. Our work describes the genetic basis of canine size determination in small and medium-sized dogs, advancing our understanding of mammalian growth.

NHGRI
Parker, Stephen
Postdoctoral Fellow

Genomics

Integrative genomic analysis of RNA and ChIP-seq data across multiple cell types identifies â€œstretch enhancersâ€ associated with type 2 diabetes

Type 2 diabetes (T2D) affects 26 million Americans and costs our health care system an estimated $174 billion annually. Genome-wide association studies (GWAS) designed to identify genetic components of T2D have provided evidence for at least 64 associated loci that are predominantly in non-coding regions. To characterize regulatory architecture in a T2D-relevant tissue, we performed ChIP-seq in human pancreatic islet samples on a set of histone modifications. Using a high-performance computer cluster and hidden Markov model approach to integrate ChIP-seq data, we produced a reference chromatin state map for islets, which includes promoter, enhancer, insulator, heterochromatic, and actively transcribed regions. To facilitate integrative chromatin and gene expression analyses, we performed RNA-seq on four islet samples that were used for chromatin studies. Combining similar ChIP-seq and RNA-seq data from the ENCODE and Roadmap Epigenomics Projects allowed us to produce a consistent set of chromatin and expression maps for an additional nine cell types. We find that GWAS loci are specifically enriched in enhancer states in trait-relevant cell types, including novel findings for islet related traits. Size stratification of enhancers identified a previously unrecognized feature: large (>3 Kb) enhancer domains that are cell-type specific and significantly enriched for high tissue-specific gene expression in their local neighborhood. These include INS, KCNJ11/ABCC8, and VPS13C/C2CD4A/B in islets, and other locus control regions (LCRs) in relevant cell types, including the beta globin LCR in the myeloid cell line K562. We have named these â€œstretch enhancersâ€, and we suggest this may be a generalizable chromatin feature of loci that play a prominent role in tissue-specific biological functions. Using transcriptional reporter experiments, we demonstrate that human islet stretch enhancers are active in a cell-type specific manner, including specific activity in the pancreatic lobes during E11.5 mouse development. Correlation of stretch enhancer activity with gene expression and transcription factor binding site motif enrichment signatures allows for the assignment of enhancer-gene links and enhancer regulators, respectively, some of which overlap T2D GWAS loci. This combined genomic, epigenomic, transcriptional, and transgenic analysis reveals several genes regulated by stretch enhancers in an islet-specific manner, and suggests pathways relevant to T2D etiology.

NHGRI

Sen, Shurjo

Visiting Fellow

Genomics

TREML4 is a candidate gene for coronary artery calcification

Background Coronary artery calcification (CAC) is a heritable marker of cardiovascular disease that has good predictive value for future adverse cardiac events. CAC-associated genes have previously been identified using genome-wide association studies; here, using a new approach for disease gene discovery that combines transcriptome, exome and protein analysis, we describe results implicating the human TREML4 gene in CAC. Results Using RNA-Seq, we first compared the peripheral blood transcriptomes of eight advanced CAC subjects and eight matched controls from the ClinSeq project. A group of 302 genes that were differentially expressed in this pilot study at a liberal p-value cutoff (p less than 0.05) were validated within the ClinSeq cohort using NanoString technology on 96 CAC subjects (48 case-control pairs). Ten of the 302 genes that validated in this larger set were validated externally in peripheral blood Affymetrix expression array data from 2461 CAC subjects enrolled in the Framingham Heart Study. The TREML4 (Triggering Receptor Expressed on Myeloid Cells - Like 4) gene was significantly upregulated in high-calcification subjects (p less than 0.05) in all three cohorts. Next, we used exome sequencing data from 1000 ClinSeq subjects to identify rs2803496, a C/T polymorphism that shows strong eQTL behavior for TREML4. In addition to its control over gene expression, analysis of

rs2803496 genotypes and CAC scores also revealed a steady increase in relative CAC risk in individuals having the C allele, with a maximum of 6.5 fold increased risk at a CAC score of 1150 (95% confidence intervals 1.9-21.7). Finally, at the protein level, we found TREML4 to be present in human atherosclerotic lesions by immunohistochemistry. Strong staining was observed in luminal endothelial cells of the tunica intima and vasa vasorum, as well as in perinecrotic core foamy macrophages in the tunica media, confirming that the protein is located is disease-relevant regions of the vascular wall. Conclusions RNA, DNA as well as protein evidence suggests that TREML4 is a new candidate gene for CAC. We suggest that the paradigm that led us to this gene may be a post-GWAS methodology for finding candidate genes in common diseases like CAC.

NHGRI
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Postdoctoral Fellow
Immunology - General
Loss-of-function mutations in CECR1, encoding adenosine deaminase 2 (ADA2), cause early-onset strokes with fever
In 4 unrelated patients we observed an unusual syndrome characterized by early-onset fevers, skin rashes, and recurrent strokes, leading to significant neurological impairment. To identify the cause of this disorder with a potentially common genetic abnormality, we performed exome sequencing using affected patients and their unaffected parents. Exome data were filtered for novel or rare variants (q<0.01) in dbSNP and other public and in-house databases. We identified 4 recessively inherited missense mutations in CECR1, a gene encoding adenosine deaminase 2 (ADA2) in 3 cases, while the fourth patient was a carrier for a single missense mutation he inherited from his father. Further analysis of CECR1 showed the presence of a 28-kb genomic deletion encompassing the 5â€™UTR of the gene in this patient and his mother. Two ADA2 disease-associated mutations are novel while 3 mutations are found at a very low frequency (=0.001) in the general population, consistent with the recessive inheritance. Two of 5 missense mutations are shared among 3 patients. Computer modeling based on the crystal structure of human ADA2 protein indicated that CECR1 mutations might either disrupt protein stability or impair ADA2 enzyme activity. Western blots for ADA2 showed a decrease in protein expression in supernatants of cultured PBMCs from patients. All patients had at least 10-fold diminished serum and plasma concentrations of active and total ADA2 protein, and reduced ADA2 activity. These data indicate that CECR1 mutations are likely loss-of-function mutations that prevent ADA2 secretion into the extracellular space, which is the main functional compartment for the protein. Toxic metabolites that accumulate in cells as a result of ADA1 deficiency were absent in RBC of patients with ADA2 deficiency, indicating that stroke and inflammation are not caused by the toxicity of unprocessed nucleotides. Animal models suggest that ADA2 is important for growth and development. In order to explore other unknown functions of ADA2 we developed a zebrafish model in which the expression of CECR1b, the gene most homologous to human CECR1, was knocked down by morpholinos (MO). MO-injected embryos presented with intracranial hemorrhages. Although the precise molecular mechanism of this potentially devastating disease is not clear, our data suggest that the causative loss-of-function ADA2 mutations may affect vascular development and the regulation of inflammation.

NHGRI
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Postdoctoral Fellow
Signal Transduction - General
Wnt5a and Wnt11 cooperate to regulate body axis elongation during embryogenesis

Cell signaling plays essential and pivotal roles in controlling embryonic morphogenesis of multicellular organisms. The planar cell polarity (PCP) pathway is an evolutionarily conserved signaling pathways required in multiple morphogenetic processes. In vertebrates, PCP has emerged as a fundamental regulatory mechanism controlling cell migration and other critical developmental processes. Mutations in PCP components cause many developmental abnormalities including shortening of the anterior posterior (A/P) axis and neural tube closure defects (NTD), the second most common birth defect in humans. However, surprisingly little is known about the molecular mechanisms that regulate PCP. In zebrafish and Xenopus embryos the absence of the ligand Wnt11 or Wnt5a leads to A/P axis defects and NTD. We have identified, that Wnt5a is a required Wnt ligand in controlling PCP in mice, as its loss results in a shortening of the limbs and the A/P axis. By contrast, the loss of Wnt11 in mice does not cause obvious PCP defects. To investigate how Wnt signaling regulates PCP and whether Wnt5a and Wnt11 play redundant roles in mammalian PCP signaling we generated mouse embryos lacking both Wnt11 and Wnt5a. We found that Wnt5a and Wnt11 double knockout (DKO) embryos displayed a severe shortening of the A/P axis and were posteriorly truncated. Interestingly, we observed an ectopic accumulation of tissue adjacent to the embryonic organizer. As the organizer is crucial for the elongation of the A/P axis, we hypothesized that the ectopic tissue accumulation caused the severe A/P axis shortening. Through gene expression analysis we showed that the ectopic cells express similar genes compared to the organizer. To further analyze the origin of the ectopic cells, we performed fate-mapping experiments by labeling cells adjacent to the organizer, prior to the appearance of the ectopic cells. In the control the labeled cells migrated anteriorly, whereas in the DKO the labeled cells moved less and in random directions. Therefore, axis elongation was impaired. Molecularly, we observed an increase of the cell adhesion molecules ß-catenin and E-cadherin in the DKO in the ectopic cells, indicating increased cell adhesion in the absence of Wnt5a and Wnt11. Thus, epithelium to mesenchyme transition (EMT), which is required for the mobility of the cells, was impaired. Our results show that Wnt5a and Wnt11 cooperate during axis formation by regulating EMT and directional migration.

NHLBI
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Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology
Hook1 Directly Sorts Clathrin-independent Cargo Proteins on Endosomes
Clathrin-independent endocytosis (CIE) mediates the internalization of many plasma membrane (PM) proteins involved in homeostasis, immune response, and signaling. Typical CIE cargo molecules, such as the major histocompatibility complex Class I protein (MHCI) and the interleukin 2 receptor a-subunit (Tac), are internalized independent of clathrin, and dynamin, and modulated by the small GTPase Arf6. The incoming CIE cargo-loaded vesicles fuse with sorting endosomes labeled with Rab5 and early endosome antigen 1 (EEA1). From here, the cargo either traffics to lysosomes for degradation or recycles back to the PM via recycling tubular endosomes. The recycling of CIE membranes is important for cell spreading, wound healing and metastasis. New CIE cargo proteins, including CD44, CD98 and CD147, which are involved in cell-extracellular interactions, follow a trafficking itinerary different from the trafficking of MHCI/Tac. Once internalized, these proteins are rapidly sorted and routed directly to the recycling tubules, avoiding EEA1-endosomes and escaping degradation in lysosomes. This results in a prolonged surface lifetime for these proteins. This divergence from the typical itinerary suggests that endosomal sorting mechanisms determine the fate of CIE cargo. Therefore, we sought to identify sorting determinants in CD147. We examined the trafficking of chimeric proteins combining sequences of Tac with those of CD147 using antibody internalization assays and immunofluorescence. We found that the cytoplasmic sequence of CD147 harbors sorting information that determines its fate. Mutagenesis analysis of this sequence identified two highly conserved acidic clusters as critical for preventing the

trafficking to EEA1- and late-endosomes. Furthermore, using a yeast two-hybrid screen, we found that Hook1, a microtubule and cargo tethering protein, recognizes the cytoplasmic tail of CD147 to help sort it into recycling tubules. Depletion of Hook1 from cells altered trafficking of CD44, CD98 and CD147 towards EEA1 compartments, and impaired their recycling back to the PM. By contrast, MHCI, which normally traffics to EEA1 compartments, was not affected. Loss of Hook1 also led to an inhibition of cell spreading, implicating a role for Hook1 sorting of specific CIE cargos away from degradation and back to the PM. This work demonstrates that endosomal-sorting signals determine the fate of CIE cargo and uncovers the first endosomal-sorting factor for CIE cargo proteins.

NHLBI
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Clinical and Translational Research
Path to the clinic: Assessment of iPSC-based cell therapies in vivo in a non-human primate model
Current efforts investigating the efficacy and risk of human induced pluripotent stem cell (iPSC)-technologies for potential clinical applications rely predominantly on in vitro- and murine xenograft models. Although these experiments provide important proof of principle information regarding pluripotency, their predictive value, in particular for questions related to tumorigenicity, immunogenicity, and engraftment, is limited. Therefore, we developed a transplantation model in non-human primates, allowing us to examine in vivo behavior of autologous iPSCs and their differentiated derivatives in the presence of an intact immune system. Transgene-free rhesus macaque iPSCs (RhiPSCs) were derived using the Cre-excisable STEMCCA vector, leaving a DNA tag for tracking. As an alternative to Matrigel, a xenogenic scaffold rejected by an intact immune system, an autologous plasma clot teratoma scaffold was developed. Cells from three independent RhiPSC clones were subcutaneously injected in plasma clots into both the donor autologous macaque and immunodeficient NSG mice to assess teratoma formation. RhiPSCs differentiated in vitro to mesodermal stromal-like cells (RhiPS-MSCs) were mixed with ceramic particles and subcutaneously implanted into the same animal to assess bone formation. All undifferentiated RhiPSCs injected into NSG mice rapidly formed teratomas. The same cells in the autologous macaques formed masses more slowly and only at high cell doses. Individual injection sites were serially explanted. Even though immature germ layer structures were found at as early as 2 weeks, there was a significant lymphocytic and eosinophilic infiltration in the tumor and the surrounding tissue. At 10 weeks, for the first time, mature teratoma structures including neural, mesenchymal, and glandular components were seen. The simultaneously implanted RhiPS-MSCs formed unequivocal bone tissue organoids in the macaque, without evidence of teratoma formation or inflammatory infiltrates. This is the first autologous large animal model for teratoma formation and iPSC-derived tissue regeneration. These results also suggest that immunogenicity of iPSCs may differ depending on their differentiation status or in vitro culture system. We believe this study and our model will open the door for any clinicians or scientists who investigate the feasibility and safety of iPSC-based therapy.

NHLBI
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Clinical Fellow
Clinical and Translational Research
Increased Transpulmonary Gradient in Patients with Sickle Cell Associated Pulmonary Hypertension Predicts Poor Prognosis and is Associated with RV Dysfunction by MRI
Background: Assessment of pulmonary hypertension (PH) in sickle cell disease (SCD) is often complicated by high cardiac output (CO) related to anemia. The transpulmonary gradient (TPG) reflects

a pressure differential across the pulmonary vascular bed that the right ventricle (RV) must overcome. According to the cardiac transplant literature, a TPG greater than or equal to 12 mmHg indicates significant pulmonary vascular disease. With significant PH, there is typically morphologic adaptation by the right ventricle (RV). We hypothesize that patients with SCD and a TPG greater than or equal to 12 mmHg have poor functional and mortality prognosis and evidence of RV dysfunction on cardiac MRI (CMR). Materials & Methods: Five hundred and twenty nine consecutive patients (age 35.5 +/- 12.5, 54% (n=283) female, 73% (n=387) HbSS) with SCD were prospectively screened for PH using echocardiography (tricuspid regurgitant jet greater than or equal to 2.5 m/s). Eighty four (age 41 +/- 13, 55% (n=46) female, 82% (n=69) HbSS)) had right heart catheterization (RHC) and 41 (age 42 +/- 15, 54% (n=22) female, 80% (n=33) HbSS) had CMR within one week of RHC. Results: Those with a TPG greater than or equal to 12 mmHg had higher mortality (p=0.01), poorer functional class (p=0.007), shorter 6-minute walk distance (exercise capacity) (p=0.003), and lower cardiac index (p=0.01). High TPG patients have abnormal CMR markers of RV dysfunction (p=0.01) and morphologic adaptation (p=0.01). Left ventricular (LV) eccentricity index (EI) at end-systole (ES) was greater in the high TPG group (p=0.026) without a significant change between ES and end-diastole (ED), which is consistent with RV pressure overload. In the low TPG group, the EI-ED was greater than EI-ES (p=0.001), which is consistent with RV volume overload. LV ejection fraction was similar in both groups. Conclusions: A TPG greater than or equal to 12 mmHg effectively identified patients with MRI evidence of RV dysfunction and overall worse prognosis. The TPG demonstrates the functional severity of pulmonary vascular disease in SCD using objective thresholds established in the cardiac transplant literature. Further, using a TPG greater than or equal to 12 mmHg enables identification of a clinically high risk population with sickle cell disease.

NHLBI
Hu, Gangqing
Visiting Fellow
Epigenetics

H2A.Z facilitates access of active and repressive complexes to chromatin in embryonic stem cell self-renewal and differentiation

The histone variant H2A.Z is conserved from yeast to humans and is implicated in multiple nuclear processes. H2A.Z is localized to promoters of active genes in various systems, suggesting a positive role in gene regulation. However, conflicting results on the genomic localization of H2A.Z have been reported in mouse ES cells. To clarify the function of H2A.Z in ES cell self-renewal and differentiation, we performed the following experiments in mouse ES (mES) cells: 1) ChIP-Seq of H2A.Z and acetylated H2A.Z; 2) ChIP-Seq of H3K4me3, H3K27me3, RbBP5 (MLL complex), SUZ12 (PRC2 complex), OCT4 and RARa (LIF withdraw and retinoic acid (RA) exposure for 3h); 3) MNase-Seq and chromatin accessibility assay; 4) RNA-Seq for mES cells and for embryonic bodies (day 3 and day 7) derived from mES cells. We found that H2A.Z co-localizes with H3K4me3 in transcriptional regulatory regions, including promoters and enhancers. H2A.Z knockdown leads to a decrease in both H3K4me3 and H3K27me3 signals. In self-renewing ESCs, H2A.Z knockdown compromises OCT4 binding to its target genes and leads to decreased binding of MLL complexes to active genes and of PRC2 complex to repressed genes. During differentiation, inhibition of H2A.Z compromises RA-induced RARa binding, activation of differentiation markers and the repression of pluripotency genes. Data analysis of nucleosome occupancy and chromatin accessibility reveals that H2A.Z deposition results in an abnormal and unstable nucleosome structure, leading to decreased nucleosome occupancy and thereby increasing chromatin accessibility, particularly at enhancers. Knockdown of H2A.Z leads to a decreased expression of many pluripotency genes and up-regulation of differentiation genes in ESCs. But H2A.Z is also required for the silencing of ESC-specific genes and the optimal activation of differentiation genes during ESC differentiation. In summary, H2A.Z facilitates expression of many pluripotency genes and also the repression of

differentiation genes by generating chromatin accessibility and thereby facilitating the efficient targeting of activating and repressive complexes, respectively. During differentiation of ESCs, optimal induction of differentiation genes and the complete silencing of pluripotency genes also require H2A.Z to facilitate access of the appropriate complexes. Therefore, H2A.Z is a general facilitator for the access of a wide variety of activating and repressive complexes.

NHLBI
Menazza, Sara
Postdoctoral Fellow
Metabolomics/Proteomics
Proteomic and metabolomic studies reveal metabolic alterations in CypD-/- hearts
Background: Cyclophilin D (CypD) is a mitochondrial chaperone that has been shown to regulate the mitochondrial permeability transition pore (mPTP), a large conductance channel that can form in the inner mitochondrial membrane allowing passage of solutes and proteins less than 1.5 kDa. CypD-/- mice have reduced activation of the mPTP and reduced ischemia-reperfusion injury, but enhanced susceptibility to heart failure. We hypothesize that the altered susceptibility to heart failure is due to a change in mitochondrial metabolism, because, CypD as a mitochondrial chaperone, is likely to have pleotropic effects in the mitochondria. To test this hypothesis, we characterized mitochondrial proteome differences in CypD-/- mice using both proteomics and metabolomics approaches. Methods: We performed a proteomic iTRAQ (isobaric tags for absolute quantitation) analysis to measure protein changes in cardiac mitochondria from CypD-/- versus WT mice , and we used metabolomic analysis to study the acyl carnitine profile in total heart from CypD-/- and WT mice . Results: Using iTRAQ approach we found 8 proteins that were significantly increased by 20% or greater and 10 proteins that were significantly decreased by 20% or greater. Pathway analysis indicated alterations in branched chain amino acid, pyruvate and fatty acid metabolism. All the analyzed pathways involve alterations in acyl CoA, therefore, we measured the acyl carnitine profile as a surrogate for acyl CoA. Metabolomic analysis showed a significant decrease in C4/Ci4, C5-OH/C3-DC, C12:1, C14:1, C16:1, and C20:3 acyl carnitines in hearts from CypD-/- mice, suggesting a decreased in fatty acid oxidation. Using iTRAQ, we also found a 14% decrease in mitochondrial 2-oxoglutarate/malate carrier protein, a component of the malateâ€“aspartate shuttle, in the CypD-/- hearts. The shuttle activity was increased in CypD-/- mitochondria, suggesting a higher glycolytic flux in CypD-/- hearts. Conclusion: Taken together, these results show that CypD-/- hearts exhibit metabolic changes, consistent with a decreased in fatty acid oxidation relative to glucose oxidation. These metabolic changes could increase susceptibility to heart failure.

NHLBI
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Postdoctoral Fellow
Stem Cells - General
QUANTITATIVE TRACKING OF RHESUS MACAQUE HEMATOPOIETIC RECONSTITUTION AT A SINGLE CELL LEVEL OFFERS NOVEL INSIGHTS INTO LINEAGE RELATIONSHIPS
Quantitative tracking of clonal output from individual hematopoietic stem and progenitor cells (HSPCs) will be valuable for understanding normal and abnormal hematopoiesis. Current techniques for tracking cell clones in vivo are not quantitative or non-physiologic. For the first time in a large animal or human we have been able to comprehensively track the clonal output of transplanted HSPCs, using a sensitive and quantitative genetic barcoding approach. High diversity DNA barcodes library was delivered via lentivirus resulting in a single barcode per target cell. Low cycle PCR followed by Illumina sequencing of the barcoded region was performed to identify and quantify barcodes. The data was analyzed using a

computer algorithm to compare individual clonal contributions over time and between lineages. We established that the relative fraction of barcode reads retrieved by sequencing accurately represented clonal abundance in polyclonal setting(r=0.97). Two macaques received transplants of barcoded autologous CD34+ HPSCs following total body irradiation. Peripheral blood from 1, 2, 3, 4.5 and 6.5 month (m) post-transplant was sorted into granulocytes(Gr), monocytes(Mo), T, B, and NK cells. Bone marrow CD34+ and mature cells were collected from left and right pelvic sites at 5m. The contribution of barcodes within each sample was assessed. To date we have tracked 988 individual barcoded clones. Clones contributing at 1m were primarily unilineage, and disappeared at later time points, formally demonstrating that polyclonal, short-lived and lineage-restricted progenitors are responsible for initial hematopoietic recovery. Beginning at 2m, there were significant (r=0.68) and increasing shared clonal contributions to Gr and Mo, formally documenting the activity of a common Gr-Mo progenitor in vivo. By 3m and later, clones contributing to myeloid, B and T appeared, increased, and stabilized, representing multi-lineage repopulating HSPCs. Surprisingly, NK clones were not correlated with any other lineage (at 6.5m with T (r=0.09), B (r=0.05), Mo (r=0.06), and Gr (r=0.04)). CD34+ cells from left and right iliac bone marrow had almost completely distinct clonal compositions(r=0.03) but had high correlation with mature cells in their respective sites(right, r=0.85 and left, r=0.86). This suggests HSPCs reconstitute local niches with little migration. This powerful approach provides multiple new insights into hematopoiesis with relevance to human biology.

NIA
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Clinical and Translational Research
Resveratrol and curcumin enhance pancreatic ?-cell function by inhibiting phosphodiesterase activity
Resveratrol and curcumin are natural polyphenols that are found in fruits and turmeric respectively and have been reported to ameliorate various diseases, such as heart disease, cancer, and type 2 diabetes. Recent studies demonstrate the therapeutic properties of these natural products may, in part, be attributed to their anti-inflammatory, anti-proliferative, and anti-caloric properties. In type 2 diabetes, cyclic adenosine-3â€™,5â€™-monophosphate (cAMP) plays a critical role in signaling pathways essential for calorie restrictive effects and pancreatic beta-cell function. More specifically, cAMP plays an important role in glucose- and incretin- stimulated insulin secretion. A potential therapeutic target in the management of type 2 diabetes lies in the regulating the activity of phosphodiesterases (PDE), which degrade cyclic AMP (cAMP. Recent evidence suggests that both resveratrol and curcumin may act as phosphodiesterase (PDE) inhibitors in various cell types, but it remains unclear if these natural polyphenols do so in pancreatic beta-cells. Therefore, we hypothesized that these natural polyphenols would inhibit PDE activity, thus elevating cAMP production and downstream insulin secretion coupling in pancreatic beta-cells. In our current study, we first investigated which of the 11 PDE family members were expressed in various pancreatic beta-cell cell lines. We found that while all 11 PDE isozymes were expressed, there was significantly high expression of PDE 3B, 8A, and 10A, which have all been linked previously with regulating insulin secretion in rodent islets. Furthermore, in beta-TC6 cells, we found that both resveratrol (0.1 â€“ 10 uM) and curcumin (0.01 â€“ 1 nM) were capable of increasing intracellular cAMP levels that either matched or surpassed the ability of exendin-4 (25 nM) to do so. When added together with exendin-4 to beta-cells, resveratrol or curcumin enhanced exendin-4-mediated increases in intracellular cAMP levels in a similar manner to IBMX, a classic PDE inhibitor. As expected from the increased cAMP levels, resveratrol and curcumin also enhanced insulin secretion compared to control. Collectively, we demonstrate a novel role for natural polyphenols as PDE inhibitors that enhance pancreatic beta-cell function.

NIA
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Postdoctoral Fellow
DNA-binding Proteins/Receptors and DNA Repair
Fanconi Anemia Protein FANCM Promotes Replication Traverse of DNA Interstrand Crosslinks
Cells are most vulnerable to DNA damage in S phase. Failure to resolve impediments to replication may result in apoptosis or senescence, with adverse consequences for healthy aging. Among the most challenging lesions are interstrand crosslinks (ICLs), which are considered absolute blocks to replication. Repair of ICLs involves the Fanconi Anemia proteins, mutations of which cause a genetic disorder characterized by bone marrow failure, cancer predisposition, and features of premature aging. The FA pathway consists of the core complex (FANC-A, B, C, E, F, G, L, M); the â€œIDâ€ complex FANC-D2/I; and the downstream group (FANC-D1, J, N, O, P). FANCM, a DNA translocase, also exists in a separate complex, independent of the FA core components. Current models of crosslink repair during replication suggest that fork collisions, either from one (single fork model) or both sides (double fork model) of an ICL, initiate repair processes required for resumption of replication. To test these models we developed a single molecule technique for visualizing encounters of ICLs with replication forks, as they occur in living cells. The new method is based on DNA fiber technology and quantum dot imaging. We found evidence for both single and double fork models; however neither was the major pathway in mammalian genomes. Surprisingly, the most frequent replication patterns were consistent with replication fork traverse of an ICL, without lesion repair. The traverse patterns were dependent on the translocase activity of FANCM, but not the FA core complex. FANCM is found in ancient lineages while the mature FA pathway is found only in vertebrates. We suggest that translocase based fork traverse pathways evolved relatively early, in support of the cellular imperative to complete S phase. Furthermore, in contrast to traditional belief, our results indicate that ICLs are not absolute blocks to replication.

NIA
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DNA-binding Proteins/Receptors and DNA Repair
Deciphering the role of APE1 protein variants in disease etiology
Base excision repair (BER) is necessary for removal of DNA damage that has occurred from spontaneous decomposition, alkylation and oxidation. Defects in BER have been associated with cancer predisposition, neurodegeneration and premature aging. BER is initiated by a damage-specific DNA glycosylase that excises a substrate base, creating an apurinic/apyrimidinic (AP) site. The AP site is recognized by the multifunctional AP endonuclease 1 (APE1), which cleaves the phosphodiester backbone, producing a DNA strand break. DNA polymerase beta incorporates the correct nucleotide, and BER is completed via ligation by a protein complex of Ligase III and XRCC1. Like many BER proteins, APE1 is essential for survival, as deletion of both alleles in mice leads to embryonic lethality. We are pursuing the hypothesis that more mild reductions in APE1 activity will contribute to disease risk and development. To address this issue, we have engaged two strategies: (1) identification and characterization of APE1 missense mutations and (2) design and characterization of genetically-altered mutant human cell lines. Related to effort (1), we have characterized eight APE1 variants found within the population or identified in endometrial cancer. Our studies reveal that while most of these proteins are normal for thermodynamic stability, AP endonuclease, exonuclease and redox regulatory activities, and intracellular localization, the endometrial cancer-associated variant Arg237Cys has decreased exonuclease, 3â€™-damage excision and AP-DNA binding activities. Since loss of efficient autonomous exonuclease proofreading and 3â€™-damage removal could lead to increased mutagenesis and

consequent carcinogenesis, our data provide evidence for an association of APE1 dysfunction and disease. Related to effort (2), we have created a heterozygous knockout HCT116 cell line, in which we have deleted one allele of APE1. The heterozygous knockout cells, which express roughly half the level of APE1 protein and show a 50% reduction in total AP endonuclease activity, are hypersensitive to the oxidizing agent H2O2 and the alkylating agent methylmethane sulfonate, consistent with the idea that partially reduced APE1 function can lead to exposure-dependent defects. We are currently characterizing these cells for proliferation efficiency, cell cycle profile, and DNA damage accumulation, and in the future will design knockin HCT116 cell lines to define the biological contributions of specific APE1functions.

NIA
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DNA-binding Proteins/Receptors and DNA Repair
Interplay between Fanconi Anemia Pathway and DNA Damage Response induced by DNA Interstrand Crosslinks
Among the most challenging DNA lesions are the interstrand crosslinks (ICLs). They are potent blocks to replication and transcription and it has been proposed that ICL accumulation over time contributes to genomic instability and aging in tissues and organs. Sensitivity to crosslinking agents is a hallmark of Fanconi Anemia (FA), a hereditary syndrome characterized by hematopoietic abnormalities, features of premature aging, and an increased risk of cancer development. A central event in the FA pathway is the monoubiquitination of FANCD2. FANCD2 is believed to play an important role in ICL repair, and deficiencies of FANCD2 are associated with cellular hypersensitivity to cross-linking agents. Although known to localize to sites of DNA damage and stalled replication forks, the protein partners required for FANCD2 localization are obscure. It has been suggested that ICLs trigger the cellular response known as the DNA Damage Response (DDR) primarily in S-phase, during which ICLs would be expected to interfere with DNA replication. In order to understand the role of the DDR network induced by ICLs we introduced laser localized ICLs in defined subnuclear regions of living cells, and monitored the recruitment of various proteins immediately after ICL formation. We found that ICLs can activate a vigorous DDR throughout the cell cycle, including members of the FA pathway, such as FANCD2. We have found that rapidly recruited effectors of the DDR, such as phosphorylated histone H2AX and MDC1, were required for recruitment of FANCD2 in G1 phase cells, but not in S phase. Histone H2A ubiquitylation (uH2A) was also important for recruitment of repair proteins, including FANCD2. We found the first evidence for an interaction between FANCD2 and uH2A following ICL formation, thus answering a longstanding question about the identity of the chromatin partner for FANCD2 localization. These results indicate that psoralen ICLs induce a DDR response, which differs from the response to other forms of DNA damage. Furthermore, we show for the first time that the FA pathway response can be distinguished by cell cycle phase.

NIA
Shamanna, Raghavendra
Visiting Fellow
DNA-binding Proteins/Receptors and DNA Repair
RECQL4 modulates the Non-Homologous End Joining pathway through its interaction with the Ku complex
RECQL4 belongs to the RecQ family of DNA helicases that participates in DNA metabolism. The five human RecQ helicases, RECQL1, Bloom, Werner, RECQL4 and RECQL5 have been show to participate in several cellular processes including DNA replication, DNA repair, transcription and chromatin

organization. Like Werner and Bloom, mutations in RECQL4 are associated with premature aging and cancer prone syndromes. Numerous DNA lesions result from exposure to endogenous and exogenous DNA damaging agents, chemicals and ionizing radiation, and the repair of damaged DNA is essential for the maintenance of genome integrity. Repair of DNA double strand breaks (DSBs), essential for the maintenance of genome integrity, occurs through homologous recombination or non-homologous end joining (NHEJ). Our results show that RECQL4 knockdown cells (~90% knockdown) retain 2.5-fold higher levels of 53BP1 foci 12 h after gamma-irradiation when compared to control cells, suggesting a possible defect in processing of DSBs. However, the exact role of RECQL4 in DSB repair is not well understood. In this study, we have investigated the functional role of RECQL4 in NHEJ-mediated DNA repair. The NHEJ pathway of DSB repair is initiated by DNA-dependent protein kinase (DNA-PK), a protein complex consisting of Ku70, Ku80 and catalytic subunit of DNA-PK (DNA-PKcs). Co-immunoprecipitation experiments indicated that RECQL4 interacts with the Ku70/Ku80 heterodimer in vitro and in vivo, but not with other components (Artemis, Ligase IV and XLF) of the NHEJ pathway. In DNA binding assays, increasing amounts of RECQL4 stimulated higher order DNA binding of the Ku70/Ku80 complex. In an in vitro NHEJ assay, extracts from RECQL4 knockdown cells displayed reduced (50-80%) end-joining activity on DNA substrates with cohesive and non-cohesive ends, relative to extracts from control siRNA-treated cells. In vivo, depletion of RECQL4 reduced end joining activity on a GFP reporter plasmid and increased the sensitivity of cells to ionizing radiation-induced DNA damage. Taken together, these results implicate the involvement of RECQL4 in the NHEJ pathway of DSB repair via its functional interaction with Ku70/Ku80 complex. This is the first direct study to demonstrate the participation of RECQL4 in NHEJ pathway.

NIA
Murphy, Rachel
Other
Epidemiology/Biostatistics - Etiology, Risk, and Prevention
Adipose tissue density, a novel biomarker predicting mortality risk in older adults
The relationship between obesity and mortality risk in old age is inconsistent, with studies reporting increased risk of mortality with obesity or no relationship. As a result, the harm of obesity and the need for its treatment in old age is controversial. Knowledge of the characteristics of adipose tissue may help clarify relationships. However, little is known regarding adipose tissue and health outcomes independent of adipose volume. We aimed to relate abdominal visceral (VAT) and subcutaneous (SAT) adipose tissue density to mortality. Plasma levels of inflammatory markers (CRP, IL-6 and TNF-alpha) were explored as potential mediators of relationships between adipose density and mortality. We used data from men and women aged 65 and older in the Health ABC study (n=2,735) and a replication cohort; the AGES-Reykjavik study (n=5,131). VAT and SAT density were determined from the Hounsfield Unit (X-ray attenuation through tissue) of computed tomography images; higher values represent denser, smaller adipocytes and less lipid filled tissue. Participants were followed for mortality for 4-13 years. Mortality risk was estimated by Cox models; the lowest quintile of density compared to the upper 3Qâ€™s. Over the follow-up period there were 1,337 deaths in Health ABC and 1,207 deaths in AGES-Reykjavik. Denser adipose was associated with significantly greater mortality risk in both studies even with adjustment for age, education, smoking, physical activity, co-morbid conditions, adipose area and BMI. Compared to women with the least dense VAT, women with the densest VAT had an almost 2-fold increased mortality risk. Similarly, women with the densest SAT had a 1.5-fold increased mortality risk. There was a 1.5-fold increased mortality risk with the densest VAT for men in Health ABC but not AGES-Reykjavik and a 1.5-fold mortality risk for men with the densest SAT in both studies. Of note, participants with the densest adipose on average had BMI in the normal range (18.5-24.9kg/m2). Comparably participants with the lowest adipose density were overweight or obese. When comparing

levels of inflammatory markers, there were no consistent associations with VAT or SAT density in either study. These results provide novel insight into the relationship between obesity and mortality in old age and suggest that adipose tissue density may confound estimation of obesity risk. Further, VAT and SAT density may be unique markers of mortality risk that are not inflammation related.

NIA
Renton, Alan
Visiting Fellow
Genomics
Exome sequencing of young onset sporadic amyotrophic lateral sclerosis trios
Amyotrophic lateral sclerosis (ALS) is a devastating and ultimately fatal neurodegenerative disease lacking effective therapy. This complex disorder is generally thought to arise from a combination of genetic and environmental factors. ~10% of cases display a family history, whereas the remainder of patients are classified as sporadic because they appear to occur randomly throughout the population. Highly-penetrant pathogenic mutations have been found in ~65% of familial ALS (FALS) and ~10% of sporadic ALS (SALS). Despite this progress, the majority of genetic factors contributing to ALS risk await discovery. In order to identify de novo variants putatively underlying SALS, we undertook exome sequencing in a cohort of 19 disease trios from Italy. Each trio consisted of two unaffected parents and one offspring diagnosed with young onset SALS. This trio-exome experimental approach has been successfully applied to intellectual disability and psychiatric illness, but not to to ALS. Moreover, the early presentation of our probands suggests that their disorder is more likely to be caused by penetrant mutations. Gene validation was performed by exome sequencing in 240 unrelated FALS probands from North America, Italy, Germany, Israel, and Saudi Arabia. Identified variants were filtered against dbSNP, 1000 Genomes database, in-house population exome controls, 6500 population controls obtained from the Exome Sequencing Project cohort, 1100 Human Genome Diversity Panel controls from diverse populations, and co-occurrence of pathogenic mutations in known ALS genes. We found eleven de novo heterozygous missense mutations in eleven genes across seven trios. All of these were single base alterations, with three trios carrying multiple changes. One of these genes harbored two unique heterozygous missense mutations in two FALS cases. Subsequent dideoxy sequencing in an affected sibling demonstrated that one of these mutations apparently segregates with disease. For three other genes, a single unique heterozygous non-synonymous mutation was detected in one FALS case. Furthermore, one of these mutations is predicted to truncate the encoded protein. These results suggest that we have tentatively identified one or more novel ALS genes, and demonstrate the power of the trio-exome paradigm for elucidating the genomic architecture of complex disorders.

NIA
PANDA, AMARESH
Postdoctoral Fellow
Molecular Biology - Eukaryotic
AUF1 promotes myogenesis by enhancing Myf5 and Mef2c expression
Objective: To investigate the role of RNA-binding protein AUF1 in muscle cell differentiation
Background: Skeletal muscle contains long multinucleated and contractile cells know as myotubes, generated from the fusion of myoblasts arising from satellite cells. Satellite cells intiate the formation of myotubes by expressing a group of muscle-specific basic helix-loop-helix transcription factors like Myf5, MyoD, myogenin, MRF4, and MEF2. Together, these transcription factors are the major drivers of the transcriptional program in place during myogenesis. Besides transcriptional changes, posttranscriptional gene regulation critically affects the timing and abundance of many myogenic proteins. Post-transcriptional control is mainly elicited by RNA-binding proteins (RBPs) and noncoding RNAs, which

interact with myogenic mRNAs and regulate their stability and/or translation. The heterogeneous nuclear ribonucleoprotein D (hnRNP D), also known as AUF1, is a well-characterized RBP that binds to AU-rich elements and influences mRNA turnover. A recent report showed that AUF1-knockout mice have reduced muscle mass and weak musculature, suggesting a role for AUF1 in myogenesis. Therefore, we set out to test the hypothesis that AUF1 affects myogenesis. Results and Conclusions: We used C2C12 myoblasts as a cultured cell model of myogenesis. C2C12 cells differentiated in the presence of 2% horse serum for 6 days to form myotubes. Downregulation of AUF1 in myoblasts by using small interfering RNA resulted in reduced myotube formation. The association of AUF1 with myogenic mRNAs, studied by using RIP (RNA immunoprecipitation) assays, revealed that AUF1 binds, among other myogenic transcripts, to myf5 and mef2c mRNAs. AUF1 knockdown reduced both myf5 and mef2c proteins and these effects were attributed to the reduced translation of these mRNAs in AUF1-silenced cells. AUF1 silencing also led to a reduction in mef2c mRNA, likely mediated by a lesser-understood role of AUF1 as an inducer of gene transcription. Importantly, overexpresstion of myf5, mef2c, or both proteins rescued the effect of AUF1 as an inhibitor of myogenesis. In sum, AUF1 regulates myogenesis by promoting the expression of myf5 and mef2c through a combination of transcriptional and translational mechanisms.

NIA
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Visiting Fellow
Neuroscience - General
Learning and memory alterations in type 1 taste receptor 3 (T1R3) knockout mice
The type 1 taste receptor 3 (T1R3) is a G protein-coupled receptor which together with the type one taste receptors 1 and 2 (T1R1, T1R2) plays an important role in transducing sweet and umami taste signals. These GPCRs have been shown to be highly expressed in the taste buds of the tongue and in the enteroendocrine cells of the gut. We and other labs have recently shown that T1R3 is also highly expressed in multiple brain areas which regulate learning and memory ability, including the hypothalamus, hippocampus and cortex. However, the functional role of T1R3 in the brain is currently unknown. To gain insight into this, we performed extensive behavioral and molecular analyses of the T1R3 KO mouse. Using the Morris Water Maze and the Novel Object Preference Tests we found that T1R3 KO mice had significantly reduced learning and memory ability, compared to age-matched WT control littermates. Interestingly, T1R3 KO mice also showed significant reductions in social behavior (assessed using the standard social mouse/object preference test). To investigate the underlying molecular mechanisms of these cognitive changes, we measured synaptic protein expression and conducted a detailed analysis of dendritic spine number in the hippocampus. We found that in the T1R3 KO mice there were significant alterations in the subcellular translocation of pre- and post-synaptic markers including synapsin I, synaptophysin, post synaptic density protein 95 (PSD95) and spinophilin. Additionally, cyclic AMP-responsive element binding protein (CREB) phosphorylation was significantly decreased in the T1R3 KO mice, which is likely linked to reduced activity of the upstream calcium/calmodulin-dependent protein kinase IV (CaMKIV). Furthermore, Golgi staining demonstrated that there were significantly less dendritic spines in the hippocampal CA1 area of T1R3 KO mice compared to WT controls. From our findings, it is clear that T1R3 plays an important role in regulating learning and memory function and social behavior and that these cognitive alterations could, in part, be caused by the underlying alterations in hippocampal pre- and post-synaptic protein subcellular translocation and in dendritic spine number. Gaining a greater understanding of the role of T1R3 and other taste receptors in the brain could lead to the development of novel treatments for neurodegenerative and neurological disorders.

NIA
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Visiting Fellow
Neuroscience - Integrative, Functional, and Cognitive
Artificial sweetener Acesulfame Potassium alters energy metabolism and impairs cognitive function in C57BL/6J mice
To ameliorate the current obesity epidemic, artificial, non-nutritive, sweeteners are widely used dietary supplements that provide sweet taste without adding extra caloric load. The currently approved non-nutritive sweeteners are all recognized as safe, according to the American Dietetic Association. Recent epidemiological data though has challenged the concept of benign non-caloric sweet-taste activation. Sweet taste of both nutritive and non-nutritive agents is primarily mediated through activation of the lingual T1r2-T1r3 G protein-coupled receptor heterodimer. We have previously demonstrated that T1r3 is not only expressed in the tongue and peripheral tissues but also in central nervous regions including the hypothalamus, cortex and hippocampus. We postulated that it would be unlikely that chronic receptor activation via non-nutritive sweeteners would not induce pathophysiological effects. We therefore investigated the peripheral and central effects of protracted exposure to the T1r2-T1r3 sweet taste activator acesulfame K (ACK). Chronic ACK exposure (40 weeks) in normal C57BL/6J mice significantly altered circulating metabolic hormone levels (insulin, leptin), pancreatic islet size, lipid levels, and most importantly, profoundly impaired the cognitive memory functions. This ACK-induced phenotype was associated with metabolic dysregulation (glycolysis inhibition. ATP depletion), neurotrophic dysregulation in hippocampal neurons and central downregulation of the T1r3 sweet taste receptor subunit. Our study demonstrates that central T1r3 receptors may play a critical role in mediating ACK-induced effects. We supported this by demonstrating the abrogation of lingual detection of ACK and the resistance to ACK-induced cognitive impairment in T1r3 knock-out mice. Our data suggests that chronic use of ACK could deleteriously affect both metabolic homeostasis and cognitive health. Our work represents a novel and important contribution to the field of biological science as well as a direct translational implication for human health.

