FARE2015 WINNERS

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CC
Scott Burks
Postdoctoral Fellow
Radiology/Imaging/PET and Neuroimaging
Pulsed focused ultrasound improves mesenchymal stem cell homing to kidneys and their therapeutic capabilies to both prevent acute kidney injury and rescue established injury
Introduction: Acute kidney injury (AKI) is a dire clinical condition with mortality rates up to 50% when comorbidities exist. It is an inflammation-driven disease that develops over several days after renal injury/toxicity. Animal studies show mesenchymal stem cell (MSC) infusions help prevent AKI when administered soon after injury, before renal function deteriorates. This has spurred several clinical trials of MSC to prevent AKI. To date however, no therapy can effectively treat clinically-obvious AKI or rescue renal function during advanced AKI. We previously showed noninvasive image-guided pulsed focused ultrasound (pFUS) alters the kidney microenvironment and upregulates chemoattractants to enhance homing of infused MSC to healthy kidneys. We investigated this targeted homing platform to improve MSC homing to kidneys during AKI. We investigated whether pFUS with MSC during early AKI would better prevent disease compared to MSC alone and whether it is a viable therapeutic modality for advanced AKI. Methods: AKI was induced in C3H mice by cisplatin (15 mg/kg) on Day (D)0. Kidneys received ultrasound-guided pFUS (8.9 MPa, 5% duty cycle, 2 min) on either D1 or D3. One million human MSC were IV injected 4hr post-pFUS. Mice treated on D1 were euthanized on D5 and those treated on D3 were euthanized on D7. Renal function (blood urea nitrogen and serum creatinine clearance) was measured from serum and kidneys were harvested for molecular and histological analyses. Results: pFUS increased chemoattractants and enhanced MSC homing at D1 post-cisplatin (prior to renal functional deficits) or at D3 (during established AKI). pFUS+MSC at D1 better prevented AKI than MSC alone, generating improved renal function and reduced tubular cell apoptosis. MSC alone during established AKI (at D3) significantly improved 7-day survival of mice from 14% to 58%. Survival was further improved to 93% with pFUS+MSC. pFUS+MSC increased CD206 (M2 phenotype marker) on kidney macrophages and Ki67 expression (a proliferation marker) in tubular cells. Conclusion: pFUS is a clinical modality to increase MSC homing to kidneys during AKI. In AKI, pFUS with MSC stimulates tubular cell regeneration (Ki67) and shifts resident macrophages from a proinflammatory M1, to an anti-inflammatory M2 phenotype. Thus, pFUS with MSC better prevents AKI than MSC alone and it represents the only viable option to rescue established AKI, which otherwise has no meaningful therapeutic options.

CC
Van Nguyen
Postdoctoral Fellow
Radiology/Imaging/PET and Neuroimaging
Detection of genetic alteration events by ionizing radiation in human embryonic stem cells via next-generation sequencing

Ionizing radiation (IR) is widely employed for various medical purposes, ranging from therapy to diagnosis. Although its cytotoxic properties have long been successfully exploited for therapeutics, the biological effects of IR from clinically relevant diagnostic doses remain unclear. We therefore seek to investigate this urgent question as the use of IR-related diagnostic tests continues to rise. A novel model based on human embryonic stem cell (hESC) culture was established to conduct the study. ESCs are pluripotent stem cells derived from the inner cell mass of a blastocyst, an early-stage embryo. As such, they are able to differentiate into all derivatives of the three primary germ layers: ectoderm, endoderm, and mesoderm. Because of their early development status and assumed extreme sensitivity to IR, they can serve as a model for studying the effects of diagnostic low-dose IR. Since low-dose IR is expected to inflict rare mutations in a very small subset of cells (if any) as a result of DNA damage and the miss-repair that follows, we employed next-generation sequencing to obtain a very high coverage to detect such rare variants. Additionally, because IR is often implicated as a risk for cancer, we performed targeted sequencing of the genomic â€œhotspotâ€ regions that are frequently mutated in 50 human cancer genes. Utilizing this deep sequencing approach, we were able to consistently sequence low-, high-dose, and control samples from 4 hESC cell lines H1, H7, H9, and H14 with reliable coverage for data analysis. Single nucleotide variants occurring at very low frequencies (Ëœ 2%) were successfully identified using two independent analysis software platforms, confirming the plausibility of our method. We did not detect any differences in the number of genetic alteration events between the low-dose samples and controls. However, as expected, the frequencies of these events trend upwards for some cell lines in the high-dose samples. Our findings suggested that in the highly sensitive hESCs, diagnostic low-dose IR did not result in a detectable increase in genetic alteration events occurring within the cancer â€œhotspotâ€ regions. As future studies are warranted to confirm the safety of medical diagnostic procedures involving IR, we plan to continue our investigation at the transcription level with RNA sequencing and expand the project to a more comprehensive panel of cancer genes.

CC

Holger Roth
Visiting Fellow
Radiology/Imaging/PET and Neuroimaging

A New 2.5D Classifier for Lymph Node Detection using Random Sets of Deep Convolutional Neural Network Predictions

Radiological imaging of lymph nodes (LNs) plays an important role in clinical practice. Diseases cause LNs to swell in size which can be measured by LN analysis in CT images. LN assessment monitors the staging of certain diseases (e.g. lung cancer), prognosis, choice of therapy, and follow-up examinations. Radiologists need to detect, quantify and evaluate LNs. Assessment is typically done manually. LNs can vary markedly in shape and size, and can have attenuation coefficients similar to surrounding organs which makes manual processing time-consuming, tedious and delays clinical workflow. Hence, automated LN detection is important but very challenging, due to the low contrast of surrounding structures in CT and their varying sizes, poses and shapes. State-of-the-art methods show performance of 52.9% sensitivity at 3.1 false-positives (FP) per volume, or 60.9% at 6.1 FP/vol. for mediastinal LN. We aim to improve automated computer-aided detection (CADe) by firstly operating a preliminary CADe system at very high sensitivity (~100%) but high FP level (~40-45 per patient) in order to generate volumes of interest (VOI) for LN candidates. Our 2.5D approach decomposes any 3D VOI by resampling 2D reformatted orthogonal views N times, via scale, random translations, and rotations with respect to the VOI centroid coordinates. These random views are used to train a deep Convolutional Neural Network (CNN) classifier, which is inspired by the visual processing of the brain. A CNN uses deep layers of neurons to learn features automatically, based on example images. In testing, this CNN classifier assigns simple binary counts for all N random views and these are then averaged per VOI to retain a final

LN classification confidence. We validate the presented approach on two CT datasets: 90 patients with 388 mediastinal LNs and 86 patients with 595 abdominal LNs. We achieve sensitivities of 78% and 86% at3 FP/vol., and AUC values of 0.944, 0.926 in the mediastinum and abdomen respectively, which markedly outperforms previous work (p=9.1E-4 and p=4.0E-16). The proposed CNN approach may be generalizable for a variety of applications in CADe for medical images. An effective 2.5D CNN classifier may allow us to solve 3D object detection tasks directly, with less computational effort than full 3D approaches. More sophisticated spatial sparse pooling or aggregating principles over simple averaging of random CNN classifications will be explored in the future.

NCATS
Michael Gormally
Postdoctoral Fellow
DNA-binding Proteins/Receptors and DNA Repair
Suppression of the FOXM1 transcriptional program via novel small molecule inhibition
Forkhead box M1 (FOXM1) is a transcription factor of considerable importance. Aberrant overabundance of FOXM1 through mutations in upstream regulators or gene amplification has been identified in most human cancers and FOXM1 expression correlates with severity of prognoses. Thus, chemical inhibition of FOXM1 has become a major goal. We designed a novel in vitro assay to detect disruption of FOXM1 DNA binding. We sucessfully miniaturized this assay for quantitative high-throughput screening (qHTS) and interrogated a novel collection of 54,211 compounds, which were assembled at the National Center for Advancing Translational Sciences (NCATS) and consisted of diverse drug-like molecules intended as starting points for medicinal chemistry lead development. We identified the small molecule FDI-6 as a potent inhibitor of the interaction of FOXM1 with its consensus DNA binding motif and characterized its interaction in detail by biophysical analyses. We confirmed that FDI-6 binds directly to FOXM1 protein, and also demonstrated that this small molecule is able to displace FOXM1 protein from promoters of target genes in MCF-7 breast cancer cells. Finally using next generation sequencing, we employed RNA sequencing (RNA-seq), to show that FDI-6 selectively down-regulates the FOXM1 transcriptional program of cell-cycle regulation. Importantly, FDI-6 is specific for FOXM1 binding and has no effect on the expression of genes regulated by other related forkhead factors, which exhibit homology with the DNA binding domain of FOXM1. FOXM1 functions by binding consensus DNA targets in gene promoters and activating their transcription, however, the contradictory tumor suppressive and oncogenic roles in different cellular contexts remain incompletely understood. Our study shows that the genomic interaction of this clinically important transcription factor can be manipulated with small molecules to regulate the expression of key gene families. This demonstrates clear potential for FOXM1 to be pursued as a therapeutic target in the future.

NCI-CCR
Susan Hamilla
Doctoral Candidate
Biophysics
Cancer cell mRNA localization during metastasis
The high mortality rate of cancer is because of metastasis formation, the ability of cancer cells to break away from the primary tumor site and form secondary tumors in distant areas. Much research has been dedicated to analyzing cancer cellsâ€™ molecular and biochemical capabilities, but less is known about regulation and localization of genetic factors during metastasis and how mechanical properties influence metastasis formation. Localization of RNAs in lamellipodial regions has been proposed to play an important role during metastatic progression, however, the identity and functional significance of these localized mRNAs has been investigated only in a few cases. RNAs accumulate at sites of new integrin

engagement, at lamellipodia, and at sites of early or persistent protrusion formation. In one pathway, the tumor suppressor protein, adenomatous polyposis coli (APC) targets RNAs to cell protrusions. APC associates with many RNAs in protrusive areas, including Ddr2, Rab13, and Pkp4. During metastasis, certain cancers are known to form secondary tumors in specific organs. For example, breast cancer commonly forms secondary tumors in the brain, lung, bone, and liver. It has been shown previously that cancer cells modulate their gene expression in response to the mechanical properties of the substrate. Additionally, mRNA localization at adhesion sites is influenced by mechanical tension, which is adjusted by cells as a function of the mechanical properties of the cell environment. Therefore, mechanical properties of tissues may play a role in cancer cell homing to specific organs in the body during metastasis and localization of mRNAs may change during this process. In this work, we used the MCF10A cell series, a well-characterized breast cancer progression model composed of four different cell lines representing different stages of pre-malignant to invasive transformation to investigate mRNA localization and how it is affected by substrate stiffness. By using in situ hybridization, and polyacrylamide gels of varying stiffness (0.87kPa â€œsoftâ€, 5kPa â€œintermediateâ€ 280kPa â€œstiffâ€ we were able to observe APC associated mRNA localization of mRNAs Pkp4, Rab13, and Ddr2. We find that the accumulation of candidate mRNAs at the cell periphery increases with increasing metastatic transformation in the MCF10A cell series. Additionally, we observe that cytoplasmic mRNAs are more localized on stiff 280kPa and glass substrates compared to soft 0.87kPa substrates.

NCI-CCR
Wei Gao
Postdoctoral Fellow
Carcinogenesis
Inactivation of Wnt signaling by a novel human monoclonal antibody therapeutically targeting glypican-3 in liver cancer
Wnt signaling is important for cancer pathogenesis, suggesting that the pathway represents an attractive therapeutic target. However, due to ubiquitous expression of Wnt genes, normal tissues could also be affected if we directly target Wnt or Wnt receptors (FZD). Therefore, we hypothesize that targeting tumor-specific partners that are involved in Wnt signaling should be a more desirable strategy. Glypican-3 (GPC3) is a glycosylphosphatidylinositol (GPI)-anchored cell surface protein comprised of a core protein and two heparan sulfate chains. In recent years, GPC3 has emerged as a candidate therapeutic target in liver cancer because of its expression in over 70% of hepatocellular carcinoma (HCC) cases but not in any adult normal tissues. Interestingly, Wnt signaling is abnormally activated in more than 95% of HCC cases. GPC3 may potentially attract Wnt molecules to the cell surface and modulate Wnt signaling in liver cancer. In this study, we demonstrated the expression of Wnt3a and its FZD receptors, as well as its ability to promote cell proliferation, in a variety of HCC cell lines. We found that GPC3 enhanced the activity of Wnt3a/ß-catenin signaling whereas GPC3 knockdown cells had reduced response to Wnt3a stimulation. GPC3 functionally interacted with Wnt3a through its heparan sulfate chains. GPC3 without heparan sulfate chains exhibited less Wnt3a binding and Topflash reporter activity, indicating the heparan sulfate chains of GPC3 is important for Wnt activation. Based on our biochemical and functional analysis of GPC3 and its role in Wnt signaling, we have successfully isolated HS20, a human monoclonal antibody against GPC3, by phage display technology. The HS20 antibody preferentially recognizes the heparan sulfate chains of GPC3; the antibody binding requires both the sulfated and non-sulfated portions of heparan sulfate. Most interestingly, HS20 inactivated Wnt3a/ß-catenin signaling via disrupting the interaction of GPC3 and Wnt3a. Furthermore, the human antibody inhibited Wnt3a-dependent cell proliferation in HCC cell models and xenograft tumor models in mice. This is one of the first examples of an antibody directed against the heparan sulfate of a proteoglycan

that showed efficacy in blocking Wnt signaling and tumor growth, suggesting a novel strategy for liver cancer therapy.

NCI-CCR
Matthew Jones
Doctoral Candidate
Carcinogenesis
miR-215 represses BMI1 expression to promote differentiation and limit self-renewal in colorectal cancer stem cells
Since the initial description of cancer stem cells (CSCs) as a self-renewing subpopulation of malignant cells with tumor-initiating capacity, a growing body of evidence has supported the existence of CSCs in virtually every tumor type. Our previous work in colorectal cancer has identified the transcription factor CDX1 as a key regulator of CSC dynamics. The expression pattern of CDX1 in the normal colon forms a gradient with its minimum in the crypt base stem cell niche, increasing to a maximum in the mature enterocytes lining the lumen, where CDX1 transactivates genes important for enterocyte structure and function. CDX1 expression is frequently lost in colorectal cancer, resulting in more aggressive, poorly differentiated tumors with higher proportions of CSCs. These CSCs share many genetic markers with normal stem cells, including BMI1, a polycomb repressive complex 1 component involved in epigenetic silencing of genes that oppose self-renewal. Many miRNAs have been implicated in tumor suppression and carcinogenesis, but the roles of miRNAs in differentiation, particularly in colorectal cancer, remain poorly understood. We hypothesized that miRNAs downstream of CDX1 function as key effectors of differentiation. To identify CDX1-regulated miRNAs, we sequenced small RNAs in two pairs of colorectal cancer cell lines: CDX1-low HCT116 and HCT116 with stable CDX1 overexpression; and CDX1-high LS174T and LS174T with stable CDX1 knockdown. Validation of candidates miRNAs in a larger cell line panel revealed miR-215 to be most significantly correlated with CDX1 expression. CDX1 ChIP-qPCR and promoter luciferase assays confirmed that CDX1 transactivates miR-215 transcription. miR-215 expression was depleted in FACS-enriched CSCs compared to unsorted samples. Overexpression of miR-215 in poorly-differentiated, highly clonogenic cell lines caused growth arrest and a dramatic decrease in colony formation. Conversely, miR-215 knockdown using a miRNA sponge increases clonogenicity and impaired differentiation in CDX1-high cell lines. Identification of genome-wide miR-215 targets indicated that miR-215 induces terminal differentiation associated growth arrest, due in part to direct silencing of BMI1 expression and de-repression of BMI1 target genes including CDKN1A. Our novel findings situate miR-215 as a link between CDX1 expression and BMI1 repression that governs differentiation in colorectal cancer.

NCI-CCR
Na-Young Song
Visiting Fellow
Carcinogenesis
Role of IKKalpha in Kras-driven lung carcinogenesis
IKKalpha (IKKa), one of subunits of IKK complex, is required for the epidermal homeostasis. We previously demonstrated that keratinocyte-specific deletion of IKKalpha induces spontaneous skin squamous cell carcinomas (SCCs) in mice. Interestingly, IKKa kinase dead knockin (IkkaK44A/K44A, KA) mice spontaneously develop lung SCCs which is associated with increased inflammation and IKKa reduction. Thus, IKKa reduction is crucial for SCC formation in skin and lung. Here, we attempted to verify whether IKKalpha inactivation plays a role in lung adenocarcinomas (ADCs) as well. We have crossed between KA mice and KrasG12D oncogenic mutant (Kras) mice. Then, these IkkaK44A/K44A;KrasG12D (KA;Kras) mice were intratracheally treated with adenovirus carrying the cre

recombinase to activate Kras. At 4.5 months after injection, the lung weight was significantly increased in KA;Kras compared to Kras mice. Moreover, the lungs of KA;Kras mice increased bronchial epithelial cells and Ki67-positive cells. At 7 months after treatment, lung tumors in KA;Kras mice were larger compared to tumors in Kras mice. To confirm whether IKKa reduction promotes Kras-driven lung carcinogenesis in a cell autonomous model, we crossed Ikkaf/f (FF) mice with Kras mice to generate FF;Kras mice. Similar to KA;Kras, FF;Kras mice showed significant increase of lung weight, hyperproliferative bronchial abnormalities, Ki67-positive cells in bronchial region and larger tumor numbers at 4 months following treatment compared with Kras only group. Finally, FF;Kras mice had shorter life span compared to Kras mice. It is well known that the activation of oncogenes such as Kras can induce a senescence program in tumors. We found that tumors derived from FF;Kras mice were Ki67-positve but senescence-associated beta-gal (SA-b-gal) negative, while Kras mice showed less Ki67-positive but strongly SA-b-gal-positive in the tumors. Moreover, the lung tumors from FF;Kras mice showed reduced expression of p53 and p21 but increased expression of cyclin D1 and E, compared to tumors derived from Kras mice. Taken together, these data suggest that IKKa loss promotes Kras-driven lung tumorigenesis via suppression of Kras-induced senescence and plays an important role in lung ADCs as well as in SCCs. Interestingly, the lung tissues of FF;Kras mice showed profound downregulation of antioxidant enzymes. The underlying molecular mechanism will be further investigated, focusing on the role of antioxidants and inflammation.

NCI-CCR
Nailing Zhang
Postdoctoral Fellow
Carcinogenesis
Exploration of basic and therapeutic mechanisms in novel models of RTK/RAS-network-driven glioblastoma
Glioblastoma (GBM) remains fatal despite intensive treatments including standard-of-care surgery, radiation, and chemotherapy. Modern targeted therapies have also failed to produce effective results in numerous clinical trials, possibly due to a lack of relevant preclinical models for drug discovery and assessment. Approximately 90% of GBMs harbor aberrations in the RTK/RAS signaling, including the following frequent events: EGFR amplification and gain-of-function mutations (57%), PDGFRalpha amplification (10%), and NF1 deletion and loss-of-function mutations (10%). K-RAS mutations also occur, but at low frequency (1%). Frequent alteration of the RTK/RAS signaling in human GBMs emphasizes a great need for RTK/RAS-network-driven preclinical tumor models. We have recently generated a genetically engineered mouse model of GBM via adult-inducible astrocyte-specific perturbation of Rb, Kras and Pten. These Rb/Kras/Pten triple mutant mice develop GBMs that faithfully recapitulate the histopathology and molecular characteristics of the most aggressive form of the human disease. Interestingly, without Ras activation, only low grade disease is induced. We established newborn astrocytes from the triple mutant mouse model [defined as Rb/Kras/Pten triple astrocytes (TAs)], as well as the Rb and Pten double mutant mice [Rb/Pten double astrocytes (DAs)]. The Rb/Kras/Pten TAs lead to high-grade brain tumors within 3 months upon orthotopic transplantation into syngeneic host mice; in contrast, the Rb/Pten DAs did not generate tumors within 6 months. This result confirms that Kras activation is essential for high-grade disease and provides an ideal platform for evaluation of multiple RAS-activating events in causation of GBM. We are currently introducing the most frequent events found in human GBMs, including EGFR and PDGFRalpha activation and NF1 deficiency, into DAs in vitro. We will assess the ability of each event to convert the Rb/Pten non-GBM-forming DAs into GBM-forming astrocytes by orthotopic introduction into syngeneic mice. This novel in vitro-to-allograft approach we have established provides direct comparisons of etiologies driven by various RAS-activating events in the same genetic background and will potentially lead to basic discovery of distinct mechanisms in GBM

tumorigenesis. In addition, this approach will generate relevant panels of RTK/RAS-network-driven GBM models for robust preclinical therapeutic discovery and biomarker development.

NCI-CCR
Fatima Ali-Rahmani
Visiting Fellow
Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology
Inhibition of collagen receptor discoidin domain receptor-1 (DDR1) tyrosine kinase enhances cytotoxicity of anti-mesothelin immunotoxin for cancer therapy
Discoidin domain receptor 1 (DDR1) is an emerging anti-cancer target that belongs to the family of tyrosine kinases that are activated by collagen, the most abundant component of extracellular matrix (ECM). Collagen mediated induction of DDR1 facilitates cell adhesion, migration, proliferation and matrix remodeling. The collagen/DDR1 axis is also thought to modulate tumor-stromal interaction and potentially can affect tumor response to therapy. Mesothelin is a cell-surface tumor-associated antigen over-expressed in several human cancers including mesothelioma, ovarian, lung, breast, and pancreatic cancers with limited expression on normal cells. RG7787 is a clinically optimized recombinant immunotoxin (RIT) being developed in collaboration with Roche that should enter clinical trials late this year. It consists of a humanized anti-mesothelin Fab fused to domain III of Pseudomonas exotoxin A in which immunogenic B cell epitopes are silenced. Activation of tyrosine kinase mediated signaling in tumor cells has been shown to modulate the activity of RITs. Therefore, we hypothesized that DDR1 regulates RIT activity and its inhibition might enhance the activity of RG7787. Knockdown of DDR1 by siRNA or its inhibition with a novel and specific inhibitor, '7rh', synergistically enhanced the cytotoxic activity of RG7787 in several cancer cell lines. Investigation into the mechanism of action showed the inhibitor acts at an early step in immunotoxin action; it enhances inhibition of protein synthesis in cells treated with combination of '7rh' and RG7787. Furthermore, stimulation of DDR1 activity by collagen treatment protected cancer cells from killing by RG7787. However, this collagen mediated resistance of cancer cells to RIT therapy can be overcome by addition of 7rh. In conclusion, we report that collagen protects cells from killing by RITs through DDR1 and that lowering DDR1 protein or inhibiting DDR1 kinase activity enhances the cytotoxic activity of RITs. Our data suggest that the combination of '7rh' and RG7787 represents a novel therapeutic strategy to target mesothelin-expressing cancers. These data also provide insight into how tumor stroma might be protecting cancer cells from therapy.

NCI-CCR
Rozenn Josse
Postdoctoral Fellow
Cell Cycle-General, Regulators and Checkpoints, Apoptotic Mechanisms
Use of ATR inhibitor in combination with topoisomerase I inhibitor kills cancer cells by disabling DNA replication initiation and fork elongation
Camptothecin and its derivatives, topotecan and irinotecan are specific topoisomerase I inhibitors and potent anticancer drugs. These agents produce well-characterized double-strand breaks upon collision of replication forks with topoisomerase I cleavage complexes. In an attempt to develop novel drugs combinations, we conducted a synthetic lethal siRNA screening using a library that targets nearly 7000 human genes. Depletion of ATR, the main transducer of replication stress-induced DNA damage response came at the top candidate gene with synthetic lethality with camptothecin. Validation studies showed that ATR siRNA exacerbated cytotoxic response to both camptothecin and the indenoisoquinoline LMP-400 (indotecan), a novel topoisomerase inhibitor in clinical trial. Inhibition of ATR by the recently developed specific inhibitor VE-821 induced synergistic antiproliferative activity when combined with either topoisomerase inhibitor. Cytotoxicity induced by the combination with LMP-

400 was greater than with camptothecin. Using single cell analysis and DNA fiber spread, we show that VE-821 abrogates the S-phase checkpoint, and restores origin firing and replication fork progression in cells treated with camptothecin or LMP-400. Moreover, the combination of topoisomerase inhibitors with VE-821 inhibited the phosphorylation of ATR and ATR-mediated Chk1 phosphorylation, while strongly inducing gamma-H2AX. Single cell analysis revealed that the gamma-H2AX pattern changed over time from well-defined focus to a pan-nuclear staining. The change in gamma-H2AX pattern can be useful as a predictive biomarker to evaluate the efficacy of therapy. The key implication of our work is the mechanistic rationale it provides to evaluate the combination of topoisomerase I inhibitors with ATR inhibitors.

NCI-CCR
Lei Sun
Postdoctoral Fellow
Cell Cycle-General, Regulators and Checkpoints, Apoptotic Mechanisms
Id2 is required for the self-renewal and proliferation of hematopoietic stem cells
The production of mammalian blood cells is sustained throughout life by self-renewal and differentiation of hematopoietic stem cells (HSCs). Dysregulation in this system leads to different pathologies including anemia, bone marrow failure and hematopoietic malignancies. The Helix-loop-helix transcriptional regulator Id2 plays essential roles in regulating proliferation and cell fate of hematopoietic progenitors; however, its role in regulating HSC development remains largely unknown. To assess the function of Id2 in HSCs, we developed two mouse models, including Id2 conditional knockout model and Id2-EYFP model, in which EYFP expression is driven by endogenous Id2 promoter. When we examined HSC function by serial transplantation, we found that mice transplanted with Id2-/- bone marrow cells died after two serial transplantations, while mice transplanted with Id2+/+ bone marrow survived 4 serial transplantations. This indicates that HSC self-renewal is impaired when Id2 is deleted. To further determine if self-renewal and maintenance of HSCs depends on the expression level of Id2, we purified HSCs with different levels of Id2 expression in the EYFP model to specifically address the role of Id2 in HSCs. First, we confirmed Id2 is highly expressed in HSCs in this model. Second, when HSCs with either low or high levels of Id2-EYFP were transplanted into irradiated mice, cells with high levels of Id2 reconstituted transplanted recipients faster than those with low levels of Id2, suggesting that Id2 expression is associated with repopulation advantage. Furthermore, ki67 staining showed that HSCs with high levels of Id2 have 15-fold more cells in G2/M phase, and fewer cells in G0, indicating that Id2 expression correlates with cell cycle progression in HSCs. In addition, the quiescence-associated gene p57 is downregulated in HSCs with high levels of Id2, and p57 is correspondingly upregulated in Id2-null HSCs. Altogether, our data demonstrate that Id2 is required for the self-renewal and proliferation of HSCs, and suggest a link between Id2 and the transcriptional regulatory networks that regulate functional hematopoietic system. Since Id2 is also expressed in other adult stem cells including muscle and neuronal stem cells, as well as cancer cells, we believe our results can improve our understanding of stem cell biology and cancer development, and result in the identification of novel molecules that can be potentially targeted to eliminate cancer stem cells.

NCI-CCR
Eswary Thirthagiri
Research Fellow
Cell Cycle-General, Regulators and Checkpoints, Apoptotic Mechanisms
BRCA2?105 (deletion of exons 4-7) splice variant of the Fanconi Anemia (FA) related BRCA2 IVS7+2T>G mutation â€“ effect on bone marrow compartment of BRCA2?105 knock-in mouse, and cell cycle response to IR induced DNA damage in vitro.

BRCA2 is indispensable for viability in mice, however, individuals with deleterious biallelic mutations are born, but succumb to hematopoietic related disorders within the first 5 years of life. The BRCA2 IVS7+2T>G mutation is found in homozygous state in FA patients. The mutation causes skipping of exon 7, protein truncation, and generates a spliced transcript variant lacking exons 4â€“7, which encodes an in-frame BRCA2 protein with a deletion of 105 amino acids (?105). The exons 4â€“7 are highly conserved, and have multiple PLK1 and Cdk1 phosphorylation sites. The BRCA2?105 protein is DNA repair proficient in vitro, but confers reduced viability in mouse embryonic stem (mES) cells. Notably, FA patients with BRCA2 mutation exhibit severe clinical symptoms; downregulated BRCA2?105 transcript was associated with disease progression in a mutation carrier. In this study, we generated both Brca2?105/?105 and Brca2?105/null knock-in mice and used mES cells carrying human BRCA2?105 to determine if other functions of BRCA2 may be abrogated in BRCA2?105, and how that may contribute to development of FA. Both Brca2?105/?105 and Brca2?105/null mice are viable, fertile and do not show any developmental defects. We found no difference in viability between wt, ?105/?105 and ?105/null bone marrow mononuclear cells (MNCs) in the absence of stress. However, after IR (6Gy), while wt and ?105/?105 MNCs showed similar growth patterns, there was an 8 fold reduction in viability of ?105/null MNCs. Furthermore, BRCA2?105 mES clones exhibited abrogation of the G2/M checkpoint in response to irradiation induced DNA damage (6Gy); ~27% phospho-H3, a mitosis marker, positive cells was seen in two BRCA2?105 mutant at G2/M compared to wt (2.82%). A 3-fold reduction in phospho-H3 positive cells was seen in mutants (~9%) following IR+PLK1 inhibitor treatment. Chromosomal analysis of irradiated (2Gy) mutants showed about 2-3-fold increase in fragments, gaps, breaks and dicentric chromosomes compared to wt. Based on these preliminary data, we hypothesize that exons 4-7 of the BRCA2 gene is dispensable for viability. The preservation of DNA repair function in BRCA2?105 may support survival of cells, however, the inability to maintain G2/M arrest in response to DNA damage, which may be due to early activation of PLK1, may lead to production of genomically unstable clones that may contribute to disease progression. This phenotype may be exacerbated in cells with reduced BRCA2?105 levels.

NCI-CCR
Ya Zhang
Postdoctoral Fellow
Cell Cycle-General, Regulators and Checkpoints, Apoptotic Mechanisms
A Replicator-Specific Binding Protein Essential For Initiation of DNA Replication in Mammalian Cells
Mammalian chromosome replication starts from distinct sites called replication origins. The principles governing origin selection are yet unclear because proteins essential for DNA replication do not exhibit sequence specific DNA binding. To better understand how replication origins are selected, we sought to identify and characterize proteins that bind an essential region of the replication origin at the human beta globin locus. Using mass spectrometry, we identified a replication initiation determinant (Rep-ID, also called DCAF14/PHIP) protein that formed a sequence specific complex with the origin. Rep-ID is a member of the DCAF family that interacts with the DDB1 and Cul4B members of the ubiquitin ligase complexes and their substrate, CDT1, via its WD40 domains. Mutations that eliminated Rep-ID binding to the replication origin disabled replication initiation, suggesting that Rep-ID binding is required for initiation of DNA replication. Chromatin-immunoprecipitation followed by sequencing (ChIP-Seq) indicated that Rep-ID binding sites frequently colocalized with a group of replication origins genome-wide. Rep-ID protein levels are cell cycle regulated: levels decreased during S phase, began to rise during G2/M, and peaked at G1. In concordance, Rep-ID interacted with beta globin origin during the G1- and early S-phase of the cell cycle in human myeloid leukemia cells. Rep-ID deficiency resulted in slow cell proliferation, reduced frequency of replication initiation events, increased replication fork stalling and accumulation of CDT1 on chromatin. In addition, chromosome conformation capture combined with

chromatin immunoprecipitation (ChIP-3C) data showed that Rep-ID participated in an essential distal interaction between the replication origin and the locus control region at early replicating beta-like globin loci. Taken together, our observations suggest that Rep-ID may play multiple roles during the DNA replication process, functioning at specific categories of replication origins.

NCI-CCR
Alexander Gorka
Postdoctoral Fellow
Chemistry
Discovery of a Near-IR Photorelease Technique for Targeted Drug Delivery
The use of tissue penetrant near-IR light to interrogate and modulate biological processes is at the forefront of modern cancer diagnosis and treatment. Emerging strategies, such as targeted fluorescence probes to guide tumor resection and antibody-targeted photoimmunotherapy (PIT) techniques highlight this recent progress. A longstanding, but as yet unrealized, goal is the use of near-IR light for targeted drug delivery. Such approaches center on â€œphotocages", or molecular scaffolds that silence drug activity prior to selective release by light-dependent chemical reactions. However, the existing repertoire only functions with high energy UV or blue light, which is unsuitable for in vivo applications. My research centers on the development and application of near-IR photocages for targeted drug delivery. We have developed and characterized a reaction that releases secondary amine- and phenol-containing small molecules utilizing near-IR light-initiated photochemical reactivity of Cy7 fluorophores. The basis of this approach is chemical reactivity previously only associated with fluorophore photochemical decomposition, or â€œphotobleachingâ€. The sequence readily occurs under physiologic conditions and over a wide concentration range. These photocages elicit light-dependent in vitro growth inhibition of tumor cells, including tamoxifen- and auristatin-sensitive lines, at similar potencies to the respective native drug. Such an effect is not seen for the same Cy7s conjugated to biologically inactive small molecules, indicating that the observed inhibition is solely a consequence of release. Our laboratory has also conjugated Cy7 to cyclofen, a potent tamoxifen analog. This compound is capable of regulating recombination-dependent beta-galactosidase expression in Cre-ER mouse embryonic fibroblasts (MEFs) in a light dependent manner. Together, these results demonstrate effective â€œcagingâ€ of both general antiproliferative activity and specific biochemical function, which are fully restored utilizing our near-IR light-initiated release platform. Modification with targeting moieties will enable enhanced tumor specificity and cellular localization. Such optimization, as well as expanding this near-IR photorelease methodology to other targeted drug delivery applications are ongoing.

NCI-CCR
Roger Nani
Postdoctoral Fellow
Chemistry
A Near-IR Ratiometric Fluorescence Sensor of Protease Activity
Fluorescence ratiometric imaging measures the ratio of two fluorescence signals that depend on biological stimuli. This approach is superior to â€œturn-onâ€ fluorescence because it corrects for uneven fluorophore distribution, fluorophore leakage and photobleaching. The measurement of tumor-specific protease activity is an emerging strategy in cancer diagnosis and of significant interest in basic research. However, few probes are available for protease sensing, and existing systems rely on UV or visible light, which limits the application to in vitro experiments due to poor tissue penetration and background autofluorescence. This study aims to develop new small molecule platforms for ratiometric sensing of protease activity using near infrared (NIR) light. NIR light is better suited for many in vivo

applications due to reduced absorption by endogenous biomolecules. We have developed a novel transformation of heptamethine cyanine (Cy7) fluorophores that is ideal for this purpose. Various oxygenâ€“linked Cy7 fluorophores were been synthesized bearing a pendant peptide sequence attached to the Cy7 backbone via a linker. Cleavage of the C-terminal amide bond initiates a rapid N-to-O rearrangement of the linker that results in the nitrogen atom being attached to the Cy7 backbone. The optical properties of the newly formed nitrogenâ€“linked Cy7 fluorophore are dramatically altered â€“ a change in absorption maxima of over 100 nm is observed. These molecules are readily synthesized, and we are creating a small library of molecules based on protease-specific sequences to broadly evaluate these fluorophores as protease substrates. Ongoing efforts include kinetic analyses of the chemical rearrangement process and fluorescence shift assay experiments of our Cy7-peptide conjugates in the presence of key cancer-specific proteases. Future studies will include activity based imaging of these Cy7 fluorophores for detection of proteolytic activity in in vitro and in vivo contexts. This approach relies on a new mechanism to alter the optical properties of Cy7 fluorophores that will offer significant benefits over existing platforms. We anticipate these tools will have utility for both cancer diagnosis and in basic research to study localization and relative activity of overexpressed proteases in a rapid and high-throughput fashion.

NCI-CCR
Rebecca Burgess
Postdoctoral Fellow
Chromatin and Chromosomes
Chromatin condensation is an integral part in the activation of the DNA damage response
Sensing and signaling of DNA damage occurs in the context of higher order chromatin structure and architectural features of chromatin contribute to genome surveillance, DNA damage signaling and repair. While the role of chromatin decondensation in the DNA damage response (DDR) is well established, we have found that chromatin condensation is an integral part of DDR signaling in human cells. Direct tracking of the dynamics of a photoactivated chromatin region revealed that upon DNA damage, chromatin first decondenses before undergoing re-condensation. These chromatin changes can be dampened by overexpression of the Set1/Ash2 methyltransferase, which contributes to chromatin expansion during transcriptional activation. To directly assess the effects of local chromatin changes on DDR signaling, we used a protein-chromatin tethering system to create defined local chromatin domains. We found that interfering with the ability to locally condense chromatin by tethering Ash2 leads to a failure to fully activate the DDR after DNA damage, reduced phospho-H2AX and decreased recruitment of 53BP1. Conversely, forced induction of local chromatin condensation promotes ATM- and ATR-dependent local phosphorylation of H2AX, and recruitment of upstream DDR proteins, but does not activate typical downstream effector kinases or cell cycle checkpoints. Upstream DDR signaling in the absence of DNA damage is also observed in prematurely condensed chromosomes and during mitotic chromosome condensation. Our results demonstrate that chromatin condensation is sufficient to activate upstream components of the DDR and contributes to the physiological DDR signaling cascade.

NCI-CCR
Koichi Utani
Postdoctoral Fellow
Chromatin and Chromosomes
Identifying DNA replication origins and binding proteins before start replication.
Genomic stability depends on accurate, sequential activation of replication origins in a strict order during the cell cycle. In metazoan cells, initiation of DNA replication at distinct sites called replication

origins requires DNA-protein interactions at the G1 phase of the cell cycle prior to replication. A 6-protein anchor termed the Origin Recognition Complex (ORC) is essential for initiation of DNA replication, but ORC proteins bind chromatin in a non-specific manner, making it difficult to identify replication origins and challenging to understand how non-specific binding translates into site-specific replication initiation. In addition, it is yet unclear if cells consistently start replication from the same replication origins on each chromosome each cell cycle. To address these issues, I developed a new technique to identify replication origins on a whole genome scale and to identify proteins that bind replication origins. To identify replication origins, I used sequential labeling with two thymidine analogs (IdU and CldU) and detected the labeling patterns on single DNA fibers. The locations of replication origins can be detected by distinct patterns of CldU and IdU labeing. Next, I asked whether the same origins are utilized during two sequential cell cycles. To that end I probed replication origins detected using single fiber CldU/IdU labeling in one cell cycle with Fluorescent In Situ Hybridization (FISH) with newly replicated nasent DNA strands prepared in a previous cell cycle. The results suggested a 50% complete overlap between the live IdU/CldU and FISH labels, suggesting that the same origins were used in the two cell cycles. It may suggest that only about half the replication origins were activated consistently in each cell cycle, or that replication origins vary on allelic regions. To distingish between these two possibilities, I studied genome-wide replication patterns in several cell lines on X chromosomes by interrogating nascent strands sequencing data. As predicted, my preliminary data suggest that female cells had more numerous origins on X chromosomes than male cells. If confirmed, these data suggest that each chromosome establishes and re-uses a distinct pool of replication origins. With this method, we enriched for known replication origins and we now combine this method with mass spectrometry to identify and characterize origin binding proteins.

NCI-CCR
Shaofei Zhang
Postdoctoral Fellow
Chromatin and Chromosomes
Functionally redundant and compensatory binding of HMGN variants to nucleosomes affect DNaseI hypersensitivity of chromatin and gene expression in mouse B lymphocytes
The dynamic features of chromatin allow cells to alter gene expression in response to various environmental or endogenous cues. The chromatin structure is regulated by dynamic interactions between DNA elements and numerous chromatin-associated factors. A key challenge in the field of gene regulation is to characterize these factors. Here we report that HMGN proteins affect the DNaseI hypersensitivity patterns of chromatin and modulate the fidelity of transcriptional profile in mouse B lymphocytes. HMGN is a family of ubiquitous proteins that bind dynamically to chromatin. B lymphocytes contain two major variants named HMGN1 and HMGN2 and a minor variant named HMGN3. Our ChIP-seq results revealed that all the HMGN variants are strongly enriched at CpG island containing promoters of transcriptionally active genes. At silenced genes, HMGN variants mark the â€˜poisedâ€™ genes that are activated in response to specific stimuli. Interestingly, despite of the strong overlapping between HMGN binding sites and DNaseI hypersensitivity sites, no significant changes in the DNaseI hypersensitivity or gene expression were detected in Hmgn1-/-/Hmgn3-/- double knockout B cells. However, simultaneous loss of HMGN1 and HMGN3 induced a dramatic increase in the binding of HMGN2 at the promoter regions, suggesting that HMGN2 may functionally compensate for loss of HMGN1 and HMGN3. To further validate the compensatory function of HMGN2, we examined the transcriptomic profiles in Hmgn1-/-/Hmgn2-/- double knockout cells using RNA-seq. Indeed, we found that simultaneous loss of HMGN1 and HMGN2 induced significant changes in the expression of 389 genes. Strikingly, analysis of DNase-seq data revealed that ~ 40% DNaseI hypersensitivity sites were lost in Hmgn1-/-/Hmgn2-/- double knockout cells as compared with wild type controls. This study

demonstrated a role for HMGN proteins in maintaining DNaseI hypersensitivity of chromatin and in modulating gene-regulatory networks. Given the ubiquitous presence of HMGN1 and HMGN2 in all vertebrate cells, it is likely that similar compensatory mechanisms are widely spread.

NCI-CCR
Nataliya Buxbaum
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Clinical and Translational Research
In Vivo T Regulatory Cell Kinetics Are Altered in a Pre-Clinical Model of Chronic Graft-Versus-Host Disease
Chronic graft-versus-host disease (cGVHD) is the main cause of late morbidity and non-relapse mortality after allogeneic hematopoietic stem cell transplantation (AHSCT). T cells are known modulators of cGVHD, while their in vivo kinetics, as defined by cell division, cell death and trafficking, remain largely uncharacterized, and current therapies for cGVHD target T cell proliferation. T regulatory cells (T regs) are of particular interest in cGVHD as adoptive transfer or in vivo expansion of this cell type has resulted in amelioration of cGVHD severity in pre-clinical models and in patients with cGVHD. We have developed a method to measure T reg and non-T reg cell kinetics using deuterated water labeling/de-labeling combined with triple quadrupole GC/MS detection of deuteurium enrichment in DNA of dividing/dying cells. Using a minor antigen mismatch murine model of cGVHD we show that in AHSCT recipients, donor CD4+ non-T regs in the spleen have a proliferative advantage over T regs, while in the syngeneic (genetically matched donor-recipient pairs) control animals cell gain rates for these populations are similar. The net result of the differential cell kinetics in the cohort with cGVHD was fewer T regs in the spleens of allogeneic versus syngeneic marrow recipients, with a suggestion for decreased trafficking out of the spleen. Evaluation of T reg and non-T reg CD4+ T cells within lymphoid compartments (lymph nodes and peripheral blood) and target organs (liver, intestines and skin) allowed assessment of T cell trafficking. Preliminary results show distinct organ-specific patterns of T cell kinetics after AHSCT. These data allow mathematical modeling of T cell behavior post AHSCT in a pre-clinical model of cGVHD, enabling interpretation of in vivo T cell kinetics in peripheral blood of patients undergoing AHSCT with and without cGVHD.

NCI-CCR
Michael Manning
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Clinical and Translational Research
Bortezomib reduces preexisting antibodies to recombinant immunotoxins in mice
Recombinant immunotoxin (RIT) therapy is limited in patients by neutralizing antibody responses. Most patients with normal immune systems make neutralizing antibodies after 1 cycle (3 doses) of RIT, preventing repeated dosing. Furthermore, some patients have preexisting antibodies from environmental exposure to Pseudomonas exotoxin, the component of the RIT that elicits the neutralizing antibody response. Bortezomib is an FDA-approved proteasome inhibitor which selectively targets and kills plasma cells which are necessary for a neutralizing antibody response. Bortezomib, in combination with methotrexate, rituximab, and cyclophosphamide, was shown to reduce antibody levels >64-fold following enzyme replacement therapy in the clinical setting of Pompe disease. We hypothesized bortezomib may abrogate the neutralizing antibody levels, making dosing of RIT possible in mice already immune to RIT. We immunized BALB/c mice with multiple doses of SS1P, a RIT whose antibody portion targets mesothelin. Antibody titers were determined by immunocapture ELISA. Mice with elevated levels were separated into groups to receive bortezomib (1mg/kg i.v., twice a week for 5.5 weeks) or saline. Bortezomib treatment lowered the initial antibody titer in mice previously immunized with RIT by an average of 57%, while antibody titer in control mice decreased by an average of 13%.

Despite success in lowering titers, bortezomib did not abrogate titer in any mice. We hypothesized a population of memory cells, not susceptible to bortezomib inhibition, was the source of remaining antibody. To further diminish preexisting antibodies, we performed a separate experiment in which mice with preexisting anti-SS1P antibodies received bortezomib with concomitant pentostatin and cyclophosphamide (P/C) therapy. P/C is an immune-depleting regimen already shown to be effective in preventing the onset of new neutralizing antibodies. Combination treatment lowered the initial antibody titer in mice previously immunized with RIT by an average of 88%, while antibody titers increased 15% in control mice. In addition we observed significantly fewer plasma cells in the bone marrow of combination treated mice. We have shown that bortezomib reduces, but does not eliminate, antibody titer in mice with preexisting antibodies to RIT. Addition of the immune-depleting P/C regimen further diminishes antibody titers. We are investigating the source of the remaining antibody following combination therapy.

NCI-CCR
Sanja Stevanovic
Visiting Fellow
Clinical and Translational Research
Complete tumor regression of metastatic human papilloma virus (HPV)-induced cervical carcinoma following adoptive transfer of autologous tumor-infiltrating lymphocytes
Cervical carcinoma is a common epithelial cancer caused by the infection with a high risk human papillomavirus (HPV), accounting for an estimated 266,000 yearly deaths worldwide of which 7,000 in the Unites States. Metastatic disease has a dismal prognosis with current chemotherapies, warranting exploration of novel treatment modalities. Adoptive T-cell therapies have emerged as a promising treatment for patients with advanced malignancies. In cervical carcinoma, HPV oncoproteins E6 and E7 are not only required for initiation and maintenance of the malignancy, but are also constitutively and selectively expressed by malignant cells, making them attractive targets for T-cell therapies. We evaluated whether adoptive transfer of autologous tumor-infiltrating lymphocytes (TIL) selected for HPV E6 and E7 antigen reactivity could be an effective treatment in metastatic cervical carcinoma. Nine patients with metastatic cervical carcinoma (2 HPV16+ and 7 HPV18+) were treated with TIL therapy in an ongoing phase II clinical trial. Following establishment of multiple TIL cultures from metastasis-derived fragments, TIL cultures were selected for expansion and administration to patients based on reactivity against autologous dendritic cells (DC) loaded with HPV-type specific E6 and E7 peptide pools, T-cell growth rate, and CD8+ T-cell frequency. Patients received a single TIL infusion (86+/-37*109 cells; average+/-SD) and 720,000 IU/kg interleukin-2 to tolerance after a non-myeloablative conditioning regimen. The treatment regimen led to objective clinical responses in 3/9 patients including two complete responses, one a HPV16+ squamous cell carcinoma and the other a HPV18+ adenocarcinoma, that are ongoing at 18 and 11 months post-treatment, respectively. Their infused TIL contained at least 20 and 5% HPV reactive T cells, respectively, as determined by IFN-? ELISPOT and expression of activation marker CD137 upon co-culture with E6 and E7 peptide pool loaded DC. No HPV-reactivity was detectable in peripheral blood pre-treatment. In contrast, HPV-reactive T cells were present at one month post-treatment, and remained detectable throughout ongoing remission. Analysis of other patients is currently ongoing. These observations indicate that TIL therapy can lead to complete regression of progressive metastatic cervical carcinoma, and warrant further study of TIL therapy as a new therapeutic modality for patients with HPV-associated carcinomas.

NCI-CCR
Michael Hall
Postdoctoral Fellow

Developmental Biology

Stat3 signaling modulates the osteochondro transcription factor Sox9 in vivo to influence endochondral ossification and is important in the pathology of campomelic dysplasia

Campomelic dysplasia (CD) is an often-fatal human congenital abnormality characterized by a hallmark bowing of the long bones and features such as short stature, cleft palate, and laryngotracheomalacia. The pathology of CD is frequently underpinned by mutations in and around the coding region of the Sry-box gene Sox9, a master regulator of multipotent osteochondro progenitor cells. Beyond this, little is known about the normal regulation of Sox9 or, in cases where Sox9 mutations have not been detected, if CD is also caused by alterations in the control of Sox9 expression. To elucidate novel regulators of Sox9, we analyzed its proximal promoter region and discovered several potential Stat DNA response elements (DRE). Stat3 has been implicated in stem cell maintenance, and disruption of Stat3 signaling through deletion of leukemia inhibitory factor (LIF) receptor causes a defect in bone mineralization, suggesting the possible involvement of Sox9. Indeed, we found that a Sox9 promoter-driven reporter is activated in cells by oncostatin M in a Stat3-dependent fashion. Further, reporter activation is mediated by the Stat DREs, and Stat3 physically binds the promoter of Sox9. In the developing mouse, Stat3 is expressed in somites and early limb buds, mesodermal compartments where Sox9 mediates endochondral ossification of vertebrae and limbs, respectively. We engineered mesodermal Stat3 loss of function mutant mice, which, as neonates, yielded a phenotype consistent with CD, i.e., limb bowing, labored breathing, and mortality. The histology of Stat3 mutants was also consistent with Sox9 heterozygous mutants, including shortening of the long bones, and expansion of the hypertrophic chondrocyte zone, suggesting modulation of Sox9 function. Furthermore, Sox9 levels were decreased in Stat3 mutant mice, as was the direct target Col2a1, a marker for a functional decrease in Sox9 in vivo. Other skeletal features such as reduced trabecular mineralization, regional restriction and death of osteoblasts, and elevated osteoclast activity were also observed, indicating a critical role for Stat3 in skeletogenesis. We posit that Stat3 instructs bone development by fine-tuning Sox9 expression in mesenchymal stem cells to drive the differentiation of osteochondro progenitors. These findings demonstrate for the first time a role for Stat signaling in regulating SRY-box factors in vivo and provide a potential alternative mechanism for CD where Sox9 mutations are not detected.

NCI-CCR

Xia Ding

Visiting Fellow

DNA-binding Proteins/Receptors and DNA Repair

PARP1 is a novel genetic interactor of BRCA2

Pathogenic mutation of BRCA2 is one of the highest risk factors of developing breast and ovarian cancer. Because BRCA2 loss causes genomic instability, which results in apoptosis or cell cycle arrest in normal cells, mutation in other genetic interactors is required for tumor development. One such interactor is p53, which is mutated in half of all BRCA2-asociated tumors. The current study aims to identify novel BRCA2 genetic interactors by using a siRNA library screen in mouse embryonic stem cells (mESC). BRCA2 loss induces mESC lethality and the screen identifies genes rescuing the lethality. We utilize mESC genetically engineered to have endogenous Brca2 deleted and carry bacterial artificial chromosome (BAC) expressing a hypomorphic mutant BRCA2. These cells are viable but are hypersensitive to poly(ADP-ribose) polymerase (PARP) inhibitor (PARPi). The screen revealed that after knocking down certain genes, the cells gained resistance to PARPi, suggesting these genes can compensate for BRCA2 loss and may be potential BRCA2 genetic interactors. Surprisingly, PARP1 itself was identified as one of the top candidates. We validated that stable knockdown (KD) of PARP1 expression rescued BRCA2-null mESC lethality. This is surprising because chemical inhibition of PARP1 specifically kills BRCA2-deficient cells and PARPi is being used to treat BRCA2-deficient tumors. BRCA2-null mESC rescued by PARP1 KD

are deficient in homologous recombination (HR) and exhibit extensive genomic instability. We found the rescued cells have increased non-homologous end joining (NHEJ) as reflected by increased 53BP1 foci number, suggesting increased NHEJ compensated for HR loss and allowed cell survival without BRCA2. This further implied that BRCA2 deficient cells rely on NHEJ for survival when PARP1 activity is suppressed. Based on this hypothesis, we treated BRCA2-deficient mouse primary breast tumor cells as well as its wild-type BRCA2 BAC reconstituted counterpart with AZD2281 (PARPi) in combination with DNA-PK inhibitor NU7441 (DNA-PK is a major kinase mediating NHEJ). We found the combination significantly exacerbated killing of only the BRCA2-deficient tumor cells, not its BRCA2 wild-type counterpart. We found AZD2281 induced more 53BP1 foci in BRCA2-deficient tumor cells than in the latter. In summary, the current study focuses on establishing PARP1 as a novel BRCA2 genetic interactor and revealing its relevance in understanding and treating BRCA2-associated tumors.

NCI-CCR
Naoki Tanaka
Postdoctoral Fellow
Endocrinology
Adipocyte-specific disruption of fat-specific protein 27 protects from high-fat diet-induced obesity but aggravates fatty liver and insulin resistance
Fat-specific protein 27 (FSP27, CIDEC in humans) is a lipid-coating protein that is highly expressed in white adipose tissue (WAT) and contributes to lipid droplet formation. WAT of obese mice shows marked up-regulation of FSP27 that is closely associated with adipocyte hypertrophy, and silencing the gene encoding FSP27 in cultured adipocytes leads to formation of multiple small lipid droplets. However, the role of FSP27 in adipocytes in vivo remains unclear. Mice with adipocyte-specific disruption of the Fsp27 gene (Fsp27delta(adipo)), generated using an aP2-Cre transgene with the Cre/LoxP system, were compared with Fsp27-floxed mice (Fsp27F/F). Upon high-fat diet (HFD), Fsp27delta(adipo) mice showed resistance to body weight gain and reduced adiposity. The WAT was atrophic, had multilocular small lipid droplets, and demonstrated enhancement of mRNA levels encoding the genes associated with lipolysis, mitochondrial beta-oxidation, and brown adipocyte properties. Actual lipolysis activities were significantly enhanced in adipocytes isolated from HFD-treated Fsp27delta(adipo) mice compared with those from Fsp27F/F mice. These results indicated alterations in adipocyte properties and disruption of WAT function as a fat/energy reservoir in Fsp27delta(adipo) mice. Additionally, marked hepatic steatosis, hypertriglyceridemia, and reduced insulin sensitivity were found in these mice, which is likely a consequence of compensatory ectopic fat accumulation due to impaired fat storage in WAT. Collectively, adipocyte-specific disruption of Fsp27 in mice protected from obesity induced by HFD but aggravated hepatic steatosis and insulin resistance. Lipodystrophy in humans is characterized by lean phenotype and severe hepatosteatosis/steatohepatitis, diabetes, dyslipidemia, and atherosclerosis, and CIDEC mutations have been recently reported as a novel cause. Lipodystrophy is a relatively rare disorder and proper therapeutic interventions have not been established. Therefore, this novel mouse line may be a useful model to clarify the pathogenesis of lipodystrophy and develop new therapeutic strategies for the patients having lipodystrophy.

NCI-CCR
Arvin George
Clinical Fellow
Epidemiology/Biostatistics - Prognosis and Response Predictions
Identification of threshold prostate-specific antigen levels to optimize the detection of clinically-significant prostate cancer by MRI/US fusion guided biopsy

Abstract Purpose The sensitivity of prostate specific antigen (PSA) increases with lower threshold values but with a corresponding decline in specificity. Magnetic resonance imaging/ultrasound (MR/US) targeted biopsy has been shown to detect prostate cancer (PCa) more efficiently and of higher grade than standard 12-core transrectal ultrasound (TRUS) biopsy, but the optimal population for its use is not well defined. We aimed to evaluate the performance of MR/US targeted versus 12-core biopsy across a PSA continuum. Materials and Methods A review of all patients enrolled in a prospective trial undergoing 12-core TRUS and MR/US-targeted biopsies from August 2007 through June 2013 was performed. Patients were stratified by each of four established cutoffs by PSA. The greatest Gleason score from either biopsy method was compared within and across groups as well as across the PSA range of the population. Clinically-significant (CS) PCa was defined as Gleason =7 (4+3). Univariate and multivariable analyses were conducted with the JMP statistical package (version 11, SAS Institute, Inc.) Results A total of 885 targeted and 12-core TRUS biopsies were performed, of which 493 diagnosed PCa for a detection rate of 55.7%. Targeted biopsy led to significantly more upgrading to CS disease as compared to 12-core biopsy overall, moreso with increasing PSA, and specifically in patients with PSA 4-10 and >10 ng/mL. A PSA of =5.4 ng/mL captured 95% of upgrading by targeted biopsy, corresponding to 40% of patients undergoing MP-MRI. Conversely, a greater proportion of clinically-insignificant disease was detected by 12-core versus targeted biopsy overall. These differences persisted when controlling for potential confounders in a multivariable analysis. Conclusions Upgrading increased significantly with PSA cutoffs. Above a threshold PSA of 5.4 ng/mL a majority of men were upgraded by targeted biopsy to clinically-significant disease. In the NCI population, this corresponded to 40% of MP-MRIs potentially being spared. Below this value, 12-core biopsy detected more clinically-insignificant cancer. Diagnostic utility for targeted biopsy, and by extension MP-MRI, is optimized in patients with a PSA of =5.4 ng/mL

NCI-CCR
Hani Ebrahimi
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Epigenetics
Location is important: New functions of nuclear microenvironments for maintenance of heterochromatin and alternative telomeres
Organization of chromosomes inside the eukaryotic nucleus correlates with gene expression levels and DNA damage repair, but the functional significance of this organization has been widely debated. Here we test directly the cause/effect relationships between localization and chromatin structure and function. Furthermore, we discuss an important role for chromosome organization in maintaining chromosome ends in absence of the reverse transcriptase telomerase. The natural ends of chromosomes (telomeres) are maintained by telomerase, which replenishes terminal sequences lost due to the inability of conventional DNA polymerases to fully duplicate linear molecules. Telomerase activity is not generally detected in somatic cells, but in 80-90% of human cancers, telomerase is activated. It is not yet clear how the telomeres are maintained in the remaining 10-20% of cancers where telomerase is not activated. We have shown that in telomerase-minus fission yeast, canonical telomere sequences can be replaced with heterochromatic ribosomal-DNA (rDNA) repeats. This alternative mechanism for maintaining linear chromosomes requires establishment of heterochromatin and is therefore named HAATI (Heterochromatin Amplification-mediated And Telomerase Independent). A component of the nuclear envelope, Bqt4, is essential for maintenance of HAATI chromosomes. Heterochromatic loci, including telomeres, position near the nuclear envelope , suggesting that positioning of these loci near Bqt4 at the nuclear periphery could be important for maintenance of heterochromatin. Using ChIP and live cell microscopy, we show that in absence of Bqt4, heterochromatic loci move away from the nuclear envelope and heterochromatin abnormally expands or shrinks. We can rescue this heterochromatin expansion by artificially tethering heterochromatin to

the nuclear envelope. In particular, we find that the peripheral localization of heterochromatic regions is important during the period in which they are replicated. These data are revealing the first detailed insights into the importance of nuclear microenvironments for maintaining heterochromatin.

NCI-CCR
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Epigenetics
DNA double strand break induced chromatin compaction promotes BRCA1 repair factor choice.
The repair of DNA double-strand breaks (DSBs) is critical for maintaining genome stability and is orchestrated by a diverse set of DNA damage response (DDR) factors. Appropriate repair factor choice is a critical step in promoting efficient DSB repair. However, the basis for selective repair factor recruitment to DSBs is not well understood. DDR mediators often occupy extensive DSB-surrounding chromatin domains, suggesting a role for chromatin structure in this process. We now show that the DSB-induced formation of a repressive chromatin environment differentially affects recruitment of BRCA1 and 53BP1, two repair factors central to homologous recombination (HR) and nonhomologous end-joining (NHEJ), respectively. Using RNA interference-based screening, we have identified a novel repair module consisting of macro-histone H2A variants and the tumor suppressor RIZ1/PRDM2 (a histone H3-K9 methyltransferase), which is required for efficient HR. DNA damage results in persistent enrichment of macoH2A1 at DSBs, which in turn promotes the recruitment of PRDM2 along with PRDM2-mediated dimethylation of H3-K9. Enrichment of macroH2A1/PRDM2 result in the compaction of DSB-proximal chromatin following an initial phase of damage-induced expansion. Loss of macroH2A1 or PRDM2 as well as experimentally induced chromatin relaxation impairs BRCA1, but not 53BP1 accumulation at DSBs consistent with a biochemical preference of BRCA1 for repressive over active histone marks. As a result, depletion of PRDM2 or macroH2A1 cause a selective and epistatic defect in homology-directed repair, a decrease in the HR-associated phosphorylation of replication protein A, and increased sensitivity to PARP inhibition, all of which are hallmarks of BRCA1 mutant cells. Together, these findings reveal dynamic, DSB-associated chromatin reorganization as a critical modulator of repair factor choice with implications for BRCA1-dependent genome maintenance.

NCI-CCR
Maayan Salton
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Epigenetics
Epigenetic control of alternative pre-mRNA splicing
Splicing of precursor mRNA (pre-mRNA) is an important regulatory step in gene expression. Alternative splicing (AS) allows the production of multiple protein isoforms from one pre-mRNA molecule, thereby contributing to proteomic diversity. Recent evidence points to a regulatory role of epigenetic marks and higher order chromatin structure in AS regulation. In order to identify novel chromatin regulators of AS, we performed an RNAi screen using a cell-based in-vivo assay for high-throughput screening. We identified 10 chromatin proteins that regulate AS of the TAU reporter-gene used in the screen. 8 of the 10 identified proteins have also been implicated in transcription. To distinguish their roles in transcription and AS, we focused on EHMT2, a H3K9 methyltransferase with a known role in transcription silencing. Genome-wide analysis of transcription and AS regulation revealed very limited overlap in affected genes indicating distinct roles for EHMT2 in these two regulatory pathways. One of the most prominent AS target of EHMT2 was VEGF. Silencing of EHMT2, as well as its heterodimer partner EHMT1, reduced VEGF189 isoform formation but did not affect VEGF total mRNA. The epigenetic regulatory mechanisms involves an adaptor system consisting of the chromatin modulator

HP1? known to bind H3K9Me1/2 and SRSF1, a splicing factor of VEGF, which we found to bind HP1?. Silencing either HP1? or SRSF1 down-regulate VEGF189. Conversely tethering EHMT2 to the VEGF promoter reduces VEGF total mRNA but up-regulates VEGF189. The epigenetic regulation of VEGF is physiologically relevant since hypoxia induces the amount of EHMT2 protein and VEGF189 isoform. These results characterize a novel epigenetic regulatory mechanism of AS and they demonstrate separate roles of epigenetic modifiers in transcription and alternative splicing.

NCI-CCR
John Pooley
Postdoctoral Fellow
Gene Expression
The Interaction of the MR and GR in the Nucleus and at DNA: Transcriptional Implications for the Glucocorticoid Response of Stress-associated Brain Regions.
Limited 11beta-hydroxysteroid dehydrogenase in brain allows glucocorticoid actions to be mediated by two nuclear hormone receptors, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). MR and GR bind endogenously circulating glucocorticoids, share a hormone response element, and are co-expressed in the same neurons in several brain areas involved in HPA axis and stress regulation (e.g. hippocampus, PVN). This arrangement opens the possibility for MR/GR cooperation in the regulation of gene expression, a prospect first reported two decades ago when MR-GR interaction was observed in vitro. We have expanded on this work utilizing microscopy in conjunction with a unique cell line (3617ChMR) to study MR-GR interactions in vivo. 3617ChMR expresses GFP-tagged GR and mCherry-MR under tetracycline regulation and includes a tandem array of the MMTV long terminal repeat driving Ras expression. Accumulation of fluorescent MR/GR at the chromatinized, stably integrated array structure (800-1200 GREs) is observable microscopically. The fluorescence lifetime (FLIM) approach to Forster resonance energy transfer (FRET) and fluctuation analysis by cross-correlation number and brightness assay (ccN&B) were used to assess MR-GR interactions in living cells. Initially confirming an interaction of MR-GR biochemically by co-immunoprecipitation, we show MR-GR interactions occur in the nucleus of living cells (FLIM-FRET, ccN&B) but crucially also at a chromatinized DNA template (ccN&B). This result strongly supports the expectation that the interacting complex plays a transcriptional role in co-expressing cells. We compared the patterns of array-associated Ras and endogenous gene expression between MR+GR (interacting) and GR only (non-interacting) conditions by RT-qPCR. Results indicate that the MR-GR interaction confers a different pattern of gene induction compared to that mediated by GR alone under a physiologically relevant hormone presentation (ultradian pulse). Curiously, examination of the stoichiometry of the complex formed in the nucleoplasm (N&B method) indicated the MR-GR interaction in the living cell may not be the anticipated heterodimer. We conclude that an interaction of MR and GR within the living cell participates in transcriptional regulation in response to endogenous glucocorticoids. More work is required to determine how hormone signals carried through the MR-GR complex differ in output from those carried by MR or GR alone.

NCI-CCR
Bau-Lin Huang
Postdoctoral Fellow
Genetics
5â€™Hoxd genes regulate an evolutionary shift in development of the primary limb axis
In tetrapods, the developmental anterior-posterior pattern of limb elements is spatially and temporally conserved. The formation of the limb axis starts with posterior elements (ulna/fibula: U/F) with a distal digit4 (d4) extension and later anterior (radius/tibia: R/T) with digit2 (d2) extension in the hand/foot

plate. In urodeles (tailed amphibians), this order is reversed: R/T-d2 appears before U/F-d4. How the appearance order is regulated is not known. 5â€™homeobox genes (5â€™Hoxd: Hoxd11, 12, 13) are key players in appendage formation and their expression has been examined from fish to mouse. Hoxd11 expression is restricted to the posterior limb bud early and later forms two distinct stripes, one proximal and one distal. However, the late stage distal stripe of Hoxd11 is absent in urodeles, suggesting that distal 5â€™Hoxd expression plays a role in posterior-anterior axis appearance order. To test this hypothesis, we examined the appearance order in 5â€™Hoxd-/- mice. Noggin, expressed in the cartilage primodia and used as an early condensation marker, reveals a normal pattern of U/F-d4 first then R/T-d2 as expected. However, in 5â€™Hoxd-/- mouse limbs, R/T-d2 forms before U/F-d4, as in urodeles. The reversed order in urodeles and 5â€™Hoxd-/- indicates that 5â€™Hoxd determines the appearance order of components of the limb axis. Cell number is a cue for mesenchymal cell condensation due to either transcriptional regulation of cell cycle targets or by a direct effect on replication. 5â€™Hoxd genes promote DNA synthesis in vivo. In a 5â€™Hoxd antagonist mutant, Gli3-/-, there is anterior expansion of 5â€™Hoxd expression and ectopic digit condensations, suggesting that 5â€™Hoxd genes directly or indirectly control cell proliferation. To test the hypothesis that reversal of appearance order in 5â€™Hoxd-/- is due to altered cell proliferation, we are analyzing the cell cycle in 5â€™Hoxd Flox/Flox using ShhCre;RosaTomato to selectively label and knockout 5â€™Hoxd genes in the posterior limb bud, combined with a BrdU pulse to measure cell cycle parameters by FACS. Since ShhCre descendent cells are restricted to the posterior half of the limb, anterior cells (remain unrecombined) serve as an internal, developmentally matched control to which the cell cycle in posterior cells will be compared, both in 5â€™Hoxd KO and wild type. This study will reveal the molecular mechanism controlling appearance order of anterior/posterior limb axis and shed light on origins of evolutionary diversity.

NCI-CCR
Lars Boeckmann
Visiting Fellow
Genomics

Genome-wide screen in S. cerevisiae reveals novel targets for specific treatment of Cse4/CENPA overexpressing tumors

The evolutionarily conserved centromeric histone H3 variant Cse4/CENPA is essential for faithful chromosome segregation. CENPA overexpression and mis-localization is observed in various cancers and is particularly associated with the most aggressive cases with a poor prognosis. Consistent with this, development of aneuploidy due to CENPA overexpression has been reported in yeast, flies and humans. Given the poor prognosis for patients with aneuploid and CENPA-overexpressing (CENPA-OE) tumors, novel treatment is needed to specifically target these tumors. Currently, one of the most promising and novel approaches for selectively treating cancerous cells is to target synthetic lethal (SL) partners of mutated or mis-regulated genes. The aim of this study is to identify novel therapeutic targets for CENPA-OE tumors by identifying SL interaction partners, an approach that has not been exploited so far for these tumors. We used budding yeast as a model to perform a genome-wide screen for gene mutations/deletions that are SL in a strain overexpressing Cse4 but not in a control strain without Cse4 overexpression. This screen identified 102 temperature sensitive alleles of essential genes and 273 deletions of non-essential genes that exhibited SL with overexpression of Cse4. Five alleles of cdc7 and dbf4 were amongst the top eight hits as the most significant SL interaction partners. Cdc7 is an evolutionary conserved, Dbf4 dependent kinase (DDK), which is essential for DNA replication. We validated the SL interaction of cdc7 and dbf4 with Cse4 overexpression using viability assays. Biochemical analysis showed that stability of Cse4 is increased in cdc7-7 and dbf4-1 mutants. Consistent with this, ubiquitination of Cse4 is reduced in these mutants. These studies, supported by further genetic analysis, revealed a novel role of DDK in regulating Cse4 levels and maintaining a balanced

stoichiometry with other histones. Interestingly, inhibitors of Cdc7 are currently being used for cancer treatment in clinical trials. Our results suggest that Cdc7 inhibitors may be especially effective in the specific treatment of CENPA-OE tumors. Taken together, our studies have led to the identification of potential therapeutic targets for CENPA-OE tumors and provide novel insights into the mechanistic role of DNA replication factors in regulating Cse4 levels. Future studies with human cells will validate these results from budding yeast for the effective treatment of CENPA-OE tumors.

NCI-CCR
Amber Giles
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Hematology/Oncology, Tumor Immunology, and Therapy
Hematopoietic Stem Cell Niche Activation and Progenitor Mobilization Mediates Cancer Associated Immunosuppression and Metastasis
The ability of tumors to metastasize to distant tissues is the most lethal aspect of cancer. Tumors impact the local microenvironment and elicit changes in distant tissues, including resident stromal cells and circulating hematopoietic cells, which permit their growth and survival. In adult mice, hematopoietic cells are primarily produced in the bone marrow. To identify tumor-induced changes in the bone marrow microenvironment, we performed microarray analysis on bones of control versus tumor-bearing mice. Notably, in tumor-bearing mice, we saw differential expression of several genes associated with the hematopoietic stem cell niche, including Ptgs2. The product of Ptsg2, Prostaglandin E2 (PGE2), expands hematopoietic stem cells in vitro and in vivo. Cultured mesenchymal stem cells, which contribute to the hematopoietic microenvironment, express Ptgs2 and secrete PGE2, and both are significantly increased in response to tumor-secreted factors. Activation of the bone marrow in tumor-bearing mice is accompanied by expansion and mobilization of hematopoietic stem and progenitor cells (HSPCs). We recapitulated this finding in breast and rhabdomyosarcoma cancer patients. Importantly, elevated levels of HSPCs can indicate metastatic risk. To determine if mobilized HSPCs enhance metastasis, we pretreated mice with AMD3100 to create a surge of circulating HSPCs. HSPC-mobilized mice had significantly shorter survival following tail-vein injection of tumor cells, and this effect was abrogated in immunodepleted mice. We tracked the developmental fate of sorted HSPCs injected into circulation and found that significantly more HSPCs developed into myeloid cells in the lungs of tumor-bearing mice than lungs of control mice. We find that myeloid cells from the lungs of tumor-bearing mice are potent suppressors of T cell activation. Together, these data demonstrate that a primary tumor activates the bone marrow microenvironment prior to successful metastasis. This enhances HSPCs production and mobilization, providing one of the earliest prognostic markers of metastatic risk. Circulating HSCPs promote metastatic progression, potentially by differentiation into immunosuppressive myeloid cells in metastatic sites. Monitoring HSPCs 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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Hematology/Oncology, Tumor Immunology, and Therapy
IKKa Modulates Primary Sclerosing Cholangitis and Intrahepatic Cholangiocarcinoma
Intrahepatic cholangiocarcinoma (ICC) is the second most common primary liver cancer after hepatocellular carcinoma (HCC). The risk of ICC is higher in patients with primary sclerosing cholangitis (PSC). To date, the cellular and molecular mechanism underlying the pathological progression of PSC and ICC is poorly understood. IKKa is part of the I?B kinase (IKK) complex, which plays an important role in

regulating inflammation- associated carcinogenesis through both NF-?B-dependent and independent pathways. Here, we show that IKKa mutant mice developed very serious PSC as early as four weeks of age. The ALT/AST and bilirubin levels were significantly increased in the serum of IKKa mutant mice along with lymphocytic and eosinophilic infiltration into the liver. Liver inflammation in the IKKa mutant mice, mediated by macrophages, neutrophils and CD4 T cells, was associated with the death of cholangiocytes and hepatocytes, and obstruction of intrahepatic and extrahepatic bile ducts, which impeded bile flow and ultimately led to biliary fibrosis and cirrhosis. Additionally, upon activation of NOTCH signaling in the liver via hydrodynamic shear, we observed that NOTCH-induced ICC, with the PSC, developed significantly faster in IKKa mutant mice. To identify whether intrinsic IKKa dysfunction in hepatocytes promotes the NOTCH-induced ICC in IKKa mutant mice, we generated IKKa hep KO mice, in which IKKa is conditionally knocked out in hepatocytes. No biliary disease or liver injury was observed in these mice. We then established an accelerated ICC model utilizing hydrodynamic delivery of NICD and AKT expression vectors. Unexpectedly, ICC development was remarkably slower in the IKKa hep KO mice compared to the IKKa floxed control mice, and this delayed ICC development was associated with reduced activation or levels of AKT, NOTCH, MAPK/Erk and c-myc. These data suggest that IKKa may play a protective role in PSC, while promoting ICC derived from hepatocytes. In contrast to ICC, we also established the cMET/ß-catenin-induced HCC model in the IKKa hep KO mice, which did not reveal any difference in tumor development between IKKa hep KO and control mice. Taken together, our findings suggest that IKKa plays complicated and important roles in PSC and ICC pathological progression.

NCI-CCR
Wonil Kim
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Hematology/Oncology, Tumor Immunology, and Therapy
Expression Levels of ID and GFI Transcription Factors Are Dramatically Changed in Disease Progression of BCR-ABL-positive Chronic Myeloid Leukemia with Imatinib Treatment
Recent therapies using imatinib and second generation drugs for chronic myeloid leukemia (CML) have successively achieved 5-year disease-free survival rate up to 70-80% in patients. However, CML patients still suffers by molecular targeting failure and disease progression from a chronic phase to a drug-resistant and accelerated phase. Therefore, to find new molecular mechanisms for maintaining CML initiating/stem cells bypassing BCR-ABL inhibition, we are interested in studying the expression levels of the Inhibitor of DNA binding gene family (ID1, ID2, and ID3) and the Growth Factor Independent gene family (GFI-1 and GFI-1B), which are required for maintaining the proper functions of hematopoietic stem cells, in BCR-ABL-positive CML patients treated with imatinib. We obtained RNA from bone marrow (BM) samples collected from 32 CML patients including two groups of 12 good responder (GR) and 12 primary resistance (PR) showing different prognostic results after 6-month treatment with imatinib (6m-GR and 6m-PR, respectively) and a group of 8 disease progression (DP) samples with accelerated CML phase, and analyzed gene expressions by real-time qRT-PCR. Compared to the expression levels in normal BM, we found that ID2 is decreased by 43% in GR (p=0.005), and then increased by 78% in 6m-GR (p<0.0001) CML samples, which resulted in dramatic increase of ID2 expression with imatinib treatment (4.4±2.50, p=0.001). However, the expression levels of ID1 and ID3 were not significantly changed in CML samples after imatinib treatment except in DP, which showed the increased expression of ID1 (p=0.01) and ID3 (p=0.001) in contrast to the decreased ID2 expression (p<0.0001). By analyzing expression levels of GFI-1 and GFI-1B, we found that GFI-1 expression was increased in PR (p=0.022), GFI-1B was decreased in GR (p=0.032), and the expression of both genes was greatly reduced in DP (p<0.016) CML samples compared to those in normal BM. These results suggest that ID2 may have a physiological role in suppressing the disease progression of BCR-ABL-positive CML. However, both ID1 and ID3 may have oncogenic functions while CML develops to the accelerated CML

progression. Also, the lowest expression levels of GFI-1 and GFI-1B in DP patients may imply a molecular mechanism to repress the disease progression of CML. Furthermore, the expression levels of ID1, ID2, ID3 and GFI-1B genes can be used as efficient prognostic markers for imatinib treatment in CML patients.

NCI-CCR
John Simmons
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Hematology/Oncology, Tumor Immunology, and Therapy
A Systems Approach Identifies Cooperative Targeting of MYC in Combined mTOR/HDAC Inhibition for Cancer Treatment
The clinical necessity of combining molecularly targeted agents for effectively treating cancer is clear. Yet, elucidation of how these combinations cooperate in the context of highly complex oncogenic and tumor suppressive networks remains challenging. We have taken a multifaceted approach to evaluate the synergistic activity of combining mTOR and HDAC inhibitors to treat multiple myeloma (MM), a plasma cell cancer. The combination of the mTOR inhibitor sirolimus and the HDAC inhibitor entinostat was active and synergistic in high-throughput matrix dose response screening across a large MM cell line library, and diminished cellular viability in ex vivo patient samples. To examine the core synergistic consequence of combining entinostat (HDACi) with sirolimus (mTORi), an integrated, systems-level approach was used. Transcriptional co-expression analysis (WGCNA) of tumor cells treated individually and in combination was used to define the contribution of each drug to an overall response network; a distinct module of 126 genes was cooperatively affected by both drugs. Of these, 37 were found to be differentially expressed in myeloma and predictive of survival. Ingenuity upstream analysis identified MYC as a potential core regulator of the synergistic transcriptional response. Subsequent analysis of ChIP-Seq datasets confirmed MYC promoter binding in 34 of these genes. Using NanoString as a read-out for gene signature expression, we experimentally validated the relationship of MYC with expression of this gene signature, by genetic and pharmacologic (JQ-1) modulation of MYC expression. Using tet-off, MYC-inducible P493 cells, the necessity of MYC for both the drop in cellular viability and response of the gene signature to the combination was evident. Upon experimental assessment of the combination action on MYC, we found levels of MYC protein expression, but not mRNA, to be decreased soon after mTORi/HDACi treatment. Further experiments showed increased degradation of MYC protein with combination treatment (~50% decrease in protein half life) via the Skp1-Cul1-F box ubiquitin ligase complex-directed proteasomal targeting. Additionally, the combination reduced tumor burden and volume and increased survival in a long-term, in-vivo study in immuno-competent mice; MYC protein expression was substantially diminished in tumors after in vivo mTORi/HDACi treatment.

NCI-CCR
Tori Yamamoto
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Hematology/Oncology, Tumor Immunology, and Therapy
Constitutive Fas expression in memory CD8+ T cell subsets augments cellular differentiation and effector function
Memory CD8+ T cells (TMem) may provide lifelong protection against intracellular pathogens and cancer. In order to maximize their protective capacity on a population basis, TMem develop into phenotypically, functionally, and anatomically diverse subsets in varying states of differentiation. Despite this diversity, the tumor necrosis factor receptor (TNFR) superfamily member Fas is held in common across multiple species among all TMem subsets. This finding presents a conundrum: why should long-lived cells required for host survival also express a death receptor? As Fas has been shown

to mediate non-apoptotic functions in other cell types, we sought to elucidate the role of Fas-signaling in defined TMem subsets, including T stem cell memory (TSCM), T central memory (TCM), and T effector memory (TEM) subsets. We found that augmenting Fas-signaling in stimulated TSCM using an oligomerized Fas ligand (lz-FasL) resulted in augmented cellular differentiation, as evidenced by an increased frequency of TCM and TEM progeny and a corresponding loss in IL-2 secretion capacity. Conversely, deprivation of Fas-signaling in TCM using a blocking non-agonistic antibody to FasL (anti-FasL) limited apoptosis, consistent with the canonical role of Fas. However, anti-FasL also delayed the loss of CD62L expression despite proliferation and retained the capacity to secrete IL-2. Microarray analysis demonstrated that TMem expanded with anti-FasL expressed greater levels of memory-associated transcription factors like Tcf7, Bach2, and Id3 relative to IgG-treated controls, which exhibited higher levels of effector transcription factors like Prdm1 (Blimp1) and Id3 and effector molecule Prf1. Moreover, preventing Fas-signaling significantly altered the metabolic state of activated TMem, most notably by limiting the acquisition of glycolytic metabolism. Following adoptive transfer, TMem expanded with anti-FasL manifested significantly increased in vivo proliferation, persistence, and antitumor efficacy against established B16 melanoma tumors compared with IgG controls. These studies demonstrate that Fas-signaling promotes both apoptotic and non-apoptotic signaling in TMem. Current studies are being designed to understand the molecular basis for this dualistic signaling by FasL-Fas. Taken together, these findings have implications for the design and execution of clinical trials using T cell-based immunotherapies and vaccine therapies for patients with cancer and infectious disease.

NCI-CCR
Gabriel Frank
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HIV and AIDS Research
Paradigm shift in the mechanism of HIV-1 core morphogenesis: Structural origins and therapeutic implications
The proteolytic cleavage of the poly-protein HIV-1 Gag, which is assembled on the surface of plasma membranes of infected cells, is the first step in the conversion of the initial immature, non-infectious form to the functionally distinct mature, infectious form. Gag cleavage results in a series of structural changes, resulting ultimately in the formation of a mature core which contains the packaged viral RNA. Current models for assembly of the mature core suggest that the cleaved HIV capsid protein (CA) nucleates in a concentration-dependent manner, and polymerizes into a pseudo-hexagonal lattice, forming the conical core in a diffusion-controlled process. These nucleation and growth models also postulate that the core begins to grow at its narrow end, and stops growing once it reaches the membrane at the opposite end; thus the size of the virus itself is expected to be the primary factor that determines core size. New findings in our lab now challenge this view. Cryo-electron microscopic analyses show that along with infectious viruses, viral isolates also comprise large membranous compartments that contain multiple, freely-floating mature cores. Numerous instances of membrane-attached assembly intermediates with partially formed â€œcore-rollsâ€ that are at different stages of conversion from a planar Gag lattice to the mature core are also observed. We show that these membranous, core-containing compartments are formed in Env-negative virions, establishing that they are not formed by fusion of mature viruses. Thus we conclude that the mechanism of core formation involves a non-diffusional, cooperative transition triggered by cleavage of the immature GAG lattice, resulting in its rolling away from the plasma membrane to form sheets that wrap around the viral RNA. Our proposed mechanism predicts that the generation of infectious HIV-1 is acutely dependent on completion of the cleavage process, and that even a small percentage of uncleaved Gag would prevent the core from rolling away. In contrast, present models for core formation by self-assembly of cleaved Gag suggest that formation of mature cores and infectious virions should be insensitive to a small

amount of uncleaved Gag. The demonstration that expression of a mixture, where only 4% represents a cleavage-resistant Gag is sufficient to cause a 50% reduction of infectivity, provides strong support for our hypothesis and challenges the current diffusion-controlled model for core formation.

NCI-CCR
Luca Sardo
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HIV and AIDS Research

DETERMINE THE DYNAMICS OF HIV-1 RNA GENOME ON THE VIRUS ASSEMBLY SITE

To generate infectious particles, HIV-1 must package its RNA genome during virus assembly. It is known that the interactions between viral RNA elements and structural protein Gag are important for the viral packaging. However, little is known about the dynamics and the events in live cells that lead to RNA packaging. To study HIV-1 RNA kinetics, we used total internal reflection fluorescence (TIRF) microscopy that allows the illumination of a shallow observation volume immediately above the glass surface, which is ideal for studying events on plasma membrane, the major HIV-1 assembly site. We visualized HIV-1 RNA by tagging it with an RNA binding protein fused with a fluorescent protein that recognized sequence engineered into HIV-1 genome. We sought to answer two experimental questions: 1) what is the window of opportunity of a HIV-1 RNA on plasma membrane to interact with Gag and be packaged into a viral particle? and 2) what proportion of the HIV-1 RNAs that reached plasma membrane is packaged into viral particles? To address these questions, we labeled the RNA with RNA binding protein fused with EOS, a photoconvertible fluorescent protein that emits green signal, and upon UV conversion, emits red signal. By selectively converting EOS near the plasma membrane and following the red and green RNA signals through time, we can determine the dwell time of the HIV-1 RNA on plasma membrane. We found that in the absence of Gag, HIV-1 RNA stays in this region with a half-life of ~2 minutes and a portion of the RNA (~20%) remains near the plasma membrane 30 minutes later. However, HIV-1 Gag stabilizes RNA genome and increases its dwell time near the plasma membrane several fold so that most of the RNAs remained on plasma membrane after 30 minutes. The stabilization effect of Gag on HIV-1 RNA is dependent upon the ability of Gag to target the plasma membrane and to specifically bind HIV-1 RNA. We further tagged HIV-1 Gag with a blue fluorescent protein and use 3-color live-cell imaging to study virus assembly. We found that at the early phase of the HIV-1 expression, when few or no Gag signals are detected, ~20-40% of the HIV-1 RNA that reached membrane is recruited into assembling particles. These studies are the first measurements of HIV-1 RNA dynamics on plasma membrane and the efficiency of RNA recruitment, which provide insights into the events leading to the generation of infectious HIV-1 virions.

NCI-CCR
Rachel Van Duyne
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HIV and AIDS Research

Characterization of HIV-1 envelope second-site compensatory changes in Alix-binding site Gag-p6 mutants which may enhance cell-to-cell infectivity

Infectious HIV-1 particle production is driven by the expression of the Gag polyprotein precursor that acts to recruit host cell factors to assist in viral budding. The p6 domain of HIV-1 Gag contains a YPXnL motif that interacts directly with the endosomal-associated protein Alix. Upon passaging in culture, replication-defective Alix-binding site mutants revert to viruses with near-wild-type replication kinetics. Sequencing of these viruses revealed novel mutations in the HIV-1 Env gene, Y61H, P81S, A556T, I744V, and R786K, which not only exhibit a full rescue of the original Gag mutants, but also exhibit replication kinetics faster than those of wild-type HIV-1 in Jurkat T-cells. To explain this phenomenon, we recently

determined that these Env mutants exhibit no effect on the processing of Gag, the incorporation of Env, or virus release efficiency, some decrease fusogenicity and all decrease single-cycle infectivity. We concluded that these Env mutations have no effect on cell-free infectivity and instead propose that these mutations enhance cell-to-cell viral transmission. Cell-to-cell HIV-1 transmission occurs more efficiently and rapidly than infection by cell-free viruses, supporting the relevance of this mode of viral dissemination, especially in vivo. To determine what role these Env mutations play in cell-to-cell transmission, we infected Jurkat T-cells with VSV-G-pseudotyped NL4-3 virus containing Env mutations and co-cultured with uninfected Jurkat-1G5-Luc T-cells, containing an integrated HIV-1-LTR-driven luciferase gene. Treatment with the HIV-1 entry inhibitor T-20 after two hours prevents any further cell-free and cell-to-cell infection of the target cells allowing us to assay for luciferase activity representative of infection due to rapid cell-to-cell transmission. Preliminary results indicate at least a 5-fold increase in cell-to-cell infectivity compared to wild-type in Env Y61H, I744V, and R786K mutants. These results suggest that the Env mutants are capable of enhancing cell-to-cell transmission without affecting cell-free infectivity. This study also implies that compensatory mutations arising in Env are capable of correcting replication defects originating in the Gag-p6 domain through enhancement of an alternate, more efficient form of viral transmission. This mechanism will help to shed light on the complexity of HIV transmission as well as to what extent cell-to-cell infection is susceptible to anti-retroviral therapies.

NCI-CCR
Joo-Young Park
Visiting Fellow
Immunology - Autoimmune
Increased gc receptor expression does not augment but paradoxically inhibits gc cytokine signaling and ameliorates lethal autoinflammatory disease
The common gamma cytokine receptor (gc) is the shared signaling unit for a series of cytokines that are collectively known as common gamma cytokines. gc expression is essential for lymphocyte development as illustrated by the absence of T- and B- cells in gc-deficient mice, and it is also critical for T cell activation as it is necessary for signaling of gc cytokines such as IL-2, IL-4, and IL-15. The current paradigm in cytokine signaling posits that increased cytokine receptor expression results in increased cytokine signaling. Consequently, we generated gc transgenic mice with the assumption that increased gc levels would result in enhanced gc cytokine signaling. Surprisingly, we found that exactly the opposite was the case. Compared to wildtype control T cells, gc transgenic (gcTg) T cells, expressing increased levels of surface gc, were profoundly impaired in gc cytokine signaling as demonstrated by significantly decreased STAT5 and Akt phosphorylation upon IL-7 signaling. Thus, increased gc expression did not promote but rather suppressed gc cytokine signaling. Mechanistically, we found that the gc-associated receptor tyrosine kinase JAK3 was expressed only at limited amounts in T cells, so that increased gc expression resulted in the appearance of gc-chains that had failed to recruit JAK3. Consequently, increased gc expression dampened gc cytokine signaling because it generated JAK3-deficient gc receptors unable to transduce gc cytokine signaling. To assess the in vivo effect of increased gc expression, we introduced the gcTg into scurfy mice, which is a mouse model of lethal autoinflammation. Scurfy mice are deficient in Foxp3 expression, which is a transcription factor required for CD4+ regulatory T cell (Tregs) generation. Scurfy mice succumb to death within 3 weeks of birth due to autoinflammation and multi-organ failure. Strikingly, gc overexpression in scurfy mice significantly improved the disease outcome and dampened inflammation with the result that gcTg scurfy mice survived up to 120 days. Importantly, gcTg scurfy mice were still deficient in Foxp3+ Treg cells, indicating that suppression of gc signaling is sufficient to ameliorate autoinflammatory disease even in the absence of Treg cells. Collectively, these results document a critical role for gc signaling in inflammation and

report a new mechanism of regulating gc cytokine signaling that is suppressed by increased expression of gc.

NCI-CCR
Rahul Roychoudhuri
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Immunology - Autoimmune
BACH2 represses effector programs to stabilize Treg-mediated immune homeostasis
Genetic polymorphisms within a single locus encoding the transcription factor BACH2 are associated with numerous autoimmune and allergic diseases including asthma, Crohnâ€™s disease, coeliac disease, vitiligo, multiple sclerosis and type 1 diabetes. A function for BACH2 in the maintenance of immune homeostasis had not been established. We proposed a direct role for BACH2 in the prevention of inflammation. To test this hypothesis, we characterized the phenotype of knockout (KO) mice in which the Bach2 gene had been disrupted. These mice developed a progressive wasting disease that resulted in diminished survival compared to wild-type (WT) littermates. We observed massive enlargement of the lungs with extensive perivascular and alveolar infiltration by lymphocytes and macrophages and infiltration of CD4+ T cells of the effector TH2 lineage. FOXP3+ regulatory T (Treg) cells suppress inflammatory reactions and are required for maintenance of immune homeostasis. Treg cells arise from precursor cells in the thymus (thymic Treg cells) or from Foxp3- naïve peripheral T cells (induced Treg cells). Remarkably, there was a near-complete cell-autonomous absence of FOXP3+ Treg cells amongst KO CD4SP thymocytes. Similarly, BACH2-deficient naïve FOXP3- CD4+ T cells only inefficiently developed into induced Treg cells in vitro. Thus, BACH2 is required for the development of both thymic and induced Treg cells. Because Treg cells maintain immune homeostasis in an immunodominant fashion, disorders resulting from their deficiency are amenable to rescue by provision of wild-type Treg cells. Lethally irradiated Rag1â€“/â€“ mice reconstituted with KO BM showed profound weight loss and diminished survival whereas co-transfer of WT BM or purified CD4+ CD25+ Treg cells prevented the induction of disease. This indicated that a defect in Treg development results in systemic inflammation in Bach2-deficient mice. We hypothesized that BACH2 functions by directly regulating gene expression. Measurement of the genome-wide function of BACH2 revealed that it represses genes associated with effector cell differentiation, including Prdm1, Gata3 and Irf4. Consequently, absence of BACH2 during Treg polarization resulted in inappropriate diversion to effector lineages. These findings identify BACH2 as a key regulator of Foxp3+ Treg differentiation that potentially explains the emergence of the BACH2 gene locus as a node of susceptibility to autoimmune and allergic disease in humans.

NCI-CCR
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Immunology - General
A novel role of the neuronal receptor TRPA1 in human skin: A double feature that regulates the generation of melanin, the skin pigment needed for protection against UV induced DNA damage and cancer
Human epidermal pigment cells produce and distribute melanin, and progressively more so through UV-induced skin cell DNA-damage to protect against the harmful parts of solar radiation. However, melanin misregulation such as Postinflammatory Hyperpigmentation (PIH) may lead to social exclusion of those being affected. For the first time, we found that allyl isothiocyanate (AITC), a chemical activator of the TRP receptor A1, known for its role as a noxious cold receptor on sensory neurons and mediator of neurogenic inflammation, induced a yet undefined potential pigmentation reaction in human skin. To determine a possible direct or indirect role for TRPA1 as a regulator for pigment generation we

conducted a clinical study with 12 subjects. In vivo reflectance spectroscopy, enzyme activity readings, gene expression and HPLC analysis identified and confirmed an inflammation and a true melanin based pigmentation reaction. To assess if TRPA1 mediates its effect on pigmentation through neurogenic inflammation, we measured 50 soluble inflammatory mediators in extracellular dermal fluid. Our data revealed several strongly regulated factors, specifically prostaglandin E2 (PGE2), VEGF and TNFa. Here for the first time, we show in vivo that PGE2 is highly upregulated in a human model that results in PIH. Interestingly, skin treated with capsaicin, an activator of the TRPV1 receptor on sensory neurons, did not result in the generation of PGE2 and also showed no pigment reaction, even though it induced a neurogenic inflammation similar to AITC. Incubating primary human melanocytes with VEGF and PGE2 resulted in increased melanin production, confirming our in vivo findings. In addition to the indirect effects of the TRPA1 mediated inflammation, primary melanocytes expressing TRPA1 responded to incubation with AITC with increased melanin generation, while the co-incubation with a specific inhibitor of TRPA1 reduced melanin production. Together these findings show a strong indication for a novel role of TRPA1 in pigmentation, not only in postinflammatory responses but also in a general regulatory function that could facilitate treatments to prevent pigmentary disorders. Our results are further supported by recent findings that showed that TRPA1 might be a UV-receptor in melanocytes. As such, modifying TRPA1 activity might provide a UV/DNA-damage independent pathway to increase melanin generation in order to play a protective role in UV-induced carcinogenesis.

NCI-CCR
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Immunology - Innate and Cell-mediated Host Defenses
Exosomes mediate LTB4 signal relay during neutrophil chemotaxis
The secretion of secondary chemoattractants represents a powerful mechanism by which chemotaxing cells maintain robustness and sensitivity to highly diffusible primary chemoattractant signals. Once secreted, these secondary chemoattractants form a gradient to recruit neighboring cells, thereby dramatically increasing the range of detection. Leukotriene B4 (LTB4) has been shown to be such a secondary chemoattractant during neutrophil chemotaxis, although the secretion and the process by which such a small molecule maintains a stable long acting gradient remain to be determined. We hypothesize that in neutrophils, secreted LTB4 is packaged into extracellular vesicles called exosomes that are periodically released to form a stable gradient. We now show that upon stimulation with the primary chemoattractant fMLP, neutrophils secrete extracellular vesicles that are enriched with the exosomal markers CD63 and HSc70 and contain LTB4. Importantly, we also find that the enzymes involved in the synthesis of LTB4, 5-lipoxygenase (5-LO) and LTA4 hydrolase, are present in purified exosomes, suggesting that active synthesis of LTB4 occurs in secreted exosomes. Furthermore, we found that 5-LO-mCherry, which primarily associates with the nuclear membrane in resting neutrophils, redistributes into CD63-positive vesicles that coalesce at the back of chemotaxing neutrophils upon activation with fMLP. Super resolution imaging of exosomes in real time shows vesicular trails originating from the posterior part of migrating neutrophils. Exogenous addition of exosomes to resting primary neutrophils leads to a LTB4 receptor-dependent polarization and migration and a concomitant activation of biochemical pathways known to be involved in migration. Finally, decrease of exosome secretion by inhibiting n-Sphingomylienase2 activity leads to reduction of neutrophil recruitment and LTB4 relay, an effect that recapitulates the pharmacological inhibition of LTB4 synthesis. Together, these findings strongly suggest that LTB4 secretion is mediated by exosome release during neutrophil chemotaxis and implicate this novel delivery mechanism to mediate signal relay in mammalian systems.

NCI-CCR

hye kyung kim
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Immunology - Lymphocyte Development and Activation
Enhanced IL-7 responsiveness provides competitive advantage to recent thymic emigrants in peripheral T cell survival and homeostasis
Interleukin-7 (IL-7) is an essential cytokine for T cell survival. Importantly, T cells themselves do not produce IL-7 and IL-7 is only available in limited amounts in vivo. Consequently, peripheral T cells are constantly competing for IL-7 to survive. T cells that migrate out of the thymus and newly arrive in peripheral tissues are known as recent thymic emigrants (RTE), and RTE are important because they are necessary to replenish T cell antigen receptor diversity and rejuvenate the peripheral T cell pool. RTE also require IL-7 for survival, but it is not known how RTE successfully compete with tissue resident T cells that are pre-occupying the IL-7 niche. To address this question, first, we catalogued cytokine receptor expression on RTE. We identified RTE as GFP+ cells in Rag2-GFP reporter mice which express GFP under Rag2 gene regulatory elements. Interestingly, we found that RTE expressed substantially lower levels of IL-7Ra than naïve T cells. Such distinct expression was specific to IL-7Ra as all other ?c family cytokine receptors were expressed at comparable levels to naïve T cells. Because IL-7 signaling downregulates IL-7 expression, these result suggest that RTE are potentially more responsive to IL-7 than naïve T cells. Indeed, in vitro IL-7 stimulation showed significantly increased STAT5 phosphorylation in RTE compared to naïve T cells. To understand the molecular basis for increased IL-7 responsiveness, next we assessed regulation of IL-7R expression on RTE. We found that IL-7-induced IL-7Ra downregulation was dramatically accelerated and that IL-7Ra re-expression during in vitro recovery culture was significantly increased in RTE. These results suggest that the kinetic of IL-7 response in RTE differs from naïve T cells in that it responds faster and more vigorously, but also recovers much more quickly to become IL-7 responsive again. In fact, in vitro re-stimulation assays revealed that RTE were significantly more reactive to secondary IL-7 stimulation than naïve T cells. Collectively, these data indicate that IL-7 responsiveness is significantly enhanced in RTE, and they provide a new mechanism how RTE can outcompete peripheral T cells for IL-7 dependent survival and homeostasis.

NCI-CCR
Prachi Mishra
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Metabolomics/Proteomics
EFFECTS OF ABERRANT ACCUMULATION OF 2-HYDROXYGLUTARATE IN HUMAN BREAST CANCER
Metabolite addiction of tumors may represent an Achilles heel for cancer cells and an opportunity for therapeutic intervention. New essential metabolites and metabolomic pathways for tumor growth have been described for cancers that define disease aggressiveness and response to therapy. 2-Hydroxyglutarate (2HG) is a novel oncometabolite that accumulates in gliomas and leukemias as a result of mutations in the isocitrate dehydrogenase (IDH) enzymes; but the presence or pathology of 2HG in breast cancer was largely unexplored. A metabolome analysis of human breast tumors in our laboratory recently observed that 2HG accumulates in a subset of breast tumors with a poor outcome phenotype. 2HG reached mmolar concentrations comparable to those in IDH-mutant gliomas, despite the absence of IDH mutations in breast cancer tissues, indicating a yet novel mechanism to be unravelled. Breast tumors with high 2HG tended to be estrogen receptor (ER)-negative and were over-represented among the African-American patients. Tumors of this subtype had a stem cell-like transcriptional signature with WNT and MYC pathway activation. 13C-labeled glutamine was metabolized into 2HG in cells, whereas inhibition of glutaminase markedly reduced 2HG, suggesting a functional relationship between glutamine metabolism and 2HG in breast cancer. Additionally, 2HG was found to aberrantly accumulate in a subset of human breast cancer cell lines, mainly the ER-negative basal-like cell lines with

mesenchymal characteristics, reaching concentrations up to 50- to 100-fold above the 2HG concentration in non-cancerous mammary epithelial cells. Exposure of cell-permeable 2HG to breast cancer cells with low endogenous 2HG in vitro, resulted in accumulation of 2HG in the cells upto 50-100 fold high, similar to the level observed in 2HG high ER-negative cells. 2HG accumulation in these cells led to phenotypic changes similar to that of the aggressive ER-negative basal like cells, such as enhanced proliferation, increased migration and invasion, induction of epithelial to mesenchymal transition and inhibition to apoptosis, suggesting a consistent oncogenic function of 2HG in breast cancer. This is the first study examining 2HG as a candidate oncometabolite associated with poor prognosis in breast cancer, and is aimed to provide a mechanistic insight of accumulation of this oncometabolite and its effects on the biology of human breast cancer, which could be a drug target in the aggressive disease.

NCI-CCR
Scott Medina
Postdoctoral Fellow
Pharmacology and Toxicology/Environmental Health
Cancer cell penetrating activity of the SVS-1 peptide and its use as a carrier for selective delivery of chemotherapeutics in vitro and in vivo
For many therapeutic molecules clinical translation is often limited due to low water solubility, poor pharmacokinetics, and an inability to cross the lipid membrane of target cells. To effectively deliver these therapeutics synthetic nanoparticles and proteins have been employed to enhance solubility, improve targeting, and control drug release kinetics. However, due to their large size these systems suffer from slow uptake kinetics, insufficient endosomal escape and poor nuclear targeting. Over the last decade cell penetrating peptides (CPP) have emerged as attractive carriers to overcome these challenges due to their small size, as well as their cationic composition which allows them to electrostatically interact with the lipid membrane of mammalian cells leading to intracellular localization. Recently, our laboratory reported on an amphiphilic peptide, named SVS-1, with the ability to preferentially fold at the surface of cancer cells leading to cell lysis. We hypothesized that at sub-toxic concentrations the folding of SVS-1 at the surface of cancer cells will allow it to non-lethally intercalate with the lipid membrane and act as a CPP. The kinetics and mechanism of SVS-1 internalization into cancer cells was studied using fluorescent microscopy and flow cytometry, respectively. Next, the SVS-1 peptide was functionalized with a hydrophilic polyethyleneglycol (PEG) spacer and conjugated to a model hydrophobic chemotherapeutic Paclitaxel (PTX) through a self-immolative di-sulfide linker, designed to be reduced by intracellular glutathione. Results show conjugation of PTX to SVS-1 increased the drugs solubility by nearly 1000 times. In addition, PTX was rapidly released from the conjugate upon incubation with physiologically relevant concentrations of glutathione. In vitro cytotoxicity of PTX-S-S-PEG-SVS-1 conjugates confirmed PTX retained its anticancer activity upon conjugation to the SVS-1 carrier. In vivo, SVS-1 showed enhanced tumor-specific uptake in a mouse lung carcinoma model compared to a non-targeted control. Furthermore, incorporation of non-natural D-amino acids into the peptide sequence resulted in enhanced long-term stability of the carrier to proteolysis, and prolonged retention of the peptide in tumor tissue in vivo. Collectively these results establish SVS-1 as a novel cell penetrating peptide with an ability to efficiently solubilize hydrophobic chemotherapeutics and preferentially deliver them to tumor tissue in vivo.