NIA
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Postdoctoral Fellow
Neuroscience - Neurodegeneration and Neurological disorders
Prognostic Classification of AD using Baseline MRI Independent Component Analysis
Predicting future diagnosis of Alzheimerâ€™s disease (AD) is an important research goal, as it will aid participant selection for enrollment in secondary prevention clinical trials and facilitate development of novel drug treatments. Patients with Mild Cognitive Impairment (MCI), a condition characterized by mild memory deficits, often convert to AD. To this end, it is important to develop biomarkers that accurately predict AD conversion from MCI. During this conversion, gray matter (GM) atrophy occurs in a systematic pattern in various brain areas including medial temporal lobe. We sought to identify early GM patterns of covariance across subjects at baseline, presumably reflecting early atrophy, and see if these patterns can predict AD conversion 2 years later. Thus, we used Independent Component Analysis (ICA) on GM volume images derived using Magnetic Resonance Imaging (MRI). ICA is a data-driven approach that produces maximally independent GM patterns of voxel-by-voxel covariance across subjects (e.g., GM volumes in both medial temporal lobes covary across subjects, constituting one GM pattern). ICA was used on baseline MRI data from 162 MCI participants enrolled in The Alzheimerâ€™s Disease Neuroimaging Initiative (ADNI), a longitudinal observational study. As a control, we derived mean baseline hippocampal GM volume, which shows pronounced atrophy during the MCI to AD transition. Using discriminant analysis, either baseline ICA GM patterns or mean hippocampal volume

were used to predict which of the 162 participants 2 years later remained MCI (N=86) or converted to AD (N=76). Age, sex, and years of education were used as covariates. ICA produced 30 GM patterns that captured 95.41% of the original volumetric variance in GM images. Mean hippocampal volume achieved 63.0% prediction accuracy, while ICA achieved 79.6%. Other MRI classification models in the literature have achieved a mean prediction accuracy of 66.8%. The most statistically significant and useful GM patterns for classification were found in medial temporal lobe, temporoparietal junction, and inferior temporal-occipital cortices, reflecting the pronounced temporal and parietal atrophy seen in AD. These results suggest that ICA-derived GM patterns found at baseline are excellent MRI biomarkers for predicting AD conversion, and currently outperform other MRI classification techniques.

NIA
Yamamizu, Kohei
Other
Stem Cells - General
Systematic discovery of transcription factors for lineage-specific ES cell differentiation
A network of transcription factors (TFs) determines cell identity, but identity can be altered by overexpressing a combination of TFs, e.g., from fibroblasts to induced pluripotent stem (iPS) cells by four transcription factors, Oct3/4, Sox2, cMyc, and Klf4, and to cardiomyocytes by three transcription factors, Gata4, Mef2c, and Tbx5. In principle, this opens up the possibility of achieving one of the goals of regenerative medicine - generating desired differentiated cells from pluripotent stem cells such as embryonic stem (ES) cells and iPS cells. However, choosing and verifying combinations of TFs for specific cell differentiation has been daunting due to the large number of possible combinations of ~2,000 TFs. To address this issue, systems biology is a powerful route to understand and predict the complex, stochastic dynamics of gene regulation. In this study, we demonstrate an unbiased approach to systematically identify TFs that can direct specific, efficient, and rapid cell differentiation. Previously, we have generated global gene expression profiles with microarrays after overexpressing single TFs using the NIA mouse ES cell bank of 137 mouse ES cell lines. We start with a correlation matrix between the global gene expression responses to the induction of single TFs and the global gene expression profiles of a variety of tissues and organs in vivo. Based on the correlation matrix, we select TFs as examples and show that their overexpression differentiates ES cells into cells of specific organs, as predicted and identified: Sfpi1 or Elf1 for multi-lineage blood cells, Hnf4a or Foxa1 for hepatocytes, and 10 TFs -- including Smad7 or Nr2f1 or Ascl1-- for neurons. By microarray and chromatin precipitation assays, we found that these TFs orchestrate global gene expression into target organ cells through direct binding a specific promoter. Also, for clinical application, a nonintegrative TF expression system is absolutely necessary. Therefore, we assessed synthetic mRNA to differentiate ES cells to specific organ cells. Thus far, the 3 tested, Sfpi1, Hnf4a, and Ascl1 generated their appropriate target cells. These results demonstrate both the wide-ranging utility of this approach to identify potent TFs for cell differentiation and also the unanticipated capacity of single TFs to directly guide differentiation to specific lineage fates. This technology can provide a new way to differentiate ES cells and iPS cells into desired lineages.

NIA
Scheibye-Knudsen, Morten
Postdoctoral Fellow
Stress, Aging, and Oxidative Stress/Free Radical Research
A high fat diet rescues the progeria phenotype of Cockayne syndrome mice
Cockayne syndrome (CS) is an accelerated aging disorder characterized by progressive neurodegeneration, growth retardation and weight loss. CS is caused by mutations in the DNA repair genes CSA or CSB. We recently discovered increased metabolism and mitochondrial dysfunction with

age-related fat loss in Csb m/m mice, however, only mild neurological deterioration was found. In other mouse models exacerbation of a neurological phenotype can be achieved with a high fat diet. To investigate if variations in dietary intake could alter the phenotype of the Csb m/m mice we compared standard diet fed mice with mice on a high fat diet, a caloric restricted diet or a resveratrol supplemented standard diet. Surprisingly, the high fat diet rescued the phenotype of the Csb m/m mouse at the transcriptomics, metabolomics, histological and behavioral level, compared to Csb m/m mice on standard chow. On the other hand, caloric restriction exacerbated the CS phenotypes. These alterations appeared to occur because of an increase in PARP1 activation due to a deficiency in nuclear DNA repair. This in turn leads to loss of NAD+, shifting the lactate-pyruvate equilibrium towards lactate. Lowered NAD+ levels then lead to decreased SIRT1 activation and subsequently hyperacetylation of p65 and p53, as well as decreased activity of PGC-1alpha. Due to the loss of PGC-1alpha, uncoupling protein-2 (UCP2) levels decrease and mitochondrial membrane potential increases. Fatty acid oxidation and ketolysis supplies energy to mitochondria independent of the lactate equilibrium and increases UCP2 expression perhaps explaining the beneficial effect of a high fat diet. In support of this human CSB patient cell lines showed increased beta oxidation, compared to the same cell line reconstituted with wild type CSB. Like the Csb m/m mice the CSB deficient cells showed increased PARP activation and decreased SIRT1 activity. Finally, the cellular, as well as the mouse, metabolic phenotype was rescued by treatment with PARP inhibitors. Thus, based on these results, we urge the clinical community to initiate trials that investigate the effect of a high fat high diet on CS patients. Further, since CS displays many features of advanced aging such as weight loss, brain atrophy, hearing loss and muscle weakness, our findings indicate that a high fat diet may be beneficial during normal late stage aging when metabolism appears to be increased, while caloric restriction may be detrimental.

NIA
Sgobio, Carmelo
Visiting Fellow
Neuroscience - General
Transgenic expression of GCaMP calcium indicator in dopaminergic neurons for optogenetic measurement of presynaptic calcium transients in striatum.
Studies conducted over the last two decades have revealed a great deal about dopamine (DA) release from presynaptic terminals in brain regions such as the striatum. However, relatively little is known about signaling within dopaminergic axon terminals that regulates dopamine release. Altered DA release is implicated in neurological disorders such a Parkinsonâ€™s disease, and thus greater characterization of terminal function is needed. To address this issue we expressed the genetically encoded calcium indicator protein â€œGCaMP3â€ (GFP-Calmodulin [CaM]- protein, type 3) in midbrain dopaminergic neurons. Using the tetracycline transactivator (tTA) system, we crossed: 1) a PITX3-IRES-tTA knock-in (KI) mouse (tTA under control of PITX3 promoter), and 2) a tetO-GCaMP transgenic (TG)mouse in which tetracycline operator controls GCaMP3-expression. The resultant KI/TG Pitx3/GCaMP mice showed normal motor phenotype compared with wild type (WT) littermate, as well as normal striatal DA release and reuptake. Striatal slices were analyzed using a photomultipier tube-based system. Single-pulse (10 msec, 120µA) stimulation induced fluorescence presynaptic calcium transients, that persisted for ~200 msec. These transients were totally blocked by application of cadmium, TTX, and by application of calcium-free aCSF. Transients were partially reduced by Quinpirole (in a dose-response relationship) and N- and P/Q type calcium channel blockers ?-agatoxin IVA and ?-conotoxin GVIA (~-40%). Applied in combination, transient was completely blocked, indicating in N- and P/Q- type channels as the main source of calcium in striatal DA terminals during electrical stimulation. L type calcium channel blocker Nifedipine did not affect calcium transient over time, confirming previous evidences about L-type channel is not directly involved in DA release in striatum. Cholinergic modulation also affects calcium

transient, in particular mAChR agonist (~-75%) and nAChR antagonist (~-50%) were able to strongly reduce calcium in DA terminals. In conclusion, this is the first report of a genetic expression of GCaMP conditioned for DA terminals. This model will help for a better understanding about calcium dynamic in presynaptic compartment during striatal DA release, not only in physiological but also in pathological conditions. Preliminary data of mutated a-synuclein overexpression effect on calcium transient in DA terminals will be presented, as a Parkinsonâ€™s disease related investigation.

NIAAA
Cao, Zongxian
Postdoctoral Fellow
Pharmacology and Toxicology/Environmental Health
Bidirectional Control over Endocannabinoids and Eicosanoids by Monoacylglycerol Lipase Inactivation Confers Protection to Hepatic Injury
Background and Aims: The endocannabinoid and eicosanoid lipid signaling pathways play important roles in inflammatory syndromes. Monoacylglycerol lipase (MAGL) was recently shown to link these two pathways through hydrolyzing the endocannabinoid 2-arachidonoylglycerol (2-AG) to generate an arachidonic acid precursor pool for prostaglandin production. Here, we investigated whether MAGL blockade provides protection against inflammation and liver damage inflicted by hepatic ischemia/reperfusion (I/R) and other insults. Methods: We performed hepatic I/R in mice given the selective MAGL inhibitor JZL184, and in MAGL-/-, cannabinoid receptor type 1 (CB1) -/- and CB2-/- mice. Liver tissues were collected and analyzed, along with cultured hepatocytes and Kupffer cells (KCs). Endocannabinoids, eicosanoids, and markers of inflammation, oxidative stress, and cell death were measured using mass spectrometry, molecular biology, histology, and biochemistry assays. Additional murine hepatic injury models induced by GalN/LPS and CCL4 were utilized to confirm the effects. Results: MAGL inactivation elicited profound protection against hepatic I/R injury, which was partially CB2 dependent and was associated with heightened endocannabinoid signaling and attenuated eicosanoid production in the liver. MAGL blockade suppressed both the inflammation and oxidative stress that subserved hepatic I/R injury. Hepatocytes represented the major source of hepatic MAGL activity and endocannabinoid and eicosanoid production. CB2 receptor was expressed primarily in non-parenchymal cells including KCs, but was not expressed on hepatocytes. Treatment with the CB2 ligand 2-AG suppressed LPS-induced TNF-alpha release by KCs, but had no effect on hypoxia/reoxygenation-induced hepatocyte cell death. Strikingly, a significant hepatoprotective effect was observed when JZL184 was administered even up to 3 hours after hepatic ischemia. MAGL inactivation also effectively protected against GalN/LPS- and CCl4-induced acute liver injury. Conclusions: MAGL blockade protects against hepatic injury through a dual mechanism involving heightened CB2-mediated endocannabinoid signaling and attenuated eicosanoid production. Pharmacological blockade of MAGL was able to not only prevent but also treat hepatic I/R injury, which implicates MAGL as a feasible therapeutic target for developing novel treatment strategies for conditions that expose the liver to oxidative stress and inflammatory damage.

NIAAA
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Visiting Fellow
Protein Structure/Structural Biology
Development of a homo-FRET based CaMKII biosensor for detection of T-site interaction in beating heart of transgenic zebrafish
Forster resonance energy transfer (FRET) based protein nanosensors allow monitoring biological functions in living animals. Most biosensors employ hetero-FRET and are rarely used in live organisms.

The Zebrafish heart is an excellent vertebrate cardiovascular model system for studying heart disease because of its small size, rapid development, genetic modifiability and transparency for optical imaging. Ca2+/calmodulin (CaM) dependent kinase II (CaMKII) has been implicated in illness including schizophrenia, heart failure, arthritis, and certain types of cancers, but the molecular basis of these diseases still needs to be elucidated. CaMKII assembles to form a holoenzyme structure with multiple catalytic domain pairs radiating out from a central core composed of oligomerization domains. Each N-terminal catalytic domain harbors the kinase catalytic site, its ATP binding site, as well as the so-called â€˜T-siteâ€™, thought to interact with the T286 autophosphorylation site in the regulatory domain. Activation of CaMKII upon binding CaM is known to initiate a biochemical cascade resulting in CaMKII translocation. The T-site is thought to be responsible for mediating the interaction of the holoenzyme with other proteins, such as L-type Ca2+ channels (LTCC), in response to activation, but this interaction has never been documented in the beating heart. Here we report the development of a homo-FRET based biosensor, V-CaMKIId (venus fluorescent protein tagged CaMKII). V-CaMKIId has been characterized in cultured HEK and HeLa cells, primary hippocampal neurons, and cardiomyocytes. Further, we generated a stable transgenic zebrafish through Tol2 transposon-mediated enhancer trap transgenic lines expressing cardiac-specific V-CaMKIId biosensor. V-CaMKIId can be used to detect structural changes associated with the CaMKII activation. Using this sensor, we demonstrate that the activation of CaMKII is a two step process in which Ca2+/CaM binding leads to catalytic domain pair extension from the core, followed by a separation of the regulatory subunits which is mediated through T-site interaction. The later is triggered by peptide inhibitors which interact with the CaMKII T-site. Interestingly, these T-site interactions are not detected in the beating heart of the zebrafish suggesting that the kinase does not interact with LTCCs. Our systematic analysis of V-CaMKII d and its advantages in reference to hetero-FRET based biosensor will be discussed.

NIAAA
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Postdoctoral Fellow
Psychiatry

The association of a functional polymorphism in CNR1 with alcohol withdrawal in a treatment-seeking alcoholic sample

Animal and human studies of alcohol dependence have illustrated the potential role of CNR1, the gene encoding cannabinoid receptor 1 (CB1), in alcohol response and contribution to dependence. A functional single nucleotide polymorphism (SNP), rs2023239, has been implicated in a variety of phenotypes, including increased risk for substance use disorders, greater alcohol-induced reward, increased response to alcohol-related cues, and higher CB1 receptor density in both postmortem and in vivo human brain studies in C allele carriers compared with non-C allele carriers. In light of these studies, we assessed the effect of this SNP on alcohol-related traits in treatment-seeking alcoholics and non-treatment-seeking controls. The sample in this study consisted of 551 treatment-seeking alcoholics and 405 non-treatment seeking participants. The association between rs2023239 and clinical alcohol-related phenotypes and measures of drinking history was assessed using linear and logistic models with sex and self-reported African or European ancestry as covariates. Variation in rs2023239 was assessed using a dominant genetic model based on the presence of the C allele (CC or CT genotype, n=326) or the absence of the C allele (TT genotype, n=509). The presence of the C allele of rs2023239 was associated with a decrease in peak alcohol withdrawal severity during the first day of treatment in the treatment-seeking sample, as measured by the Clinical Institute Withdrawal Assessment-Alcohol Revised (CIWA-Ar) tool (p=0.039). The C allele had a nominal effect on the alcohol withdrawal component of the Addiction Severity Index (ASI) (p=0.086) and peak overall CIWA score (p=0.061) in the same direction of effect in the treatment-seeking sample. The SNP was not significantly associated with other clinical phenotypes

or drinking history in the non-treatment-seeking participants. This study suggests a potential involvement of the functional polymorphism in the CNR1 gene in alcohol withdrawal. This pattern is consistent with prior studies in rodents reporting a lack of alcohol withdrawal responses in CB1 knockout mice, as well as with human studies showing a gain of function in C allele carriers for CB1 ligand binding. Because an increase in number of CB1 receptors may provide a neuroprotective effect against excitotoxic cell death during alcohol withdrawal, the C allele of rs2023239 may confer a downstream protective effect against clinical alcohol withdrawal severity.

NIAID
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Visiting Fellow
Cell Biology - General
Emerging role of CD300f in clearance of apoptotic cells, and its consequences for autoimmunity
The rapid and efficient removal of apoptotic cells prevents the release of the potentially toxic or immunogenic components from dying cells, thereby reducing inflammatory and autoimmune responses. Phagocytosis is the major means of clearing apoptotic cells, which is dependent on the interaction between â€œeat-meâ€ signals on apoptotic cells and receptors on phagocytes. Newly externalized phosphatidylserine (PS) on apoptotic cells is the best-known â€œeat-meâ€ signal for phagocytes. CD300f is expressed on myeloid cells and a subset of B cells. We demonstrate that mouse CD300f (CLM-1) recognizes outer membrane-exposed PS and is important for regulating the phagocytosis of apoptotic cells. Its over-expression promotes phagocytosis, whereas both primary alveolar and peritoneal macrophages from CD300f gene-deficient mice are impaired in phagocytosis of apoptotic cells. This defect in phagocytosis likely explains why CD300f gene-deficient mice are prone to develop autoimmune diseases like experimental autoimmune encephalomyelitis (EAE) and systemic lupus erythematosus (SLE). We further investigated how CD300f regulates the phagocytosis of apoptotic cells. Mouse CD300f possesses five cytoplasmic tyrosine phosphorylation sites residing in motifs that have the potential to initiate signaling. We demonstrate that upon incubation of phagocytes with apoptotic cells, Y276 is phosphorylated to the highest extent, and is required for recruiting the p85a regulatory subunit of phosphatidylinositol 3-kinase (PI3K). The association of CD300f with PI3K leads to downstream Akt phosphorylation, and enhanced phagocytosis in an Y276-dependent manner. Using cell imaging analysis, we show that CD300f accumulates at the phagocytic cup contact site with apoptotic cells, along with p85a, which enzymatically converts PI(4,5)P2 to PI(3,4,5)P3. Consistent with decrease of PI(4,5)P2 levels, cortical F-actin is cleared from the bottom of phagocytic cups, thereby facilitating apoptotic cell internalization. At the same time, CD300f-induced and PI3K-mediated increase in PI(3,4,5)P3 levels promotes activation of the Akt and Rac/CDC42 pathway(s); this, in turn, results in actin polymerization along the edges of phagocytic cups, thereby driving the membrane around the apoptotic cells to promote their engulfment. Our study is the first report identifying the CD300f-mediated signaling pathway that is required for efficient engulfment and clearance of apoptotic cells.

NIAID
Han, Kyu Lee
Visiting Fellow
Hematology/Oncology, Tumor Immunology, and Therapy
Nonmyeloablative Conditioning regimen plus A2AR agonist oral administration promote engraftment on allogeneic bone marrow transplantation mouse model
Although non-myelaoblative conditioning regimens have proven successful in obtaining engraftment and reducing the toxicity of allogeneic transplant, improvements are still needed to improve engraftment rates as well as limit the incidence of graft versus host disease. Methods to improve

engraftment without added toxicity are especially desirable in the non malignant setting. Agonists of the Gs-coupled Adenosine A2A receptor (A2AR) have been reported to promote T cell tolerance in several in vivo models, particularly in the GVHD setting; however itâ€™s role in engraftment is unclear. We examined the ability of an A2AR agonist to promote engraftment in a murine allogeneic bone marrow transplantation model. B6D2F1/J recipients were conditioned with 300 cGy and transplanted 24 hours later via lateral tail vein infusion with 2×106 T-cell depleted bone marrow cells from C57BL/6J donor. Recipients received an A2AR agonist orally in a chow formulation (1000 mg/kg/day) beginning 2weeks before cell infusion and continuing for 9 weeks post transplantation. A second cohort also received the A2AR agonist using osmotic pumps (100 ng/kg/min) placed subcutaneously 48 hours prior to cell infusion, for 28days post transplantation as well as orally via the chow for the same time as the first group. A non-treatment transplant group was used as a control. There was no toxicity from the radiation or the drug, and all animals maintained normal blood counts throughout. Engraftment was observed in only the A2AR agonist treated groups and a 13 fold higher level of engraftment in the mice treated both subcutaneously and orally was observed. This engraftment appeared to be long term as the mice were followed out 5 months post transplant with all mice surviving. As a possible mechanism of this effect, we measured the level of CXCR4 expression on the donor bone marrow cells in vitro and observed that A2AR agonist treatment increased CXCR4 expression 1.6 fold compared to non-treatment on donor cells in vitro. To further investigate the effect of the A2AR agonist on engraftment, we are now repeating the same type of transplant using an A2AR Knock out mouse strain as the donor. We are also observed higher level of SDF-1 in the serum of the A2AR agonist treated recipients. These results demonstrate that A2AR agonist may improve engraftment by increasing homing of the donor cells without increasing the risk of GVHD and may be a significant addition to transplant regimens.

NIAID
Guzzo, Christina
Visiting Fellow
HIV and AIDS Research
XCL1/Lymphotactin: A novel CD8-derived anti-HIV-1 chemokine
The replication of HIV-1 is regulated in vivo by a complex network of cytokines and chemokines expressed by immune and inflammatory cells. However, despite significant progress, major gaps remain in our knowledge of how immune cells control HIV-1. Key players in the mechanisms of HIV-1 control are CD8+ T cells, which, in addition to their cytolytic activity, secrete soluble factors that suppress HIV-1 in a non-lytic fashion. Early studies identified three CCR5-binding chemokines produced by CD8+ T cells (MIP-1alpha, MIP-1beta, RANTES) as major components of CD8-derived anti-HIV activity. However, evidence indicates that CD8+ T cells produce additional soluble factors that suppress HIV-1 infection. Indeed, observations that CD8-derived factors can inhibit CXCR4-using HIV-1 strains (resistant to previously described CCR5-binding chemokines) support a role for additional, uncharacterized anti-HIV factors produced by CD8 cells. In this study, we characterize a novel CD8-derived anti-HIV chemokine, XCL1 (lymphotactin), which may be clinically relevant for HIV-1 control in vivo. XCL1 is a member of the C-chemokine family and is particularly unique because it can interconvert between two structurally distinct conformations. XCL1 is primarily produced by activated CD8+ T cells and NK cells, and its physiological function includes recruitment of lymphocytes and dendritic cells via activation of a specific receptor, XCR1. We found that XCL1 inhibits a broad range of HIV-1 strains, irrespective of their coreceptor-usage phenotype and genetic subtype. We investigated structural correlates of XCL1 anti-HIV activity and found the all-beta sheet conformation was the key XCL1 structure mediating anti-HIV activity. Since this XCL1 structure is unable to activate XCR1 and induce signal cascades, our focus shifted away from an effect on host cells to a direct effect on virions. Accordingly, we showed XCL1 acts at an early stage of HIV-1 infection, via blockade of viral attachment and entry. Furthermore, we

demonstrated that XCL1-mediated inhibition occurs through direct interaction with the HIV-1 envelope, as seen with virion capture assays and co-immunoprecipitation of XCL1 with the viral envelope glycoprotein, gp120. More precise characterization of XCL1-mediated mechanisms of HIV-1 inhibition may reveal new molecular targets for the design of HIV-1 inhibitors and vaccines. This study aids our understanding of HIV-host interactions and informs design of novel therapies.

NIAID
Hajduczki, Agnes
Postdoctoral Fellow
HIV and AIDS Research
Investigating HIV Env/receptor interactions using a soluble mimetic of the co-receptor CCR5
The HIV envelope glycoprotein mediates virus entry by initiating fusion of the viral envelope with the plasma membrane upon receptor engagement. The bulky, surface-exposed gp120 subunit is a flexible, dynamic molecule that undergoes a series of stepwise conformational changes in response to interactions with the receptor CD4, and co-receptors, chemokine receptors CCR5 or CXCR4. Obtaining structural information on the various intermediates during viral entry is a key focus of antiviral and vaccine research and could open the doors for more effective treatment and prevention. The coreceptors are G-protein-coupled receptors, anchored in the membrane with seven membrane-spanning helixes, leaving the N-terminus and 3 extracellular loop regions exposed outside the plasma membrane and available for interactions. Due to the inherent insolubility of membrane proteins, working with the intact co-receptors outside of the context of the membrane, as soluble proteins, is not a viable option. This project aims to generate a soluble homologue for the HIV co-receptor CCR5, by using a globular, stable scaffold protein to orient the relevant extracellular regions of CCR5 in an optimal conformation to recreate the binding interaction with gp120. There have been reports of successful use of the B1 domain of Streptococcus Protein G to display extracellular portions of chemokine receptors in studies investigating ligand interactions. We have decided on a stabilized variant of Protein G B1 domain as our scaffold, with some additional modifications to optimize expression in mammalian cells. The N-terminus of CCR5 has been shown to contain sulfated tyrosines that are necessary for co-receptor function; therefore, it is important to optimize the post-translational processing of the expressed proteins. We have successfully overexpressed and purified our scaffolded CCR5 co-receptor determinants from mammalian cells. Since Protein G is an IgG-binding protein, in order to be able to use this scaffold in assays involving antibodies we also sought to eliminate the IgG-binding properties of the B1 domain. Variants bearing a single point mutation demonstrated only negligible background binding to a variety of IgG molecules. The soluble variant of CCR5 will be used in biochemical assays to induce the conformational changes in gp120 that take place immediately preceding membrane fusion, and potentially for collaborative high resolution structural analyses of the gp120-coreceptor complex.

NIAID
Jelicic, Katija
Visiting Fellow
HIV and AIDS Research
Inhibition of B cell function by HIV-1 envelope: Role of binding to integrin Alpha4Beta7
During the early stages of HIV infection the immune system of the infected individual is already somewhat impaired. Among the defects described is the impairment of normal B cell function that includes a significant delay in the development of the anti-HIV humoral immune response. The mechanisms underlying this delay are not fully understood. We previously demonstrated that the HIV envelope protein gp120 binds to and signals through integrin Alpha4Beta7 on T cells and NK cells. In the present study, we asked whether gp120-induced signaling through Alpha4Beta7 on B cells could disrupt

their function. Peripheral blood B cells, which typically express intermediate levels of Alpha4Beta7, were treated in vitro with HIV-1 gp120s derived from viral isolates that exhibited high-affinity for Alpha4Beta7. Alternatively, these cells were treated with HIV-1 gp120s that exhibit low-affinity for Alpha4Beta7. Treated B cells were tested for alterations in their transcriptional program as determined by RNA microarray analysis. HIV-1 gp120 signaling through Alpha4Beta7 modified the expression of genes involved in inflammation, cell cycle, apoptosis and B-cell activation. Several of these genes have previously been associated with B cell dysfunction in HIV-infected patients. B cells were also tested for proliferative capacity and changes in the expression of surface receptors. HIV-1 gp120s with high affinity for Alpha4Beta7 mediated a strong inhibition of B cell proliferation. Among the genes upregulated by gp120 was FcRL4, an inhibitory receptor. Increased FcRL4 expression is observed in HIV-infected individuals, but its expression normalizes upon initiation of antiretroviral therapy. We observed induction of FcRL4 surface expression by treatment with soluble HIV-1 gp120 and by exposure of B cells to CD4+ T cells that had been infected in vitro. Taken together these results suggest that HIV-1 gp120s with a high affinity for Alpha4Beta7, have the potential to disrupt B-cell function. These studies have implications for understanding the immunopathogenic mechanisms of HIV-1 infection, particularly the ability of high levels of viremia observed during acute HIV infection to blunt an appropriate antibody response to the virus.

NIAID
Tang, Wanhu
Visiting Fellow
Immunology - Autoimmune

The NF-kB modulator Bcl-3 is a novel type of regulator of T helper cell plasticity and is required for T cell transfer-induced colitis

Bcl-3 is an atypical member of the IkB family with unique properties. Unlike classic IkBs, Bcl-3 readily enters nuclei where it can modulate gene expression by interacting with p50/NF-kB1 or p52/NF-kB2 homodimers on DNA. Depending on the cellular context and target gene, Bcl-3 appears to be able to promote or inhibit gene expression, although the molecular mechanisms involved have remained largely obscure. Mice lacking Bcl-3 are highly susceptible to various pathogens, including T. gondii infection, but the underlying cellular mechanisms have also remained largely unclear. To begin to unravel cell-specific roles for Bcl-3 in immune responses, we began to investigate its potential functions in T cells using a T cell transfer-induced colitis model. Wild type or Bcl-3 knockout naïve CD4 T cells were transferred into Rag-1 knockout mice. Mice receiving wild type CD4 T cells developed severe wasting disease, whereas mice receiving Bcl-3 deficient T cells failed to develop any disease symptoms. The inability of Bcl-3 deficient T cells to induce pathology correlated with markedly reduced numbers of IFNg-producing Th1 cells and markedly increased numbers of Th17 cells. We furthermore showed that Bcl-3 was required within in vitro differentiated Th1 cells to induce wasting disease upon adoptive transfer. In contrast to WT in vitro differentiated Th1 cells, similarly differentiated Bcl-3-deficient Th1 cells were not stable and readily converted to IL-17-producing cells in vivo. We also demonstrated that antigen-specific TCR engagement was required for efficient conversion of Bcl-3 deficient Th1 cells in vivo. Experiments using NF-kB1 deficient and NF-kB2 deficient mice suggested that Bcl-3 coordinated with p52 to regulate Th1 cell stability in vivo. Investigations into the mechanisms underlying the ability of Bcl-3 deficient Th1 cells to convert into Th17 cells revealed, among other changes, a higher level of expression of RORgt in mutant Th1 compared to WT Th1 cells, which appeared to contribute to the heightened plasticity of mutant Th1 cells. While Th17 cells are known to readily convert to Th1-like cells in vivo, the reverse has never been noted. Our data suggest that Bcl-3 functions as a novel kind of regulator of T helper cells, one that locks in the Th1 phenotype and blocks conversion to the Th17 phenotype. Our results also

suggest that Bcl-3 may be a potential therapeutic target for clinical intervention in Th1/IFNg-mediated diseases.

NIAID
Gerner, Michael
Postdoctoral Fellow
Immunology - General
The role of lymphoid micro-architecture in dendritic cell-mediated antigen capture and initiation of cellular adaptive immunity
Dendritic cells (DCs) specialize in antigen (Ag) sampling and presentation and are the principle cell population involved in promoting T cell-mediated immune responses. DCs are composed of multiple subsets with divergent functional capabilities, with lymph node (LN)-resident CD8+ vs. CD11b+ DC preferentially processing captured Ags for MHC-I vs. MHC-II presentation, respectively. Utilizing a novel microscopy method, â€œHisto-Cytometry,â€ we have recently described differential spatial residence of these subsets within murine LN, with preferential but not exclusive localization of CD8+ DCs to the central T cell zone and CD11b+ DCs to the peripheral regions. These findings raised the possibility that LNs are subdivided into specialized micro-domains with differential functional capacities for Ag sampling and subsequent T cell activation. Here, we show that DC spatial positioning has profound effects on Ag capture and downstream MHC processing after protein immunization, with poor Ag penetration deep inside the LN paracortex leading to decreased Ag sampling by the centrally localized CD8+ DCs and absence of CD8+ T cell responses at low levels of immunized Ag, as compared to the more effective capture of such Ag by peripheral CD11b+ DC and CD4+ T cell priming. Moreover, we found that DC subset regionalization directly influences the location of early CD8+ vs. CD4+ T cell activation, with centrally localized CD8+ T cell vs. predominantly peripheral CD4+ T cell priming. Collectively, these findings indicate that spatial organization by DC subsets creates micro-anatomical domains with specialized functional capacities affecting the development of cell-mediated immunity. This research was supported by the Intramural Research Program of the NIH, NIAID.

NIAID
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Immunology - General
EMR1: a novel therapeutic target for eosinophilic disorders
Eosinophils are implicated in the pathogenesis of a wide variety of disorders, including asthma, parasitic helminth infection, and hypereosinophilic syndromes (HES). Although glucocorticoids are the first line therapy for many of these disorders, long-term toxicity and/or resistance are common. Despite clinical trials demonstrating the safety and efficacy of novel monoclonal antibodies targeting eosinophils (EOS) in asthma and HES, none has shown 100% efficacy in reducing tissue EOS or clinical symptoms. Human epidermal growth factor (EGF)-like module containing mucin-like hormone receptor (EMR1) is a surface receptor of unknown function. Unlike its murine analog, F4/80, expressed on monocytes and macrophages, human EMR1 has been reported to be expressed only on EOS. To explore the potential of EMR1 as a target for the treatment of eosinophilia, the pattern of EMR1 expression was assessed in blood and tissue specimens from eosinophilic subjects and normal controls, as well as in various cell lines and CD34+ cells differentiated in vitro to promote EOS development. The ability of afucosylated anti-EMR1 IgG1 to target EOS was evaluated in vitro by NK killing assay and collaborators assessed in vivo efficacy in cynomolgus monkeys. Flow cytometric and real-time PCR analysis of blood and bone marrow cells from normal (n=16) and eosinophilic donors (n=21), as well as CD34+ cells cultured in vitro, confirmed that human EMR1 is expressed exclusively on mature EOS. EMR1 expression was also

detected by immunostaining on EOS in skin and nasal polyp biopsies. Whereas EMR1 was highly expressed on blood EOS from all donors tested, surface expression was negatively correlated with absolute eosinophil count (AEC) (r = -0.46, P<.001), suggesting modulation of EMR1 in vivo. Incubation of purified EOS in vitro with interleukin-5 revealed a similar pattern of EMR1 regulation. Plasma levels of soluble EMR1 were positively correlated with AEC (r= 0.69, P<0.001), consistent with receptor shedding. Afucosylated anti-EMR1 antibody enhanced EOS killing by NK cells from 15.1 to 62.3% (n=6, P<0.05). Afucosylated anti-EMR1 (1 mg or 5 mg iv) was well-tolerated and induced a rapid (8 hrs) and sustained (>1 month) decrease in AEC in 4 cynomolgus monkeys. These data suggest that EMR1 is highly and selectively expressed on blood and tissue EOS, and that targeting eosinophils using afucosylated anti-EMR1 may be a promising strategy for the treatment of eosinophilic disorders.

NIAID
Brickley, Elizabeth
Doctoral Candidate
Immunology - Infectious Disease
Elevated levels of Th1 inflammatory mediators at birth are protective against severe malarial anemia
Severe malarial anemia (SMA) remains a major cause of pediatric illness and mortality in Sub-Saharan Africa. Although the pathogenesis of SMA is not fully understood, evidence suggests that perturbations in the timing and magnitude of the innate immune response may influence whether a Plasmodium falciparum infection triggers a protective or pathogenic outcome for the host. To understand whether specific individuals are predisposed to SMA due to a cytokine production pattern established from birth or if the dysregulation in inflammatory mediators develops acutely during an infection, the associations between cord blood cytokines and SMA, defined as a clinical severe malaria episode with hemoglobin <60g/L, during the first four years of life were evaluated in a clinic-based cohort in Muheza, Tanzania. Levels of tumor necrosis factor (TNF), TNF receptors I and II, interleukin- (IL-) 1, IL-4, IL-5, IL-6, IL-10, and gamma-interferon were measured in cord blood samples obtained from 879 participants, including 71 who experienced SMA episodes. Cox proportional hazard models with shared frailties were used to calculate floating absolute risks to assess the shapes of associations between SMA and cytokine, receptor, or cytokine ratio levels. SMA risk decreased progressively across increasing levels of cord blood TNF, TNF-RI, IL-1, and, in contrast to the findings consistently observed during acute SMA episodes, the ratio of TNF to IL-10. The risk for SMA did not vary substantially over levels of TNF-RII, IL-5, IL-6, and IL-10 nor between undetectable and detectable levels of IL-4 and gamma-interferon. The fully adjusted hazard ratios for SMA per 1 standard deviation change of log-transformed TNF, TNF-RI, IL-1, and TNF/IL-10 were respectively: 0.83 (0.70, 0.99), 0.74 (0.64, 0.87), 0.59 (0.49, 0.70), and 0.79 (0.62, 1.02), and these associations did not vary substantially when stratified across the participant level characteristics of sex, thalassemia, sickle cell trait, birth weight, maternal gravidity, maternal age, placental malaria, birth season, and insecticide-treated bed net use. In summary, these findings suggest that infants with high cord blood levels of the Th1 inflammatory mediators TNF, TNF-RI, and IL-1 are protected against SMA in early life and that there may be a specific role for inflammatory cascades in the chronic prevention of SMA that is independent of the cytokine imbalance that occurs during the acute development of SMA.

NIAID
Iwamura, Chiaki
Postdoctoral Fellow
Immunology - Infectious Disease
NOD1 expression is critical for host resistance to Toxoplasma gondii
NOD1 and NOD2 are pattern recognition receptors that trigger NF-kB through a CARD/RIPK2 signaling pathway and are involved in the intracellular sensing of pathogens and commensals. Muropeptides

derived from cell walls of gram-positive and gram-negative bacteria are thought to be the major microbial agonists of the NOD receptors. Toxoplasma gondii is an obligate intracellular protozoan parasite that infects a wide variety of cell types across many different animal species. Infection is controlled mainly by IL-12 induced IFN-gamma derived from natural killer cells, Th1-polarized CD4 T effectors and CD8+ T lymphocytes. To assess the possible role of NODs in innate recognition of T. gondii we compared intraperitoneal infection in WT and NOD1/NOD2 double deficient mice. Unexpectedly, we found that NOD1/NOD2 KO animals display rapid acute mortality comparable to that seen in mice that lack IL-12 or IFN-gamma. Interestingly, this high susceptibility to infection was recapitulated in single NOD1, but not NOD2, deficient mice. To determine whether NOD1 expression in hematopoietic cells is critical for host resistance to toxoplasma, we reconstituted irradiated WT mice with bone marrow (BM) cells from either WT or NOD1 KO animals and found that WT recipients of NOD1 KO BM cells were more susceptible to T. gondii infection than mice reconstituted with WT BM cells. We next asked whether T cell expression of NOD1 might be important for host resistance. To this end, we showed that CD4 T cells express NOD1 mRNA and that its expression is increased following T. gondii infection. In addition, we demonstrated that in vitro NOD1 KO CD4 T lymphocytes display defective Th1 cell differentiation and are more susceptible to apoptosis than WT CD4 T cells following activation. Finally, we observed that T cell recruitment into the peritoneal inoculation site is impaired in infected NOD1 KO mice versus WT animals. These results suggest that NOD1 expression is critical for control of T. gondii infection. Whether this function is dependent on the sensing of parasite ligands and/or reflects a requirement for NOD1 in CD4+ T cell responsiveness is currently under study.

NIAID
Meissner, Eric
Clinical Fellow
Immunology - Infectious Disease

Alteration in the Endogenous Interferon Response in Patients with Chronic Hepatitis C, Genotype 1 Treated with an HCV RNA Polymerase Inhibitor and Ribavirin

Chronic hepatitis C virus (HCV) infection affects 200 million people worldwide and is a leading cause of liver-related mortality by causing cirrhosis and hepatocellular carcinoma. Endogenous interferons (IFNs) play a significant role in the resolution of acute HCV infection while exogenous IFN-alpha (IFNA) has remained the backbone of HCV treatment for two decades. Treatment is evolving from pegylated IFNA-based to IFN-free directly acting antiviral (DAA) regimens, although mechanisms of treatment relapse for DAA regimens are not understood. We treated sixty treatment-naïve, chronic HCV genotype-1 patients with an HCV RNA polymerase inhibitor (Sofosbuvir) combined with ribavirin for 24 weeks and used paired liver biopsies, peripheral blood mononuclear cells (PBMCs), and serum to understand dynamics of host-viral interactions and mechanisms of treatment relapse for an IFN-free regimen. All patients experienced rapid viral decline and normalization of hepatic markers of inflammation early in treatment, although 31% relapsed within 12 weeks of completing therapy. Gene expression in liver biopsies obtained pre-treatment and at end of treatment in 8 patients revealed downregulation of IFN-stimulated genes and genes associated with antigen presentation. Intriguingly, expression of IFN-lambdas (IFNL1-4) and IFN-gamma (IFNG) decreases with treatment, suggesting they are drivers of chronic hepatic inflammation, while endogenous IFNA was surprisingly upregulated at the end of treatment in most patients. Rapid downregulation of the endogenous IFN response was also detected in PBMCs with normalization of IFN-stimulated genes within weeks of starting therapy. Serum IP-10 (CXCL10), chronically elevated in HCV for decades and a ligand for CXCR3, is normalized within 10 days of starting therapy, correlating with an increase in peripheral B cells, T cells, NK cells, and neutrophils which peak 2 weeks after starting therapy. Detailed examination found that peripheral B cells express higher levels of CXCR3 (but not CCR4 or CXCR4) 10 days into therapy compared to baseline. Together,

our data support a model whereby HCV replication in the liver drives continuous production of IFNLs, IFNG, and pro-inflammatory chemokines that mediate hepatic migration of immune cells with dynamic and not static kinetics. They further suggest that reactivation of endogenous IFNA expression may be important to achieve HCV eradication with an IFN-free regimen.