NCI-CCR
Fahu He
Research Fellow
Protein Structure/Structural Biology
A novel ubiquitin-binding domain in myosin VI with specificity for Lys63 linked ubiquitin chains

Myosins are actin-based motor proteins that use energy derived from ATP hydrolysis to generate force and move along actin filaments. Humans have ~40 known or predicted myosins that participate in diverse activities, including conventional skeletal myosin IIs for muscle contraction and unconventional myosins that function in intracellular trafficking, cell division and motility, actin cytoskeletal organization, and cell signaling. Myosin malfunction has been implicated in hypertrophic cardiomyopathy, Usher syndrome, deafness, Griscelli syndrome, and cancer. Myosin VI moves along actin filaments in the opposite direction compared to all other myosins and has recently been implicated in endocytosis and autophagy. We report here that myosin VI joins the growing family of ubiquitin receptors with a unique ubiquitin-binding element, which we name Myosin VI Ubiquitin-binding domain (MyUb). MyUb adopts a compact 42-amino acid helix-turn-helix-like motif with an N-terminal extension that is critical for structure integrity. This structural domain includes the RR(1117)L sequence that has been reported to be required for myosin VI recruitment to autophagosomes. We find R1117 to be essential for MyUb structural integrity, suggesting that the MyUb intact structure is required for myosin VI function in autophagy. We have found MyUb to prefer K63 linked ubiquitin chains and we solved the MyUb:K63 linked diubiquitin structure, which places MyUb wedged between neighboring ubiquitins with unique interactions to each. MyUb binds to the hydrophobic patch composing L8, I44, and V70 on the ubiquitin linked via K63 (distal ubiquitin) and a unique surface that includes A46, F45, and T66 on ubiquitin linked via G76 (proximal ubiquitin). Myosin VI contains an MIU element N-terminal to MyUb that was predicted to bind ubiquitin. We demonstrate that this region binds to ubiquitin and in the longer MIU-MyUb fragment that the two ubiquitin-binding domains bind simultaneously to ubiquitin components of K63-linked diubiquitin. MyUb retains its position between the two ubiquitin components as MIU binds to the exposed I44-centered surface of the proximal ubiquitin. Altogether, these finding suggest that myosin VI function is ubiquitin-dependent.

NCI-CCR
Doreen Matthies
Visiting Fellow
Protein Structure/Structural Biology
High-resolution structure determination by single particle cryo-electron microscopy
High-resolution structures of proteins and protein complexes are currently determined using either X-ray crystallography or NMR spectroscopy, and in selected instances, from cryo-electron microscopy (cryo-EM) of ordered protein assemblies. A prerequisite for structure determination by X-ray crystallography is that the proteins form well-ordered 3D crystals, while it is challenging to use NMR to determine structures of proteins larger than ~50 kDa. The highest resolutions achieved by cryo-EM have been typically restricted to large, well-ordered entities such as helical or icosahedral assemblies or 2D crystals. However, we show that emerging methods in single-particle cryo-EM, after recent developments in microscope hardware and image processing software, now allow structure determination at near-atomic resolution, even for much smaller protein complexes with low symmetry. Rather than simply using cryo-EM maps, typically in the 6â€“20 Å resolution range, as an envelope in which to fit structures obtained by X-ray crystallography, there is the exciting prospect of using cryo-EM to derive de novo, high-resolution structural models of protein complexes. In detail, we solved the structure of the ~465 kDa Escherichia coli ß-galactosidase at ~3.2 Å resolution using single-particle cryo-EM. The majority of the side-chains, the N-termini, and the geometry of the active sites, including a catalytic Mg2+ ion, can be clearly discerned in the density map. The cryo-EM structure closely matches the 1.7 Å crystal structure with a global RMSD of ~0.66 Å, although there are significant local differences throughout the protein, with clear evidence for conformational changes resulting from contact zones in the crystal lattice. Inspection of the map reveals that while densities for residues with positively charged and neutral side-chains are well resolved, systematically weaker densities are observed for residues with

negatively charged side-chains. We show that negatively charged residues exhibit more pronounced effects of radiation damage as determined by comparison of density maps obtained using electron doses ranging from 10 to 30 electrons/Å2. In addition to establishing the feasibility of determining structures, at near-atomic resolution, of protein complexes (<500 kDa) with low symmetry, our analyses also provide a measure of the effects of radiation damage in high-resolution cryo-electron microscopy.

NCI-CCR
Muhammad Alam
Visiting Fellow
Signal Transduction - General
Selective inhibition of the p38 alternative activation pathway in tumor infiltrating T cells inhibits pancreatic cancer progression
Pancreatic ductal adenocarcinomas have a tumor-promoting inflammatory microenvironment, and manipulation of local cytokine production is an attractive, if unrealized, therapeutic approach. My work has focused on signaling mechanisms that lead to tumor-promoting factor production by tumor infiltrating T cells (TIL), in particular in pancreatic cancer. p38, a member of the MAP kinase family, is a critical signaling intermediary in the production of many of these pro-inflammatory factors. Unlike all other cell types, in which p38 is activated by a kinase cascade that phosphorylates T180 and Y182 in the activation loop, T cell possesses a unique pathway in which Y323 is phosphorylated downstream of T-cell receptor (TCR) by Zap70 (the alternative pathway). My studies have shown that the growth of subcutaneously inoculated murine Panc02 pancreatic tumors is enhanced by TNFa produced by CD4+ TIL. Inactivation of the alternative pathway by replacing endogenous p38a and p38ß with mutants carrying a Tyr -> Phe substitution at residue 323 (double knockin mice) resulted reduced TNFa secretion from CD4+ TIL and slower tumor growth. To translate this genetic approach to one that might have clinical application, studies were undertaken to develop an inhibitor of the p38 alternative pathway. The minimal region of Gadd45a (the physiological inhibitor of the alternative pathway) that was sufficient to inhibit the alternative pathway was found to be a 15 amino acid sequence. This was fused with 11 arginines to produce a cell-permeable peptide. Intra-tumor injection of this peptide inhibited TIL production of TNFa and halted growth of established Panc02 tumors. Importantly, in an oncogene-driven pancreatic cancer mouse model (LSL-KrasG12D/+; LSL-trp53R172H/+; Pdx-1-Cre) that closely mimics human pancreatic ductal adenocarcinoma, preinvasive lesions were markedly reduced and none of the peptide-injected mice developed adenocarcinoma, whereas 43% of control peptide-injected mice did. Of note, the presence of a high percentage of CD4+ TIL in human pancreatic tumors that had p38 Y323 phosphorylation defined a subgroup of patients with particularly aggressive disease. Thus, alternative p38 activation in CD4+ TIL leads to pro-inflammatory cytokine production and enhanced pancreatic tumor growth, and can be targeted for therapeutic benefit.

NCI-CCR
Zhenyu Cai
Visiting Fellow
Signal Transduction - General
Plasma membrane translocation of trimerized Mixed lineage kinase domain-like (MLKL) protein is required for TNF-induced necroptosis.
Tumor necrosis factor (TNF) is an important inflammatory cytokine and induces many cellular responses, including inflammation, cell proliferation, apoptosis, and necrosis. It is known that receptor interacting protein (RIP) kinases, RIP1 and RIP3, are key effectors of TNF-induced necrosis, but little is known about how these two RIP kinases mediate this process. Through screening a kinase/phosphatase shRNA library

in human colon adenocarcinoma HT-29 cells, we identified that lineage kinase domain-like, MLKL, is a key RIP3 downstream component of TNF-induced necrosis. We found that MLKL is phosphorylated by RIP3 and is recruited to the necrosome through its interaction with RIP3. Remarkably, MLKL forms a homotrimer through its amino-terminal coiled-coil domain and locates to the cell plasma membrane during TNF-induced necroptosis. By generating different MLKL mutants, we demonstrated that the plasma membrane localization of trimerized MLKL is critical for mediating necroptosis. Importantly, we found that the membrane localization of MLKL is essential for Ca2+ influx, which is an early event of TNF-induced necroptosis. Furthermore, we identified that TRPM7 (transient receptor potential melastatin related 7) is a MLKL downstream target for the mediation of Ca2+ influx and TNF-induced necroptosis. Therefore, our study not only identify that MLKL is a key RIP3 downstream component of TNF-induced necroptosis but also reveals a crucial mechanism of MLKL-mediated TNF-induced necroptosis.

NCI-CCR
Chih-Shia Lee
Postdoctoral Fellow
Signal Transduction - General
Identification of RAS effector network that specifically mediates KRAS oncogene addiction
Oncogenic mutations in the small GTPase KRAS occur in ~30% of human cancers, with particularly high prevalence in pancreatic (>60%), colorectal (30-40%) and lung adenocarcinomas (20-30%). Despite intense efforts at developing KRAS inhibitors, KRAS has proved â€œundruggableâ€ so far. Kinase inhibitors targeting Ras effectors including MEK, ERK, PI3K and AKT are currently in clinical trials, but none has demonstrated clear therapeutic efficacy in KRAS mutant tumors. Thus KRAS mutant tumors represent a class of â€œrecalcitrant cancerâ€ with urgent and unmet therapeutic needs that affects a large number of patients. An important, unanswered questions in this field is which of the ~60 Ras effectors are good drug targets in KRAS mutant cancer. It is likely that such effectors need to be targeted in combinations in order to deliver therapeutic efficacy. To identify target combinations rationally, we need to delineate which Ras effector combinations mediate functional addiction to the KRAS oncogene in cancer cells. To answer this critical question, we have established a validated siRNA library targeting all RAS effectors using the new â€œSensorâ€ technology. Importantly, we have optimized and selected our siRNAs to achieve higher-order combination of gene knockdown with high efficiency and minimum off-target effect, which has not been previously possible with unvalidated, low potency siRNAs. As a proof of principle, using these siRNAs we analyzed a panel of KRAS-mutant colorectal cancer (CRC) cell lines for their dependency on A-, B-, and C-RAF kinases downstream of the KRAS oncoprotein. We show that KRAS wild-type cells are largely insensitive to various combination of RAF knockdown, whereas KRAS-mutant CRC cells are only sensitive to the combined knockdown of BRAF and CRAF. Unlike small molecule RAF inhibitors, our siRNAs experiment clearly indicates that co-targeting of BRAF and CRAF is necessary to phenocopy KRAS depletion in KRAS-mutant cells. Furthermore, we are able to validate our findings in vivo using nanoparticle-based siRNA therapy against KRAS-mutant CRC xenografts in mice. Our approach presents a rational and effective approach to identify target combinations for KRAS mutant cancers. We are currently screening through 600 combinations of Ras effectors with our Sensor siRNA pools to identify optimal combinations of Ras effectors that mediate KRAS addiction. Our finding will guide target selection and therapeutic development.

NCI-CCR
Yixing Han
Postdoctoral Fellow
Stem Cells - General

The role of Lsh in Regulating Proliferation and Differentiation of NSCs
The cerebral cortex develops from multipotent neural stem cells (NSCs) that begin as neuroepithelial cells in the ventricular zone (VZ). After initial symmetric divisions to self-expand, NSCs divide asymmetrically to give rise to differentiated progeny and maintain a copy of themselves. Epigenetic regulations plays pivotal role in the cell identity maintenance as well as the stepwise cell differentiation guidance. Chromatin factors that regulate NSCs self-renewal and neurogenesis in the central nervous system remain to be explored. Lsh is crucial for normal development and Lsh-deficient mice show multiple developmental defects. At molecular level, Lsh deletion leads to genome-wide DNA hypomethylation in brain, liver and whole embryos. To clarify the function of Lsh in NSCs self-renewal and differentiation, we performed the following experiments in mouse embryonic NSCs and mouse brain tissue: 1) Cell-based analysis and IHC; 2) RNA-seq for proliferating NSCs and RT-PCR for proliferation and differentiation NSCs; 3) Image-based time-lapse immunofluorescence analysis. We found that Lsh knockdown resulted in a profound decrease in neural progenitor proliferation and an increase in cell death even in early embryonic stage cells. The proliferation decrease and apoptosis increase of Lsh-/- NSCs are accompanied with cell cycle alteration through p21 expression elevation in a p53-independent manner. During self-renewal, p21 works at downstream of Lsh to control NSCs expansion by regulating Sox2 expression. Our data suggest that Lsh represses p21 and that acute reduction of Lsh relieves this repression, allowing p21 transcription and thereby promoting cell cycle exit. By using combined time-lapse imaging and immunofluorescence, we show that Lsh deletion does not affect the asymmetric division (ACD) rate as well as the cell types both in vitro and in vivo, which indicates that Lsh-/- NSCs are of the ability to differentiation into functional subtypes. However, by checking the expression level of intermediate markers and neural progenitor markers at different time points, we found that Lsh-/- NSCs differentiation was delayed than WT, which suggests that Lsh affect the NSCs differentiation by promoting neural linage specification. In summary, we demonstrate functional requirements for Lsh in self-renewing proliferation of NSCs and cell lineage specification.

NCI-CCR
Madhusudhanan Sukumar
Research Fellow
Stem Cells - General
Low mitochondrial membrane potential (MP) is an intrinsic property of stem-like cells
Identification of various functional T cell subsets has historically relied on cell surface markers. Here, we show that combining such immunophenotyping approaches with an assessment of mitochondrial activity enables significant enrichment for the in vivo biological activity of T cells. When CD8+ T cells were purified based on mitochondrial membrane potential (MP), CD8+ T cells exhibiting low MP demonstrated markedly improved proliferative potential following acute in vivo reconstitution. While low and high MP cells did not differ by classical cell surface markers, the gene expression and metabolomic profiles of purified low MP CD8+ cells closely resembled the characteristics of stem cell-like memory T cells. Direct pharmacological uncoupling of the mitochondrial membrane potential, lowered MP and was, by itself, sufficient to skew naïve CD8+ T cells towards a memory T cell phenotype. Low MP T cells also exhibited reduced mitochondrial activity, lower levels of reactive oxygen species and reduced constitutive DNA damage. These properties contributed to a remarkable augmentation of long-term T cell activity. In particular, when assessed 300 days after adoptive transfer, low MP CD8+ cells displayed a 150-fold increase in abundance and significantly increased biological activity. Similarly, low MP CD8+ cells demonstrated an enhanced ability to destroy large, established tumors. Finally, the ability of low MP sorting to enrich for improved functionality and stem cell-like properties was not restricted to CD8+ cells, as we saw similar discriminatory powers in other lymphocyte populations including CD8 and CD4 T cell subsets such as central memory (TCM), Tc17 cells, Th1 and Th17 cells as well as in

hematopoietic stem cells (HSC). Our findings therefore establish low MP as a hallmark of stem cell-like behavior and provide a simple, general and robust enrichment strategy based on intrinsic cellular metabolism that could have widespread applications in both regenerative medicine and cancer therapies.

NCI-CCR
Myriem Boufraqech
Postdoctoral Fellow
Tumor Biology and Metastasis
Tumor suppressor miR-30a inhibits LOX expression and progression in anaplastic thyroid cancer.
Anaplastic thyroid cancer (ATC) is one of the most aggressive human malignancies with a median survival time of less than one year. A better understanding of the molecular events involved in ATC initiation and progression that could be targeted for therapy is gravely needed. MicroRNA (miRNA) profiling of thyroid tissue samples showed significant downregulation of miR-30a in ATC compared to differentiated thyroid cancer and normal tissue. Ectopic expression of miR-30a in vitro decreased invasion and migration of ATC cells with downregulation of epithelial-mesenchymal transition (EMT) markers and Lysyl Oxidase (LOX) expression, a predicted target of miR-30a. Using an in vivo mouse model of ATC metastases, miR-30a significantly decreased lung metastasis with decreased vimentin, CD44 and LOX expression in lung metastases tumor tissue. We found that LOX and its downstream target TWIST-1 significantly upregulated in ATC, and the expression of both were inversely correlated with miR-30a expression (p= 0.0026). In addition, analysis of 454 primary thyroid cancer samples from The Cancer Genome Atlas dataset demonstrated upregulation of LOX and downregulation of miR-30a in high-risk thyroid cancer. To determine whether LOX is a direct effector of miR-30a, the 3â€™UTR region of LOX was cloned into a luciferase reporter plasmid. Transient transfection of 3â€™UTR-LOX along with miR-30a in ATC cell lines led to decreased luciferase activity, demonstrating that LOX is a direct target of miR-30a. In vitro treatment of ATC cell lines with ß-aminoproprionitrile (BAPN), an irreversible inhibitor of LOX, inhibited cell invasion and migration, and significantly decreased EMT markers and CD44 expression. Moreover, the inhibition of LOX using BAPN or siLOX in mice with metastatic human ATC significantly decreased metastases. Our data taken together support a critical role of miR-30a in ATC progression, which is mediated by LOX, and suggests that miR-30a and LOX is possible therapeutic target in ATC.

NCI-CCR
Min-Hyung Lee
Postdoctoral Fellow
Tumor Biology and Metastasis
CDCA7L functions as a male-specific oncogene in astrocytoma
The most common types of primary central nervous system (CNS) tumors, astrocytoma and glioblastoma multiforme (GBM), are currently incurable. Both astrocytoma and GBM show male predominance, with a male to female ratio of 1.42:1 and 1.58:1, respectively, suggesting differences in the biology or susceptibility of male vs. female brain tumors. We performed linkage analysis in the Nf1-/+;Trp53-/+cis (NPcis) mouse model of astrocytoma/GBM to identify a male-specific gliomagenesis modifier and further used combinatorial bioinformatics approaches as well as cross-species comparisons to prioritize male-specific candidate gene(s). Here, we showed that Cell division cycle-associated 7-like (CDCA7L), a myc co-transcription factor, is a male-specific oncogene in astrocytoma/GBM. CDCA7L expression is up-regulated in astrocytoma cells compared to normal brain or wild-type primary astrocytes, with males showing higher levels than females in both human and mouse. shRNA-mediated knockdown of CDCA7L led to the decreased cell growth and viability of male-derived astrocytoma cells,

but not female tumor cells in both human and mouse. Further mechanistic studies showed that CDCA7L depletion in male-derived astrocytoma cells led to the induction of cleaved Caspase-3 and p27 expression and reduction of Cyclin D1 expression. Furthermore, Cdca7l overexpression promoted the growth of wild-type mouse primary astrocytes only in males by inducing Cyclin D1 expression, but not in female astrocytes, suggesting a male-specific oncogenic role for CDCA7L in astrocytoma/GBM. Strikingly, CDCA7L depletion in human female U87MG GBM cells caused the increase of both growth and viability opposite to what is seen in male cells. Because male-female differences in CDCA7L action are hormone-independent, we examined whether the male-specific histone demethylase KDM5D regulates the effects of CDCA7L. We found that knockdown of KDM5D inhibits male-specific effects of CDCA7L on p27 and Cyclin D1 expression. Our data highlight the sex-specificity of CDCA7L in astrocytoma/GBM tumorigenesis and show that CDCA7L signaling pathways can be oncogenic in males, while being neutral or tumor suppressive in females. This identification of the first male-specific oncogene in GBM has important implications for the application of new personalized sex-specific therapies to human GBM patients.

NCI-CCR
Pravin Mishra
Postdoctoral Fellow
Tumor Biology and Metastasis
Integrated embryonic transcriptome analyses identify key melanoma metastasis regulator
The enhanced ability of melanoma cells to metastasize is reminiscent of the innate propensity of melanoblasts to migrate to distant sites during embryonic development - from the neural crest to their eventual niche in the skin. Once transformed, melanoma cells mimic migratory and growth capabilities similar to those of embryonic melanoblasts. This putative relationship between tumorigenesis and developmental processes was first suggested by Rudolf Virchow more than 150 years ago. While this theory is largely unproven, there are mechanistic links between the processes regulating development and malignancy. Here we investigate this age-old puzzle using a mouse model with melanocyte-specific GFP expression to capture and sequence embryonic melanoblasts. We have, for the first time, isolated and sequenced the transcriptomes of murine embryonic melanoblasts at several key representative developmental stages. To uncover the overall classes of gene expression and to identify and characterize genesets whose expression is common and equally important to melanomagenic and developmental processes, we have devised a novel cross-species multi-dimensional embryonic-onco-genomics analyses (MEGA) approach and show that late stage melanomas reactivate genes used during embryonic development to achieve a more aggressive metastatic phenotype (which we refer to as metafetal genes). One such identified gene, a KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum (ER) protein retention receptor (KDELR) family member, was expressed in advanced mouse melanomas relative to normal skin or benign nevi. We confirmed this in human melanoma by showing that this metafetal gene was expressed at high levels in metastatic patient samples relative to benign lesions, and also predicted patient survival. Next, we determined the consequences of RNAi-based knockdown on experimental metastasis in mouse models. We validated the functional significance in human and mouse melanomas by showing that this KDELR plays a key role in melanoma metastasis through adaptation to chronic ER stress for survival by modulating the unfolded protein response (UPR), which can be targeted. Our study identifies key hardwired pathways associated with melanocyte development that can be co-opted by opportunistic metastatic melanoma cells. This approach offers a novel perspective on melanoma therapeutics and intervention and offers both mechanistic as well as prognostic insights into our understanding of this fatal disease.

NCI-CCR

Nellie Moshkovich
Postdoctoral Fellow
Tumor Biology and Metastasis
Estrogen-related-receptor ß (ESRRB) as a novel transcriptional co-factor that may contribute to TGF-ß regulation of breast cancer stem cell dynamics
Breast cancer (BC) is a worldwide problem that accounts for almost a quarter of all cancers in women; however, better therapeutic approaches are required since it is estimated that one to three deaths from overtreatment occur for every one death avoided. Our lab is interested in transforming growth factor beta (TGF-ß) signaling and its dual role as tumor suppressor/promoter in BC and its therapeutic applications. We previously performed genome-wide ChIP/Chip analysis to identify TGF-ß-activated Smad3 target genes and discovered estrogen-related-receptor ß (ESRRB) as a transcription factor binding motif enriched in Smad3 binding regions in BC cell lines, suggesting a functional interaction between ESRRB and the TGF-ß pathway that may influence BC progression. ESRRs (a, ß, ?) are members of the nuclear orphan receptor family that share significant homology with the estrogen receptors (ERs) but are not activated by natural estrogens. Additionally, ESRRB maintains pluripotency in embryonic stem cells (ESCs) and is activated by Wnt signaling to promote self-renewal in ESCs. Thus, we hypothesized that mechanistically ESRRB/TGF-ß may affect BC progression through effects on dynamics of a subpopulation of tumorigenic cancer cells known as cancer stem cells (CSCs) and cancer cell differentiation. In order to test our hypothesis, we performed ESRRB knockdown (KD) and our preliminary data demonstrate that ESRRB may enhance inhibitory effects of TGF-ß on CSCs as measured in vitro by tumorsphere formation assay in multiple BC cell lines. More importantly, in vivo ESRRB KD reduces tumorigenicity of several different BC lines. These results suggest that down-regulation of ESRRB by TGF-ß may contribute to tumor suppressive effect of TGF-ß on CSCs and that endogenous overexpression of ESRRB in tumor cells may override the inhibitory effect of TGF-ß on CSCs. Our current studies are focused on exploring the effects of ESRRB KD and ESRRB overexpression in multiple BC cell lines using a novel lentiviral-based stem cell reporter system that efficiently marks CSCs. Furthermore, we are investigating the therapeutic potential of ESRR antagonists by pharmacologic targeting of ESRRB. In conclusion, our preliminary findings suggest that ESRRB may be a novel transcriptional co-factor that antagonizes TGF-ß and its inhibitory effects on CSCs and BC progression, making it an attractive novel therapeutic target to eliminate the tumorigenic breast CSC population.

NCI-CCR
Suman Vodnala
Postdoctoral Fellow
Tumor Biology and Metastasis
Micro RNA mediated reprogramming of myeloid cells by targeting TGFß signaling and its associated molecular network
Tumor derived soluble factors result in accumulation of Gr-1+CD11b+ immature myeloid cells at tumor site and premetastatic organ. These cells are known to promote tumor progression and metastasis. Gr-1+CD11b+ immature myeloid cells and tumor-associated macrophages display a type 2 phenotype (M2) and produce immune suppressive cytokines including TGFß. We have previously shown that immature myeloid cells are vicious producers of TGFß ligand and express high levels of TGFßR2 receptor for eliciting immune suppressive function. Specific deletion of TGFßR2 in myeloid cells resulted in increased antitumor immunity and decreased metastasis in the lungs of cancer mouse models. microRNAs (miRs) are abundant class of small non-coding RNAs that have recently emerged as powerful epigenetic regulators of gene expression in pathophysiological conditions. One of the critical properties of miR is to target a large number of genes frequently within the context of a network, making them very efficient in regulating distinct biological cell processes. In the current project, we have identified three miRs that

target myeloid TGFßR2 associated molecular network including p38 and cAMP pathways, which are critical in type 2 cytokine production. We have successfully transduced bone marrow stem cells with lentivirus expressing these miRs driven by a myeloid specific promoter CD11b. Transplantation of these modified bone marrow into lethally irradiated mice decreased breast cancer metastasis. The underlying mechanisms involve the reduction in type 2 cytokines such as IL-4, IL-10 and IL-13 and activation of IFN? producing CD8 cytotoxic lymphocytes. Interestingly, the miRNAs are also differentially regulated by TGFß signaling in TGFßR2+/+ and TGFßR2-/- myeloid cells, suggesting a feedback loop between TGFß and miRNA. These results demonstrate that the candidate miRNA can reprogram tumor-associated myeloid cells by altering the cytokine milieu and enhance host antitumor immunity. Our work points out that endogenous miRNA targeting myeloid TGFß signaling and its molecular network provides an important therapeutic option.

NCI-CCR
Nishi Sharma
Postdoctoral Fellow
Virology - DNA
KSHV lytic infection maintains RNA-granules-free environment by expressing ORF57
KSHV belongs to the family of gamma-Herpes viruses and encodes more than 90 proteins, several non-coding RNAs and microRNAs. KSHV lytic infection leads to cell stress and host shutdown, but viral RNA and protein production survive. The efficient KSHV replication requires the efficient viral translation and concomitant suppression of translation inhibitory RNA granules which are non-membranous compartments containing mRNAs and associated proteins. GW182-positive P-bodies (PBs) and TIA-positive stress granules (SGs) are two common RNA granules and play major roles in translational repression during physiological stress and viral infections. We recently discovered that B cells with lytic KSHV infection do not exhibit visible SGs, but the B cells without lytic KSHV infection display 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 affects the formation of SGs through inhibition of PKR and EIF2a phosphorylation and the formation of PBs via its interaction with Ago2 and XRN2. Thus ORF57 inhibits both Dicer-dependent formation of PBs and Dicer-independent, arsenite-induced formation of SGs. By co-immunoprecipitation, we found that ORF57 interacts with N-terminal region of PKR encompassing the RNA binding domain (RBD1 and RBD2) and inhibits binding of poly:IC to the PKR RNA binding domain, thus blocking PKR phosphorylation. Moreover, we found that ORF57 homologous protein HSV-1 ICP27, but not EBV EB2, exhibits the similar function in inhibition of PKR/EIF2a phosphorylation and eventual inhibition of SGs. Altogether, this study provides deep insight into ORF57 roles in inhibition of RNA granule formation to benefit viral gene expression during stressful lytic KSHV infection.

NCI-CPFP
Oscar Vidal
Visiting Fellow
Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology
A Patient-Specific iPSC Model Reveals Nuclear Retention of PPARg mRNA in Patients with Familial Partial Lipodystrophy
Laminopathies are a collection of rare genetic disorders caused by mutations in the genes that code for nuclear lamina proteins. Familial Partial Lipodystrophy Type 2 (FPLD2) is an autosomal dominant laminopathy characterized by the abnormal distribution or loss of adipose tissue, frequently manifesting at the onset of puberty. While it is known that this disease is caused by a mutation in the LMNA (Lamin A) gene, the functional consequences and subsequent aberrant biology associated with these mutations

are poorly understood at a molecular level. However, the advent of iPCS technology and deriving patient-specific iPSCs has led to the development of an in-vitro disease modeling system for various human disorders that otherwise would be difficult to investigate. To understand the molecular defects that lead to the loss of adipose tissue in patients with FPLD2, we derived iPSCs from 3 patients with a R482W missense mutation in the LMNA gene. iPCSs derived from non-affected individuals were used as a control. We show that while the differentiation of iPSCs to mesenchymal stem cells is normal, terminal adipocyte differentiation leads to increased apoptosis and a surviving cell population trapped in the G0 phase of the cell cycle. To understand this phenotype further, we used a combination of RNA in-situ hybridization and immuno-fluorescence to track mRNA specific sub-cellular localization. We found that a master regulator of adipogenesis, the transcription factor PPARG2, gets trapped in the nuclear envelope in patients with the R482W LMNA mutation. This disrupts the downstream regulatory pathway necessary for terminal adipocyte differentiation. Confocal analysis further shows co-localization of PPARG2 mRNA and Lamin A protein at the 1 hour-time point after acute induction of PPARG2 mRNA expression in FPLD-MSCs while there was no co-localization in control-MSCs. A physical interaction between PPARG2 mRNA and the mutated Lamin A protein was further confirmed by PAR-CLIP. Interestingly, we found that reconstitution of cytoplasmic PPARG2 levels rescues adipocyte differentiation and allows MSCs from FPLD-specific patients to differentiate into adipocytes. Our results provide new pathogenic insights into how the Lamin A protein may play a role in the adipogenesis process in FPLD2 patients. Future use of this in-vitro patient-specific model could be used to address knowledge gaps and lead to new strategies to potentially therapy in these individuals.

NCI-CPFP
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Cancer Prevention Fellow
Cultural Social and Behavioral Sciences
Self-Rated Health and Biomarker Risk Profile: Impact on the Measurement of Racial/Ethnic Health Disparities in the U.S.
Self-rated health (SRH), which is measured by asking individuals to rate their overall health on a scale from poor to excellent, is extensively used to monitor population health. However, using SRH to estimate health disparities may be problematic if different race/ethnic groups have systematically different health expectations and/or reporting standards. Biomarkers that reflect aggregate effects of a variety of health conditions can be used as an alternative and objective measure of health to evaluate reporting heterogeneity. Thus, the aim of this study was to test whether associations between biomarkers and SRH vary by race/ethnicity. Nine biomarkers were used to create a summary index: C-reactive protein, albumin, glycated hemoglobin, total cholesterol, high density lipoprotein cholesterol, waist-to-height ratio, systolic and diastolic blood pressure, and resting heart rate. Biomarker concentrations were dichotomized into above or below clinically recommended levels; indicators were then summed to generate a biomarker score. We used weighted cumulative ordinal logistic regression to compare the distribution of biomarker scores by SRH between non-Hispanic white (NHW) (n=6,346), non-Hispanic black (NHB) (n=2,263), and Mexican Americans (MA) (2,434) from the 2001-2006 National Health and Nutrition Examination Survey. The Fairlie decomposition method was used to estimate the relative contributions of physiologic and sociodemographic factors to race/ethnic differences in SRH. NHB participants were more likely to have unhealthy biomarker scores than NHW participants among persons who rated their health as â€œfair/poorâ€ [OR=1.62 (CI=1.30, 2.02)], â€œgoodâ€ 1.18 (1.01, 1.37)], and â€œexcellent/very goodâ€ 1.29 (1.07, 1.56)]. MA were more likely to have healthy scores than NHW participants among persons who rated their health as â€œfair/poorâ€ [0.75 (0.61, 0.94)] and â€œgoodâ€ 0.71 (0.60, 0.85)], with no difference for â€œexcellent/very goodâ€ 1.11 (0.90, 1.38)]. Sociodemographic factors accounted for about 30% of the observed differences in SRH between

NHW and minority participants, compared to about 15% from physiologic factors. Gender and marital status did not contribute to the observed disparities in SRH. This novel approach of using a biomarker score to measure health indicates that SRH might be underestimating health disparities in black adults. Caution should be exercised in using SRH to guide policy and programmatic decisions involving health disparities.

NCI-CPFP
Holly Nicastro
Cancer Prevention Fellow
Epidemiology/Biostatistics - Etiology, Risk, and Prevention
Comparison of NHANES 2007-2010 methodologies for assessing dietary supplement use
BACKGROUND: Over half of adults in the US use dietary supplements (DS). DS intake in the National Health and Nutrition Examination Survey (NHANES) was traditionally measured using a DS questionnaire (DSQ) that assessed usage over the past 30 days. However, in NHANES 2007-2010, DS intake was also measured by 24-hour dietary recalls (24HR). Estimating total nutrient intake requires combining intake from food sources (assessed by 24HRs) with DS intake. Accurate assessment of nutrient intake by DS is important for measuring the true macronutrient, vitamin, and mineral intake of the US population. AIM: We aimed to determine the impact on the distributions (including mean, % below the estimated average requirement (EAR), and % above the upper limit (UL)) of total nutrient intake using food values plus DSQ values alone or food values plus DSQ values calibrated to the relative frequency on the DS 24HR (ADJ method) in NHANES 2007-2010. METHODS: Participants >1 year old who were not pregnant/lactating and for whom DS intake information was available were included. Distributions for 33 nutrients were calculated for DS users, DS non-users, and the total population by age-sex groups. Distributions were modeled using food intake from 24HR plus DS intake from either DSQ values alone or DSQ values and frequency of use on the 24HR. RESULTS: ADJ distribution values showed similar means but different percentages of individuals with nutrient intakes above the UL or below the EAR. Differences were more pronounced among DS users than non-users or the overall population. For example, among females aged 51-70 who used calcium supplements, mean DSQ calcium intake was 1566+27 and mean ADJ calcium intake was 1518+26 mg (p<0.0003). 8+1% (DSQ) vs 7+1% (ADJ) had intake below the EAR (p=0.046), and 23+1% (DSQ) vs 18+2% (ADJ) had intake above the UL (p<0.00001). These differences also significantly affected the overall population distribution. CONCLUSIONS: Our findings suggest that using the reported DSQ intake as truth potentially erroneously extends both the left and right tails of nutrient distribution curves, leading to overestimation of the percentage of the population who have too low or too high intake. Combining information from DSQ and 24HR with our calibration technique may improve estimates for total intake distributions and may aid in estimation of true nutrient intake in populations

NCI-CPFP
Krystle Kuhs
Cancer Prevention Fellow
Epidemiology/Biostatistics - Prognosis and Response Predictions
Reduced Prevalence of Vulvar HPV16/18 Infection Among Women Who Received The Bivalent Vaccine: A Nested Analysis Within The Costa Rica Vaccine Trial
Although rare, the incidence of vulvar cancer is increasing, particularly among young women. This increase is thought to be due to human papillomavirus (HPV). However, vaccine efficacy (VE) against vulvar HPV infection for either prophylactic HPV vaccine has yet to be reported and information regarding the epidemiology of vulvar HPV infection is sparse. Women (N=5,404) at the final 4-year study visit of the Costa Rica Vaccine Trial, a randomized double-blind controlled trial designed to assess VE of

the bivalent HPV vaccine, provided a vulvar sample. A subset (N=1,044) was tested for vulvar HPV (SPF10/LiPA25 version 1). VE against one-time detection of vulvar HPV16/18 infection at the 4-year study visit among HPV vaccinated versus unvaccinated women was determined among the subset (regardless of doses received, HPV DNA or antibody status) and compared to VE at the cervix. Prevalence of and risk factors for vulvar (and cervical) HPV were evaluated in the control arm (N=536). VE against vulvar and cervical HPV16/18 infection was 54.1% (95%CI: 4.9%-79.1%) and 45.8% (95%CI: 6.4%-69.4%), respectively; p for interaction by anatomical site=0.6. Prevalence of any vulvar HPV infection in the control arm was 29.5%; 16.8% had carcinogenic HPV and 3.0% had HPV16 (compared to 4.7% at the cervix). Independent risk factors for vulvar HPV were similar to cervix and included: age (adjusted odds ratio [adjOR] 0.5 [95%CI:0.3-0.9] women =28 versus 22-23]); marital status (adjOR 2.3 [95%CI:1.5-3.5] single versus married/living-as-married); and number of sexual partners (adjOR 3.6 [95%CI:1.9-7.0] =6 versus 1). In this intention-to-treat analysis, VE against prevalent vulvar HPV16/18 at the 4-year study visit was comparable to the cervix. Therefore, vulvar VE among the target vaccination population of HPV-naïve adolescents would be expected to be high as previously reported for the cervix. Factors that elevate a womanâ€™s risk for cervical HPV similarly increase risk of vulvar HPV.

NCI-DCEG
Catherine Lerro
Doctoral Candidate
Epidemiology/Biostatistics - Etiology, Risk, and Prevention
Use of acetochlor and cancer incidence in the Agricultural Health Study Cohort
Background: Acetochlor, a chloroacetanilide herbicide, has been registered by the US Environmental Protection Agency for use on corn since 1994, with re-registration conditioned on reduction in the use of other herbicides of known health concern. It has become one of the most commonly used pesticides in the US; over 32 million pounds of acetochlor were applied from 1997-2001 on approximately 25% of corn crops. We performed the first ever analysis of acetochlor use and cancer incidence in the Agricultural Health Study, a prospective cohort of licensed pesticide applicators. Methods: During a telephone interview administered from 1999-2005, participants provided information on use of acetochlor and other pesticides, farming and pesticide application practices, demographic information, family and personal health history, and other factors. Incident cancer cases were obtained from North Carolina and Iowa state registries through 2010 and 2011, respectively. Total lifetime days of acetochlor use were calculated and an intensity-weighting algorithm was applied, accounting for potential confounders such as tobacco use, age, and family history of cancer. We used Poisson regression to estimate relative risks (RR) and 95% confidence intervals (CI). We examined all cancer sites together, and individual sites with >5 exposed cases. Results: Among 33,484 men, 3,234 incident cancers and 304 acetochlor-exposed cases occurred. An increased risk of lung cancer was observed among ever users of acetochlor (n=23) (RR=1.57; CI=0.95-2.59) compared to never users, but there was no evidence of an exposure response trend (p-trend=0.30). Acetochlor ever-use was also associated with increased risk of pancreatic cancer (RR=2.65; 95% CI=1.03-6.84, p-trend=0.01), however there were few exposed pancreatic cancer cases in our cohort (n=7). Also, there was increased risk of colorectal cancer (n=25) with high acetochlor use (RR=1.60; CI=0.97-2.65, p-trend=0.14). Conclusions: The associations between acetochlor use with lung, pancreatic, and colorectal cancer are novel. However, due to lack of consistent exposure-response trends, small number of exposed cases, and relatively short time between acetochlor use and cancer development, these findings warrant caution in interpretation and further investigation. Future studies should attempt to replicate these findings in novel populations with large sample sizes, and also to elucidate possible mechanisms of carcinogenesis for acetochlor.

NCI-DCEG

Jason Liu
Postdoctoral Fellow
Epidemiology/Biostatistics - Etiology, Risk, and Prevention
Subsequent gastrointestinal cancer risks of childhood and early adulthood cancer survivors
Introduction: The number of childhood/early adulthood cancer survivors has been growing due to improved cancer treatment, but subsequent malignant neoplasms (SMN) in the digestive system are an important cause of morbidity and mortality. Few studies have examined these survivors' risks of developing various subsequent primary gastrointestinal (GI) cancers. In addition, although there have been SMN studies exclusively on Hodgkin lymphoma (HL) survivors, none have compared the GI cancer risks of young HL survivors with that of other young first primary cancer survivors. We used the National Cancer Instituteâ€™s Surveillance, Epidemiology and End Results (SEER) Program registries to perform the largest analysis on the risks of developing various GI cancers in childhood/early adulthood cancer survivors. Methods: Among the patients reported to 9 SEER registries from 1973 to 2010, there were 27,790 children/young adults diagnosed with first primary cancer before 21 years of age and survived a minimum of 5 years. From these survivors, 83 GI SMNs were included in this analysis, of which 35 developed from 3,239 HL survivors. Standardized incidence ratios (SIR) were calculated using age-, sex-, race-, and calendar year-matched SEER incidence rates. Results: Relative to the general population, childhood/early adulthood cancer survivors had significantly higher risks of developing GI cancers (N=83, SIR=3.3, 95% CI: 2.6-4.1), esophageal cancer (N=6, SIR=6.9, 95% CI: 2.5-15.0), stomach cancer (N=13, SIR=5.5, 95% CI: 2.9-9.4), pancreatic cancer (N=13, SIR=5.9, 95% CI: 3.2-10.1), and colorectal cancer (N=32, SIR=2.2, 95% CI: 1.5-3.1). HL survivors had 7-fold higher risk of developing GI cancers (N=35, 95% CI: 4.9-9.7), whereas survivors of other first primary cancers had 2.4-fold higher risk (N=48, 95% CI: 1.8-3.2) (P-homogeneity<0.001). Those who received radiation treatment for first primary cancer had 5.3-fold higher risk of developing GI cancers (N=50, 95% CI: 3.9-7.0), with 8.1-fold higher risk for HL survivors (N=29, 95% CI: 5.4-11.6) versus 3.6-fold higher risk for survivors of other primary cancers (N=21, 95% CI: 2.2-5.5) (P-homogeneity<0.001). Conclusion: Childhood/early adulthood cancer survivors have significantly higher risks of developing GI cancers relative to the general population, with particularly high risks for upper GI sites and HL survivors. These novel findings may contribute to the clinical follow-up guidelines for young cancer survivors.

NCI-DCEG
Han Zhang
Visiting Fellow
Epidemiology/Biostatistics - Etiology, Risk, and Prevention
A fast and powerful tree-based association test for detecting complex joint effects in case-control studies
Motivation: The logistic regression model is widely used for studying the relationship between a binary outcome and a set of explanatory variables. It is a convenient tool for studying the joint effect of multiple risk factors if their effects are nearly additive. But the logistic regression model can be less efficient if the joint effect cannot be approximated adequately by the additive model. The tree-structure model, which hierarchically partitions the data into multiple exclusive subsets and models each subset individually, can be an attractive alternative to the linear model approach in the situation where the joint effect of multiple risk factors is non-additive. However, the flexible tree-structure model is used most commonly for prediction but seldom for hypothesis testing, mainly due to the computational burden associated with the resampling-based procedure required for evaluating the significance level. Methods: We designed a fast algorithm for building the tree-structure model by adopting the Boolean operation method, which performs numerical calculations using CPU-efficient logic operations, and dramatically increases the computational efficiency. Building upon this new tree-building algorithm, we proposed a robust TREe-based Association Test (TREAT) that can be used as a multivariate test for the

association between a set of risk factors and a binary outcome. The new test incorporates an adaptive model selection procedure to identify the optimal tree model representing the joint effect, and relies on a permutation procedure to evaluate its significance level. Results: TREAT takes 45 seconds to analyze a gene with 20 SNPs using 10^5 permutation steps. We applied TREAT as a gene-based test on over 20,000 genes/regions in a genome-wide association study of esophageal squamous cell carcinoma (ESCC) involving 1942 cases and 2111 controls. This new test detected a highly significant novel association between the gene CDKN2B and ESCC (p = 6.0E-8), while other commonly used multi-locus tests failed to detect the signal (p > 1.0E-5). Exome sequence data on ESCC tumors revealed that the CDKN2B gene had a significantly high somatic mutation rate (Lawrence et al. Nature, 2014). We also demonstrated, through simulation studies, the power advantage of TREAT in detecting complex joint effect. Conclusions: The TREAT is a valuable multivariate testing procedure, and is complementary to those regression-model-based tests that target additive joint effect.

NCI-DCEG
Elizabeth Yanik
Postdoctoral Fellow
Epidemiology/Biostatistics - Prognosis and Response Predictions
Oral leukoplakia, risk of oral cancer, and oral cancer outcomes among elderly adults in the U.S.
Oral leukoplakia is the most common precursor lesion for oral cancers. However, the rate of progression of leukoplakia to cancer and the benefits of early oral cancer detection through screening for leukoplakia are currently unclear. We conducted a cohort study of US residents >=65 years old in SEER-Medicare, a linkage between cancer registries and Medicare that includes all SEER cancer cases and a subcohort of 5% of the US Medicare population. We identified oral leukoplakia diagnoses through Medicare claims, and cancer diagnoses (ICD-O-3: C000-C148) and deaths through cancer registries. The Kaplan-Meier method was used to estimate cumulative cancer incidence following a leukoplakia claim. Cox regression was used to estimate associations of leukoplakia with cancer incidence in the 5% subcohort. Among all SEER cancer cases, we estimated associations between leukoplakia and cancer stage at diagnosis using logistic regression, and between leukoplakia and death after cancer diagnosis using Cox regression. All associations were adjusted for age, race, sex and calendar year. Associations with death were also adjusted for cancer stage at diagnosis. Among 622,138 Medicare recipients in the 5% subcohort, 2,409 (0.4%) had a leukoplakia diagnosis claim, and there were 1633 incident oral cancers. Among those with leukoplakia, the cumulative incidence of oral cancer was 0.9% at 3 months (95%CI=0.5-1.3), 2.7% at 5 years (95%CI=2.0-3.5), and 4.5% at 10 years (95%CI=3.0-6.0). Individuals with leukoplakia had 11.5 times higher incidence of oral cancer than individuals without leukoplakia (95%CI=8.9-14.8). Oral cancer risk was most increased <3 months after a leukoplakia claim (hazard ratio [HR]=83.5, 95%CI=54.7-127.6), but risk was also significantly increased in subsequent follow-up (HR=7.8, 95%CI=5.7-10.7). Among all SEER cancer cases (N=31,487), a prior leukoplakia claim was associated with lower cancer stage at diagnosis (odds ratio for regional/distant vs. localized=0.4, 95%CI=0.4-0.5), as well as a lower risk of death (HR=0.9, 95%CI=0.9-1.0). Individuals with leukoplakia have substantially higher risk of oral cancer. Many cancer diagnoses were made shortly after identification of leukoplakia perhaps reflecting increased diagnostic work-up. The associations of leukoplakia with down-staging of cancer and better survival after cancer diagnosis imply that identification of these lesions often leads to earlier detection of oral cancer and better cancer outcomes.

NCI-DCEG
Bari Ballew
Postdoctoral Fellow
Genomics

Dyskeratosis congenita caused by a TEL patch mutation in TPP1
Dyskeratosis congenita (DC) is an inherited telomere biology disorder resulting in bone marrow failure and high cancer risk. Mutations in 1 of 9 telomere genes account for 70% of patients. Identification of causal mutations is complicated by multiple inheritance patterns, incomplete penetrance, variable expressivity, and genetic anticipation. Gene discovery is important for DC patients, to confirm diagnosis and for family planning, and provides insight into potential cancer susceptibility loci: polymorphisms in 6 DC genes are associated with cancer risk in the general population. We performed whole exome sequencing on 43 mutation-negative DC families in our longitudinal cohort study, revealing 300,000 variants per family. We utilized a custom filtering algorithm to identify likely causal variants. With data from the NHLBI Exome Sequencing Project, 1000 Genomes, and an in-house database of 1400 exomes, we identified rare variants in DC families. We further filtered variants by family structure, using telomere length as the phenotype of interest to minimize the effect of variable expressivity (e.g., siblings with short telomeres should share potentially causative variants, even if one sibling is less profoundly affected). We then combined our naïve analysis with a candidate-gene approach, highlighting variants in any of 3000 genes involved in telomere biology, DNA damage repair, or that are in genome-wide association with DC-related phenotypes. Finally, we analyzed variantsâ€™ impact using predictive algorithms, protein structure, evolutionary conservation, and published literature. This led to the discovery of novel mutations in TPP1, part of the telomeric shelterin complex, in a DC family. The proband, his older sister, and his father have telomeres <1st percentile for age and harbor a single amino acid deletion in TPP1. His healthy twin sisters and mother have normal telomeres and lack this mutation. The deletion affects a conserved solvent-accessible charged loop: a likely site of molecular interaction. This is supported by publications describing the TEL patch of TPP1, a cluster of residues encompassing the deletion that mediates interactions required for telomerase recruitment and telomerase processivity. Our collaborators have shown that in cell lines bearing this mutation, both functions are compromised. This data suggests that germline mutation of TPP1 results in DC, further advancing understanding of the consequences of aberrant telomere biology.

NCI-DCEG
Jason Hoskins
Postdoctoral Fellow
Genomics
Functional analysis of the chr13q22.1 pancreatic cancer risk locus suggests allele-specific effects on DIS3 expression with prognostic implications
Pancreatic cancer is the 4th leading cause of cancer mortality in the U.S. with a 5-year survival rate of ~6%. Surgical resection of low stage tumors is the most effective treatment, but early detection is rare. Therefore, improved risk assessment and screening modalities could prove instrumental in reducing the mortality rate. A genome-wide association study (GWAS) of pancreatic cancer conducted within the NCI Cohort Consortium (PanScan I and II) identified pancreatic cancer susceptibility loci on chromosomes 1q32.1/NR5A2, 5p15.33/CLPTM1L/TERT, 9q34.2/ABO, and 13q22.1. The most significant single-nucleotide polymorphism (SNP) identified on 13q22.1 lies in a 600kb gene desert. Imputation using the 1000 Genomes and DCEG reference datasets did not improve the GWAS signal, but produced a set of highly correlated SNPs for functional follow-up. We performed eQTL analysis to test for associations between the genotypes of these functional candidate variants and expression of nearby genes (KLF5, KLF12, PIBF1, DIS3, and BORA). Among 100 normal derived pancreatic tissue samples, DIS3 expression showed the strongest association with a novel indel variant (rs76366896) in the risk locus (P = 0.0004), indicating risk alleles associate with reduced DIS3 expression. Mutations in DIS3 have previously been identified in acute myeloid leukemia and multiple myeloma, and its expression has been correlated with metastatic potential in colorectal cancer, suggesting DIS3 is relevant to cancer biology. Chromosome

conformation capture identified a physical interaction between the indel-containing locus and a region near the DIS3 promoter. Luciferase assay for regulatory function revealed allele-specific silencer activity for the insertion allele. Supershift electromobility shift assay (EMSA) demonstrated binding of the LEF1 transcriptional activator/repressor specifically to the insertion allele of the indel, which contains two in silico predicted LEF1 binding elements. Finally, through immunohistochemical analysis, high DIS3 protein levels associated with better survival for pancreatic cancer patients (hazard ratio = 2.87, 95% CI = 1.49â€“5.53, P = 0.001, n = 52). Our results suggest that at least one target gene for the pancreatic cancer risk variants on chr13q22.1 may be DIS3, and that the underlying biology is likely mediated by the novel indel through a long-range, risk allele-specific repressive effect on DIS3 expression.