NIAID
Tran, Tuan
Clinical Fellow
Immunology - Infectious Disease
A novel method for assessing malaria risk reveals a correlation between Plasmodium falciparum RH5-specific IgG and protection from malaria
Vaccines strategies targeting Plasmodium falciparum asexual blood stages, which cause the clinical manifestations of malaria, have yet to show protective efficacy in clinical trials. PfRH5, an essential P. falciparum merozoite protein involved in erythrocyte invasion, is an attractive candidate for a blood-stage vaccine. We investigated the association between naturally acquired PfRH5-specific IgG present before the malaria season in Mali and the prospective risk of malaria using time to first clinical malaria episode after PCR-confirmed P. falciparum blood-stage infection as the primary outcome. Baseline IgG responses to PfRH5 and another blood-stage antigen P. falciparum apical membrane antigen 1 (PfAMA1) were determined by enzyme-linked immunosorbant assay in 333 children and adults aged 6 months to 25 years who began the study free of blood-stage Plasmodium infection. 275 participants subsequently became infected with P. falciparum by PCR detection during the ensuing malaria season. A significant delay in median time from blood-stage infection to first clinical malaria episode was observed among individuals with positive IgG responses to PfRH5 (n = 54; 70 days; 95% lower confidence limit, 33 days) compared to negative responders (n = 221; 18 days, 95% CI, 14 â€“ 26 days) by log-rank test (P = 0.0014). After adjustment for age, sickle cell trait, transmission intensity and IgG responses to PfAMA1 using a Cox proportional hazards model, the protective effect of PfRH5-specific IgG on malaria risk remained (HR 0.64, 95% CI, 0.44 â€“ 0.94, P = 0.02). In addition, positive responses to PfRH5 were associated with lower parasite densities at first PCR-positive P. falciparum infection. We are currently assessing the ability of naturally acquired PfRH5-specific antibodies to neutralize parasite invasion of erythrocytes in vitro using growth inhibition assays. These findings provide evidence for the role of naturally acquired PfRH5-specific antibodies in clinical immunity to P. falciparum infection. In addition, our methodology for assessing malaria risk improves the ability to detect associations between immune responses to blood-stage infection and clinical protection and may prove useful for evaluating potential correlates of blood-stage immunity in vaccine trials.

NIAID
Vilar Portugal, Silvia
Postdoctoral Fellow
Immunology - Infectious Disease
Host and parasite factors underlying asymptomatic Plasmodium falciparum infection in Malian children
In Mali, Plasmodium falciparum transmission is seasonal during the 6-month rainy season, when almost all children below 8 years old experience one or more febrile malaria episodes. On the other hand, during the 6-month dry season virtually no children experience symptomatic malaria, even though ~50% carry blood stage P. falciparum parasites. To determine the host and parasite factors that underlie asymptomatic infection, we performed a one-year longitudinal study of 580 children between 1 and 11 years old. Subjects found to be infected with P. falciparum by standard rapid diagnostic testing (RDT) at the end of the dry season were treated with standard anti-malarial drugs while RDT negative but PCR positive individuals for P. falciparum were left untreated; the third group included those who were

uninfected at the end of the dry season (RDT and PCR negative). The risk of P. falciparum infection and clinical malaria was assessed prospectively during the subsequent malaria season and various immune parameters were compared in the three groups. Individuals infected with P. falciparum infection at the end of the dry season who were left untreated showed a decreased risk of clinical malaria during the ensuing malaria season compared with non-infected individuals, and interestingly, treatment of asymptomatic infection at the end of the dry increased the subsequent risk of malaria compared to the treated group, indicating that chronic asymptomatic -infection protects from febrile malaria. By analyzing polymorphic regions of P. falciparum genes we also compared the risk of super-infection in children whose asymptomatic infection at the end of the dry season was treated or not. In addition we are exploring parasite factors that underlie chronic asymptomatic infection by comparing the metabolome and transcriptome of parasites collected from asymptomatic children at the end of the dry season versus parasites collected during acute febrile malaria during the transmission season. These data provide critical insights into the host and parasite factors that underlie asymptomatic P. falciparum infection, as well as the risks associated with treating chronic asymptomatic P. falciparum infectionâ€"information that may have important public health implications for proposed malaria control strategies such as mass anti-malarial drug administration of populations in endemic areas.

NIAID
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Postdoctoral Fellow
Immunology - Innate and Cell-mediated Host Defenses
Localized production of IL-10 by CD8+ T cells in virus-infected skin
CD8+ T cells can control acute viral infection through a variety of effector mechanisms including the production of pro-inflammatory cytokines such as IFN-gamma and TNF-alpha. While often necessary for viral control, excessive inflammation can lead to tissue damage and enhanced morbidity. As a counter-balance, the anti-inflammatory cytokine interleukin 10 (IL-10) is often induced, modulating the immune response and dampening inflammation. Tight control of these two opposing signals is necessary to strike an accord between excess inflammation and viral containment. The primary sources of IL-10 are generally thought to be CD4+ T cells along with antigen non-specific dendritic cells, macrophages and granulocytes. However, CD8+ T cells have recently been shown to produce IL-10 after virus infection, serving to limit excessive inflammation. Despite knowledge of the existence of IL-10-producing CD8+ T cells, nothing is known about their precise location in situ. We used ex vivo analyses combined with multiphoton microscopy to assess the dynamic behavior of IL-10 producing CD8+ T cells during epicutaneous (ec.) vaccinia virus (VV) infection of the skin. After infection, highly activated CD8+ T cells are the main source of IL-10 and peak in number concurrently with maximal virus replication. IL-10+ CD8+ T cells within the virus-infected skin vary in motility, but exhibit average speeds of 5 um/minute. Rather than being dispersed throughout infected tissue, mobile IL-10 producing CD8+ T cells are concentrated directly adjacent to virus-infected keratinocytes. These results demonstrate the spatial concentration of IL-10 production at the site of the heaviest immune response, and suggest that localized cytokine production is a key feature of not only effector immune responses but also anti-inflammatory immune responses.

NIAID
Laemmermann, Tim
Visiting Fellow
Immunology - Innate and Cell-mediated Host Defenses
Neutrophil swarms require the lipid LTB4 and integrins at sites of cell death in vivo

Neutrophil recruitment from blood to extravascular sites of sterile or infectious tissue damage is a hallmark of early innate immune responses. Once outside the vessel, individual neutrophils often show strikingly coordinated chemotaxis and cluster formation reminiscent of the swarming behavior of insects. The molecular players that direct this â€œswarmingâ€ response on the single cell and population level within the complexity of an inflamed tissue are unknown. Here we have investigated extravascular neutrophil swarming at very small focal sites of sterile tissue injury in the skin and in infected lymph nodes by intravital two-photon microscopy. We found that local cell death initiates dramatic swarm-like interstitial neutrophil recruitment and clustering with a key role of the lipid leukotriene B4 (LTB4) as a unique intercellular communication signal between neutrophils. LTB4 acutely amplifies limited primary chemotactic cues and DAMP signaling at the lesion to allow rapid integrin-independent, long-distance neutrophil recruitment through the tissue. At the injury site, congregating neutrophils rearranged collagen bundles, leading to an environment that required integrin activity for neutrophil participation in dense clusters, with neutrophil-generated lipid and other chemoattractant stimuli enhancing cell-cell interactions to maintain motility while eliminating cell debris. These findings provide an initial map of the molecular actors that regulate the interstitial phase of neutrophil responses to tissue damage, revealing how local events are propagated over large distances, and how auto-signaling produces co-ordinated, self-organized swarming behavior that isolates the wound from surrounding viable tissue. Such insights should prove useful for studies where local sterile or pathogen-induced cell death characterizes the early innate immune responses, while the molecules identified may serve as potential targets for therapeutic intervention in destructive neutrophil-dependent inflammatory processes.

NIAID
Riteau, Nicolas
Postdoctoral Fellow
Immunology - Innate and Cell-mediated Host Defenses
A Caspase-8 dependent pathway for ER stress mediated IL-1beta maturation
Maintaining normal cell physiology requires constant protein synthesis and proper folding, processes that occur in the endoplasmic reticulum (ER). Improperly folded proteins accumulate within the ER and generate perturbations known as ER stress that engage the unfolded protein response (UPR). Albeit not fully understood, downstream effector mechanisms of the UPR promote a wide range of inflammatory pathologies such as neurodegenerative disorders, diabetes and obesity. The present work aims to better characterize the link between the ER stress response and the production of interleukin-1beta (IL-1b), a pro-inflammatory cytokine known to play a role in those conditions. IL-1b is produced in an inactive form that must be cleaved notably within cytoplasmic protein complexes termed inflammasomes to become a functional cytokine. Here, using a well-established model of ER stress induced by treatment with tunicamycin or thapsigargin, we show in vitro that macrophages undergoing ER stress are able to potently activate pro-IL1b in response to LPS stimulation. When the ER stress response is engaged, LPS is sufficient to drive both the induction and maturation of IL-1b without further stimulation. Interestingly, the classical Nlrp3 inflammasome is dispensable since maturation of pro-IL1b does not require the adaptor protein ASC or the effector Caspase-1. In contrast, our results indicate that processing of pro-IL1b is dependent on Caspase-8 since pro-IL1b maturation is completely abrogated in macrophages lacking this non-inflammasome associated protease. Intriguingly, we found that the UPR proteins XBP1 and CHOP are not required for Caspase-8 activation, nor is the TLR4 adaptor molecule MyD88. Instead, we observed that the alternative TLR4 adaptor TRIF is fully required and potentially could recruit Rip1, a signaling intermediate involved in Caspase-8 activation. We are currently attempting to decipher the mechanism by which ER stress enables TRIF/Rip1-mediated Caspase-8 activation. This pathway is of interest as it could underlie the induction of IL-1-driven tissue pathology in different disease settings.

NIAID
Lee, Jinmin
Visiting Fellow
Immunology - Lymphocyte Development and Activation
Mapping the B cell receptor on B cell surfaces using super-resolution fluorescence microscopy
Cell surface immuoreceptors for antigens, including the B cell receptor (BCR), T cell receptor (TCR), and high affinity IgE receptor (FceRI), induce intracellular signals that are critical for translating extracellular environmental cues into cellular behavior and activation. Previous observations suggest that these receptors and their associated signaling molecules are not uniformly distributed in the plasma membrane but rather are spatially organized to facilitate the initiation of receptor signaling and its regulation. At the resolution of diffraction-limited light microscopy, BCRs appear to be diffusely expressed over the B cell surface in resting cells and upon antigen binding the BCRs form signaling-active microclusters containing hundreds of receptors. Even though these microclusters have been studied extensively the spatial organization of BCRs in resting cells and how this organization influences signaling in response to antigen are yet to be established. A fundamental issue, namely whether BCRs exist as monomers or oligomers on the surface of resting B cells remains controversial. Recently developed super-resolution imaging techniques that bypass the diffraction-limit of light might provide valuable insights into understanding these fundamental questions. Using direct stochastic optical reconstruction microscopy (dSTORM), we showed that in resting IgM-expressing naïve human B cells the majority of IgM BCRs appear as monomers (66%) with a small fraction of BCRs that are clustered into dimers or trimmers (13%), or larger clusters containing hundreds of receptors (9%). Both of these clusters likely appear as microclusters at the diffraction-limited resolution. Following antigen binding a larger fraction of IgM BCRs are clustered (33%) with a decrease in monomeric fraction (52%). Interestingly, on resting IgG-expressing human memory B cells surfaces, IgG BCRs appear to be more pre-clustered (52% monomeric, 18% dimers or trimmers, 14% larger clusters) than do IgM BCRs on the surface of naïve B cells. More robust, larger BCR clusters form on IgG-expressing B cells following antigen binding (40% monomeric, 45% clustered). These observations suggest that intrinsically different spatial organization between IgM BCRs and IgG BCRs might in part explain their functional outcome. More detailed analysis on the spatial organization of BCRs, and activating co-receptor CD19 and inhibitory receptor Fc?RIIB in relation to BCRs are currently under investigation.

NIAID
Kumar, Krishan
Visiting Fellow
Metabolomics/Proteomics
A systems biology approach for the discovery of drug and/or vaccine targets in Plasmodium falciparum using irradiated long-lived merozoites
Malaria caused by Plasmodium falciparum causes several hundred million cases of clinical disease and nearly 1 million deaths each year. Passive transfer of hyper-immune human IgG from an endemic area protects children against clinical disease. Efforts to replicate this clinical immunity using blood stage recombinant protein vaccines have not been successful. Thus new discovery efforts are important. To this end, we are using a systems biology approach to identify the biological basis for a gamma-irradiated parasite line to have a long-lived invasive merozoite phenotype. Cell-sieve purified long-lived merozoites significantly retain their capacity to invade RBCs at approximately 3 to 5 times that of the parent line. Analysis of the genomes has identified several single nucleotide polymorphisms leading to a stop codon in the genome of the irradiated parasite line which are currently be verified by qPCR. Using microarrays, an overall comparison of the transcriptomes of schizonts (2 - 4 nuclei) versus purified merozoites

identified a greater difference (greater or less than 2-fold) in the transcript levels in schizonts (>1400 transcripts) as compared to merozoites (4 transcripts). In parallel using a label-free quantitative proteomic approach (LC/MSE or LC/HDMSE), long-lived merozoites appear to have higher protein abundance after normalization to a set of 5 house-keeping proteins. Furthermore, the relative molar concentration of merozoite proteins has been determined for approximately 1300 qualified proteins which range from 0.171 pecomoles to 0.850 femtomoles. Using this information, a reconstruction of the merozoite is underway. Finally, using TransOmicsâ„¢ analysis of two biological replicates evaluated by LC/MSE or LC/HDMSE, a total of 446 and 1196 proteins were identified as significantly different in protein abundance (p-value less than 0.05) using Principle Component Analysis, respectively. Possibly of greater biological significance, a total 212 proteins were shared between the two groups, including 48 proteins currently identified as of unknown function. Taken all together our findings provide a unique opportunity to improve our understanding of merozoite invasion of RBCs which holds promise to aid in the discovery of new vaccine or drug targets.

NIAID
BROOKE, CHRISTOPHER
Postdoctoral Fellow
Microbiology and Antimicrobials
Viral evolution drives changes in the gene expression potential of individual influenza A virions that alter the population-level genome structure
The influenza A virus (IAV) genome is divided between eight negative-sense RNA segments. Recent studies have suggested that the individual segments are co-packaged with high efficiency, thus ensuring that the vast majority of virions express the full set of gene products required for productive infection. By analyzing viral protein expression in cells infected with single IAV virions by flow cytometry, we complicate this picture by revealing that most infectious virions fail to express one or more essential viral proteins. For the PR8 strain of IAV, we examined the HA, NA, NP, and NS1 proteins, and found that each was expressed by only 70-85 percent of infectious virions within a population. Under selective pressure, the virus selectively modified the expression frequencies of these genes, resulting in dramatic effects on the behavior of the virus and demonstrating the mutability of viral gene stoichiometry at the population level. Different H1N1 strains also exhibited striking diversity in expression frequency profiles, indicating that the modulation of population-level expression frequencies is a common feature of influenza virus biology. Together, our results illuminate a hidden dimension of IAV genetics that has profound implications for understanding influenza replication, transmission, and evolution.

NIAID
Chouikha, Iman
Visiting Fellow
Microbiology and Antimicrobials
Silencing the insecticidal activity of Yersinia pestis urease was important for the evolution of flea-borne transmission.
Transmission of Yersinia pestis, the plague bacillus, by fleas is a recent evolutionary adaptation acquired during its divergence from the food-borne pathogen Y. pseudotuberculosis less than 20,000 years ago. To produce a transmissible infection, Y. pestis colonizes the flea midgut in the form of a biofilm in the proventricular valve that blocks the fleas blood feeding and enhances the transmission of the bacteria during a flea bite. In contrast, Y. pseudotuberculosis is transmitted by the oral route; however it can infect the flea gut but is orally toxic to fleas. Immediately after feeding on a blood meal containing Y. pseudotuberculosis, ~50% of fleas show signs of acute toxicity including diarrhea. The loss of toxic activity was certainly a fundamental step to the evolution of Y. pestis from Y. pseudotuberculosis,

however the entomotoxic factor has yet to be identified. Using a subcellular fractionation method and our flea infection method, we first localized the bacterial entomotoxic protein of Y. pseudotuberculosis toward the rat flea Xenopsylla cheopis in the membrane fraction. We then compared the two dimensional gel electrophoresis profiles of Y. pestis and Y. pseudotuberculosis membrane fractions and sequenced 33 protein spots that differed in intensity. The sequences showed 97 to 100% identity between the two species except for one spot identified as the urease accessory protein UreD. In Y. pseudotuberculosis UreD is encoded by the ureD gene part of the urease locus (URE), while its homologue in Y. pestis has been characterized as a pseudogene. Two Y. pseudotuberculosis mutants deleted of the entire URE cluster or of the ureD gene alone, showed a significantly decreased toxicity to fleas compared to the wild type strain. The complementation of the URE mutant was able to restore the toxic phenotype. To confirm the toxic role of urease, we reactivated the silenced urease activity in Y. pestis. The urease positive clones showed a toxicity rate toward fleas similar to that of Y. pseudotuberculosis. These results clearly demonstrate that the Yersinia urease cluster is responsible for flea toxicity and suggests that silencing of this cluster during the evolution of Y. pestis was positively selected because it enhanced flea infectivity and flea-borne transmission. The silencing of the urease activity may confer other advantage to the plague bacillus, we are currently evaluating its impact in biofilm formation and proventricular blockage in the flea.

NIAID
Oteng, Eugene
Doctoral Candidate
Microbiology and Antimicrobials
DISCOVERY OF CONSERVED PLASMODIUM ANTIGENS ON THE SURFACE OF INFECTED RED BLOOD CELLS USING DNA APTAMERS
Malaria continues to present a major human and economic challenge causing over 1.2 million deaths in 2010 and billions in lost economic potential. A vaccine targeting the leading causative agents, Plasmodium falciparum or P. vivax, would reduce much of the poverty associated with malaria and greatly assist in eradication efforts. Yet despite decades of work, no licensed malaria vaccine exists and some of the few candidates that have been evaluated elicit strain specific responses due to extensive polymorphism. During its intraerythrocytic stages (IE), P. falciparum remodels the host red cell membrane with a complex and poorly defined assortment of parasite-encoded proteins that undergo antigenic variation. Despite the requirement for immunologic stealth, exported parasite proteins also mediate strain-independent functions such as endothelial sequestration that are critical for parasite survival and pathogenesis. Based on this observation, we hypothesized that P. falciparum displays novel structurally conserved proteins on the IE surface and these proteins may serve as useful antigens for a broadly effective anti-malarial vaccine. In order to test this hypothesis, we developed an in vitro evolution technique that sequentially incorporates unique P. falciparum isolates as the target for Systematic Evolution of Ligands by EXponential enrichment (Serial-SELEX) to generate nucleic acid molecular probes, aptamers, capable of recognizing conserved cell surface determinants. Of the 11 aptamers identified, 10 demonstrated parasite-specific binding with nanomolar dissociation constants. Examination of the binding specificity of radiolabeled aptamers revealed a subset of aptamers that recognized all laboratory-adapted clones and clinical isolates of P. falciparum tested. Remarkably, these aptamers also recognized all tested laboratory and clinical isolates of P. vivax and P. knowlesi, a human zoonotic infection, but not the murine malaria parasites, P. chabaudi and P. berghei. Competition studies showed that the aptamers bound a single target which was confirmed as an IE membrane protein using biochemical techniques and confocal microscopy. Aptamer-mediated affinity purification and tandem mass spectrometry enabled the definitive identification of the aptamer target. Discovery of

a protein conserved between the major human malarias may have implications for vaccine development and validates the Serial-SELEX technique as a powerful tool for antigen discovery.

NIAID
Huestis, Diana
Postdoctoral Fellow
Physiology
Identifying phenotypic markers of aestivation (dry-season survival) of malaria mosquitoes (Anopheles gambiae) in the Malian Sahel
The African malaria mosquito, Anopheles gambiae, is widespread across sub-Saharan Africa and inhabiting variable environmental conditions. Its lifespan is only 6-8 weeks and fresh water is required for breeding, yet it persists without water during the long Sahelian dry season (5-7 months). Although the mechanisms of survival during the dry season are not yet known, targeting dry-season mosquitoes could provide novel opportunities for vector control and reducing malaria. In the Sahel region of Mali, previous research showed that A. gambiae likely persist via aestivation (dormancy). To assess the role of ecophysiological changes associated with dry-season survival, a series of novel experiments was conducted to measure metabolic rate, body size, flight activity, and oviposition rate of wild-caught mosquitoes throughout 1 year in a Sahelian locality without permanent water and at a riparian control site adjacent to the Niger River. Additionally, cuticular hydrocarbon (CHC) and lipid-content analyses were performed to test the hypotheses that mosquitoes would increase CHCs for enhanced desiccation resistance and increase lipid reserves to allow for prolonged starvation tolerance. We found significant seasonal variation in metabolic rate and body size at both the Sahelian and riparian sites, with greater changes in the Sahel. However, after controlling for biotic and abiotic covariates, significant seasonality remained only in the Sahel, indicating that changes in metabolic rate can be associated with dry-season survival. Significant seasonality in flight activity was only observed in the Sahel, with increased flight activity (4.8-fold) in the wet season when compared with that just prior to and throughout the dry season, supporting our hypothesis that mosquitoes will decrease energy usage to prolong survival during the Sahelian dry season. Oviposition rate of Sahelian females decreased 3.5-fold in the dry season as compared with the wet season and we observed an increase in the proportion of females that developed eggs but resorbed them instead of laying them. Finally, we also found a 5-fold increase in CHCs when comparing dry-season with wet-season mosquitoes from the Sahel. Lipid assays have been successfully performed on field and laboratory mosquitoes, but this analysis is still underway. In conclusion, these novel assays yielded many phenotypic changes which may enable the survival of A. gambiae during the dry season, and additional studies are ongoing.

NIAID
Sweeney, Colin
Postdoctoral Fellow
Stem Cells - General
Targeted correction of X-CGD iPSCs by TALEN-mediated gene transfer to the CYBB locus
X-linked chronic granulomatous disease (X-CGD) is a defect in neutrophil production of microbicidal reactive oxygen species (ROS), caused by mutations that can occur throughout the >30 kb CYBB gene. We previously described gene therapy of X-CGD induced pluripotent stem cells (iPSCs) by targeting a therapeutic CYBB minigene to the â€œsafe harborâ€ AAVS1 locus using zinc finger nucleases, restoring ROS production in differentiated neutrophils through constitutive expression of CYBB from a CAG promoter. To achieve gene therapy while restricting CYBB minigene expression to neutrophils, we have now targeted insertion of the CYBB minigene to the endogenous CYBB locus under the control of the endogenous CYBB promoter. TAL effector nucleases (TALENs) were developed to target the start codon

of CYBB, and a donor gene transfer plasmid vector was constructed containing the CYBB minigene and a loxP-flanked CMV promoter and puromycin resistance gene surrounded by sequences homologous to approximately 1 kb upstream and downstream of the start site of the normal endogenous CYBB locus. iPSCs were established from peripheral blood CD34+ cells of an X-CGD patient with CYBB exon 3 mutation using loxP-STEMCCA lentivirus, which was subsequently excised by transient Cre expression. Upon transfection with donor and TALEN plasmids and after puromycin selection, 1 out of 22 (4.5%) iPSC clones contained a targeted insertion of the donor vector at the start codon of the endogenous CYBB locus without additional random insertions. The CMV-puromycin cassette was then excised, resulting in a corrected iPSC clone containing only the targeted therapeutic CYBB minigene insert. Upon neutrophil differentiation in vitro, this clone exhibited ROS production by dihydrorhodamine flow cytometry assay, with a >300-fold increase in ROS activity over the uncorrected X-CGD iPSC neutrophils by chemiluminescence assay. Our findings show that safe minigene targeting to the endogenous CYBB locus can be achieved in iPSCs for X-CGD correction while maintaining CYBB promoter regulation, with no other foreign gene or promoter elements remaining after Cre excision. Since this approach targets gene transfer to the start site of exon 1 of CYBB, it may be applied for therapy of the majority of CYBB mutations causing X-CGD.

NIAID
Wan, Wuzhou
Research Fellow
Vascular Disease and Biology
Reduced Susceptibility to Atherosclerotic Plaque Formation in ApoE-deficient Mice Lacking the Atypical Chemokine Receptor Darc
The Duffy antigen receptor for chemokines (Darc) is an atypical chemokine receptor expressed by erythrocytes, venular endothelial cells and cerebellar neurons. Darc binds to a broad range of inflammatory CC and CXC chemokines, and is known to be used by P. vivax as an entry factor in malaria infection. Up to 99% of West Africans and 68% of African Americans lack Darc on their red blood cells, which reduces their susceptibility to P. vivax malaria infection. However, the role of Darc in other inflammatory diseases, such as atherosclerosis, is still unclear. Atherosclerosis involves recruitment of numerous leukocyte subsets into vascular lesions, especially monocytes and T cells. Here, we investigated the role of Darc in atherogenesis by using the apolipoprotein E-deficient (ApoE-/-) mouse model of atherosclerosis. First, we identified Darc in atherosclerotic lesions at both the RNA and protein level, and found that Darc deficiency in ApoE-/- mice resulted in markedly reduced atherosclerotic plaque formation. Darc-/-ApoE-/- mice showed ~35% less atherosclerotic lesion development in both the whole aorta and the aortic root compared with Darc+/+ApoE-/- mice. However, Darc deficiency did not affect mouse serum cholesterol level or collagen/smooth muscle cell/macrophage content in the aortic root area. Instead, loss of Darc resulted in a significant decrease of T cells in the blood, spleen, aorta and aortic root lesions of ApoE-/- mice. Also, the ratio of inflammatory /resident monocytes (Ly6Chi/Ly6low cells) in the blood and aorta of Darc-/-ApoE-/- mice was markedly reduced compared with Darc+/+ApoE-/- mice, although they had similar levels of total monocytes. Finally, we found that Darc deficiency significantly reduced the expression of Ccl2, Cxcl2, Icam-1 and Vcam-1 in the whole aorta of ApoE-/- mice. In conclusion, our data suggest that Darc deficiency impedes atherosclerosis in ApoE-/- mice by 1) skewing the balance of inflammatory/resident monocytes; and by 2) reducing T cell accumulation in the vessel wall, which may be caused by reduced expression of inflammatory chemokines and adhesion molecules in these mice.

NIAID
Liu, Shin-Wu

Visiting Fellow
Virology - DNA
The decapping activity of vaccinia virus is required for viral replication in cells and in mice
All cellular and most viral mRNAs have a 5â€™-methylated cap structure that protects mRNA from degradation by exonucleases and enhances translation. Vaccinia virus (VACV), a member of the poxvirus family, is a large DNA virus that replicates exclusively in the cytoplasm of host cells. It encodes enzymes for mRNA synthesis and processing, including a multisubunit RNA polymerase, a capping enzyme and a polyA polymerase. Remarkably, VACV also encodes decapping enzymes D9 and D10 that can destabilize viral and host cell mRNAs. Previously, we have demonstrated that D9 and D10 proteins contained intrinsic decapping activities, and that replacement of D10 with GFP increased stability of viral and cellular mRNAs and reduced viral replication in BS-C-1 cells. Here, we further analyzed the role of decapping activities of D9 and D10. Three mutant viruses were characterized: D10mu contained point mutations in the catalytic domain of D10 that abolished its decapping activity; D10stop contained stop codons that abolished D10 synthesis; the double mutant D9muD10mu contained point mutations that abolished the decapping activities of both D9 and D10. D10mu and D10stop displayed milder phenotypes in cells compared to the previous D10 deletion mutant. Nevertheless, viral early and late transcripts and cellular GAPDH mRNA persisted longer, consistent with the decapping role of D10. Interestingly, the double mutant D9muD10mu displayed significantly impaired replication. Western analysis showed that expression of viral intermediate and late proteins was blocked. Furthermore, electron microscopy showed there were no assembled viral particles formed in infected cells. Surprisingly, these phenotypes of severely impaired replication of D9muD10mu only displayed in BS-C-1 cells, but not BHK21 cells. In the mice study, groups of BALB/c mice were infected intranasally with various doses of D10mu, D10stop, D9muD10mu, or wild-type viruses. Daily monitoring for mice weight loss revealed that D10mu and D10stop were less virulent than the wild-type virus and had higher mice survival rates, and D9muD10mu displayed no virulence at all even with the highest lethal dose. Taken together, VACV requires at least one functional decapping enzyme for its replication in BS-C-1 cells and in mice. The host range effect of D9muD10mu suggests that an important role of VACV decapping activity is to prevent expression of antiviral factors that are more potent in BS-C-1 cells than in BHK21 cells.

NIAID
Baz, Mariana
Postdoctoral Fellow
Virology - RNA and Retroviruses
A Live Attenuated Avian Influenza A H3 Virus Vaccine for Pandemic Preparedness
An integral part of influenza pandemic preparedness is the generation and evaluation of vaccines against influenza viruses with pandemic potential, prior to the emergence of a pandemic. However, it is not possible to predict which influenza virus subtype will cause a pandemic and we learnt from the 2009 pandemic that a novel antigenically distinct H1N1 virus could cause a pandemic despite the circulation of H1N1 viruses in humans since 1977. H3 influenza viruses infect pigs, horses, dogs, cats, seals and numerous avian species and these viruses are antigenically and genetically distinct from human H3 viruses. Several sporadic cases of cross-species transmission of H3 viruses have been documented and importantly, in 2011 an avian H3N8 influenza virus caused an outbreak of pneumonia with a high mortality in New England harbor seals. This virus had mutations that reflect adaptation to mammalian hosts, raising concerns about potential spread to humans. Based on the ability of 11 geographically and temporally distinct H3 influenza viruses isolated from pigs, horses and birds to induce cross-reactive antibody responses and their ability to replicate in the respiratory tract of mice and ferrets, we selected A/blue-winged teal/Texas/Sg-00079/2007 (tl/TX/079/07) H3N8 virus to produce a live attenuated H3N8

vaccine containing the hemagglutinin (HA) and neuraminidase gene segments from the H3 virus and the six internal protein gene segments from A/Ann Arbor/6/60 ca, the backbone of the licensed seasonal live attenuated influenza vaccine. Intranasal immunization of mice and ferrets with one dose of the tl/TX/079/07 vaccine induced significant neutralizing antibody (NtAb) titers against the homologous virus with a further increase in titer following a second dose of vaccine. A robust NtAb response was observed after two doses of tl/TX/079/07 ca vaccine against a heterologous avian virus and A/harbor-seal/MA/1/2011, which shared 96.6 and 97.2% amino acid sequence identity in the HA, respectively with the vaccine virus. One dose of the tl/TX/079/07 vaccine conferred complete protection from replication of the homologous and heterologous viruses in the upper and lower respiratory tract of mice and ferrets. In summary, the H3N8 vaccine was highly immunogenic and effective in two animal models and warrants further evaluation in humans.

NIAID
de Wit, Emmie
Visiting Fellow
Virology - RNA and Retroviruses
Fulfillment of Kochâ€™s postulates for the novel coronavirus hCoV-EMC/2012 in a macaque model
In 2012, a novel coronavirus (CoV) associated with severe respiratory disease emerged in the Arabian Peninsula. So far, 13 human cases have been reported, including several probable cases of human-to-human transmission. The first human isolate, EMC/2012, was classified as a betacoronavirus, placing it in the same genus as SARS-CoV. This alarming situation is reminiscent of the SARS-CoV pandemic in 2003, when this virus caused ~8000 cases with a case fatality rate of 10%. Thus, the emergence of EMC/2012 calls for an immediate public health response. The availability of an animal disease model is an important aspect of developing effective countermeasures. First, we tried to establish a disease model in Syrian hamsters, a suitable model for SARS-CoV. Animals were inoculated via intratracheal instillation or via exposure to aerosols containing EMC/2012. No clinical signs were observed; virus replication or cytokine upregulation could not be detected; animals had not seroconverted by 21 days post inoculation (dpi). After receiving information that other small animal models such as ferrets and mice did not result in a suitable model, we attempted to establish a model in rhesus macaques. Upon a combination of intratracheal, ocular, oral and intranasal inoculation, macaques developed clinical signs within 24 hours. Radiographically, varying degrees of localized infiltration and interstitial markings of the lungs were observed. Post mortem examinations on 3 dpi showed lesions throughout the lower respiratory tract indicative of acute pneumonia. These lesions progressed into dark red purple areas of pulmonary inflammation by 6 dpi. qRT-PCR analysis of tissues revealed widespread presence of hCoV-EMC/2012 in the nasal mucosa, trachea, mediastinal lymph nodes and all six lung lobes on 3 dpi, with viral loads decreasing over time. Virus was re-isolated from lung tissue collected on 3 and 6 dpi. Histologically, lesions were characterized as multifocal, mild to marked, interstitial pneumonia. Using in situ hybridization and immunohistochemistry we demonstrated the presence of viral RNA and proteins in respiratory tissues. This rhesus macaque model establishes the causal relationship between EMC/2012 and respiratory disease, thus fulfilling Kochâ€™s postulates. The model enables detailed studies on pathogenesis and transmission of this virus and will be a critical component for the evaluation of potential intervention strategies for this newly emerging coronavirus.

NIAID
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Postdoctoral Fellow
Virology - RNA and Retroviruses

TRAK2 control of mitochondrial dynamics and type I interferon expression is antagonized by the flavivirus NS5 protein

Flaviviruses include globally significant human pathogens such as tick-borne encephalitis virus (TBEV), West Nile virus and dengue virus that cause millions of human infections annually with high mortality and morbidity. To replicate, these viruses must avoid the antiviral effects of host type I interferon (IFN-I). Accordingly, flaviviruses delay early expression of IFN-I following infection although the virus-host interactions underlying this are largely unknown. Here, we show that the nonstructural protein, NS5, from TBEV inhibits IFN-I expression following infection with Sendai virus (SenV), a potent inducer of IFN. To understand the mechanism, we conducted a yeast 2-hybrid study that revealed an interaction between TBEV NS5 and a mitochondria-associated protein called trafficking kinesin-binding protein 2 (TRAK2). This interaction was confirmed by immunoprecipitation and immunofluorescence in virus-infected cells. Following virus infection, mitochondria serve as critical signaling platforms that transmit signals from cytosolic RIG-I-like helicases to downstream transcription factors resulting in production of IFN-I. To determine if TRAK2 was important for IFN-I production, three clonal knockdown HeLa cell lines were created by RNAi for TRAK2, the closely related protein TRAK1, and a non-targeting (NT) control. Following SenV infection, loss of TRAK2, but not TRAK1, resulted in 10 fold higher IFN-I expression compared to the NT control suggesting that TRAK2 is a negative regulator of IFN-I signaling. TRAK2 is known to interact with mitofusion proteins that mediate fusion of mitochondria, and mitochondrial fusion is required for early IFN-I expression. Hence, we examined the effects of TRAK2 knockdown on mitochondrial dynamics, which revealed that loss of TRAK2 delayed the fusion and elongation of mitochondria at 12 h post SenV infection. Finally, we examined the importance of TRAK2 during flavivirus replication. Loss of TRAK2, but not TRAK1, resulted in increased IFN-I production during TBEV infection and reduced the ability of flaviviruses to replicate by approximately 90% at 72h post infection. These findings demonstrate that TRAK2 is a novel regulator of the antiviral response by controlling mitochondrial dynamics and IFN-I expression. Furthermore, our results suggest that TRAK2 is a proviral factor utilized by the flavivirus NS5 protein to delay early IFN-I production and facilitate virus replication.

NIAID
Parra, Gabriel
Visiting Fellow
Virology - RNA and Retroviruses

Overcoming Antigenic Diversity in the Design of Novel Norovirus Vaccines

Noroviruses are important pathogens of acute gastroenteritis that can result in life-threatening dehydration, and an effective vaccine would save thousands of lives per year. A major challenge in norovirus vaccine development is the absence of an in vitro cell culture system for the virus, making it difficult to assess the role of neutralizing antibodies in protection. The norovirus major capsid protein (VP1) is divided into two structural domains, shell (S) and protruding (P), with the highly variable P domain linked to the marked antigenic diversity among strains. We have developed libraries of monoclonal antibodies (MAbs) and employed a molecular and structural approach to define functional epitopes on the norovirus capsid important in the design of vaccines. Ten distinct epitopes in the VP1 from Norwalk virus (prototype Genogroup I.1 [GI.1] virus) and six in GII.4 viruses (the most prevalent strain) were defined. Most MAbs were Genogroup-specific, but one recognized a highly conserved region in the S domain of the VP1 that represented the first known cross-reactive epitope conserved across the genus Norovirus. Using hemagglutination inhibition (HI) assays (a surrogate neutralization test for the noroviruses), competition assays and chimeric rVLPs with S and P domain swaps, we identified MAbs that targeted major protective antigenic sites within the P domain. One such GI.1-specific MAb that presented the highest HI titer competed against our newly-developed therapeutic scFab that protected chimpanzees from Norwalk virus infection in a proof-of-concept study for

norovirus neutralization, validating our approach. An effective vaccine will need to provide broad coverage against both GI and GII strains. Because the predominant norovirus strain is GII.4, we designed a consensus GII.4 rVLP as a novel vaccine candidate to increase coverage against emerging GII.4 strain variants. Reactivity patterns of the consensus VLP with our library of GII.4 MAbs confirmed the presence of epitopes from different strains that are likely involved in neutralization and protection. The consensus rVLPs were tested in experimental animals and shown to broaden immune responses against past and present GII.4 variants. In summary, our work has provided useful insight into the antigenic topology of the noroviruses that will facilitate the design of new norovirus vaccines.

NIAID-VRC
Lynn, Geoffrey
Doctoral Candidate
Hematology/Oncology, Tumor Immunology, and Therapy
Polymer Carriers of Toll-Like Receptor-7/8 Agonists Provide a Defined Chemical Approach for Regulating the Magnitude and Duration of Local Innate Immune Responses
Toll-like receptors (TLR) 7 and 8 are expressed by multiple dendritic cell subsets, monocytes and B cells. Therefore, small molecule agonists of TLR 7 and 8 (TLR-7/8a) provide a potent means for enhancing innate cytokine production and DC maturation, as well as killing by NK cells through indirect mechanisms. These characteristics make TLR-7/8a potentially useful for cancer immunotherapy and as vaccine adjuvants. Despite their potential, small molecule TLR-7/8a undergo rapid clearance following injection leading to transient (< 24 hours) and weak immune cell activation at target sites (e.g., tumor and draining lymphatics). To address these limitations and improve local activation, we have developed chemical strategies for linking multiple TLR-7/8a to biocompatible polymer carriers, referred to as Poly-TLR-7/8a, to retain the agonist within the tumor and proximal draining lymphatics following intratumoral injection. Combinatorial synthesis was used to prepare a number of unique Poly-TLR 7/8a. These were fluorescently labeled to facilitate tracking in vivo and administered into the footpads of mice. At serial time points, proximal draining lymph nodes were isolated and evaluated for polymer persistence and local innate immune stimulation using flow cytometry. We show that Poly-TLR 7/8a is retained in the proximal draining lymphatics for up to 23 days and is taken up by ~ 20-40% of all dendritic cells at peak response (~ 96 hr). Poly-TLR 7/8a enhanced local dendritic cell recruitment (> 50 fold, relative to the small molecule TLR-7/8a), upregulated CD80 and CD86 co-stimulatory molecule expression and induced high levels of Th1-polarizing cytokines (IFN-gamma and IL-12p40). No systemic toxicity was observed, as serum cytokines (IL-6, IL-12p40, etc.) were undetectable in mice that received Poly-TLR-7/8a. Finally, varying only the molecular weight of Poly-TLR-7/8a from 5 to 70 kilodaltons enhanced polymer persistence and duration of local innate immune activation over a range of 4 to 23 days. Altogether, we have shown that Poly-TLR-7/8a is more potent and less toxic than currently available small molecule TLR-7/8a and that polymer carriers provide a chemically tunable means for modulating the magnitude and persistence of local innate immune stimulation at target sites. Our current efforts are focused on evaluating whether persistence of local innate immune stimulation by Poly-TLR 7/8a can reverse immunosuppression and promote tumor clearance in mice.

NIAID-VRC
Wheatley, Adam
Visiting Fellow
Immunology - Infectious Disease
Prime-boost vaccination against influenza H5N1 elicits the expansion of potentially neutralising, cross-reactive memory B cells.

Immune protection against influenza is primarily mediated via antibodies raised against the major viral surface proteins; neuraminidase and the viral entry receptor hemagglutinin (HA). However, the rapid emergence of H5N1 â€œavianâ€ and H1N1 â€œswineâ€ demonstrates the susceptibility of human populations to novel virus serotypes despite previous cycles of infection and/or vaccination, which might be expected to favour cross-protective responses. To study the generation of antibody responses to novel HA serotypes in the context of widespread pre-existing influenza immunity, we examined longitudinal samples from the VRC310 clinical trial where subjects were primed with DNA expressing H5 and boosted with H5N1 monovalent influenza vaccine. Using novel HA probes and flow cytometry, we found the memory B cell response to H5 was dominated by cross-reactive B cells that bound both H5 and H1 antigens. In most subjects, a small population of H5/H1 cross-reactive B cells was identified at baseline that underwent significant expansion in response to H5 vaccination. Broadly neutralising antibodies (bNAbs) described to date target one of two conserved regions of HA; the sialic acid receptor binding site at the tip of the globular head of the HA trimer or alternatively, an exposed region within the HA stem. Based upon epitope-specific inhibition with scFv fragments of the prototypic bNAb F10, we established the majority of cross-reactive B cells target the HA stem. A selection of recombinant antibodies were cloned from single cross-reactive B cells, confirmed to be stem-binding and mediated broad neutralisation by in vitro infectivity assays. In common with previously described bNAbs, H5/H1 cross-reactive memory B cells displayed preferential usage of VH1-69 germlines. Disappointingly, within the sera of vaccinees we observed only a transient increase in the proportion of antibodies directed at the HA stem as measured by competition ELISA, which rapidly returned to baseline levels post-vaccination. We suggest the transient nature of cross-reactive, potentially neutralising antibody responses may contribute to the puzzling lack of broadly protective influenza immunity in the general populace. Further clarification of the mechanisms driving the persistence of memory B cell responses may significantly speed the development of a long-sought universal influenza vaccine that overcomes the narrow protection afforded by currently available products.

NIAID-VRC
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Visiting Fellow
Informatics/Computational Biology

HIV-1 Antibody Epitope Prediction Based on Neutralization of Diverse Viral Strains

Objective: Identification of the precise epitopes targeted by HIV-1 broadly neutralizing antibodies is a key step to the development of epitope-based HIV-1 vaccines. Ideally, epitope residues are determined in the context of antibody-antigen complex structures, though structure determination may in many cases be infeasible. Variation of antigen sequence within an antibody epitope is more likely to affect the antibody neutralization potency than variation outside of the epitope. Hence, neutralization data on diverse viral strains may encode substantial information about an antibody epitope. The goal of this research is to develop a bioinformatics tool to identify antibody epitope residues by analyzing the neutralization activity of a given antibody against a set of viral strains. Methods: The algorithm ranks antigen residues based on the mutual information score between the amino acid type of each residue position and neutralization potency for each strain. The prediction accuracy of the algorithm is enhanced by incorporating structural information of the unbound antigen. Specifically, antigen structure information was used to filter out: (1) surface inaccessible residues and (2) top-ranking (mutual information) residues that were not in the vicinity of other residues with high mutual information scores. Results: The algorithm was evaluated on a neutralization panel of 181 HIV-1 strains and 19 antibodies, for which antibody-antigen structures were available. When ranking each residue by mutual information alone, the method gave an average of 4.2-fold enrichment of accurately predicted epitope residues at a 5% false positive rate, compared to random selection (p<0.01). Incorporating structural

information of the unbound antigen, the method gave an average of 7.7-fold enrichment of accurately predicted epitope residues at a 5% false positive rate compared to random selection (p<0.001) and performed significantly better than ranking with mutual information alone (p<0.001). Conclusion: Since neutralization assays are a standard step in the evaluation of antibodies against viruses such as HIV-1 and influenza, the method described here could serve as an efficient screening tool to predict antibody epitope residues.

NIAMS
Fontana, Juan
Visiting Fellow
Protein Structure/Structural Biology
Low pH induces a conformational change in the Influenza virus M1 matrix protein that provokes the dissociation of the M1 layer from the viral envelope, rendering the envelope more pliable and therefore conducive to fusion
The envelope of Influenza A virus has three integral proteins (hemagglutinin HA, neuraminidase NA, and ion channel M2) embedded in a lipid bilayer. Underlying the viral envelope, the M1 protein forms a matrix layer that has been assigned multiple roles in virion assembly and infection. However, previous studies by cryo-electron microscopy (cryo-EM) have given differing accounts of the number of layers in the M1/envelope complex and their thicknesses and compositions. Additionally, exposure of virions to low pH, which promotes membrane fusion by inducing conformational changes in HA, has been correlated with release of the M1 layer from the viral envelope. With the goals of (i) clarifying the conflicting accounts of the M1/envelope complex; and (ii) establishing how many distinct states of this complex exist at both neutral and acidic pH (4.9), I performed cryo-EM and cryo-electron tomography on influenza virions mixed with liposomes (used to provide lipid bilayer references). To reduce the impact of virion heterogeneity on the comparison, the same fields were imaged by both methods. Subtomogram classification and averaging was subsequently performed to afford preciser measurements of the thicknesses of the M1/envelope components and the distances between them, and to probe for alternative conformations of the complex. At neutral pH, there are two kinds of complexes, corresponding to a lipid bilayer with embedded glycoproteins, with and without a closely associated, 4 nm-thick, sheet of M1 protein. The reported discrepancies arose from differences in imaging conditions; i.e. in defocus and in whole-particle projections vs. thin tomographic slices. After 5 min at pH 4.9, the proportion of virions lacking an M1 layer increases from 10% to 50%. In pH 4.9/5 min virions retaining an M1 layer, the M1/envelope complex exhibits two states: viz., the original, neutral pH state and one in which the M1 layer appears thinner and/or closer to the membrane. We infer that the tight state is a manifestation at the virion level of a conformational change in M1 that disrupts the M1-M1 and M1-envelope interactions and preludes dissociation of the M1 layer from the viral envelope. Taken together, these observations extend previous indications that acidic pH causes the M1 layer to dissociate, leaving the envelope more pliable and consequently fusion-compatible, and show that dissociation is preceded by a conformational change in M1.

NICHD
Hammond, Gerald
Research Fellow
Biochemistry - General and Lipids
A novel role for phosphatidylinositol 4-phosphate at the plasma membrane
Defects in the metabolism of phosphatidylinositol 4-phosphate (PI4P) and phosphatidylinositol 4,5-bisphosphate (PIP2) cause a litany of diseases, from cancer to ciliopathies. These lipid molecules act as membrane-docking sites, cofactors or substrates for proteins involved in many cellular processes, from

membrane traffic to the recruitment of signalling proteins. Therefore, understanding how cells synthesize and distribute these molecules is crucial to understanding basic cellular function as well as the etiology of many diseases. PI4P acts on endomembranes, but is also the intermediate in the synthesis of PIP2, which acts at the plasma membrane (PM). Since mammalian cells contain equivalent amounts of PI4P and PIP2, the PM was hypothesized to contain a relatively small proportion of cellular PI4P, dedicated solely to PIP2 synthesis; here, we test this hypothesis. Using a newly designed GFP-fused PI4P biosensor in live cells, we found that PI4P is localized to the PM and Golgi. Specificity of the probe was confirmed using chemical genetic-induced recruitment of a PI4P-degrading phosphatase to either the PM or Golgi, which caused the release of the biosensor selectively from the targeted organelle. Recruitment of a catalytically inactive control phosphatase was without effect. Surprisingly, depletion of PI4P in any membrane had no effect on the PM localization of a PIP2 biosensor. Furthermore, using a novel pharmacological inhibitor of a kinase that synthesizes PI4P, we found depletion of the PI4P biosensor from the PM, with no effect on PIP2 biosensors. We confirmed these results by biochemical extraction and measurement of total cellular PI4P and PIP2 levels after treatment with the inhibitor, which caused large reductions (>80%) in PI4P but comparatively small (<20%) changes in PIP2. The localization of several signalling proteins thought to be targeted to the PM by an interaction between polybasic motifs and PIP2 were unaffected by chemical genetic-induced recruitment of PIP2 or PI4P-degrading phosphatases. However, a novel fusion protein carrying both phosphatases efficiently depleted these proteins from the PM, whereas control proteins targeted to the PM via other motifs (such as palmitoylation) were unaffected. We conclude that the major fraction of cellular PI4P is in fact present in the PM, although it does not serve as a precursor for PIP2 synthesis. Instead, PI4P co-operates with PIP2 to recruit polybasic-motif containing proteins to the PM.

NICHD
Arimbasseri, Gopalakrishnan
Visiting Fellow
Biochemistry - Proteins
The non-template DNA strand plays a major role in transcription termination by RNA polymerase III
How eukaryotic RNA polymerases switch from elongation to termination is unknown. Transcription termination by RNA polymerase III (pol III) is unique as a model because the termination signal is a simple tract of 6-9 T residues on the non-template (NT) strand (oligo-dA on the template) and because pol III is the best characterized of the eukaryotic pols for the final step, disengagement of the active center from the transcript. Elongation complexes (EC) are extremely stable due to stability of the RNA/DNA hybrid that forms during transcription. The oligo-rU.dA hybrid is known to be very much weaker than other RNA/DNA hybrids and this instability was believed to be a trigger for pol III termination. We tested this and also whether the NT strand plays a role in termination by purified (S. cerevisiae) pol III. We reconstituted ECs scaffolds from pol III, NT and template strand DNAs, and an RNA primer. This approach provided unique opportunity to alter the identity of NT strand terminator residues while maintaining oligo-dA in the template strand. Initial data indicated termination was significantly reduced in efficiency and the position of RNA chain termination within the oligo-dA tract, suggesting multiple roles for the NT strand in termination. Experiments with NT strands bearing mismatches in various regions of a 9T terminator suggest three distinct functional regions of the NT strand. The first is duplex DNA 5 nucleotides (nt) downstream of the active site. Interaction of this region of DNA and polymerase were previously found to increase EC stability and consistent with this we show that mismatches here enhance termination. Second is the strand separation region (SSR) immediately downstream of the catalytic center where the incoming template and NT strands are melted to create the transcription bubble. SSR mismatches decreased termination, we propose by facilitating separation or stabilizing the melted structure, thereby enhancing elongation. The third region is just upstream of

the active site. Since this region is already in the single stranded bubble, these data suggest the novel possibility that base-specific recognition of dT may enhance termination. We used several nt analogs differentially positioned in the terminator NT to distinguish between the potential for base-pairing and sequence-specific recognition of thymine. The results of these experiments support a model of sequence-specific effects of the NT as important for pol III termination.

NICHD
Visweswaraiah, Jyothsna
Visiting Fellow
Biochemistry - Proteins
Role of an eukaryotic 40S ribosome exit-channel protein (Rps5) in accurate start codon selection
In eukaryotic translation initiation, the 43S pre-initiation complex scans the mRNA leader for an AUG triplet in favourable sequence context. When an AUG occupies the ribosomal P-site, the upstream â€œcontextâ€ nucleotides at -3 to -1, which are highly enriched for adenines in yeast, occupy the mRNA exit channel. Hence, 40S ribosomal proteins near the exit channel might play a role in accurate AUG selection. To explore this possibility, we mutated conserved residues in the ß-hairpin of yeast Rps5, located near the exit channel, and screened the mutants in vivo for altered fidelity of AUG selection: either increased initiation at near-cognate UUG codons (Sui- phenotype) or decreased UUG initiation in cells harbouring a Sui- mutation in another factor (Ssu- phenotype). Interestingly, we identified Sui- substitutions in the upper portion of the ß-hairpin that increase the ratio of UUG to AUG initiation by 4 to 5-fold, measured with matching HIS4-lacZ reporters differing in these start codons. It is known that Sui- mutations in eIF1 mask the effect of poor context and thereby increase recognition of AUGs in poor context, including the initiation codon of its own (SUI1) mRNA. Surprisingly, however, we found that eIF1 expression is markedly reduced in the rps5 mutants, and assaying SUI1-lacZ reporters with either native or optimized context showed that SUI1 translation was reduced specifically with the native (poor) context surrounding the AUG codon. Furthermore, restoring eIF1 to wild-type levels eliminated the elevated UUG:AUG initiation ratio of the rps5 mutants, indicating that their Sui- phenotypes result from reduced eIF1 translation. Assaying GCN4-lacZ reporters revealed that the rps5 mutations also increase â€œleaky scanningâ€ past an upstream out-of-frame AUG codon. Together, these results imply that the rps5 mutations reduce AUG recognition by impairing recognition of the context nucleotides (or another unknown feature of the initiation region) rather than the AUG itself. Remarkably, we also identified Ssu- substitutions affecting residues in the lower portion of the hairpin that specifically discriminate against near-cognate (UUG) start codons and do not reduce recognition of AUGs in either optimum or poor context. Thus, different regions of the Rps5 ß-hairpin influence distinct aspects of start codon recognition. These results provide the first functional evidence implicating a structural component of the 40S ribosome mRNA exit channel in AUG recognition.

NICHD
Avram, Alexandru
Visiting Fellow
Biophysics
Whole-brain assessment of mean axon diameters using multiple pulse-field gradient (mPFG) diffusion MRI
Measuring microstructural features of neurons, e.g. axon diameters, provides valuable neurophysiological and functional information. The recently proposed multiple pulsed-field gradient (mPFG) diffusion MRI technique applies concatenated diffusion blocks with varying orientations to exclusively quantify the motions of water molecules trapped in restricted compartments, thereby providing a unique measure of average cell geometry that cannot be obtained with conventional

diffusion MRI methods such as diffusion tensor imaging (DTI). Advances in diffusion encoding strategies and theoretical modeling have recently enabled the first in vivo application of mPFG MRI to measuring axon diameters of white matter pathways in the human corpus callosum. In this study we optimize the pulse sequences and clinical scan parameters to conduct mPFG MRI with whole-brain coverage and assess the variability of the axon diameter measurements along specific fiber pathways. Within a 25 minute scan, mPFG MRIs with whole-brain coverage at 2.8mm isotropic resolution were acquired in healthy volunteers using a newly developed 3D mPFG diffusion encoding scheme. Intra-voxel fiber orientations measured from co-registered DTIs were incorporated into our white matter tissue model which approximates myelinated axons with parallel impermeable cylinders, and the mPFG MRI data were fit to obtain estimates of mean axon diameters, intra- and extra-axonal diffusivities. Median values of the estimated parameters along individual fibers fell within the expected physiological ranges and were in great topographical agreement with the results of the recently published mPFG MRI assessment of fibers in the corpus callosum. Variations of mean axon diameters along individual fiber pathways were restricted to regions of high curvature and fiber crossings where the intra-voxel fiber orientation distribution deviated significantly from that assumed in our model of parallel fibers. Our results establish the clinical feasibility of using whole-brain mPFG diffusion MRI to measure neuronal microanatomical information complementary to DTI, with potential clinical significance. Upon further validation, mPFG MRI may provide a non-inasive, whole-brain histological assessment that could prove transformative to neuroimaging and neuroscience.

NICHD
Cohen, Sarah
Visiting Fellow
Cell Biology - General
Cells respond to starvation by increased flux of lipids through lipid droplets
Lipid storage in cells is crucial because it allows organisms to survive variations in the availability of energy. The storage and utilization of lipids are impaired in a variety of disorders, including obesity and diabetes. Within the cell, energy is stored in the form of neutral lipids surrounded by a phospholipid monolayer, in organelles called lipid droplets (LDs). It is likely that LDs play a key role in shuttling lipids between cellular compartments in response to changing metabolic needs. It has been shown that in response to excess fatty acids cells store more lipids. LDs associate with the endoplasmic reticulum, a major site of lipid synthesis, resulting in LD growth. However, what happens to LDs during the opposite condition â€“ starvation â€“ is unknown. We hypothesized that the size and quantity of LDs would decrease as cells used stored energy. Surprisingly, when we quantified LDs in response to starvation of mouse embryonic fibroblasts we found that after 24 hours of starvation the number of LDs increased while the LD size remained constant compared to un-starved cells. To investigate the cellular mechanism feeding lipids into LDs we included inhibitors of autophagy, a cellular recycling process that breaks proteins and organelles down into their constituent parts. This prevented the increase in LD number during starvation, suggesting that autophagy supplies fatty acids for continued LD biogenesis. We next asked how the rate of lipid utilization is affected under starvation conditions. We developed a pulse-chase assay in which cells were pulsed with a fluorescent fatty acid (FFA). Immediately following the pulse, thin layer chromatography and fluorescence microscopy revealed that the majority of FFA had been incorporated into neural lipids inside LDs. After chasing for 24 hours with complete medium, the FFA remained in LDs, while after starvation the FFA localized to mitochondria, the primary site of fatty acid metabolism. We also found that reducing the metabolic potential of the mitochondria through genetic mutations that cause mitochondrial fragmentation prevented the transfer of FFA from LDs to mitochondria. In conclusion, we have found that cells respond to starvation by increasing the flux of lipids through LDs. Fatty acids are supplied by autophagy and then transferred from LDs to

mitochondria, where they are metabolized to provide energy for the cell. These results reveal an important role for LDs in the cellular response to starvation.

NICHD
Ritter, Alex
Doctoral Candidate
Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology
A novel mechanism regulating cortical actin density controls secretion in cytotoxic T lymphocytes.
Cytotoxic T Lymphocytes (CTL) play a vital role in the immune system as they are responsible for destroying virally infected and tumorigenic cells. CTL kill their targets through polarized secretion of lytic granules, which are specialized lysosomes that contain a potent cocktail lytic proteins. CTL are serial killers, able to destroy multiple target cells in quick succession. Although secretion of only a few granules is sufficient to kill a target cell, each CTL contains upwards of 25 lytic granules, which it uses to terminate sequential targets. When a CTL encounters a target cell, it secretes only a fraction of its lytic granules before moving on to the next one. However, it is unclear how CTL mediate selective release of only a subset of their granules. The cortex of mammalian cells consists of a thick layer of branched actin which has been proposed to act as a barrier to secretion. We hypothesized that CTL manipulate the density of this cortical actin barrier to control the number of lytic granules released toward a target cell. Using high-resolution live cell imaging techniques to image fluorescently-labeled actin in CTL, we have observed that upon CTL recognition of a target cell, the density of cortical plasma membrane actin at the interface between the CTL and the target is greatly diminished. In order to more clearly image the activation interface of the CTL, we used a technique to direct CTL interaction and secretion toward a glass coverslip. Imaging CTL interacting with the coverslip, we simultaneously monitored actin organization and lytic granule secretion at the activating interface of the CTL with high spatiotemporal resolution. We found that the density of cortical actin decreases as the CTL interacts with the glass, but just after granule secretion, a wave of actin polymerization emanates from the degranulation site, increasing the density of actin covering the interface. This actin â€œwallâ€ appears to prevent further granule secretion, as lytic granules that are visible close to the plasma membrane do not fuse. When cells are then treated with latrunculin A, a small molecule which destabilizes actin polymers, the actin â€œwallâ€ disappears and large granules immediately and continuously fuse with the plasma membrane. This data suggests that secretion of a few lytic granules induces actin polymerization at the CTL-target interface that acts as a barrier preventing further secretion, thus preserving lytic granules for future CTL targets.

NICHD
Sampley, Megan
Postdoctoral Fellow
Chromatin and Chromosomes
Molecular Mechanisms of Transcription Regulation of the Imprinted Genes H19 and Igf2
The Igf2 and H19 genes are coordinately regulated, imprinted genes in which defective transcription is implicated in several cancers and growth disorders such as Silver-Russell Syndrome and Beckwith-Wiedemann syndrome. Igf2 encodes a paternally expressed growth factor and is positioned next to H19, a maternally expressed non-coding RNA linked to tumor suppression. In addition to implication in disease pathogenesis, the Igf2/H19 imprinted locus is a prime model of the interplay between epigenetic and genetic elements in transcription. An imprinting control region (ICR) located between Igf2 and H19 marks the alleleâ€™s parental origin. On the maternal allele, the ICR is bound by CTCF, which insulates and silences Igf2. On the paternal allele, the ICR is marked by CpG methylation, which spreads to and silences the H19 promoter. In myocytes, expression depends on a muscle-specific

enhancer (ME). To determine how the ICR and ME interact to direct gene expression, we analyzed two mutations at the Igf2/H19 locus: 1.) a mesoderm enhancer deletion (delta-ME) and 2.) an insertion of the ICR at an ectopic location that insulates both Igf2 and H19 on the maternal allele (H19R). Strikingly, ChIP-chip microarray analysis of RNAPII-binding and mRNA expression analysis show extensive enhancer-dependent polymerase activity and transcription that is contained within, but broadly active across the entire 120kb locus with a surprising amount of intergenic transcription between H19 and Igf2 that is completely lost in delta-ME cells. Allele-specific chromatin immunoprecipitation (ChIP) in mutant and wild type myoblasts further show high bi-allelic enrichment of myogenic transcription factors, CTCF, and RNAPII at the ME. Furthermore, in the H19R mutants where the enhancer is blocked from looping with the H19 promoter, binding of these factors accumulates compared to wild type. Altogether, these data suggest that the ME is a scaffolding platform that recruits transcription machinery and transfers it to looped promoters during gene activation through a facilitated tracking model of enhancer action whereby the ME tracks within the 120kb locusâ€"generating non-coding intergenic transcripts as a byproductâ€"and assembles target promoters into an ordered transcription unit dictated by the methylation status of the ICR. These studies add information to the model of how two alleles with the same genetic code can form different higher-order chromatin structures in the same cell.

NICHD
Ahrens, Katherine
Postdoctoral Fellow
Epidemiology/Biostatistics - Etiology, Risk, and Prevention
The effect of physical activity on reproductive hormones across the menstrual cycle and the risk of sporadic anovulation
Background: High-intensity physical activity (PA) is associated with menstrual dysfunction and subfertility in female athletes, but the effect of PA on menstrual cycle function among more moderately active women is unclear. Methods: We evaluated the associations between PA, reproductive hormones, and anovulation using data from the BioCycle Study (2005-2007), which followed 259 premenopausal, healthy women prospectively for up to 2 menstrual cycles (N=509 cycles). Serum levels of estradiol, progesterone, luteinizing hormone, follicle-stimulating hormone, testosterone and leptin were measured up to 8 times per cycle. The long-form International Physical Activity Questionnaire (IPAQ, administered at baseline) assessed habitual PA, the short-form IPAQ (administered 4 times per cycle) assessed past-week PA and daily diaries assessed minutes spent each day in vigorous activity. Linear mixed models were used to estimate the effect of each PA measure on average hormone concentrations across the menstrual cycle. Risk ratios (RR) for sporadic anovulation (n=42 cycles) were estimated using log-binomial models. All analyses adjusted for body mass index, race, age, and perceived stress. Time-varying PA models further adjusted for concurrent reproductive hormone levels (using inverse probability weighing) and habitual PA. Results: Habitual PA was not associated with differences in hormone levels. High past-week PA was associated with lower leptin (-5.7%, 95% confidence interval [CI]: -10.0, -1.3) and luteal phase progesterone levels (-18.9% [-33.9, -0.5]) as compared to the lowest tertile. Any vigorous PA during the previous day was also associated with lower leptin (-5.8% [-8.0, -3.2]) and luteal phase progesterone levels (-18.0% [-28.4, -6.0]) compared to no vigorous PA. High habitual PA showed a lower risk of anovulation compared with the lowest tertile (RR 0.54 [0.23, 1.29]), while cycles with high past-week PA (RR=1.5 [0.6, 3.4]) and high previous day vigorous PA (RR 1.66 [0.76, 3.65]) showed a higher risk of anovulation. However, none of these RRs were statistically significant. Conclusions: Overall, PA was modestly associated with selected reproductive hormones and was not observed to influence sporadic anovulation in healthy, premenopausal women. It is important to understand the effects of moderate exercise on reproductive hormones since it may have implications for fecundability as well as for chronic disease risk.

NICHD
Hinkle, Stefanie
Postdoctoral Fellow
Epidemiology/Biostatistics - Etiology, Risk, and Prevention
The association between parity and birthweight in a longitudinal consecutive pregnancy cohort
Background: Nulliparity has been associated with lower birthweight, however, in most prior studies data on important confounding variables are unavailable, and longitudinal studies are rare. Differences observed in birthweight by parity may be partially due to selection bias in prior cross-sectional studies, which include some nulliparous women who do not go on to have other children and therefore are not fully comparable to multiparous women. Methods: We used longitudinal electronic medical record data from a hospital-based cohort of 39,499 women with parity 0-6 at study entry, and who had at least 2 singleton deliveries >=37 weeks from 2002-2010 in Utah. We calculated infant gestational age and sex-specific birthweight z-scores with infants of nulliparous women as the reference. Using linear mixed models to account for repeated data within women, we estimated the association between parity and birthweight z-score. All models were adjusted for pregnancy-specific variables including maternal prepregnancy body mass index, gestational weight gain, sociodemographics, smoking and alcohol use, chronic diseases, and pregnancy complications. Results: Based on the cross-sectional assessment at the first pregnancy in the study, infants of nulliparous women were lighter on average by 31.7 g [95% confidence interval (CI) 21.4, 42.0] or 0.21 z-score units [95% CI 0.18, 0.23] than infants of primiparous women. Within nulliparous women, the difference in birthweight between the first and second pregnancies was 16.8 g [95% CI 11.0, 22.6], about half that observed in the cross-sectional analysis, but the z-score change was similar, 0.19 [95% CI 0.18, 0.21]. Using longitudinal adjusted models, there was a significant departure in linearity over parity 2 to 7 (P=0.01). Compared to infants of nulliparous women, infants of primiparas were larger by 0.21 z-score units [95% CI 0.19, 0.22] and parity was associated with an increase in z-score only up to parity 4, (parity 4 vs. 0 ß =0.37, 95% CI [0.34, 0.41]). Beyond parity 4, there was no significant additional increase in birthweight z-score with increasing parity (parity 7 vs. 0 ß =0.34, 95% CI [0.22, 0.47]). Conclusions: The association between parity and birthweight is independent of maternal demographic, medical, or weight-related changes that occurred between pregnancies, and non-linear with the greatest increase observed between first and second-born infants of the same mother.

NICHD
RAY, PAYAL
Visiting Fellow
Epigenetics
Identification of Combgap as a novel DNA-binding protein necessary for epigenetic silencing by Polycomb group proteins
Polycomb group proteins (PcG) are a class of transcriptional regulators that mediate the epigenetic repression of genes involved in development, cellular differentiation and cell proliferation. Disruption of PcG repression has been implicated in several types of cancer. In Drosophila, where PcG proteins were first identified, members of the PcG protein family form multi-protein complexes that interact with chromatin via cis-elements known as Polycomb Response Elements (PRE). These complexes, known as PRC1 and PRC2 (PcG Repressive Complex 1 and 2), are recruited to the PREs by the DNA-binding protein Pho (Pleiohomeotic). There are several DNA-binding proteins that function in association with Pho to recruit the PRCs. However, the identities of some of the DNA-binding proteins and the exact mechanism of recruitment of PRC1 and PRC2 to the PREs remain unknown. PREs range from several hundred to a few thousand base pairs in length and are made up of binding sites for multiple DNA-binding proteins.

We aimed to identify the DNA-binding proteins that interact with the Drosophila engrailed PRE. Engrailed is an essential gene for Drosophila development and a well-established PcG target. Previous studies from our group have identified a 139 bp region that contains binding sites for the PcG proteins Pho, Spps (Sp1-like factor for Pairing Sensitive-silencing), GAF (GAGA Factor) and acts as a PRE. This fragment also contains binding sites for two unknown proteins and mutation of these sites abrogates PRE activity in functional studies. To identify these proteins we performed a biotin-streptavidin pull-down coupled with Mass Spectrometry (MS). MS analysis identified Combgap as one of the candidate proteins that binds to the 139 bp PRE fragment. Combgap was reported to represses the expression of a PcG target gene, Cubitus interuptus but the mechanism of this repression is unknown. In immunostaining experiments we observed a colocalization of Combgap with the PRE-binding protein Spps on polytene chromosomes, which suggests an interaction of Combgap with the PcG machinery. A genome wide ChIP-seq study showed Combgap binding at many PREs within the Drosophila genome, including the 139bp engrailed PRE. We validated these results by quantitative PCR on a subset of the target genes identified in the ChIP-seq. Our studies have identified Combgap as a novel protein that binds to the PREs. We are currently investigating the role of Combgap at PREs and in PcG recruitment.

NICHD
Singh, Parmit
Visiting Fellow
HIV and AIDS Research
A new high-density procedure for sequencing positions of HIV-1 integration identified a preference of insertion near the 5â€™ end of transcription units and at splice junctions
Safety concerns now exist with retrovirus-based vectors used in gene therapy because the integration of murine leukemia virus vectors next to oncogenes induced these genes and resulted in cases of leukemia in patients. Now that lentiviruses are being adopted for use in gene therapy there is great need to determine whether these vectors are safe to use. It is therefore essential to create a high-density profile of HIV-1integration. Previous studies of 40,000 unique HIV-1 integration sites revealed that transcribed genes are preferred targets. However, the number of insertions was insufficient to address important question such as does HIV-1 integrate into specific sets of genes and what are the genomic features that influence the selection of integration sites? The existing methods of ligation mediated PCR for sequencing insertion sites are limited because they are unable to distinguish multiple insertions at single nucleotide positions from PCR amplification of a single insertion event. To greatly increase the numbers of insertions sequenced I developed a serial number system that tags each insertion event with a unique 5 bp sequence ID. A collaborator isolated genomic DNA from 293T and HOS cells infected with a one-round HIV-1 vector. I digested the genomic DNA with restriction enzyme Mse I and then ligated these fragments to a serial number linker that contained 5 bps of randomized sequence. I then used 96 PCR reactions to make libraries for sequencing. By converting the format of our PCR primers from 454 to the Illumina platform I substantially increased the read capacity of the existing methods. Using these innovations I obtained a dense profile of 2,661,143 and 582,345 independent insertions in 293T and HOS cells, respectively. My results are not only a substantial improvement over the previously published report of HIV-1 integration with 40,000 sites but also are the first integration data for any system to quantify how much integration occurs at single nucleotide positions. The integration efficiency at the sites varied widely with 50% of the total sites containing 2 to over 1000 insertions. My analysis of this data revealed HIV-1 has a unique preference for the 5â€™ end of transcription units as well as splice sites. These data shed new light on the integration mechanism of HIV-1 and will help evaluate how safe lentivirus vectors are for use in gene therapy.

NICHD

Gupta, Monica
Visiting Fellow
Immunology - General
Interferon Regulatory Factor 8 (IRF8), a master regulator of IFN-gamma/TLR stimulated Autophagy in Macrophages and promotes clearance of self proteins and bacterial pathogens
Autophagy plays a pivotal role in innate and adaptive immunity. The process involves the sequestration of cytoplasm and organelles into double-membrane autophagic vacuoles which subsequently breakdown within lysosomes. Autophagy is also involved in the clearance of intracellular bacteria and viruses in dendritic cells (DC`s) & macrophages (Mphages), and facilitates efficient MHC II presentation to T helper cells by these cells. Till now, more than 20 genes have been identified to be involved in the execution of autophagy, however it`s regulation in immune specific cells is not well understood. In the present study we found that Interferon Regulatory Factor 8 (IRF8), a transcription factor essential for myeloid cell function, plays a critical role in Interferon-gamma (IFN-gamma)/Toll like receptors (TLR`s) signal induced autophagy in bone marrow derived Mphages and DC`s. Microarray and ChIP on chip analyses on IRF8+/+ and IRF8-/- DC`s revealed that IRF8 regulates expression of some autophagy related genes. The extension of these analyses led to the observations that in response to IFN-gamma/TLR signaling IRF8 stimulates expression of at least 17 autophagy genes in Mphages. Further tests found that out of 17 genes, 12 genes could be rescued for expression in IRF8-/- Mphages upon Irf8 gene transfer. Moreover, we show that IRF8 directly binds to the promoter regions of 7 of these genes, demonstrating that IRF8 is a major regulator of autophagy gene expression. Accordingly, biogenesis and maturation of autophagosomes, a unique structure formed during autophagy, is profoundly impaired in IRF8-/- Mphages as compared to WT Mphages. Furthermore, we found that intracellular degradation of ubiquitinated proteins/organelles is defective in IRF8-/- Mphages, a pathological hallmark of defective degradation processes including autophagy. Interestingly, WT Mphages when infected with bacterial pathogen, Listeria monocytogenes, showed increase in autophagy and most of the bacteria were found to be localized within autophagosomes. On the other hand, in IRF8-/- Mphages induction of autophagy was not observed in response to the bacterial infection explaining the reason behind poor clearance of bacteria in IRF8-/- Mphages. Taken together, our results provide new insights into the mechanism of regulation of autophagy in Mphages. We have identified IRF8 as an important regulator of IFN-gamma/TLR signal induced autophagy, thereby critically contributing to innate immunity.

NICHD
Jovic, Marko
Visiting Fellow
Intracellular Trafficking
An Unsuspected Role for a Lipid Kinase in SNARE-mediated Vesicle Fusion
Soluble NSF attachment proteins (SNAREs) facilitate vesicular fusion, a process driven by interaction of complementary SNAREs present on a vesicle and a target compartment. This event is ubiquitous to all trafficking processes, ranging from synaptic transmission to the uptake of nutrients such as glucose, cholesterol and iron. However, there is a lasting question regarding regulation of the SNARE-SNARE pairing preference. Although highly promiscuous in vitro, all 63 SNAREs exhibit amazing binding specificity in vivo, resulting in a limited number of pairing combinations. Our study addresses this important question of how the SNARE specificity is achieved in vivo. Using tandem mass-spectrometry we identified phosphatidylinositol 4 phosphate (PI4P)-kinase IIa (PI4K2A) as a novel binding partner of VAMP3, a small SNARE mediating fusion of endosomes with the Golgi and recycling compartment. We tested the role of the kinase on VAMP3 localization using a PI4K2A knockdown approach that resulted in increased VAMP3 missorting into late endosomes, as determined by increased co-localization with a late-endosomal protein LAMP-1. To further explore this effect in live cells we used a photobleaching

approach, measuring the rate of fluorescence recovery of GFP-VAMP3 at the post-fusion membranes. VAMP3 appearance at target membranes was significantly delayed in PI4K2A knockdown cells, but not in cells treated with control oligos. As an additional control we found that VAMP3 kinetics was not affected by inhibition of another PI4K, PI4KB, which does not interact with VAMP3. Next we addressed the potential role of the PI4K2A product, PI4P, on VAMP3 localization using a novel approach of targeted, acute PI4P depletion in live cells. To this aim we used the PI4P-metabolizing enzyme Sac1 engineered to rapidly re-localize from cytosol to specific organelles upon stimulation. While acute depletion of PI4P at the Golgi or plasma membrane (i.e. VAMP3 target compartments) had little effect, PI4P depletion in endosomes, from which VAMP3 facilitates fusion, led to a dramatic delay in VAMP3 appearance at post-fusion target membranes. Taken together, our findings uncover an unsuspected new role for PI4K2A and its lipid product PI4P in regulation of SNARE distribution. As the first in vivo link between lipid regulation and vesicle fusion, direct PI4K2A-SNARE interaction opens up new prospects in exploring the broader coordination between SNAREs and numerous lipid-modifying enzymes.

NICHD
Schindler, Christina
Visiting Fellow
Intracellular Trafficking

Identification of the endosome-associated recycling protein (EARP) complex, a novel complex implicated in endocytic recycling to the plasma membrane

Correct sorting of cargo proteins within cells and maintenance of organelle integrity is crucial for cell and organism health and survival. Underlying this process is a series of tightly regulated transport carrier generation and fusion events. The Golgi-associated retrograde protein (GARP) complex composed of Ang2, Vps52, Vps53 and Vps54 is responsible for tethering of retrograde carriers to the trans-Golgi network (TGN), a process that is poorly characterized despite its implication in motoneuron degeneration in the wobbler mouse model of Amyotropic Lateral Sclerosis (ALS). In the present study, we establish that the GARP components Ang2, Vps52 and Vps53 and an uncharacterized protein CCDC132 form a novel heterotetrameric endosomal complex that we termed endosome-associated recycling protein (EARP) for its crucial role in the recycling of the transferrin receptor (TfR) to the cell surface. CCDC132 was identified as a top hit in a mass spectrometry analysis upon tandem-affinity purification using Ang2, Vps52 and Vps53 as baits. Sedimentation and gel filtration analysis showed that CCDC132 is part of a complex with similar biophysical properties to GARP. Co-immunoisolation revealed mutual interactions of CCDC132 with all GARP subunits except for Vps54. In addition, RNAi-mediated knockdown of CCDC132 resulted in a partial reduction in the levels of Ang2, Vps52 and Vps53, but not Vps54. Co-localization with EEA1, Rab4 and Rab11 placed CCDC132 on early endosomes. CCDC132 depletion by RNAi caused a dramatic delay in TfR recycling to the cell surface, a process in which both Rab4 and Rab11 have been previously implicated. No defects in the initial endocytosis of TfR or retrograde transport to the TGN were detected. The Ang2, Vps52 and Vps53 subunits co-localize with CCDC132 on endosomes. Upon overexpression of Vps54 these subunits however re-localize to the TGN pointing to Vps54 as a crucial determinant for GARP function at the TGN. Based on our findings we conclude that there are two functionally distinct heterotetrameric complexes sharing Ang2, Vps52 and Vps53: GARP additionally contains Vps54, whereas EARP contains CCDC132 instead. To expand our analysis of EARP function, we depleted primary cultured hippocampal neurons of CCDC132 using shRNA and observed severe dendrite branching defects and eventually neuron death. This phenotype is currently being investigated by studying the recycling of neuronal receptors and synapse formation upon EARP depletion.

NICHD

Sarai, Naoyuki
Visiting Fellow
Molecular Biology - Eukaryotic
The methyltransferase WHSC1 links transcription elongation to HIRA-mediated histone H3.3 deposition in activated genes
Most of the canonical histones are expressed at S phase, and are incorporated into chromatin in conjunction with DNA replication. On the other hand, expression and incorporation of histone variants occur in a replication-independent manner. One of the histone H3 variants, H3.3, is synthesized throughout the cell cycle and mainly deposited into chromatin in a transcription-coupled manner, and is thought to play a role in epigenetic control. However, mechanisms controlling H3.3 deposition have remained elusive. We investigated the role of the histone methytransferase, Wolf-Hirshhorn syndrome candidate 1 (WHSC1, also known as NSD2 or MMSET) in H3.3 deposition in transcription activated by interferon (IFN). IFN stimulation triggered rapid and prolonged H3.3 incorporation into multiple IFN stimulated genes (ISGs), which accompanied robust ISG mRNA induction in wild type cells. The deposition of H3.3 is mainly observed in the distal end of coding region, and H3.3 replacement continued for days even after IFN induced transcription ceased. In contrast, H3.3 deposition in ISGs was absent in Whsc1-/- cells, combined with a marked reduction in ISG mRNA induction. Similarly, UV-activated transcription and H3.3 incorporation were both strongly diminished in Whsc1-/- cells. Moreover, recruitment of the elongation factor, P-TEFb and the elongating form of RNA polymerase II was decreased in Whsc1-/- cells. We found that WHSC1 is recruited to the ISGs by interacting with BRD4, a bromodomain protein that localizes to the ISGs by binding to acetylated histones. BRD4 and WHSC1 were required for the recruitment of P-TEFb and the subsequent productive ISG elongation. During ISG transcription, WHSC1 also interacted with HIRA, the H3.3 specific histone chaperone, independently of BRD4 and P-TEFb. WHSC1 and HIRA co-occupied ISGs and supported prolonged H3.3 incorporation into activated genes, leaving a lasting transcriptional mark on them. We found that inhibition of P-TEFb did not completely prevent IFN induced H3.3 deposition, suggesting that WHSC1 directs P-TEFb mediated elongation and HIRA dependent H3.3 deposition through molecularly separable mechanisms. Our results reveal a previously unrecogninzed role of WHSC1which links transcription elongation and H3.3 deposition in activated genes through molecularly differentiable processes.

NICHD
Thouennon, Erwan
Visiting Fellow
Neuropharmacology and Neurochemistry
Anti-tumoral and neuroprotective effects of rosiglitazone (Avandia) is mediated through Carboxypeptidase E expression.
The antidiabetic drug Rosiglitazone (Avandia), a ligand for peroxisome proliferator-activated receptor-gamma (PPARG) has been shown to act on a number of other pathophysiological conditions. In particular, RGZ exerted anti-tumoral effects on breast cancer and hepatocellular carcinoma cells by inducing apoptosis. On the contrary, it has been demonstrated that RGZ was able to protect neurons against apoptosis. This dichotomy between the effects of RGZ on cell survival in different cell types remains poorly understood. Carboxypeptidase-E (CPE) a prohormone processing enzyme and its splice variant, CPE-deltaN have been shown to promote neuronal survival and tumor growth, respectively. Here we investigated whether RGZ could mediate its effects on promoting neuronal survival and its anti-tumoral effects by modulating the transcription of CPE and CPE-deltaN, respectively. We found that CPE mRNA/protein levels were significantly increased in the hippocampus of RGZ-fed mice ($p=0.0276$ for mRNA) and in rat hippocampal neurons treated with RGZ ($p=0.0317$ for mRNA and $p=0.0082$ for protein). In contrast, we showed that RGZ treatment strongly reduced CPE-deltaN expression in highly

metastatic breast cancer (p=0.0006) and hepatocellular carcinoma cell lines (p=0.05). We previously showed that CPE-deltaN was able to induce tumor metastasis. Down-regulation of CPE-deltaN by RGZ could contribute to its anti-tumoral effects. To investigate whether RGZ could regulate CPE expression at the promoter level, we performed bioinformatic analysis revealing the presence of evolutionary conserved PPARG-binding sites in the CPE promoter. We designed a luciferase construct containing those PPARG-binding sites and transfected it into embryonic (E13.5) primary neurons. Treatment by RGZ was able to increase the luciferase activity (p=0.0286) whereas RGZ failed to modify the luciferase activity when the binding sites were removed. This indicated that RGZ was able to regulate CPE/CPE-deltaN expression by directly binding to the promoter of the gene and provides a mechanism underlying the effects of RGZ observed in neuronal cell survival and tumor cell apoptosis. PPARG and CPE/CPE-deltaN are involved in many physiological functions such as food intake, glycemia and bone metabolism. Our novel finding that CPE expression is directly regulated by PPARG, whether negatively or positively, suggests that CPE may underlie the mechanism of other physiological functions triggered by PPARG activation.

NICHD
Vargish, Geoffrey
Doctoral Candidate
Neuroscience - General
Persistent inhibitory circuit defects following chronic in utero exposure to cannabinoids
Several studies have shown that in utero exposure to exogenous cannabinoids (CBs) may lead to significant cognitive defects in humans. Disruption of cholecystokinin expressing interneuron (CCK INTs) development may underlay these defects as CCK INTs express cannabinoid receptor 1 (CB1R) early in development and account for much of the CB1R expression in the cortex and hippocampus. Indeed endocannabinoid (eCB) signaling has been implicated in aspects of development such as specification, migration and circuit integration. Previous fate mapping experiments performed by our lab have shown that CCK INTs proliferate and migrate beginning around embryonic day 10.5 in mice, indicating maternal exposure to CBs such as marijuana, the most widely used illicit drug during pregnancy worldwide, could interfere with CCK INT eCB signaling in utero. Thus, we examined the outcome of chronic prenatal exposure to exogenous CBs by comparing the offspring of pregnant mice treated from E10.5 to birth with CBs (either ?9-tetrahydracannibinol, the major psychoactive ingredient of marijuana, or WIN55,212-2 (WIN), a CB1R agonist) or a vehicle control (VH). Treatment was done via intraperitoneal injection as CBs, both natural and synthetic, are highly lipophilic molecules allowing them to easily cross the placental barrier. Immunohistochemical characterization revealed a deficit in CCK INT density in mice prenatally exposed to WIN when compared to VH controls (55.5% decrease). This deficit was specific to CCK INTs as the density of cells expressing other INT markers was comparable between the two treatment groups. Consistent with a loss of CCK INTs, electrophysiological recordings revealed a striking deficit in inhibitory tone within the hippocampal circuit. Recordings of spontaneous inhibitory postsynaptic currents (sIPSCs) in the dentate gyrus show a reduction in basal sIPSC amplitude in mice prenatally treated with WIN (VH=63pA, n=64; WIN=54pA n=61). Further, recordings of disynaptically evoked feedback inhibition in CA1 of WIN treated animals exhibit a significantly reduced sensitivity to acute WIN application when compared to VH (VH=54% n =20; WIN=30% n=17; p=0.01). Overall, our data reveal deficits in CCK INT mediated inhibitory drive in mice exposed to exogenous CBs in utero. This loss of CCK INTs could precipitate a change in the balance of excitation and inhibition, providing a potential mechanistic basis for cognitive defects associated with prenatal CB exposure.

NICHD
Gupta, Nitin

Visiting Fellow
Neuroscience - Integrative, Functional, and Cognitive
Understanding the relevance of temporal patterning in sparse neural codes
A fundamental goal of neuroscience is to understand how neural circuits process sensory information and store memories. To optimize processing, different brain areas use different neural representations to encode sensory objects. These representations range from dense, time-varying patterns of spikes in overlapping sets of neurons (dense codes) to a few spikes in a few selective neurons distributed across a large population (sparse codes). Sparse codes may be advantageous for retrieving and writing memories as they require few synapses to be accessed and altered. In this scheme, sensory objects are thought to be encoded by the identities of active neurons. But, whether the temporal order of activation of these neurons also carries useful information remains unclear. To test this, we are using the locust olfactory system, which shares many organizational principles with the human version. The locust brainâ€™s small size and amenability to in-vivo electrophysiology make it useful for testing ideas about neural coding. Kenyon cells (KCs) in the mushroom body, thought to be the site of olfactory memories in the insect brain, use sparse codes for odors. To test whether activation order of KCs is consequential, we manipulate their spiking patterns while recording from their follower cells, the beta-lobe neurons (bLNs). To identify and categorize bLNs we made intracellular recordings in awake animals while delivering odors, followed by dye-fills and immunohistology. We found several morphological classes of bLNs, some staining for the inhibitory neurotransmitter GABA, some forming local circuits, some projecting outside of the mushroom body. We found that, in sharp contrast to KCs, individual bLNs respond to nearly all odors; thus, the identity of responsive bLNs cannot represent sensory objects. Rather, we found that information about the stimulus can be decoded by analyzing the temporal patterns of spikes in bLNs. These temporal patterns are likely derived from the order of activation of KCs, but they may also be generated independently by the local circuits within the mushroom body. To tease apart these possibilities, we are delivering different temporally structured patterns of extracellular electrical stimulation to KCs, and testing whether the responses of bLNs can be used to tell these patterns apart. Our results will help us understand the relevance of temporal patterning in the context of sparse coding and memory.