NCI-DCEG
Maki Inoue-Choi
Postdoctoral Fellow
Pharmacology and Toxicology/Environmental Health
Ingestion of nitrate and nitrite from drinking water and diet: A prospective analysis of epithelial ovarian cancer risk among postmenopausal women in Iowa
Background: Ovarian cancer has the highest mortality rate among female reproductive organ cancers, but its etiology is poorly understood. It is thus critical to identify modifiable risk factors that can be targeted for prevention. Nitrate and nitrite are precursors in endogenous formation of N-nitroso compounds (NOCs), potential human carcinogens, and exist in foods. Nitrogen from agricultural fertilizers and other sources can contaminate drinking water with nitrate. Ingested nitrate/nitrite have been associated with several cancers but few studies evaluated ovarian cancer. Objective: To evaluate the association of nitrate and nitrite ingestion from drinking water and diet with ovarian cancer risk. Methods: Dietary nitrate/nitrite intakes were assessed using a food frequency questionnaire among 28,555 cancer-free postmenopausal women enrolled in the Iowa Womenâ€™s Health Study in 1986. Historical drinking water source was obtained in a 1989 survey. Nitrate-nitrogen (NO3-N) and total trihalomethanes (TTHMs), major disinfection byproducts, levels were estimated for Iowa public water utilities; average levels were computed for the womenâ€™s duration of use of each public supply. We computed multivariable-adjusted hazard ratios (HR) and 95% confidence intervals (CI) using Cox proportional hazards regression. We tested interactions of nitrate with TTHMs and dietary intakes known to inhibit NOC formation, such as antioxidants. Results: We identified 315 incident invasive epithelial ovarian cancers (1986-2010). Ovarian cancer risk was 2.2 times higher (95%CI=1.3-3.6, ptrend=0.001) among women in the highest quartile (=2.98 mg/L) compared with the lowest quartile (=0.47 mg/L) of NO3-N in public water, regardless of TTHM levels. Risk among private well users was also elevated (HR=1.5, CI=0.9-2.5). Associations were stronger when vitamin C intake was < median, but the interaction was statistically significant only for private well use (pinteraction=0.01). Ovarian cancer risk increased by 11% with each 0.1 mg/d increment in nitrite from processed meats (CI=1.0-1.2). Dietary nitrate, mostly from fruits and vegetables high in antioxidants, was associated with lower risk (HRQ5 vs. Q1=0.6, CI=0.4-0.9, ptrend=0.01). Conclusion: This is the first study to show nitrate from drinking water, especially with low vitamin C intake, is associated with increased ovarian cancer risk. Replication of our findings could result in a novel target for ovarian cancer risk reduction.

NEI
Suddhasil Mookherjee
Visiting Fellow
Molecular Biology - Eukaryotic
Adeno-Associated Viral (AAV) Vector-Mediated Gene Therapy for X-linked Retinitis Pigmentosa (XLRP): A Long-Term Efficacy Study in Mouse Models of RPGR or RP2 Deficiency

Retinitis pigmentosa (RP) refers to a diverse group of hereditary retinal dystrophies characterized by progressive loss of photoreceptor cells, leading to vision impairment or blindness. It affects 1 in 4,000 live births and is inherited as autosomal dominant, recessive or X-linked form. Mutations on RPGR and RP2 genes account for ~90% of all XLRP cases, with no treatment available. The mutant proteins in most XLRP patients are loss-of-function, affecting the viability of both rod and cone photoreceptors and could be corrected by gene replacement therapy. To develop a treatment, we designed, generated and characterized AAV vectors carrying human RPGR or RP2 cDNA. A combination of appropriate AAV serotype and gene regulatory elements was employed to ensure photoreceptor-specificity of the transgene expression. These vectors were tested in genetically engineered Rpgr or Rp2-null mouse models resembling XLRP. Mice receiving different doses of the vectors were monitored periodically by electroretinography (ERG) and optical coherence tomography (OCT) for retinal function and structural changes for up to 18-23 months of age, due to the slow disease progression. Optomotor test was used to evaluate the visual acuity. On termination, mice were euthanized for histological analyses. Rpgr-null mice receiving 1e9 vector genomes (vg) of the RPGR vector preserved a long-term RPGR expression and displayed significantly larger scotopic (rod) and photopic (cone) ERG amplitudes, much thicker outer nuclear layers and more corrected opsin localization, demonstrating the effectiveness of the treatment. Interestingly, mice receiving vector administration at an advanced age were also benefited functionally and morphologically, suggesting that patients with advanced RP could be amenable to the therapy. Similarly, Rp2-null mice receiving 1e8 vg RP2 vector retained long-term RP2 expression and exhibited a significantly higher photopic (cone) ERG response and cone density, along with an improved visual acuity. However, high vector doses were found to be toxic to the retina in both animal models, highlighting the importance of appropriate vector dosing in future clinical study. In conclusion, AAV-mediated RPGR and RP2 gene delivery preserve the retinal function and structure in mouse models of XLRP. Our study represents the first comprehensive pre-clinical dose-efficacy study for the treatment of XLRP and paves the way for future clinical trials in patients.

NHGRI
Kelly O'Brien
Postdoctoral Fellow
Cell Biology - General

Autosomal recessive Diamond-Blackfan anemia: Identification of mutations in MCM2, SEMA7A, and FLNB

Diamond-Blackfan anemia (DBA) is a rare, congenital bone marrow failure syndrome characterized by severe anemia. Approximately 65% of DBA patients have heterozygous mutations or deletions in ribosomal protein (RP) genes encoding both large and small subunit proteins, resulting in autosomal dominant DBA. The causal abnormalities in the remaining ~35% of DBA patients are unknown. We hypothesize that mutations in non-RP genes may account for some of these cases of DBA. We used whole exome sequencing (WES) to identify candidate gene mutations in DBA patients with normal RP gene sequences and no copy number variants. We sequenced the proband, an unaffected sibling and their parents in four unrelated DBA families. We found 3-5 variants/proband consistent with either a sporadic autosomal dominant or an autosomal recessive pattern of inheritance in three of the four families. In one family, we identified potential autosomal recessive mutations in the Mini Chromosome Maintenance Complex Component 2 (MCM2) and Polymerase RNA III beta subunit (POLR3B). In another family, we identified mutations in the Filamin B (FLNB) gene, and mutations in Semaphorin 7a (SEMA7A) in a third family. RNA-Seq analysis of normal erythroid cells at defined stages of differentiation revealed that MCM2, POLR3B, SEMA7A, and FLNB are expressed in developing erythrocytes. We transduced normal CD34+ progenitor cells with lentiviral vectors containing shRNAs targeting MCM2, POLR3B, SEMA7A, or FLNB mRNA, resulting in 60-90% mRNA knockdown. After differentiation, we found that

POLR3B knockdown cells showed no inhibition in the differentiation of erythroid cells compared to control, indicating POLR3B is an unlikely DBA candidate gene. In contrast, MCM2, SEMA7A, and FLNB depletion resulted in significant reductions in the number of erythroid cells, indicating these genes play important roles in erythropoiesis. Furthermore, when MCM2, SEMA7A, or FLNB shRNA transduced CD34+ progenitor cells were plated in semi-solid medium, CFU-GM (myeloid) colony numbers were normal, but BFU-E (erythroid) colony formation was significantly reduced, suggesting an erythroid-specific role for these genes. In conclusion, we have identified mutations in the non-ribosomal protein genes MCM2, SEMA7A, and FLNB in patients with DBA and demonstrated an important role for these gene products in erythropoiesis. These findings would represent the first autosomal recessive mutations identified in DBA patients.

NHGRI
Gaurav Varshney
Visiting Fellow
Genetics
High-throughput Targeted Mutagenesis using CRISPR-Cas9 in Zebrafish
The zebrafish genome is now completed and is the only third vertebrate to have a fully annotated reference genome, which facilitates systemic large-scale functional genomic studies. The number of large-scale mutagenesis projects has increased in last few years, further enhancing the utility of zebrafish as a model organism. Most of these projects are using random mutagenesis approaches thus limiting the number of genes that can be mutagenize with this approach. However, the development of targeted mutagenesis approaches such as TALENs and CRISPR-Cas9 have opened up new avenues to mutagenize genome in a systematic fashion. The bacterial CRISPR-cas9 derived RNA-guided Cas9 endonuclease has emerged as a very powerful genome-editing tool in various cells and organisms. The guideRNA binds to the target genome and recruits endonuclease cas9 to generate double stranded break which then gets repaired using error-prone non-homlogous end joining (NHEJ) mechanism thus generating indels in the target genome. We developed an inexpensive high-throughput method of multi-allelic targeted mutagenesis using CRISPR/cas9 system, which allows synthesis of guide RNA in 96-well format thus bypassing laborious cloning procedure of guide-RNAs. As a proof-of principle, we targeted all zebrafish genes known or believed to cause deafness in humans (63), and all genes encoding tyrosine kinases (125). We are in the process of targeting genes involved in lateral line migration, glycosylation pathway, glycans and TGF-B pathway (total ~300 genes). We also generated a highly fecund lab strain NHGRI-1.0 and mapped all the polymorphisms in this strain. By having all polymorphisms identified, it allows us to computationally design CRISPR targets without additional target validation. All these mutants will be released to the community through Zebrafish International Resource Center (ZIRC).

NHGRI
Kendra Williams
Postdoctoral Fellow
Genetics
Germline variation modulates susceptibility to metastasis in a mouse model of prostate tumorigenesis
Although prostate cancer is common, with over 233,000 new cases being diagnosed in the US in 2013, it typically runs an indolent course with most men succumbing to unrelated disease processes. This is reflected in the low prostate cancer mortality, with approximately 29,000 men succumbing in the same period. The vast majority of these deaths are a consequence of metastasis. Identification of germline modifiers modulating susceptibility to metastasis will allow physicians to accurately identify the subset of men at risk for the fatal disease forms, and is of critical importance. The goal of this work is to map prostate metastasis modifier loci mapping using the C57BL/6-Tg(TRAMP)8247Ng/J (TRAMP) mouse

model of aggressive prostate carcinoma. We hypothesize that germline variation influences metastasis in prostate cancer. The effect of germline variation in TRAMP mice was investigated by crossing it to different inbred mouse strains and quantifying metastasis in transgene-positive F1 males. Strains with the greatest phenotypic variation from the wildtype TRAMP C57BL6/J mice were chosen for modifier mapping using an F2 intercross approach. F2 mice were genotyped using a linkage panel of 1,449 SNPs and modifier loci were analyzed using a java-based quantitative trait loci (QTL) mapping package (j/QTL). The greatest number of loci achieving genome-wide significance were observed in the TRAMPxNOD/ShiLtJ F2 cross (n=232). Modifier loci associated with metastasis were observed on chromosomes 1, 6 and 11. We performed microarray analysis of primary tumors derived from the TRAMPxNOD/ShiLtJ F2 cross (n=122) and correlated metastasis with the expression of all transcripts within the three metastasis susceptibility loci on chromosomes 1, 6 and 11. These analyses allowed for the identification of 22 novel metastasis susceptibility candidate genes. We have therefore identified multiple novel metastasis susceptibility candidate genes using both QTL mapping and tumor expression profiling. The importance of these 22 genes is being further evaluated by expression QTL (eQTL) mapping. The relevance of those genes that display a cis-eQTL to aggressive human prostate cancer will be investigated in publically-available human prostate cancer genome-wide association study cohorts. This approach will facilitate the identification of novel germline factors driving metastasis susceptibility and allow for new insights into this deadly form of prostate cancer.

NHGRI
Matthew LaFave
Postdoctoral Fellow
Genomics
DNA integration mapping reveals that MLV integration site selection is driven by a subset of strong enhancers and active promoters
Retroviruses integrate into the host genome in patterns specific to each virus. Understanding the causes of these patterns can provide insight into viral integration mechanisms, pathology and genome evolution, and is critical to the development of safe gene therapy vectors. We generated murine leukemia virus (MLV) integrations in human HepG2 and K562 cells and subjected them to second-generation sequencing, using a DNA barcoding technique that allowed us to quantify independent integration events. We characterized >3,700,000 unique integration events in two ENCODE-characterized cell lines. We find that integrations were most highly enriched in a subset of strong enhancers and active promoters, relative to 10,000 matched random control datasets. In both cell types, approximately half the integrations were found in <2% of the genome, demonstrating genomic influences even narrower than previously believed. The integration pattern of MLV appears to be largely driven by regions that have high enrichment for multiple marks of active chromatin. Integration enrichment in these regions was 40x the amount expected by random chance, and was significantly greater than the ~12x enrichment detected in open chromatin in general (ANOVA with Tukey's test; P < 10^-7). The combination of histone marks present was sufficient to explain why some strong enhancers were more prone to integration than others. The approach we used is applicable to analyzing the integration pattern of any exogenous element and could be a valuable preclinical screen to evaluate the safety of gene therapy vectors. We have extended our mapping approach to the analysis of gene traps and additional retroviral vectors, and have used experimental results to fine-tune a command-line pipeline designed to provide optimal integration recovery. This software is freely available at http://bit.ly/1fxhJ66.

NHGRI
Senta Kapnick

Other
Immunology - Innate and Cell-mediated Host Defenses
Inducible T cell kinase regulates CD8+ T lymphocyte effector function
CD8+ cytotoxic T lymphocytes (CTLs) are critical for killing virally infected cells, and defects in CTL responses can lead to secondary lymphoproliferative syndromes. Patients with mutations in Inducible T cell Kinase (Itk), a kinase that serves as an amplifier of T cell receptor (TCR) signaling, develop a fatal lymphoproliferative disorder associated with ineffective responses to Epstein-Barr virus (EBV), a virus that infects B cells. These phenotypes are similar to those seen in X-linked lymphoproliferative disease (XLP), where we have found specific defects in T:B cell interactions and the specific cytolysis of B cells. To evaluate whether Itk-deficiency resembles XLP, we examined killing using CTLs from WT and Itk-/- mice. Unlike murine WT CTLs, we found that CTLs from Itk-/- mice exhibit impaired cytotoxicity against multiple cell types, suggesting Itk-deficiency, unlike XLP, leads to a global defect in cytolysis. These killing defects were not due to altered T cell development because they could be reproduced by treating WT CTLs with an Itk-specific inhibitor during the cytolysis assay. To better understand the roles of Itk and TCR signaling in regulating cytolytic activity and the development of lymphoproliferative disorders, we examined how Itk-deficiency affects the discrete stages of CTL function. Killing by CTLs is initiated when the TCR triggers activation and adherence of CTLs to targets, accumulation and centralized clearance of actin into a ring at the T cell:target interface leading to centrosome polarization to the target, and finally the release of secretory granules containing effector proteins that induce cytolysis of targets. Using immunofluorescence confocal microscopy of T:target cell conjugates, we found that adhesion and actin ring clearance were intact in the absence of Itk. Itk-deficient CTLs were also able to polarize their lytic machinery toward the target contact site as well as their WT counterparts. However, using a FACS-based degranulation assay, we found defects in granule secretion in Itk-deficient CTLs, suggesting that proximal signaling components such as Itk play a previously unappreciated role in the final stages of cytolytic effector function. Together these experiments provide clues to novel roles for Itk and TCR signaling in regulating late stages of cytolytic activity, and why mutations in Itk lead to defective CTL function.

NHGRI
kai ying
Visiting Fellow
Informatics/Computational Biology
GSVseq - Detection of Genomic Structural Variation Using Multiple Features of NGS Data
Genomic structural variation (SV), and especially copy number variation (CNV), play an important role in many complex diseases such as autism, Alzheimerâ€™s disease and cancer. Recent advances in next generation sequencing (NGS) technologies have enabled us to sequence the whole genomes (WGS) or whole exomes (WES) of many samples at a reasonable cost. However, current analytical methods for CNV detection are still unsatisfactory in both sensitivity and accuracy. These methods generally utilize one feature of sequence, e.g. read depth (RD), allele frequency (AF), paired end distributions (PE), soft clipped ends (SC) and split reads (SR) to call CNVs. They assume that those features follow certain probability distributions, which may not be accurate due to sampling bias caused by many well known (e.g. GC-content, low complexity region) or uncertain factors. Every feature has its advantages and weaknesses and may complementary to each other e.g. PE is sensitive to small CNV, while RD is more powerful for large CNV. A combination of the two or more features would increase CNV detection power. Here we propose an integrated model that combines different sequence features in a unified statistical model to increase CNV detection power. First, the genome is divided into small bins, and sequence features of each bin are fed into a local Bayes model to estimate the likelihood of different local copy numbers. Then, a Hidden Markov Model (HMM) is applied to combine data from neighboring

bins to identify contiguous genomic regions that show consistent abnormal copy number states. Finally, smoothing and statistical testing are performed to mark only high confidence CNV with a controlled false discovery rate (FDR). When family pedigree information is available (e.g. sequence from family trios), our method can perform a joint analysis of sequence from all members of the family based on Mendelian inheritance laws. When samples are from an unrelated population, our method can benefit from prior population information such as the allele frequencies of known mutations. Our method can also improve SNP calling in duplicated regions. Both simulated and benchmark data show that our method can give reliable detection of moderate sized (5-10Kb) CNV with high coverage (>30x) WGS sequence, or large size CNV (>100Kb) with either low coverage (<=10X) WGS or high coverage WES data. Our method shows significantly improved accuracy comparing to several widely used tools.

NHGRI
Erica Bresciani
Postdoctoral Fellow
Stem Cells - General
CBFß and RUNX1 are required at two different steps during the development of hematopoietic stem cells
RUNX1 and CBFß form a transcription factor complex that is essential for the hematopoietic development. Both genes, however, are also targets of recurrent chromosomal translocations in human leukemia. Multiple evidences suggest that misregulation of their normal transcriptional program is an important mechanism for leukemogenesis. Thus, a better understanding of their roles during physiological hematopoiesis can improve our understanding of their involvement in leukemogenesis. Knockout mice for either Runx1 or Cbfb showed essentially identical phenotypes with lack of definitive blood lineages and embryonic lethality. Studies in mouse and zebrafish demonstrated that Runx1 is required for the emergence of hematopoietic stem cells (HSCs) during the early phases of definitive hematopoiesis. Since CBFß is considered the obligate partner of RUNX1, it was suggested that CBFß is also required for HSC formation. Using zebrafish as a model, we generated two independent cbfb knockout lines (cbfb-/-) by zinc-finger-nuclease-mediated targeted mutagenesis. Fish from these two lines presented identical hematopoietic phenotypes and died around 14 days-post-fertilization. The analysis of cbfb-/- embryos revealed an uncoupled function of Runx1 and CBFß during HSC development. Unlike the runx1-/- mutants embryos, where HSCs are absent, the emergence of the runx1+/c-myb+ HSCs appeared unaffected in the cbfb nulls embryos. Further support for this finding was provided by the pharmacological inhibition of the RUNX1-CBFß interaction by a novel compound, Ro5-3335, on wild type embryos, as the emergence of HSCs was not affected by Ro5-3335 treatments. However, in the cbfb-/- mutants the HSCs fail to translocate from their site of origin, the aorta-gonad-mesonephro (AGM) region, to their subsequent niche as evidenced by live imaging analysis of cbfb-/-/tg(c-myb:eGFP) transgenics and the accumulation of runx1+ HSCs in the AGM. As a result cbfb-/- mutants lacked all differentiated blood lineages. The inhibition of Runx1-Cbfß interaction by Ro5-3335 recapitulated the hematopoietic phenotype observed in cbfb-/- embryos and caused a severe impairment in the release of HSCs from AGM in wild type embryos. Overall our data indicate that CBFß and functional CBFß-RUNX1 heterodimers are not required for the emergence of HSCs, but are essential for the release of HSCs during early definitive hematopoiesis.

NHGRI
Alberto Rissone
Postdoctoral Fellow
Stress, Aging, and Oxidative Stress/Free Radical Research
Antioxidant Treatment Rescues Hematopoietic Phenotypes in Different Models of Adenylate Kinase 2 Deficiency

Adenylate kinase 2 (AK2) is a mitochondrial enzyme critical for cellular energy homeostasis. Mutations of the AK2 gene are responsible for reticular dysgenesis (RD), one of the most rare and severe forms of severe combined immunodeficiency (SCID). RD is characterized by a total lack of granulocytes and lymphocytes, leaving RD patients vulnerable to recurrent serious infections and death early in life. The only recognized treatment for RD is hematopoietic stem cell transplant. To gain insights into the pathophysiology of RD, we performed a comprehensive study of the effects of AK2 deficiency using the zebrafish model and the induced pluripotent stem cells (iPSCs) system. Loss of zebrafish AK2 function obtained by zinc finger nucleases-mediated gene knockout and ENU-induced mutants resulted in severe impairment of hematopoietic stem cells development. We also observed increased levels of reactive oxygen species and apoptosis in hematopoietic tissues of AK2-deficient embryos. To support the hypothesis that oxidative stress is responsible for defective zebrafish hematopoietic development in the absence of AK2, we tested the efficacy of an antioxidant treatment on our mutant lines. Notably, the treatment of AK2 mutant embryos with serial concentrations of N-Acetyl-L-Cysteine (NAC) or Glutathione (GSH), not only specifically reduced the oxidative stress, but also resulted in the rescue of the hematopoietic phenotypes in more than 50% of null mutants. To begin to explore the utility of antioxidants as therapeutics for RD, we used an in vitro hematopoietic differentiation model based on iPSCs derived from fibroblasts of a RD patient. In this model, myeloid differentiation of mutant iPSCs recapitulated the characteristic maturation arrest of the myeloid lineage observed in the bone marrow of RD patients. Notably, the treatment of AK2 mutant iPSCs with 3 mM GSH induced a 14-fold increase in the generation of mature neutrophils. In summary, our findings of marked rescue of hematologic development in different AK2 mutant systems exposed to antioxidant drugs may prove relevant to the clinical management of RD patients and support the investigation of antioxidant treatment in sustaining patient conditions prior and/or following hematopoietic stem cell transplant.

NHLBI
Jinwei Zhang
Research Fellow
Biophysics
Crystal structure and mechanisms of the T-box riboswitches, bacterial sensors of amino acid starvation
The T-box riboswitches are phylogenetically conserved gene-regulatory RNAs widespread in Gram-positive bacteria including many clinically devastating pathogens such as Clostridium and Listeria. Discovered in 1993, T-boxes are key players in monitoring and regulating amino acid metabolism, and were the first described riboswitches. Comprised of two domains connected by a variable linker, the T-box uses its 5' "Stem I" domain to decode tRNA anticodon and a 3' "antiterminator" domain to sense tRNA aminoacylation and cause transcription readthrough or termination based on that readout. However, despite 20 years of research, it has remained largely unknown how the T-boxes specifically recognizes its cognate tRNA substrate, how it reads the aminoacylation status of that tRNA, and how the tRNA aminoacylation status is translated into the genetic decision. We have determined the first co-crystal structure of a T-box riboswitch Stem I domain in complex with its cognate tRNA at 3.2 Å resolution. Our structure reveals how the C-shaped T-box stem I wraps around the iconic L-shaped tRNA, making a series of intimate contacts via mutually induced fit. These not only include canonical base-pairing interactions, but also critical stacking interactions between precisely positioned elaborate RNA modules such as the interdigitated T-loops and the characteristic tRNA elbow. Structural elucidation of this mRNA-tRNA complex not only allowed us a rare glimpse into how non-coding RNAs recognize each other, but may permit design of next-generation antibiotics to combat the rising epidemic of antibiotic resistance, To dissect the mechanisms by which the T-boxes sense tRNA aminoacylation status, we developed a novel, facile method to prepare homogeneous aminoacyl-tRNA and a panel of aminoacyl-tRNA analogs. These allowed us to show that the Bacillus subtilis GlyQS T-box detects the molecular

volume of tRNA 3'-substituents. Further, calorimetry and fluorescence lifetime analysis of labeled RNAs show that the tRNA acceptor end co-axially stacks on a helix in the T-box 3' domain. This intimate intermolecular association, selective for uncharged tRNA, stabilizes the antiterminator conformation of the T-box. Taken together, the structural and mechanistic elucidations of the T-box system have shed light on general principles of RNA-RNA recognition and may open the door towards understanding the regulatory network interconnecting many non-coding RNAs.

NHLBI
Jordan Beach
Postdoctoral Fellow
Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology
Non-muscle Myosin II Isoforms Co-assemble in Living Cells
Non-muscle myosin II (NM II) powers myriad developmental and cellular processes, including embryogenesis, cell migration, and cytokinesis. To exert its functions, monomers of NM II assemble into bipolar filaments that produce a contractile force on the actin cytoskeleton. Mammalian cells express up to three isoforms of NM II (NM IIA, IIB and IIC), each of which possesses distinct biophysical properties and supports unique, as well as redundant, cellular functions. Despite previous efforts, it remains unclear if NM II isoforms assemble in living cells to produce mixed (heterotypic) bipolar filaments, or if filaments consist entirely of a single isoform (homotypic). Resolving this question is critical for interpreting both past and future studies on NM II, and for clarifying the distinct functions of the NM II isoforms. We addressed this question using fluorescently-tagged versions of NM IIA, IIB and IIC, isoform-specific immunostaining of the endogenous proteins, and a novel super-resolution imaging technique (two-color total internal reflection fluorescence structured-illumination microscopy, or TIRF-SIM) to visualize individual myosin II bipolar filaments inside cells. We show that NM II isoforms co-assemble into heterotypic filaments in a variety of settings, including various types of stress fibers, individual filaments throughout the cell, and the contractile ring of dividing cells. Additionally, it is well established in polarized cells that NM IIA is enriched in peripheral lamella relative to NM IIB, while NM IIB is enriched in central and posterior regions relative to NM IIA. We show that the differential distribution of NM IIA and NM IIB typically seen in well-polarized cells is reflected in the composition of individual bipolar filaments. Interestingly, this differential distribution is less pronounced in freshly-spread cells, arguing for the existence of sorting mechanism acting over time. Together, our work answers a longstanding question in the myosin field and argues that individual NM II isoforms are potentially performing both isoform-specific and isoform-redundant functions while co-assembled with other NM II isoforms.

NHLBI
Colleen Skau
Postdoctoral Fellow
Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology
A novel actin-adhesion structure involved in nuclear positioning requires the formin FMN2
Active asymmetric positioning of the nucleus in nondividing cells is critical to a variety of cell functions, including cell migration, particularly in complex 3D environments. Previous studies have highlighted the importance of integrin-FAK and Rho signaling in controlling nuclear orientation in migrating fibroblasts. Although these studies establish that integrins, actin, and actin-binding proteins at the nuclear envelope are critical for nuclear positioning in adherent cells, it is not known if this is mediated by linking the nucleus to the previously-characterized adhesion/actin system used for migration, or if nucleus-ECM coupling is accomplished via a specialized cytoskeletal system. We sought to test the hypothesis that a dedicated adhesion-actin system is specifically responsible for maintenance of nuclear position in

adherent and migrating cells. To test this, we examined the interplay between actin, adhesions, and the nucleus in mouse embryonic fibroblasts using fluorescence microscopy. We identified novel adhesive structures located underneath the nucleus termed subnuclear adhesions that are dynamically and compositionally distinct from the canonical focal adhesions found near the leading edge of migrating cells. Unlike focal adhesions at the leading edge, the long-lived subnuclear adhesions assemble in bursts and slide toward the front of the cell. Evidence from nuclear displacement experiments indicates that the number and placement of these adhesions is controlled by the nucleus. Additionally, we find that subnuclear adhesions have reduced levels of several signaling and actin-binding proteins found in leading edge adhesions, and have elevated levels of the IIB isoform of myosin. We also show that a specific set of actin fibers connecting two subnuclear adhesions can physically impinge upon the nucleus and help control nuclear shape. We have previously identified the actin nucleation factor formin FMN2 in a screen of adhesion components. We now find that the formin FMN2 localizes underneath the nucleus and is essential for organization of these subnuclear actin fibers. In fact, cells lacking FMN2 migrate poorly in 3D environments and exhibit uncoordinated changes in nuclear shape during migration. Together, our data reveal a previously unidentified mechanism for control of nuclear position via a novel adhesive structure linking the actin cytoskeleton, which connects to the nucleus, to the extracellular matrix underneath the cell body.

NHLBI
gang ren
Doctoral Candidate
Chromatin and Chromosomes
High-resolution analysis of genome-wide long-distance chromatin interactions using sureHi-C
Transcription is often regulated by promoter-enhancer (P-E) interactions. Currently, there are two popular techniques to map genome-wide P-E interactions: one is Hi-C and the other is ChIA-PET. The former is unbiased but suffers from low-resolution of about 20 to 50kb and thus not very useful to pin-point specific P-E interactions; the latter has higher resolution but is biased due to the requirement of a specific antibody against a chromatin binding protein. In order to achieve unbiased and high-resolution mapping of P-E interactions, we have developed a new technique termed super-resolution Hi-C (sureHi-C) by using a combination of different restriction enzymes for digesting chromatin, followed by proximity-mediated re-ligation and next generation sequencing. Using this technique, we analyzed the genome-wide long-distance chromatin interactions in mouse ES cells and CD4 T cells. With limited sequencing depth, we achieved a resolution of about 1kb. We identified 2000 and 2015 topological domains in ES and CD4 T cells, respectively. Comparison between these domains indicated that 90% of them are conserved between ES and T cells. However, the intradomain interactions are related to gene expression in such a way that higher interaction is correlated with higher gene expression. Among the 66,941 interaction clusters identified in CD4 T cells, 98% are intra-chromosome interactions, and only 2% interactions are inter-chromosome interactions. Comparison with H3K27ac and p300 ChIP-Seq data revealed that these interactions are highly enriched in potential enhancer regions, suggesting that these interactions may be critically involved in regulating transcription in T cell. Indeed, by analyzing gata3- and stat6-deletion-induced gene expression changes, we found that the more interactive a factor binding site is, the more likely it regulates gene expression. Our high-resolution interaction data predict numerous potential enhancers. To experimentally test the enhancer activity, we employed CRISPR to delete the genomic regions. One example is a potential enhancer located 120kb away from the Bcl2 promoter. We found that deletion of this region in EL4 cells significantly decreased the Bcl2 expression and increased apoptosis, indicating a critical role of this region as an enhancer. In summary, sureHi-C is an unbiased high-resolution technique for mapping chromatin interactions.

NHLBI
Benjamin Kidder
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Epigenetics
Abstract & Title removed at request of author

NHLBI
Christopher Jones
Postdoctoral Fellow
Protein Structure/Structural Biology
Towards a high-resolution structure of the cyclic-di-AMP riboswitch, a master regulator of bacterial gene expression
Invasion of human cells by pathogenic bacteria characterizes some of the most damaging diseases worldwide (e.g., tuberculosis, pneumonia). Alarmingly, current antibiotic treatments are becoming outdated due to the development of widespread antibiotic resistance, which threatens to turn once controllable diseases into untreatable life-threatening infections. In human cells, a critical line of defense against invading pathogens is the cytoplasmic detection of bacterial DNA or small molecules, which trigger production of interferons and an immune response. Cyclic-di-adenosine monophosphate (cyclic-di-AMP) is a recently discovered marker of bacterial infection that is detected by human cells via the stimulator of interferon genes (STING) protein. In bacteria, cyclic-di-AMP is a second messenger that regulates protein expression via a cyclic-di-AMP-binding riboswitch in the mRNAs of many bacterial genes. The cyclic-di-AMP riboswitch is widespread in bacteria, with over 3,000 examples identified, but the mechanism by which these riboswitches recognize cyclic-di-AMP and alter gene expression is unknown. Using isothermal titration calorimetry to measure the binding affinity of cyclic-di-AMP to cyclic-di-AMP riboswitch variants, we have determined which regions of the riboswitch are necessary for high affinity ligand binding. Through this approach, we have constructed a minimal RNA domain that binds to cyclic-di-AMP with a dissociation constant of ~10 nM, similar to that for the wild-type RNA. Using small-angle X-ray scattering (SAXS), which reports on the overall shape of macromolecules in solution at an ~15 Å resolution, we have examined the minimal riboswitch in the presence and absence of cyclic-di-AMP. From this data, we have found that the riboswitch undergoes a conformational change upon ligand binding that leads to RNA compaction. To better understand how the cyclic-di-AMP riboswitch recognizes its ligand, we have also screened RNA variants for crystallization in the presence of ligand. We have recently obtained cocrystals of the riboswitch bound cyclic-di-AMP that diffract to 3.5 Å resolution. Our current efforts are focused on improving crystal diffraction and obtaining phase information essential to solving the structure. With a more detailed understanding of the cyclic-di-AMP riboswitch, future plans include designing cyclic-di-AMP derivatives and assaying their efficacy as potential antibiotics.

NHLBI
Katherine Warner
Doctoral Candidate
Protein Structure/Structural Biology
Lead compounds from fragment-based drug discovery specifically bind and remodel the TPP riboswitch
Microbial resistance to the current clinical arsenal of antibiotics is emerging at an alarming rate, and multidrug-resistant infections are a major world health concern. Novel classes of antibiotics that target cellular processes distinct from the current repertoire of antibiotics offer a promising route for drug development. One attractive group of cellular targets for the development of novel antibiotics are riboswitches, which are structured regions of mRNA that modulate gene expression in response to the

intracellular concentration of a variety of small molecules. Thiamine pyrophosphate (TPP) riboswitches regulate essential genes in pathogenic bacteria by changing conformation upon binding intracellular TPP. Previous studies using fragment-based approaches identified small molecule â€œfragmentsâ€ that bind this gene-regulatory mRNA domain. Crystallographic studies now show that, despite having micromolar Kds, four different fragments bind the TPP riboswitch site-specifically, occupying the pocket that recognizes the aminopyrimidine of TPP. Unexpectedly, as visualized in the fragment co-crystals, the unoccupied site that would recognize the pyrophosphate of TPP rearranges into a structure distinct from that of the cognate complex. In each case, a guanine nucleobase coordinates either one or both of the divalent cations previously seen to mediate binding of the anionic moiety of TPP by the riboswitch. This idiosyncratic fragment-induced conformation, also characterized by small-angle X-ray scattering (SAXS) and chemical probing (SHAPE), represents a possible mechanism for adventitious ligand discrimination by the riboswitch, and suggests that off-pathway conformations of RNAs can be targeted for drug development. Our structures, together with previous screening studies, demonstrate the feasibility of fragment-based drug discovery against RNA targets.

NHLBI
Allison Ta
Research Fellow
Radiology/Imaging/PET and Neuroimaging
Discriminating dark rim artifacts from true perfusion defects with fully quantitative perfusion analysis at the pixel resolution of cardiovascular MRI perfusion in humans
Purpose: Pixel-wise quantitative analysis of Cardiac perfusion MRI (CMR) is a new noninvasive test for diagnosing coronary artery disease (CAD) by measuring myocardial blood flow (MBF) that was developed in the intramural NHLBI. A severely stenotic epicardial artery can induce a downstream perfusion defect that is easily detected on CMR pixel maps. A dark rim artifact (DRA) that mimics a perfusion defect is one of the most important limitations in CMR. This study used fully quantitative analysis of MBF at the pixel resolution to characterize regions of DRA vs. true perfusion defects. Methods: 199 consecutive patients had a regadenoson perfusion CMR study using a steady state free precession sequence and 0.05 mmol/kg gadolinium. To study DRA, we excluded 139 cases without correlative invasive coronary angiography (CATH) or computed tomography angiogram (CTA) and 18 cases with coronary artery bypass, 3-vessel disease, or technical issues. Classification as a true perfusion defect required CATH with significant CAD (>70% stenosis). Classification as DRA required no significant CAD (<30% stenosis) by CATH or CTA and presence of DRA on CMR. CMR perfusion pixel maps were generated by a model-constrained Fermi deconvolution. Regions of interest were drawn in the subendo-, mid-, and subepicardial myocardium. Results: Patients (n=42) average age 55±11 years and CAD was present in 23% (n=10). In patients with DRA, MBF in the subendocardium was lower than the midwall and epicardium (2.40±0.64 vs 2.94±0.72 and 3.15±0.78 ml/min/g respectively, p=0.001) as well as normal myocardium (p<0.001). However, the endocardial MBF of true perfusion defects was much lower than endocardial MBF in DRA (0.81±0.35 vs 2.40±0.64 ml/min/g; p<0.001). Endocardial MBF could separate CAD from DRA with an area under the receiver operator curve of 0.997(p<0.001). Conclusion: Although DRA are present and have statistically lower MBF in the subendocardium than the midwall and epicardium, the endocardial MBF in true perfusion defects is much lower. Thus, fully quantitative analysis of MBF was able to discriminate DRA from true perfusion defects. These new methods may reduce false positive results of CMR due to DRA an d reduce unnecessary referral for additional tests.

NIA
Taraswi Banerjee

Visiting Fellow
DNA-binding Proteins/Receptors and DNA Repair
Conserved Aromatic Loop of Human RECQ1 Helicase is Required for Catalytic Strand Separation During DNA Unwinding or Branch-Migration of Recombination Intermediates and Necessary for a Robust Replication Stress Response
RECQ1 is a DNA helicase belonging to a family of conserved enzymes important for genomic stability. Mutations in three (WRN, BLM, RECQ4) of the five RecQ helicases are linked to genetic disorders characterized by premature aging and high incidence of cancer. Although RECQ1 is the most abundant RecQ helicase and displays elevated expression in a spectrum of cancers, its precise roles are poorly understood. Here we used single molecule DNA fiber analysis along with genetic and biochemical assays to dissect the role of RECQ1 in DNA replication and chromosomal stability. Two highly conserved residues in the aromatic loop of the RECQ1 helicase core domain were replaced by site-directed mutagenesis (F231A, W227A) and the mutant recombinant proteins were purified from insect cells. Gel-mobility shift assays showed the mutant proteins retained DNA binding similar to RECQ1-WT; however, unwinding of forked duplex DNA substrates was severely compromised and branch-migration to resolve replication repair intermediates (mobile D-loops or Holliday Junctions) was completely abolished. In contrast to their negative effects on strand separation activities, the RECQ1 mutations only partly reduced ATPase (kcat) 2-fold. RECQ1-W227A or RECQ1-F231A retained their ability to oligomerize, bind ATP, and catalyze efficient ATP-regulated strand annealing between ssDNA molecules. Collectively, the biochemical studies demonstrate the aromatic loop of RECQ1 is critical for efficient coupling of ATPase to DNA unwinding or branch-migration. Genetic rescue experiments performed in RECQ1-depleted HeLa cells expressing either RECQ1 aromatic loop mutant revealed a markedly reduced replication rate and consequent random firing of dormant replication origins as demonstrated by single molecule DNA fiber assays. These cells displayed significantly elevated spontaneous double-strand breaks and sensitivity to the DNA damaging agents camptothecin or H2O2. Moreover, expression of either mutant exerted a dominant negative effect on replication rate and resistance to exogenously induced DNA damage. Taken together, our results demonstrate that the aromatic loop of RECQ1 is crucial for DNA strand separation activities required for normal replication and a robust DNA damage response. These RECQ1 separation-of-function mutants, representing the first class of RECQ1 mutants to exert dominant negative phenotypes, serve as valuable tools to interrogate its role in genome homeostasis and tumorigenesis.

NIA
Evandro Fei Fang
Postdoctoral Fellow
DNA-binding Proteins/Receptors and DNA Repair
Defective Mitophagy in Accelerated Aging Disorders via PARP1 Hyperactivation and Impairment of the NAD+/SIRT1-axis
Mitochondrial dysfunction is emerging as a common feature of neurodegeneration, cancer and aging. Several DNA repair disorders show neurodegeneration but the pathogenesis has remained unclear, and mitochondrial alterations may represent an attractive explanation. Xeroderma pigmentosum (XP) is a rare autosomal recessive disorder with severe sun sensitivity leading to a greatly increased risk of skin cancers. Apart from the dermatological ailments XP group A (XPA) patients often suffer from neurodegeneration and hearing loss of unknown etiology. Here, we sought to determine whether XPA-deficient cells had mitochondrial alterations and further whether these changes could be reversed. We found that cells deficient in XPA, either patient samples or knockdown cells, accumulated damaged mitochondria and had higher reactive oxygen production due to defective mitophagy, compared with wild type cells. The mitochondrial abnormalities were caused by decreased activation of the NAD+/SIRT1 axis triggered by increased activation of the DNA damage sensor poly(ADP-ribose) polymerase 1 (PARP1)

which utilizes NAD+. Thus, we tested whether the XPA mitochondrial phenotype could be rescued by up-regulation of NAD+ bioavailability through pharmacological inhibition of PARP1 or supplementation with NAD+ precursors. Using three different models, human cell lines deficient in XPA, XPA null (xpa-1) C. elegans and a mouse model, we documented that up-regulation of NAD+ levels rescued the mitochondrial function in XPA-deficient samples. Most encouragingly, NAD+ supplementation greatly extended the lifespan in xpa-1 C. elegans, and rescued the mitochondrial phenotype in an Xpa-/-/Csa-/- mouse model, whereas it had little impact on normal controls. Thus NAD+ levels profoundly impact mitochondrial function and syndromes which elicit activation of PARP1 are at risk for mitochondrial alterations. It is our hypothesis that the DNA repair defect triggers high NAD+ utilization and secondarily mitochondrial dysfunction which then predisposes these individuals to neurological impairment due to mitochondrial dysfunction. Importantly, our findings reveal a previously unknown nuclear-mitochondrial cross-talk that is critical for maintenance of mitochondrial health, and further that DNA repair deficient patients may benefit from NAD+ supplementation.

NIA
Magdalena Misiak
Postdoctoral Fellow
DNA-binding Proteins/Receptors and DNA Repair
Evidence for the involvement of DNA polymerase-beta in the regulation of neurogenesis in aging and Alzheimerâ€™s disease
Alzheimerâ€™s disease (AD), the most common and feared form of dementia, involves the progressive degeneration of neurons critical for learning and memory. In addition, findings from animal models and postmortem human brains suggest that neurogenesis in the hippocampus and olfactory bulb (OB) is impaired during aging and in AD. Olfactory deficits occur during normal aging, and a more profound olfactory deficit is an early symptom of AD. The present study is a part of our broader investigation of the role of DNA base excision repair (BER) in aging and AD. We tested the hypothesis that inefficient DNA repair due to DNA polymerase-beta (PolB) deficiency can disrupt proliferation, differentiation and cellular metabolism in neural stem cell (NSC) during adult neurogenesis. In the absence of PolB, neurons in the brain of mice undergo massive perinatal apoptosis. In fact, even when apoptosis is prevented by the concomitant deficiency of the pro-apoptotic protein p53, mice still display major brain abnormalities, suggesting that PolB is required for normal NSC differentiation. PolB levels in the brain decrease with normal aging, and in Down syndrome patients who invariably develop AD-like pathology, cognitive impairment and olfactory deficits. We noticed that PolB-/- mouse embryos lack a discernible OB. To study the role of PolB in neurogenesis during aging and in AD, we crossed PolB+/- mice with 3xTgAD mice (an AD mouse model). We then injected PolB+/+, PolB+/-, 3xTgAD and PolB+/-/3xTgAD mice (cohorts of 6 and 14 month-old mice) with bromodeoxyuridine (BrdU) to monitor neurogenesis. Preliminary analysis suggests a decrease in newly formed neurons in the hippocampus and OB of PolB+/- mice compared to age-matched controls, which was further reduced in the PolB+/-/3xTgAD mice. The decline in BrdU-positive cells was paralleled by increased apoptosis. Moreover, the PolB+/-/3xTgAD mice showed the most severe memory impairment in behavioral tests. Currently we are testing olfaction in the mice by multiple behavioral tests. We are also performing studies aimed at establishing the molecular mechanisms by which deficiency in a DNA repair enzyme adversely affects cellular energy metabolism and neurogenesis. Since hippocampal dysfunction and loss of olfaction are the early events in AD, understanding the role of PolB and DNA repair in plasticity and function of the hippocampus and OB may lead to new approaches for early therapeutic intervention in neurodegeneration.

NIA
Susan Walker

Postdoctoral Fellow
Endocrinology
Peripheral Blockade of the Cannabinoid-1 Receptor Exerts Beneficial Effects on Pancreatic Beta Cell Function
Type 2 diabetes mellitus is characterized by the progressive loss of pancreatic beta cell function, resulting in decreased insulin secretion and uncontrolled hyperglycemia. With more than 25.8 million individuals suffering from diabetes in the U.S. alone and another 79 million with pre-diabetes, there is an urgent need to develop novel therapies to treat this rapidly expanding metabolic disease. One attractive target for diabetic therapeutics is the endocannabinoid system, which is composed of both lipid-derived ligands and their receptors. Over-activation of the endocannabinoid system has been linked with obesity, insulin resistance and impaired glucose homeostasis. Moreover, both rodent and human pancreatic beta cells express the cannabinoid-1 receptor (CB1R), and inhibition of this receptor in mice has been shown to stimulate beta cell proliferation, increase insulin secretion, and improve insulin sensitivity. However, whether these beneficial effects also occur in islets of Langerhans of a diabetic model and what role(s), if any, CB1R plays in insulin secretion from human islets is unknown. The objectives of the current study were therefore to determine if a novel, peripherally-selective CB1R inverse agonist (JD-5037) would influence beta cell function in the db/db diabetic mouse model and impact insulin secretion in human islets. Chronic treatment of db/db mice with JD-5037 (1 mg/kg) significantly decreased body weight and blood glucose levels compared to vehicle-treated mice while also increasing total beta cell area and insulin content within the pancreata. Utilizing mouse insulinoma cell lines, we demonstrate that JD-5037 stimulates beta cell proliferation and increases glucose-stimulated insulin receptor signaling in vitro. Importantly, perfusion of human islets with JD-5037 increased glucose-mediated insulin secretion in a dose-dependent manner. These results collectively demonstrate that peripherally-selective CB1R blockers may be a useful therapeutic to improve beta cell function in diabetic individuals.

NIA
Salman Tajuddin
Visiting Fellow
Genetics
Carotid intima-media thickness: a genome-wide association analysis among African Americans
Although the age-adjusted death rate from coronary heart disease (CHD) continues to decline in the population overall, there remain significant mortality disparities for African Americans (AAs). The atherosclerotic process of CHD leads to thickening of the intimal and medial layers of the common carotid artery. Carotid intima-media thickness (CIMT), measured by B-mode ultrasound, is a non-invasive assessment of subclinical atherosclerosis and has been shown to predict cardiovascular events. Genetic factors influence CIMT. Identification of genetic factors linked with CIMT may facilitate early detection of atherosclerosis. Recent genome-wide association studies have identified genetic loci associated with CIMT in European ancestry and Hispanic populations. However, the association between genetic variants and CMIT has never been investigated among AAs. We conducted a genome-wide association analysis of CIMT in AAs in the Healthy Aging in Neighborhoods of Diversity across the Life Span (HANDLS) study, an epidemiological, longitudinal study of health disparities. CIMT in the left common carotid artery was measured by high-resolution B-mode ultrasound. Genotyping was done using Illumina 1M genotyping platform. Single nucleotide polymorphisms (SNPs) with minor allele frequency greater than or equal to 0.05, genotyping call rate > 95%, and in Hardy-Weinberg equilibrium (P> 0.00001) were included in the analysis. Complete mean CIMT and genotype data were available for 584 AA (55% men) study participants. We estimated the beta coefficients of each SNP in additive model adjusting for the first 10 principal components. Two suggestive SNPs were found to be associated with

mean CIMT: rs9871833 (beta=-0.06; P=2.3 x 10superscript-6), and rs9381309 (beta=-0.04; P=2.7 x 10superscript-6). The SNP rs9871833 is located in chromosome 3p13 region while rs9381309 is located in chromosome 6p21.1 near alanyl-tRNA synthetase 2 (AARS2). The enzyme encoded by this gene aminoacylates the alanine amino acid with its cognate transfer RNA. Translational inaccuracy of AARS2 such as incorporation of non-protein amino acid homocysteine into endothelial proteins has been suggested to contribute to the development of atherosclerosis via protein damage and altered high density lipoprotein level. These findings may shed light on atherosclerosis development and provide a biomarker of susceptibility in AAs. Replication studies in other AA cohorts will be conducted to validate these findings.

NIA
Guobing Chen
Postdoctoral Fellow
Immunology - Lymphocyte Development and Activation
Histone methyltransferase Ezh2 is critical for activation-induced CD8 T cell proliferation and survival
Ezh2, an enzymatic component of polycomb repressive complex 2 (PRC2), catalyes trimethylation of lysine 27 of histone 3 and regulates transcriptional repression. It plays a vital role during stem cell development and cell differentiation. Although it is well documented that epigenetic change is involved in gene expression regulation, the precise epigenetic mechanism underlying the transcriptional changes during CD8 T cell differentiation are not fully understood. Here, we assessed the role of Ezh2 in CD8 T cell differentiation using Ezh2 deficient mice in T cells (Ezh2-loxp/CD4-Cre, Ezh2 KO). In the absence of Ezh2, the number of CD8 single positive thymocytes and peripheral CD8 T cells in the young Ezh2 KO were similar to wild-type (WT) mice. However, a gradual loss of peripheral CD8 T cells was observed in Ezh2 KO mice with age (~50% less at 40 weeks old), suggesting Ezh2 play a role in CD8 T cells homeostasis. When infected with listeria-OVA strain, OVA+ CD8 T cells were not increased in Ezh2 KO compared to WT mice. This deficiency was further confirmed in the adoptive transfer experiments that transferred same OVA+ naïve CD8 T cells into congenic or Rag2 knockout host mice with infection. To determine the mechanism underlying this defect, we examined CD8 T cell differentiation after antigen activation. We found that 1) CD8 T cells expressed similar levels of activation markers CD69, CD25 and CD44 and produced similar levels of effector molecules IFN-gamma, IL-2, TNF-alpha, and Granzyme B between Ezh2 KO and WT mice; 2) activation-induced CD8 T cell proliferation was impaired in Ezh2 KO compared to WT mice; and 3) more than 20% of ANXV+ and Caspase 3/7+ apoptotic population was observed in Ezh2 KO while less 1% of them in WT CD8 T cells in vivo. Furthermore, genome-wide transcription analysis showed that Ezh2 KO CD8 T cells expressed higher level of CDK inhibitor genes, such as Cdkn2a and Cdkn2b (~5 folds), and apoptosis related genes, such as Bim and caspase 3 (~20 folds), providing a transcriptional basis of activation-induced proliferation defect and increased apoptosis. Collectively, these findings demonstrated that Ezh2 is required for repression of the transcriptional programs of genes involving cell proliferation and apoptosis during CD8 T cell differentiation. The identification of these repressed genes will allow further characterizing their role in memory cell formation and may serve as potential targets to modulate vaccination efficiency.

NIA
JI HEON NOH
Postdoctoral Fellow
Molecular Biology - Eukaryotic
Molecular regulatory mechanism and function of mitochondrial lncRNAs
Introduction: Mitochondrial dysfunction is associated with various aging-related diseases including neurodegeneration, cancer, and cardiovascular disease. Although the mitochondrial genome encodes 13

proteins, two rRNAs, and 22 tRNAs, a large portion of mitochondrial proteins necessary for maintaining mitochondria structure and function are imported from the nucleus. In addition, nuclear-encoded long noncoding RNAs (lncRNAs) are required to maintain mitochondrial function. However, it is still unclear how RNAs are transported into mitochondria from the nucleus and what is the cellular role of the mitochondria-localized RNA species. Results and Discussion: From RNA-seq analysis, we recently identified nuclear-encoded lncRNAs localized in the mitoplast, the soluble compartment within the mitochondrial inner membrane. Among those lncRNAs, RPPH1 and RMRP were elevated in senescence fibroblasts compared to proliferating 'young' fibroblasts and were found to be the targets of RNA-binding proteins (RBPs) HuR, AUH, and GRSF1. Importantly, silencing HuR increased oxygen consumption rate (OCR), while depleting AUH or GRSF1 reduced OCR, highlighting key functions for these RBPs in mitochondrial respiration. Based on these data, we hypothesized that the mitochondrial lncRNA import can be regulated by RBPs. To test this hypothesis, we conducted experiments that (1) showed that RBPs regulate lncRNA import into mitochondria by observing lncRNA distribution in situ in RBP-depleted cells using microscopy, (2) identified other protein or RNA molecules that physically interact with RPPH1 and RMRP, and (3) discovered that changing mitochondrial lncRNA distribution and molecular interactions potently affected mitochondrial function and metabolism. Through this work, we have uncovered new roles for RBPs in the transport of lncRNAs into mitochondria and have gained critical knowledge of the mechanisms of through which regulatory RNAs are mobilized among cellular organelles.