NICHD
Chandra, Goutam
Visiting Fellow
Neuroscience - Neurodegeneration and Neurological disorders
Oxidative-Stress via TFEB Upregulates Cathepsin D but its Defective lysosomal Maturation Contributes to a Neurodegenerative Lysosomal Storage Disease
In multicellular organisms, lysosomes play pivotal roles in the degradation and clearance of damaged macromolecules and organelles. Cathepsin D (CD), a major lysosomal aspartyl protease, facilitates proteolysis of intracellular long-lived proteins, accumulation of which contributes to pathogenesis of many neurodegenerative diseases. Paradoxically, both CD-overexpression and CD-deficiency are reported to underlie neurodegenerative diseases although the mechanism of this paradox remains unclear. We report here that CD is overexpressed in a mouse model of a childhood neurodegenerative lysosomal storage disease (LSD), Infantile Neuronal Ceroid Lipofuscinosis (INCL), caused by palmitoyl-protein thioesterase-1 (PPT1) deficiency. PPT1 is a lysosomal enzyme that cleaves thioester linkages in palmitoylated (S-acylated) proteins required for their degradation and its deficiency causes abnormal accumulation of these S-acylated proteins in lysosomes. In the present study, we demonstrate that in the brain tissues of Ppt1-knockout (Ppt1-KO) mice, CD-overexpression is mediated by oxidative-stress via upregulation of transcription factor-EB (TFEB), a master regulator of lysosomal proteins. Unexpectedly, despite CD-overexpression, lysosomal maturation of pro-CD to active-CD was disrupted

due to increased lysosomal pH that suppressed cathepsin B and cathepsin L activities, which are required for CD-maturation. Consequently, due to markedly reduced CD activity in lysosomes the degradation and clearance of intracellular long-lived proteins were disrupted leading to lysosomal storage that contributed to neurodegeneration in INCL. Importantly, an anti-oxidant thioesterase-mimetic small molecule, N-tert Butyl Hydroxylamine (NtBuHA), ameliorated CD processing defect in lysosome by suppressing the elevated lysosomal pH in Ppt1-KO cells, reduced lysosomal protein accumulation, afforded neuroprotection and extended lifespan of Ppt1-KO animals. Our findings provide insight into a lysosomal processing defect, which, despite CD-overexpression, may lead to a functional CD-deficiency in lysosomes contributing to INCL pathogenesis. We propose that varying mechanisms adversely affecting lysosomal CD-activity may underlie other neurodegenerative LSDs.

NICHD
Fero, Kandice
Postdoctoral Fellow
Neuroscience - Neurodegeneration and Neurological disorders
Coding of postural information by otoliths in larval zebrafish
Vestibular dysfunction is one of the most common medical complaints but 60-80% of them have an unidentifiable cause. This poor understanding stems from the complexity of vestibular processing; to sense relative position and motion in space, information from the ear is integrated with that from many other sensory modalities including vision. Thus, a pressing need exists for examining neural mechanisms underlying vestibular function. Using zebrafish, we aim to reveal how the otoliths code vestibular cues and identify the signal to motor system pathways involved in postural control. To test how the otoliths code vestibular cues, we disrupted utricular and saccular otoliths in larvae and then analyzed behavioral responses to vestibular stimulation. We disrupted otoliths via morpholino knock-down of GP96, which was previously found to cause only one of the two otoliths to form in either ear in zebrafish. Larvae that did not possess either utricle (Utr-) showed poor balance, often rolling sideways or upside-down. But under illumination, Utr- balance improved showing increased reliance on visual cues to maintain upright roll balance. Utr- also showed decreased routine turn (R-turn) initiation during normal swimming. Intact controls also reduced R-turns during momentary vestibular disruption following exposure to intense vibration. These results indicate that R-turns may largely depend on utricular sensory input. Larvae were then exposed to a controlled vestibular stimulus in the form of a slow horizontal acceleration; all groups except Utr- increased R-turns when oriented perpendicular to the motion. Utr- instead increased scoots (forward swims) but only when oriented with the motion. Finally, as fish navigate in a 3D environment, we examined vertical swimming and found that larvae controlled body pitch via passive drifting to orient downward, scoots to orient upward and R-turns for large angle pitch changes. While the utricle is known to be necessary for roll balance, the vestibular function of the saccule has remained unknown. These results are the first evidence that saccular otoliths are directionally sensitive to vestibular stimuli and are associated with scoots, while the utricle may stimulate R-turns. Taken together, these results indicate that R-turns and scoots have distinct roles in ongoing pitch and roll corrections during swimming. Understanding how otoliths code vestibular stimuli opens the door to identifying vestibular neural circuits.

NICHD
Murthy, Saravana
Research Fellow
Neuroscience - Neurodegeneration and Neurological disorders
CARBOXYPEPTIDASE E: A NOVEL NEUROTROPHIC FACTOR PROTECTS HIPPOCAMPAL NEURONS DURING STRESS IN MICE THROUGH THE AKT/BCL2 PATHWAY

Chronic physiological stress due to prolonged elevation in glucocorticoid (GC) levels could lead to the development of several disease pathologies including neurological diseases. Allostasis, a homeostatic mechanism of adaptive response to stress can delay this process. However, the delicate balance of allostasis, before neuronal damage, is poorly understood. We have previously shown that Knock-Out (CPE KO-/-mice) of carboxypeptidase E (CPE) in mice resulted in hippocampal neuronal degeneration in the CA3 region after weaning stress. Recently, CPE was identified as a novel neurotrophic factor against oxidative stress in primary hippocampal neurons (PHN). Here, we investigated the neuroprotective role of CPE in vivo in C57BL/6 and CPE KO-/- mouse brains during stress, using a mild chronic restraint stress (mCRS) paradigm. We found that after mCRS (n=12 each group) for 1 h/day for 7 days (chronic) and after 24 hrs recovery, CPE protein levels were increased (p>0.001 and p>0.0002 for C57BL/6 chronic and recovery groups, respectively, compared to the naïve group) in the hippocampus. In situ hybridization studies show increased levels of Cpe mRNA (p>0.001) in all of the hippocampal subfields (CA1-CA3) and no evidence of gross neuronal cell death after mCRS, indicating a neuroprotective effect of CPE in vivo. This increase could be due to enhanced GC secretion after mCRS, since bioinformatic analysis and luciferase reporter assays (p>0.05) identified 3 potential GC response elements (GREs) in the CPE promoter and studies on PHN culture exhibited an increase in CPE (>60%) after treatment with dexamethasone, a synthetic GC. In addition, PHN over-expressing CPE showed elevated BCL2 protein (p>0.001) a neuronal survival marker. These findings were corroborated in vivo, where C57BL/6 mice, chronic stress and recovery groups showed elevated BCL2 anti-apoptotic protein (p>0.01), and decreased BAX, pro-apoptotic protein (p>0.05) expression. In contrast, mCRS on CPE KO-/- mice revealed a 3-fold (p>0.002) reduction in BCL2 and 2-fold increase in BAX expression. Studies on the mechanism demonstrated an increase in p-AKT phosphorylation (p<0.01) in CPE WT mice after mCRS, whereas CPE KO-/- mice showed a decrease in AKT phosphorylation and an increase in activated Caspase3 (p>0.01). Hence, our studies have uncovered a novel key player, CPE, in neuroprotection of hippocampal neurons, which acts through the p-AKT/BCL2 signaling pathway to maintain allostasis during stress.

NICHD
Barksdale, Elizabeth
Postdoctoral Fellow
Neurotransmission and Ion Channels
From synapses to disease: the role of neuronal pentraxins in building functional inhibitory networks
A growing number of neurological disorders, from autism to epilepsy to schizophrenia, are hypothesized to result from an imbalance of excitation and inhibition in the brain. Mechanisms behind this imbalance, however, vary widely and are still not fully understood. One area of intense scrutiny is the role of gamma oscillations in the pathophysiology of disease. Gamma oscillations have been implicated in higher cognitive functions including storing and recalling information and combining sensory inputs into unified percepts. Generation of gamma oscillations is driven by GABAergic parvalbumin-expressing basket cells (PVBCs); interconnected networks of PVBCs synchronize firing of glutamatergic principle cells (PCs) via thousands of inhibitory synapses, and it is this coordinated firing that is picked up as gamma. Factors affecting PVBCsâ€™ ability to regulate the timing, extent and synchrony of PC firing - and thus alter excitation/inhibition dynamics - could provide potential therapeutic targets for associated disorders. Neuronal pentraxins (NPs), a family of three calcium-dependent lectins: NARP, NP1 and NPR, present an intriguing possibility in this capacity. Localized to excitatory synapses on PVBC dendrites, NPs promote surface clustering of the GluA4 subunit of AMPA receptors (AMPARs), the primary excitatory synaptic receptor utilized for network recruitment of PVBCs. Using knockout mice we found that deleting individual (NARP or NPR) or multiple (NARP and NPR) NPs resulted in reduced or absent immunoreactivity for GluA4 on hippocampal PVBCs, respectively. Interestingly, only double NARP/NPR

knockouts (dKOs) showed physiological manifestations of these alterations to normal AMPAR composition. dKOs exhibited impaired AMPAR-mediated synaptic drive onto PVBCs, as evidenced by significant deficits in evoked release and in the amplitude and frequency of spontaneous events (sEPSCs) in PVBCs, compared to WT controls. This reduction in excitatory drive onto PVBCs translated into depressed feedforward inhibition, indicating less temporal precision in the PVBC-PC network in dKOs which, in turn, led to a significant reduction in the power of gamma oscillations as compared to WT. Altogether these results demonstrate the importance of NPs in controlling the integration of excitatory synapses on PVBCs within cortical circuits.

NICHD
Mitchell, Robert
Postdoctoral Fellow
Neurotransmission and Ion Channels
Neuregulin reduces synaptic GABAA currents independent of canonical ErbB4 receptor tyrosine kinase activity
Neuregulin (NRG)-ErbB signaling is critical for neuronal differentiation and development, modulates plasticity and intrinsic neuronal excitability, and has been implicated in neuropsychiatric disorders. In addition, NRG-ErbB4 signaling has been genetically linked to schizophrenia and regulates hippocampal dopamine levels and neuronal gamma oscillations. Importantly, ErbB4 expression is restricted to subsets of inhibitory GABAergic interneurons, including fast spiking parvalbumin positive, that are critical for gamma frequency oscillations and have also been implicated in schizophrenia. Therefore, most of NRG's effects reported in principal neurons are indirect; however, little is presently known about ErbB4â€™s direct targets in GABAergic interneurons expressing the receptor. To address this issue, in this study I have employed an unbiased proteomics approach, in combination with electrophysiological, biochemical and cell biological techniques, to identify the GABAA receptor alpha 1 (GABAR1a) subunit as a biologically important ErbB4-interacting protein. Moreover, I demonstrate a novel, NRG-mediated but receptor tyrosine kinase (RTK)-independent pathway, which couples ErbB4 to decreased postsynaptic GABARa1 currents (mIPSCs) onto inhibitory interneurons. NRG induces its effects on mIPSCs by promoting the biochemical association of ErbB4 with GABARa1, which occurs independently of ErbB4 tyrosine kinase activity. To confirm the effects of NRG are independent of ErbB4â€™s canonical RTK activity, we generated an AAV virus expressing a kinase-dead (KD) ErbB4 mutant. I show that GABARa1+ interneurons cultured from ErbB4 null mice and infected with the KD mutant functionally regain NRGâ€™s effect on mIPSCs. Mechanistically, NRG reduces surface GABARa1 colocalization with the inhibitory postsynaptic scaffolding protein gephyrin and requires clathrin mediated endocytosis and PKC activity. In addition, ErbB4 and GABARa1 are extensively coexpressed in hippocampal interneurons in vivo, as well as in vitro, and ErbB4 null mice have decreased cortical GABARa1 subunit expression. I posit that this RTK-independent mechanism critically modulates postsynaptic inhibitory control of ErbB4-expressing hippocampal interneurons and may govern hippocampal network activity and plasticity. Future studies will employ transgenic models to probe the relevance of this pathway to neuropsychiatric disorders and potential therapeutic interventions.

NICHD
Lee, Bonggi
Postdoctoral Fellow
Physiology
Knock-in mice with a mutated (Thr6Lys and Val81Ile) human MC3R exhibit greater fat mass by increasing food intake and feeding efficiency

Our previous studies have shown that children who are homozygous for a pair of function-decreasing missense melanocortin 3 receptor (MC3R) sequence variants (Thr6Lys and Val81Ile) have significantly greater BMI and fat mass than control children. To further characterize the effects of these mutations in MC3R, we generated two novel knock-in mouse models replacing the murine MC3R with either the wild type human MC3R (hWT) or the Thr6Lys and Val81Ile human MC3R (hMU). On both chow and high fat diet, homozygous hMU mice had significantly greater body weight (p<0.05) and body fat mass (p<0.001), and significantly decreased fat-free mass (p<0.001) compared to homozygous hWT. However, as observed for heterozygous children, heterozygous hMU mice did not exhibit any differences in body composition. Homozygous hMU mice had altered energy balance: body weight-matched young homozygous hMU mice had increased food intake (p<0.05) as well as increased feeding efficiency (p<0.05) compared to homozygous hWT mice. In addition, energy expenditure was also slightly reduced in homozygous hMU vs. hWT when studied at thermoneutrality (p<0.05). Measurements of food intake and body weight after leptin injection (1 μg/g) in body weight-matched young mice indicated that leptin sensitivity was not different, suggesting that altered leptin signaling may not be the main mechanism for altered food intake and energy expenditure in hMU mice. Interestingly hMU mice have significantly increased serum adiponectin levels despite their notable increase in fat mass, which may explain why hMU mice have similar insulin sensitivity to lean hWT mice. In vitro preliminary data using isolated adipocytes suggest that glucose uptake is higher in homozygous hMU adipocytes. Together, these data suggest that the MC3R sequence mutations Thr6Lys and Val81Ile may play an important role in regulating energy homeostasis in both humans and mice at least partially through controlling food intake and energy expenditure. In addition, hMU white adipose tissue may be metabolically healthy despite higher fat mass. Further studies examining how such mutations drive the marked increase in adiponectin levels and how increased adiponectin may affect food intake and energy expenditure signaling may further reveal mechanisms through which MC3R affects energy homeostasis.

NICHD
Lomash, Suvendu
Visiting Fellow
Protein Structure/Structural Biology
Novel ligand binding mechanisms in Adineta vaga glutamate receptors
Ionotropic glutamate receptors (iGluRs) relay the majority of rapid excitatory neurotransmission in the vertebrate central nervous system and have been studied extensively. Homologs of mammalian iGluRs are now known to be widespread in species from prokaryotes to other complex eukaryotes, but in contrast, they remain poorly characterized. A recently discovered glutamate receptor from a primitive eukaryote Adineta vaga (AvGluR1) was proposed to be an evolutionary link between the bacterial and vertebrate iGluRs. We crystallized and performed sedimentation analyses on the ligand binding domain (LBD) of AvGluR1 and observed a prokaryotic-like secondary structure arrangement and dimer packing but a eukaryotic-like affinity for dimer assembly, supporting its placement as an evolutionary intermediate. Surprisingly electrophysiological experiments on the intact receptor and competitive [3H]-glutamate displacement assays for the LBD showed that unlike mammalian iGluRs, AvGluR1 also binds to and is activated by a number of hydrophobic and neutral polar amino acids, producing rapid and fully desensitizing responses. Insight in to this promiscuous ligand-binding pattern was obtained by solving high-resolution crystal structures of the AvGluR1 LBD complexed with Glu, Asp, Ser, Ala, Met and Phe that revealed two Arg residues in the binding site directly coordinating the gamma-carboxyl group of Glu/Asp; a feature not previously observed for any other iGluR or its parent superfamily. Binding of Ser, Ala and Met also utilized a unique mechanism wherein a chloride ion substitutes for the missing Glu gamma-carboxyl, facilitating ligand interactions by acting as a countercharge for the two Arg residues. Indeed, binding assays in the absence of Cl- show a drastic reduction in the receptorâ€™s affinity for

Ser/Ala/Met but not Glu/Asp. Phe being much bulkier displaces the two arginines and binds independent of any counterion but with a much lower affinity. Given previous data on the broad ligand selectivity of bacterial GluR0, the present work suggests that the evolution of glutamate as neurotransmitter may have required pruning of the receptor sensitivity to other ligands. At a more fundamental level, it illustrates a novel ligand-binding scheme that highlights the role of ions/solvent molecules in ligand-receptor interactions and presents a mechanism to diversify the ligand repertoire for a receptor that may find implications in protein biochemistry and pharmacology.

NICHD
Krispin, Shlomo
Postdoctoral Fellow
Vascular Disease and Biology
BMP Signaling Regulates Angiogenic Sprouting
The complex body design of vertebrates requires efficient transport of gases, metabolites, nutrients, hormones and circulating cells between tissues and organs, all of which depend on proper assembly and function of the circulatory system. The formation of new blood vessels during development depends mainly on angiogenesis, the sprouting of new vascular tubes from preexisting vessels. Sprouting must occur at defined places and times in order to assemble a properly functioning vascular network, however the cues responsible for initiating this angiogenic sprouting remain largely unknown. The metameric arrangement and relative anatomical simplicity of the trunk angiogenic network in the developing zebrafish makes it ideally suited to study the cues and mechanisms leading to the ordered formation of new vascular networks. As we have described previously, trunk intersegmental vessels (ISV) sprout from the dorsal aorta, the major trunk axial artery, in a synchronous rostral- to- caudal wave, with a pair of ISV emerging at the each somite (future trunk muscle block) boundary. The positive and negative cues constraining ISV sprouting to this particular time and place are not known. We have now found that BMP signaling is playing a key role in directing angiogenic sprouting. Using a bmp reporter line, we demonstrate bmp activity in endothelial cells just before the onset of ISV sprouting. We show that growth differentiation factor 6a (gdf6a) is the main bmp ligand regulating arterial sprouting, whose activity is initially inhibited by antagonist noggin1. As this inhibition is relieved (in a rostral to caudal fashion) sprouting is allowed to take place. In gdf6a loss of function or noggin1 ectopic gain of function, ISV fail to sprout and elongate on time. We further demonstrate that gdf6a controls angiogenic sprouting by regulating vascular endothelial growth factor receptor 3 (vegfr3) expression in endothelial cells â€“ the receptor for vegfc ligand. These results are in line with recent report from our lab demonstrating vegfc as a key cue regulating ISV sprouting. Although previous work had suggested BMPs might play a role in angiogenesis, this role was poorly defined. Our results demonstrate for the first time a specific role for BMP ligands and their antagonists in regulating arterial angiogenic sprouting. This work also highlights BMPs and their antagonists as possible therapeutic targets to either promote or suppress angiogenesis.

NIDA
Bakare, Oluyomi
Postdoctoral Fellow
Chemistry
Structure Activity Relationship Studies of Novel Modafinil Analogs at the Monoamine Transporters: Elucidation of Structural Elements for Selectivity at DAT versus SERT
The dopamine (DA), serotonin and norepinephrine transporters (DAT, SERT and NET, respectively) terminate synaptic neurotransmission and serve as targets for drug action. For example, inhibition of DA reuptake via the DAT is the primary mechanism underlying the reinforcing effects of abused drugs such

as cocaine. However, modafinil (2-[(diphenylmethyl)sulfinyl] acetamide, MOD), a wake-promoting medication, selectively inhibits DA reuptake, but with no evidence of abuse liability in humans, and thus may have potential as a treatment for psychostimulant abuse. Both computational and molecular studies using DAT mutants demonstrate that MOD binds the DAT in a unique fashion as compared to cocaine. Other reports have suggested mechanisms involving indirect targets such as orexin, glutamate and serotonergic transmission. Investigation of MOD in vitro and in vivo is confounded by its relatively weak affinity for the DAT and poor water solubility. Herein, we synthesized a series of MOD analogs wherein substituents were added to the phenyl ring(s), the sulfoxide (S=O) function was reduced, and the primary amide group was replaced with secondary amides or amines. In general, these modifications gave analogs with higher binding affinities (lower Ki values) at DAT compared to MOD (Ki = 2520 nM). The S=O motif was not critical for affinity at all three monoamine transporters (MATs). The amine analogs, in addition to being more water soluble, had higher binding affinities than the amide analogs at the MATs. Halogen-substitution of the phenyl rings of MOD gave amide analogs with improved Ki values for DAT over SERT and NET, whereas affinity was improved at SERT over DAT and NET for the amine analogs. Overall, we identified one highly DAT-selective amide analog (2700-fold over SERT) and two highly SERT-selective amine analogs (Ki values less than 30 nM). Computational modeling of DAT and SERT led to the identification of key amino acid residues (T497 in SERT and A480 in DAT), and preliminary data using DAT (A480T) and SERT (T497A) mutants validated a critical role of these residues in the observed selectivity. This is the first report of highly DAT- or SERT-selective MOD analogs that also provide a molecular basis for their selectivity. These novel tools will be useful in further elucidating mechanisms underlying MODâ€™s therapeutic effects and may be exploited toward the development of medications to treat drug abuse.

NIDA
Kumar, Vivek
Postdoctoral Fellow
Chemistry

Design and synthesis of high affinity and enantioselective D3 receptor antagonists

The neurotransmitter dopamine is synthesized in dopaminergic neurons and released to stimulate G-protein coupled receptors, affecting movement, cognition and emotion. The five dopamine receptor subtypes are classified into D1-like and D2-like subfamilies, based on sequence, G-protein coupling and pharmacology. The D3 receptor (D3R) is a member of the D2-like family and is elevated in the ventral striatum of both laboratory animals and humans upon exposure to cocaine and methamphetamine. Recently, a significant effort to target the D3R for therapeutics to treat drug abuse and other neuropsychiatric disorders has identified templates for the design of most high affinity and selective D3R antagonists, which have been studied in numerous animal models of addiction. However, these molecules are typically highly lipophilic and poorly water soluble, limiting their usefulness as in vivo tools. Structure-activity relationships (SAR) in the 4-phenylpiperazine class of D3R antagonists has revealed that a terminal heteroaryl amide attached by a functionalized 4-carbon linking chain results in highly selective D3R antagonists. The first enantioselective D3R antagonist (R)PG-648 was discovered to be ~400-fold D3R selective and about 15-fold higher affinity at D3Rs than its S-enantiomer, whereas there was <2-fold enantioselectivity at D2 receptors. Replacement of the 3-OH substituent with 3-F provided one of the most selective D3R ligands in the series (BAK2-66; D3R/D2R > 1000 fold). In the present study, our objectives were to 1) improve the enantioselective synthesis of both R- and S-PG648 to synthesize multigram quantities of ~100% ee products for in vivo testing and 2) to develop the enantioselective synthesis of R- and S-BAK2-66 to determine enantioselectivity at D3 vs. D2 receptors. Enantiomeric separation of BAK2-66 was carried out using a new chiral resolution method (ee = 90%). Interestingly, when binding affinities were determined using [3H]N-methylspiperone radioligand binding

in HEK293 cells expressing dopamine D2 or D3 receptors, opposite enantioselectivity at D3Rs was demonstrated with the 3-F (BAK 2-66) analogues as compared to their 3-OH (PG 648) analogues (e.g. S>R). Further investigation into the difference in OH vs. F-interactions at the D3R protein level using computational methods is underway, as this is a pivotal position for D3 vs. D2 receptor selectivity.

NIDA
Keck, Thomas
Postdoctoral Fellow
Neuropharmacology and Neurochemistry
Novel dopamine D3 receptor antagonists as tools for in vivo investigation of methamphetamine-induced cognitive deficits and abuse
Dopamine D3 receptors (D3Rs) are enriched in mesolimbic circuits of the brain that are involved with drug addiction. Excessive stimulation of D3R signaling is implicated in addiction to psychostimulants like methamphetamine (METH), while blockade of D3Rs attenuates drug-seeking behavior in rats trained to self-administer METH. D3R antagonism has also been reported to enhance cognition in animal models, and thus may have therapeutic potential to treat cognitive deficits caused by METH abuse. Our goal is to develop novel D3R antagonists as medicines to treat METH addiction and its associated cognitive dysfunction. Highly selective D3R antagonists have been discovered using small molecule structure-activity studies. These efforts have been augmented by the recently published crystal structure of the D3R protein, providing guidance for novel drug design. We explored several unique chemical templates to identify new compounds with high affinity and selectivity for D3Rs, while possessing drug-like physical properties (e.g., desirable water solubility, blood-brain barrier penetration). Our novel compounds feature refinements to the classic 4-phenylpiperazine pharmacophore, which occupies the orthosteric (dopamine) binding pocket. Additional structural modifications focused on a second binding pocket-recently described as unique to D3R-that can be targeted by a second pharmacophore conjugated to the 4-phenylpiperazine variants. This strategy resulted in a library of new bitopic compounds that bridge two binding sites, improving binding stability and receptor selectivity. Binding affinities for these compounds were determined using [3H]N-methylspiperone in membranes prepared from HEK293 cells expressing dopamine D2, D3, or D4 receptors. A subset of compounds with high affinity and selectivity for D3R were evaluated in vivo in a D3R agonist-induced yawning model in rats. Three candidate compounds that demonstrated potent, D3R-selective blockade of yawning were chosen for testing in models of METH addiction. Rats were trained in a long-access METH self-administration model and METH-induced neurotoxicity and cognitive dysfunction are currently being assessed. We hypothesize that our novel D3R antagonists will protect against METH-induced neurotoxicity and improve cognitive deficits, supporting attenuation of D3R signaling as a promising pharmacological approach to treatment of METH abuse and other neurological conditions in which cognitive dysfunction is evident.

NIDA
Wakabayashi, Ken
Postdoctoral Fellow
Neuropharmacology and Neurochemistry
Rapid, experience-dependent changes in nucleus accumbens glutamate release induced by repeated intravenous cocaine
Repeated cocaine (COC) exposure has a widespread impact on glutamate (Glu), the major excitatory brain neurotransmitter. However, in brain areas such as the nucleus accumbens (NAc), a region heavily implicated in addiction, alterations in Glu release have been largely inferred by post-synaptic changes because detection of release in awake rats has been limited to techniques with poor time resolution. High-speed amperometry with enzyme-based biosensors can successfully detect small, structure-

specific, second-by-second changes in extracellular Glu. With this technique, we examined changes in Glu in the NAc shell induced by repeated intravenous (iv) COC injections (1 mg/kg). In drug-naive rats, COC only induced a small, rapid rise in Glu within the 20-s injection. Subsequent COC injections dramatically changed the Glu response. While the initial injection-related peak persisted, a second, slower and longer lasting (~30-40 min) tonic Glu increase appeared. This tonic rise became greater with each next injection. Importantly, this increase was not detected using control sensors that were equally sensitive to all other interferents but were fully insensitive to Glu. As well, this sensitized Glu response occurred before significant increases in locomotion, another behavioral index of COC-induced changes. The rapidity of the initial Glu peak with each injection suggested its link with neuronal Glu release via COCâ€™s actions in the periphery. To test this mechanism, drug naive rats were repeatedly injected with COC-methiodide (CMet), a COC analog that does not enter the brain. Like COC, CMet induced an initial Glu peak, confirming that it was related to synaptic input from the periphery. However, this peak rapidly habituated with subsequent CMet injections. Also in contrast to COC, no secondary tonic rise in Glu was seen with CMet, suggesting that this slow rise in Glu was related to COCâ€™s direct action on the brain. To test if COC experience altered the Glu response to the peripheral actions of COC, rats pretreated with 4 iv injections of COC were challenged with CMet. Unlike acute CMet injections in naive rats, CMet in COC-experienced rats showed a persistent sensitized initial and tonic Glu response to the drug, but no COC-like increase in locomotion. Thus, this work reveals rapid and dynamic neuroplasticity in Glu release to COC, which may have critical implications for elucidating our understanding of the mechanisms underlying COC addiction.

NIDA
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Neuroscience - Cellular and Molecular

Glutamate neurons from the ventral tegmental area inhibit lateral habenula neurons

The ventral tegmental area (VTA) is required for the self-administration of abused drugs. The VTA is a cellularly diverse structure, consisting of dopamine, GABA, and glutamate neurons. Whereas the importance of dopamine and GABA neurons in various addiction-related phenomena are known, recent reports have suggested VTA glutamate neurons might play a role in addiction. In order to gain insight into their function, we first identified the lateral habenula (LHb) as a major recipient of VTA glutamate neuron innervation. The LHb is an important epithalamic structure that powerfully modulates midbrain dopamine and serotonin neurons. By combining retrograde tract-tracing (iontophoresis of 1% FluoroGold into the LHb) and in situ hybridization in six rats, 92.95% (1134/1220) of retrogradely labeled neurons were positive for vesicular glutamate transporter 2 mRNA (VGluT2), the marker for VTA glutamate neurons. We next sought to identify how VTA VGluT2 neurons might affect LHb neurons (henceforth referred as the mesohabenular pathway). By combining in vivo electrophysiological and optogenetic methods, we unexpectedly observed a rapid, long duration inhibition of LHb firing upon optogenetic stimulation of the mesohabenular pathway. Using vglut2::cre mice, selective 10 millisecond optical stimulation of mesohabenular terminals [expressing channelrhodopsin (ChR2) under vglut2 promoter] induced inhibition of firing in 16/17 cells within 15 ms of light application. Mesohabenular inhibition of LHb neurons was replicated in rats expressing ChR2 in this pathway. Given that rodents readily respond to avoid excitation of the LHb, and mesohabenular stimulation inhibits LHb, we are currently investigating whether or not optogenetic stimulation of the mesohabenular pathway is rewarding. Preliminary data from a single rat observed that the animal spent at least 67% of its time of a two-chamber compartment on the optogenetically-stimulated side, compared with a nonstimulated side, over three consecutive days. These data show that VTA VGluT2 neurons project to and inhibit LHb. In summary, we found that the LHb is a major target of VTA glutamate neurons and the LHb is

surprisingly inhibited by mesohabenular optical stimulation. We are continuing behavioral examinations of the mesohabenular pathway as well as testing the hypothesis that the VTA contains a subset of neurons that preferentially innervate the LHb and have the capability to signal via glutamate or GABA.

NIDA
Belcher, Annabelle
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Neuroscience - General
Resting-State Functional Connectivity in the Awake Marmoset Brain
Traditionally, neuroimaging researchers have explored potential differences in brain function between patient and control populations by asking subjects to lie still in a scanner and perform a relevant cognitive task. Frequently, differences in brain activity yield clues regarding the origin of brain dysfunctions. Although this approach has yielded critical information regarding functional brain alterations in a variety of disease states, a complementary approach has taken the field by storm, that of assessing resting-state functional connectivity (rsFC). The human brain exhibits spontaneous task-independent signal fluctuations, and research suggests these fluctuations correlate between anatomically-connected brain regions. This passive measure of assessing connectivity has important potential applications, as it allows researchers to assess system-wide differences between groups with the implication that these differences may serve as a biomarker of disease. Recent reports suggest that drug-addicted individuals have altered patterns of mesocorticolimbic connectivity. Despite its continued application, there are few animal studies exploring the basis of rsFC. A significant challenge in resting state analysis is the need to use anesthesia to immobilize animals during scanning. Here we report successful use of a method to train common marmoset monkeys to tolerate restraint in an MRI machine in an effort to explore these rsFC patterns in an awake nonhuman primate. Four animals were exposed to a three-week period of behavioral acclimation and scanned in several sessions to obtain a high-resolution anatomical scan as well as 4-8 functional EPI scans. Following standard AFNI (NIMH) data pre-processing and motion correction steps, the datasets were registered to a common space (Riken, Japan) and submitted to a Group Independent Component Analysis (Melodic, FSL). We identified 8 relevant networks with good correspondence to rsFC reported in human subjects, centering on several primary and association cortical regions. Here we report for the first time, using functional imaging, patterns of rsFC in the common marmoset. These components each have a functional significance, regions within the identified components having strong anatomical connections. Future work will explore these patterns of connectivity, particularly those within the mesocorticolimbic system, as they relate to changes in response to drugs of abuse.

NIDA
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Research Fellow
Neuroscience - Integrative, Functional, and Cognitive
Prefrontal cortex input uniquely influences nucleus accumbens physiology
The nucleus accumbens (NAc) plays a major role in the generation of motivated behaviors. It is where dopaminergic reinforcement signals integrate with glutamate-encoded environmental stimuli. Prominent glutamate inputs to the NAc come from the hippocampus, amygdala, and prefrontal cortex (PFC). Pathway-specific activation of these inputs is known to produce distinct behavioral responses, but mechanistic explanations for pathway-specific effects are lacking. Here, we sought to uncover differences in innervation patterns between the glutamatergic inputs to the NAc. We combined viral-mediated red fluorescent protein expression with pathway-specific photostimulation techniques to probe the synaptic properties of each pathway in electrophysiology experiments. Because NAc output

neurons can be classified into groups based on efferent connectivity, we recorded from brain slices of transgenic mice that expressed GFP in different neuronal subpopulations. Recordings of these labeled neurons showed that both types of NAc output neurons were innervated to a similar extent by each glutamatergic input. The PFC input was unique, however, in that it targeted distinct patches of neurons and elicited especially robust feed-forward inhibition. Feed-forward inhibition is a polysynaptic effect that involves local microcircuitry. Thus, PFC input engages NAc circuitry in a distinct manner, and we are presently trying to determine if a specific interneuron subtype is preferentially targeted by this pathway. The small patches of the NAc where PFC fibers localized to were also areas that had low calbindin expression, which are sites that are known to contain output neurons that directly inhibit midbrain dopamine neurons. This anatomy suggests the PFC input could ultimately lead to lower dopamine levels and reward-seeking. To test this hypothesis, we asked if mice would actively avoid environments associated with photostimulation of PFC fibers in the NAc. We unexpectedly found that mice preferred to be in locations associated with enhanced activity of this pathway. This finding raises the possibility that PFC input engages NAc circuitry in a way that enhances downstream dopamine signaling, possibly via feed forward inhibition of dopamine-projecting NAc neurons. Overall, this work highlights the distinctiveness of the PFC input to the NAc by demonstrating how it innervates specific patches of neurons and triggers an uncharacteristically robust amount of feed-forward inhibition.

NIDA
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Postdoctoral Fellow
Neuroscience - Integrative, Functional, and Cognitive
Affective role of ventral tegmental area and substantia nigra dopamine neurons: An optogenetic study
Previous studies show that activation and suppression of dopamine systems elicit reward and aversion respectively. These effects are usually attributed to dopamine neurons in the ventral tegmental area (VTA) and the mesolimbic dopamine system. On the other hand, dopamine neurons in the substantia nigra pars compacta (SNc) and the nigrostriatal system often linked with motor and cognitive control, including the learning of stimulus-response associations. Here we show that excitation and inhibition of dopamine neurons in the SNc elicit reward and aversion. TH-Cre transgenic mice received adeno-associated viral vectors encoding channelrhodopsin2 (ChR2), halorhodopsin (NpHR), or no opsin into the VTA or SNc. Mice with ChR2 quickly learned to respond on the lever that delivered light into the VTA or SNc. And, when the assignment of active and inactive levers were changed, they shifted their lever preference. They also showed place preference to the compartment in which they concurrently received intracranial light. In addition, mice with NpHR avoided the compartment in which they concurrently received intracranial light into the VTA or SNc, while control mice did not. Our findings confirm that the excitation and inhibition of SNc dopamine neurons induces reward and aversion similar to the VTA dopamine neurons. The present data challenge the dichotomous notion that affective processes are mediated by dopamine neurons in the VTA, but not SNc and suggest the role of nigrostriatal projections in motivation and affect.

NIDA
Wang, Dong
Research Fellow
Neuroscience - Integrative, Functional, and Cognitive
Neural activity of nucleus accumbens induced by rewarding optogenetic stimulation of VTA dopamine neurons
Brain reward systems have long been a widely regarded topic ever since the discovery that animals lever press to stimulate certain brain areas, known as self-stimulation. Since then, numerous studies using

anatomy, pharmacology and electrophysiology methods have established the ventral tegmental area (VTA)-nucleus accumbens (NAc) projection as a key reward circuit. Recent studies using optogenetics found that optical stimulation of VTA dopamine neurons supported robust self-stimulation, suggesting that simply activating dopamine neurons is highly rewarding. The question remains is that why is activation of VTA dopamine neurons rewarding, and how dopamine influences downstream brain areas. We hypothesize that activation of VTA dopamine neurons cause neural firing pattern changes in the NAc, and our goal is to identify the firing patterns that encode â€˜dopamine neuron-mediated rewardâ€™. We injected the AAV-ChR2 viruses and implanted optical fibers in the VTA area of TH-Cre transgenic mice, which allowed us to specifically activate VTA dopamine neurons through optical stimulation; meanwhile we implanted a bundle of 8 tetrodes (32 wires) in the NAc shell to record multiple neural activities. Our results showed that VTA optical stimulation in free-behaving mice evoked fast excitatory local field potential (LFP) responses in the NAc, and the amplitudes of this LFP correlated well with the animalâ€™s self-stimulation rates. Similar to the LFP activity, 35% of the recorded NAc neurons showed fast phasic excitations, suggesting an excitatory input to the NAc after optical stimulation of VTA dopamine neurons. We also recorded neurons that showed phasic inhibitions (17%). To determine whether these firing pattern changes were mediated by dopamine, we injected mice with a dopamine D1 receptor antagonist. Although the antagonist decreased most basal firing in the NAc, it did not abolish optical stimulation-evoked neural responses, suggesting that transmitters other than dopamine were released by VTA dopamine neurons. In light of recent in vitro studies showing that dopamine neurons can also release glutamate and GABA, our results provide the first evidence that VTA dopamine neurons in vivo may employ multiple neural transmitters in activating, as well as inhibiting different groups of NAc neurons that are responsible for mesolimbic dopamine reward signaling.

NIDCD
Mann, Zoe
Postdoctoral Fellow
Developmental Biology
A gradient of Bmp7 regulates tonotopic development in the chick basilar papilla
Tonotopy is the fundamental organizing principal of the auditory system. It refers to the spatial separation of sounds based on component frequencies in the auditory periphery and the subsequent reassembly of those sounds in the brain. Spatial separation in the periphery is achieved through phenotypic gradations along the longitudinal axis of vertebrate auditory organs, such as the mammalian cochlea or avian basilar papilla (BP). Within the BP, hair cell phenotypes show morphologic and physiologic changes in phenotype along the long axis that are consistent with tuning to different frequencies. Despite the significance of tonotopic organization for auditory function, the molecular mechanisms underlying its development remain unknown. To investigate the mechanisms involved in specifying tonotopy, we used the chick BP because of its more obvious tonotopic metrics. The timing of acquisition of tonotopic identity was determined using organotypic explants and markers for tonotopy, including hair cell density, and expression of Calbindin. Based on the results, we used exome analysis to compare gene expression between low and high frequency halves of the BP at E6.5. Many genes were differentially expressed but we were most intrigued by Bmp7 which was much greater in the low frequency region, a result confirmed by in situ hybridization and qPCR. By contrast, two Bmp antagonists, Chordin and Follistatin, were expressed in an opposing gradient along the BP. To investigate the role of Bmp7 in tonotopy, the normal Bmp7 gradient was disrupted in vitro. Explants were incubated in control media, or media supplemented with Bmp7 or a Bmp antagonist, Noggin. Control explants developed with normal tonotopic gradients, whereas those maintained in Bmp7 media showed changes in tonotopic phenotypes consistent with conversion to a uniform low frequency identity. Conversely, treatment with Noggin caused the BP to develop with a high frequency phenotype. These effects were

specific to the Bmp7 pathway as treatment with other Bmps had no effect. Additional analysis suggests that Bmp7 functions through activation of the pJNK and p38 MAPK signaling pathways. In summary, Bmp7 and two Bmp7 antagonists are expressed in counter gradients along the length of the BP. Disruption of these gradients induces phenotypic changes consistent with alterations in positional identity along the tonotopic axis, suggesting that Bmp7 signaling plays a major role in specifying tonotopy.

NIDCD
Nakanishi, Hiroshi
Visiting Fellow
Genetics

A dominant mutation in NLRP3 causes nonsyndromic hearing loss DFNA34

Hearing loss is a common sensory disorder that affects 28 million Americans. It is estimated that more than 100 genes at different chromosomal locations underlie nonsyndromic hearing loss (NSHL), in which hearing loss is the only phenotypic manifestation. To date, up to 63 of NSHL genes have been identified and therefore many deafness genes remain to be identified. Identification of novel deafness genes improves diagnostic and prognostic care of patients and provides critical insights into the molecular and cellular basis of hearing. In this study, we ascertained LMG113, a North American family of Swiss German descent, segregating autosomal dominant NSHL. Their hearing loss initially begins in the 2nd to 4th decade of life, affects middle and high frequencies, and typically progresses to mild to moderate severity. A genome-wide scan with 440 short tandem repeat (STR) markers revealed probable linkage on chromosome 1q44 with a maximum two-point LOD score of 3.15. There were no other autosomal dominant NSHL (DFNA) loci mapped to this region, so it was defined as DFNA34. A genome-wide linkage analysis with 6090 single nucleotide polymorphisms confirmed the STR linkage result. Meiotic recombinations in affected family members narrowed the DFNA34 interval to 3.93 Mb that included 36 known and putative genes. One of these genes was NLRP3, in which mutations were known to cause autosomal dominant systemic autoinflammatory syndromes with recurrent fevers, episodic urticaria and hearing loss. A focused rheumatologic evaluation found no evidence to support a diagnosis of the allelic autoinflammatory syndromes. Sanger dideoxy sequencing of NLRP3 detected a heterozygous missense substitution, c.2753G>A (p.R918Q), in the leucine-rich repeat domain of NLRP3. p.R918Q co-segregated with NSHL in the family and was not present in 574 ethnically matched control chromosomes. Whole exome sequencing of DNA samples from 3 affected subjects identified no other probable pathogenic variants in the DFNA34 interval except for p.R918Q in NLRP3. These results indicate that NLRP3 is the gene underlying DFNA34 hearing loss. Current studies are under way to determine if DFNA34 is due to disruption of NLRP3 function within the cochlea or if it is secondary to systemic autoinflammation.

NIDCD
Rehman, Atteeq
Postdoctoral Fellow
Genetics

Mutations of CLPP, encoding a mitochondrial ATP-dependent chambered protease, cause Perrault syndrome

Perrault syndrome (PS) is an autosomal recessive condition characterized by sensorineural hearing loss and premature ovarian failure. Additional clinical features in some affected individuals include cerebellar ataxia, learning disability and peripheral neuropathy. To date, mutations of HSD17B4 and HARS2 have been described as underlying causes of this genetically heterogeneous disorder. However, there are families segregating PS that are genetically unlinked to these two genes, indicating other causes for PS remain to be identified. We used linkage analysis and homozygosity mapping in three

families to identify a novel PS locus on chromosome 19p. Exome sequencing of one affected individual from each of the three families revealed mutations in CLPP as the cause of this phenotype. In each family, affected individuals were homozygous for a different pathogenic CLPP mutation: p.Thr145Pro, p.Cys147Ser, and c.270+4A>G. These mutations were not present in public SNP databases or in 483 ethnically matched controls. CLPP encodes an evolutionarily conserved chambered endopeptidase. It is a component of a mitochondrial ATP-dependent proteolytic complex that forms an element of the ancient mitochondrial unfolded-protein response stress signaling pathway. Crystal structure modeling predicts that p.Thr145Pro and p.Cys147Ser alter the structure of the CLPP chamber that captures unfolded proteins and exposes them to proteolysis. An in vitro exon splicing assay demonstrated that the c.270+4A>G mutation weakens the splice donor site of exon 2 of CLPP. By immunostaining in mouse, we found that CLPP localized to granulosa cells and oocytes of the adult mouse ovary. In the mouse organ of Corti, CLPP localized predominantly to the supporting cells while a relatively weak signal was detected in adjacent sensory hair cells. The organ of Corti is the part of the mammalian inner ear that contains mechanosensory hair cells that transduce sound into electrical signals conveyed to the brain. The higher levels of CLPP in supporting cells flanking hair cells and in granulosa cells surrounding oocytes of the mature ovary suggest a specific need for proteolysis of particular unfolded or misfolded proteins to preclude damage in these cells. We conclude that dysfunction of mitochondrial protein homeostasis due to mutations in CLPP is another cause of PS. The exact mechanisms of pathogenesis in the ovary and auditory system are the subjects of further studies using mouse models.

NIDCD
Roy, Soumen
Visiting Fellow
Stress, Aging, and Oxidative Stress/Free Radical Research
Inner ear specific, targeted sound therapy to prevent ototoxic drug induced hearing loss: A preclinical study
Background: 360 million people worldwide suffer from hearing loss. It is often caused by exposure to life saving drugs with ototoxic side effects, which result in permanent hearing loss for over 500,000 patients annually in the USA. Ototoxic drugs like aminoglycosides and cisplatin are mainly used to treat diseases like bacterial infections and cancer respectively. Ototoxic drugs damage mainly the outer hair cells and cause high frequency hearing loss. Currently no effective treatment option exists to prevent aminoglycoside and cisplatin ototoxicity; and an urgent clinical intervention is needed. Objective: Our objective was to develop an ear specific, noninvasive co-therapy which would protect the hearing against the exposure of ototoxic drugs. Experimental approach: We developed an inner ear specific sound conditioning strategy that would induce heat shock proteins (HSPs), mainly HSP70 expression by non-traumatic stress in the inner ear. An 8-16 kHz octave band of 94 dB sound was exposed to the mouse for 2 hours. Expression of inducible HSP70 in cochlea was tested by RT-PCR. Hearing threshold shift was checked by auditory brainstem response and distortion product otoacoustic emission. Hair cell survival was quantified by confocal microscopy. Results: Sound exposure was able to induce a temporary threshold shift (TTS) but not a permanent threshold shift, which indicated that sound conditioning can induce mild stress in the cochlea to produce HSPs. TTS was back to normal within 24 hours after sound exposure. A distinct protection pattern was seen at different frequency levels and hearing has significantly (P<0.03, t-test with Welchâ€™s correction) rescued in the 32 kHz frequency region in the cochlea. We have tested sound therapy against cisplatin administration and it was significantly protected at the 16 kHz (P<0.001) and 22.4 kHz (P<0.001) frequency region. Outer hair cells were protected in the sound exposed frequency region in both the kanamycin and cisplatin mice group. Conclusion: This sound conditioning strategy would be clinically feasible as it is non invasive, showed no toxicity in the cochlea and most importantly is inner ear specific, which highly reduces the chance of

interacting with other primary drugs. Taken together these data shows that sound therapy may have an important application to treat against kanamycin and cisplatin induced ototoxicity and has a high potential to translate this therapeutic strategy to the clinic.

NIDCR
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Doctoral Candidate
Carcinogenesis
A novel Trio-regulate Rho GTPase network links Gaq and Gaq-coupled GPCRs to the nuclear activation of AP-1 and YAP: A key oncogenic driver in uveal melanoma.
Activating mutations in GNAQ and GNA11, which encode members of the Gaq family of heterotrimeric G protein a subunits, are the driver uveal melanoma oncogenes, while mutations in Gaq-linked G protein-coupled receptors (GPCRs) have been identified recently in numerous human malignancies. How Gaq and its coupled receptors transduce mitogenic signals is still unclear, due to the complexity of signaling events perturbed upon Gaq activation. Using of a synthetic biology approach and a genome-wide RNAi screen, we found that a highly conserved guanine nucleotide exchange factor, Trio, is essential to activate Rho- and Rac-regulated signaling pathways acting on JNK and p38, thereby transducing proliferative signals from Gaq to the nucleus. Indeed, while many biological responses elicited by Gaq depend on the transient activation of second messenger system, Gaq utilizes a hardwired protein-protein interaction-based signaling circuitry to achieve the sustained stimulation of proliferative pathways, thereby controlling normal and aberrant cell growth. Of interest, many of the hits in our RNAi screen involved members of YAP oncogenic signaling pathway, which controls organ size in mammals. Indeed, we have recently found that Gaq activating mutations stimulate YAP potently, leading to YAP translocation into the nucleus and the activation of YAP-dependent transcription of downstream genes. Furthermore, we found that Gaq stimulates YAP through the Trio-dependent Rho-GTPs family activation, and showed that YAP activation is an integral component of the pathway by which Gaq initiates uveal melanoma formation. Remarkably, we also found that targeting YAP by recently described small molecule inhibitors prevents uveal melanoma growth in vitro and in vivo, thus representing a novel molecular target for uveal melanoma treatment.

NIDCR
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Postdoctoral Fellow
Developmental Biology
WNT signaling initiates the neuronal-epithelial communication essential for organogenesis.
Communication among multiple progenitor cell types at distinct locations and times during embryogenesis initiates and exquisitely coordinates organogenesis. Submandibular gland (SMG) organogenesis involves the development and association of the parasympathetic ganglion (PSG) with the initial epithelial duct. Cholinergic signals from the PSG maintain epithelial progenitor cells as a reservoir for organogenesis, and the epithelial progenitors in turn produce neurotrophic factors for PSG survival and axon outgrowth. However, the signals that establish the initial interaction and association between the PSG and the epithelial duct are unknown. Here we used microarray analysis to identify secreted factors produced by the initial duct. We identified that multiple Wnts were produced by the duct and used organ culture to show that WNT signaling promotes PSG cell survival, proliferation, and association with the duct. Since increasing FGF signaling has been previously reported to decrease WNT signaling we treated SMGs with exogenous FGFs, which reduced Wnt expression within 2 hours. Decreasing WNTs disrupted the PSG-epithelial association, which depleted the epithelial progenitor cells. To confirm these findings in vivo, we reduced WNT signaling by deleting the FGF signaling

antagonists Sprouty1 and Sprouty2 (Spry1/2 KO), which increased FGF signaling. The Spry1/2 KO SMGs have reduced epithelial Wnt expression and a striking loss of PSG formation. This also led to depletion of epithelial progenitor cells and impaired gland development. To confirm that the phenotype was due to an FGF-dependent reduction in WNT signaling we either reduced FGF gene dosage or used a WNT activator to increase WNT signaling. Neither alone was sufficient to rescue the Spry1/2 KO phenotype, but combined they were able to restore PSG formation and reestablish the association of the PSG with the duct, rescuing SMG development. These results suggest that WNT signaling controls the development and association of the PSG with the duct, establishing the neuronal-epithelial communication that is required for progenitor cell maintenance and organogenesis.

NIDCR
Konkel, Joanne
Visiting Fellow
Immunology - Autoimmune
Role of TGFß signaling in the development of unconventional T cell populations
The gastrointestintal (GI) tract presents challenges not faced at other sites, immune regulation must be maintained concomitant with a degree of tonic inflammatory signaling. One way this is achieved in the GI tract is through the presence of unique T cell populations nestled between epithelial cells that are referred to as intra-epithelial lymphocytes (IELs). Populations of IELs are similar to T cells found in the periphery but others are unique to the GI epithelium. One unique population is the TCRalphabeta+CD4+CD8alpha+ IEL, a population expressing both the CD4 co-receptor and homodimers of CD8alpha. This unique IEL population has been suggested to have a regulatory function as transfer of these IEL reduces severity of colitis in a T cell transfer model of the disease.
TCRalphabeta+CD4+CD8alpha+ IEL are thought to acquire expression of CD8alpha in the TGFbeta-rich GI tract once peripheral lineage committed CD4+ T cells have trafficked there from the periphery, as transfer of CD4+ T cells into T cell deficient hosts leads to the generation of CD4+CD8alpha+ T cells specifically in the IEL compartment. In line with this it was recently demonstrated that in vitro culture of CD4+ T cells with TGFbeta induces expression of CD8alpha. We made use of these in vitro and in vivo models of CD4+ T cell acquisition of CD8alpha expression to probe the molecular events downstream of the TGFbeta receptor required for TCRalphabeta+CD4+CD8alpha+ IEL development. Canonical TGFbeta signaling is mediated by Smad proteins, yet here we demonstrate that non-canonical, E2A-dependent signals are required for the development of this population of IELs. Genetic deletion or siRNA knockdown of E2A in CD4+ T cells resulted in an inability to express CD8alpha in response to TGFbeta. In complimentary experiments we employed CD4+ T cells in which E2A activity was enhanced through genetic deletion of its inhibitor Id3. Id3-/- CD4+ T cells gave rise to increased frequencies of CD4+CD8alpha+ T cells in response to TGFbeta in vitro and, importantly, TCRalphabeta+CD4+CD8alpha+ IEL in vivo. Collectively, we show that E2A is a downstream mediator of TGFbeta in the induction of CD8alpha on CD4+ T cells and in the generation of TCRalphabeta+CD4+CD8alpha+ IEL. These data not only begin to elucidate the molecular events which allow the expression of CD8alpha homodimers on CD4+ T cells, but also shed light on the developmental pathway of a population of IEL shown to regulate colitogenic responses.

NIDCR
Mays, Jacqueline
Clinical Fellow
Immunology - Autoimmune
Salivary Mediators Suggest New Mechanisms in Chronic-Graft-versus-Host Disease Sicca Patients

Oral mucosa and salivary glands are involved in ~50% of patients affected with chronic graft-versus-host disease (cGVHD), an autoimmune-like disorder afflicting a majority of long-term survivors of allogeneic hematopoietic stem cell transplantation. We assessed the levels of inflammatory, tissue-modulatory and chemotactic factors in saliva and plasma of cGVHD patients with reduced salivary flow. These levels were correlated with clinical assessments of oral cGVHD and minor salivary gland histopathology to evaluate the use of saliva as source of relevant biomarkers of disease severity and pathogenesis. Subjects enrolled in an ongoing cGVHD Natural History Protocol (NCT00331968) were evaluated for mucosal cGVHD (Oral Mucositis Rating Scale, OMRS) and salivary flow. In 33 patients selected for sicca symptoms, matched plasma and whole saliva samples were assayed for inflammatory factors(IL-6, TNFRII), chemokines associated with Th1(IP-10, MIG), Th2 (MDC), Th17(CCL20), and myeloid (MCP-1) recruitment, and factors involved in fibrosis (MMP3, MMP9, TIMP1) using multiplex ELISA assays. A subset of 10 patients consented to minor salivary gland biopsy, graded by Greenspan scale. Data were log transformed then compared using multiparameter statistical analysis. Significant correlations (p<0.05) were found in a multiparameter analysis between clinical OMRS score and salivary levels of MIP3a (CCL20), associated with monocyte and Th17 infiltration into mucosal tissue and BAFF, a known plasma marker for cGVHD activity. Patients with high Greenspan score (3-4) had significantly increased IP-10 in saliva vs those with low Greenspan scored (0-2). Significant associations were not found between clinical oral GVHD scores and plasma protein levels, nor between salivary and plasma protein levels. In this study, salivary proteins were more targeted indicators of important parameters in clinical oral disease than were plasma proteins. Minor salivary gland infiltration was positively correlated with saliva IP-10, a chemotactic factor for activated T cells. OMRS score was significantly associated with BAFF, a known marker in plasma for systemic cGVHD activity not previously associated with oral mucosal disease, and chemokine (CCL20) associated with dendritic cell and Th17 recruitment and signaling. Expression of these markers in saliva implicates new pathways for future work on the immunological mechanisms of oral cGVHD.

NIDCR
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Neuroscience - Cellular and Molecular
The cellular code for mammalian thermosensation
Mammalian somatosensory neurons are exquisitely sensitive across a wide range of temperatures. This allows for attraction to optimal, as well as avoidance of potentially dangerous temperatures. In this study we dissected the neuronal inputs responsible for both innocuous and noxious temperature sensation, and discovered that a few types of neurons can encompass the full range of mammalian temperature sensation. Previously, we generated mutant mice that are insensate to all thermal cues because they lack many different classes of nociceptive cells. To dissect the function of nociceptors within this population, we initially focused on inputs from the two distinct populations of neurons expressing either TRPV1 or TRPM8 ion channels. To examine the thermosensory functional roles of these neurons, we engineered mice where these cell populations can be conditionally ablated with diphtheria toxin treatment. We found that TRPV1-expressing neurons are responsible for aversion to temperatures from 40 to 50°C while TRPM8 neurons are responsible for most cold aversive behaviors. In addition, ablation of TRPM8 neurons caused a shift in the preferred temperature towards cooler temperatures and an increased aversion to high temperatures. Upon ablation of both TRPV1 and TRPM8 cells, mice lose their preference for mild warmth and avoidance of high and low temperatures, but still avoid extremes. These data suggest that all temperature sensation between 10 and 50°C results from non-overlapping aversive inputs from TRPM8 and TRPV1 neurons. We propose that attraction to pleasantly warm temperatures may in fact be due to minimal aversive inputs from both of these cell

populations. In addition, our data show that avoidance of extreme temperatures is the result of an additional population of nociceptors that express Mrgprd, and that these responses are likely mediated through an additional, non-thermally gated, mechanism. Together these studies give us a better understanding of how humans and other mammals are able to navigate a wide range of environments effectively and safely.

NIDCR
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Visiting Fellow
Vascular Disease and Biology
Rho Kinase Inhibitors Protect from Anaphylactic Shock
Histamine-induced vascular permeability is an integral component of many highly prevalent human inflammatory diseases, including asthma and sepsis, and plays a central role in the atopic syndrome and anaphylaxis, which result in ~1500 deaths per year in the US alone. By the use of genetically defined animal models, pharmacologic inhibitors, and a synthetic biology approach we show here that the small GTPase RhoA mediates histamine-induced endothelial permeability. Histamine disrupted the endothelial barrier in vitro and in vivo, which was verified by electron microscopy. Pharmacological inhibitors confirmed that H1 histamine receptors are responsible for the induction of endothelial cell permeability. The coupling of H1 receptors to the G protein a subunits Gaq and Ga11 was verified by monitoring increased [Ca2+] levels following histamine stimulation and by the abrogation of in vivo permeability in endothelial-specific Gaq/11 KO mice. Of interest, disruption of the endothelial barrier function and vessel leakiness required the activation of RhoA, rather than being mediated by diffusible second messenger generating systems, such as PLC activation, calcium mobilization, and phospholipid turnover. RhoA activation upon histamine stimulation is rapid and RhoA knock down abolished endothelial permeability. The same effect was observed by the pre-treatment with the Rho inhibitor C3 exoenzyme from Clostridium botulinum and with small molecule pharmacological inhibitors of Rho kinase. The effect of small molecule Rho kinase inhibitors was also confirmed in vascular permeability assays in vivo. The ability of H1 receptors to trigger a Gq/11-RhoA pathway leading to endothelial permeability was recapitulated by the use of synthetic biology approach consisting in the expression of mutant GPCRs that couple to Gq/11 in response to an artificial ligand, CNO, in endothelial cells. Furthermore, small molecule Rho kinase inhibitors abrogated the inflammatory response underlying passive cutaneous anaphylaxis and rescued mice from PAF-induced anaphylaxis and death. Ongoing experiments using conditional deletion of RhoA in endothelial cells in vivo may further support the key biological role for this GTPase and its regulated signaling network in histamine-induced endothelial permeability, thereby identifying novel pharmacological targets for the treatment of a myriad of human diseases characterized by aberrant vascular leakage, ranging from allergic reactions to septic shock.

NIDDK
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Postdoctoral Fellow
Biochemistry - General and Lipids
O-linked N-acetylglucosamine is critical for the Caenorhabditis elegans innate immune response to S. aureus
The dynamic posttranslational O-linked N-acetylglucosamine (O-GlcNAc) modification of serine and threonine residues plays a critical role in cell signaling and is implicated in many human diseases. Its addition to proteins is governed by the enzyme O-GlcNAc transferase (OGT). Data from our lab reveal that deregulation of O-GlcNAc addition results in the deregulation of stress- and immune-responsive genes. Moreover, literature data show that mammalian OGT physically interacts with evolutionarily

conserved proteins key for the innate immune response, including p38 MAPK (PMK). Given these findings and the modificationâ€™s role as a signaling molecule, we hypothesized that O-GlcNAc plays a role in â€œfine-tuningâ€ the innate immune response. Utilizing the bacteriovore C. elegans, we explored whether OGT null animals would stimulate effective immune responses to pathogenic S. aureus (SA). First, we identified that while OGT and PMK mutants are healthy under normal conditions, both genotypes have a 20% decreased viability in comparison to wild type (WT) when fed SA. Importantly, SA-fed PMK null animals in the OGT null background have lifespans over 44% shorter than WT animals. These data suggest that OGT and PMK act synergistically to stimulate the immune response to pathogenic SA. Furthermore, we generated genome-wide microarray data to analyze the OGT and PMK null animals' transcriptional response to SA. These microarray data indicate that there is little overlap between the genes regulated by OGT and PMK supporting that they act in different pathways to promote immunity. Indeed, both OGT and PMK control the expression of candidate antimicrobials including C-type lectins, antimicrobial peptides, and CUB-like genes. Our data highlight that O-GlcNAc plays a novel, indispensable role in the C. elegans innate immune response to SA and that the modificationâ€™s addition to proteins may be key for immune regulation. Additionally, our microarray data support that OGT is an essential component for infection-induced expression of SA-triggered immune response genes. Our findings provide the first insight into O-GlcNAcâ€™s role in immunity and underscore that in order to combat pathogen infection we need to bolster our knowledge of signaling pathways involved in the innate immune response.

NIDDK
Hwang, Ling Chin
Visiting Fellow
Biophysics
Protein pattern formation drives bacterial plasmid segregation: an analysis of ParB mutations
DNA segregation is a vital process that ensures every daughter cell inherits a copy of genomic DNA. Low-copy-number plasmids such as P1 in Escherichia coli have partitioning systems to separate and transport plasmids towards opposite cell-halves before cell division. Only three components are required to partition plasmids: a parS sequence on the plasmid and two proteins, ParA and ParB. It was previously believed that P1 plasmids segregate similar to a mitotic mechanism in eukaryotes. We proposed that the partition proteins pattern the bacterial nucleoid using it as a track for plasmid motion. We recently reconstituted the P1 partition system in a DNA-coated flowcell as an artificial nucleoid, and visualized with TIRF microscopy. ParA and ParB coated the DNA surface uniformly and ParA, an ATP-dependent DNA-binding protein, formed a transient depletion zone surrounding the plasmid prior to its motion on the DNA surface. ParB binds specifically to parS site and stimulates ParA ATP-hydrolysis but how this generates ParA patterning and plasmid motion on DNA is unclear. The N-terminus of ParB is responsible for interacting with ParA and with itself but it has been difficult to study because this region is flexible and is likely unfolded. Two mutations in the N-terminus of ParB were studied to understand its role of ParA stimulation in protein patterning. When ParA-GFP, ParB[G10E] mutant, parS-Alexa647 and ATP were infused into the flowcell, the plasmids were observed to freely diffuse in solution while ParA-GFP coated the DNA surface, indicating that the G10E mutation is defective for ParA-ParB interactions that are needed to bridge the plasmid onto DNA. A second mutant ParB[L13P], displayed a partition-deficient phenotype and was unable to stimulate ParA. When all reaction components were infused into the flowcell, the plasmids were bridged onto the DNA surface and remained stably bound without any ParA depletion zones or plasmid dynamics. Photobleaching a region of ParA on DNA surface in the presence of L13P revealed a fluorescence recovery time constant 1.4-fold slower than with wild-type ParB, indicating that ParA exchange on DNA is influenced by ParB stimulation. These data show that the G10 and L13 residues in the N-terminus of ParB are involved in ParA-DNA interactions and stimulating ATP-

hydrolysis, which are prerequisites for ParA patterning on the bacterial nucleoid and for generating the driving force for plasmid segregation in live cells.

NIDDK
Iyer, Jyoti
Visiting Fellow
Cell Biology - General
PP1 is necessary for the fidelity of centrosome duplication
Centrosome duplication (CD) is a highly regulated process. Centrosomes must be duplicated only once during each cell cycle. Deregulation of this process yields an abnormal centrosome number. This can result in aneuploidy, a hallmark of cancer cells. The nematode C. elegans is an excellent model system to study the process of CD because the core components of the CD pathway in C. elegans are conserved in humans. Genome-wide RNAi and forward genetics screens have thus far identified only six proteins as being necessary for CD in C. elegans. One such protein is the serine-threonine kinase ZYGote defective -1 (ZYG-1). Mutation of the zyg-1 gene results in a failure to duplicate centrosomes and thereby causes embryonic lethality. Since the genome-wide screens mentioned previously have been able to identify only six proteins involved in CD, an alternative approach is required to identify additional proteins that play a role in CD. Therefore, we have utilized a different approach to screen for novel proteins in the CD pathway. This approach involves screening for genes that when mutated, can restore normal CD in the zyg-1 mutants. These genes thereby encode novel candidate proteins of the CD pathway. Through our study, the protein phosphatase PP1 has been identified as a novel component of the C. elegans CD pathway. Importantly, the depletion of PP1 as well as its regulatory subunits- I-2 and SDS-22 using RNA interference can rescue the CD defect of zyg-1 mutants. Thus, under normal conditions, PP1 functions to inhibit ZYG-1 mediated CD. Significantly, the removal of either SDS-22 or I-2 promotes the overduplication of centrosomes in C. elegans. Thus, under endogenous conditions, PP1 seems to act as a molecular brake for CD. Current efforts are being directed to identify the substrate of PP1 through which it regulates CD. Notably, the depletion of an RNA-binding protein, R09B3.3, also rescues the embryonic lethality of zyg-1 mutants. Furthermore, R09B3.3 interacts physically with the PP1 regulators, I-2 and SDS-22. These data make R09B3.3 an attractive candidate substrate through which PP1 could regulate CD in C. elegans. In summary, our findings highlight PP1 as a novel core component of the C. elegans CD pathway.

NIDDK
Walters, Alison
Visiting Fellow
Cell Biology - General
Regulators of nuclear envelope expansion in yeast
Nuclei of cells of a particular cell-type have a distinctive size and shape. There are several disease states in which abnormalities in nuclear size and shape are observed, including cancer and the premature aging syndrome, progeria. Despite these long-standing observations, our understanding of the factors determining nuclear morphology remains poor. The nuclear envelope (NE) is a double membrane structure that encloses the nucleus. In higher eukaryotes, the NE expands at the end of mitosis to allow for chromosome decondensation. In yeast, which undergo closed mitosis where the NE remains intact throughout the cell cycle, the NE must expand during mitosis to allow chromosome segregation and the formation of two daughter nuclei. The mechanisms by which NE expansion is achieved and controlled are unknown. To understand the causes and consequences of abnormal nuclear morphology in disease states, we must first understand how normal nuclear shape is regulated. I am using the yeast S. cerevisiae as a model system to discover genes involved in the process of NE expansion. Recent work in

our lab has shown that when wild-type yeast cells delay in mitosis, despite the fact that chromosome segregation is stopped, NE expansion continues and a membrane extension, or â€œflareâ€, is formed at the site of the nucleolus. I used this observation to design a screen to identify mutants that do not form a flare during a mitotic arrest, and therefore have defects in regulating NE expansion. I generated a collection of 1500 temperature sensitive mutants and screened them, using fluorescence microscopy, for mutants where nuclei remain round during mitotic delay. Through this screen, I have identified 8 genes in which point mutations alter NE expansion. These genes include several genes involved in lipid synthesis, as well as genes involved in tRNA metabolism, transcriptional regulation and spliceosome activation. I have also identified a point mutation in the kinase domain of a key cell cycle regulator, CDC5, which causes altered NE expansion. CDC5 is homologous to polo kinases in humans and it plays multiple known roles in the regulation of mitosis. However, I found that Cdc5's involvement in NE expansion occurs through a novel pathway. My analysis of the cdc5 mutant phenotype indicates that Cdc5 plays a role in sequestration of excess NE to the nucleolar region. This is the first time that Cdc5 has been implicated in the control of nuclear architecture.

NIDDK
Goeres, Jacqueline
Postdoctoral Fellow
Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology
Centrosome duplication is regulated by the Ras signaling pathway and the DP/E2F transcriptional network
The formation of a bipolar mitotic spindle organized by centrosomes at each pole is a key determinant for the accurate segregation of chromosomes during cell division. In order to ensure spindle bipolarity, centrosomes must be duplicated once and only once per cell cycle. Precise regulation of centrosome duplication is therefore essential for proper cell growth and division. Misregulation of the centrosome duplication pathway leads to the formation of aberrant spindles, which cause defects in chromosome segregation and contribute to aneuploidy and tumorigenesis. Several lines of evidence have indicated that signaling through the Ras oncogene and MAP kinase pathway causes centrosome amplification. However, the molecular mechanism underlying these events has not been elucidated. Genetic analysis in C.elegans has identified a basic framework of five conserved factors that are required for centrosome duplication, which include the kinase ZYG-1 (PLK-4 in humans), a master regulator of this pathway. Mutation or depletion of these factors leads to a failure in centriole duplication and results in the formation of monopolar spindles in the C. elegans embryo. The zyg-1(it25) mutant fails to duplicate its centrioles, resulting in embryonic lethality. We found that mutation of the transcription factor dpl-1 restores centrosome duplication in the zyg-1(it25) mutant and suppresses its embryonic lethality, indicating a genetic interaction between the transcriptional regulator and the centrosome duplication pathway. dpl-1 is the homolog of human DP, a binding partner of the E2F transcriptional regulator that is responsible for the G1-to-S phase transition in higher eukaryotes. Mutation of the E2F homolog, efl-1, also suppresses zyg-1(it25) lethality, implicating that the DPL-1/EFL-1 complex negatively regulates centrosome duplication. Consistent with this, ZYG-1 protein levels are increased in the dpl-1 mutant indicating that DPL-1 normally represses zyg-1 expression. Notably, dpl-1 has been shown to be negatively regulated by Ras signaling. We found that overactivation of the Ras signaling pathway through the expression of a gain-of-function allele of Ras also suppresses zyg-1(it25) lethality. This work elucidates a transcriptional network that is regulated by Ras signaling and controls expression of the centrosome duplication factors, providing a mechanistic link between Ras and centrosome amplification.

NIDDK

Lee, Su-Lin
Postdoctoral Fellow
Chemistry
Design, Synthesis, and Target Validation of Novel FtsZ Inhibitors as Anti-MRSA Agents
Objective The methicillin-resistant Staphylococcus aureus (MRSA) is one of the most life-threatening pathogens and is now causing more deaths than AIDS in the U.S. since 2005. There is urgent need for novel selective antibiotics with low resistance probability. One of the emerging targets for anti-MRSA therapy is the cell-division protein, FtsZ. FtsZ is conserved in almost all known pathogenic bacteria and plays a key role in bacterial cell division. FtsZ is a filament-forming GTPase that localize to the bacterial cell-division site to form the Z-ring prior to cell division. A number of FtsZ-interacting compounds have been reported, some of which have powerful antibacterial activity. Previously, we discovered a marine natural product, Chrysophaentin A, that was isolated from the chrysophyte alga Chrysophaeum taylori. This macrocycle compound demonstrated strong anti-bacterial activities against a panel of gram-positive bacteria (e.g. MRSA MIC50: 1.5 ± 0.7 μg/mL; vancomycin-resistant Enterococcus faecium MIC50: 2.9 ± 0.8 μg/mL) through the inhibition of FtsZ. The objective of this work is to use Chrysophaentin A as a lead to develop novel potent small molecule inhibitors of FtsZ. Methods Through the process of structure-based drug design, we first analyzed the binding interactions between the crystal structures of S. aureus FtsZ and chrysophaentin analogs from molecular docking simulations. A new series of compounds were designed after two generations of in silico structure optimization. The new designed compounds were further synthesized through an 8 step synthetic pathway. Two key reactions were optimized for constructing the backbone structure of these compounds, including Suzuki coupling and aldol condensation. This compound library was then screened by using a disc diffusion assay for anti-bacterial activities. FtsZ inhibition activity will be evaluated using a FtsZ polymerization assay. Results & Conclusions The original macrocycle structure of chrysophaentin A was simplified through our molecular modeling simulation by keeping half of the backbone structure with moderate modifications. Ten compounds were generated via the synthetic pathway with reasonable yields (~10%). Among these compounds, f-NH2 demonstrated unique selectivity against MRSA without affecting S. aureus, B. subtilis, E. coli, and VREF in the initial screening. We will further focus on the structure modification of f-NH2 and its mechanistic studies.

NIDDK
Lee, Ji Eun
Visiting Fellow
Chromatin and Chromosomes
H3K4 mono-/di-methyltransferase MLL4 marks adipogenic enhancers and controls adipocyte differentiation
Spatiotemporal regulation of gene expression is critical for animal development. Enhancer is one of the essential regulatory elements that control tissue- and developmental stage-specific gene expression. Recent genome-wide association studies indicate that enhancers are marked by high levels of H3K4me1 and H3K4me2 but low levels of H3K4me3. Active enhancers are further marked by H3K27ac. However, several questions remain open regarding chromatin modification on enhancers. First, which methyltransferases are responsible for H3K4me1/2 on enhancers? Second, how are these methyltransferases recruited to enhancers? Third, what is the functional significance of H3K4me1/2 on enhancers? We previously purified the MLL3/MLL4-containing histone H3K4 methyltransferase complex. We further showed that PTIP, a component of MLL3/MLL4 complex, is critical for adipocyte differentiation (adipogenesis). Here we show that MLL4 is a major H3K4 mono/di-methyltrasferase during adipogenesis, that MLL4 is essential for adipogenesis, and that MLL4 directly controls the induction of adipogenesis genes. Genome-wide profiling reveals extensive co-localization of MLL4 with

the master adipogenic transcription factors C/EBPalpha, C/EBPbeta and PPARgamma on enhancers during adipogenesis, indicating that MLL4 marks adipogenic enhancers. Consistently, MLL4 physically interacts with C/EBPbeta and PPARgamma. Further, deletion of MLL4 leads to dramatic decreases of H3K4me1/2 and H3K27ac levels on adipogenic enhancers, indicating that MLL4 is the dominant H3K4 mono/di-methyltransferase required for enhancer activation during adipogenesis. Finally, overexpression of C/EBPbeta alone in the absence of differentiation markedly increases MLL4-mediated H3K4me1 on adipogenic enhancers, suggesting that C/EBPbeta recruits MLL4 to adipogenic enhancers to establish enhancer chromatin signature. Together, our data suggest that the master adipogenic transcription factors recruit H3K4 mono/di-methyltransferase MLL4 to establish and activate enhancers critical for adipocyte differentiation.

NIDDK
Lee, JongJoo
Visiting Fellow
Chromatin and Chromosomes
LDB1 and CTCF cooperatively mediate chromatin looping
Long range interactions between functional DNA elements such as insulators, enhancers, promoters and silencers are important for transcriptional regulation. LDB1 is known to be involved in enhancer long range interaction in the ß-globin locus by self interaction. CTCF is also known to be involved in long range interaction at several different loci, primarily as an enhancer blocking insulator. Here we find that CTCF and LDB1 are cooperatively involved in chromatin loop formation and gene transcription. First we demonstrate direct interaction between LDB1 and CTCF using pull down assays with purified protein and co-IP with nuclear extract of MEL cells. Further analysis with FPLC shows that LDB1 and CTCF co-elute in the same fractions. This result suggests that CTCF and LDB1 may cooperatively regulate gene expression. Second, to demonstrate transcriptional regulation, we analyzed the carbonic anhydrase (CAR) gene locus in erythroid cells. CAR1 and CAR2 are preferentially down-regulated by knockdown of either LDB1 or CTCF and we confirmed the occupancy of these factors in the CAR locus using chromatin immunoprecipitation (ChIP) assays. Finally by performing 3C in the CAR locus, we confirmed that an enhancer LDB1 binding site interacts with a CAR2 promoter CTCF binding site but not in LDB1-KD or CTCF-KD cells. To verify this interaction we performed dual cross-linking ChIP with EGS crosslinker and showed the occupancy. We conclude that LDB1 and CTCF can cooperate directly in chromatin loop formation and gene transcription.

NIDDK
Fufaa, Gudeta
Postdoctoral Fellow
Clinical and Translational Research
Effect of Losartan on Prevention and Progression of Early Diabetic Nephropathy in American Indians with Type 2 Diabetes
Angiotensin receptor blockers (ARB) reduce the rate of diabetic kidney disease progression in hypertensive, azotemic patients with type 2 diabetes (T2D). Their efficacy in slowing progression of early kidney disease, however, is less certain, and use of surrogate endpoints complicates interpretation of most studies. We performed a randomized placebo-controlled clinical trial in 169 Southwestern American Indians with T2D to test whether the ARB losartan offered renoprotection in those who had either normal urinary albumin excretion (albumin/creatinine ratio (ACR) <30 mg/g) or microalbuminuria (ACR=30-299 mg/g) at baseline. Participants received 100 mg losartan or placebo daily for a median of 5.9 years (IQR=5.0-6.0 years). Glomerular filtration rate (GFR) and ACR were measured annually, and 111 participants underwent kidney biopsies after 6 years. Primary endpoint was decline in

GFR to =60 ml/min or to half the baseline value in subjects entered with GFR <120 ml/min. Other endpoints were progression to macroalbuminuria and differences in glomerular structure at end of treatment. Baseline characteristics of the treatment groups were similar, except for higher GFR (P=0.02) and HbA1c (P=0.01) in subjects with normoalbuminuria who received losartan, and longer duration of diabetes (P=0.02) in subjects with microalbuminuria who received placebo. Only 9 subjects reached the GFR endpoint, and the hazard ratio (losartan vs. placebo) was 0.50 (95% CI 0.12-1.99). Differences in mesangial fractional volume and progression to macroalbuminuria were not estimated in the combined albuminuria groups because of a significant interaction with treatment assignment. Mesangial fractional volume was lower in subjects treated with losartan in the microalbuminuria group (18.8% vs. 25.6%, P=0.02), but not in the normoalbuminuria group (19.6% vs. 17.8%, P=0.86). The hazard ratio (losartan vs. placebo) for progression to macroalbuminuria was 8.12 (95% CI 1.02-64.98) in the normoalbuminuria group and 0.54 (95% CI 0.26-1.10) in the microalbuminuria group. These results suggest that treatment with losartan preserves some features of normal kidney structure in American Indians with T2D and microalbuminuria. This benefit does not extend to those with normoalbuminuria at baseline, in whom treatment was associated with a significant increase in the incidence of macroalbuminuria.

NIDDK
Huang, ChenChe
Visiting Fellow
Endocrinology
Thyroid hormone-mediated adrenal regression/remodeling
Most hormone disorders of the adrenal cortex occur in the context of overgrowth or underdevelopment of the adrenal gland. At embryonic stages, the fetal adrenal cortex already has the ability to synthesize and secrete steroid hormones that are critical for fetal development. These functional fetal cortical cells then undergo regression and are replaced by continuously renewing adult-like cortical cells after birth. The continuously renewing adult cortical cells grow from the most outer layer of the cortex and form the â€œadult zoneâ€, whereas fetal cortical cells stay in the inner portion of the cortex, losing the steroid synthesis ability overtime and form the â€œfetal zoneâ€. During development, the fetal zone regresses while the adult zone grows. In humans, failure of fetal cortex regression is accompanied by poor differentiation of adult cortical cells, resulting in steroid hormone deficiency. Also, adrenocortical carcinoma has been proposed to originate from fetal cortical cells. However, the mechanisms that regulate the differential development and the regression of the adrenal cortex remain unclear. Here, we first report that thyroid hormone receptors are expressed in the adrenal cortex, especially in the adrenal fetal zone. X-gal staining shows that a knockin of lacZ in the endogenous TRb1 gene is specifically expressed in the fetal zone. To further study how thyroid hormone elicits its function in the fetal zone, we used thyroid hormone treatment and knockout mouse models with disruption of genes involved in the thyroid hormone signaling pathway. We found that the thyroid hormone-mediated fetal zone hypertrophy and the increased steroid synthesis activity relies on TRb1 and thyroid hormone transporters, Mct8 and Mct10. Furthermore, loss of functional thyroid hormone receptor, TRa, delays the formation and regression of the fetal zone. TRb1 KO mice also have a disturbed expression profile of the fetal zone specific gene. Our results point to the novel role of thyroid hormone in adrenal regression/remodeling. This study helps in the understanding of the mechanism that controls the cell fate of adrenal cortical cells and could further provide novel therapeutic interventions for adrenal cancer and adrenal insufficiency.

NIDDK
Katz, Liora
Visiting Fellow

Endocrinology

Reprogramming adult human dermal fibroblasts to islet-like cells by epigenetic modification coupled to transcription factor modulation

In type 1 diabetes (T1D) there is a loss of insulin producing ß cells and patients are dependent on daily insulin injections for their survival. Several protocols to transplant islets from cadaveric donors have been developed, however, their use is limited mainly because of a shortage of donors. Hence, generation of islet-like ß-cells from other cell types may be used in place of bona fide islets and could result in significant improvement over current therapeutic approaches for patients with diabetes. We describe novel conditions for culture, expansion and transdifferentiation of primary human dermal fibroblasts (hDFs) to induce expression of transcription factors (TFs) and hormones characteristic of the islets of Langerhans. We show that histones associated with the insulin gene are hyper-acetylated and that insulin gene DNA is less methylated in islet cells compared to cells that do not express insulin. Using two compounds that alter the epigenetic signature of cells, romidepsin (Romi), a histone deacetylase inhibitor, and 5-Azacytidine (5-AzC), a chemical analogue of cytidine that cannot be methylated, we show that hDFs exhibit a distinctive regulation of expression of TFs involved in islet development as well as of induction of glucagon and insulin. Overexpression of Pdx1, a TF important for islet differentiation, and silencing of MafB, a TF that is expressed in mature glucagon producing cells, result in induction of insulin to a higher level compared to Romi and 5-AzC alone. The cells obtained from this protocol exhibit glucose-stimulated insulin secretion, and lower blood glucose levels of diabetic mice. These data show that fully differentiated non-islet derived cells could be made to transdifferentiate to islet-like cells and that combining epigenetic modulation with TF modulation leads to enhanced insulin expression.

NIDDK

Lateef, Dalya

Postdoctoral Fellow

Endocrinology

Bombesin Receptor Subtype-3 in Cardiovascular Regulation and Brown Adipose Thermogenesis

Bombesin receptor subtype-3 (BRS-3) is an orphan G-protein coupled receptor implicated in the regulation of energy metabolism. BRS-3 knockout (Brs3 KO) mice have increased food intake, reduced metabolic rate, reduced body temperature and increased body weight. Synthetic BRS-3 agonists reduce food intake, increase metabolic rate and body temperature and reduce body weight in mice, rats and dogs. BRS-3 agonists also increase heart rate in dogs, increase both blood pressure and heart rate in rats, and transiently increase blood pressure in humans. Here, we investigate the role of BRS-3 in blood pressure, heart rate and body temperature. Body temperature is a component of energy metabolism and, under defined conditions, can be used as a surrogate of metabolic rate. We measured blood pressure in anesthetized Brs3 KO mice and wild type controls. Brs3 KO mice had a lower mean arterial, systolic and diastolic blood pressure compared to controls, although there was no difference in heart rate between groups. Treatment with MK-5046, a synthetic BRS-3 agonist, increased both blood pressure and heart rate in wild type but not Brs3 KO mice. Clonidine, a centrally acting a2-adrenoceptor agonist that reduces sympathetic autonomic outflow from the brain, attenuated the increase in blood pressure by MK-5046, suggesting that MK-5046 activates BRS-3 receptors in the brain that, in turn, increase sympathetic outflow to the periphery to increase blood pressure and heart rate. In addition, we found that ambulatory Brs3 KO mice had lower BAT temperatures under fed and fasted conditions compared to controls, although Brs3 KO and wild type mice showed a similar increase in BAT temperature in response to acute 6-hour cold expose, suggesting the presence of functional BAT in the Brs3 KO mice. Furthermore, MK-5046 increased body and BAT temperature in anesthetized wild type but not Brs3 KO mice. Taken together, these data provide evidence that BRS-3 may be involved in the

regulation of BAT thermogenesis. In addition, our data demonstrate that central activation of BRS-3 may have a role in cardiovascular regulation by acting through a sympathetic pathway.