NIA
Krisztina Marosi
Postdoctoral Fellow
Neuroscience - Cellular and Molecular
Beta-Hydroxybutyrate Stumulates BDNF Signaling and Bolsters Neuronal Bioenergetics
Dietary energy restriction (ER) and exercise enhance the functional capabilities of the brain by inducing molecular and structural changes in the neuronal circuits. In response to ER and exercise a range of intracellular pathways are activated that modify metabolism, plasticity and stress resistance of the neurons. Brain derived neurotrophic factor (BDNF) plays a prominent role in the mediation of adaptive responses of neurons to energetic challenges. Aerobic exercise and intermittent fasting can increase BDNF expression and signaling in several brain regions, although the underlying cellular and molecular mechanisms remain to be determined. Beta hydroxybutyrate (BHB), a ketone produced by the liver during fasting and vigorous exercise can supply over 50% of the brain metabolic energy needs. We found that BHB enhances BDNF expression in primary cerebral cortical neurons. The protein level of intracellular BDNF and the activation of its downstream target ERK were higher in the neurons treated with BHB for 6 hours. Cellular bioenergetics was evaluated by measuring mitochondrial oxygen consumption and the glycolytic capacity when glucose was supplied to the treated and control neurons. The data show that BHB treatment increases the oxygen consumption rate and the production of ATP, possibly by supplying reducing equivalents (NADH) to the electron transport chain via the conversion of BHB to acetoacetate and acetyl CoA and its subsequent oxidation in the Krebs cycle. BHB also improved mitochondria health by reducing the amount of reactive oxygen species in the mitochondria. The glycolytic capacity in the BHB-treated neurons was higher compared to the controls. These data suggest that ketones could be beneficial in neurological disorders associated with disrupted cellular energy metabolism. Besides metabolic changes, long-term incubation of neurons with BHB resulted in an increase of mitochondrial mass that can be linked to the activation of BDNF- and PGC1alpha-mediated pathways. The increased number of mitochondria can sustain cellular energy substrates required for synapse formation; therefore, ketone bodies might promote axonal growth and synaptic plasticity. Our findings suggest that by increasing BDNF signaling and modifying energy metabolism of neurons, ketone

bodies produced in response to fasting, exercise and ketogenic diets may promote neuronal plasticity and resilience.

NIA
Emmette Hutchison
Postdoctoral Fellow
Neuroscience - General
An Approach for the Replacement of Adult Neurons through Direct Conversion of Reactive Astrocytes into Neurons
Loss of neurons from aging, injury or disease in the central nervous system (CNS) is devastating due to the relative lack of regenerative potential in the mammalian CNS. Mammalian astrocytes serve a number of functions during injury that make them an ideal therapeutic vehicle, including increased expression of the GFAP protein and migration to sites of injury or degeneration in the CNS. Recently a number of groups have demonstrated direct conversion of human and mouse astrocytes into neurons both in-vitro and in-vivo using defined transcription factors (TFs). Recombinant adeno-associated virus (rAAV) is a promising gene therapy tool presently undergoing clinical trials and is ideal given the specificity of genomic integration and lack of viral pathogenicity. Different serotypes of rAAV also display selective tropism for distinct cell populations, including neurons and astrocytes. Some serotypes have additional beneficial features such as the ability to cross the Blood Brain Barrier, a major biological barrier that prevents peripheral delivery of therapeutics from reaching the CNS. To investigate the potential of rAAV to convert reactive astrocytes into neurons we have designed viral vectors that drive expression of specific transcription factors, Neurogenin 2 (NGN2), Neurogenic Differentiation 2 (NeuroD2) and microRNAs (miR-124, miR-9) under the GFAP promoter and a fluorescent reporter under a universal promoter. We found that contrary to the literature, rAAV-driven expression of NGN2 does not convert astrocytes into functional neurons. We have had some success at conversion of astrocytes into neurons through expression of miR-124 and miR-9, two of the highest enriched microRNAs in CNS neurons. MicroRNA-124 in particular has been demonstrated by other groups to induce a pan-neuronal transcriptional profile on cells of non-CNS origin. Future AAV variants will test these two microRNAs in combination with NeuroD2 both in-vitro and in-vivo. These experiments seek to utilize reactive astrocytes to deliver replacement neurons to sites of injury or disease-related pathology in the CNS. If successful, this approach could have wide-ranging therapeutic potential for stroke, Alzheimerâ€™s disease and other neurodegenerative diseases.

NIA
Jie Ding
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Neuroscience - Neurodegeneration and Neurological disorders
Location of cerebral microbleeds: a new and specific neuroimaging marker predicting dementia risk in older people
Commonly observed in healthy older people, cerebral microbleeds (CMBs) have emerged as a new and important diagnostic marker of bleeding-prone small vessel disease in the brain. Importantly, CMBs resulting from amyloid deposits in the walls of cerebral blood vessels generally occur in lobar regions, whereas CMBs attributable to hypertension or atherosclerosis are located in the deep gray matter. However, little is known regarding the clinical impact of CMBs on cognitive decline and risk of dementia in late life, especially according to their location. We aimed to relate the presence and location of CMBs to newly developed dementia. We used data from men and women who were aged 65 years and older and had no dementia at the baseline exam (2002-2006) of the population-based AGES-Reykjavik study (n=2,612). Participants had brain MRI at baseline and again in 2007 to 2011. New dementia was

identified using a three-step screening and comprehensive diagnostic workup. We estimated the relative risks by Cox models while accounting for age, sex, education, depression, cardiovascular risk factors and the presence of other small vessel disease in the brain. Over the follow-up period there were 121 people who developed dementia, of whom 87 had Alzheimerâ€™s Disease (AD) and 22 had vascular dementia. Compared to people without CMBs at baseline, people with deep CMBs had almost 4-fold increased risk of developing vascular dementia but not AD. Moreover, there was a 2-fold increased risk of AD in people who developed new lobar CMBs over time and an 8-fold risk of vascular dementia in people who had new deep CMBs. Of note, these associations with new CMBs were independent of baseline CMBs. These results provide novel insight into the underlying mechanisms of cerebral small vessel disease in dementia and suggest an independent role for CMBs-associated vascular changes in dementia development. Further, lobar and deep CMBs may be unique markers of different dementia subtypes.

NIA
Chinmoyee Maharana
Visiting Fellow
Neuroscience - Neurodegeneration and Neurological disorders
Presenilin 1 Mutations Impair Neuronal Bioenergetics and Dietary Energy Restriction Ameliorates Cognitive Deficits Caused by a Presenilin 1 Mutation
Missense mutations in presenilin 1 (PS1) are the most common cause of early onset familial Alzheimerâ€™s disease (EOFAD) inherited in an autosomal dominant manner. PS1 is a transmembrane protein that forms the catalytic site of gamma-secretase complex, but PS1 may also have gamma-secretase independent functions in cells. AD patients, including those with a PS1 mutation, exhibit a deficit in brain cell energy metabolism that occurs relatively early in the disease process. We previously generated and characterized PS1 mutant M146V knock-in (PS1mutKI) mice which exhibit no amyloid-beta pathology, but show abnormalities in neuronal calcium homeostasis, synaptic plasticity, and increased neuronal vulnerability to energetic and excitotoxic stress. The present work aims to understand the roles of PS1 in cellular energy metabolism and the pathogenesis of EOFAD. To elucidate the impact of PS1 mutations on cellular bioenergetics, we used a cell-based model with a human neuroglioma cell line engineered to express in an inducible manner wild type (WT) or AD-causing mutant forms of PS1 that includes a range of ages of disease onset. We observed that PS1 mutations caused a reduction in mitochondrial membrane potential, increased vulnerability to glucose deprivation-induced degeneration, and an altered cellular respiratory capacity measured using an extracellular flux analyser (Seahorse). We next asked whether manipulations of dietary energy intake would modify cognitive function in PS1mutKI mice. WT and PS1mutKI mice were divided into different diet groups: control (C), intermittent fasting (IF) or high calorie diet (HCD). After 3-4 months of dietary intervention behavioral tests were performed. We found that WT and PS1mutKI mice on the IF diet exhibited improved spatial memory retention in a water maze test and greater preference for the novel object in an object recognition test compared to mice on the control diet. Contrarily, WT and PS1mutKI mice maintained on the HCD exhibited increased anxiety, measured using open-field and elevated plus maze when compared to WT and PS1mutKI mice on C or IF diets. Blood serum analysis further suggests a shift in energy metabolism in PS1mutKI mice on the IF diet indicated by elevated levels of serum ketones, and reduced levels of glucose, cholesterol and triglycerides compared to C or HCD mice. Thus, targeting neuronal bioenergetics by dietary intervention may have potential to counteract pathogenic actions of PS1 mutations.

NIA
Sarah Mitchell

Postdoctoral Fellow
Physiology
Sex, health and lifespan: untangling the response to calorie restriction in mice
Calorie restriction (CR) is the only established laboratory intervention that consistently delays the onset of aging and age-related diseases. However, recent evidence suggests that CR may not universal and that the response to CR in terms of health and lifespan may be dependent on more than just a reduction of calories without malnutrition. Understanding the molecular and genetic mechanisms underlying the effects of CR presents unique possibilities to further our understanding of normal aging. To investigate the role of CR in lifespan and healthspan we subjected two inbred mouse strains to lifelong CR at either 20% or 40%. We used male and female DBA/2J (D2) and C57BL/6J (B6) mice, which are classically considered unresponsive and responsive to CR, respectively, and performed longitudinal assessments of healthspan and assessed lifespan. Despite reduction in calories of 20 and 40% relative to ad libitum (AL)-fed mice, there was not the same proportional reduction in bodyweight across strain and sex of the mouse. Interestingly, AL mice lost body fat over time, while CR mice were able to preserve their body fat, in a sex- and strain-specific manner. One of the hallmark features of CR-mediated lifespan extension is preservation of insulin sensitivity. Here, serum insulin and glucose levels were proportionally reduced with increasing dose of CR in D2 mice, whereas 20% CR was the most effective in lowering these two parameters in B6 mice. D2 mice exhibited lifespan extension when maintained on 20% and 40% CR; however, 40% CR was detrimental to female B6 mice, with 20% CR having a beneficial effect on lifespan in both male and female B6 mice. DNA microarray analysis was then performed on the livers of 22-month-old mice. Principal component analysis showed clear separation between experimental groups based on strain, sex and diet (AL versus CR). Interestingly, no clear separation was observed between 20 and 40% CR in females of either mouse strain despite large difference in their lifespan, indicating that a discrete subset of genes could be implicated in CR-mediated lifespan extension instead of having a global, multidimensional alteration in gene expression profile. Thus, the principal tenets of CR may not be as universal as previously thought. Our data highlight the importance to systematically examine the contributions of sex and strain of the mouse vis-à-vis CR actions to further our understanding of normal aging.

NIA
Jessica Curtis
Postdoctoral Fellow
Stress, Aging, and Oxidative Stress/Free Radical Research
Regulation of expression of the citrate transporter Iâ€™m Not Dead Yet by the proinflammatory cytokine Interleukin-6
Metabolism is a highly coordinated process balancing extracellular energy supply with intracellular energy production and storage. Transport of circulating metabolites, such as tri- and di-carboxylic acids, is accomplished via movement though plasma membrane transporters encoded by the solute carrier (SLC) gene family. This diverse class of membrane-embedded transporters includes SLC13A5, a protein with high specificity for citrate and lower affinity for succinate. The orthologue of this gene in lower organisms is named Iâ€™m Not Dead Yet (INDY) since inactivation of INDY expression leads to a 50% increase in lifespan in flies and worms. To investigate the role of this protein in mammals, INDY knockout (KO) mice were generated. INDY KO mice display a phenotype, which resembles that of a calorie restricted animal, with smaller body size, improved glucose tolerance, and enhanced lipid homeostasis. By blocking the import of extracellular citrate, the cell depends more heavily on mitochondrial production of citrate, enhancing mitochondrial efficiency and reduces lipid synthesis and storage. INDY KO mice have elevated expression of mitochondrial fatty acid oxidation genes protecting them from deleterious consequences of a high fat diet such as insulin resistance. Interestingly, in human

liver biopsies, the expression of INDY is positively correlated with body mass index and insulin insensitivity, conditions associated with higher circulating levels of the proinflammatory cytokine interleukin (IL) â€“ 6. To investigate the role of IL6, primary human liver cells were treated in vitro with recombinant IL6. This led to an upregulation of INDY expression. Preliminary in silico analysis of the promoter region of the INDY gene indicates a potential binding site for the transcription factor Signal Transducer and Activator of Transcription (STAT)-3, indicating that expression of INDY may be mediated by the proinflammatory signaling pathways typically induced by diet-induced obesity. Studies are currently underway to determine STAT3 binding to the INDY promoter by chromatin immunoprecipitation. In conclusion, inhibition of INDY expression may be beneficial for human metabolic health, with implications for the treatment of hyperlipidemia, insulin resistance, and obesity by pharmacologically inhibiting the expression or function of INDY.

NIA
Huiming Lu
Postdoctoral Fellow
Stress, Aging, and Oxidative Stress/Free Radical Research
Senescence triggered by a deficiency of RECQL4 contributes to clinical features of Rothmund-Thomson Syndrome in mice
Senescence, defined as an irreversible cell cycle arrest, contributes to the decline of stem cells, which results in degeneration of tissues and aging-related diseases. RecQ helicase RECQL4 plays an important role in maintaining genome integrity. Defects in RECQL4 cause three human diseases, Rothmund-Thomson Syndrome (RTS), RAPADILINO and Baller-Gerold Syndrome. These diseases share clinical features, like short stature, skeletal abnormalities and radial ray defects. RTS patients also have skin maladies, sparse hair and hematological disorders. However, how RECQL4 loss causes these features is unclear. RECQL4 is highly expressed in proliferative cells, indicating its importance in these cells. We hypothesized that senescence may be triggered by RECQL4 dysfunction in proliferative cells like stem cells, contributing to pathological features of RTS. We first investigated senescence in control and RTS mice and identified two novel features in the RTS mice. Relative to their wild type littermates, the RTS mice only have 50% of hair intensity on their tails and 54% of normal white blood cells. The RTS mice accumulated more 53BP1 foci, a DNA damage marker, and senescence-associated beta-galactosidase (SA-b-gal) staining in their hair follicles and bone marrow cells than wildtype mice. These cells are responsible for hair growth and blood cell production, indicating that a RECQL4-deficiency predisposes these cells to senescence. To delineate RECQL4â€™s role in prevention of senescence in human cells, we depleted RECQL4 with shRNA in human primary fibroblasts and measured senescence features. Relative to control cells, RECQL4-depleted cells displayed increased SA-b-gal staining, cell proliferation defects, and increased persistent DNA damage foci, suggesting that RECQL4 inhibits senescence in human cells. Moreover, RECQL4 loss causes cells to senesce via ATM/p53/p21 and Rb/p16 pathways. We further identified that ectopic expression of the N-terminal and helicase domains of RECQL4, involved in replication and DNA repair, can rescue RECQL4 knockdown-induced senescence. These results have implications for patients with mutations in the N-terminal or helicase domains of RECQL4 as they are at greater risk for senescence-induced tissue degeneration. In conclusion, loss of RECQL4 increases persistent DNA damage and triggers premature senescence, which results in degeneration of tissues and may contribute to the progress of clinical features in RTS patients.

NIAAA
Dechun Feng
Visiting Fellow
Immunology - General

A Cre-inducible human CD59 mediates rapid and efficient cell lineage ablation model after intermedilysin administration

Conditional and targeted cell ablation is a powerful and widely used approach for studying specific cellular functions as well as tissue repair in vivo. Currently, the most commonly used approach for cell ablation involves cell specific transgenic and/or Cre recombinase (Cre)-inducible expression of the diphtheria toxin (DT) receptor gene in mice. However, this method has several inevitable shortcomings. First, it cannot be used for ablating cells that are not actively synthesizing proteins, such as erythrocytes because DT toxicity is mediated by protein synthesis inhibition. Second, overexpression of DT receptor may cause abnormalities in mice. Finally, it takes a relatively long time (several days) to ablate target cells. Here we used the proven concept that intermedilysin (ILY) specifically lyses human CD59 (hCD59)-expressing cells by exclusively binding to hCD59 rapidly and potently lyses cells by forming large-diameter (250â€“300 A) irreversible transmembrane pores, which disrupts the membrane permeability barrier and causes cell lysis in a few seconds. To streamline this approach, we established floxedSTOP-hCD59 (ihCD59) knock-in mice where hCD59 expression only occurs following Cre-mediated recombination. To further characterize the ihCD59 mice, we crossed ihCD59 mice with Lysozyme-Cre (Lyz-Cre) or Albumin-Cre (Alb-Cre) mice, and demonstrated that ihCD59/Lyz-Cre mice expressed specifically hCD59 only on myeloid cells (neutrophils, monocytes and dendritic cells) but not on other cell types, while ihCD59/Alb-Cre mice expressed hCD59 only in hepatocytes. In ihCD59/Lyz-Cre mice, ILY injection ablated ~90% hCD59 expressing myeloid cells in blood within 10 minutes. In ihCD59/Alb-Cre mice, ILY treatment dramatically increased the serum ALT (a marker for hepatocyte death) levels in as early as 10 min post injection by lysing hCD59 expressing hepatocytes. The peak of ALT levels occurred 2h after 150 ng/g ILY injection with >10000 U/L serum ALT levels (compared with peak levels of ALT at 500 U/L 24h after DT treatment in DT system), while the serum ALT levels ihCD59 mice without Alb-Cre expression remained in baseline levels after ILY injection. These results demonstrates the specificity and potency as well as the lack of off-target effects in our model. In summary, we established a novel rapid and efficient Cre-inducible cell ablation system, which represents an alternative model applicable to studies of cell function, tissue regeneration and differentiation.

NIAAA
Ozge Gunduz Cinar
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Neuropharmacology and Neurochemistry

Antidepressant treatment inhibits fear via brain endocannabinoids

Endocannabinoids are key modulators for protection and recovery from stress and traumatic experiences. Previous work has shown that augmenting endocannabinoid anandamide, via inhibition of the catabolic enzyme fatty acid amide hydrolase (FAAH), facilitates the learned inhibition of fear (i.e., extinction) in mice. This effect is mediated via CB1 receptors in basolateral amygdala and is associated with facilitation of synaptic plasticity (long-term depression of inhibitory transmission). Interestingly, recent studies demonstrate that chronic treatment with the selective serotonin reuptake inhibitor (SSRI) fluoxetine, a first-line therapeutic treatment for many anxiety and stress-related neuropsychiatric conditions, facilitates fear extinction and promotes amygdala plasticity in a similar manner to FAAH inhibitors. However, it is currently unclear whether fluoxetineâ€™s effects on fear extinction involve functional interactions with endocannabinoids. To investigate this, we first assessed changes in endocannabinoids in basolateral amygdala and other extinction-mediating brain regions following chronic fluoxetine. This revealed a significant elevation in levels of the endocannabinoid anandamide in the basolateral amygdala as well as dorsal striatum and dorsal hippocampus following chronic fluoxetine treatment. We next tested whether the increased endocannabinoid tone after fluoxetine mediated the drugâ€™s extinction-facilitation effects by blocking CB1 receptors, via Rimonabant, either systemically

or only in the basolateral amygdala. Systemically delivered Rimonabant abolished the extinction-facilitation effects of fluoxetine. Intra-amygdala microinjection of Rimonabant also blocked these effects. Future experiments will examine the effects of fluoxetine on CB1 receptor-mediated synaptic plasticity in the basolateral amygdala, and identify key downstream signaling pathways (including protein phosphatases) that underlie fluoxetine-endocannabinoid interactions in the amygdala. Together, these findings demonstrate a novel, obligatory role for endocannabinoids in the fear inhibiting effects of a major pharmacotherapy for anxiety disorders.

NIAAA
Lauren Dobbs
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Neuroscience - Cellular and Molecular
Hypersensitivity of D1R signaling following deletion of striatal D2R facilitates cocaine sensitization and reward
Cocaine is a highly addictive psychostimulant drug that increases extracellular levels of dopamine in the nucleus accumbens (NAc). The NAc is a part of the mesolimbic pathway that is critically involved in reward motivated behaviors and it expresses a high density of two subtypes of dopamine receptors: Gs-coupled D1 receptors (D1R) and Gi-coupled D2 receptors (D2R). Low levels of D2R in the NAc are found in humans that abuse cocaine and are thought to render subjects vulnerable to developing addiction. However, the mechanism by which low levels of D2R predispose subjects to abuse cocaine is unknown. So far it has been difficult to determine the contribution of D1R and D2R to the behavioral response to cocaine because D2R are expressed on many different neuronal types within the striatum, and pharmacological agents, such as agonists and antagonists, act on D2R on all neuronal types. In this study, we used a Cre-loxP approach to generate transgenic MSN-D2 KO mice, which lack D2R only in one subpopulation of GABAergic projection neurons, the indirect pathway medium spiny neurons (iMSN). Deletion of D2R from iMSNs unmasked a robust collateral inhibition on the other major subpopulation of GABAergic projection neurons, the direct pathway MSNs, and resulted in a severe blunting of the locomotor response to acute cocaine, which is rescued by restoring Gi-signaling in iMSNs. Surprisingly, MSN-D2 KO mice acquired conditioned cocaine reward twice as fast as wild type controls and showed a sensitized locomotor response to repeated cocaine exposure that was enhanced relative to wild type littermates. This was accompanied by D1R hypersensitivity, shown by a leftward shift in the locomotor response and an upregulation of ERK signaling in the NAc in response to a D1R agonist. We hypothesized that D1R hypersensitivity mediates cocaine sensitization and this mechanism is increased in the MSN-D2 KO mice. Indeed, after repeated cocaine injections, mice that show locomotor sensitization had a larger locomotor response to a D1R agonist compared to cocaine naïve mice. Altogether, this study demonstrates that D2R in iMSNs are necessary for the acute locomotor response to cocaine, possibly by relieving the collateral inhibition that restrains activity of direct pathway MSNs. This study also reveals that low levels of D2R in the NAc cause D1R hypersensitivity, which we speculate facilitates the development of cocaine locomotor sensitization and reward.

NIAAA
Hadley Bergstrom
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Neuroscience - Integrative, Functional, and Cognitive
The dorsolateral striatum competes with visual discrimination learning
Brain processes of reward learning are essential for shaping and guiding decision-making. Dysfunctions of reward learning can lead to maladaptive behaviors such as addiction. The dorsolateral striatum (DLS) is required for mediating various forms of reward learning. Recent evidence suggests that either lesions

or pharmacological inactivation of the DLS attenuates reward learning. Despite a known role for the DLS in reward learning, precisely how the DLS contributes to reward learning, and which of the various components of reward learning it mediates is unknown. Here we investigated DLS-mediated reward learning using optogenetic tools in vivo. To probe reward learning, we used an operant task in which mice must first visually discriminate objects presented on a touchscreen display and then physically touch the one that predicts an appetitive reward. Prior to visual discrimination learning, an adeno-associated viral construct (rAAV8/CAG-ArchT-GFP) was bilaterally expressed in a population of DLS neurons in C57BL/6J mice. ArchT-infected DLS neurons were then illuminated with green light for rapid and reversible DLS inhibition only when the mouse made a response or collected a reward. We found DLS inhibition at the time when mice made a response facilitated the rate of learning, as compared to a control group expressing an inactive virus (rAAV8/CAG-GFP). This surprising result demonstrates that the DLS interferes with reward learning (stimulus-response) associations. Facilitation of learning was already evident at the first (of multiple) training sessions, suggesting the DLS engages reward-learning processes at its earliest stages. By contrast, DLS inhibition at the time when mice collected the reward did not impact performance, indicating the DLS does not process (at least not directly) response outcome signals (reward receipt). Overall, these findings support a dynamic model of DLS-mediated functioning in which the DLS competes with stimulus-response relationships at the earliest stages of learning. Optogenetics in vivo combined with a challenging task of higher-order cognition in mice was essential for unmasking a previously unidentified role for the DSL in visual discrimination learning. Given that addictive drugs directly target and modify DLS circuits, these data provide new fundamental insight into both normal and abnormal DLS functioning.

NIAAA
Nicholas Jury
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Psychiatry

Effects of chronic intermittent ethanol vapor exposure in adolescent and female mice

It is estimated that 10% of the U.S. population has an alcohol use disorder. The medical and economic consequences are estimated to cost the U.S. economy over $185 billion annually. Despite the economic ramifications, the etiology of alcoholism is not currently known. Many studies have attempted to identify the cause(s) of alcohol dependency. However, the majority of these clinical and pre-clinical studies have only utilized adult, male subjects. Recent reports have demonstrated that alcohol abuse is on the rise in women and adolescents. In order to fully understand the etiology of alcohol dependency and the effects of chronic alcohol abuse future studies should not be restricted to adult male subjects, but should also include female and adolescent subjects as well. A growing body of literature has demonstrated various behavioral and neural effects of chronic intermittent ethanol (CIE) exposure in mice. The majority of these studies have only investigated these effects in adult, male mice, despite known differences in ethanolâ€™s effects in young and female rodents. The goal of the current study was to examine the behavioral effects of CIE in male and female adolescent and adult C57BL/6J mice. Mice were exposed to CIE through vapor inhalation over 4 weeks and then tested for ethanol tolerance (via measurement of the loss of righting reflex) and ethanol preference (2-bottle drinking). Adolescent mice were CIE exposed from 4 to 8 weeks of age; adults were exposed from 9 to 13 weeks of age. All mice were therefore tested post-CIE as adults (i.e. at least 8 weeks of age). Results showed that CIE produced ethanol tolerance (as compare to controls), irrespective of age or sex. In the absence of CIE exposure, adolescent and female mice showed greater ethanol consumption and/or preference than their corresponding adult or male controls. CIE exposure led to an increase in ethanol consumption and/or preference in adult male mice, but not in adolescents or females. These novel findings suggest there are differential effects of CIE on ethanol drinking in adolescent and female mice. The current

results establish evidence for future studies on how age and sex modify the lasting consequences of chronic ethanol exposure, with potential implications for understanding how these factors influence risk for alcoholism in humans.

NIAID
Andrezza Campos-Chagas
Visiting Fellow
Biochemistry - Proteins
Sicpin, the first immunomodulatory salivary protein described in black flies significantly reduced T and B cell proliferation and directly binds to soluble CD4 receptor
Hematophagy is key to blood feeding arthropods reproductive success and an important link in pathogen transmission cycles. Salivary gland homogenates from blackflies have been shown to contain immunomodulatory activity on murine splenocytes. However, the molecule(s) responsible for this salivary activity remains elusive thus far. Here, we report the first immunosuppressive protein from blackfly salivary glands. Sicpin (Simulium cell proliferation inhibitor) was produced in E. coli and purified using size exclusion and ion exchange chromatography. Purified Sicpin was LPS-decontaminated and its sequence verified by N-terminal sequencing and LC-MS/MS analysis. Sicpin inhibited cell proliferation in a dose-response manner independently of the mitogen utilized (ConA, LPS, CD3/CD28 and Pokeweed). LPS or ConA stimulated cells had a significant lower proliferation rates (P<0.001) in the presence of Sicpin (IC50=0.5uM) with 10uM completely abrogating cell proliferation. Flow cytometry analysis showed that Sicpin inhibited proliferation of CD19+ B-cells and CD4+/CD8+ T-cells. Sicpin did not induce apoptosis or necrosis in mitogen-induced proliferative responses by murine splenocytes as determined by flow cytometry. Sicpin also inhibits antigen-specific cell proliferation without inducing apoptosis in resting or mitogen-induced splenocytes. We demonstrate that the production IFN-alpha, IL4, IL5, IL6 and IL10 by splenocytes stimulated by ConA or LPS was dose-dependently reduced by Sicpin. Reduction of cytokines in presence of Sicpin could lead to a retardation of B and T cell activity. Carrageenan-induced paw-edema model showed that the intensity of edema significantly decreases when Sicpin was administered at 5 and 15 ug/animal. The molecular mechanism of Sicpin on cell proliferation inhibition is currently under investigation; however, initial binding experiments using SPR analysis showed a direct binding to soluble CD4 receptor with a calculated KD of 17.77 nM. Direct binding of Sicpin to CD4 could inhibit the subsequent TCR ligation-induced T cell signaling at the earliest steps including tyrosine phosphorylation of the receptors, downstream effector proteins, and lipid raft reorganization. The immune suppressive and anti-inflammatory properties of Sicpin should be explored as a strategy to modulate immune responses in infection and tumor proliferation as well as its involvement in parasite transmission

NIAID
Dongying Ma
Visiting Fellow
Biochemistry - Proteins
Desmolaris, a novel factor XIa anticoagulant from the salivary gland of the vampire bat
Since the early 1930s, it has been reported that the saliva of vampire bats exhibits anticoagulant properties. However, the identity of the saliva anticoagulant remained elusive for the past 80 years. Here we have finally identified the coagulation inhibitor from the salivary gland of the bat Desmodus rotundus, which is designated as Desmolaris. Desmolaris is a novel 21.5-kDa naturally deleted (Kunitz 1-domainless) form of human tissue factor pathway inhibitor (TFPI). Recombinant Desmolaris was expressed in HEK293 cells and characterized as a slow, tight, and noncompetitive inhibitor of FXIa by a mechanism modulated by heparin (KD in pM range). Desmolaris also inhibits FXa with lower affinity,

independently of protein S. In addition, Desmolaris binds kallikrein and reduces bradykinin generation in plasma activated with kaolin. Comparison the activities of truncated- and mutated (R32L)-forms of Desmolaris determined that Arg32 in the Kunitz-1 domain is critical for protease inhibition. Moreover, Kunitz-2 and the C-terminus domains mediate interaction of Desmolaris with heparin and are required for optimal inhibition of FXIa and FXa. Notably, Desmolaris (100 µg/kg) inhibited FeCl3-induced carotid artery thrombus without prolonging bleeding time and reduced collagen/epinephrine mediated thromboembolism in mice. Desmolaris also reduced the polyphosphate (polyP)-induced vascular permeability and blocked the inflammatory effects of FXa in vivo. The results elucidate that Desmolaris is the anti-hemostatic and anti-inflammatory molecule which is helpful to fulfill the blood sucking process of vampire bat. In the future, Desmolaris could potentially be used as an anticoagulant or as a template for the development of new therapies to prevent and treat thrombosis. Desmolaris is fast-acting and effective at lower concentrations. In addition, Desmolaris does not promote bleeding at concentrations needed to prevent clots formation in mice suggests that Desmolaris-based therapies may be safer than current clinical anticoagulant drugs, which carry side effects of bleeding.

NIAID
ERI SAIJO
Visiting Fellow
Cell Biology - General
Using antibodies to reveal conformational differences between prion strains
Accumulation of misfolded proteins in the brain can cause various fatal neurodegenerative disorders including prion diseases. The mammalian prion diseases involve the conversion of the normal, cellular, form of prion protein (PrPC), into the disease-causing PrP (PrPSc), and PrPSc provides the template that induces this conversion. The misfolding of PrPC (an alpha-helix rich structure) results in largely beta-sheet conformation, which can form into fibrils and aggregates. However, the structures of the disease-associated PrPSc are poorly understood. Even though the existence of prion strains have been observed and classified based on various properties of prions, e.g. different incubation time, and variable neuropathology and biochemical properties, the conformational differences between prion strains have not been clearly understood. In order to investigate the structures of the disease-associated PrPSc and their strain variations, we studied the structure of PrPSc using antibodies to different epitopes by comparing purified PrPSc from brains of mice with either the Chandler or 22L scrapie strains or hamsters with 263K scrapie. Epitope mapping of PrPSc was performed under native and guanidine-denatured conditions by indirect-ELISA. Our results using different antibodies showed that only a small subset of antibodies recognized epitopes in the native structure of PrPSc, specifically, near the N-terminus. The purified PrPSc from the Chandler and 22L strains carries the same primary structure of mouse PrP but has different reactivities to the C-terminal antibody. Although it has long been clear that prion strains can have different conformations in the N-terminus of PrPSc, this is the first study that shows that the conformational-dependent C-terminal PrPSc antibody can distinguish two different strains. Further, our results suggest that the conformational differences in the C-terminus of PrPSc might contribute to the differentiation of prion strains as well.

NIAID
Pragyan Acharya
Visiting Fellow
Gene Expression
VAR2CSA, a placental malaria specific cytoadherent antigen, is involved in severe malaria of children.
Severe malaria (SM) is a debilitating and often fatal manifestation of Plasmodium falciparum infection in African children under 5 years, with features such as coma, severe anemia, metabolic acidosis, renal

failure, convulsions and hypoglycemia. The large majority of malaria episodes, even in children, have only mild sequelae, and the host-parasite interactions that cause severe disease are unknown, although parasite sequestration in deep vascular beds and host inflammation are thought to be key virulence factors. Our study seeks to identify genes that are specifically upregulated in SM parasites. We investigated SM and MM patients from 4 cohorts from 2 geographically distinct locations in Africa- Tanzania and Mali. Microarray and RNA seq were performed on parasite RNA from Tanzania and RNA Seq and qRT-PCR were performed on parasite RNA from Mali. Parasite gene expression data were compared in two different ways (i) All SM parasites were compared against all MM parasites, (ii) SM parasites and MM parasites from different infection episodes for the same child were compared with each other to minimize differences due to host variability. Meta-analysis of gene expression data revealed a consistent transcriptomic signature for SM parasites irrespective of geography, study design or methodology. SM parasites showed a distinct upregulation of several hypothetical proteins and some proteins with known functions. Interestingly, we found that SM parasites consistently upregulated VAR2CSA, a variant surface antigen that binds chondroitin sulfate A linked proteoglycans on the placenta leading to placental sequestration of parasites. Placental sequestration causes localized inflammation associated with severe maternal anemia and low birth weight. We speculate that VAR2CSA expression during severe disease may be involved in localized inflammation and explain some aspects of disease pathogenesis. We are now confirming that gene expression differences translate to functional differences by detecting VAR2CSA protein in SM and MM parasites by IFA, FACS and western blot. In summary, we have identified a parasite factor that may be involved in the pathogenesis of SM and could be a potent vaccine candidate for two devastating forms of malaria- severe malaria as well as placental malaria.

NIAID
Agnes Hajduczki
Postdoctoral Fellow
HIV and AIDS Research
Developing soluble co-receptor mimetics for the study of HIV Env/receptor interactions
The HIV envelope glycoprotein mediates virus entry by initiating fusion of the viral envelope with the cell membrane upon receptor engagement. The surface-exposed gp120 subunit is a dynamic molecule that undergoes a series of stepwise conformational changes in response to interactions with the primary receptor CD4, and co-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 with seven membrane-spanning helices, 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 the context of the membrane is not a viable option. This project aims to develop a recombinant soluble co-receptor mimetic featuring the two critical co-receptor determinants of CCR5 (N-terminus and extracellular loop 2) using two approaches: fusing the CCR5 moieties to soluble two-domain CD4 by flexible polypeptide linkers and using a globular, stable scaffold protein to orient the co-receptor determinants in an optimal conformation to recreate the binding interaction with gp120. We have successfully overexpressed and purified both variants from mammalian cells. The N-terminus of CCR5 has been shown to contain sulfated tyrosines necessary for co-receptor function. ELISA experiments confirmed that sulfotyrosines are present in our recombinant proteins. Characterization of the gp120-binding properties of the variants is underway using a vaccinia-based cell fusion assay where soluble CD4 has been shown to induce membrane fusion between Env-expressing effector cells and target cells bearing CCR5, but no CD4. When soluble CD4 fused co-receptor mimetic is added to the fusion reaction, there is strongly

impaired activation of membrane fusion compared with sCD4 alone, suggesting that the CCR5-derived portion of the protein competes with cell-surface CCR5 for binding to gp120. Conversely, the mimetic displays stronger neutralization activity than sCD4 in a standard fusion assay. The soluble variants of CCR5 will be used to elucidate the conformational changes in gp120 that immediately precede membrane fusion, and potentially for collaborative high resolution structural analyses of the gp120-coreceptor complex.

NIAID
Tej Pratap Singh
Visiting Fellow
Immunology - Autoimmune

IL-23-induced psoriasis-like inflammation in mice depends on CCR6-sufficient blood monocytes and the accumulation of monocyte-derived dendritic cells in the skin

Human studies have strongly implicated IL-23 in psoriasis, and injecting IL-23 in mouse skin every other day over six days produces psoriasis-like inflammation. IL-23 injection leads to accumulation of dendritic cells (DCs), which are also abundant in psoriatic skin. In analyzing myeloid cells, we found monocyte-derived langerin-positive cells (moLCs) appearing in the epidermis, and increased numbers of other monocyte-derived cells, including moDCs and macrophages, plus non-monocyte derived DCs in the dermis after IL-23 injection. Depletion of all CD11c+ cells in diphtheria toxin-treated CD11c-DTR mice blocked IL-23-induced up-regulation of IL-22, IL-19, IL-17, IL-36, IL-1beta and s100A, as well as the psoriasis-like changes. IL-22 and IL-17 are known to be important mediators of pathology in this model, and using Il1r1-/- mice, we also identified a major role for IL-1. Despite the dramatic effects of depleting CD11c+ cells, Batf3-/-, diphtheria toxin-treated Bdca2-DTR, and Flt3l-/- mice, which lack CD103+ cDCs, pDCs, and all cDCs and pDCs, respectively, showed, in each case, only modest protection against IL-23-induced changes. Although depleting conventional LCs alone had no effect, inflammation was much diminished in langerin-DTR mice also depleted of moLCs. Together, these data suggest that CD11c+ moDCs and moLCs contribute significantly to pathology. Previously, we have reported that Ccr6-/- mice are resistant to IL-23-induced skin inflammation, and the skin of Ccr6-/- mice showed no changes in numbers of CD11c+ cells in dermis or epidermis after IL-23 injection. Repeated and selective depletion of Ccr6+/+ (but not Ccr6-/-) CD11C+ cells using mice reconstituted with a mixture of bone marrows from Ccr6-/- and (Ccr6+/+) CD11c-DTR mice revealed that CCR6 was required for the recruitment and/or activity of the pathologic CD11C+ cells or their immediate precursors. Using Ccr6-EGFP mice, we identified expression of Ccr6 in blood monocytes and we demonstrated that monocytes migrate to the CCR6 ligand, CCL20. Importantly, we found that although both Ccr6+/+ and Ccr6-/- monocytes restored inflammation when injected, together with IL-23, into ears of Ccr6-/- mice, only the Ccr6+/+, but not Ccr6-/-, monocytes were effective if the cells were injected intravenously. Together, our data suggest that blood monocytes are recruited to inflamed skin using CCR6 and give rise to moDCs and moLCs, which are critical for IL-23-induced psoriasis-like pathology.

NIAID
Gautam Sule
Visiting Fellow
Immunology - Autoimmune

Identification of a suppressor of autoimmune disease that affects deoxynucleotide synthesis in T cells

FcgRIIB-deficient mice represent a well-characterized animal model of systemic lupus erythematosus. They develop spontaneous anti-nuclear antibodies (ANA) and fatal glomerulonephritis when on the C57BL/6 (B6) background (B6.FcgRIIB -/- mice). In contrast, the same mutation on the BALB/c background (BALB.FcgRIIB -/- mice) is phenotypically benign, indicating differences in lupus susceptibility

between the BALB/c and B6 strains. After extensive backcrossing, we narrowed down the genomic interval to a 151KB genomic fragment on chromosome 12 responsible for the suppressive/protective effect in BALB/c mice. We generated a transgenic mouse line expressing this BALB/c genomic region directly in the C57BL/6 background (A12 Tg) and subsequently crossed to B6.FcgRIIB -/- mice to test the putative protective effect. The BALB/c-derived A12 transgene was able to suppress the spontaneous disease that normally develops in B6.FcgRIIB -/- mice: it reduced spontaneous germinal center formation, serum autoantibody titers and proteinuria. The A12 transgene also reduced the number of activated and memory T cells, as well as the number of follicular helper T cells when compared to the B6.FcgRIIB -/- mice. Additional studies suggest that reduction in the autoimmune phenotype is T cell-intrinsic. The A12 protective interval contains a single gene, Rrm2, which encodes for a subunit of the ribonucleotide reductase responsible for the synthesis of deoxyribonucleotides. We hypothesize that reduced levels of this enzyme affect the ability of T cell to expand upon activation and that is why allelic differences can alter tolerance in mouse models of lupus. In the future we will test the ability of this genetic element to suppress autoimmunity in other susceptible strains/lupus models to identify the translatability of this element as a target for therapy.

NIAID
Jing Wang
Visiting Fellow
Immunology - General
The autoinhibitory cSH2 domain of phospholipase gamma 2 stabilizes B cell receptor early signalosomes
Antigen binding to the B cell receptor (BCR) triggers the assembly of a signaling complex composed initially of the kinases Lyn, Syk, and Btk and the adaptor, BLNK, that together recruit and activate PLC gamma 2 (PLCg2), a critical effector that triggers increases in intracellular calcium and activates a variety of vital, downstream signaling pathways. Individuals with one copy of a mutant of PLCG2 gene, lacking the autoinhibitory domain, cSH2, show PLCg2-associated antibody deficiencies and immune dysregulation (PLAID). Here we shows that the B cells from PLAID patients only transiently phosphorylate Syk, Btk and BLNK in response to BCR crosslinking resulting in dysregulation of downstream signaling to the MAP kinase, Erk, and NF-?B even though the phosphorylation of the BCRâ€™s Ig-alpha and activation of PI3K are normal. In addition, although the BCRs on PLAID B cells internalized normally into early endosomes following antigen binding, they fail to enter antigen-processing compartments, suggesting an additional mechanism for immune dysregulation in PLAID individuals. By TIRF microscopy, in response to anti-IgM incorporated into fluid lipid bilayers human peripheral blood B cells transiently expressing PLAID mutant (PM) PLCg2 fail to form stable BCR clusters and to accumulate BCRs in the contact area between the B cell and the bilayer. Two-color time-lapse TIRF imaging showed that PM PLCg2 was recruited to the B cell plasma membrane following BCR crosslinking but only transiently colocalized with BCRs. Similarly, pBtk and pSyk showed only transient association with activated BCRs in contrast to the stable colocalization of pLyn and pPI3K. Lastly, although B cells expressing WT and PM PLCg2 both recruit the negative regulator of BCR signaling, pCbl, to the immune synapse, in B cells expressing WT PLCg2, pCbl was excluded from the central areas in which the BCRs accumulated in contrast to B cells expressing PM PLCg2, in which pCbl colocalized with the BCR. Taken together these data provide evidence for a novel role of the autoinhibitory cSH2 domain of PLCg2 acting in a dominant negative fashion to destabilize early BCR signaling complexes resulting in the recruitment and colocalization of pCbl and dysregulation of both downstream signaling and antigen transport.

NIAID
Christoph Wilhelm

Research Fellow
Immunology - General
Adaptation of Intestinal Barrier Immunity in Response to Nutrition
The intestinal immune system has developed to maintain the mucosal barrier in the face of a diversity of challenges such as the commensal microbiota, chronic worm infections and recurrent infections with pathogens. Innate lymphoid cells (ILC) play important roles in the maintenance and defense of the intestinal barrier. Type 2 ILC (ILC2) produce the cytokines IL-5 and IL-13 and mediate tissue repair, mucus production and anti-helminth responses. In contrast type 3 ILC (ILC3) facilitate anti-bacterial responses via the expression of IL-22. Over the course of evolution maintenance of barrier immunity had to adapt to periods of unstable nutritional uptake. Interestingly, despite impaired adaptive immune responses associated with malnutrition humans can often survive extended periods of severe dietary restrictions. This suggests the existence of compensatory mechanisms to maintain barrier function in such settings. To investigate how the intestinal immune system can adapt to the loss of adaptive immune responses during malnutrition, we used an experimental model of vitamin A deficiency, still one of the most common nutrient deficiencies worldwide. Intriguingly, in mice fed a vitamin A deficient diet the accumulation of intestinal ILC3 and their production of IL-22 was significantly impaired if compared to animals fed vitamin A sufficient diets. Paradoxically, we also observed a dramatic increase of ILC2 coinciding with elevated levels of IL-13 in Vitamin A deficient (VAD) mice. This effect was mediated by the absence of retinoic acid (RA), the main vitamin A metabolite, since treating mice with a pharmacological inhibitor to RA signaling recapitulated our initial findings. In line with impaired IL-22 production and ILC3 numbers in the absence of vitamin A or RA, mice showed impaired immunity upon infection with an intestinal bacterial pathogen. Strikingly, immunity to helminths was unaffected or even accelerated upon infection with worms, but not in mice treated with depleting anti-IL-13 antibody. Since helminths still represent the major form of parasitic infection worldwide and nematodes compete with the host for nutritional resources, the reinforcement of anti-helminth immunity could provide a substantial advantage to the host in periods of malnutrition. Thus, our findings identify ILC2 mediated barrier immunity as a potential compensatory mechanism in the context of malnutrition to facilitate prolonged survival in the absence of acute bacterial infections.

NIAID
Denise Fonseca
Visiting Fellow
Immunology - Infectious Disease
Long-term consequences of infection-induced structural changes secondary lymphoid organs
The gastrointestinal tract (GI) needs to tolerate constant exposure to innocuous antigens while simultaneously maintaining the capacity to respond to pathogens. Defined GI infections have been proposed as triggering factors in the initiation of inflammatory diseases such as inflammatory bowel disease (IBD). Further, children exposed to repeated GI infections fail to respond to oral vaccination. However, the mechanistic link between these severe sequels of mucosal infections remains unclear. To study the long-term impact of GI infections in the mucosal immunity, we developed an experimental model of acute mucosal infection, by using Yersinia pseudotuberculosis that closely mimics the human disease. Patients infected with Y. pseudotuberculosis develop massive inflammation of the mesenteric lymph node (MLN) characterized by a central necrotic granuloma with coincident MLN enlargement, and predisposition to IBD development. Similarly, mice orally infected with the bacteria develop a significant inflammation in the MLN, spleen and liver, marked by neutrophils/inflammatory monocytes recruitment and activation of Th1/Th17 adaptive immunity. Mice develop massive fibrotic granulomas and abscesses in the MLN that persist months after the resolution of the infection and clearance of the bacteria. The structure of the MLNs is severely compromised, with substantial changes in the B- and T-cell zones, in

the organization of lymphatic vessels, fibroblastic reticular cells and CCL21 chemokine production. As a result of these structural changes, a specific subtype of migratory dendritic cell subpopulation (CD103+CD11b+ DCs) cannot reach the MLN and are attracted to the perinodal adipose tissue due to changes in the inflammatory status of this mesenteric fat tissue. The absence of CD103+CD11b+ DCs in the MLN is associated to impaired development of regulatory response to oral antigen, defects in IgA production and Th17 adaptive immunity to oral vaccine. Together, our results support the idea that defined GI infections can be associated with severe and permanent remodeling of secondary lymphoid structures leading to impaired acquisition of oral tolerance and response to vaccine. Such scaring of the immune system can have severe implications for GI tract integrity and predisposition to inflammatory diseases.

NIAID
Jonathan Linehan
Postdoctoral Fellow
Immunology - Infectious Disease
The role of bacteriophages in driving a unique skin commensal-specific immune response
The skin is home to a diverse array of microbes termed commensals. Commensals play a fundamental role in the education and function of the host immune system. Our laboratory recently demonstrated that skin-resident commensals control immunity to pathogens. However, little is known about the mechanisms by which this occurs. Moreover, whether individual commensals have the ability to modulate immunity remains unclear. We found that genetically distinct strains of the ubiquitous bacterial human skin commensal Staphylococcus epidermidis (S.epi) stimulated a robust S.epi-specific T cell response, with a unique immunological signature, when applied to the skin of mice. Comparative genomic analysis of stimulatory strains with non-stimulatory strains revealed that stimulatory S.epi strains share three genetic loci, each containing groups of less than 20 genes. Of particular interest two of these loci encode for putative prophages. From these data, we hypothesized that phages could be responsible for the observed T cell response. To test this, we treated one of the stimulatory strains with a low concentration of the DNA crosslinking agent Mitomycin C. This treatment induced a stress response in S.epi causing the prophages to be excised from the genome and bacteriophages (phages) to be secreted into the culture medium. A non-stimulatory strain of S.epi was then infected with purified phage supernatant. Subsequently, we isolated a pure culture of a clonal, phage-infected, non-stimulatory S.epi strain. Surprisingly, when this bacterium was applied to the skin of mice, the T cell response phenocopied the response to a stimulatory S.epi strain. These results reveal that specific phages, residing in distinct strains of a common human skin commensal, can drive a unique adaptive immune response in the skin. This striking finding is the first to link phages with a tissue-specific immune response and has important implications for our understanding of tissue immunity and the future development of novel vaccines aimed at inducing protective immunity to pathogens.

NIAID
Scott Nash
Postdoctoral Fellow
Immunology - Infectious Disease
An Elite Infection-Control Phenotype with Immunological Correlates in a Tanzanian Birth-Cohort exposed to Intense Malaria Transmission
Malaria infection and disease is highly heterogeneous even in hyperendemic areas, although no conclusive evidence exists that innate or naturally acquired resistance can prevent infection. Resistance that prevents infection might target the â€œpreerythrocyticâ€ sporozoite and liver stages, or the blood stages to prevent patent parasitemia. Our aim was to examine immunoparasitological evidence for an

elite infection-control phenotype from a prospective birth-cohort followed 2002-2006 in Muheza, Tanzania, an area of intense malaria transmission. Children (n=688) provided blood smears every 2 weeks their first year of life and monthly thereafter. Maternal and childhood demographic and clinical characteristics were obtained, cord-blood cytokines were measured, and antibody responses to a panel of common blood- (MSP1, AMA1) and preerythrocytic (LISP1, SLARP, among others) malarial antigens were assayed every six months as measures of stage-specific exposure. Antibody seroprevalance comparisons were assessed through generalized estimating equations, and the association of cytokine levels at birth with elite infection-controller status was estimated through multivariate logistic regression. Statistical significance was assessed at p<0.05. Sixty (8.7%) children had no blood-smear positive slides over an average of two years of follow up (range: = 1 to 3.5 years, ~ 121 person-years) and were identified as elite infection-controllers. Elite infection-controllers were similar to non-controllers with respect to completeness of follow-up and most maternal and childhood behavioral and biomedical risk factors. Antibody seroprevalence was similar between elite and non-controllers for five of six preerythrocytic antigens tested, and increased with age in both groups. Elite controllers had a lower seroprevalence to MSP1 (5.6% vs. 29.3%; P<0.0001) and AMA1 (25.9% vs. 60.3%; P<0.0001) compared with non-elite controllers. In addition, elite controllers were over two times as likely as non-elite controllers to have cord-cytokine levels of IL1-beta, TNF-alpha, or TNF-R1 in the top third of the distribution. These data suggest that a subset of children living in highly endemic areas are exposed to malaria but are able to control infection before patent parasitemia, and this control is associated with a unique immunologic profile at birth. Further research with such resilient children may identify mechanisms for highly effective naturally acquired immunity to malaria.