NIDDK
Li, Yong-Qi
Visiting Fellow
Endocrinology
The G Proteins Gq/11 alpha and Gs alpha mediate distinct physiological responses to central melanocortins
Mutations of the G protein-coupled melanocortin-4 receptor (MC4R) are the most common cause of monogenic obesity. MC4R deficiency in both humans and mice leads to several physiological defects, including increased food intake, fat mass and body length and reduced sympathetic nerve activity (SNA), energy expenditure, and insulin sensitivity. Pseudohypoparathyroidism type 1A is another monogenic obesity resulting from heterozygous inactivating mutations of Gs alpha, the ubiquitous G protein that couples receptors, including MC4R, to intracellular cAMP production. Mice with brain-specific disruption of the Gs alpha maternal allele (mBrGsKO) develop obesity associated with reduced SNA and energy expenditure, as well as peripheral insulin resistance prior to the onset of obesity, but do not show a primary defect in food intake or body length. The ability of mBrGsKO mice to increase their energy expenditure in response to the MC4R agonist MTII is impaired, while MTII inhibition of food intake is unaffected. It therefore appears that MC4R mediates its effects on body length and food intake via Gs alpha-independent pathways. We hypothesized that these Gs alpha-independent MC4R effects may be mediated by another G protein family Gq/11 alpha that primarily couples receptors to activation of phospholipase C. As MC4R controls food intake primarily in the paraventricular nucleus of hypothalamus (PVN), we generated a mouse line with PVN-specific loss of Gq/11 alpha (PVNGq/11KO:Sim1-cre+, Gq alpha flox/flox, G11 alpha-/-). Like MC4R knockout mice and in contrast to mBrGsKO mice, PVNGq/11KO mice had marked obesity due to hyperphagia and increased body length without primary effects on energy expenditure or insulin sensitivity. In contrast to mBrGsKO mice, MTII inhibition of food intake was impaired while MTII stimulation of energy expenditure was unaffected. Sim1 and CRH expression in PVN was significantly reduced, and serum ACTH and corticosterone levels were markedly reduced. Our results show that physiological effects of central melanocortins are mediated by distinct G protein pathways: food intake and body length via Gq/11 alpha in PVN; SNA, energy expenditure and insulin sensitivity via Gs alpha in areas outside PVN. Gq/11 alpha signaling in PVN is critical in regulation of the HPA axis. A biased MC4R agonist that activates Gq/11 alpha but not Gs alpha has the potential to suppress appetite without untoward cardiovascular effects observed with non-selective agonists.

NIDDK
Xu, Lingyan
Visiting Fellow
Endocrinology
Forkhead box protein A3 deficient mice display an age associated lean phenotype and enhanced thermogenesis
Aging is associated with increased adiposity and severe decline of thermogenesis and mitochondrial function, which are critical determinants of the onset of obesity, type 2 diabetes and other disorders. It has been reported that aging leads to decreased incidence of cold-activated BAT and programmed loss of brown adipocytes in subcutaneous white adipose tissue. To date, only few transcription factors have been shown to affect this process. The forkhead box (FOX) transcriptional regulators belong to an evolutionarily conserved family of factors binding to DNA through a winged-helix binding motif and regulating organ development, metabolism and aging. Among them, Foxa3 belongs to FOXA subfamily

and has been shown to be essential for the maintenance of glucose homeostasis during a prolonged fast. However, adult old Foxa3 null mice show no apparent anomalies in weight gain, fed blood glucose levels, triglycerides, or cholesterol levels. We report here that aging Foxa3 null mice have significantly reduced adiposity, improved insulin sensitivity and serum profiles. In addition, our physiological analysis revealed that Foxa3 deficiency enhanced the thermogenic capacity and energy expenditure in aging mice by inducing thermogenic and mitochondrial genes in brown adipose tissue and brown remodeling of white adipose tissue. Interestingly, further examinations showed that Foxa3 expression levels were increased in brown and inguinal fat during aging, which were negatively correlated with decreased expressions of PGC1a, a master regulator of mitochondrial biogenesis and adaptive thermogenesis. Via PGC1 promoter analysis, we identified a Foxa responsive element in the proximity of the cAMP response element (CRE), which is an important regulator of the induction of PGC1 in response to cAMP treatment. Through Chromatin IP assays and mRNA analysis, we revealed that ectopic expression of Foxa3, or increased Foxa3 levels in BAT of aged mice, disrupted CREB binding on the CRE site and suppressed PGC1a transcription. Thus, our data demonstrates that Foxa3 ablation in mice prevents aging-associated obesity and insulin resistance by driving thermogenesis through increased PGC1a expression in brown and inguinal fat, suggesting Foxa3 as a novel therapeutic target to treat aging associated metabolic disorders.

NIDDK
Coulon, Antoine
Visiting Fellow
Gene Expression

In vivo transcription and splicing kinetics revealed by fluctuation analysis of single-molecule nascent-RNA measurements in live human cells

Eukaryotic transcription involves the coordination of many multi-subunit complexes, including the pre-initiation complex, the polymerase, the spliceosome and elongation and termination factors. Most knowledge in the field is inferred from ensemble and/or in vitro assays (e.g. ChIP, ChIP-seq or RNA-seq), giving a detailed but steadystate picture. How these macromolecular machines coordinate in vivo remains unknown. Recently, transcription was observed in live yeast cells by monitoring fluorescently-tagged nascent transcripts over time. Here, in collaboration with experimentalists, we extend this method to a dual-color system in human cells, allowing one for the first time to resolve the kinetics of initiation, elongation, splicing and termination at the same gene. Using the RNA-binding MS2 and PP7 proteins, we fluorescently labeled with two distinct colors the largest intron and the 3â€™UTR of a stably integrated human beta-globin reporter gene. By tracking in both colors the transcription site in space and time, I obtained time traces of fluorescence fluctuations reflecting events taking place on the gene: initiation of pre-mRNA synthesis, elongation dynamics of the polymerase, and release of introns and transcripts. To extract these kinetics from the fluorescence time traces, I developed an approach based on auto- and cross-correlation functions (i.e. curves reflecting how much a fluctuation in one color is statistically correlated with a fluctuation in either the same or the other color after a given delay). To that end, I derived a stochastic mathematical model relating the shape of the correlation functions with the nature and timing of the underlying mechanisms. This allowed us to extract quantitative information and to test hypotheses about splicing and polymerase progression/pausing. We observe an elongation speed of 1.5 kb/min, which is measured independently of initiation and termination. Intron removal was measured ~500 sec after transcription of the splice acceptor. We found no evidence for a kinetic checkpoint between splicing and transcription and were not able to detect transcriptional pausing in the gene body. However, termination involved a stochastic pause, taking on average ~200 sec, during which co-transcriptional splicing was observed only for a fraction of

transcripts. These results indicate that transcription and splicing are two independent processes that can complete in either order as a result of their proper stochastic kinetics.

NIDDK
SERTI, ELISAVET
Visiting Fellow
Immunology - Innate and Cell-mediated Host Defenses
Monocytes Sense Hepatitis C Virus-Replicating Cells and Induce Natural Killer cell Antiviral Activity in an IL-18-Mediated Manner
The innate immune system is the bodyâ€™s first line of defense against pathogens. Natural killer (NK) cells are the main effector population of the innate immune defense and elicit their protective effects through cytotoxicity and release of antiviral proteins such as interferon-gamma (IFN-g) and tumor necrosis factor-alpha (TNF-a). NK cells are activated during hepatitis C virus (HCV) infection but it is not known how they sense HCV-infected cells given that HCV suppresses the production of the NK cell-activating cytokine IFN-a. Using an in vitro co-culture system with hepatoma cells that harbor a subgenomic HCV replicon, we show that NK cells are more activated, produce more IFN-g and exert a greater antiviral effect when peripheral blood mononuclear cells (PBMCs) were added to the co-culture. The increased concentration of monocyte-derived cytokines in the PBMC/HCV-replicon co-culture supernatants suggests that monocytes contributed to NK cell stimulation. This was confirmed by the abrogation of IFN-g production of NK cells when monocytes were depleted from the PBMC/HCV-replicon coculture. We further show that monocytes directly sense replicating HCV, which results in the induction of their NALP-3 inflammasome and the release of monokines, including interleukin-12 (IL-12), TNF-a, IL-1ß and IL-18. SiRNA-mediated silencing of the NALP-3 inflammasome in monocytes or neutralization of IL-18 in the culture supernatant significantly decreased the HCV-specific IFN-g production and the antiviral effect of NK cells. The combined neutralization of NK cell-derived IFN-g and monocyte- and NK cell-derived TNF-a almost recovered HCV replication in our co-culture system, indicating that the main antiviral effect is mediated by cytokines rather than cytotoxicity. Finally, we show that the function of NK cells from patients with chronic HCV infection can be improved in our co-culture system by replacing the autologous monocytes with monocytes from healthy uninfected blood donors. In summary we provide evidence that the antiviral activity of NK cells is dependent on the activation of the NALP-3 inflammasome in monocytes and the secretion of monocyte-derived IL-18. The findings point to a monocyte rather than NK cell defect in patients with chronic HCV infection.

NIDDK
Lee, Jin-Gu
Visiting Fellow
Molecular Biology - Eukaryotic
Reversible inactivation of deubiquitinating enzymes by oxidative stress
Ubiquitination is a widespread posttranslational modification that controls many essential cellular processes in eukaryotes. Ubiquitin is conjugated to substrates by three types of enzymes, an activating enzyme (E1), a conjugating enzyme (E2), and a ligase (E3). Once attached to substrates, ubiquitin can also be removed by a family of isopeptidases termed deubiquitinase (DUB), which reverses the function of ubiquitination on substrates and thereby alters their stabilities, localizations, or activities. The human genome encodes ~100 DUBs that belong to five subfamilies: USP, UCH, OTU, Josephin and JAMM. Little is known about how DUBs are regulated. All deubiquitinases, except for those in the JAMM subfamily (12 in human), are cysteine proteases whose activities are dependent on an active site cysteine residue. This catalytic cysteine is situated in a cleft proximal to a His and an Asp residue, forming a triad that lowers the pKa of the catalytic cysteine for catalysis. Here we characterize 34 human DUBs including 25

USP, 4 OTU, 1 Josephin and 4 UCH subfamily members and show that many of these enzymes are reversibly inactivated when oxidized by reactive oxygen species (ROS) in vitro and in cells. Oxidation occurs preferentially on the catalytic cysteine, abrogating the isopeptide-cleaving activity without affecting these enzymesâ€™ affinity to ubiquitin. Sensitivity to oxidative inhibition is associated with the activation of the DUBs wherein the active site cysteine is converted to a deprotonated state prone to oxidation. . We further demonstrate that this redox regulation is essential for mono-ubiquitination of proliferating-cell nuclear antigen in response to oxidative DNA damage, which initiates a DNA damage-tolerance program. In normal cells, USP1 serves as a â€˜brakeâ€™ that keeps PCNA in the unmodified form. Oxidative stress not only induces DNA damages, but also inactivates USP1, allowing ubiquitinated PCNA to rapidly accumulate in cells to launch the damage tolerance translesion synthesis (TLS) program. These findings establish a novel mechanism of DUB regulation that may be integrated with other redox-dependent signaling circuits to govern cellular adaptation to oxidative stress, a process intimately linked to aging and cancer.

NIDDK
Vecchiarelli, Anthony
Visiting Fellow
Molecular Biology - Prokaryotic
Surfing the Bacterial Nucleoid: A key role for spatial confinement in intracellular transport
Protein gradients play key roles in intracellular spatial organization. In bacteria, ParA ATPases pattern the nucleoid surface and position chromosomes, plasmids, and large protein assemblies prior to cell division. The increasing scope of cargo directed by ParAs has raised interest in the novel mechanism of transport. Protein patterning is considered a critical feature, but how the ATPase interacts with the nucleoid to generate a pattern that yields the pulling force for cargo movement has been unclear. ParA-mediated plasmid partition systems are key models for understanding this type of cargo transport. Here, the F plasmid partition system, SopABC, was reconstituted from purified fluorescent-labeled components, and the system dynamics were visualized using TIRFM on a DNA carpeted flowcell, which acted as a nucleoid biomimetic. SopA (the ParA-type ATPase) colocalizes with the nucleoid in vivo. SopB forms a â€˜partition complexâ€™ on the plasmid centromere (sopC) and also stimulates SopA ATPase activity. In vivo, the partition complex appears to chase and redistribute SopA on the nucleoid. We recapitulated many aspects of the in vivo dynamics. However, we did not recapitulate the persistent protein patterning and directional movement of cargo observed over the nucleoid. The gap distance between the inner membrane of a bacterial cell and its nucleoid surface is submicron. We proposed our flowcell geometry did not provide the spatial confinement necessary for a persistent interaction between the plasmid cargo and the DNA carpet. We therefore used a magnet above the flowcell to artificially confine one micron magnetic beads, coated with the sopC centromere, on the DNA carpet. As shown in vivo, SopA-ATP bound the DNA carpet. The artificial confinement, generated by the magnet, allowed the SopB-coated sopC-beads to persistently stimulate the release of SopA from the DNA carpet in the vicinity of the beads. This locally stimulated release resulted in the formation of SopA depletion zones on the carpet. Strikingly, protein patterning of the ATPase resulted in directional movement of the beads across the DNA carpet. Spatial confinement of the bead was essential for both SopA patterning and for directed bead transport. Our results show how the extreme spatial confinement within a bacterial cell is an under-appreciated parameter in ParA-mediated transport, and likely in other forms of intracellular spatial organization.

NIDDK
Souza, Ana
Postdoctoral Fellow

Physiology

CD36 receptor: a new therapeutic target for chronic kidney disease progression

Background: Chronic kidney disease (CKD), a progressive decline in kidney function, leads to end-stage renal disease (ESRD) with the need for dialysis or kidney transplantation. CKD affects 26 million Americans. Increasing rates of diabetes and hypertension, important risk factors for CKD, suggest that CKD prevalence will continue to increase in the US and worldwide. Whereas Renin-Angiotensin-Aldosterone System inhibitors are widely used to slow progression, a significant number of treated patients still progress to ESRD. Other therapies are needed. Scavenger receptor CD36 is a widely expressed cell surface receptor important in lipid metabolism and metabolic syndrome. CD36 is upregulated in blood monocytes of dialysis patients, and soluble CD36 is increased in the plasma of patients with advanced CKD. To determine the role of CD36 in CKD progression, we compared WT versus CD36 knockout mice using a model of CKD that is AngiotensinII-dependent. Methods: Sixteen weeks old WT and KO mice (C57BL6 background) were subjected to 5/6 nephrectomy (5/6Nx) and continuous infusion of AngII (0.75 µg/kg/min) by osmotic minipump. We previously showed that C57BL6 mice are resistant to CKD progression after 5/6Nx, and AngII overcomes this strain-dependent resistance. Thus, WT mice subjected to 5/6Nx without AngII infusion do not progress to CKD and were used as controls. All groups (N=10) were monitored for 4 weeks, with weekly urine collection and body weight record. At 4 weeks mice were euthanized for blood collection and kidney harvesting. Serum BUN was measured by colorimetry, serum creatinine (Scr) by HPLC, and albuminuria by ELISA. Kidney sections were fixed in formalin, paraffin embedded, and stained with Massonâ€™s trichrome and PAS reagent for histological analysis. Statistical analysis was performed by ANOVA. Results: At 4 weeks kidney function was protected in KO mice (BUN control 94.7+/-8.5, WT 187+/-36 and KO 119+/-16 mg/dl; Scr control 0.33+/-0.32, WT 0.64+/-0.13 and KO 0.38+/-0.04 mg/dl,p<0.05). KO mice had less weight loss (p<0.01), albuminuria (p<0.0001), glomerulosclerosis score (control 1.78+/-0.17, WT 3.55+/-0.27 and KO 2.31+/-0.18,p<0.0001), and interstitial fibrosis score (control 1.18+/-0.19, WT 3.01+/-0.46, KO 1.81+/-0.27,p<0.01). Conclusions: CD36KO mice are protected from CKD in our model, suggesting that AngII may signal through the CD36 receptor. Therefore, CD36 is a possible novel therapeutic target for slowing progression of CKD.

NIDDK

Roche, Julien

Visiting Fellow

Protein Structure/Structural Biology

The early steps of HIV-1 infection: an atomic view of gp41 pre-fusion intermediate states by solution NMR

The 344-residue HIV-1 coat protein, gp41, is anchored to the viral membrane, together with its covalently bound partner gp120. Binding of gp120 to the host cell receptor CD4 and co-receptors CCR5-CXCR4 induces a cascade of conformational changes that result in anchoring of gp41's N-terminal fusion peptide into the host cell membrane. Trimers of gp41 were shown to rapidly fold into a symmetric 6-helix bundle, characterized by X-ray crystallography, that pulls together the N-terminus and its C-terminal transmembrane helix, anchored to the viral membrane, thereby bringing the host and viral membranes into close proximity. Very little structural information is known about the pre-fusion state of gp41, notwithstanding its critical importance for the design of fusion inhibitor drugs. To investigate the internal dynamics and structural properties of such metastable states by solution NMR, we designed a set of protein constructs mimicking the extracellular ectodomain of gp41. We find that the secondary structure of the ectodomain is pronouncedly impacted by the presence of phospholipid vesicles or detergents containing phosphatidyl choline (PC) headgroups, but not by other detergents such as maltose-neopentyl glycol, which lacks a PC headgroup. Our NMR and multi-angle light scattering data

attribute these changes to the transition between a trimer in the absence of detergent to a monomer in the presence of a membrane mimicking environment. The structures of gp41 trimeric and monomeric ectodomains were determined by state-of-the-art 1H, 15N and 13C NMR techniques, including measurements of classical NOE distance restraints and residual dipolar couplings, using weakly aligned solutions. 15N relaxation data provide a detailed view at the internal dynamics of both the trimeric and monomeric states. Next to the shortened ectodomain, we also provide the first atomistic view of the full ectodomain that includes the important immuno-dominant loop and the membrane proximal regions, two domains of pivotal importance for vaccine-based therapeutic strategies.

NIEHS
Weaver, Jeremy
Postdoctoral Fellow
Biochemistry - General and Lipids
Kinetic evaluation of an inositol pyrophosphate kinase reveals its signaling credentials.
IP7 and IP8, the pyrophosphorylated members of the inositolphosphate family, are synthesized by ATP-dependent kinases (IP6K and IP7K respectively). IP6Kâ€™s catalytic activity impacts metabolic homeostasis by its regulation of both the secretion and the actions of insulin. In contrast, IP7K has been considered unfit to participate in rapid signaling events. Early reports that IP7K is catalytically feeble seemed to reflect the high-energy constraints impeding synthesis of IP8 - Natureâ€™s most crowded phosphate array. Moreover, a consensus belief that IP7K is fully reversible as an ATP-synthase has pictured the enzyme as being at the mercy of fluctuating cellular ATP/ADP ratios. The fact that no laboratory has shown IP8 synthesis is receptor-regulated in vivo has further diminished IP7Kâ€™s signaling credentials. We now overhaul all of these opinions. We isolated highly pure recombinant IP7K from E. coli with a continuous-flow cell disrupter. Substrates that were not commercially available were prepared enzymatically, and then purified electrophoretically. We ascertained IP7Kâ€™s fundamental biochemical parameters: reaction rates, substrate affinities, and equilibrium conditions. We determined that the IP7K Vmax (190 +/- 10 nmol/mg/min) is actually comparable to that of IP6K. We further established the equilibrium point for IP7K favors 80-90% IP8 accumulation. Contrary to the paradigm with higher inositol phosphate kinases (IP5K and ITPK1), our data also show IP7K is not reversible in a physiologically-relevant bioenergetic environment (i.e. [ATP]>[ADP]). IP7K activity was insensitive to physiologically-relevant changes to either [AMP], [ATP]/[ADP] ratio, or to [ATP] itself (since the ATP Km = 20-40 uM). Thus we show for the first time, independent of the bioenergetic context, IP7K is kinetically well equipped to participate in rapid cell-signaling events. To interrogate this novel viewpoint, we selected a metabolically active cell model with high levels of expression of IP7K: the L6 skeletal myoblast cell-line. IP8 turnover was studied by HPLC analysis. We discovered that either insulin, IGF or PDGF each raised IP8 levels 2-3 fold. Thus, despite the size of the energetic investment, cells place IP8 synthesis under receptor regulation. Precedents suggest this is a valuable observation: demonstrations of receptor-regulation of levels of IP3 - and, later, PIP3 - were critical in fermenting widespread interest in these molecules as cell-signals.

NIEHS
Palii, Stela
Postdoctoral Fellow
Cell Cycle-General, Regulators and Checkpoints, Apoptotic Mechanisms
Combined disruption of ATM and CHK1 functionalities reveals redundancies in the DNA damage response pathways and results in synthetic growth inhibition following gamma-irradiation
Exposure of proliferating cells to genotoxic stresses activates a cascade of signaling events termed the DNA damage response (DDR). The DDR preserves genetic stability by detecting DNA lesions, activating

cell cycle checkpoints, and promoting DNA damage repair. Ataxia Telangiectasia-Mutated (ATM), ATM and Rad 3-related kinase (ATR), and DNA-dependent Protein Kinase (DNA-PK) are crucial for lesion sensing and signal transduction. The checkpoint kinase-1 (CHK1) is a canonical ATR substrate and a pharmacological target for anti-cancer regimens. We hypothesized that ATM-deficient cells depend on signaling through CHK1 for survival after genotoxic exposures. Our goal was to develop isogenic cell lines that are stably depleted for CHK1, ATM, or both and examine cross-talk and compensatory effects on cellular growth and the G2/M checkpoint after treatment with ionizing radiation (IR). Individual depletion of CHK1 and ATM rendered cells radiosensitive without abrogating their IR-mediated G2/M checkpoint. CHK1-deficiency led to enhanced ATM phosphorylation post gamma-IR and conversely, ATM-deficiency resulted in enhanced CHK1 phosphorylation, thus revealing a signaling co-dependency. Our data indicates a feedback regulatory loop with the phosphatase PP2A as one of the underlying mechanisms: compared to wild-type cells, the nuclei of CHK1-depleted cells show a lower abundance of PP2A catalytic subunit in conjunction with increased inhibitory PP2A phosphorylation on tyrosine-307. Stable depletion of CHK1 in an ATM-deficient background showed only a 50% reduction from wild-type CHK1 protein expression levels, suggesting that loss of CHK1 by ATM-deficient cells may be synthetically lethal. Combined ATM depletion and CHK1-deficiency resulted in an additive attenuation of the G2/M checkpoint response compared to the individual knockdown, whereas pharmacological ATM inhibition in conjunction with 90% CHK1 depletion abrogated the early G2/M checkpoint. Furthermore, at 48 hours post-IR the individually-depleted cell lines continued to proliferate, whereas the double-deficient cell line ATM-CHK1 halted proliferation. In conclusion, dual targeting of ATM and CHK1 functionalities disrupts the compensatory response to DNA damage, abrogates the G2/M checkpoint, and leads to synthetic growth inhibition. Combined functional disruption of ATM and CHK1 may be a worthwhile approach for developing more efficient anti-neoplastic treatments.

NIEHS
Fromm, George
Postdoctoral Fellow
Developmental Biology
Pausing of RNA Polymerase II Regulates Mammalian Developmental Potential
The regulation of transcription is vital in the coordination of events during development, and gene misregulation is often associated with developmental disorders and disease states. Thus, there is great interest in elucidating the mechanisms that underlie transcription regulation to identify novel targets and therapies. A critical point of gene regulation occurs during early transcription elongation, when pause-inducing factors such as the NELF complex cause RNA polymerase II (Pol II) to halt just downstream of the promoter. Release of Pol II into the gene and dissociation of NELF are triggered by recruitment of the kinase P-TEFb, which mediates productive elongation and subsequent gene activation. Pausing is widespread in metazoans and is associated with genes involved in development and th¬¬e response to stimuli. This finding led to an appealing model; that pausing poises genes for rapid activation pending a signal, and consequently when pausing is lost, developmental genes could be activated resulting in a phenotypic change such as differentiation. The dynamics of pausing and pause release have therefore been proposed to play key roles in many cellular processes; however our understanding of these events is lacking. To address these questions, we knocked out (KO) the NELF subunit NELF-B in the mouse. Loss of NELF-B is embryonic lethal emphasizing an essential developmental role. To gain further insight, we generated induced pluripotent stem cells (iPSCs) from conditional NELF KO mice. Contrary to the model proposed above, we find that NELF-B KO iPSCs resist differentiation due to dysregulation and dampening of Mapk/ERK signaling pathway activity. We observe that many genes in the Mapk/ERK pathway are directly bound by NELF and harbor paused Pol II, and that the loss of NELF-mediated pausing results in a redistribution of Pol II at these genes and

aberrant gene expression. Strikingly, we find that the majority of genes misregulated upon NELF loss are signaling pathway components as opposed to the downstream inducible target genes. Inhibition of Mapk/ERK signaling has been shown to prevent primitive endoderm specification in the embryo. Remarkably, we find that NELF-B KO embryos share a similar fate. Thus, the lack of lineage differentiation during embryonic development contributes to lethality, and provides evidence that NELF-mediated pausing plays a critical role in establishing Mapk/ERK signaling pathway activity during development.

NIEHS
Lichti-Kaiser, Kristin
Postdoctoral Fellow
Developmental Biology
Transcription Factor Glis3 Plays a Critical Role in the Development of Functional Pancreatic beta-cells and Diabetes
Gli-similar (GLIS) 3 belongs to a subfamily of Krüppel-like zinc transcription factors related to members of the Gli and Zic family. Several genes including GLIS3 have been associated with risk for type-1 and type-2 diabetes and genetic aberrations in the GLIS3 gene have been associated with a syndrome characterized by neonatal diabetes and congenital hypothyroidism. We previously showed that Glis3 KO mice die at post-natal day 3 from neonatal diabetes as evidenced by hyperglycemia and hypoinsulinemia. This phenotype is due to a dramatic loss of insulin-secreting beta-cells in contrast to a smaller relative loss of other endocrine cell types. In addition, Glis3 regulates insulin gene expression in mature beta-cells, indicating that Glis3 plays a key role in both the development and function of mature pancreatic beta-cells. However, the spatial and temporal expression of Glis3 during pancreas development and the mechanism by which Glis3 contributes to the development and maintenance of functional beta-cells are unknown. In order to further study the precise role of Glis3 in pancreas development and function, we have generated a pancreas-specific Glis3 KO mouse model. Glis3fx/fx mice were crossed with mice expressing cre-recombinase under the control of the Pdx1 gene promoter, which is activated at an early time-point during pancreas development. Glis3fx/fx;Pdx-cre KO (PGlis3KO) mice develop a delayed-onset diabetic phenotype at 2-3 months of age as evidenced by hyperglycemia, hypoinsulinemia, and loss of white adipose tissue. Gene expression profiling and immunofluorescence analysis demonstrated that the expression of insulin was significantly decreased at 2 months, but not at 2 weeks, of age in PGlis3KO mice. The expression of other pancreatic hormones and transcription factors important for endocrine cell development, including Ngn3 and Pdx1, was not significantly different than that of WT littermates, indicating that, unlike in the whole-body KO, there may not be a significant loss of beta-cells in the PGlis3KO mice. However, the expression of several genes that are critical for beta-cell function, including Glut2, MafA, and G6pc2, is decreased in the PGlis3KO mice compared to WT controls. The PGlis3KO mice provide an excellent model to examine various therapeutic strategies for diabetes. Our study shows that Glis3 has multiple critical functions in the pancreas and suggests that Glis3 may provide a new therapeutic target to intervene in diabetes.

NIEHS
Ungewitter, Erica
Postdoctoral Fellow
Developmental Biology
GLI-similar 3 Maintains Sexually Dimorphic Germ Cell Development in Mouse Embryos
Germ cells are a uniquely important cell type because they give rise to gametes, the cells responsible for sexual reproduction and the propagation of all higher species. In the mouse, programs of germ cell development are identical in male and female embryos until around embryonic day (E)13.5. At this time,

germ cells in the ovary first express Stra8 and initiate meiosis. Germ cells in the testis, by contrast, arrest at the G0/G1 phase of mitosis and do not enter meiosis until early postnatal life. This dimorphic pattern of meiotic entry is mediated by the meiosis-inducing agent retinoic acid (RA). In the fetal testis, the RA-degrading enzyme Cyp26b1 inhibits RA action, thereby preventing male germ cells from entering meiosis. Fetal ovaries do not express Cyp26b1, and consequently, RA is able to induce Stra8 expression and subsequent meiosis in female germ cells. In this study, we set out to uncover new players involved in the dimorphic development of germ cells using mice as a model organism. We found that Gli-similar 3 (Glis3), a zinc finger transcription factor essential for lineage specification in endocrine organs such as thyroid and pancreas, has a testis-specific role in germ cell survival and meiosis. Glis3 expression is low in fetal ovaries at all stages of development, but high in fetal testes for a brief window of time around E13.5, coincident with the normal dimorphic initiation of meiosis. To identify the functions of Glis3, we examined the gonads of global Glis3 knockout animals. Germ cells in ovaries developed normally in the absence of Glis3; however, knockout testes had reduced numbers of germ cells and decreased expression of germ cell markers (Oct3/4 and Vasa). Defects in germ cell development are not the result of impaired somatic cell differentiation as somatic cell markers in knockout testes (Sox9 and Cyp11a) remain normal. Intriguingly, Stra8, the critical regulator of meiotic entry in germ cells, was ectopically elevated in knockout testes despite the fact that neither of the known regulators of Stra8 (Cyp26b1 and Nanos2) were changed. These findings reveal a novel role for Glis3 in germ cell survival and meiosis, whereby Glis3 prevents male germ cells from entering meiosis prematurely through a pathway independent of RA. We are currently investigating factors upstream and downstream of Glis3 with the goal of identifying the mechanisms underlying sexually dimorphic establishment of the germlines in mammals.

NIEHS
Dang, Huaixin
Visiting Fellow
Endocrinology
TAK1/TR4 regulates cold induced thermogenesis by inhibiting CREB-PGC1a pathway
Brown adipose tissue (BAT) can disperse stored energy as heat. Promoting BAT function is an attractive, if elusive, therapeutic approach to staunch the current obesity epidemic. Our previous study showed that nuclear receptor TAK1/TR4/NR2C2 -/- mice are resistant to the development of obesity, glucose intolerance and insulin resistance. In this study, we found that TAK1-/- mice consume more oxygen and produce more carbon dioxide than wild type mice fed a normal diet suggesting a higher metabolic rate. This is consistent with our observations that exposed to 4 degree temperature, TAK1-/- mice are more tolerant to cold stress compared to wild type littermate. Microarray analysis showed that PGC1a, DIO2, adenylate cyclase 3(Adcy3) and CEBPa are expressed at significantly higher levels in TAK1-/- mice brown adipose tissue than WT tissue. Q-RTPCR analysis showed that this difference is significantly enhanced when mice are challenged with cold exposure. Knockdown of TAK1 expression in brown fat progenitor cells induced increased PGC1a, DIO2 and UCP1 expression and a better response to cAMP. Oil Red O staining suggests that BAT differentiation is enhanced in cells in which TAK1 is knocked down. Although over-expression of TAK1 in BAT progenitor cells has no significant impact on lipid accumulation, the cAMP-dependent induction of PGC1a, DIO2 and UCP1 was significantly reduced. Further studies will determine whether TAK1 directly inhibits the binding of CREB to the PGC1a and DIO2 promoter. Given the important role of PGC1a and DIO2 in metabolism, TAK1 may provide a new target in the management and prevention of obesity and related pathologies, including diabetes.

NIEHS
Englert, Neal

Postdoctoral Fellow
Epigenetics
Epigenetic Modification of Histone (H3) and CYP2C9 Regulation: Involvement of Med25 as the Key Regulator
Cytochrome P450 (CYP450) enzymes are important for the hepatic metabolism of exogenous chemicals including common pharmaceutical drugs such as Warfarin and Ibuprofen, as well as the metabolism of endogenous substrates. Some CYPs have been found to be regulated by DNA methylation, however, little is known about the epigenetic regulation in human tissues. The CYP2C9 gene is regulated by liver-specific nuclear receptor HNF4alpha, which has been shown to recruit Med25, a subunit of the Mediator complex. Mediator complex is involved in the transcriptional regulation of most genes by recruiting Polymerase II, thus revealing its importance in controlling cellular function. Mediator has also displayed an ability to recruit histone modifiers such as G9a, a histone methyltransferase, which leads to gene repression by methylating H3K9. Polycomb repressive complex (PRC2), which represses expression by methylating H3K27, may also be recruited by Mediator complex. Here, we examined whether Med25 is involved with epigenetically regulating the expression of CYP2C9, through modifying histone markers in the chromatin of the CYP2C9 promoter. We hypothesize that Med25 is important not only for Polymerase II recruitment, but for controlling the chromatin architecture, and thus the expression, of target genes such as the CYP450s. We performed chromatin immunoprecipitation in HepG2 cells to identify histone modifications at the HNF4alpha binding site in relation to Med25 protein levels. Results indicated that altering Med25 expression modified H3K27 status; in the presence of Med25, the HNF4alpha binding site was enriched for activating markers H3K27ac and H3K27me1, with progressive demethylation. When Med25 expression was silenced, the H3K27 residue was highly methylated (H3K27me3), which is a prototypical gene-silencing marker. CYP2C9 transcript levels were reduced after silencing Med25 expression. These results suggest that Med25 induces a permissive chromatin state at the CYP2C9 proximal promoter. Additionally, confocal microscopy revealed that Med25 colocalized with activating histone markers (H3K27ac) in the nucleus of HepG2 cells while it did not colocalize with repressive markers (H3K27me2, H3K27me3). Our data indicate that Med25 is important for changing the epigenetic landscape to allow for increased levels of transcriptional activation of highly inducible genes such as the hepatic cytochrome P450s.

NIEHS
Joubert, Bonnie
Research Fellow
Epigenetics
Maternal smoking and DNA methylation in newborns: An in utero effect or epigenetic inheritance?
Maternal smoking in pregnancy is related to multiple adverse health outcomes in children and underlying mechanisms may include epigenetic modifications. We previously identified differential DNA methylation related to maternal smoking during pregnancy at 26 CpG sites (CpGs) in 10 genes including those involved in the metabolism of the components of tobacco smoke (AHRR, CYP1A1) or developmental processes (e.g., GFI1 and MYO1G). However, it is unclear whether these methylation signals in the infant epigenome reflect in utero exposure only or possibly epigenetic inheritance of smoking-related modifications from the mother. To investigate this, we evaluated the relationship between the timing of motherâ€™s smoking (before or during pregnancy) as well as the combined effect of grandmotherâ€™s and motherâ€™s smoking in pregnancy on methylation at the 26 previously identified CpGs. We measured DNA methylation with Illuminaâ€™s Infinium HumanMethylation450 Beadchip in 1,042 newborn cord bloods from the Norwegian Mother and Child Cohort Study (MoBa). Both self-report and cotinine measured in pregnancy were used to categorize the timing of motherâ€™s smoking (never smoked, formerly smoked, smoked in pregnancy but quit by 18 weeks, and actively

smoked throughout pregnancy). Grandmotherâ€™s smoking was assessed by motherâ€™s response to â€œDid your mother smoke when she was pregnant with you?â€ Grandmother and mother smoking information were combined (neither smoked, only grandmother smoked in her pregnancy, only mother smoked in her pregnancy, and both grandmother and mother smoked in their pregnancies). The association between smoking category and methylation was assessed using robust linear regression, adjusting for covariates. Bonferroni correction was used to adjust the level of statistical significance to p-value (p)<0.0019. Only active smoking throughout pregnancy was statistically significantly associated with differential cord blood DNA methylation (p<1.6x10-5 for all 26 CpGs). We did not observe statistically significant associations for former smoking or quitting early in pregnancy (minimum p=0.010). There was no statistically significant effect of grandmother smoking alone and if mother smoked, the additional effect of grandmother smoking was not significant (minimum p=0.078). Our findings suggest that DNA methylation at these locations in the infant epigenome reflects in utero exposure rather than epigenetic inheritance of smoking-related modifications.

NIEHS
Kadmiel, Mahita
Visiting Fellow
Gene Expression
Glucocorticoid receptor action at the interface with the environment
The cornea is the transparent outermost layer of the eye. It forms the first barrier of the eye, and as such it is exposed to the environment. Allergies and infections of the eye can result in corneal diseases that may lead to blindness if left untreated. Glucocorticoids have been used to treat eye diseases pertaining to the cornea. However, the use of glucocorticoids sometimes comes with adverse effects. For example, chronic use of glucocorticoids is associated with the risk of development of cataract and glaucoma. Thus, further understanding of the role of glucocorticoid receptor (GR) signaling in the eye and specifically the cornea is required to improve therapeutic strategies. In this study, we utilized an immortalized human corneal epithelial cell line (HCE) to examine glucocorticoid function. By qRT-PCR and immunoblotting techniques, we found that GR is expressed in HCE cells. Confocal imaging revealed that stimulation of GR with the synthetic glucocorticoid dexamethasone was able to induce nuclear translocation of the receptor, indicating that corneal epithelial cells express a functional GR. Genome-wide expression profiling in HCE cells revealed that glucocorticoids significantly regulated 8399 probes, of which 97% of the probes were repressed, suggesting that transrepression is a predominant function of glucocorticoids in corneal epithelial cells. Ingenuity Pathway Analysis identified G-protein coupled receptor pathway as a highly-ranked pathway altered in HCE cells by dexamethasone treatment. Moreover, cell development, movement, morphology and cell-to-cell signaling were ranked as the top significant glucocorticoid-regulated biological functions. Consistently, in vitro wound healing assay in HCE cells exhibited a remarkable delay in wound healing when treated with dexamethasone. This effect was reversible by treating with RU486, an inhibitor of GR, indicating that dexamethasone-induced delay in wound healing is mediated via GR. Taken together, our results demonstrate that glucocorticoids significantly alter the gene expression profile of corneal epithelial cells, which may be manifested in biological outcomes such as delayed wound healing.

NIEHS
kazgan, nevzat
Postdoctoral Fellow
Gene Expression
Intestine-specific deletion of SIRT1 alters systemic lipid and bile acid homeostasis in mice

SIRT1, the most conserved mammalian NAD+-dependent protein/histone deacetylase and the orthologue of yeast anti-aging protein Sir2, is a key metabolic sensor in various tissues in mammalian species. However, the role of SIRT1 in small intestine, an essential metabolic organ that is primarily involved in nutrient absorption and sensing, is still largely unknown. To elucidate the function of SIRT1 in intestinal metabolism, we generated a novel intestine-specific SIRT1 KO mouse model, SIRT1 IKO mice, by breeding SIRT1 floxed mice with Villin-Cre mice. Utilizing this mouse model, we demonstrate that intestinal SIRT1 is an important regulator of intestinal lipid and (ileal) bile acid absorption that feedback modulates systemic lipid and bile acid homeostasis. Deletion of SIRT1 only in the mouse intestinal epithelial cells results in decreased expression levels of lipid transporters SRB1 and CD36, proteins that are essential for prechylomicron complex formation. As a result, SIRT1 IKO mice have reduced total body fat percentage. Moreover, loss of SIRT1 in the intestinal epithelial cells leads to decreased intestinal HNF1a/FXR signaling pathway, transcription factors that play an important role in systemic bile acid metabolism, resulting in reduced expression of ileal bile acid transporter genes, Asbt and Osta/Ostß. This defect decreases the ileal absorption of bile acids, leading to increased fecal bile acid output. We provide evidence that SIRT1 regulates the HNF1a/FXR signaling pathway through deacetylation of DCoH2, a dimerization cofactor of HNF1a. Deacetylation of DCoH2 facilitates dimerization and DNA binding function of HNF1a. Furthermore, in contrast to deletion of hepatic SIRT1, intestinal SIRT1 deficiency stimulates hepatic bile acid synthesis, leading to decreased contents of cholesterol and triglyceride in the liver and protecting against liver damage upon atherogenic diet feeding. Taken together, our findings uncover novel functions of SIRT1 in intestinal lipid and bile acid absorption and reveal a previously unknown molecular mechanism by which SIRT1 regulates the HNF1a/FXR signaling pathway. Our studies further point out that the same molecular mechanism can yield distinct pathophysiologies in the same metabolic pathway in different tissues, and suggest that tissue specificity should be considered when applying SIRT1 small molecule modulators-based therapeutic strategies to bile acid and cholesterol diseases.

NIEHS
ZeRuth, Gary
Postdoctoral Fellow
Gene Expression

The Krüppel-like protein Gli-similar 3 (Glis3) functions as a key regulator of insulin transcription

Transcriptional regulation of insulin in pancreatic beta cells is mediated primarily through enhancer elements located within the 5â€™ upstream regulatory region of the preproinsulin (INS) gene. Recently, we demonstrated the Krüppel-like transcription factor, Glis3, can bind the insulin promoter and positively influence insulin transcription while genome-wide association studies (GWAS) have identified GLIS3 as a risk locus for both type 1 and 2 diabetes. In this study, we examined in detail the activation of insulin transcription by Glis3 with the co-regulators, CBP/p300, Pdx1, NeuroD1, and MafA. Using quantitative RT-PCR and luciferase reporter assays, we demonstrate that Glis3 acts synergistically with Pdx1, NeuroD1, and MafA to activate the insulin promoter. We further show by means of co-immunoprecipitation assays that both the Glis3 N-terminus and C-terminal transactivation domain associate with CBP/p300 and that the observed synergism between the different transcription factors relies in large part on the recruitment of CBP/p300 by Glis3 to the insulin promoter. Our data show that Glis3 expression, the binding of Glis3 to GlisBS, and its recruitment of CBP are required for optimal activation of the insulin promoter not only by Glis3, but also by Pdx1, MafA, and NeuroD1 in pancreatic beta cells. Mutations in the GlisBS or siRNA-directed knockdown of GLIS3 diminished insulin promoter activation by Pdx1, NeuroD1, and MafA and chromatin immunoprecipitation (ChIP) analysis indicated that neither Pdx1 nor MafA were able to stably associate with the insulin promoter when the GlisBS were mutated. In addition, we showed that a single nucleotide mutation within the human INS

promoter implicated in the development of neonatal diabetes similarly abated activation by Pdx1, NeuroD1, and MafA in the absence of exogenously expressed Glis3, which likely overcomes the reduced affinity for the mutated binding site to restore INS promoter activation. We therefore propose a model whereby recruitment of CBP/p300 by Glis3 provides a scaffold for the formation of a larger transcriptional regulatory complex that stabilizes the binding of Pdx1, NeuroD1, and MafA complexes to their respective binding sites within the insulin promoter. Taken together, these results indicate that Glis3 plays a pivotal role in the transcriptional regulation of insulin and may serve as a potential therapeutic target for the treatment of diabetes.

NIEHS
Cinghu, Senthilkumar
Postdoctoral Fellow
Informatics/Computational Biology
Meta-analysis identifies key determinants of embryonic stem cell identity and homeostasis
Embryonic stem cells (ESCs) can self-renew indefinitely and can differentiate into all derivatives of the 3 germ layers, making them an attractive model for regenerative medicine and disease modeling. Successful development of ESC-based therapies, however, largely depends on understanding the genetic network governing ESC self-renewal and differentiation. To characterize the genetic network controlling the ESC identity, we developed a novel bioinformatics pipeline for a systematic meta-analysis of 68 published gene expression datasets, and have rank-ordered all mouse genes based on their likelihood to have a role in ESC maintenance. Not surprisingly, master ESC regulator Oct4 is ranked number one, followed by Nanog and Sox2. Moreover, several other known regulators of ESC maintenance were ranked atop our gene list, along with a number of genes that have not been previously implicated in ESC biology. To identify novel regulators of mESC identity, we used RNAi-mediated loss-of-function experiments and phenotypic assays to test 49 candidate genes ranked within the top 2%. Depletion of 17 candidates showed cellular and molecular changes consistent with mESC differentiation, suggesting that these genes are critical for ESCs to maintain their self-renewal characteristics. To understand the roles of these potential regulators of ESCs, we studied our top-hit Nucleolin (Ncl). Ncl codes for a highly conserved protein abundant in stem and cancer cells. We show that Ncl depletion elevates endogenous reactive oxygen species (ROS) levels and p53 activity, resulting in p53-mediated suppression of Nanog and subsequent ESC differentiation. We demonstrate that p53 depletion, Nanog overexpression, or antioxidant treatment restores the phenotype due to Ncl depletion. Furthermore, we show that Nanog's positive regulation of Ncl provides the means for Nanog to suppress p53 activity in a Ncl-dependent manner to maintain ESCs. Together, these findings support a conceptually novel mechanism involving a Ncl-dependent Nanog-p53 bistable switch regulating the homeostatic balance between self-renewal and differentiation in ESCs. Given that many cancers have elevated levels of Ncl and Nanog in addition to impaired p53 signaling, our findings have profound implications for understanding tumorigenesis. Altogether, our studies unearth a wealth of novel ESC regulators and reveal a previously unknown regulatory circuitry involving genes associated with traits in both ESCs and cancer.