NIAID
Audrey ROMANO
Visiting Fellow
Immunology - Infectious Disease
Genetic and immunologic analyses of experimental interspecies hybrids between visceral and cutaneous strains of Leishmania.
Leishmania (L) species produce a spectrum of clinical outcomes in their mammalian hosts. Following transmission to skin by the bite an infected sand fly, L. major produces localized cutaneous lesions while L. infantum can disseminate to the liver and spleen in susceptible hosts. The parasite genes and host responses implicated in these tissue tropisms remain largely unknown. We have shown previously that different sub-strains of L. major are capable of a sexual cycle in the sand fly vector, with various parental phenotypes segregating differentially in the hybrid progeny, suggesting that forward genetic approaches are possible. Genetic exchange between two different species has not been experimentally demonstrated. To explore the possibility of mating between L. major and L. infantum, we co-infected Lutzomyia longilpalpis sand flies using parasite lines engineered to express distinct antibiotic resistance markers. Eleven double antibiotic resistant diploid, triploid, and tetraploid progeny were selected that were shown to be full genomic hybrids by multi-locus genotyping. For the polyploid progeny, the species origin of the extra-genome influenced the outcome of infection in C57Bl/6 mice, with the extra copy of L. major or L. infantum associated with stronger growth in the skin or viscera, respectively. Amongst the diploid progeny, intermediate phenotypes were observed. In BALB/c mice, which are normally susceptible to uncontrolled growth of L. major in the footpad or ear, none of the hybrid progeny produced lesions, indicating complete dominance of the L. infantum genes controlling this phenotype. To better understand the nature of this dominance, mice were simultaneously infected with both parental lines in the same or different dermal site. Co-infected animals were in each case more resistant to L. major. Preliminary data suggest that L. infantum actively alters the subsets of inflammatory cells, including neutrophils, which condition the site for L. major growth or elimination. These data are the

first demonstration of sexual recombination between different Leishmania species, and further analysis of the hybrids and backcross lines should allow positional cloning of the genes, and identification of the host responses, controlling visceral and cutaneous tissue tropisms.

NIAID
Diego Costa
Visiting Fellow
Immunology - Innate and Cell-mediated Host Defenses
Antibiotic treatment of Mycobacterium tuberculosis infection leads to early alterations in innate and adaptive immune responses to the pathogen
Although antibiotics have had a major impact on the global control of tuberculosis, both the required 6 month treatment course and the emergence of multi-drug resistant bacterial strains have emphasized the need for more effective therapies that do not rely solely on conventional pharmacologic approaches. Combined immuno/chemotherapeutic intervention represents a major strategy to achieve this end. In this regard, it is important to know whether the immune response to Mtb is altered during antibiotic treatment and if it participates in antibiotic induced bacterial control. To begin to address these questions, we used a drug treatment model in which C57BL/6 mice are treated perorally with isoniazid, pyrazinamide and rifampicin initiated at 24d following aerosol infection with H37Rv M. tuberculosis strain. We then evaluated alterations in cytokine and chemokine expression patterns in lungs and in Mtb antigen specific CD4+ T cells, in mice that received antibiotic treatment compared to non-treated animals. At 60d post-treatment, animals displayed the expected 4-5 log reduction in pulmonary bacterial loads together with reduced lung pathology and inflammatory cell infiltration. Pulmonary RT-PCR revealed major decreases in pro-inflammatory cytokine and chemokine but not anti-inflammatory or Th2 cytokine gene expression as early as 10d after treatment, a time point before significant reductions in bacterial load were observed. IL-12p40 protein levels measured by ELISA in lung homogenates were also dramatically reduced during this early period. We also characterized tetramer positive CD4+ T cell responses during treatment, and observed that while the frequency of ESAT-6 specific CD4+ T cells decreased, Ag85 specific CD4+ T cells actually increased in the lungs and draining LN of antibiotic treated mice, as early as 7 days post-treatment initiation. A similar outcome was observed when CD4+T cell IFN-gamma and TNF-alpha production was measured by intracellular staining following restimulation with ESAT-6 versus Ag85 specific peptides. These observations indicate that antibiotic treatment, even in its early phase leads to important changes in the immune response to Mtb. Thus, interventions that sustain immune responsiveness may prove to be beneficial adjuncts to conventional antibiotic treatment.

NIAID
Jose Ramirez
Postdoctoral Fellow
Immunology - Innate and Cell-mediated Host Defenses
Molecular Mechanisms of Innate Immune Memory in An. gambiae mosquitoes
An innate immune priming response is triggered when Plasmodium ookinetes invade the mosquito midgut and the microbiota comes in direct contact with injured cells. This is a long-lasting response that confers the mosquito enhanced ability to control subsequent Plasmodium infections. The immune priming response involves hemocyte differentiation, in particular an increase in the granulocyte population. A hemocyte differentiation factor (HDF) is released into the hemolymph and transfer of cell-free hemolymph from challenged mosquitoes can induce hemocyte differentiation and enhanced immunity in recipient naïve mosquitoes. In this study, we have characterized the biochemical nature of HDF. We found that it consists of a bioactive lipid that is transported in the hemolymph by a lipocalin

(ApoD-like) carrier protein. RNAi-based silencing of this Apo-D-like abolished the release of HDF activity in the hemolymph extract, indicating that it is a critical component of the immune priming response. In turn, LC/MS/MS analysis showed that the HDF lipid component is an eicosanoid in nature. Injection of synthetic eicosanoid analogs recapitulates the phenotype observed in Plasmodium-infected mosquitoes as well as those observed when transferring cell-free hemolymph from challenged to naïve mosquitoes. Currently, we are further characterizing the identity of the bioactive lipids that are critical in mediating innate immune memory in An. gambiae mosquitoes.

NIAID
Sonia Majri
Doctoral Candidate
Immunology - Lymphocyte Development and Activation
Abstract & Title removed at request of author

NIAID
Mina Seedhom
Postdoctoral Fellow
Immunology - Lymphocyte Development and Activation
Increased Protein Translation in Bystander Bone Marrow T Cells Early After Virus Infection
Protein translation is an essential and intricately regulated cellular process. We recently described the RiboPuromycylation Method (RPM) to measure protein translation in cultured cells. The RPM is based on ribosome-catalyzed addition of puromycin to nascent chains followed by detection of the ribosome-bound nascent chains with a monoclonal antibody specific for puromycin in fixed and permeabilized cells. To examine protein translation in vivo by the RPM, we administer puromycin intravenously to mice and measure puromycin incorporation ex vivo via flow cytometry. This adaptation of the RPM allows for the measurement of protein translation in multiple immune subsets after cell isolation. After puromycin injection, we are able to detect a robust signal that is blocked by ribosome targeting antibiotics that prevent nascent chain puromycylation. After we infect mice with either vaccinia virus (VACV) or influenza virus, we find profound increases in protein translation in activated B and T cells. In the bone marrow, we find that over 30% of CD4+ T cells and over 40% of CD8+ T cells are translating at high levels 1 day after VACV infection, days before peak protein translation occurs in T cells in either the draining lymph node or the spleen. Bone marrow CD4+ and CD8+ T cells that upregulate protein translation early after VACV infection express high levels of CD44+, which suggests that these T cells are of an innate-memory phenotype. Stimulation of these bone marrow T cells with VACV-infected dendritic cells or with peptides that make up the major epitopes of VACV does not result in cytokine secretion, which suggests that these T cells are not VACV-specific. In an infection model where we transfer OT-1 T cells to act as bystander T cells, bone marrow OT-1â€™s have significantly increased protein translation after VACV infection as opposed to no increase in protein translation of these bystander T cells in the same mice in the spleen. These results suggest that the bone marrow, a known reservoir for memory phenotype T cells, is a unique environment where protein translation is upregulated early after virus infection. We hypothesize that this may be to place memory T cells and memory-like T cells in a state where virus-specific cells can be more rapidly activated and expanded.

NIAID
silvia vilar portugal
Visiting Fellow
Immunology - Lymphocyte Development and Activation
A systematic characterization of malaria-associated atypical B cells

Antibodies (Abs) play a critical role in malaria immunity, but Ab-mediated protection is only acquired after years of repeated infections, leaving children in endemic areas vulnerable to repeated bouts of febrile malaria. Many P. falciparum antigens are diverse and clonally variant, likely contributing to the inefficient acquisition of protective Abs. However, mounting evidence suggests that Plasmodium-induced dysregulation of B cell function may also play a role. Several recent studies have shown that malaria exposure is associated with an expansion of atypical B cells, which are distinguished from classical memory B cells by the expression of high levels of inhibitory receptors. A similar subset of B cells has been described in individuals infected with HIV and HCV, yet the origin and function of this B cell subset remains unclear. We performed a comprehensive, systematic investigation of atypical B cells collected from individuals exposed to intense malaria in Mali. Sorted naïve B cells (CD19+ CD21+ CD27-), classical memory B cells (CD19+ CD21+ CD27+) and atypical B cells (CD19+ CD21- CD27-) were subjected to genome wide expression profiling, VDJ sequence analysis (VDJ heavy and light chain usage and somatic hypermutation rates), KREC analysis (replicative history), as well as proliferative and cytokine production analysis following in vitro stimulation We found that classical memory B cells and atypical B cells have distinct genome-wide expression profiles, but are similar in terms of VDJ heavy and light chain usage and somatic hypermutation rates as well as replicative history. We further show how these B cell subsets differ in their proliferative and cytokine production capacity, and how the expression of inhibitory receptors on atypical B cells impairs their proliferative capacity. This thorough characterization of B cell subsets in malaria-exposed individuals has generated new hypotheses on how chronic Plasmodium exposure leads to B cell dysregulation and the inefficient acquisition of protective antibodies. Further investigation of B cell differentiation pathways in the context of malaria could shed light on how malaria immunity could be accelerated through vaccination.

NIAID
Virginie Sjoelund
Postdoctoral Fellow
Metabolomics/Proteomics
Phosphoproteome profiling of toll-like receptor response to different ligand stimulation in macrophages identifies differentially activated pathways and phosphorylation kinetics
Toll-like receptors are among the first sensors that detect and drive immune responses to pathogens. Macrophages that encounter a pathogen are usually stimulated not by one, but by a combination of TLR engaged by distinct ligands on the microbe. As a first step to understanding the integrated signaling under such complex conditions, we have investigated the differences in the phosphoprotein signaling cascades triggered by individual TLR4, 2 and 7 ligands using a single responding cell population. We performed a global quantitative and early post-stimulation kinetic analysis of the phosphoproteome of mouse macrophages using SILAC coupled to phosphopeptide enrichment and high-resolution mass spectrometry. For each ligand-TLR pair, we found marked elevation of phosphorylation of components of the cytoskeleton, GTPases in the Rho family, and proteins involved in phospholipase C signaling. Phosphorylation of elements involved in phagocytosis was only seen in response to TLR2 and 4 ligands but not TLR7 ligands. Changes in proteins involved in endocytosis-related signaling pathways were delayed in response to TLR2 versus TLR4 ligands. In particular, using a PKC inhibitor, we investigated the role of MARCKS, a cytoskeletal phosphoprotein that is differentially phosphorylated in response to the three different ligands, in macrophage migration and in TLR4 endosome trafficking. These findings reveal that the phosphoproteomic response to engagement of distinct TLRs varies both in the major targets within the cell that undergo modification and the kinetics of such changes. Our results have important implications for understanding how macrophages sense and respond to pathogens that confront the immune system with a diverse set of TLR stimuli.

NIAID
Shu Hui Chen
Postdoctoral Fellow
Microbiology and Antimicrobials
Subtelomeric gene regulation in cryptococcosis of solid organ transplant recipients
Cryptococcus neoformans (Cn) is a major cause of fungal meningitis in solid organ transplant (SOT) recipients and causes over 600,000 deaths annually in individuals with HIV/AIDS. Upon gaining entry to the host, Cn establishes infection in either the lung where the infection is usually successfully treated or the central nervous system (CNS), often leading to a lethal meningoencephalitis. Several Cn virulence factors, such as a polysaccharide capsule and the enzymes laccase and urease, have been extensively studied. However, no correlation has been found between expression levels of these factors and disease outcome. Thus, we conducted a discovery study to identify genes associated with brain dissemination in a cohort of SOT recipients. For these studies, a newly-designed Cn microarray was used to compare expression profiles of strains causing either lethal meningitis (M) to those of non-lethal pulmonary (P) infection in strains collected from SOT recipients infected with Cn. Our findings demonstrated a significant variation in subtelomeric gene expression levels between the two disease states-M vs P, with a large proportion involved in sugar metabolism or transport, suggesting that these genes confer a selective advantage in the low glucose environment of the brain. We then transformed representative pulmonary strains with plasmids overexpressing identified subtelomeric gene candidates and assessed dissemination to brains using a mouse model. These studies demonstrated gene-dependent brain dissemination of several identified candidates, suggesting a role for subtelomeric expression plasticity in microbial virulence within human hosts. In contrast, molecular genotyping using multi-locus sequencing found no relationship between the strainsâ€™ underlying genetic makeup and a propensity to disseminate to brain. This suggests that small numbers of genetic changes contribute a disproportionate effect on human virulence in this patient population. Further studies will seek to identify subtelomeric regulatory mechanisms involved in the acquisition of virulence and will suggest molecular targets for drug discovery or diagnostic tests for human cryptococcosis.

NIAID
Jessica Hostetler
Doctoral Candidate
Microbiology and Antimicrobials
Production and characterization of a library of full-length P. vivax parasite proteins
Plasmodium vivax continues to receive much fewer research resources than the more deadly Plasmodium falciparum despite its wider geographic reach and role in causing malaria in 70â€“391 million people each year. A vaccine targeting the illness-inducing blood stage of parasite development is an essential component of any worldwide malaria eradication campaign, but major gaps in our understanding of P. vivax biology, including the protein-protein interactions that mediate erythrocyte invasion, hinder the search for an effective vaccine. Only a single parasite ligand-host receptor interaction is presently known, that between P. vivax Duffy Binding Protein (PvDBP) and Duffy Antigen Receptor for Chemokines (DARC), and strain-specific immune responses to PvDBP make this antigen a challenging vaccine target. Given that P. vivax lacks an in vitro culture system and the production of full-length antigens presents a host of technical challenges, previous studies considered only a handful of P. vivax parasite antigens. We are carrying out a comprehensive study of P. vivax proteins that mediate erythrocyte binding and invasion in order to identify additional vaccine candidates. As a first step, we produced a library of 39 full-length recombinant P. vivax proteins to test for erythrocyte binding and immunoreactivity. This represents the largest full-length P. vivax antigen set ever assembled. Candidates were selected based on predicted localization to the merozoite surface or invasive secretory organelles,

and on homology to P. falciparum vaccine candidates. 37/39 P. vivax recombinant proteins were expressed in the HEK293E cell system, which has been successfully used for expression of full-length P. falciparum invasion ligands such as PfRH5. Known or predicted functions, such as the interaction between merozoite surface proteins Pv12 and Pv41, were confirmed and several novel parasite protein-protein interactions were identified. Pilot immunoreactivity screens using sera from Cambodian patients with P. vivax malaria showed that IgG variously recognize the majority of antigens tested. The large-scale initial screenings of this library will be presented through protein expression, protein interaction and seroreactivity data, as well as immuno-epidemiological studies.

NIAID
Aaron Neal
Doctoral Candidate
Microbiology and Antimicrobials
Identifying novel trafficking components of the Plasmodium falciparum virulence factor PfEMP1 through QTL analysis
Over 207 million cases of malaria occurred in 2012, resulting in 627,000 deaths worldwide. Of the five protozoan parasites that cause the disease, Plasmodium falciparum is responsible for 40% of episodes and nearly all fatalities. This enhanced virulence is due to the ability of asexual blood-stage parasites to adhere to the host microvasculature, leading to the mass sequestration of infected red blood cells (iRBCs) in vital organs. Efforts to ameliorate the morbidity and mortality of P. falciparum have focused on the primary parasite virulence factor, P. falciparum erythrocyte membrane protein 1 (PfEMP1), due to its central role in mediating cytoadherence. While directly targeting PfEMP1 as a malaria therapy is greatly limited due to the proteinâ€™s hypervariable nature, interfering with the trafficking of PfEMP1 to the iRBC surface is an attractive strategy, as previous studies have demonstrated that reducing PfEMP1 surface expression significantly weakens cytoadherence, likely lessening the severity of symptoms and permitting parasite clearance by the spleen. Interestingly, the in-vitro culture-adapted parasite line 3D7 is inherently defective in exporting PfEMP1, resulting in reduced PfEMP1 levels on the iRBC surface. Presuming that PfEMP1 export from the parasite to the iRBC surface is controlled at the genetic level, we hypothesized that 3D7 harbors one or more genetic determinants of impaired PfEMP1 trafficking. To test this possibility, we examined the surface PfEMP1 levels of 17 progeny clones from a genetic cross between 3D7 and the â€˜trafficking-competentâ€™ parasite line HB3. This was assessed using Western blotting and a two-color, triple-layer flow cytometry assay with plasma from malaria-immune Malian adults. Normalized to HB3, we found that 3D7 displays 75% less PfEMP1 on the iRBC surface, with progeny phenotypes ranging from 37% more to 88% less PfEMP1. QTL analysis using our phenotypes and 3,597 genome-wide SNP markers identified a significant locus on chromosome 12 that explains approximately 50% of the phenotypic variance. This locus contains a single gene, PF3D7_1245600, encoding a putative kinesin that has not yet been characterized. The role of this gene in the trafficking of PfEMP1 is being confirmed in allele-exchange experiments, where the trafficking defect is rescued in 3D7 and introduced in HB3. The results of this work will strengthen our understanding of malaria pathogenesis and provide new targets for much needed therapeutics.

NIAID
Jian Wu
Visiting Fellow
Microbiology and Antimicrobials
Strain-specific innate immune signaling pathways determine malaria parasitemia dynamics and host mortality.

Malaria infection triggers vigorous host immune responses; however, the parasite ligands, host receptors, and the signaling pathways responsible for these reactions remain unknown or controversial. Malaria parasites primarily reside within RBCs, thereby hiding themselves from direct contact and recognition by host immune cells. Host responses to malaria infection are very different from those elicited by bacterial and viral infections and the host receptors recognizing parasite ligands have been elusive. Here we investigated mouse genome-wide transcriptional responses to infections with two strains of Plasmodium yoelii (N67 and N67C) and discovered differences in innate response pathways corresponding to strain-specific disease phenotypes. Using in vitro RNAi-based gene knockdown and KO mice, we demonstrated that a strong type I IFN (IFN-I) response triggered by RNA polymerase III and melanoma differentiation-associated protein 5, not Toll-like receptors (TLRs), binding of parasite DNA/RNA contributed to a decline of parasitemia in N67-infected mice. We showed that conventional dendritic cells were the major sources of early IFN-I, and that surface expression of phosphatidylserine on infected RBCs might promote their phagocytic uptake, leading to the release of parasite ligands and the IFN-I response in N67 infection. In contrast, an elevated inflammatory response mediated by CD14/TLR and p38 signaling played a role in disease severity and early host death in N67C-infected mice. In addition to identifying cytosolic DNA/RNA sensors and signaling pathways previously unrecognized in malaria infection, our study demonstrates the importance of parasite genetic backgrounds in malaria pathology and provides important information for studying human malaria pathogenesis.

NIAID
Charles Larson
Doctoral Candidate
Molecular Biology - Prokaryotic
Subversion of clathrin-mediated vesicular transport by the Q fever pathogen, Coxiella burnetii
Coxiella burnetii is an intracellular bacterial pathogen and the causative agent of the flu-like illness Q fever. Macrophage colonization during acute infection requires C. burnetii replication within a parasitophorous vacuole (PV) resembling a large phagolysosome. Cumulative evidence indicates C. burnetii actively directs PV biogenesis, but the historical requirement of C. burnetii growth in host cells hampered genetic approaches commonly used to define mechanisms of bacterial pathogenesis. However, the development of a host cell free growth medium now makes efficient genetic manipulation possible. Indeed, it was recently shown that C. burnetii growth in host cells requires a Dot/Icm type 4B secretion system (T4BSS) that delivers bacterial effector proteins into the host cell cytosol. Multiple C. burnetii Dot/Icm effectors likely regulate vesicle traffic to promote PV biogenesis, and functional characterization of these secreted proteins is critical to understanding C. burnetii virulence. Based on bioinformatic criteria, such as the presence of eukaryotic-like coiled-coil domains, a list of predicted T4BSS substrates was assembled and screened for Dot/Icm-dependent export during C. burnetii infection. Of the 14 Dot/Icm substrates identified, five proteins termed Coxiella vacuolar protein A (CvpA), CvpB, CvpC, CvpD, and CvpE labeled the PV membrane when ectopically expressed fused to mCherry fluorescent protein. C. burnetii cvpA, cvpD, and cvpE mutants exhibited severe defects in intracellular replication and PV formation rescuable by genetic complementation. Detailed examination of CvpA revealed multiple endocytic sorting motifs predicted to interact with clathrin adaptor proteins, such as AP2, implying the effector could modulate clathrin-mediated vesicular transport. Ectopically expressed mCherry-CvpA localized to recycling endosomes positive for Rab11 and transferrin receptor, an intracellular trafficking itinerary that was disrupted by mutation of endocytic sorting motifs within CvpA. Moreover, in pull-down assays CvpA interacted with AP2 and clathrin heavy chain, and depletion of cellular AP2 or clathrin by siRNA severely inhibited C. burnetii growth. Collectively, these data suggest CvpA modulates clathrin-mediated transport to promote C. burnetii intracellular growth. Functional

characterization of the remaining Cvp effectors should reveal other host cell functions that contribute to C. burnetii infection.

NIAID
Stephen Dollery
Postdoctoral Fellow
Virology - DNA
Identification of a virion-associated B cell entry determinant for Kaposiâ€™s sarcoma associated herpes virus.
Kaposiâ€™s sarcoma herpesvirus (KSHV) is an HIV-associated lymphotropic herpes virus. In addition to Kaposiâ€™s sarcoma, KSHV causes two devastating B-cell lymphoproliferative disorders: multicentric Castlemanâ€™s disease (MCD) and primary effusion lymphoma (PEL). These disorders are frequent in populations where HIV is endemic. For many viruses the identification of cell-tropism determinants has greatly facilitated many arms of intervention research. For KSHV no such tropism determinants have been identified. During KSHV infection of the host, B cell infection is critical. B cells are believed to be the reservoir of KSHV during lifelong infection. As such, B cells are a likely origin of reactivation and dissemination, and of KSHV-associated diseases. In spite of all the evidence for B cell tropism in vivo, KSHV infection of B cells is remarkably inefficient in vitro. In activated primary cells, infection is limited to around 5%, and transformed B cell lines are notoriously refractory to infection. This puzzling discrepancy of between in vivo and in vitro B cell infection has stymied research into KSHV tropism for this critical cell type. In a directed search for a susceptible B cell line, we identified the MC116 B cell line as markedly more susceptible to KSHV infection than any others. MC116 cells phenotypically resemble early immature B cells like those believed to be important in MCD and PEL. Analysis of viral and reporter genes revealed that KSHV latently infected the majority of susceptible cells (LANA dots, GFP). Lytic replication was induced by traditional agents such as HDAC inhibitors and by cross-linking of the B-cell receptor (gH, K8.1A, RFP), strongly implicating a novel role for immune function in KSHV reactivation. In studies of virus entry we identified a KSHV glycoprotein critical for KSHV infection of MC116 B cells but dispensable for fibroblast and endothelial cell infection. Using a recombinant knockout virus and a panel of antibodies, we demonstrate that this glycoprotein has a mechanistic role in viral attachment to cell surface heparan sulphate proteoglycans; interestingly the glycoprotein is also needed in a post-attachment step. We verified the glycoproteinâ€™s importance for infection of primary B cells from tonsil cultures. As for other gamma herpesviruses, elucidation of KSHV B cell tropism determinants will be integral to understanding biology and pathogenesis of this virus, as well as to developing therapeutic interventions.

NIAID
Darryl Falzarano
Visiting Fellow
Virology - RNA and Retroviruses
Infection with MERS-CoV Causes Lethal Pneumonia in the Common Marmoset
The availability of a robust disease model is essential for the assessment of countermeasures for Middle East respiratory syndrome coronavirus (MERS-CoV). While a rhesus macaque model of MERS-CoV has been established, the lack of uniform, severe disease in this model complicates the analysis of countermeasure studies. Variation in the dipeptidyl peptidase 4 (DPP4), the receptor for MERS-CoV, appears to play a major role in the ability of MERS-CoV to infect certain animal species. To predict the ability of MERS-CoV spike protein to bind to common marmoset DPP4 in silico, analyses were performed using human (known to bind MERS-CoV spike glycoprotein) and ferret DPP4 (unable to bind MERS-CoV spike glycoprotein) using data from co-crystallography studies that mapped the interface between

human DPP4 and the receptor-binding domain (RBD) of the MERS-CoV spike glycoprotein. No differences between human and marmoset DPP4 were identified within the region that interacts with the MERS-CoV RBD, whereas between human/marmoset and ferret nine amino acid residues were different within this region. In addition the binding potential calculations for the DPP4 â€“ MERS-CoV RBD interaction revealed no significant differences between the human (-981) and marmoset (-978), whereas the binding energies for ferret was significantly higher (-601). Taken together, MERS-CoV spike is predicted to bind to marmoset DPP4 and as such marmosets should be susceptible to MERS-CoV infection. Therefore, nine marmosets were inoculated via combined intratracheal, intranasal, oral and ocular routes with MERS-CoV. Most of the marmosets developed a progressive severe pneumonia leading to euthanasia of two of six remaining animals on day 4 post inoculation. Necropsies on days 3, 4 and 6 revealed extensive gross lesions (up to 85%) in the lungs of all animals. Histological analyses of lungs confirmed coalescing moderate to marked acute bronchointerstitial pneumonia tightly correlated to the presence of viral antigen. Animals were also viremic, had 1000X higher viral loads in the lungs than rhesus macaques and total RNAseq of lungs revealed the induction of immune and inflammatory pathways. This serves as the first description of a severe, partially lethal, disease model for MERS-CoV and as such will have a major impact on the ability to assess the efficacy of vaccines and treatment strategies as well as allowing more detailed pathogenesis studies.

NIAID-VRC
Katija Jelicic
Research Fellow
HIV and AIDS Research
The HIV-1 envelope protein gp120 effects on naïve and memory B cells
During the early stages of HIV infection, 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 demonstrated that the HIV-1 envelope protein gp120 binds to and signals through integrin alpha4beta7, the gut mucosal homing receptor 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. The data show that gp120 also binds and signals through alpha4beta7 on naïve B cells, resulting in an abortive proliferative response. Also, gp120 induced an alpha4beta7-dependent pattern of gene expression in B cells that suggested an inhibitory effect. In primary naïve B cells with T cell-independent or T cell-dependent stimulation, gp120 signaling through alpha4beta7 resulted in impaired B cell function by activating inhibitory pathways that resulted in the production of the immunosuppressive cytokine TGF-beta1, induction of B cell inhibitory receptor FcRL4 and low surface expression of CD80. Increased FcRL4 expression is observed in HIV-infected individuals, but its expression normalizes upon initiation of antiretroviral therapy. Co-culture of B cells with HIV-1-infected autologous CD4+ T cells also induced the B cells to upregulate FcRL4 expression. Treatment of CD27+ memory B cells with alpha4beta7-reactive gp120, resulted in a 5-20% inhibition of proliferation, compared with 45-65% inhibition of proliferation for naïve B cells. The alpha4beta7-reactive gp120 affect the responses of memory B cells to T cell-independent and T-cell-dependent in a relatively modest way compared with a suppressive effect observed for naïve B cells. These findings indicate that, in addition to mediating chronic immune activation, viral proteins can contribute directly to HIV-associated B cell dysfunction. Subsequently was analyzed TGF-beta1 concentration in serum from both SIV-1 infected Rhesus Macaques and HIV-1 infected patients in the acute stage of infection. Preliminary data show TGF-beta1 levels increased during peak viremia in the acute phase of SIV infection. 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 early antibody response to the virus.

NIAID-VRC
Syed Moin
Postdoctoral Fellow
Immunology - General
A new high throughput assay for measuring RSV neutralization activity and its potential application in candidate vaccine evaluation
Respiratory syncytial virus (RSV) is the leading cause of severe acute lower respiratory tract infection in infants worldwide . A licensed safe RSV vaccine is not available yet and therefore, an efficient and standardized neutralization assay is essential for evaluating vaccine candidates. Neutralizing activity (NT) is a known correlate of protection against severe RSV disease and primary immunogenicity endpoint for vaccine studies. The conventional NT measurement takes a week and relies on visual readout. The flow cytometry based NT assay we previously developed was fast, efficient and reliable but is difficult to adapt to a low-cost, high-throughput format for vaccine studies. We have developed a plate-reader based, high-throughput method for measuring NT against a panel of viruses that can be used for assessing vaccine immunogenicity. This assay employing mKate2, a far-red fluorescent protein, uses a florescence plate-reader based approach that can be automated. We constructed representative, recombinant mKate-RSV subtype A (strain Line19, D46/6120), and subtype B (strain 18537, 9320) viruses by recombineering. NT was measured as reduction of fluorescence intensity compared to virus control. A time point of ~24 hours produced dose responsive and reproducible neutralization curves and titers. The NT measured from 55 serum samples of human, monkey, mouse, rabbit and cotton rat, and their linear regression analysis demonstrated significant correlation (R2=0.9039, Pearson r =0.9505, P< 0.0001) between EC50 from plate-reader and flow cytometry based neutralization assay. The BEI (Biodefense and Emerging Infections Research Resources Repository) panel of human antiserum to RSV were also used for assay validation. The 96-well plate method was then adapted to a 384-well format and the EC50 of two showed significant correlation (R2 =0.8969, Pearson r = 0.9470, P< 0.0001). Further, the fully automated, robotic 384-well format was developed and the NT was measured for RSV subtype A and B from15 human samples. The antibody titers measured from automation system were statistically similar to the manually performed plate-reader assay (R2 =0.9365, Pearson r =0.9678, P< 0.0001). The new fluorescence plate-reader based assay is low-cost, fast, efficient and high throughput, and could either be employed in research laboratory or adapted for evaluating the comparative neutralizing activity and breadth of candidate RSV vaccines in a completely automated fashion.

NIAID-VRC
Meghan Altman
Postdoctoral Fellow
Virology - RNA and Retroviruses
Lamprey Anti-Viral Antibody Responses Support Universal Rules of Influenza A Antigenicity
Immunoglobulins (Igs) are a crown jewel of vertebrate evolution. Due to combinatorial shuffling and somatic mutation, antibodies can respond to nearly all biological compounds. Remarkably, it was recently discovered that jawless vertebrates evolved a parallel antibody system, variable lymphocyte receptors (VLRs), which recognize antigens by a non-Ig structural platform. The convergent evolution of Ig and VLR antibodies allowed us to probe whether intrinsic chemical features of inactivated influenza A virus (IAV) determine its antigenicity. We collected blood from lamprey larvae immunized 3 times with inactivated, purified H1N1 PR8. ELISAs revealed that each immunized lamprey generated Abs which bound the PR8, but not parainfluenza-3, a distinct virus with a similar architecture. PR8-immunized lamprey plasma had hemagglutinin inhibition (HI) titers of 1:30 against PR8, but less than 1:5 against a serologically distinct H3N2 IAV. As with HA-specific Igs with HI activity, immunized VLRs neutralized viral

infectivity in standard microneutralization assay using MDCK cells. These activities suggest that, as with the mammalian IAV response, hemagglutinin (HA) is a major target of lamprey antibodies. We confirmed this by flow cytometry using cells expressing HA from transfected cDNA. We next examined the fine specificity of lamprey anti-HA Abs using a panel of sequentially (Seq) selected viruses with an increasing number of amino acid substitutions in the classical H1 antigenic sites. Testing lamprey plasma against Seq 3, 6, 9 and 12 viruses revealed modification of epitopes that prevent Ig binding, similarly affect VLR binding. Importantly, comparing the area under the curves from wild-type versus Seq 12 demonstrates that approximately 60 percent of the antibody response is directed to the HA globular domain. This is despite the presence of large amounts of the other IAV structural antigens in the virus used to coat the ELISA wells. Further, competition experiments with monoclonal mouse antibodies showed that VLRs recognize IAV HA in a manner highly similar to mammalian antibodies. In particular IAV VLRs compete with monoclonal antibodies recognizing each of the five major antigenic sites on the HA globular head, but not the HA stem. The striking similarity between lamprey and mammalian antibody responses to IAV suggests that antigenicity is governed by common chemical principles.

NIAMS
Laura Vian
Postdoctoral Fellow
Cell Biology - General
Defining CTCF physiological roles: rules of the â€œCTCF codeâ€
CTCF is a multifunctional 11-zinc-finger (ZF) DNA binding protein implicated in the establishment and maintenance of higher-order chromatin architecture and gene regulation. However, its precise role in development is unknown because CTCF deletion invariably leads to embryonic lethality. In the mammalian genome, there are ~50,000 CTCF binding sites, displaying in some cases significant sequence variability. By ectopically expressing ZF mutants in activated B cells, we recently showed that CTCF uses different ZF combinations to recognize these diverse DNA motifs. For instance, we found that ZFs 9-11 are required for CTCF binding to sites carrying a particular 20-bp sequence known as U (Upstream). As this motif is only present in a relatively small fraction of CTCF binding sites, the overall genomic profile of CTCF is unaffected in ZF9-11 mutants. This observation raised the interesting possibility of being able to develop partial CTCF knockouts, defective in CTCF activity at specific loci but without compromising cell viability. To this end we have used TALEN and CRISPR-Cas9 genome editing technology to generate ZF mutants in B cell lines. Following our prediction, ZF11 mutants were viable whereas targeting of ZF3, which is required for CTCF binding to most sites, led to lethality. Thus, we have begun a comprehensive deep-sequencing analysis of ZF11 mutants, including recruitment of polymerase II and cohesin (a CTCF cofactor), and transcriptome profiles. Our preliminary data shows localized transcriptional defects in ZF11 mutant cells. The validation of these results and its implications vis-à-vis CTCF nuclear architecture will be discussed.

NIAMS
Brian Foster
Research Fellow
Developmental Biology
Developmental analysis of genes controlling dental cementum mineralization and regeneration
Periodontal disease affects 47% of U.S. adults, and 70% in those over 65, causing destruction of tooth root cementum, periodontal ligament (PDL), and alveolar bone, and leading to tooth loss if untreated. Current periodontal therapies are unpredictable, few are truly regenerative, and many lack a biologic foundation, in part due to poor understanding of cementum formation. We aimed to elucidate developmental mechanisms of cementum formation, focusing on regulators of mineralization. By

mapping developmental expression, we found that pro-mineralization enzyme, tissue nonspecific alkaline phosphatase (TNAP), is expressed at the onset of cementum formation. The TNAP knock-out (KO) mouse features lack of cementum and loss of PDL attachment, mimicking severe hypophosphatasia (HPP). In order to better understand effects of reduced TNAP, we studied a knock-in of TNAP mutation A116T, found in dominant adult HPP. A116T mice featured 45% reduction in serum TNAP activity, and mineralization defects were discovered in the alveolar bone, and manifested as thin cementum on molars and incisors. Serum TNAP was significantly correlated with incisor cementum thickness ($p=0.03$). Orphan phosphatase PHOSPHO1 also functions in early mineralization, as evidenced by bone and dentin defects in its absence. We found expression of PHOSPHO1 in the early periodontia, and a thin, poorly mineralized cementum layer in the PHOSPHO1 KO mouse, showing PHOSPHO1 works parallel with TNAP in cementogenesis. Pyrophosphatase phosphodiesterase 1 (NPP1), negatively regulates mineralization by producing mineral inhibitor pyrophosphatase (PPi). Unlike TNAP and PHOSPHO1, NPP1 is specifically up-regulated in cementoblasts only after establishment of cementum. NPP1 KO mice feature significantly (more than 10-fold) increased cementum, confirming that NPP1 regulates cementum thickness. Early TNAP expression and later, mineralization-induced NPP1 induction in cementoblasts were confirmed in vitro, and in an in vivo mouse model of cementum repair. These studies identify cementum as extremely sensitive to mineralization regulators, and implicate early intervention with TNAP or antagonism of NPP1 as promising novel strategies for cementum regeneration. Administration of recombinant alkaline phosphatase (RecAP) to TNAP KO mice shows promise for rescuing dental defects, including in bone, dentin, and periodontal attachment, while also improving overall skeletal function and significantly prolonging life.

NIAMS
Marei Dose
Postdoctoral Fellow
Genomics
AID targets clustered regulatory elements in the B cell genome
Approximately 95% of lymphomas are of B cell origin. This overrepresentation is thought to reflect misrepair of DNA lesions introduced by the cytidine deaminase AID, an enzyme that is required for antibody gene recombination and hypermutation. Although AID works primarily at the immunoglobulin (Ig) gene loci, it can also cut the DNA at oncogenes such as cMyc. Damaged Ig and cMyc or other oncogene loci are sometimes aberrantly joined to create chromosomal translocations that deregulate the oncogene and promote tumor development. However, what attracts AID activity to non-Ig genes remains an unresolved mystery in the field. To begin addressing this question we generated comprehensive maps of AID targets in mouse and human B cells. We did this with novel deep-sequencing techniques that visualize AID-induced mutations (SHM-seq) or DNA breaks (RPA-seq). Our study revealed that AID targets are not randomly distributed in the B cell genome but are instead clustered within nuclear structures defined by PolII long-range interactions. In some instances damaged genes interact over 2Mb of linear chromatin. Unexpectedly, we find that AID activity within these networks is not restricted to promoters but extends to linked enhancers. To deduce defining features of targeted promoters and enhancers we interrogated multiple large-scale genomic data sets from primary B cells. The analysis identified long-range interactions and transcription levels as the best predictors of AID targeting of a given regulatory element. To test these results we tethered a highly transcribed promoter that does not recruit AID on its own into a targeted promoter cluster by genome editing. We find de novo AID deamination of the inserted element. At the same time, altering the sequence of one promoter affected AID targeting of other linked elements. These observations show that topologically linked elements cooperate to recruit AID, demonstrating that nuclear architecture may play a key role in the formation of oncogenic lesions in primary B cells.

NIBIB
Yan Fu
Research Fellow
Biophysics
Axial Super Resolution via Even Illumination Multi-Angle total internal reflection fluorescence
An even illumination multi-angle total internal reflection fluorescence (EI-MA-TIRF) microscope was constructed to achieve super-resolution optical sectioning capability in axial direction. A dual-axis scanning galvo mirror system was added to an objective-based TIRF microscope and controlled by a waveform generator to generate a hollow cone incident beam. The total internal refection in continuous azimuthal directions resulted in a uniform evanescent field for illumination for TIRF images free of interference fringes and shadow artifacts. The incident beam angle was adjusted by manipulating the amplitudes of two sine wave outputs from the waveform generator and controlled the penetration depth of the evanescent field. Selective z-sectioning TIRF was achieved by decreasing incident beam angles towards the critical angle. Layer-by-layer TIRF images from the sample above the surface of the coverslip were obtained by combining selectively z-sectioning TIRF imaging and sequentially photobleaching. Reconstruction of these TIRF images showed a 3D image of biological sample with each layer around 20 nm apart. This technique can be used to identify the axial position changes of cellular structure within 20 nm. In addition, two color images of two proteins allow differentiating their relative localizations in axial direction.

NIBIB
Orit Jacobson Weiss
Research Fellow
Radiology/Imaging/PET and Neuroimaging
PET Imaging of Tenascin-C with ssDNA Aptamer
Background: Tenascin-C (TsC) is an extracellular matrix glycoprotein that is expressed by injured tissues and by various cancers. Recent publications showed that TsC expression by cancer lesions support tumor growth, metastasis and angiogenesis, suggesting TsC as a potential therapeutic target. However, currently there is no non-invasive alternative to determine TsC expression by tumor cells in vivo. Positron emission tomography (PET) is a molecular nuclear imaging technology that is becoming increasingly important for the measurement of physiologic, biochemical, and pharmacological functions at cellular and molecular levels in patients. In order to address the need for an agent to image TsC noninvasively, we report the development of a radioactive PET tracer that will allow quantification of TsC expression. The PET tracer reported here was developed based on a TsC specific single stranded DNA aptamer (ssDNA AP), which is a short strand of DNA that folds by itself into a three-dimensional structure that specifically binds TsC. Methods: ssDNA AP targeting TsC was radiolabeled by conjugation to either N-succinimidyl-4-18F-fluorobenzoate or to a NOTA chelator and labeled with 64Cu. The stability of radiolabeled AP was evaluated in vitro in phosphate-buffer saline (PBS) and mouse serum and in vivo in the blood and urine. PET studies were performed in mice bearing TsC-positive tumor (U87 glioma) and TsC-negative tumor (H460 lung carcinoma). Results: The ssDNA AP was successfully radiolabeled with 18F and 64Cu and purified to give radiochemical yield >99%. 64Cu-NOTA-AP was stable in PBS and mouse serum up to 24 h. In vivo stability was tested in the urine and blood up to 1 h post-injection (p.i.) and no significant amount of metabolites was found for 64Cu-NOTA-AP. PET studies using AP labeled with either 18F or 64Cu demonstrated clear visualization of U87 tumors but not H460 tumors. The labeled AP had fast clearance from the blood and other nonspecific organs through the kidneys which results in high tumor contrast. 64Cu-NOTA-Ap had some retention in the liver (3-4 %ID/g) which was not observed in the 18F labaeled AP probably due to transchelation of 64Cu. Conclusion:

radiolabeled ssDNA AP can be used as a PET tracer to image tumor expression of TsC with a high tumor-to-background ratio and might provide insightful and personalized medical data that will help determine appropriate treatment and monitoring.

NICHD
Yi-Han Lin
Postdoctoral Fellow
Biochemistry - Proteins
Exploitation of the host cell lipidation machinery by the Legionella pneumophilla U-box E3 ligase GobX
Upon infection, bacterial pathogens release effector proteins into host cells to support their intracellular replication. Knowing the function and host target of those bacterial effector proteins is a key step to uncover bacterial pathogenesis in general, and for therapeutic advancement to emerge. Legionella pneumophilla, the causative agent of Legionnaireâ€™s disease, represents an ideal model organism to study bacterial effector proteins. L. pneumophilla translocates over 300 effector proteins into host cells during infection. Most effector proteins have no known functions due to lack of primary sequence homology. We used a bioinformatics approach (HHpred) that identifies remote protein homologies to search for L. pneumophilla effector proteins that contain E3 ubiquitin ligase domains. Mimicking E3 ubiquitin ligases to hijack the host ubiquitination pathway is a strategy many bacteria use during infection, but only 1 L. pneumophilla effector has been confirmed to be an E3 ligase. We identified 17 additional L. pneumophilla effectors of unknown function as putative E3 ligases, 5 of which we confirmed experimentally in vitro or in vivo. Among them, GobX, a 209-amino acid (aa) effector protein, contains a U-box E3 ligase domain within aa51-100. Interestingly, in transiently transfected COS-1 cells, GobX showed intense localization to the Golgi compartment depending on the region between aa161-200. Site-directed mutagenesis identified that Cys175 and its neighboring residues are critical for Golgi localization, and their substitution with alanine either completely abolished or strongly reduced GobX localization to the Golgi. Biochemical analyses using the metabolic labeling agent 17-ODYA followed by click chemistry revealed that GobX is S-palmitoylated on Cys175. Transfection of HEK293T cells with GobX and 23 mammalian DHHC acyl transferases indicated that GobX is preferentially palmitoylated by DHHC12, 20, 21, 22, and 23. Our study represents the first example of a Legionella pneumophilla effector protein that utilizes host-mediated palmitoylation for its subcellular localization. It also shows that E3 ligases are more abundant in bacterial pathogens than predicted, and that GobX can simultaneously exploit two host machineries, ubiquitination and lipidation.

NICHD
SANTOSH VERMA
Visiting Fellow
Cell Biology - General
CELL FUSION STAGE IN OSTEOCLAST FORMATION
Little is known about mechanisms of cell-cell fusion that generates osteoclasts, multinucleated cells that resorb our bones in their continuous remodeling. One of the key challenges in characterizing these complex and relatively slow membrane fusion events is to uncouple actual fusion stage from the preceding differentiation processes, which prepare macrophages for fusion. In this study we isolated fusion stage from preceding stages of osteoclast formation and for the first time identified proteins involved in different fusion steps. To trigger osteoclastogenesis, we applied RANKL to RAW macrophage-like cells or M-CSF and then RANKL to human monocytes. We blocked fusion by applying a reversible hemifusion inhibitor lysophosphatidylcholine (LPC), accumulated the ready-to-fuse macrophages for 16h and then removed LPC. This fusion-synchronization approach has allowed us to accumulate the ready-to-fuse macrophages and concentrate cell fusion events that would normally develop within 16h to

develop within 30-90 min. Fusion-committed macrophages expose at their surface phosphatidylserine (PS), lipid that is normally found only in the inner leaflet of the plasma membrane. CaCCinh-A01, an inhibitor of TMEM16F, protein implicated in non-apoptotic PS externalization, suppressed synchronized macrophage fusion suggesting that transient exposure of PS regulates macrophage fusion. We also established that initial merger of two cell membranes detected as lipid mixing involves PS-binding proteins - annexins (Anxs) A1 and A5. Antibodies against these Anxs and peptides derived from their N-terminal domains inhibited both membrane merger and syncytium formation. Transition from Anx-dependent membrane merger to formation of multinucleated osteoclasts depended on cell metabolism and dynamin activity. ATP depletion as well as dynamin inhibitors dynasore and MitMAB and siRNA to dynamin 2 did not interfere with membrane merger but inhibited syncytium formation by macrophages. Mechanistic motifs uncovered in our work on osteoclast formation are apparently shared by another important cell-cell fusion process - generation of multinucleated myofibers. We found that synchronized fusion between primary murine myoblasts also involves TMEM16F-dependent PS exposure, Anxs and dynamin activity. Our results suggest a striking conservation of cell-cell fusion mechanisms in development and regeneration of bones and muscles.

NICHD
SHAOFEI ZHANG
Doctoral Candidate
Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology
Spatial organization of mitotic spindle assembly
During mitosis, eukaryotic sister chromatids segregate into daughter cells with the help of the spindle, a microtubule (MT)-based structure. Abnormal spindle MT assembly may cause chromosome missegregation and aneuploidy, the condition of having an incorrect number of chromosomes. Aneuploidy is associated with carcinogenesis, and is observed in many solid tumors. Proper spindle formation requires a chromatin-based gradient of the small GTPase Ran, so that RanGTP levels are high in vicinity of chromosomes and RanGDP predominates in distal regions. This gradient is established through the activity of Ranâ€™s GTPase activating protein (RanGAP), which is dispersed in mitotic cytosol, and its guanine nucleotide exchange factor (RCC1), which concentrates on chromatin. Notably, a substantial pool of RCC1 is also found in mitotic cytosol, causing us to wonder why this pool does not cause inappropriately high levels of RanGTP in distal regions of the cell. We studied the mitotic dynamics of RCC1 using the Xenopus Egg Extract (XEE) in vitro system, where RCC1 exists both on chromatin and in cytosol. We hypothesized that cytosolic RCC1 may be inhibited to maintain a steep RanGTP gradient. Previous in vitro studies have shown that RCC1 forms a heterotrimeric complex with Ran and RanBP1, and the nucleotide exchange activity of RCC1 is inhibited in this complex. After RanBP1 depletion, we found that spindle MTs did not form in a manner that was correctly oriented to chromatin in mitotic XEE. Rather, spontaneous chromatin-independent MT nucleation occurred, indicating a disrupted RanGTP gradient. Restoration of physiological RanBP1 levels using recombinant proteins completely rescued spindle assembly. We also measured the enzymatic activity of endogenous RCC1 in mitotic XEE and found it strongly inhibited. Notably, identical levels of RCC1 in the XEE after immunodepletion of RanBP1 showed more than 10-fold increase in its activity, and the addition back of RanBP1 reproduced physiological RCC1 inhibition completely. Finally, binding of RCC1 to chromatin released RanBP1 and allowed RCC1 to become fully active for nucleotide exchange. We thereby concluded that the Ran-GTP gradient is sharpened through the formation of a complex of free RCC1 with RanGTP and RanBP1. The RanBP1/Ran complex sequesters the pool of RCC1 that is not chromatin associated and inhibits its enzymatic activity to restrict RanGTP production to chromosomes.

NICHD

Youheng Wei
Visiting Fellow
Cell Cycle-General, Regulators and Checkpoints, Apoptotic Mechanisms
The Drosophila Nprl2/Nprl3 complex controls the response to nutrient stress by modulating TORC1 activity
Target of rapamycin complex 1 (TORC1) is master regulator of metabolism in eukaryotes that integrates information from multiple upstream signal pathways to control cell growth and proliferation. Recent studies in yeast and mammalian cells have shown that Nitrogen permease regulators like 2 and 3 (Nprl2 and Nprl3) mediate an essential response to amino acid limitation upstream of TORC1. Like other TORC1 inhibitors, Nprl2 is a putative tumor suppressor gene that suppresses cell growth and enhances sensitivity to numerous anticancer drugs including cisplatin. However, the precise role of Nprl2 and Nprl3 in the regulation of metabolism in metazoans remains poorly defined. Here we demonstrate that the central importance of Nprl2 and Nprl3 in TORC1 inactivation and the response to nutrient stress has been conserved from single celled to multicellular animals. To determine the role of Nprl2 and Nprl3 in metazoans, we used Drosophila oogenesis as a model system, which is highly sensitive to nutrient input. We find that in the female germline, Nprl2 and Nprl3 physically interact and target to lysosomes and autolysosomes, the sites of TORC1 activation. When females are starved for amino acids, older egg chambers undergo apoptosis and degenerate. In contrast, young egg chambers are resistant to apoptosis and remain intact but stop growing. Knocking down Nprl2 and Nprl3 in germline cells triggers young egg chambers apoptosis during amino acid starvation. Additionally, the recovery of female fertility is markedly delayed after amino acid starvation in nprl2 RNAi and nprl3 RNAi females. Thus Nprl2/3 is critical to the preservation of female fertility during times of protein scarcity. We further determine whether the Nprl2 and Nprl3 protection role relates with TORC1 inactivation. Rapamycin, a TORC1 specific inhibitor, inhibits the young egg chamber apoptosis and the egg recovery defect in npr2 RNAi and npr3 RNAi flies. Furthermore, knocking down Tsc1, a well-known TORC1 inhibitor, in germline cells also causes the young egg chambers death during amino acid starvation. These results confirm that the young egg chamber death in nprl2 RNAi and nprl3 RNAi flies resulted from TORC1 hyperactivation during nutrient stress. Thus, our data suggest the presence of a metabolic checkpoint that initiates a cell death program when TORC1 activity remains inappropriately high during periods of nutrient scarcity.

NICHD
Josefina Ocampo
Postdoctoral Fellow
Chromatin and Chromosomes
Dynamic interplay between the ISW1, ISW2, CHD1 and RSC chromatin remodeling complexes determines nucleosome spacing and phasing in Saccharomyces cerevisiae
Genome-wide nucleosome maps for yeast have revealed that nucleosomes are regularly spaced and show a global phasing relative to the transcription start site (TSS). In addition, most genes have a nucleosome depleted region (NDR) at the promoter. We have addressed the roles of four different chromatin remodeling complexes in nucleosome organization in vivo: ISW1, ISW2, CHD1 and the essential RSC complex. We constructed strains with the essential RSC8 subunit under the control of the GAL promoter and isw1, isw2 or chd1 null mutations in all possible combinations in the same genetic background. We used paired-end sequencing to map nucleosomal DNA obtained by micrococcal nuclease digestion of yeast nuclei. In the absence of RSC, all the nucleosomes shift towards the TSS with consequent narrowing and filling in of the NDR, with no change in nucleosome spacing, which is maintained at ~165 bp. Others have shown that the combined action of ISW1 and CHD1 is required to maintain nucleosome phasing. Here, we confirm this observation and show that nucleosome spacing in the isw1 mutant is reduced by 5 bp, to ~160 bp, whereas the chd1 mutant shows little change. In

contrast, the isw2 mutant does not show any obvious changes in global chromatin structure. In most cases, the chromatin structures of the double, triple and quadruple mutants represent the sum of the effects observed in the individual mutants, indicating that these remodeling complexes have distinct functions in chromatin organisation. We propose that RSC determines the position of the +1 nucleosome, which is then used as a reference nucleosome by CHD1 and ISW1 to build nucleosomal arrays on genes, such that CHD1 sets a 160 bp spacing, which is increased to 165 bp by ISW1. Although 5 bp is only a small change in linker DNA length, a profound effect on chromatin higher order structure is predicted, due to a major change in the relative orientation of neighbouring nucleosomes.

NICHD
Maeve Wallace
Postdoctoral Fellow
Cultural Social and Behavioral Sciences
Preterm birth in the context of increasing income inequality
Income inequality may have a deleterious impact on population health. Preterm birth is a leading cause of infant morbidity and mortality in the US and rates are consistently higher among socioeconomically disadvantaged women. Little is known about the contextual effect of income inequality on preterm birth, an issue of increasing concern in the US where the current economic divide is the largest it has been since 1928. As income and resources are increasingly concentrated among a smaller segment of the US population, we sought to examine whether changes in income inequality over time were related to the preterm birth rate. Gestational age, clinical and demographic data were based on electronic medical records for singleton deliveries (n=223,512) from 11 US states and the District of Columbia from 2002-2008. Income inequality was determined using state-level Gini index, a measure of resource distribution in a population. Increasing income inequality was defined as a positive change in Gini index from the year prior to birth. Multi-level models were used to estimate independent effect of increasing inequality on preterm birth < 37 weeks controlling for differences in individual-level demographics (age, race, parity) and health behaviors (smoking or alcohol use), insurance and marital status, chronic medical conditions, pre-pregnancy body mass index, and state-level poverty and unemployment during the year of birth. In the fully-adjusted model, increasing inequality was significantly associated with preterm birth (adjusted odds ratio=1.08; 95% confidence interval=1.04, 1.12). An 8% increased risk translates to approximately 37,500 preterm births each year that could be attributable to increasing income inequality. Importantly, living in an area where inequality expanded over the course of the year leading up to delivery increased preterm birth risk regardless of the degree of initial inequality and the amount of increase was less important than any increase itself. Our findings were not explained by poverty or unemployment. Contrary to our hypotheses, we observed no significant interactions for maternal race or insurance status suggesting that income inequality has a broad effect across the population, although preterm rates were higher for disadvantaged women. Understanding mechanisms by which increasing income inequality impacts preterm delivery risk and identifying modifiable risk factors should be priorities for future reproductive health research.

NICHD
Jianxin Yu
Postdoctoral Fellow
Developmental Biology
Single Cell Analysis Reveals Multiple Cellular Mechanisms of Endothelial Morphogenesis in vivo
Angiogenesis is critical for vertebrate organogenesis and plays an essential role in pathological processes such as cancer. Although vessel formation has been extensively studied at the tissue level, difficulty of in vivo imaging and lack of suitable genetic tools have hampered study of the cellular architecture and

dynamic cell behaviors during endothelial morphogenesis. Therefore, basic questions such as how individual endothelial cells (ECs) coordinate their motility and interactions during sprouting angiogenesis and how cords of EC undergo differential shape changes to form lumenized tubes still remain poorly understood. We have now developed endothelium-specific transgenic zebrafish and high-speed two-photon confocal imaging methods to examine in vivo endothelial morphogenesis at single cell resolution. New fluorescent reporter transgenic tools that simultaneously mark both EC nuclei and the plasma membranes, or cell-cell junctions have been generated to monitor the morphology and dynamic behaviors of individual ECs. These transgenes, which we are using in injected mosaics, permit definitive identification and imaging of individual ECs in live animals. Single cell morphometric analysis reveals the heterogeneity of EC morphology in developing vessels. 3D visualization analysis of single cell shape and cell-cell junction patterns indicates the coexistence of both seamless transcellular lumens and single or multicellular enclosed lumens with autocellular or intercellular junctions. Time-lapse confocal imaging of individual ECs further suggests that vacuolar fusion events contribute to the initial lumenization. These results from single cell analysis strongly indicate multiple cellular mechanisms during endothelial tubulogenesis. The newly developed transgenic tools, combined with sophisticated high-speed high resolution imaging methods, allow us visualize complex cellular and subcellular dynamics of single ECs during vascular sprouting and lumen formation with an unprecedented new level of resolution. Extensive application of this approach will help us further understanding not only the concerted EC behaviors during normal vessel development, but the underlying cellular mechanisms of abnormal endothelial phenotype in zebrafish models of human vascular disease.

NICHD
Aikaterini Nella
Clinical Fellow
Endocrinology
Abstract & Title removed at request of author

NICHD
EVA SZAREK
Visiting Fellow
Endocrinology
A cAMP-specific phosphodiesterase, phosphodiesterase 8B (PDE8B), affects Sertoli cell proliferation in adult mice

Phosphodiesterases (PDEs) play a critical role in regulating cAMP levels and signaling. Among them PDE8B, a cAMP-specific PDE, is highly expressed in the testis. Genetic aberrations in cAMP-signaling predispose to endocrine tumors but they are also known to affect reproduction. We examined testes isolated from wild-type (WT) and Pde8b-/- or knock-out (KO) mice at 6, 9, and 12 months (n=3-8/group). Pde8b-/- testis revealed regressive changes in seminiferous tubules (ST), containing increased numbers of atrophied tubules by 12 months (WT: 0±0.001% vs. KO: 11±0.012%) with ST diameter significantly decreased (WT: 209.3±6.65um vs. KO: 169.6±4.22um). Atrophied tubules resembled Sertoli-cell only (SCO) syndrome. Using Sox9 immunostaining we examined Sox9+ cell numbers, revealing a significantly higher number of Sertoli cells (SC) in Pde8b-/- testes (KO: 27.68±0.15 vs. WT:19.20±0.05 Sox9+ cells/tubule); SC in Pde8b-/- testes are maintained in an immature state. Since spermatogonial differentiation or accumulation of spermatogonia in ST has been shown to induce germ cell death, we hypothesized that germ cell loss resulted from increased apoptosis due to accumulation of spermatogonia undergoing defective spermatogenesis. We performed TUNEL to assess cell death and observed significantly higher numbers of TUNEL+ cells in Pde8b-/- testes (6mo: WT 4±1.0 vs KO 30±8.2 cells/tubules; 12mo: WT 9±1.9 vs KO 64±5.4 cells/tubules). Examination of junctional proteins, Cx43 and

N-Cadherin, revealed a failure to establish junctional characteristics of the blood testis barrier, a likelihood substantiated by the abnormal localization of germ cells. Evaluation of blood biochemistry revealed a significant increase in LDH in Pde8b-/- mice (KO: 265.5±81.33 vs WT: 182.50±7.50U/L; P<0.05); LDH is known to be elevated in non-seminomatous germ cell tumors. Serum estradiol was elevated, but there was no difference in serum testosterone. In our further analysis of the hypothalamicâ€“pituitaryâ€“gonadal axis in Pde8b-/- mice, it is evident that there are fertility issues in both males and females, the causes of which are currently under investigation. We conclude that PDE8B has a previously unknown role in SC function and proliferation; its wider role in fertility remains poorly characterized. PDE8B may be a therapeutic target for male infertility, especially that related to SC dysfunction.

NICHD
Emily Mitchell
Postdoctoral Fellow
Epidemiology/Biostatistics - Prognosis and Response Predictions
A novel statistical tool for regression analysis of a skewed biomarker subject to pooling
Pooling biospecimens prior to performing laboratory assays has various benefits, such as reducing laboratory costs and meeting minimum volume requirements. When measurements are taken on pools instead of individual specimens, specialized statistical techniques are often required for proper analysis. While existing methods to analyze pooled specimens have focused primarily on characterizing the distribution of a pooled exposure or calculating odds ratios in a logistic regression setting, we consider regression of a continuous biomarker subject to pooling. Since many biomarkers (e.g. cytokines) exhibit a positive and right-skewed distribution, a log-transformation on the outcome is often applied prior to performing linear regression. Applying this same technique to pooled measurements, however, can result in statistically biased estimates. We propose an alternative regression model in which a log-transformation on pooled outcome measurements will still provide reliable regression estimates. This method is straightforward but is only theoretically justified when pools are formed from specimens with identical predictor values (homogeneous pools). When pools are not homogeneous, we develop a Monte Carlo Expectation Maximization (MCEM) algorithm to calculate maximum likelihood estimates of the regression coefficients. This algorithm, while requiring more computational time and proficiency, is a reliable alternative when pools are not homogeneous with respect to all predictor variables included in the regression model. We use simulation studies to demonstrate the importance of applying appropriate analytical methods to pooled measurements. We then test the proposed methods on a substudy of the Collaborative Perinatal Project, containing measurements on 672 biospecimens, as well as 418 pools, with a maximum of 2 specimens per pool. This analysis demonstrates the efficacy of the proposed methods to closely approximate results from regression on the individual specimens. While this dataset has the unique characteristic of containing measurements on individual as well as pooled samples, the methods developed in this study will aid in the analysis of future datasets, when only pooled measurements may be available. Our methodological contribution to the base of available statistical methods to analyze pooled specimens will empower researchers to more confidently consider pooling as a potential study design.

NICHD
SANDIP DE
Visiting Fellow
Epigenetics
The importance of chromosomal neighborhood in Polycomb group repression

Epigenetic regulation by Polycomb group proteins (PcGs) is critical for organism development. Recent studies have linked deregulation of PcG-proteins to cancer. PcG proteins, first identified in Drosophila as repressors of homeotic genes, are now known to regulate several hundred genes including engrailed (en) and invected (inv). en and inv are juxtaposed genes required for segmentation, CNS, PNS, and head development in the fly. In Drosophila, cis-regulatory DNA elements- Polycomb response elements (PREs) recruit PcG proteins, and can also regulate three-dimensional (3D) genome organization most likely via protein-protein interactions with PcG complexes. en/inv form a co-regulated gene complex, covered by the PcG-repressive H3K27me3 mark. 4 PREs have been characterized in the en/inv complex, 2 at inv, and 2 at en. Surprisingly, deletion of either the inv or en PREs did not disrupt development or accumulation of H3K27me3 in the en/inv domain. This shows the PREs behave redundantly in the laboratory. More surprisingly, flies that had a deletion of all 4 PREs survived and were fertile with no obvious derepression of en/inv expression suggesting that PcG repression is intact. Consistent with this, H3K27me3 covered the en/inv domain in the PRE(4?) mutant, albeit at a reduced level in the region of inv. ChIP-seq analysis with antibodies against 2 PcG proteins [Pho and Ph] uncovered 6 potential weak PREs in the en/inv domain and strong PREs in the flanking genes- Enhancer of Polycomb [e(Pc)] and toutatis (tou). We suggest that these weak PREs, along with PREs in the neighboring regions maintain the epigenetic mark and 3D structure of the en/inv domain in the PRE(4?) mutants. Chromosome conformation capture (3C) experiments showed an interaction between PREs in neighboring genes and the en promoter in both the wild-type and PRE(4?) mutants. We suggest these interactions are crucial for en repression. Consistent with this hypothesis, a large transgene containing 79-kb of en/inv DNA (marked with HA-en), inserted in another place in the genome, correctly regulates HA-en expression; but deletion of the 2 en PREs from the transgene leads to a dominant disruption of segmentation in the adult abdomen, suggesting En misexpression. These data show that chromosomal neighborhood is important for PcG repression and that surprisingly, PREs from flanking genes can regulate en/inv expression.

NICHD
Maria Bagh
Visiting Fellow
Intracellular Trafficking

Subunit a1 of V-ATPase Requires Dynamic Palmitoylation for Lysosomal Localization and is Disrupted in Ppt1-/- mice

Dynamic palmitoylation (palmitoylation-depalmitoylation) is an important mechanism that regulates the functions of many proteins, especially in the brain. Palmitoylation is catalyzed by palmitoyl-acyltransferases (PATs) while palmitoyl-protein thioesterases (PPTs) catalyze depalmitoylation. Genetic deficiency of lysosomal PPT1 causes infantile neuronal ceroid lipofuscinosis (INCL), a fatal neurodegenerative lysosomal storage disease (LSD). PPT1-deficiency impairs degradation of palmitoylated proteins (constituents of ceroid) by lysosomal hydrolases causing lysosomal ceroid accumulation leading to INCL. Despite the facts that lysosomal hydrolases require acidic pH for activity and in virtually all LSDs the lysosomal pH is elevated, the molecular mechanism(s) remains unexplained. Lysosomal pH is regulated by vacuolar H+-transporting ATPase (V-ATPase), which is a multi-subunit protein-complex with a membrane-bound V0 sector and a cytosolic V1 sector. The reversible assembly of V0V1 is suggested to regulate pH of intracellular compartments including the lysosomes. Using Ppt1-/- mice, a reliable animal model of INCL, we tested a hypothesis that one or more subunits of V-ATPase require dynamic palmitoylation for lysosomal targeting and are disrupted in INCL. We found that Cys-25 in subunit a1 of the V0 sector (V0a1) requires palmitoylation for lysosomal localization. Unexpectedly, in Ppt1-/- mouse brain V0a1 was predominantly localized to the plasma membrane instead of the lysosomal membrane. Furthermore, plasma membrane-localized V0a1 was transported via clathrin/AP2-dependent pathway to early endosomes where lack of PPT1 suppressed depalmitoylation of V0a1,

which impaired dissociation of the clathrin/AP2/V0a1 complex. Failure of V0a1 dissociation from the complex prevented its repalmitoylation essential for binding AP3, which is required for its transport to lysosomes. Consequently, V0a1 was delivered to recycling endosome via clathrin/AP2 and was recycled to the plasma membrane. Thus PPT1-deficiency disrupted V0a1 trafficking to lysosomes impairing V0V1 assembly, essential for lysosomal pH regulation. Importantly, a PPT1-mimetic small molecule, NtBuHA, ameliorated these defects. Our findings for the first time demonstrate that PPT1 functions to promote lysosomal targeting of V0a1 required for V-ATPase activity, which regulates lysosomal pH and suggest that varying mechanism(s) impairing V-ATPase activity may underlie elevated lysosomal pH in other LSDs.

NICHD
Ginny Farias
Postdoctoral Fellow
Intracellular Trafficking
A Pre-axonal Exclusion Zone for Vesicles Containing Somatodendritic Proteins in Neurons.
Neurons are polarized cells having distinct somatodendritic and axonal domains. The ability to sort proteins to these domains is critical for neuronal function and is impaired in a variety of diseases including neurodevelopmental and neurodegenerative disorders. The Axon Initial Segment (AIS) has been shown to function as a barrier for the diffusion of plasma membrane proteins between the somatodendritic and axonal domains. We now report that transport vesicles containing somatodendritic proteins are prevented from entering the axon not at the AIS but at a preceding pre-axonal exclusion zone (EZ). Confocal and z-stack reconstruction analysis of the distribution of a somatodendritic marker, the transferrin receptor (TfR), and an AIS marker, ankyrin-G, in rat hippocampal neurons revealed the EZ as a region of the soma prior to the AIS that was devoid of these two proteins. The EZ also excluded all other somatodendritic proteins examined. Live-cell imaging and fluorescence recovery after photobleaching (FRAP) of TfR labeled with fluorescent proteins showed that organelles containing this protein were unable to enter the EZ. Moreover, synchronization of TfR transport in the biosynthetic pathway using an ER-retention-release system showed similar results for the biosynthetic pool of TfR, indicating that the EZ, rather then the AIS, is the filter for segregation of somatodendritic from axonal vesicles. We hypothesized that the exclusion of somatodendritic organelles at the EZ might be due to their inability to acquire a specific microtubule motor involved in axonal transport. We focused on Kinesin-1 because of its well-known role in this process. Indeed, we found that a Kinesin-1 â€œrigorâ€ mutant that binds to microtubules but is unable to â€œwalkâ€ decorated a track crossing the EZ from the soma to the proximal AIS. Importantly, fusion of a Kinesin-1-binding sequence to the cytosolic domain of the TfR resulted in missorting of the resulting chimera to the axon. Other somatodendritic proteins were similarly missorted to the axon in neurons expressing the TfR chimera, indicating that addition of the Kinesin-1-binding sequence to the TfR redirected the whole somatodendritic vesicles with all of their cargos to the axon. These findings thus uncovered a novel mechanism for the sorting of somatodendritic and axonal vesicles at a pre-axonal EZ that is based on the interaction of axonal, but not somatodendritic, vesicles with the microtubule motor Kinesin-1.

NICHD
Lori Griner
Postdoctoral Fellow
Molecular Biology - Eukaryotic
TOWARD A RATIONAL TREATMENT FOR HUNTINGTONâ€™S DISEASE
Huntingtonâ€™s disease (HD) is an autosomal dominant, neurodegenerative disease in which an expanded polyglutamate tract in the huntingtin protein results in toxic aggregates that induce neuronal

cell death. This impairs the patientâ€™s behavior, movement, and speech, and eventually dementia. Current therapy for HD is directed at slowing the effects of symptoms; there is no cure. A promising approach is to promote autophagy, a process in which cells degrade internal cellular components that have become damaged, thereby maintaining metabolic homeostasis. HD cells are deficient in autophagy. Promotion of autophagy in both cultured cells and animal models for HD has been shown to increase clearance of the toxic protein aggregates with concomitant reduction of HD symptoms and increased behavioral performance. Conversely, down regulating autophagy in HD models worsens the disease. Unfortunately, currently available drugs that stimulate autophagy are too toxic for long term care. Fortunately, through high throughput screening, we identified six small molecules that promote autophagy far more effectively than currently used drugs. These molecules increase the cardinal autophagic biomarker, LC3 lipidation linked to an increased autophagy flux. Furthermore, we have shown by transmission electron microscopy (TEM) that the large scale vesicles induced by these novel molecules are functional autophagosomes and autosomes, and they are superior in size and number to the to those produced by rapamycin, the current â€˜gold standardâ€™. Our TEM ultrastructure analysis and biochemical data supports the conclusion that six small molecule therapeutics do, in fact, promote autophagy. We are now testing these molecules in an in vitro HD cell model to determine whether or not they are suitable therapeutics. We are also testing their ability to improve the survival of induced pluripotent stem cells (iPS) from HD patients. Our studies suggest that our novel molecules offer superiority in autophagy induction in comparison to rapamycin. Our data identifies new small molecule therapeutics that promote autophagy and autophagy flux, suggesting great potential for the therapy for HD patients.

NICHD
David Young
Visiting Fellow
Molecular Biology - Eukaryotic
The ATP binding cassette (ABC) protein RLI1 is required for ribosome recycling in vivo in Saccharomyces cerevisiae
Ribosome recycling is a vital cellular process that involves dissociation of the post-termination 80S ribosome (with the mRNA stop codon positioned at the A site of the ribosome) into a 60S subunit and a tRNA/mRNA-bound 40S subunit, followed by dissociation of the tRNA and mRNA from the 40S subunit. In vitro studies showed that ABC protein RLI1 catalyzes dissociation of the 60S subunit from the tRNA/mRNA-bound 40S subunit, but it is unknown if RLI1 catalyzes ribosome recycling in vivo. To address this question, we performed ribosome profiling of a wild-type (WT) yeast strain and a strain depleted of RLI1 (RLI1 is essential). Ribosome profiling involves deep-sequencing of mRNA fragments protected by 80S ribosomes from nuclease digestion and reveals the exact locations and occupancies of translating ribosomes in the transcriptome. In the RLI1-depleted strain, ribosomes are found stalled at the stop codons of most genes, consistent with a defect in translation termination or ribosome recycling. Remarkably, the 3â€™UTRs of most genes contain large numbers of ribosome footprints, consistent with a recycling defect that allows ribosomes to proceed into the 3â€™UTRs. The distribution of footprints strongly suggests that the ribosomes in the 3â€™UTR are translating, with ribosome peaks occurring at 3â€™UTR termination codons, often in a reading frame different from that of the main coding sequence. These novel 3â€™UTR translation products were directly detected by Western blot analysis in RLI1-depleted strains where Myc tags were inserted upstream of 3â€™UTR stop codons that showed large ribosome occupancy peaks. The size of the observed 3â€™UTR translation products is consistent with a model wherein following translation termination un-recycled 80S ribosomes undergo a frameshift and reinitiate translation of the 3â€™ UTR in a different frame. Ribosome profiling of the RLI1-depleted strain treated with a drug (3-AT) that evokes histidine starvation resulted in increased

ribosome occupancy at His codons both in CDS and 3â€™UTRâ€™s, further confirming 3â€™UTR translation. Thus, by ribosome profiling we have provided the first evidence that RLI1 is required for ribosome recycling in vivo, and that a defect in recycling is accompanied by reinitiation and translation of 3â€™UTRs. Such aberrant reinitiation should be disastrous for cell metabolism, owing to production of abnormal peptides and a reduced pool of ribosomal subunits available for translating the correct coding sequences.

NICHD
Taylor Updegrove
Postdoctoral Fellow
Molecular Biology - Prokaryotic
A sE regulated small RNA dedicated to repressing synthesis of the abundant Lpp protein
The outer membrane (OM) of gram-negative bacteria is the first line of defense against the environment, as it is a barrier against antibiotics and other stresses. A key component of this barrier is the lipoprotein, Lpp which connects the OM to the peptidoglycan layer and is the most abundant protein in the cell. sE is the major transcription factor in E. coli dedicated to maintaining cell envelope homoeostasis. sE induces ~100 genes involved in OM modification and repair and represses the synthesis of OM proteins through induction of two regulatory RNAs (sRNAs) MicA and RybB. We set out to identify other sRNAs that are induced by sE using tiling arrays to monitor transcript levels following sE overexpression. Surprisingly, a transcript originating from the 3â€™ end of the known cutC gene was highly induced. Northern blot analysis and RACE experiments confirmed this transcript, now denoted MicL, originates within the cutC coding sequence and terminates with the cutC 3â€™UTR, and assays of a micL promoter-GFP promoter fusion verified that the region upstream of micL contains a strong sE promoter. The MicL transcript was found to co-immunoprecipitate with the RNA binding protein Hfq suggesting the sRNA acts by base pairing. Remarkably, deep sequencing after MicL overexpression revealed that MicL has a single target, the lpp mRNA. lpp mRNA and protein levels were elevated in a cutC deletion strain and down regulated after sE overexpression. Repression of wild type and mutant lpp-lacZ translational fusions showed that MicL represses Lpp by direct base pairing. We also found that while lpp overexpression enhances sE activity, MicL overexpression represses it - thus establishing a regulatory feedback loop. The cutC gene was identified by mutations that caused cells to become sensitive to copper. To test whether this phenotype was due to the CutC protein as originally reported, or the previously unknown MicL RNA, we examined the copper sensitivity of strains carrying various mutations. We found that deleting the 5â€™ end of the cutC gene, while leaving the micL gene intact, causes no copper sensitivity, while deleting the lpp gene or overexpressing MicL represses the copper sensitivity in the cutC deletion strain. These results show that MicL regulation of lpp is important for cell adaptation to heavy metal stress and raise the question whether other mutations thought to affect proteins actually impair regulatory RNAs.

NICHD
Yong Cheng
Postdoctoral Fellow
Neuroscience - General
Carboxypeptidase E/(Neurotrophic Factor-a1) is key for preventing depression through increasing hippocampal FGF2 levels and neurogenesis during chronic stress.
Major depressive disorder is associated frequently with stress. Prolonged chronic stress leads to depressive-like behavior in mice, but short-term chronic stress does not, indicating that there is an allostatic mechanism at play. This mechanism that prevents depression during short-term stress is unknown. Here we show that mice subjected to short-term (1 h/day for 7-days) chronic restraint stress

(CRS), do not exhibit depressive-like behavior. These mice showed co-ordinately increased expression of carboxypeptidase E (CPE) (n=6, p<0.01) and fibroblast growth factor 2 (FGF2) (n=6, p<0.01) and enhanced neurogenesis in the hippocampus (n=6, p<0.05). Prolonged CRS (6 h/day for 21-days) resulted in decreased hippocampal CPE (n=5, p<0.05) and FGF2 (n=5, p<0.05) levels and depressive-like behavior (n=10, p<0.001). These results are consistent with previous reports that FGF2 mediates anti-depressive activity. Using cultured hippocampal neurons we show that exogenous CPE (also known as Neurotrophic Factor-a1, NF-a1) directly up-regulated FGF2 expression (n=8, p<0.001) through the ERK-Sp1 signaling pathway, independent of its protease activity. Genetic deletion of CPE in mice resulted in severely decreased hippocampal FGF2 levels (N=5, p<0.05) and reduced immature neuron counts (n=5, p<0.05) in the subgranular zone and depressive-like behavior (n=10, p<0.01). This aberrant mouse phenotype was rescued by peripheral administration of FGF2 (n=10, p<0.01), which increased hippocampal neurogenesis (n=5, p<0.05). Moreover, we show that the drug rosiglitazone, which has been reported to have anti-depressive activity in mice and humans, up-regulated endogenous CPE (n=10, p<0.05) and FGF2 (n=10, p<0.001) in hippocampal neurons. Our results further demonstrate that the rosiglitazone induced up-regulation of CPE and FGF2 were blocked by siCPE. These data identify CPE as a key modifier during short-term CRS to establish allostasis and prevent depressive-like behavior onset through enhancement of hippocampal FGF2 expression and neurogenesis. As such, CPE/NF-a1 is a novel drug target for treatment of depression.

NICHD
Celine Cluzeau
Visiting Fellow
Neuroscience - Neurodegeneration and Neurological disorders
Generation and characterization of a zebrafish model of Smith-Lemli-Opitz syndrome
Smith-Lemli-Opitz syndrome (SLOS) is an autosomal recessive disorder with a broad phenotypic spectrum. It is characterized by multiple malformations, cognitive impairment and abnormal behavior including autistic traits. It is caused by mutations of the DHCR7 gene, encoding the enzyme 7-dehydrocholesterol (7DHC) reductase, which result in decreased cholesterol and accumulation of 7DHC. With the exception of cholesterol supplementation that has shown modest clinical improvement, no therapy is available to date. Several rodent models have proven useful for elucidating some aspects of this syndrome, but they do not offer the ability to study postnatal brain development. The long-term goal of this work is to gain insight into the pathological processes underlying SLOS, and identify and test new targets for therapeutic interventions. We produced a novel SLOS zebrafish model using Transcription Activator-like Effector Nucleases, which induce double-strand DNA breaks at a selected position and introduce small insertion/deletion mutations. Six frameshift and three in-frame mutations in two different exons of dhcr7 were selected to derive lines. We showed that all mutant fish present the characteristic accumulation of 7DHC and decreased cholesterol in both liver and brain from 3 weeks of age, with variable severity depending on the mutation. As observed in both human and mouse, dhcr7-deficient fish growth is delayed. Mutant fish are able to breed. However, the yolk from eggs produced by mutant females contains 7DHC, and the 1-week-old mutant progeny of the mutant females already present with high levels of 7DHC. In spite of this early exposure to 7DHC, this second generation of mutant fish surprisingly survives past 5 weeks of age. However, initial results of cartilage stain with alcian blue in one-week-old larvae showed that about one third of these mutant fish have lower jaw anomalies. Interestingly, the spontaneous movement of 3-day-old mutant embryos is decreased compared to control and heterozygous embryos. We are working on determining whether this decreased activity could be a consequence of a developmental delay or a behavioral defect. In both cases, it might be a useful trait to develop an in vivo assay for screening potential therapeutic drugs.

Future work will continue the characterization of dhcr7-deficient fish development. We will also pursue the characterization of the motor and cognitive functions as well as the behavior of dhcr7-deficient fish.

NICHD
Kathryn Tabor
Postdoctoral Fellow
Neurotransmission and Ion Channels
Direct activation of central motor command neurons by electric field pulses drives rapid escape responses
Electric shock is widely used in biomedical research to study stress, pain and learning and memory in model species throughout the animal kingdom. Research in fish models have used electric field pulses (EFPs) for forty years as noninvasive stimuli to investigate behavior, sleep and basic forms of learning. However, the sensory modality through which EFPs trigger behavioral responses in fish remains unknown. We used high-throughput behavioral analysis in combination with genetic and physiological techniques to elucidate the neuronal mechanism by which EFPs elicit behavioral responses in larval zebrafish. Remarkably, we found that EFPs triggered well-coordinated escape behaviors closely resembling the escape responses initiated by the Mauthner cells, a pair of giant motor command neurons. To monitor the activity of the Mauthner cells during EFP responses we used the Gal4/UAS system to target expression of the calcium indicator, GCaMP6s, to Mauthner cells. The natural transparency of larval zebrafish allowed for calcium imaging in behaving larvae, which revealed robust activity in the Mauthner cells during the EFP response. Next, to test if Mauthner cells were required for the EFP response we used targeted genetic or laser ablation. EFP responses were lost after bilateral ablation of the Mauthner cells, demonstrating that the Mauthner cells were necessary for EFP responses. Surprisingly, the reaction time to EFPs was extremely short, with many escape responses initiated within 2 ms of the EFP, faster than any other sensory-evoked escape response. We asked if the rapid reaction time to EFPs was due to direct activation of the Mauthner cells, bypassing delays imposed by stimulus detection and transmission by sensory cells. To address this we developed a novel approach to isolate the Mauthner cells from other brain activity by using the Gal4/UAS system to target expression of the tetrodotoxin (TTX) resistant voltage-gated sodium channel, SCN5. Mauthner cells transgenically expressing SCN5 retained responsiveness to EFPs despite TTX-induced suppression of action potentials in the rest of the brain. Thus, EFPs directly activate the Mauthner cells. This surprising discovery that EFPs are not detected by a sensory modality but instead selectively trigger a pair of central motor command neurons to drive an escape response will compel researchers to re-interpret the large body of research using EFPs to interrogate behavior.

NICHD
Mark Ziats
Doctoral Candidate
Psychiatry
Altered Processing of the Mitochondrial Genome May Link Known Molecular and Synaptic Abnormalities in Autism Spectrum Disorder
Both gene expression and mitochondrial function are known to be abnormal in the brains of individuals with autism spectrum disorder (ASD). However, no work has specifically investigated the expression of the unique mitochondrial genome (mtDNA) in ASD brain. Therefore, I hypothesized that altered expression of the mitochondrial transcriptome may represent a point of convergence between previously reported defects in gene transcription and known synaptic abnormalities related to dysfunctional mitochondrial-initiated apoptosis in ASD. To test this hypothesis, I performed mitochondrially-focused gene expression profiling on post-mortem brain tissue from pediatric autistic

patients (n =9), and matched control samples (n = 9). The prefrontal cortex (PFC) and the cerebellum, both previously associated with ASD, were assessed from each donor. A human mitochondria-focused cDNA microarray was designed, which contained all 37 mitochondrialâ€“encoded genes, 1,098 nuclearâ€“encoded genes that function specifically in the mitochondria, and 225 controls. Microarray labeling, hybridization, and bioinformatic data analysis were performed following standard protocols. Our results demonstrate substantial, brain-region specific changes in both mtDNA and nuclear-encoded genes with mitochondrial function in ASD. Specifically, all mtDNA genes that are up-regulated in autistic PFC encode for transfer RNAs (tRNAs). In the cerebellum, most of the same tRNAs are also up-regulated, but so are components of the NADH dehydrogenase complex. Remarkably, nuclear-encoded mitochondrial genes with significantly increased expression in ASD prefrontal cortex (274 genes) are enriched for the process of apoptosis, whereas significantly down-regulated genes in autistic PFC (197 genes) are enriched for the process of oxidative metabolism. However, autistic cerebellum does not have apoptosis-related gene expression changes. In summary, autistic brains have alterations of mitochondrial genes involved in oxidative metabolism and in tRNAs in both PFC and cerebellum, yet apoptotic functions appear up-regulated only in ASD prefrontal cortex. This supports other cell-level work in ASD of PFC-specific mitochondrial abnormalities. Collectively, our results suggest that altered processing of the unique mitochondrial genome may partially reconcile known transcriptional and mitochondrial/apoptosis abnormalities in ASD, and these molecular results recapitulate known cell-level region-specific pathology.

NICHD
Julia Rodiger
Visiting Fellow
Stem Cells - General
The histone methyltransferase PRMT1 regulates adult intestinal stem cell development in mice
Adult organ-specific stem cells are essential for organ homeostasis, tissue repair and regeneration. Since the intestinal epithelium is constantly renewed every 2-6 days throughout mouse adulthood, the intestinal epithelial stem cells (ISC) are often used as a stem cell model system. Furthermore, uncontrolled proliferation of ISC can lead to intestinal cancer. However, the molecular mechanisms regulating the formation of adult stem cells during development are poorly understood, indeed elucidating this is important to understand how stem cell activity leads from normal renewal to cancer development. During mouse development, formation of adult ISC takes place around birth when plasma thyroid hormone (TH) levels peak. TH is essential for postembryonic development in several species and functions by binding to nuclear TH receptors (TRs), which in turn recruit coactivators and promotes gene transcription. One of these coactivators is the protein arginine N-methyltransferase 1 (PRMT1) that mediates post-transcriptional modification of target proteins such as histone H4 through arginine methylation. We observed that PRMT1 is also upregulated around birth in the developing stem cells and thus hypothesize that PRMT1 is involved in ISC formation in mice. As the homozygous knockout (KO) of PRMT1 in the whole mouse is embryonic lethal, we generated a conditional KO of PRMT1 only in the intestinal epithelium to investigate the participation of PRMT1 in ISC development via the Cre-Flox System using Villin-Cre as a specific intestinal promoter. Our results indicate that conditional PRMT1 KO animals altered intestinal crypt development by the time when the adult intestine is developed. The crypts, where the intestinal stem cell population resides, are elongated and thinner compared to wild type control. Further, proliferation in this crypt area was significantly reduced, indicative of a reduction in stem cell activity. In addition, when TH was administered around birth to promote adult stem cell development, we observed similar effects due to intestine-specific PRMT1 KO. In summary, these findings indicate that PRMT1 is important for the TH dependent formation of adult intestinal stem cell

development during mouse intestinal maturation. This raises new insights into the development of ISC and intestinal diseases, clarifying the role of PRMT1 dysregulation in intestinal cancers.

NICHD
Parmit Singh
Visiting Fellow
Virology - RNA and Retroviruses
A dense-profile of 3 million HIV-1 insertion events reveals targeting in cancer genes and identifies a role of mRNA splicing in integration
Integration of cDNA plays a key role in HIV-1 infection. Interaction of HIV-1 integrase and chromatin-binding factor LEDGF preferentially targets HIV-1 to actively transcribed genes. Still, it is not clear how LEDGF is recruited to active genes. Although HIV-based vectors are popular in gene therapy to avoid oncogene activation by other retroviral vectors, it is not known whether HIV favors oncogenes. To address this, I developed a novel method using ligation mediated PCR and Illumina sequencing in cultured cells to map 3-million independent HIV insertions at single nucleotide positions. Of 1 million integration positions, 597,453 had multiple insertions. In total, 17,384 genes were targeted. I obtained reproducible integration densities per gene in 9 independent PCR libraries (R2>0.9) and 2 different cell lines (R2=0.85), indicating this technique accurately measures integration frequency of each gene. 1000 genes with the highest integration densities showed 5 times more integration than would occur by chance. Cancer genes were five times more common in these top 1000 genes than expected based on their abundance in the genome, indicating that HIV-1 can impact the growth behavior of infected cells. I calculated the distribution of insertions in all transcription units divided in 15 equal segments; found a unique pattern with preference for the 5â€™ ends of transcription units. RNA-Seq analysis showed that integration density in genes correlated strongly with the number of alternative transcripts (R2=0.97) indicating splicing increases integration frequency. This correlation is independent of transcription levels (R2=0.00013). Integration density in genes also correlated strongly with numbers of introns in the gene (R2=0.73), as intron-containing genes are more targeted than intron-less genes. This correlation is independent of intron sequence as both introns and exons showed the same integration density. Integration in intron-less genes favored the 3â€™ end of transcription units, suggesting a role of splicing in the 5â€™ end integration preference of genes with introns. An analysis of published data in mouse showed that LEDGF-dependent HIV-1 integration has this preference for the 5â€™ end of transcription units in intron-containing genes and the 3â€™ preference in intron-less genes. The high correlation between integration amounts, number of isoforms and number of introns suggests a role of co-transcriptional splicing in LEDGF dependent HIV-1 integration.

NIDA
Vivek Kumar
Visiting Fellow
Chemistry
Novel and High Affinity Fluorescent Ligands for the Serotonin Transporter based on (S)-Citalopram
The serotonin transporter (SERT) is a member of the Neurotransmitter:Sodium Symporter (NSS) family of transporters and regulates serotonin signaling and homeostasis. The important classes of selective serotonin reuptake inhibitors (SSRIs) and tricyclic antidepressants (TCAs) that are prescribed for the treatment of anxiety and major depressive disorders work through inhibition of serotonin reuptake via the SERT. Despite clinical success, drug-protein interactions at the molecular and cellular levels that result in inhibition of serotonin reuptake have not been characterized fully. One approach to studying native protein function, regulation, oligomerization and distribution, in live cells or brain tissue, is to use fluorescently labeled small molecules. We have previously synthesized tropane-based fluorescent

probes (e.g. JHC1-064) that have proven highly useful for visualizing monoamine transporters in both transfected cells and live neurons. However, JHC1-064 binds to all three monoamine transporters and therefore would have limited usefulness in brain tissue for specific visualization of the SERT. Hence we designed and synthesized several rhodamine-labeled ligands based on the selective serotonin reuptake inhibitor (S)-citalopram. This series of N- and 5-subsituted-(S)-citalopram analogues were evaluated for uptake inhibition at the SERT, dopamine and norepinephrine transporters (DAT and NET, respectively) and for binding at SERT, in transiently transfected COS7 cells. All four fluorescent analogues demonstrated moderate to high potency for inhibition of serotonin uptake at SERT and none for the analogues were active at DAT or NET, at a concentration of 10 micromolar. The 5-substituted analogues were generally more potent at SERT and (R)-2-(6-(diethylamino)-3-(diethyliminio)-3H-xanthen-9-yl)-5-(N-(6-(((1-(3-(dimethylamino)propyl)-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-yl)methyl)amino)-6-oxohexyl)sulfamoyl)benzenesulfonate (VK 02-83) demonstrated the highest binding affinity and selectivity, in the set, with Ki=3.0 nM. This is comparable to the parent compound (Ki=2.6 nM), indicating that rhodamine does not interfere with binding of the pharmacophore to the SERT binding site. VK 02-83 also demonstrated highly specific binding to cells transiently transfected with EGFP-SERT as observed by confocal microscopy in HEK293 cells. Visualization and transporter trafficking studies with VK 02-83 are underway in raphe neurons.

NIDA
Melody Furnari
Postdoctoral Fellow
Cultural Social and Behavioral Sciences
Real-time stress, craving and mood differences in drug treatment responders and nonresponders
Although stress is widely believed to influence drug use, the exact relationship has been difficult to elucidate. Much of the existing research has been limited by the recall bias inherent in retrospective assessment. We used a real-time data-collection method called ecological momentary assessment to study drug use and stress during addiction treatment. This allows a truly prospective look at stress and drug use. We evaluated whether stress and drug use differed between outpatients who reduced their drug use while in treatment (responders; R) and those who did not (Nonresponders; NR). Opioid-dependent volunteers receiving methadone maintenance were given smartphones for 16 weeks of ecological momentary assessment. They were asked to complete 3 randomly prompted entries (RP) per day and to initiate an event-contingent entry (EC) each time they used a drug or felt more stressed than usual. Urine drug screens (3 x/wk) and EC opioid-use reports were used to identify Rs (negative for opioid use >50% of wks) and NRs (negative for opioids <50% of wks). RP and EC data were then compared between the two groups using multilevel models. Rs (N=50) and NRs (N=47) had 11% and 72% opioid-positive urines, respectively. NRs had higher craving ratings for heroin (d = .61) and lower positive mood ratings (d = 1.21) compared to Rs. There was no difference in RP stress ratings or numbers of EC stress events between Rs and NRs, though NRs gave higher ratings of ability to deal with stress in both types of entries (RPs, d = .48; ECs, d = .54). The groups also reported different causes of stress. Rs reported higher rates of interpersonal conflict, having too much to do (d = .81) and health problems (d = .68), while NRs reported higher rates of thinking about stressful things (d = .74) and being in unsafe surroundings (d = .48). Thus, although Rs and NRs rated their levels of ongoing stress and frequency of stressful events similarly, they differed in types of stress and in mood and craving. These results are an important first step in understanding the nature of stress in the lives of drug users and its relationship to drug use, and how treatment interventions should be individualized.

NIDA
Xuan Li

Postdoctoral Fellow
Neuropharmacology and Neurochemistry
Role of histone deacetylase 5 in dorsal striatum in incubation of methamphetamine craving
A key feature of methamphetamine addiction is high relapse rates during abstinence. We previously have demonstrated that cue-induced methamphetamine seeking in rats progressively increases after withdrawal from extended access intravenous methamphetamine self-administration. Here, we studied the mechanisms underlying this â€˜incubation of methamphetamine cravingâ€™. Emerging evidence suggests that histone deacetlyases (HDACs) play a key role in addiction-related behaviors. For example, HDAC5 in mice nucleus accumbens has been shown to negatively regulate cocaine reward. To assess the role of HDAC5 in incubation of methamphetamine craving, we first measured HDAC5 expression in rat dorsal striatum 2 and 35 days after withdrawal from either saline or methamphetamine self-administration training. Compared to saline rats, HDAC5 mRNA and protein level in dorsal striatum of methamphetamine rats significantly increased on withdrawal day 35 but not day 2, suggesting that HDAC5 in dorsal striatum may contribute to the progressive increase of cue-induced methamphetamine craving. Next, we injected adeno-associated virus (AAV) bilaterally into dorsal striatum to over-express a constitutively active form of HDAC5 (MUTANT-HDAC5). Control rats received bilateral AAV-GFP injection into dorsal striatum. After virus injection, we trained all rats to self-administer methamphetamine and tested cue-induced extinction responding on withdrawal day 2 and 35. We also examined re-escalation of methamphetamine intake after abstinence, and progressive-ratio responding, a measure of motivation to take methamphetamine. During self-administration training, methamphetamine intake did not differ between two groups. While both groups showed incubation of methamphetamine craving after withdrawal, cue-induced extinction responding in MUTANT-HDAC5 group was significantly higher than GFP group on withdrawal day 35 but not day 2. Moreover, MUTANT-HDAC5 group showed accelerated re-escalation of methamphetamine intake after abstinence and significantly elevated progressive ratio responding compared to GFP group. Overall, our data suggest that potentiation of HDAC5 function in dorsal striatum positively regulates incubation of cue-induced methamphetamine craving, re-escalation after withdrawal and progressive ratio responding. Studies are underway to examine the behavioral effects of attenuation of endogenous HDAC5 function in rat dorsal striatum during incubation of methamphetamine craving.

NIDA
Francisco Rubio Gallego
Other
Neuropharmacology and Neurochemistry
Unique molecular alterations in dorsal striatal neuronal ensembles selectively activated by environmental cues associated methamphetamine seeking in rats
Drug addicts learn to associate environmental stimuli (cues) with drug taking, and over time these cues can promote relapse. We hypothesize that these drug-related memories are encoded by unique alterations induced within sparsely distributed patterns of neurons called neuronal ensembles that are selectively activated during drug taking. Here we assessed unique gene expression alterations induced within behaviorally activated dorsal striatal neurons in a rat model of drug relapse called context-induced reinstatement of drug seeking. We double immunolabeled cells that express NeuN and Fos protein to identify neurons that were strongly activated during reinstatement. We used fluorescence-activated cell sorting (FACS) to isolate activated neurons and assessed mRNA expression using RT-qPCR from Fos-positive neurons. Rats were trained over 12 days to press a lever to receive methamphetamine infusions in one environment (training context) that became associated with drug reward. Lever pressing was extinguished over 12 more days in a different environment (extinction context) where lever pressing did not produce drug infusions. On test day, we induced context-induced reinstatement

of drug seeking by re-exposing the rats to their training context in the absence of drug infusions. Reinstatement increased the number of Fos-positive neurons 2-3 folds in lateral and medial portions of the dorsal striatum, relative to control rats not re-exposed to the training context. We then isolated RNA from 5-487 FACS isolated Fos-positive neurons and assessed gene expression of 20 genes using gene-targeted pre-amplification and qPCR. Reinstatement increased expression of the gene encoding NMDA-type glutamate receptor subunit GluN2A (and other genes) in the activated Fos-expressing neurons, but not in Fos-negative neurons. Quantitative in situ hybridization RNAscope® assay for both Fos and GluN2A RNA transcripts confirmed that GluN2A mRNA was preferentially increased in Fos-positive neurons, but not Fos-negative neurons, located in the lateral, but not medial, part of the dorsal striatum. Intracerebral injections of GABA agonists (muscimol and baclofen) were used to inactivate the dorsal striatum during reinstatement. Lever pressing decreased 50% following injections into the lateral, but not medial, dorsal striatum. Future experiments will examine the relevance of increased GluN2A expression in these selectively activated dorsolateral striatal neurons in our relapse model.

NIDA
Dong Wang
Research Fellow
Neuroscience - Integrative, Functional, and Cognitive
Median raphe nucleus regulates hippocampal ripple oscillation and memory consolidation
Sleep promotes memory consolidation, a process known as reorganization and progressive integration of newly acquired information into pre-existing neural networks for long-term memory storage. The hippocampus is critical for memory consolidation and its damage leads to amnesia, or the inability to form new memory of events or facts. Recent studies found that hippocampal ripple activity, a discrete fast oscillation (~200 Hz) that occurs primarily during slow-wave sleep, plays an essential role in memory consolidation: 1) newly formed hippocampal neural firing patterns tend to reactivate coinciding with ripple events, a phenomenon referred as â€˜memory replayâ€™; and 2) disruption of ripple activity impairs memory consolidation. However, little is known about how hippocampal ripple activity is regulated by other brain regions. Median raphe nucleus is thought to regulate brain states, and sends a direct projection to the hippocampus, thus we sought to examine its role in regulating hippocampal activity. We performed in vivo recording in the median raphe and hippocampus simultaneously, and found that majority raphe neurons displayed activities correlated with ripple events during slow-wave sleep. Notably, activity of one distinct group of raphe neurons (~20%) was accompanied by suppression of ripple events. To determine whether there was a causal link, we stimulated raphe neurons using an optogenetic procedure, and found that the ripple occurrence was largely suppressed, suggesting a strong regulation of hippocampal ripple activity by the raphe nucleus. We hypothesized that this regulation of hippocampal activity could lead to regulation of memory consolidation. Therefore, mice were trained with a hippocampus-dependent contextual fear conditioning, followed by optical stimulation of the raphe nucleus during sleep/rest. We found that these mice showed significantly reduced freezing behavior when tested the next day, suggesting that stimulation of the raphe nucleus impairs fear-memory consolidation. Together we provide a novel neural mechanism involving the median raphe nucleus in regulating hippocampal ripple oscillation and memory consolidation processes.

NIDA
Marco Pignatelli
Research Fellow
Neurotransmission and Ion Channels
Synaptic plasticity of dopamine neurons and behavioral profile in the DATcre Thorase mice

Dopamine (DA) neurons in the ventral tegmental area (VTA) are crucial for the 'stamping-in' of memory, thus attaching motivational salience to otherwise neutral stimuli. Stress, substance of abuse and release of DA to reward predictive cues parallels an enhanced synaptic strength onto DA neurons. The latter process develops over the course of cue learning, supporting the idea that enhance synaptic strength might be the event supporting the transformation of neutral stimuli to salient cues. Synaptic efficacy is mainly defined by two phenomena: long-term potentiation (LTP) and depression (LTD) of excitatory synaptic transmission. The tuning of both directions of plasticity, LTP and LTD, is mainly regulated by synaptic insertion or removal of post-synaptic AMPA receptors (AMPAR). These processes are recognized as cellular mechanisms for learning and memory in the brain. The cellular events underlying this processes are poorly understood, particularly within the DA neurons. To understand the significance of synaptic plasticity within DA neurons of the VTA we have taken advantage of the â€œplasticity-relatedâ€ protein Thorase. We developed a mouse line carrying a conditional deletion (cKO) of the ATPase Thorase protein in a DAT-cre dependent fashion. Thorase protein mediates, in an ATPase-dependent manner, the internalization of AMPAR by disassembling the AMPAR-GRIP1 complex. Using in vitro electrophysiology we found that genetic ablation of Thorase from DAT+ cells produces a dramatic increase in surface AMPAR expression both measured as AMPA/NMDA ratio and amplitude of mEPSCs. We also found that this deletion impact plasticity properties of the VTA, reducing the ability of glutamatergic synapses to undergo LTD. We suspected that this synaptic phenotype can affect general mechanisms of associative learning. To address the latter question we designed two sets of behavioral experiments falling at opposite ends of a salience spectrum : reward (appetitive pavlovian learning) and aversion (fear conditioning test). At this point our preliminary data suggest that cKO animals exhibited a propensity to learn fearful experiences (fear conditioning) and a severe impairment to perform an associative reward learning task (appetitive pavlovian learning) compared to their respective littermates controls. In conclusion our data support the fascinating idea that a â€œrigidâ€ synaptic state within DA neurons can produce several and remarkable consequences at the behavioral level.

NIDA
Leslie Whitaker
Postdoctoral Fellow
Neurotransmission and Ion Channels
Associative learning drives the formation of silent synapses in neuronal ensembles of the nucleus accumbens
Learned associations between rewards and reward-predictive stimuli are critical to the production of motivated, goal-directed behavior that is necessary for gaining sufficient nutrients, surviving, and reproducing. However, drugs of abuse such as cocaine are capable of driving maladaptive learning of such associations that contributes to the development of addictive disorders. The learning of such associations requires alterations in specific neuronal ensembles, or small populations of sparsely distributed neurons. These neuronal ensembles have been previously shown to play a causal role in the learning of drug-associated behavior. Previous assessments of functional alterations induced by learning have been unable to distinguish neurons specifically activated by learning from non-activated neurons. In this study, cfos-GFP transgenic mice in which the c-fos promoter is fused with the GFP coding sequence were used. Strong neural activity causes the induction of the immediate early gene c-fos, thus we were able to identify cells that were strongly activated during the learning of a particular behavior by using c-fos-GFP transgenic mice. We examined both context independent and context specific cocaine-induced locomotor sensitization, two forms of drug-mediated associative learning. Following activation of a specific population of neurons by cocaine-induced locomotor sensitization, whole cell electrophysiological recordings were performed in brain slices to assess synaptic alterations in specifically activated neurons compared to surrounding non-activated neurons. We found that following

learning of context-independent cocaine sensitization, there was an increase in silent synapses in strongly activated (GFP+) neurons of the nucleus accumbens shell regardless of training context. However, in animals that displayed context-specific cocaine sensitization, this increase in silent synapses was observed exclusively in animals trained and tested in the same context. Silent synapses contain functional NMDA-type glutamate receptors, but no functional AMPA-type glutamate receptors and are thought to reflect an increased capacity of the cell to undergo subsequent plasticity. Our results indicate that the learning of an association between environmental stimuli and drug reward may be encoded by the formation of silent synapses in specific neuronal ensembles that play a causal role in behavior.