NIEHS
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Informatics/Computational Biology
T-KDE: A method for analyzing genome-wide protein binding patterns from ChIP-seq data
A protein may bind to its target DNA sites constitutively, i.e., in more than 90% of cell lines regardless of cell type. Intuitively, constitutive sites should be biologically functional. For many proteins, however, what proportion of their binding sites are constitutively bound remains unknown. The lack of both ChIP-

seq data across multiple cell types and a robust analytic method made addressing this question difficult. Recently, the ENCODE consortium has profiled genome-wide binding patterns of many transcription factors (TF) in multiple cell lines. A conventional approach to identify constitutive binding sites would divide the genome into fixed width bins and count the number of peak centers within each bin. However, this binning method suffers from several problems including a boundary effect. We propose a method, T-KDE, which combines a binary range tree, a kernel density estimator and a mode finding algorithm to identify the locations of constitutive peak centers from multiple cell lines. The binary tree algorithm partitions the locations of all peak centers throughout the genome into smaller subgroups. Then, within each subgroup, T-KDE estimates a density curve for the peak-center locations and finds its mode. We define a mode as a constitutive site when its neighborhood contains peak centers from more than 90% of the cell lines. We applied our method to all ENCODE human TF binding site datasets with six or more cell lines. We found that the number of constitutive sites for different TFs varied from hundreds to tens of thousands. Rad21 had the highest proportion of constitutive sites (8.0%). Not surprisingly, the proportion of constitutive sites for the transcriptional co-activator protein p300 was negligible because many p300 sites are tissue-specific enhancers. Interestingly, many Pol II sites (4,733) were also constitutive. Gene ontology (GO) analysis showed that genes with constitutive Pol II binding sites in their promoters are highly enriched with GO terms such as cellular metabolic process (p=8e-177) and cell cycle (p=1e-46), suggesting that these constitutive sites are biologically meaningful. In conclusion, T-KDE is general and can be applied directly to ChIP-seq peak data to identify constitutive sites that are bound by a protein either directly or indirectly across multiple cell lines. Besides the constitutive sites for a given TF, T-KDE can be used to identify genomic "hot spots" that were bound by different proteins.

NIEHS
Robertson, Sabrina
Postdoctoral Fellow
Neuroscience - Cellular and Molecular
Developmental origins of central norepinephrine neuron diversity
Central norepinephrine (NE)-producing neurons comprise a diverse population of cells that modulate many disparate behaviors and physiological processes such as attention, stress and appetite. Reflected in this diversity of function, central NE neurons differ in their anatomical location, connectivity, and response to disease and environmental insult. At present, the mechanisms that generate this diversity are unknown, as are molecular markers capable of distinguishing individual NE subtypes. Such knowledge is fundamental to understanding the basis of selective NE neuron subtype vulnerability to disease and environmental insult, and for gaining selective access to individual subpopulations of NE neurons for experimental study. To begin filling this gap, we used an intersectional recombinase-based genetic approach to elucidate the lineal relationship between molecularly distinct NE progenitor populations in the developing hindbrain and mature NE neuron subtype identity. We have identified four genetically separable subpopulations of NE neurons that differ in their anatomical distribution and have revealed previously unappreciated lineal relationships between NE neurons located in distant regions of the brainstem. We have also found that these subpopulations differ in their axon morphology and that each projects to a unique set of targets. Our ability to visualize these subpopulations in complete isolation has uncovered provocative details of the structure and organization of the NE efferent system, including the identification of a projection to the prefrontal cortex that challenges current dogma that the locus coeruleus is the sole source of NE projections to the cortex. This novel molecular classification of the NE system provides, for the first time, multiple molecular points of entry for functional manipulation of individual NE circuits with unprecedented precision, at all stages of development. Phenotypes resulting from such manipulation promise unique insights into complex NE-

related behavioral and physiological processes including arousal, attention, mood, memory, appetite, and homeostasis.

NIEHS
Wang, Qingshan
Visiting Fellow
Neuroscience - Neurodegeneration and Neurological disorders
Endogenous substance P regulates microglial density in substantia nigra through neurokinin-1 receptor/NADPH oxidase axis-mediated chemotaxis
The distribution and density of microglia differ greatly among brain regions. We have previously reported the density of microglia in the substantia nigra (SN) is 5 fold higher than other brain regions, implicating why the SN is selectively more sensitive to the neuroinflammation-mediated pathogenesis of Parkinsonâ€™s disease (PD). However, the reason for the high density of nigral microglia is not known. We hypothesized that substance P (SP), a major endogenous pro-inflammatory peptide stored at high concentrations in the SN, is a major regulator for the high density of nigral microglia. Developmental studies revealed that nigral microglial density peaked around postnatal 30 (P30). In contrast, SP was detected at high levels in SN as early as P1. Transgenic mice incapable of producing endogenous SP (TAC1-/-) exhibited reduced nigral microglial density compared to wild type (WT) controls. This finding led us to speculate that SP may attract the migration of microglia toward to the SN. We confirmed the chemotactic potential of SP in vitro by demonstrating that SP induced the migration of microglia in a transwell culture system. In vivo studies further showed facilitated directional migration of transplanted enhance-green fluorescent protein (EGFP)-labeled microglia towards the brain region injected with SP in TAC1-/- mice. Additional studies on the signaling pathways mediating chemotaxis by SP revealed that both neurokinin-1 receptor (NK1R), the G-protein coupled receptor for SP, and NADPH oxidase (NOX2, a key superoxide-producing enzyme on microglia) are necessary for the chemoattractant properties of SP. Whereby SP-induced migration of microglia prepared from either NK1R-/- or NOX2-/- mice was greatly reduced. Furthermore, pharmacological inhibition of NK1R or NOX2 showed a similar inhibition. Evidence suggesting a cross talk between NK1R and NOX2 was provided by showing SP-stimulated NOX2 activation, as measured by membrane translocation of p47phox (a cytosolic subunit of NOX2) and release of superoxide were mediated through NK1R/ß-arrestin1-dependent pathways. In summary, these results strongly suggest that SP is capable of recruiting microglia to the SN through a novel NK1R-NOX2 axis-mediated pathway, accounting for the high microglial density in the SN. These findings further suggest that intimate interactions between SP and microglia in the SN play a critical role for the pathogenesis of PD.

NIEHS
Campos, Christopher
Postdoctoral Fellow
Pharmacology and Toxicology/Environmental Health
transient barrier disruption increases transport function at the blood-brain barrier
The blood-brain barrier (BBB) resides within the brain capillary endothelium and regulates the exchange of endogenous solutes and xenobiotics between blood and brain. The BBB is a physical barrier characterized by the presence of tight junctions (TJ) as well as a metabolic barrier consisting of ATP-driven drug efflux pumps including P-glycoprotein (P-gp) and breast cancer resistance protein (Bcrp). The molecular organization and intracellular communication required to maintain this dynamic barrier is complex and poorly understood. Characterizing this communication may reveal a novel therapeutic target allowing for improved brain drug delivery. Clinically, hyperosmolar agents including mannitol are used to transiently disrupt the TJ in patients with malignant glioma, facilitating the administration of

chemotherapeutics. Additionally, hyperosmolar agents are used in the treatment of brain edema in subjects with acute ischemic stroke. We hypothesize that the transient disruption of the TJ by hyperosmotic stress will lead to compensatory increases in drug efflux transporter function at the BBB, thereby limiting the penetration of therapeutic drugs into the brain. To confirm hyperosmotic stress-induced transient disruption of TJ, rat brain capillaries were loaded with Texas Red (TR), a small molecule membrane impermeable dye and imaged during a one-minute exposure to 100mM mannitol. This treatment resulted in leakage of TR from the capillary lumen into the surrounding media, indicating a mechanical disruption of TJ. Next, we evaluated the consequence of this transient disruption of TJ on BBB transporter function. For these studies, capillaries were exposed to hyperosmotic mannitol media and after 30 minutes normal media was replaced. Protein expression and transport function were assayed 3 hours later. Here, we report that transient disruption of the TJ following hyperosmotic stress increased specific transport function of both P-gp and Bcrp and western blot analysis indicated increased protein expression of both transporters, indicating for the first time that transport function may be mechanistically connected to TJ disruption. These results suggest that hyperosmotic stress-induced increases in transport function and expression may limit the efficacy of therapies administered following transient barrier disruption.

NIEHS
Makia, Ngome
Visiting Fellow
Pharmacology and Toxicology/Environmental Health
Activator Protein 1 Regulation of Human CYP2C9 Expression by Electrophilic Stress Involves MAPK Activation and DNA Looping
CYP2C9 is an important human enzyme that metabolizes both commonly used therapeutic drugs and physiologically important endogenous compounds. CYP2C9 expression is induced by drugs such as rifampicin, hyperforin, phenytoin and dexamethasone. Exposure to these drugs is partly responsible for the inter-individual variability in CYP2C9 expression and metabolism of CYP2C9 substrates. For example, studies in humans have reported enhanced clearance of drugs such as tolbutamide and phenytoin by treatment with rifampicin. Most drugs and foreign compounds are metabolized in the liver to electrophilic and reactive metabolites. Moreover, drugs capable of undergoing redox cycling induce oxidative stress by generation of semiquinone radicals and reactive oxygen species. It not yet known whether the induction of CYP2C9 expression by drugs and xenobiotic is due to secondary effects such as formation of the electrophilic metabolites and induction of oxidative stress. Both CYP2C9 and CYP2C19 expression were induced by tert-butylhydroquinone (tBHQ) in primary human hepatocytes. As a pro-oxidant, tBHQ regulates the expression of cytoprotective genes by activation of redox-sensing transcription factors, such as the nuclear factor E2-related factor 2 (Nrf2) and activator protein 1 (AP-1). The promoter region of CYP2C9 contains putative AP-1 sites at positions -2201 and -1930 which are highly conserved in CYP2C19. Ectopic expression of Nrf2 had no effect on CYP2C9 promoter activity. We demonstrate by promoter luciferase assay that CYP2C9 is transactivated by cFos and JunD heterodimer. Using inhibitors of mitogen activated protein kinases, we showed that ERK and JNK are essential for tBHQ-induced expression of CYP2C9 by increased expression of cFos and phosphorylation of JunD, respectively. Binding assays demonstrate that cFos distinctly interacts with the distal and JunD with the proximal AP-1 site. Because cFos regulates target genes as heterodimer with Jun proteins, we hypothesize that DNA looping is required to bring together the distal and proximal AP-1 sites. Chromosome conformation capture (3C) analyses confirmed the formation of DNA loop at the CYP2C9 promoter mediated by cFos bound to the distal and JunD on proximal site for activation of CYP2C9 transcription. These results suggest that oxidative stress generated by exposure to drugs and xenobiotic

may induce the expression of CYP2C9 and alter the metabolic clearance of numerous CYP2C9 drug substrates.

NIEHS
Smith, Lindsay
Postdoctoral Fellow
Pharmacology and Toxicology/Environmental Health
Glucocorticoid Receptor Regulation of P-glycoprotein at the Blood-Brain and Blood-Spinal Cord Barriers
The blood-brain (BBB) and the blood-spinal cord (BSCB) barriers, comprised of the brain and spinal cord capillary endothelia, constitute a primary obstacle to CNS drug delivery. An important element of barrier function is the ATP-driven drug efflux transporter, P-glycoprotein (P-gp), which exhibits broad substrate specificity and high luminal plasma membrane expression in brain and spinal cord capillaries. We previously demonstrated that several ligand-activated nuclear receptors regulate the expression and activity of P-gp. Here, we examined whether the glucocorticoid receptor (GR), a ligand-activated nuclear receptor targeted by both natural and synthetic glucocorticoids, regulates P-gp at CNS barriers. Naturally occurring glucocorticoids regulate a wide range of physiological effects including gluconeogenesis, homeostasis, and apoptosis. However, the role of these hormones in maintenance of CNS barriers remains unresolved. Furthermore, the effects of synthetic glucocorticoids, a broad class of widely prescribed anti-inflammatory drugs, on CNS barriers are poorly understood. However, these potent anti-inflammatory synthetic glucocorticoids are a mainstay in the treatment of cerebral edema and spinal cord injury. We hypothesize that both natural and synthetic glucocorticoids alter the expression and activity of P-gp at CNS barriers, thereby modifying drug delivery to the CNS. We confirmed the expression of GR in both CNS barriers by qPCR and immunoblotting and demonstrated that depletion of corticosterone via adrenalectomy significantly decreased the expression and activity of P-gp in both the BBB and the BSCB. In-vivo treatment of both intact and adrenalectomized rats with the synthetic glucocorticoid dexamethasone significantly increased P-gp activity and protein expression in both CNS barriers. In-vitro exposure of brain and spinal cord capillaries to dexamethasone also increased P-gp expression and activity and the GR antagonist, RU-486, abolished these increases. These results demonstrate that the endogenous glucocorticoid, corticosterone, maintains P-gp expression and activity at the BBB and BSCB while the synthetic glucocorticoid, dexamethasone, in the presence or absence of endogenous glucocorticoids, increases the activity and expression of P-gp in a GR-dependent manner. Thus, natural glucocorticoids have a protective role in maintaining CNS barriers, while synthetic glucocorticoids may hinder delivery of therapeutic drugs to the CNS.

NIEHS
Freudenthal, Bret
Postdoctoral Fellow
Protein Structure/Structural Biology
The Polymerase Reaction Exposed: Observing a DNA Polymerase Choose Right from Wrong
DNA polymerase (pol) ß is a model polymerase involved in gap-filling DNA synthesis utilizing two metals to facilitate nucleotidyl transfer. Previous structural studies have trapped catalytic intermediates by utilizing substrate analogues (dideoxy-terminated primer or non-hydrolysable incoming nucleotide). To identify novel intermediates during catalysis, we now employ natural substrates (correct and incorrect nucleotides) and follow product formation in real time with thirteen different crystal structures. We are able to observe molecular adjustments at the active site that hasten correct nucleotide insertion and deter incorrect insertion not appreciated previously. A new metal binding site is transiently formed during correct, but not incorrect, nucleotide insertion. Additionally, long incubations indicate that pyrophosphate more easily dissociates after incorrect, compared to correct, nucleotide insertion. This

appears to be coupled to subdomain repositioning that is required for catalytic activation/deactivation. The structures provide insights into a fundamental chemical reaction that impacts polymerase fidelity and genome stability.

NIMH
Harel, Assaf
Visiting Fellow
Neuroscience - General
Behavioral goals are directly reflected in visual object representations in human occipito-temporal cortex
Adaptive behavior depends on the integration of incoming sensory information with the observerâ€™s behavioral goals. How and where this integration occurs, however, is unclear. Visual object recognition is thought to proceed along an occipito-temporal pathway with increasingly complex representations of objects emerging as the pathway progresses. According to some models, this pathway produces abstract representations of objects that can then be used in any given task, with the interaction between current goals and object information occurring at the level of the prefrontal cortex. Here we used functional magnetic resonance imaging (fMRI) to test an alternate view, that behavioral goals affect object representations throughout the occipito-temporal visual pathway. In six diverse tasks, participants made judgments about the same set of objects, focusing on either physical (e.g. whether the object is tilted to the left or right) or conceptual (e.g. relative size) properties. In each trial, participants were first presented with a cue, indicating the task to be performed, and then after a delay, one of the objects. Following a second delay, a response screen appeared instructing the participant which button to press for a given answer, ensuring that no response planning could occur during the object presentation. Focusing on the patterns of response in different regions of the occipito-temporal pathway as well as lateral prefrontal cortex, we found that both the high-level type of task (physical versus conceptual) and the specific task being performed were reflected in the patterns of response and could be decoded even in early stages of visual processing. Critically, behavioral goal interacted with object information, resulting in reduced object decoding across tasks in all regions but early visual cortex. This interaction demonstrates that the specific representations of objects are different across different tasks. Together, these findings demonstrate that behavioral goals are directly reflected in representations not only in prefrontal but also throughout visual cortex, suggesting that even representations within regions thought to be largely driven by bottom-up sensory signals are shaped by the high-level intent of the observer.

NIMH
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Postdoctoral Fellow
Neuroscience - General
Signaling by tuberoinfundibular peptide of 39 residues in the amygdala modulates long-term fear memory
Individuals with post-traumatic stress disorder have exaggerated responses to stimuli that remind them of the fear-provoking event. Impaired extinction or overgeneralization of fearful memories are thought to contribute to the disorder. We are investigating the mechanism(s) by which tuberoinfundibular peptide of 39 residues (TIP39) and the parathyroid hormone 2 receptor (PTH2R), a peptide-receptor neuromodulator system, modulate long-term fear memory. First, PTH2R knockout (KO) and wild-type (WT) male mice were exposed to a single 1.5mA foot shock and long-term fear-recall was assessed by measuring freezing behavior after re-exposing mice to the place they were shocked (shock context) 28 days later. Compared to WT, KO mice exhibited increased freezing in the shock context, suggesting enhanced fear recall by mice lacking TIP39 signaling. The medial amygdala (MeA), which contains a high

density of PTH2Rs and TIP39 containing terminals, projects to the basolateral and central amygdala, areas that have well established roles in fear memory. To evaluate whether TIP39 signaling in the MeA modulates fear memory, we stereotaxically injected viruses encoding a secreted PTH2R antagonist (HYWH) + GFP or GFP only (control) into the MeA of WT male mice. Mice then received a single foot shock as described above and were tested for fear recall 28 days later. Similar to the KO results, mice injected with HYWH in the MeA had higher levels of freezing than controls. We then used the designer receptor exclusively activated by designer drug (DREADD) pharmacogenetic technique to examine whether TIP39 signaling in the MeA is required during initial coding and/or recall of fear memory. A Cre-dependent Gi-coupled DREADD virus that can suppress neuronal activity was injected into the MeA of mice that express Cre in PTH2R neurons. Saline or clozapine-N-oxide (CNO), an agonist for DREADDs that has no effect on endogenous receptors, was administered (1) 1 hour before foot shock or (2) 1 hour before fear recall testing. Mice were tested for fear recall 28 days after foot shock. We found that inhibiting PTH2R neurons with CNO at the time of foot shock increased freezing levels during fear recall, but inhibition at the time of recall had no effect, compared to saline treated mice. Taken together, these findings demonstrate that TIP39 signaling within the MeA at the time of aversive event contributes to modulation of long-term fear recall of traumatic experiences.

NIMH
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Visiting Fellow
Neuroscience - Integrative, Functional, and Cognitive
Emergence of neural specialization for species-specific vocalizations along the ventral auditory cortical stream
The auditory cortex underlies our effortless ability to discriminate complex sounds. Vocalizations are an important class of natural sounds that are critical for conspecific communication in a wide range of animals, including macaque monkeys. The auditory cortex in the macaque monkey consists of several interconnected subdivisions on the supratemporal plane (STP) of the lateral sulcus. Here we investigated the nature and emergence of specialization for vocalizations by measuring auditory evoked field potentials to species-specific vocalizations simultaneously from primary and higher-level auditory cortex of macaques. This approach exploited three high-density micro-electrocorticographic arrays (totaling 96 channels) chronically implanted on the STP in each of three macaque monkeys. Examination of characteristic frequency maps, derived from high-gamma band responses to pure tone stimuli, revealed tonotopic maps that reversed frequency direction at putative areal boundaries. These boundaries divided the STP into four sectors. To assess the differential encoding of vocalization stimuli across these sectors, we used evoked broadband waveforms within each sector to predict stimulus identity. Classification analysis was performed using a multivariate regularized classifier trained by an early-stopping algorithm. The early-stopping allowed us to estimate the classifier by including all recording sites within each sector and multiple time points. We found that neural discrimination performance among vocalizations, compared to matched control stimuli in which only the frequency spectra or temporal content was preserved, was highest in the most rostral sector, while this difference was minimal in the caudally-located primary auditory area. Moreover, the most rostral sector had greater representation for stimuli in particular vocalization categories, further supporting this sector's functional specialization for vocalizations. There were also differences among the temporal scales that encoded vocalizations along the STP, with the theta-band (4-8 Hz) component of the evoked potential contributing most strongly to discrimination performance in the rostral sector. Together, these results reveal a caudorostral progression of specialization in the ventral auditory pathway, a mode of signal encoding that results in the neural discrimination of conspecific vocalizations.

NIMH
Jarcho, Johanna
Postdoctoral Fellow
Psychiatry
Developmental differences in brain response to expected and unexpected social feedback in anxious patients
Background: Adolescence is a developmental period when the approval of peers is particularly salient, and prevalence of social anxiety disorder increases dramatically. Socially anxious in adolescents and adults is associated with fronto-striatal dysfunction in response to social stimuli. Because the importance of peer acceptance declines with normal maturation, it is unclear if both age groups would show similar fronto-striatal dysregulation when receiving expected or unexpected social feedback. Methods: To address this issue, we conducted an fMRI study using a â€œChatroomâ€ paradigm with healthy (N=32) and anxious (N=19) adults (M=26.83 years), and healthy (N=24) and anxious (N=15) adolescents (M=13.24 years). Participants viewed pictures of 60, age-matched peers and sorted these photos into high- and low-interest groups, reflecting peers the participant selected, or rejected for an on-line chat at the end of the study. Participants believed these choices would be revealed to their peers before they sorted the participantâ€™s photo in a similar manner. During an fMRI scan 2-weeks later, participants viewed photos of high- and low-interest peers, and predicted if each peer was interested in chatting with them, prior to receiving expected or unexpected social feedback (selection or rejection). Results: Regardless of age, anxious patients predicted their peers would be less interested in chatting with them than healthy individuals (F=4.21, p<05). Striking interactions in fronto-striatal activity emerged (Fâ€™s>19.00, pâ€™s<.001) when participants received expected, relative to unexpected feedback from high-interest peers who expressed mutual interest in the participant. Specifically, anxious adolescents exhibited heightened activity in caudate and ventrolateral prefrontal cortex (VLPFC; BA 47) when receiving unexpected, relative to expected feedback from mutually interested peers. This pattern of activity was not observed in healthy adolescents, or in healthy or anxious adults. Conclusion: These findings suggest that expectations about social feedback from mutually interested peers are uniquely salient among anxious adolescents. Thus, while anxious adults and adolescents exhibit biases in their predicted interest of peers, dysregulated engagement of fronto-striatal circuits suggest that such biases may have a particularly potent effect on adolescents. Expectation biases may therefore be an advantageous target for treatment in anxious adolescents.

NIMH
Schmitz, Anja
Visiting Fellow
Psychiatry
Anxiety-potentiated startle as a Source of Heterogeneity of Mood Disorders
Mood disorders are the 2nd leading cause of disability worldwide. However, identification of their genetic and biologic underpinnings has been challenged by their heterogeneity characterized by multiple subtypes and components, and comorbidity with other emotional conditions such as anxiety. Recent evidence suggests that there may be two subgroups of people with Bipolar Disorder, with one(BIP-1) being more strongly associated with psychosis and the other(BIP-2) more associated with anxiety states and Major Depressive Disorder(MDD). We took advantage of advances in basic research that distinguish between fear responses to a specific threat cue that are mediated by the amygdala (fear-potentiated startle[FPS]) and more sustained anxiety states that are mediated by the bed nucleus of the stria terminalis (anxiety-potentiated startle[APS]) to determine whether the proposed subgroups can be differentiated in terms of fear and anxiety by employing potentiated startle in a human sample. The purpose of this study was to evaluate the potential utility of these two startle measures to

distinguish between the putative subtypes of bipolar disorder and their association with anxiety states. We assessed whether FPS and APS distinguished participants with a range of mood disorder subtypes(BIP-1, BIP-2, and MDD) from unaffected participants with no history of mental disorders. We also examined familial correlations in FPS and APS as an index of genetic susceptibility factors. We designed a novel startle experiment with developmentally appropriate stimuli to assess FPS and APS to predictable and unpredictable aversive events(e.g. a blast of air to the neck; a picture of a frightened woman accompanied by a scream). We measured FPS and APS in 66 children and 150 adults within a population based family study. In adults, measures of APS were related to BIP-2(F=26.5, p<.001) and MDD(F=4.75, p=.03) but not BIP-1. In addition, APS was correlated between parents and their adult or youth offspring (N pairs =48; r=.36, p=.01). Taken together these findings suggest that APS is familial and may comprise a marker that distinguishes between mood disorder subgroups. This study is the first to assess the potential utility of FPS and APS to distinguish between the subgroups of bipolar disorder in a population based family study. The finding that APS is familial also suggests that startle reactivity may comprise an endophenotype, or intermediate biologic marker for BIP-2 and MDD.

NIMH
Lohith, Talakad
Visiting Fellow
Radiology/Imaging/PET and Neuroimaging
Increased brain phosphodiesterase type 4 (PDE4) in DISC1 gene locus impairment mouse model imaged by [11C](R)-rolipram PET
Purpose: Phosphodiesterase type 4 (PDE4), which selectively hydrolyzes the second messenger cyclic adenosine monophosphate in brain, is regulated by protein kinase A (PKA) and disrupted in schizophrenia 1 (DISC1). DISC1 suppresses the activity of PDE4, and mutations in DISC1 are involved in schizophrenia and mood disorders. Using [11C](R)-rolipram, a PDE4 inhibitor, and positron emission tomography (PET) imaging, PDE4 regulation by PKA has been shown before in rats. However, the role of DISC1 on PDE4 activity has not been shown in living animals. To investigate interactions between DISC1 and PDE4, this study compared [11C](R)-rolipram binding in brain of DISC1 gene locus impairment mouse model lacking genomic regions between exon 1 and 3 (KO) and wild type (WT) mice. Methods: [11C](R)-rolipram binding was measured by PET scanning under equilibrium achieved by bolus plus constant infusion for 90 min of the PET tracer in age-matched (3 months) DISC1 KO (n = 11) and WT (n = 9) C57BL/6 mice. Plasma levels of [11C](R)-rolipram were measured in heart blood at the end of scan. [11C](R)-rolipram binding in the whole brain was measured as total distribution volume VT which is ratio of brain radioactivity in PET images to [11C](R)-rolipram concentration in plasma at equilibrium. As only free ligand enters brain, VT was corrected for plasma free fraction (fP) of [11C](R)-rolipram measured in a separate group of age-matched (3 months) DISC1 KO (n = 6) and WT (n = 7) mice. VT / fP equals Bmax / KD (binding site density / dissociation constant) plus nonspecific binding. Results: Average change in brain radioactivity per hour after 50 min scan time was â€“5 ± 6% for both mouse groups indicating equilibrium was achieved within the scan time. DISC1 KO mice showed a 41% significant increase in VT (18 ± 6 vs. 13 ± 4 mL/cm3, P = 0.04) compared to WT. The VT/fP, which more accurately reflects [11C](R)-rolipram binding than VT, showed a 73% significant increase (90 ± 31 vs. 52 ± 15 mL/cm3, P = 0.004) in DISC1 KO compared to WT mice suggesting increased PDE4 activity in the presence of dysfunctional DISC1 protein. Conclusion: This study is the first to demonstrate that levels of PDE4, a key enzyme that regulates cAMP, is regulated by DISC1 in living animals. PET imaging of PDE4 activity could facilitate development of drugs such as PDE4 inhibitors or monitor treatment in individuals affected with DISC1 gene variants.

NIMH

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Radiology/Imaging/PET and Neuroimaging
Decreased Cannabinoid Cb1 Receptor Binding In Tobacco Smokers Examined With Positron Emission Tomography
Objectives: Cannabinoid CB1 receptors are involved in the rewarding effects of nicotine. In addition, tobacco smoking is an important confounding factor in clinical studies of psychiatric disorders, because many patients with such disorders smoke tobacco. We previously found that both cannabis abuse and alcohol dependence are associated with reduction of brain cannabinoid CB1 receptor binding. In this study, we tested whether people who smoke tobacco also have reduced cannabinoid CB1 receptor binding, using positron emission tomography (PET). Methods: We measured CB1 receptors in 11 healthy male subjects who smoked tobacco (age 33 ± 10 years, BMI 27 ± 4 kg/m2), and in 31 healthy male subjects who did not smoke tobacco (age 34 ± 9 years, BMI 28 ± 7 kg/m2). Smokers had an average Fagerström Test for Nicotine Dependence score of 4 (denoting mild to moderate nicotine addiction) and smoked an average of 9 cigarettes per day (range: 1â€“20). We used positron emission tomography (PET) with [18F]FMPEP-d2, a radioligand for CB1 receptors. Arterial blood was sampled during PET scans to estimate receptor binding as distribution volume (VT), which is the ratio at equilibrium of the concentration of radioactivity in brain to that of the parent radioligand in plasma. Results: VT of [18F]FMPEP-d2 ¬was about 20% lower in tobacco smokers than in non-smokers throughout the brain (main effect of smoking: F=5.1, p=0.030; smoking by region interaction: F=2.1, p=0.098). The plasma free fraction of [18F]FMPEP-d2 was not lower in smokers (0.46 ± 0.1%) than non-smokers (0.41 ± 0.2%), and therefore did not reduce VT. Among smokers, VT did not correlate with the Fagerström score (R=0.38, p=0.29), number of cigarettes smoked per day (R=-0.21, p=0.53), years of tobacco smoking (R=0.25, p=0.46), or age at onset of tobacco smoking (R=0.17, p=0.61). Conclusions: Our results suggest that tobacco smoking is associated with a reduction of brain cannabinoid CB1 receptors. In contrast to the regionally specific downregulation in cannabis smoking, the reduction in tobacco smoking occurred throughout the brain, while being similar in magnitude. This reduction is smaller in magnitude than that seen in patients with alcohol dependence. This pattern of results suggests that different substance use disorders may differentially involve brain CB1 receptors.

NINDS
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Gene Expression
The cis-regulation of POU-domain transcription factor genes, pdm-1 & pdm-2
One of the major goals in biomedical research 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. Genome-wide association studies have shown that mutations in regulatory DNA 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, which are required for Drosophila neurogenesis. These genes are related to the human TF genes Oct-1 & Oct-2, which have been implicated in glioma, the most common malignant brain tumor. During neurogenesis, 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 the pdm genes. 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. We next tested whether this region contains the pdm-1 NSC enhancer. Enhancer-reporter analysis showed that the DNA fragment recapitulates pdm-1 expression in NSCs. To delimit the enhancer boundaries, we used our

program EvoPrinter, which reveals highly conserved DNA. We found that the enhancer is a 1 kb cluster of conserved sequences (CSC) that contains Cas-binding sites. To locate the pdm-2 NSC enhancer, we performed a genome-wide search using the pdm-1 enhancer sequences and our database of ~100,000 CSCs. The identified pdm-2 NSC enhancer also contains Cas sites and regulates expression similar to the pdm-1 enhancer. We next showed that the loss of Cas expression or removal of Cas sites from the pdm-1 enhancer triggers ectopic expression during neurogenesis, indicating that Cas is a direct regulator of pdm. Given that the identified enhancers do 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. From these results, we developed a novel program that decodes regulatory DNA by linking sequence and image data. Together, defining the principles governing cis-regulation will provide a framework for understanding gene regulation.

NINDS
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Visiting Fellow
Genetics
siRNA-Mediated Allele-Specific Silencing of a Dominant Negative COL6A3 Mutation Causing Ullrich Congenital Muscular Dystrophy
Congenital muscular dystrophy type Ullrich (UCMD) is a severe disorder of early childhood onset, characterized by proximal or generalized muscle weakness, distal joint hypermobility, progressive joint contractures, and skin changes. Progressive scoliosis and diaphragmatic weakness may also develop and contribute to respiratory insufficiency, which may require ventilatory support. At present, there are no pharmacological treatment options available for UCMD patients. UCMD is caused by mutations in the genes coding for collagen type VI, a major microfibrillar component of the extracellular matrix surrounding the muscle fibers that is produced and secreted by the interstitial fibroblasts. UCMD mutations are more commonly acting as dominant, where the production of a mutant collagen VI isoform interferes with its normal counterpart, prevents normal collagen VI secretion and impairs extracellular matrix formation, a phenomenon referred to as the dominant-negative effect. Achieving allele-specific silencing of the mutant mRNA transcript would convert this dominant-negative state into a haploinsufficient state, which is clinically asymptomatic. To explore the value of RNAi as a potential therapy for dominant UCMD, we designed a series of siRNA oligos specifically devised to target a mutant mRNA transcript lacking the exon 16 of the Collagen VI a3 gene (COL6A3). The skipping of exon 16 in COL6A3 is one of the most frequently observed mutations among dominant UCMD patients. We tested this series of siRNA in UCMD-derived dermal fibroblast cells lines. Transcript analysis by semi-quantitative and quantitative RT-PCR showed that a subset of these siRNAs significantly reduced the expression of the mutant transcript, without affecting the normal allele, showing the potency of the allele-specific silencing. In HEK293T cells these siRNA oligos selectively suppressed protein expression from a reporter construct carrying the mutation, but not from a wild-type construct, suggesting that the collagen VI protein levels are as significantly reduced in an allele-specific manner. Furthermore, we found that treating UCMD fibroblasts with these siRNAs considerably improved both quantity and quality of the collagen VI extracellular matrix in cell culture, as measured by confocal microscopy of immunostained collagen VI. Our current study serves as a proof-of-principle and establishes RNAi as a promising molecular approach for treating UCMD caused by dominant negative mutations.

NINDS
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Neuroscience - Cellular and Molecular
Real-time in vivo two-photon microscopy reveals rapid and continuous plasticity of sensory input to the mouse olfactory bulb
Olfactory bulb (OB) glomeruli are the initial sites for processing of odor information in the brain, and are thought to represent functional units of odor coding. Odor information is transmitted from the nose to the OB by olfactory sensory neurons (OSNs): OSNs expressing a particular odorant receptor project their axons to specific glomeruli, where they synapse with dendrites of projection neurons and local interneurons. OSNs have lifespans of 1-2 months and turn over throughout life, posing a challenge to the maintenance of functional glomerular circuitry. Nevertheless, the degree of structural plasticity of OSN presynaptic terminals is unknown. This question is key to understanding how OB circuits balance stability and plasticity to maintain their function, and how this equilibrium is disrupted in disease. Here, we used two-photon imaging to follow the structural dynamics of mature OSN presynaptic terminals for the first time in living mice. Using the tetracycline transactivator system, we expressed GFP-tagged synaptophysin (sypGFP), a presynaptic marker, specifically in mature OSNs. We then imaged these sypGFP clusters in juvenile (postnatal day (P)21) and adult (P56) mice at 30-minute intervals through cranial windows implanted over the OB. Novel ImageJ analysis routines were developed for automated, unbiased detection and characterization of syp-GFP clusters. We analyzed 28 glomeruli from 6 P21 mice, and 18 glomeruli from 6 P56 mice. At P21, OSN presynaptic terminals were highly dynamic, with both gain and loss of sypGFP clusters being evident. For individual glomeruli, changes in total sypGFP ranged from +35% to -49% over 3 hours. However, synapse gain and loss were balanced: the mean (+/- sem) change in sypGFP per glomerulus was -1 +/- 3% in 3 hours. Surprisingly, at P56, OSN terminals remained dynamic (change in total sypGFP between +93% and -54% over 3 hours). sypGFP turnover rates were again balanced (-1 +/- 4%), and were similar to those at P21 (P=0.85, t-test). Control experiments, in which sypGFP clusters were imaged at very short time intervals, showed that respiration and heartbeat-induced movement could account for only a +/-7% change in sypGFP per glomerulus. Our findings provide the first evidence that continuous high-level turnover of OB sensory inputs occurs even in adult mice, providing an important substrate for lifelong plasticity. Nevertheless, balanced synapse gain and loss enable the maintenance of stable glomerular function.

NINDS
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Neuroscience - Cellular and Molecular
Activity-dependent recruitment of dynamin 1 and 3 for different kinetics of synaptic vesicle endocytosis
After exocytosis, synaptic vesicles undergo endocytosis and recycling, which is critical for sustaining supply of releasable vesicles at the nerve terminal. It has been widely observed in various synapses that endocytosis occurs either rapidly with a time constant of ~1 second or slowly with a time constant of tens of seconds. However, the molecular mechanism underlying the kinetic difference remains poorly understood. We explored the precise function of distinct isoforms of dynamin, a large GTPase essential for vesicle fission, at the calyx of Held synapse. The calyx of Held is a mammalian glutamatergic synapse with a large nerve terminal which allows presynaptic patch-clamp recording and membrane capacitance measurement with high temporal resolution. We generated tissue-specific knockout of dynamin 1 or dynamin 3 at the calyx of Held by breeding Krox20 superscript-Cre/+ mice with dynamin 1 superscript-loxP/loxP or dynamin 3 superscript-loxP/loxP mice. In wild-type mice, single action potential evoked rapid endocytosis. Increasing the number and/or firing frequency of action potential induced a clathrin-dependent slow endocytosis. Interestingly, deletion of dynamin 1 dramatically impaired slow endocytosis while single action potential-evoked rapid endocytosis remained intact, suggesting a specific

function of dynamin 1 in clathrin-mediated slow endocytosis. In dynamin 3 knockout mice, however, rapid endocytosis was completely blocked while slow endocytosis was unchanged as compared to wild-type littermates, suggesting a selective involvement of dynamin 3 in rapid endocytosis. Thus, our findings provide strong genetic evidence showing that dynamin 1 and dynamin 3 are crucial in mediating slow and rapid endocytosis, respectively. Further, we propose that dynamin 1 is recruited during intense neuronal activity when a large amount of exocytosis increases endocytic load, consistent with its predominant expression level at the synapse.

NINDS
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Neuroscience - Neurodegeneration and Neurological disorders
ZASP mutations cause skeletal muscle Z-disc disruption by disassembling a-actinin cross-linked skeletal actin filaments in a myofibrillar myopathy
Myofibrillar myopathies (MFM) are characterized by early and prominent disruption of the Z-disc with focal dissolution of myofibrils and ectopic accumulation of myofibrillar proteins. The molecular mechanisms underlying the Z-disc disruption are not yet delineated. ZASP A147T and A165V mutations at or within an evolutionarily conserved 26 amino acid motif (sZM) are associated with a prototype MFM, zaspopathy. ZASP is anchored by a-actinin at the Z-disc. The proteins that interact with ZASP-sZM are not yet known. Our research focus was to investigate the functional interactions of ZASP-sZM, which may play a role in the structural integrity of the Z-disc, and to determine how mutations alter these functions and cause degeneration of skeletal muscle fibers. A yeast two-hybrid (Y2H) screen of a human skeletal muscle cDNA library using ZASP sZM-132aa as bait (WT and A165V) identified skeletal muscle a-actin (247-375aa) as a putative interactor. Pairwise Y2H assays and co-IP studies showed that ZASP-sZM was important for actin-binding as deletion of sZM abolished the ZASP-actin interaction. GST-tagged ZASP proteins interacted with G-actin monomers and skeletal actin filaments (F-actin) in vitro. ZASP-sZM mutations did not alter the binding of ZASP to skeletal actin or a-actinin-2. In vitro studies showed that mutant ZASP disassembled actinin-crosslinked F-actin, the core structure of skeletal muscle Z-disc. To examine the effects of the ZASP-sZM mutations on F-actin in skeletal muscle, WT and A165V ZASP-GFP were expressed in mouse tibialis anterior (TA) muscles of opposite limbs by electroporation. The architecture of the Z-disc and F-actin was preserved in the mouse TA muscle fibers expressing ZASP-GFP-WT. In contrast, there was a loss of a-actinin-2 in muscle fibers expressing ZASP-GFP-A165V as early as 1 week after the electroporation. By 4 weeks, phalloidin staining showed focal accumulations of F-actin that co-localized with ZASP-GFP-A165V in the sarcoplasm. ZASP and F-actin accumulations were also observed in skeletal muscle fibers of a patient with zaspopathy due to A165V mutation. Ultrastructural studies of the mouse TA muscle expressing ZASP-GFP-A165V confirmed discrete disruption of the Z-discs and adjacent actin filaments reminiscent of the human disease. These results suggest that ZASP-sZM mutations have deleterious effects on the core structure of the Z-discs in striated muscle and support a toxic gain-of-function disease mechanism.

NINDS
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Neurotransmission and Ion Channels
The cell-autonomous role of glutamatergic synaptic transmission in neuronal development
In the central nervous system, glutamate mediates the majority of excitatory synaptic transmission by acting on AMPA receptors and NMDA receptors. The role of excitatory synaptic transmission in neuronal development has been extensively studied by using pharmacological blockade both in vitro and in vivo.

While pharmacological reagents globally block AMPA- and NMDA- receptors mediated synaptic transmission, they do not differentiate the cell-autonomous function of excitatory synaptic transmission from the indirect effects on networks activity (non-cell-autonomous) associated with global blockade. They may also eliminate competition among neurons, and thus mask potential roles that synaptic transmission might play in neuronal development. To investigate cell-autonomous role of glutamatergic synaptic transmission in neuronal development, we employed a quadruple conditional knockout mouse line in which three genes encoding AMPA receptor subunits (GluA1, A2 and A3) plus the gene encoding GluN1, the obligatory NMDA receptor subunit, are all conditional alleles (GRIA1-3fl/flGRIN fl/fl). By utilizing in utero electroporation method, Cre recombinase fusion to mCherry (mCherry-Cre) was sparsely expressed in the neural progenitors of hippocampal CA1 pyramidal neurons at embryonic days 14. Whole-cell patch clamping experiments demonstrated that excitatory synaptic transmission mediated by both AMPA- and NMDA- receptors was completely eliminated from mCherry-Cre positive hippocampal neurons as early as postnatal days 6 in vivo (acute slice recording) or days 6 in vitro (dissociated culture recording). Morphological and electrophysiological analyses were performed to examine the difference between mCherry-Cre positive neurons devoid of AMPA- and NMDA- receptors and neighboring intact control neurons. Surprisingly, in contrast to the classic dogma that dendrites and synaptic structures undergo experience-dependent plasticity, no overt changes in dendritic arborization and density of dendritic spines were detected. Further studies are ongoing to examine more details in both pre- and post- synaptic specifications in mCherry-Cre positive cells. Our data demonstrates a functional segregation of excitatory synaptic transmission from neuronal morphological development.