NIDA
Lindsay De Biase
Postdoctoral Fellow
Physiology
Microglia within adjacent basal ganglia nuclei exhibit distinct membrane properties and divergent responses to pathology
Microglia are ubiquitous, stellate-shaped glial cells with motile processes that survey surrounding brain tissue. These immune-like cells remove debris and foreign elements from the CNS and exert both neuroprotective and neurotoxic effects during pathology. Recent findings indicate that microglia can also influence the activity of surrounding neurons through release of inflammatory and trophic signaling factors. Microglia are best studied in the hippocampus, cortex, and spinal cord, and are assumed to possess equivalent properties throughout the rest of the CNS. We investigated microglia within the basal ganglia (BG), a collection of brain nuclei that play critical roles in motor and reward-based learning. Immunohistochemical analysis in transgenic mice that express EGFP within microglia revealed that microglial density is dramatically elevated in the substantia nigra pars reticulata (SNr) and significantly depressed in the ventral tegmental area (VTA) compared to better studied brain regions. SNr microglia also display highly complex morphologies compared to VTA microglia. In these same microglial-EGFP mice, we performed the first electrophysiological recordings of BG microglia in acute brain slices and found that SNr microglia have significantly larger membrane capacitance and more negative resting membrane potential than VTA microglia. In addition, 60% of microglia within the SNr exhibited delayed rectifier potassium currents (Kdr), whereas only 10% of VTA microglia displayed these currents. Kdr expression is thought to distinguish injury-responsive, or â€œreactiveâ€ microglia, yet control experiments indicate that Kdr expression is an intrinsic feature of SNr microglia. In addition, when mice were treated with single injections of bacterial endotoxin, which causes a systemic inflammatory response, SNr microglia did not exhibit significant changes in morphology. In contrast, VTA microglia displayed reduced morphological complexity, a hallmark of microglial reactivity. This is the first report of regional variability in microglial electrophysiological properties and responses to an inflammatory insult. Collectively, these findings indicate that microglia tailor themselves to their specific brain region and cannot be considered equivalent throughout the brain. These data also raise important questions about how VTA and SNr microglia impact neurons within their vicinity, both under basal conditions and during drug abuse and Parkinsonian neurodegeration.

NIDA
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Postdoctoral Fellow
Psychiatry
Discrete-choice procedure: methamphetamine versus an alternative nondrug reward
In humans, drug addiction is characterized by intense behavioral allocation toward the procurement and use of the substance at the expense of other non-drug related activities. The Diagnostic and Statistical

Manual of Mental Disorders-V identify 6 out of 11 criteria for the diagnosis of addiction in relation to this behavioral crystallization, indicating that it is a defining feature of drug addiction. However, in most animal behavioral models of addiction, the subject exists in a â€œchoice vacuumâ€ with no alternative behavioral options but to take the drug. Therefore in these settings animals are not provided with the opportunity to choose between taking the drug and, for example, an alternative reward. This is matter of concern because of the impossibility to discern between addiction and a mere drug taking driven by lack of choice. Only recently, some researchers have addressed these concerns using a discrete-choice procedure. The authors surprisingly found that when given a choice between cocaine or heroin and a palatable reward, 90% of male rats prefer the non-drug reward. The finding emphasizes that the â€œdrug- usurpation hypothesisâ€ is insufficient to account for the addiction, conceptualized as pathology of choice. Here, we used a similar discrete-choice procedure to assess whether the non-drug preference generalizes to rats with a history of methamphetamine (Meth) self-administration. We trained non-food-deprived rats to lever press on a Meth- or palatable food-associated lever during alternating daily 3-hour sessions (0.1 mg/kg/infusion or 5 food pellets). We then gave the rats discrete choice sessions under different conditions: following limited (3 h/day) or extended (6 h/day) access to Meth self-administration, training with a higher unit dose of Meth (0.2 mg/kg/infusion), priming injections of Meth (0.5 and 1.0 mg/kg), or food pellet exposure in the ratsâ€™ home cage (a satiety manipulation) prior to choice testing and 21 abstinence days. We found that regardless of the condition, 95% of our rats strongly preferred the palatable food reward over Meth. These results extend the previous findings with cocaine-trained rats and, importantly, demonstrate that only a minority of individuals/subjects can be characterized as â€œaddictedâ€ (e.g., 5% in our experiment). We are currently examining several parameters that might contribute to these individual differences, as well as the circumstances that can make the â€œaddictedâ€ rat â€œnon-addictedâ€

NIDA
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Psychiatry

Clonidine, a novel adjunct therapy to buprenorphine-maintenance, increases the length of opiate abstinence

Despite effective pharmacological treatments (e.g., buprenorphine) for opiate dependence, relapse rates remain alarmingly high. Investigations of relapse in animal models have shown that relapse has multiple causes, such as drug priming and stress. Buprenorphine is effective in blocking drug-primed relapse, but has no effect on stress-induced relapse. Alpha-2 agonists block stress-induced relapse. Thus, the clinical efficacy of buprenorphine might be enhanced by adjuvant treatment with an alpha-2 agonist. Clonidine is an alpha-2 agonist uniquely suited for such an examination, as it is used within the population to treat withdrawal symptoms. In this randomized, double-blind, placebo-controlled, clinical trial we examined the ability of the novel drug combination of clonidine and buprenorphine to decrease time to relapse and increase abstinence duration. Participants, n=207, received buprenorphine and psychological counseling throughout the study. In the first 6 weeks, participants also received contingency management to facilitate initial abstinence, a crucial step, as relapse cannot be studied without abstinence. Participants, n=115 (56%), successfully maintained abstinence for two weeks and were randomized to receive clonidine or placebo for 12 weeks, followed by 8 weeks of buprenorphine only. Participants, n=110 (53%), who were still attending the clinic after a 2-week clonidine/placebo induction and who tolerated the study medication were included in the final analysis. Urine toxicology was done thrice weekly. Relapse was defined as two consecutive opiate positive or missed urines; the longest period of abstinence was defined as the most days of consecutive opiate-negative urines. Time to relapse was examined using a Cox regression survival analysis and longest period of abstinence with

an independent samples t-test. Survival analyses revealed no between-group differences in time to relapse. However, participants taking clonidine achieved more continuous days of abstinence than those taking placebo (mean (SE): 37.8 (3.7) vs. 28.1 (2.8), respectively; p = .04). This suggests that clonidine has utility to reduce opiate use when given as an adjunct pharmacological treatment to buprenorphine. This indication would be a novel and important one for clonidine, adding to its current uses in hypertension and opioid detoxification. Future analyses will examine the proposed mechanism by determining if stress-induced lapses were less frequent in the active group.

NIDCD
Ho Ming Chow
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Neuroscience - Integrative, Functional, and Cognitive
Temporal and spatial patterns of brain activity preceding and following stuttering
Developmental stuttering is one of the most common speech disorders, characterized by involuntary disruptions during continuous speech. Severe stuttering can have a devastating impact on a personâ€™s social, emotional, academic, and vocational development. However, very little is known about the neural responses that initiate stuttering events during continuous speech. The main obstacle to studying this issue is speech-related artifacts that contaminate data acquired by all kinds of non-invasive neural imaging techniques including functional magnetic imaging (fMRI). To tackle this problem, we developed a method that uses independent component analysis (ICA) to decompose fMRI data acquired during speech production into a number components representing the underlying sources. Based on the spatial features of each component, noise components were identified by an automatic classifier and their variances were removed from the original data set. This method allows us to study continuous speech using fMRI to collect a whole brain image every 2 seconds. We recruited 26 adults who stutter and instructed them to tell stories while their neural response was recorded using fMRI. To identify their stuttering events, 2 MRI-compatible cameras and a microphone were placed inside the scanner to capture participantsâ€™ facial expressions and speech. After using ICA to remove speech-related artifacts from the fMRI data, we used a finite impulse model to capture the development of neural response from 6 seconds before and 4 seconds after the onset of stuttering events. The preliminary results show that compared with fluent speech, there was no significant difference at 6 seconds before stuttering onset, but at 4 seconds, the putamen became significantly activated bilaterally. At two seconds prior to stuttering onsets, activation of putamen decreased, while at the same time activity in the motor areas increased. The motor activation peaked at stuttering onset and was maintained in the primary motor areas during the course of stuttering. This study shows that for the first time, we are able to disambiguate neural responses associated with the initiation of stuttering and responses associated with stuttering-related orolaryngeal movements. The results indicate that the putamen plays an important role in initiating stuttering, and suggest that therapeutic interventions aimed at regulating activity in the putamen should be useful in the treatment of stuttering.

NIDCR
Ramiro Iglesias-Bartolome
Visiting Fellow
Carcinogenesis
A novel Gas-PKA tumor suppressor pathway in the skin
The integrity of the epidermis is highly dependent on resident self-renewing stem cells, which are controlled by diverse signaling networks that ensure proper tissue homeostasis. Many cell surface receptors control these processes, coordinating stem cell proliferation with the activation of differentiation programs in response to local and environmental cues. Epithelial stem cells express

multiple G protein-coupled receptors (GPCRs), many of which are predicted to couple to the heterotrimeric G protein Gas. The function of these GPCRs and their downstream signaling targets are still largely unknown. Thus, we deleted Gnas (the gene encoding for the GaS subunit) using a floxed Gnas allele and cytokeratin 14 (K14) promoter-driven tamoxifen-inducible CRE to explore the role of this G protein on epithelial stem cell fate decisions. Surprisingly, conditional Gnas deletion was sufficient to promote the massive amplification of a GLI+ hair follicle stem cell compartment, and the rapid development of basal cell carcinoma within weeks. As Gas stimulates cAMP formation, we next investigated whether this effect was dependent on a reduced activity of PKA, taking advantage of the PKA-inhibitor protein (PKI). We generated mice expressing GFP-PKI under the control of the tet-responsive elements and bred them with mice expressing the reverse tetracycline-activated transactivator (rtTA2) under the control of the K5 promoter in order to target epithelial stem cells. Mice in which PKA was inhibited in the skin developed basal cell carcinoma with an early onset and widespread disease manifestation. We also engineered mice expressing the constitutively active form of Gas (GasR201C) in epithelial progenitors, and found that overactivation of this pathway results in hair follicle stem cell exhaustion and hair loss. Using these animal models, gene expression analysis, and in vitro stem cell function assays, we found that a Gas-PKA axis limits the transcriptional activity of hair follicle stem cell regulators, including GLI transcription factors and the YAP1 co-transcriptional activator, in order to coordinate epithelial stem proliferation with proper differentiation, preventing tumor formation These findings position Gas and PKA as master regulators of stem cell fate in the skin, and highlight their ability to function as tumor suppressor genes, in particular for malignancies driven by GLI and YAP transcriptional activity.

NIDCR
Imran Khan
Visiting Fellow
Chemistry
ATF-4 mediates Near-Infrared Laser Phototoxicity
Photobiomodulation (PBM) is a non-invasive therapy which uses absorption of photonic energy by molecular photoacceptors leading to photobiological effects for a broader range of clinical applications such as pain relief and wound healing. However, this therapy shows a biphasic response (Arndt-Schulz) curve where low doses of light are beneficial while high doses generate phototoxicity, highlighting the importance of defining maximal clinical therapeutic dose. In an attempt to establish the therapeutic dose limits of near infrared light therapy (810nm laser), its absorption through mice skin was assessed. We noted 50% attenuation of the light dose in mice skin while remaining light gets absorbed by the underlying tissues. Increasing laser dose treatment on mice skin demonstrated increased erythema and cutaneous damage that correlated with increased (= 60C) skin temperature. To simulate these laser effects in vitro on a monolayer of keratinocyte cells, laser treatments were performed on clear (10% absorption) and dark (100% absorption) well plates. This led us to develop an in vitro model of skin phototoxicity where a specific laser dose (300J/cm2) was noted to be cytotoxic in dark wells that correlated with increased surface temperature while clear wells showed no phototoxicity even at higher doses. The molecular pathways mediating laser phototoxicity were then examined. We observed the ability of laser to generate reactive oxygen species (ROS) that acts synergistically with a rise in temperature (that inactivates endogenous ROS scavengers) resulting in significant endoplasmic reticulum (ER) stress. Laser treatment in presence of ROS scavengers (N-Acetyl cysteine or Catalase) or at low temperature (4C) were noted to rescue cells from laser phototoxicity. Further, laser phototoxicity-induced ER stress appears to be driven by Activating Transcription Factor-4 (ATF-4) that mediates heat shock response and autophagy. ATF-4 knock down with siRNA, inhibitors of heat shock response (HSP70 but not 90) and autophagy (3-methyladenine and Bafilomycin) were noted to sensitize

the cells to laser phototoxicity. In contrast, pre-treatment with autophagy-inducers (Rapamycin and Tamoxifen) were able to rescue these cells. Interestingly, in this study we established the precise mechanism of laser-induced phototoxicity without generating any genotoxic effects (Ames test). Ongoing studies are exploring ER stress transgenic mice models for laser phototoxicity.

NIDCR
Patricia Forcinito
Postdoctoral Fellow
Clinical and Translational Research
In vivo Analysis of the Novel Anti-Angiogenic Factor Fbln7-C, with Therapeutic Potential for Treatment of Age-Related Macular Degeneration (AMD)
AMD is the leading cause of blindness in the western hemisphere in patients older than 60 years due to choroidal neovascularization, which is caused by the abnormal angiogenesis of the choroidal vasculature, leading to vision loss. We recently identified a recombinant c-terminus fragment of Fibulin-7 (Fbln7-C) with anti-angiogenic properties in vitro. Interestingly, the recombinant full-length protein (Fbln7-FL) has no effect on angiogenesis in vitro, providing us with a useful negative control. Fbln7 is an extracellular matrix secreted glycoprotein expressed in eyes, teeth, and blood vessels, among other tissues. To date, the Fbln7-C fragment has not been found in vivo. In this study, we hypothesize that Fbln7-C has anti-angiogenic properties in vivo and could be a potential therapeutic compound for AMD. Therefore, we chose a novel and reliable method to study ocular angiogenesis using Brown Norway rats. The method consists of placing an implant containing a pro-inflammatory and angiogenic lipid, 7-KetoCholesterol (7KCh), within the anterior chamber of the eye. The 7KCh implant causes an inflammatory response 4 days after implantation, and induces neovascularization (NV) that peaks at day 7. Our strategy is to place implants containing either a combination of 7KCh and Fbln7-FL or 7KCh and Fbln7-C, and compare the levels of NV. To investigate the half-life of proteins in this in vivo system, we prepared implants using Alexa Fluor 488-labeled BSA, which has a similar MW as Fbln7-FL, and implanted them. Daily inspection using a fluorescent microscope showed that the protein is present in the eye for at least 9 days, which is long enough to assess proteins effect on NV. In the absence of 7KCh the implant is reabsorbed and its size decreases by day 7, with no signs of NV, leaving it â€œfree floatingâ€ in the secondary chamber of the eye. In the presence of 7KCh, inflammatory cells attach and increase the implantâ€™s size, and the NV causes the implant to be â€œclampedâ€ between the cornea and the iris from which the blood vessels originate. We predict that Fbln7-FL will have no effect on the inflammation or NV caused by 7KCh. However, due to the anti-angiogenic properties of Fbln7-C in vitro, we expect the implants containing 7KCh and Fbln7-C to be enlarged due to the presence of inflammatory cells, but â€œfree floatingâ€ in the secondary chamber due to inhibition of NV. In conclusion, we expect Fbln7-C to be a promising therapeutic compound for treating AMD patients.

NIDDK
David Libich
Visiting Fellow
Biophysics
The Dark State of GroEL: Detection of Transient Chaperonin Substrate Binding by Solution NMR
Chaperonins, a sub-class of molecular chaperones, are ubiquitous to all three kingdoms of life and are absolutely essential for cellular functions such as signaling, protein transport and immune and stress responses. Despite intensive study, the details of the structure, dynamics and kinetics of substrate interactions with chaperonins are not well understood as the bound substrate is generally invisible to conventional biophysical and structural techniques. Previous investigations of the prototypical group I chaperonin GroEL, a 780 kDa complex, have provided conflicting reports on the state of GroEL-bound

polypeptides, due in part to the transient nature of GroEL-substrate binding. We have made combined use of four complementary relaxation-based NMR experiments that together provide direct kinetic, dynamic and structural information at atomic resolution on the sparsely populated (2%) NMR-invisible dark state of amyloid-beta (Abeta) bound to GroEL. The data reveal that the primary sites of interaction with GroEL are located in two hydrophobic segments and that Abeta remains disordered in the bound state. Abeta rapidly exchanges (<700ms) between binding sites and binding modes within the GroEL cavity increasing substrate avidity, a feature that is crucial for the correct in vivo functioning of chaperonins. Simultaneously, the lack of structural ordering enables Abeta to retain considerable entropy and conformational plasticity facilitating rapid dissociation. These results offer new insights to the currently understood model of GroEL-substrate interactions by providing previously unobservable, residue specific details of exchange kinetics, structure and dynamics of polypeptides in the GroEL-bound â€˜darkâ€™ state. Currently, this methodology is being extended to examine, at atomic resolution, the kinetics and dynamics of a multistate amyloid-forming protein model. Preliminary results are indicative of GroEL binding the partially folded amyloid-prone intermediate, possibly to alter the kinetics of amyloid formation and direct the equilibrium to favor the correctly folded functional state. These results will provide a molecular description of a potential protective mechanism against amyloidogenesis, a process at the heart of numerous neurodegenerative diseases.

NIDDK
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Visiting Fellow
Biophysics

Designing glycopeptides as oligomannoside mimics: exploring the structural basis of glycan recognition by the potent antiviral lectin griffithsin.

According to the WHO there are more than 35 million people infected with HIV, and more than 2 million are infected yearly. Clearly, new approaches are needed to prevent HIV transmission. A logical target for drug design is the virus envelope glycoprotein gp120, responsible for fusion of the virus to the cell membrane. Most of the 24 N-linked glycosylation sites on gp120 contain high mannose oligosaccharides (Man5-Man9). Carbohydrate binding agents (CBA) capable of binding the glycan shield of gp120 have been shown to block virus infection, but the mechanism by which this occurs is largely unknown. Understanding the biophysical basis of recognition of CBA to glycans is fundamental for development of novel microbicides and for understanding how the virus evades the immune system. As an example, the strong potency and low cytotoxicity of the lectin griffithsin (GRFT) has excelled it as one of the strongest microbicide candidates. GRFT is a homodimeric protein with three mannose-binding sites in each subunit, which makes it an interesting yet challenging model system for studying carbohydrate-mediated interactions. Here, we report the design and synthesis of a series of synthetic mannose-rich glycopeptides capable of mimicking gp120 glycans. By systematically varying number, distance and flexibility between mannoses we show that the strongest binding occurs when all three binding sites on each subunit are simultaneously engaged. In contrast, we demonstrate that the biophysical behavior changes dramatically when all three mannose binding sites are not simultaneously occupied: while the overall affinity constant decreases 1-2 orders of magnitude, resulting weaker binding, the dissociation rate constant unexpectedly also decreases by the same magnitude, resulting in molecules that stay bound for a longer time. Long residence time upon binding to gp120 is a common feature among anti-HIV lectins. Slow off rate is also observed for the Man9-GRFT interaction. Our NMR titration experiments suggest that slow dissociation rate and oligmerization are concurrent. We propose that the outstanding ability of GRFT to block HIV is a consequence of simultaneously engaging more than one high-mannose oligosaccharide on each subunit. This interaction translates into long residence time and

oligomerization. Ongoing work is focused on understanding how slow dissociation rate and/or favored oligomerization become relevant in the context of gp120-lectin binding.

NIDDK
Jyoti Iyer
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Cell Biology - General
CHD-1 is a novel inhibitor of centrosome duplication in C. elegans
Centrosome duplication (CD) is a highly regulated process. Centrosomes must be duplicated once and only once during each cell cycle. Deregulation of this process yields an abnormal centrosome number. This can result in aneuploidy, which is 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 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 gene -1 (ZYG-1). A mutation in the zyg-1 gene that reduces its activity, prevents CD. Thus, ZYG-1 is absolutely required for CD. We have utilized an innovative approach to identify novel proteins in the CD pathway. This approach involves screening for genes that when mutated, can restore normal CD in zyg-1 mutants. These genes thereby encode novel candidate proteins of the CD pathway. Through our study, we have identified the chromatin remodeling protein Chromodomain Helicase DNA-binding-1 (CHD-1) as a novel component of the CD pathway. We determined that, the depletion of CHD-1 using both RNAi as well as a deletion allele restored normal CD in zyg-1 mutants. Therefore, CHD-1 inhibits ZYG-1-mediated CD. CHD-1 has been shown to regulate protein levels by affecting gene transcription. Therefore, we questioned whether CHD-1 might regulate CD by controlling the expression levels of various CD proteins. Hence, to dissect the molecular mechanism by which CHD-1 regulates CD, we performed quantitative western blot analysis for extracts from wild-type (WT) and chd-1 deletion mutants. The total protein levels of both ZYG-1 and SAS-6 (a core component of the CD pathway) remained unchanged in the chd-1-deletion mutants. However, interestingly, the proportion of a faster-migrating band of SAS-6 was greatly increased in the chd-1-deletion mutants as compared with WT worms. The function of this post-translationally modified SAS-6 in CD is currently unknown. We expect that this modification represents the dephosphorylated form of SAS-6. Therefore, based upon our data, we hypothesize that CHD-1 inhibits CD by controlling the phosphorylation status of SAS-6. Efforts are ongoing to test this hypothesis. In summary, our study has identified the chromatin remodeler CHD-1 as a novel inhibitor of CD in C. elegans.

NIDDK
Su-Lin Lee
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Chemistry
Structure-based drug design of novel FtsZ inhibitors starting from the marine natural product chrysophaentin A
The increase in bacterial infections caused by drug resistant bacteria has led to there being an urgent need for novel selective antibiotics with low resistance probability toward currently prescribed antibiotics. The filamenting temperature-sensitive mutant Z protein (FtsZ) is a prokaryotic homologue of tubulin that plays an essential role in bacterial cell division and is an attractive target for new antibiotics. We recently discovered that chrysophaentin A 1, a novel marine natural product and its substructure 2, demonstrated strong antimicrobial activities against a broad spectrum of Gram-positive pathogens. We further identified this class of compounds as GTP competitive FtsZ inhibitors. Herein, we applied structure-based drug design by using 2 as a lead to develop novel, potent and synthetically feasible FtsZ

inhibitors. From molecular docking results, we first identified the possible binding interactions between the GTP-binding site in the crystal structure of S. aureus FtsZ and compound 2. A new series of compounds were designed to fill up the space of the binding pocket with maximum hydrogen-bonding interactions. These compounds were then synthesized through an 8-9 step synthetic pathway. The FtsZ inhibition activities of these compounds were assessed by an in vitro FtsZ GTPase assay. The antimicrobial activities toward a panel of Gram-positive/negative pathogens were determined through disk diffusion and microbroth dilution assays. The toxicology profile of these compounds against human HCT116 colon cancer cells versus BSC40 kidney epithelial cells were evaluated by colorimetric (MTT) assays. Twenty-six derivatives with a wide range of substitutions were synthesized with reasonable yields (~10%) to form a small FtsZ inhibitor library. Among these compounds, 18 demonstrated superior FtsZ inhibitory activities over our starting compound 2, with a 3-fold increase in in vitro activity against FtsZ (IC50: 7.7 μg/mL) and exhibiting strong antibacterial activity against S. aureus, MRSA, and MDRSA (MIC50 values of 17.3 μg/mL, 15.9 μg/mL, and 11.4 μg/mL, respectively). Furthermore, the structure-activity relationship (SAR) analysis revealed a stringent structural requirement of the two amino substitutions on both the alkyl chain and the 4-phenyl moiety. Ongoing studies are focused on determining the affinity and kinetics of binding of 18 to FtsZ using isothermal titration calorimetry (ITC) and saturation-transfer difference (STD) NMR techniques.

NIDDK
Matteo Avella
Postdoctoral Fellow
Developmental Biology
Shifting paradigms in reproductive biology: mouse transgenesis documents novel molecular mechanisms for gamete recognition and acrosome exocytosis in mouse and human fertilization
Gamete recognition and acrosome exocytosis are essential for successful fertilization. Ovulated eggs are surrounded by an extracellular zona pellucida that contains three glycoproteins in mouse (moZP1-3) and four in human (huZP1-4). ZP3 has long been considered the zona ligand for sperm binding and induction of acrosome exocytosis. This model is challenged by the recent observation that human sperm bind to mouse eggs when human ZP2 replaces the endogenous mouse protein (huZP2Rescue) and sperm binding to the zona matrix is not sufficient to induce acrosome exocytosis. The sine qua non for an essential component that regulates fertilization is sterility after genetic ablation. Ablation of ZP2 or ZP3 precludes zona matrix formation and has prevented assessment of either as the ligand for gamete recognition. We now report loss-of-function assays in transgenic mice that express huZP4 in lieu of ZP2 and form a zona matrix. We document that human and mouse sperm do not bind to a zona pellucida lacking huZP2 and moZP2, respectively, and female mice are sterile. The same phenotype is observed with a mutant ZP2 that lacks ZP251-149. Using heterologous human sperm to inseminate huZP2Rescue and huZP3Rescue mice, we observe sperm penetrating the zonae pellucidae of huZP2Rescue, but not huZP3Rescue rescue eggs. In transgenic mice expressing chimeric human-mouse ZP2 proteins, we confirm that the N-terminus of ZP2, found to be essential for fertilization, also accounts for the taxon specificity of human gamete recognition (i.e., human sperm bind to human but not mouse zona pellucida). To investigate molecular mechanisms leading to acrosome exocytosis, we have established mouse lines that ejaculate sperm with EGFP-tagged nuclear protamines and soluble mCherry that is released upon acrosome exocytosis. Using time-lapse imaging, we observe only acrosome-intact sperm on the surface of the zona pellucida and, after penetration through the zona pellucida, only acrosome-reacted sperm in the perivitelline space between the inner aspect of the zona matrix and the egg plasma membrane. In the absence of either mouse or human ZP1, a looser zona matrix is formed to which mouse and human acrosome-intact sperm bind and penetrate, but remain acrosome-intact. From these observations, we conclude that the N-terminus of ZP2 is the ligand for mouse and human gamete

recognition and acrosome exocytosis is induced by sperm passage through the extracellular zona pellucida.

NIDDK
Yong-Qi Li
Visiting Fellow
Endocrinology
Gs alpha Deficiency in Fat Tissues Leads to Improved Glucose Metabolism and Insulin Sensitivity without Effects on Body Weight
Gs alpha, the G protein which mediates receptor-stimulated cAMP generation, has been implicated as a modulator of various adipose tissue functions, such as sympathetic nervous system (SNS) - stimulated lipolysis, browning of white adipose tissue (WAT) and brown adipose tissue (BAT) thermogenesis. These processes have been thought to be major targets for treating obesity and diabetes. In this study, we generated a mouse line with adipose tissue-specific Gs alpha deficiency (Adipoq-GsKO: adiponectin-cre+, Gs alpha flox/flox). Adipoq-GsKO mice had impaired BAT function, as they were unable to maintain body temperature or induce the expression of Ucp1 during cold exposure and had reduced oxygen consumption after treatment with a beta-3-adrenergic-receptor agonist, CL-316,243. They also lost the ability of browning their WAT. However, adipose tissue specific Gs alpha deficiency caused neither hyperphagia nor obesity in mice fed on either a standard chow or a high-fat diet. Both lipolysis and de novo lipogenesis in Adipoq-GsKO mice were attenuated, which was accompanied by an improvement of glucose metabolism, increase of insulin-stimulated glucose uptake in skeletal and cardiac muscles, and enhancement of insulin sensitivity. While leptin, RBP4 and FGF21 levels remained relatively unchanged, levels of circulating aP2, a newly identified adipokine markedly elevated in obese/diabetic patients, was significantly reduced in Adipoq-GsKO mice. Altogether, specific inhibition of Gs alpha signaling in adipose tissue might be a plausible strategy in the treatment of obesity-related insulin resistance and diabetes without disturbance in body weight.

NIDDK
Ivan Krivega
Postdoctoral Fellow
Gene Expression
Role of LDB1 in the transition from chromatin looping to transcription activation
Chromatin looping between enhancers and gene promoters is a well established mechanism underlying enhancer function but little is known about the mechanism of looping formation and its relationship with nuclear architecture and gene expression. In the beta-globin locus, the LCR enhancer and beta-globin gene promoter are occupied by a multi-protein complex that includes LDB1 and erythroid proteins LMO2, FOG1, GATA-1 and TAL1 (LDB1 complex). The LDB1 complex is required for beta-globin gene activation, LCR/promoter looping and the locus migration away from the nuclear periphery. LDB1 homodimerizes through its N-terminal dimerization domain (DD) and provides loop formation when artificially tethered to the inactive beta-globin promoter. The mechanistic details of LDB1 complex function in vivo remain unclear. We tested whether the DD plays a key role in LDB1 complex activity by analyzing the ability of different deletions and fusions of the LDB1 protein to rescue LDB1 gene knock-down (KD) in mouse immortalized proerythroblast cells and primary E14.5 fetal liver cells. In silico and biochemical analysis showed that the N-terminal part of the DD is required for LDB1 protein homodimerization, LCR/beta-globin promoter looping and beta-globin gene activation. In contrast, deletion of the C-terminal part (4/5 region) of the DD yields an LDB1 mutated form (LDB1delta4/5) capable of homodimerization and loop formation but incapable of migration away from the nuclear periphery and beta-globin gene activation. Using coimmunoprecipitation and ChIP assays we revealed

that 4/5 region is required for the recruitment of the co-regulators FOG1 and NuRD complex. Lack of 4/5-region alters histone acetylation and RNA polymerase II recruitment to the beta-globin gene promoter. Comparative RNA-seq analysis of LDB1 KD cells expressing full-length LDB1 or LDB1dela4/5 and cells with FOG1 knock-down confirmed that LDB1-activated genes depending on the 4/5 region of the DD are also FOG1-dependent. Analysis of the OMIM database showed that the 4/5 region of the LDB1 DD is critical for regulation of blood disease-associated genes. These results uncouple enhancer-promoter looping from nuclear migration and transcription activation and reveal new roles of LDB1 in regulation of blood disease-associated genes.

NIDDK
Anup Nair
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Genetics
Common variation in a putative regulatory region of STAM2 is associated with skeletal muscle expression of STAM2 and increased risk for type 2 diabetes in American Indians.
A recent genome-wide association study (GWAS) identified the single nucleotide polymorphism (SNP) rs10930939 as being associated with type 2 diabetes (T2D) in full heritage Pima Indians. In this study we investigated the genomic region near this signal. Whole genome sequence data from 234 Pima Indians were used to identify linkage disequilibrium (LD) patterns across this region. The lead GWAS SNP (rs10930939) was in high LD with a number of SNPs that mapped within the STAM2 locus. To identify potential functional SNPs, we merged these SNP location with data from the ENCODE project and identified rs4368329, rs72864765, rs6740224 and rs60252671 that mapped within predicted transcription factor binding sites (TFBS). These 4 SNPs and 5 additional tag SNPs (for complete coverage of STAM2) were genotyped in 3604 full heritage Pima Indians and nominal associations with T2D (P=0.06 â€“ 0.0045, OR=1.12-1.9) were observed for 3 promoter variants (rs4368329, rs6740224, rs60252671) and one intronic SNP (rs7603232). We further replicated these T2D associations by genotyping the 4 SNPs in a non-overlapping sample of 3950 mixed heritage American Indians (P=0.03 â€“ 0.0004, OR=1.14-1.29). The strongest evidence for T2D association came from the combined analysis of both data sets for rs7603232 and rs60252671 (P=0.00001, OR=1.23[1.12-1.35] and P=0.0002, OR=1.19[1.12-1.27] respectively). Since rs60252671 maps to a predicted TFBS, we further analyzed mRNA expression data from skeletal muscle (N=207) and percutaneous abdominal adipose (N=197) tissue biopsies from Pima Indians. Genotypes of rs60252671 were significantly associated with the expression of STAM2 in skeletal muscle (P=0.0006, ß= 0.34 SD unit per copy of the risk allele) where the T2D risk allele showed an increased expression of STAM2 mRNA. STAM2 along with STAM1 and HRS forms the major part of the ESCRT-0 complex involved in the sorting of ubiquitinated receptors for degradation. STAM2 has two ubiquitin binding domains and a USP8/UBPY (deubiquitinating protein) binding domain. Recent studies indicate that STAM2 forms a complex with USP8 and plays a role in determining the cell surface expression and degradation of activated receptor tyrosine kinases (RTK) like EGFR. The insulin receptor is a RTK and undergoes ubiquitination upon ligand stimulation. We propose that STAM2 may have a role in determining the cell surface concentration of the insulin receptor and thereby contributes to the development of T2D.

NIDDK
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Immunology - Infectious Disease
GLOBAL MICRORNA PROFILING REVEALS MULTIPLEX INTERACTIONS AMONG HEPATITIS C VIRUS INFECTION, INNATE IMMUNE RESPONSE, AND HEPATIC MICRORNA REGULATION

MicroRNAs (miRNAs) are small non-coding RNAs that fine tune gene expression to control essential biological processes through down-regulation of translation/transcription of mRNAs. Host miRNAs, like miR-122, have been shown to play an important role in hepatitis C virus (HCV) replication. HCV, on the other hand, may manipulate miRNA expression in infected hepatocytes to create a favorable host environment for productive infection and propagation. We conducted a genome-wide functional screen and identified a repertoire of cellular miRNAs that are associated with the complete life cycle of HCV. To further investigate interactions between host miRNAs, HCV and immune response, we performed global miRNA expression analyses in both primary human hepatocytes and Huh.7.5.1 human hepatoma cell line. Cells were infected with HCV at various time points or treated with interferon-alpha (IFN-alpha) or interferon-lamda (IFN-lambda) either in the presence or absence of HCV infection. Applying Nanostring miRNA profiling technology, we identified multiple miRNAs that were significantly regulated by HCV infection or interferon treatment. HCV treated cells showed an overall decrease in general microRNA expression at all time points, albeit several miRNAs were considerably up-regulated by HCV. These HCV-induced miRNAs include miR-122-5p, miR-1915-3p, miR-204-5p and miR-27b-3p. Increased expression of miR-122 in HCV-infected cells aligns with a proviral role of the miRNA in HCV replication. Interestingly we showed that IFN-alpha generally decreased the overall miRNA expression levels, whereas IFN-lambda increased the general microRNA expression, suggesting that distinct mechanisms may be engaged by these two families of IFNs to regulate miRNA profiles in hepatocytes. Among the specific IFN-modulated miRNAs are miR-23a-3p, miR-155-5p and miR-34a-5p. In addition, bioinformatics analysis suggested that multiple certified HCV-associated interferon-stimulated genes (ISGs) may be targeted by these miRNAs that were modulated by HCV infection or IFN treatment. Conclusion:HCV infection induces a unique response in miRNA expression to facilitate productive infection. This response may result from a complex interplay among cellular mechanisms, including innate immunity responses, in infected hepatocytes. A comprehensive study of host miRNA expression and regulation associated with HCV infection may provide crucial insights into HCV-host interactions and mechanisms of interferon response.

NIDDK
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Intracellular Trafficking
A dynamin mutant defines a super-constricted pre-fission state
Dynamin belongs to a family of large GTPases that mediate fission and fusion events in the cell. Dynamin regulates plasma membrane fission during clathrin-mediated endocytosis, a process often high jacked by pathogens to gain entry into the cell. Thus, understanding the molecular mechanisms that regulate this process is of outmost importance to elucidate novel ways to prevent infection and disease. Dynamin organizes into helical assemblies at the base of nascent clathrin-coated vesicles. Formation of these oligomers stimulates the intrinsic GTPase activity of dynamin, which is necessary for efficient fission. Recent evidence suggests that the transition-state of dynaminâ€™s GTP hydrolysis reaction serves as a key determinant of productive plasma membrane fission. We have solved the structure of a transition-state-defective dynamin mutant, K44A, assembled on a lipid template, at 12.5 Å resolution using cryo-electron microscopy. In the presence of GTP, K44A-dynamin constricts the underlying lipid to an inner luminal diameter of 3.7 nm, reaching the theoretical limit for spontaneous membrane fission. To identify the organization of K44A-dynamin in this â€˜super-constricted stateâ€™, we docked the crystal structures of individual dynamin domains into our 3D density map. This revealed that in the super-constricted state, only the ground state conformation of the G domain fits into the density map. We further show that K44A-dynamin lacks stimulated GTPase activity, which further suggests that super-constricted dynamin is trapped in a pre-hydrolysis, GTP-bound ground state. These findings indicate that

the ground state conformation of the dynamin polymer is sufficient to achieve super-constriction and possibly spontaneous fission. We also find that the super-constricted dynamin polymer adopts a 2-start helical symmetry. The 2-start helical assembly allows for the most efficient packing of the subunits within the polymer and specially generates the highest possible number of G domain interfaces. This suggests that assembly of a 2-start helix allows dynamin to super-constrict the lipid neck while maximizing the G domain dimerization to achieve the highest possible GTPase activity, resulting in a highly-efficient minimal fission machinery. The subsequent GTP hydrolysis may bring about additional conformational changes and/or disassembly of the dynamin polymer, releasing the destabilized lipid bilayer, further facilitating the fission reaction.

NIDDK
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Metabolomics/Proteomics
Beta-arrestin-2 is an important regulator of hepatic glucose production and whole body glucose homeostasis
Type 2 diabetes (T2D) has emerged as one of the major threats to human health in this century. Typically, increased hepatic glucose production (HGP) is a major contributor to hyperglycemia characteristic for T2D. Factors that underlie increased HGP in T2D are hepatic insulin resistance and enhanced signaling through hepatic glucagon receptors. Hepatic glucose fluxes are regulated by the activity of G protein-coupled receptors (GPCRs), including the Gs-coupled glucagon receptor, and downstream signaling networks. GPCR signaling is regulated by many cellular factors including proteins of the beta-arrestin family (barr1 and barr2). At present, little is known about the roles of barr1 and barr2 in different tissues and cell types in regulating whole body glucose homeostasis. In the present study, we decided to explore the role of barr2, which is abundantly expressed in the liver, in regulating HGP and whole body glucose homeostasis. By using Cre-loxp technology, we generated mutant mice that selectively lacked barr2 in hepatocytes (hep-barr2-KO mice). We found that hep-barr2-KO mice showed impaired glucose tolerance and that the ability of glucagon to raise blood glucose and hepatic cAMP levels was greatly enhanced in the mutant mice. Moreover, in vitro and in vivo studies demonstrated that HGP was significantly increased in hep-barr2-KO mice. We also noted that the hepatic expression levels of the key gluconeogenic enzymes, PEPCK and G-6-Pase, were significantly increased in hep-barr2-KO mice. In vitro studies demonstrated that insulin receptor signaling was not impaired in hepatocytes from hep-barr2-KO mice. Our findings support the concept that barr2 deficiency in the liver enhances glucagon signaling, resulting in increased HGP and impaired glucose tolerance. This is the first study in which a member of the arrestin family has been deleted in a cell type-specific fashion in mice. Our data provide novel insights into the in vivo roles of barr2 in regulating HGP and maintaining blood glucose levels in a physiological range. Strategies aimed at enhancing the activity of barr2 in hepatocytes could prove useful to suppress HGP in T2D.

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Molecular Biology - Prokaryotic

Identification of Pex30 and Pex31 as novel ER shaping proteins in S. cerevisiae

The endoplasmic reticulum (ER) is a continuous membrane bound organelle with a common luminal space. ER membranes include the nuclear outer and inner membranes, flat sheet-like cisternae and highly dynamic interconnected tubules that extend into the cell periphery. In all eukaryotes, ER sheets have similar luminal thickness to tubules but can extend and curve at the edges. In S. cerevisiae, evolutionary conserved ER membrane protein families like the reticulons (Rtn1 and Rtn2) and Yop1/DP1, tubulate ER. These proteins have conserved domain containing two long hydrophobic segments that maintains the ER shape by stabilizing the high curvature of tubules and edge of the sheets. Interestingly, in cells lacking Rtn1, Rtn2, and Yop1 (rtn1rtn2yop1delta), the peripheral tubular ER mainly appears as sheets with few tubules suggesting potential role for additional tubulating proteins in maintaining ER shape. We designed a genetic suppressor screen to identify the proteins that tubulate ER in rtn1rtn2yop1delta. We identified that overexpression (OE) of Pex30p and Pex31p restores ER tubules in rtn1rtn2yop1delta suggesting that Pex30 and Pex31 are ER tubulating proteins. Similar to reticulons and Yop1 proteins, Pex30p and Pex31p are localized mainly in the peripheral ER and absent from nuclear membranes. Unlike reticulons and Yop1p, endogenous levels of Pex30p and Pex31p are low suggesting that these proteins might have a minor role in shaping a sub domain of ER. In S. cerevisiae, Pex30p and Pex31p maintain the number and size of peroxisomes respectively. Peroxisomes are independent organelles derived from ER membrane that replicate by fission. We speculate that Pex30 and Pex31 regulate de novo peroxisome biogenesis by altering ER shape. In yeast, Pex30p and Pex31p contain dysferlin domain at the C-terminal end. Even though studies have shown that mutation in human dysferlin domain leads to dysferlinopathy, a muscular dystrophy, the function of dysferlin domain remains unknown. In this study, we show that the dysferlin domain is functionally conserved from humans to yeast emphasizing the importance of this study. To determine the role of dysferlin domain we made C-terminal truncations of Pex30p. OE of truncated Pex30p lacking dysferlin domain failed to restore the ER shape in rtn1rtn2yop1delta mutant suggesting that dysferlin domain is required for membrane tubulation. The mechanism of dysferlin domain in tubulating ER membrane remains to be investigated.

NIDDK

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Visiting Fellow

Molecular Biology - Prokaryotic

Spatial Regulators for Bacterial Cell Division Self-organize into Patterns in vitro

In the bacterium Escherichia coli, the Min proteins oscillate between the cell poles to select the cell center as the division site. This dynamic pattern arises by self-organization of these proteins. The MinD ATPase binds the membrane in its ATP-bound form and recruits the cell division inhibitor MinC. MinE interacts with membrane-bound MinD, stimulates its ATPase activity, and releases MinD (and MinC) from the membrane. The perpetual chase of MinD by MinE creates the pole-to-pole oscillator, which maintains a low level of the division inhibitor at mid-cell. Here we successfully reconstituted and visualized the spontaneous self-organization of fluorescent labeled MinD and MinE proteins on a flat membrane in vitro. At high concentrations of surface-bound Min proteins, the system formed uniform waves. The formation and maintenance of these patterns, which extended for hundreds of micrometers, required ATP, and persisted for hours. Min waves were robust over a wide range of lipid compositions making up the membrane. At high anionic lipid density, the waves were narrow and travelled slowly. At lower density, the waves were thicker and faster. The findings show that charge density of the membrane strongly influences Min patterns in vivo. Under monomer depletion conditions, the system formed oscillating â€œfireworksâ€. The fireworks consisted of radially expanding MinD centers that

imploded following the formation of a MinE-ring that confined and disassembled the MinD-core. The MinD-MinE fireworks capture the in vivo system dynamics and provide a mechanistic framework to build a model based on reaction-diffusion principles. By visualizing the biochemistry involved in membrane-mediated protein patterning, we can now apply this cell-free technique to a number systems that utilize a biological surface for subcellular positioning and spatial organization.

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Neuroscience - Integrative, Functional, and Cognitive
Optically stimulating indirect pathway neurons increases anxiety
It has been well established that activation of the indirect pathway of the basal ganglia inhibits movement. However, indirect pathway neurons also play a role in negative affect. Animals will avoid a chamber paired with the stimulation of indirect pathway neurons, and so we hypothesized that stimulating these neurons may have an anxiogenic effect. Using optogenetic techniques, we have found that directly stimulating indirect pathway neurons in the dorsal striatum not only decreases movement but also induces anxiety as demonstrated by a reduction in the amount of time spent in the open arms of an elevated zero maze. This reduction of time in the open arms is separate from the movement effect, since limiting analysis to periods of movement still reveals a decrease in the percentage of time spent in the open arms. Furthermore, we found that stimulating indirect pathway neurons during an acoustic startle task increases the startle response, another measure of anxiety. These results demonstrate that the anxiety effect is distinct from the movement effect, since activating indirect pathway neurons induces an anxiety behavior which increases movement, in direct contrast to the predicted movement effects of the stimulation. Our results indicate a significant role of indirect pathway neurons in the striatum in anxiety, which could have serious implications for anxiety disorders, OCD, Parkinsonâ€™s disease and obesity.

NIDDK
Chia Li
Visiting Fellow
Physiology
Unraveling neural circuits underlying homeostatic processes of appetite
In animals, nutrient intake is essential for survival and requires food seeking and consumption behaviors, however overeating has led to a growing obesity endemic. Thus, identifying the neural circuits controlling feeding is imperative. The agouti-related peptide (AGRP) neurons in the arcuate nucleus of the hypothalamus promote feeding following acute soma activation. Moreover, stimulation of arcuate AGRP terminal fields in the paraventricular nucleus of the hypothalamus (PVH) orchestrates food intake, although the target cell type within the PVH is unknown. Arcuate AGRP neurons are thought to synapse and guide feeding through inhibition of satiety-promoting melanocortin-4 receptor (MC4R) neurons in the PVH, though this functional connectivity has never been demonstrated. Additionally, the explicit role of PVH MC4R neurons in mediating appetite has yet to be elucidated. Utilizing slice electrophysiology and optogenetics, a technique that combines genetics and optics to

manipulate neural activity with spatial and temporal precision, we have recently demonstrated monosynaptic arcuate AGRP to PVH MC4R connections. As a follow-up to this result, we assessed behavioral function by performing AGRP to MC4R cell type-specific occlusion studies. This technique involves the artificial expression of the light-activated cation channelrhodopsin (ChR2) into molecularly circumscribed cells, followed by the implantation of optical fibers for light delivery deep into defined brain regions to activate specific cell types while observing behavior in freely moving mammals. We found that the elevated feeding response following activation of the inhibitory arcuate AGRP terminals to the PVH was completely abrogated by simultaneous activation of the entire PVH neural population (marked by the transcription factor single-minded1). Importantly, similar attenuated food intake was observed with simultaneous stimulation of arcuate AGRP terminals to the PVH and MC4R neurons, but not cells marked by oxytocin, which have been indirectly implicated in mediating feeding. In accordance with these findings, we have also demonstrated that photoactivation of PVH MC4R neurons alone drives satiety, reducing food intake in hungry mice. These results establish direct modulation, connectivity and function of the arcuate AGRP to PVH MC4R circuit, helping reveal the precise wiring diagram of these complex neural networks directing motivated behaviors such as feeding.

NIDDK
Christine Jao
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Protein Structure/Structural Biology
Structural Analysis of a Bacterial Zinc Transporter
Nosocomial infections, or hospital-acquired infections, are increasingly becoming serious public health issues. There is an urgent need to study host-pathogen interactions in order to develop new antimicrobial therapies. Metal homeostasis, especially of transition metals such as iron and zinc, is important for the survival of all organisms. Transition metals are incorporated in proteins that have diverse functions and are important for host-pathogen interactions. Host defense mechanisms involve the sequestration of these transition metals in order to prevent bacterial growth and survival. Bacterial survival depends on uptake of trace metals that is conducted through specialized metal-specific receptors and transporters found on the bacterial surface that mediate import. Several recent studies have explored iron uptake in pathogenic bacteria, but much less is known about bacterial uptake of other trace metals. A zinc uptake system in Acinetobacter baumannii was recently identified, and inhibition of zinc acquisition was shown to enhance the efficacy of carbapenem, one of the frontline antibiotics used. Using structural biology to understand how these bacteria specifically utilize zinc for survival, we determined the crystal structure of an A. baumannii zinc transporter, ZnuD1 (zinc uptake), an outer membrane bacterial protein. ZnuD1 (691 residues, 76kDa) is a 22-stranded beta-barrel protein. This is the first high-resolution structure of a TonB-dependent zinc transporter structure that has been solved. The TonB box, the region that recognizes the TonB protein in the bacterial inner membrane, is located at the N-terminus at the periplasmic side. Our data show that zinc is transported as an ion, unlike iron, which is transported in complex with a chelator, or siderophore. The ZnuD1 structure reveals at least two zinc binding sites along the surface of the receptor with the potential for several other sites along an extended surface loop, suggesting that the transporter may saturate zinc along its surface prior to transporting it across the outer membrane into the cell. Guided by the crystal structure, mutations in the protein will be made, and tested for zinc uptake and transport in bacterial cells. Our structure will serve as a starting point for structure based screening of small molecule inhibitors of bacterial zinc uptake.

NIDDK
Soumyadeep Dey

Visiting Fellow
Signal Transduction - General
Erythropoietin controls POMC expression and cross talks with leptin response via JAK2/STAT3-signaling in mouse hypothalamus
Obesity is a critical public health problem associated with comorbities like type 2 diabetes, metabolic syndrome, and cardiovascular diseases. A common problem leading to obesity is the disruption of balance between food intake and energy expenditure, a process controlled by the hypothalamus. Most well known among the regulatory signals are peripherally produced leptin and insulin, which control neuropeptide production in the hypothalamic neurons. Previous work from our lab has shown that similar to leptin, erythropoietin (Epo) signaling through Epo receptor (EpoR) in hypothalamus directly regulate production of proopiomelanocortin (POMC), a major anorexigenic neuropeptide. Epo-treatment of wild-type (WT) mice increased POMC, reduced body weight gain on high fat-diet (HFD), and increased energy expenditure. Conversely, mice lacking non-hematopoietic EpoR (delta EpoRE) have low POMC, become obese, and develop metabolic syndromes. Importantly, Epo-treatment of delta EpoRE mice increased hematocrit like WT mice, but did not reverse weight gain on HFD or increased POMC expression. This suggests that Epoâ€™s metabolic effects are independent of its hematopoietic effects. Based on this background data, we carried out follow up mechanistic studies using ex vivo neural progenitor cell (NPC) culture system, and in vivo mice model. Immunocytochemical analysis of hypothalamus NPC showed co-localization of EpoR and POMC expression. Similar to leptin, both in vivo and ex vivo Epo-treatment induced POMC expression via activation of JAK2/STAT3-signaling in WT hypothalamic neurons. Moreover, this effect can be abrogated by adding a STAT3-inhibitor WP1066. Surprisingly, Epo- but not leptin-treatment, induced leptin receptor (long-form) expression, the form that is active in hypothalamus. This suggests a possible role of Epo in priming hypothalamic neurons for leptin response. On the other hand, delta EpoRE hypothalamus showed reduced baseline JAK2/STAT3-signaling which also explained the lower POMC expression. Interestingly, leptin treatment of delta EpoRE mice showed a blunted STAT3-activation compared to WT mice, and could induce POMC expression only up to untreated WT levels. This further suggests a novel role of Epo in regulating leptin response in hypothalamic neurons. Collectively, these data show that Epo regulates POMC expression in hypothalamus via JAK2/STAT3-activation, and provide a previously unknown link between Epo and leptin response in hypothalamus neurons.

NIDDK
Netra Meena
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Signal Transduction - General
A Novel Secreted Protein that Promotes Cell Motility, Chemotaxis, and Cell-Cell Interaction
Chemotaxis is a fundamental process whereby cells sense and migrate in chemoattractant gradients. Chemotaxis plays pivotal roles in embryogenesis, inflammation, wound healing, and renewal of skin and intestinal cells. It also mediates development of chronic inflammatory diseases, as well as, cancer, angiogenesis, and metastasis. The molecular mechanisms that regulate chemotaxis/migration under different biological and pathological conditions are complex, but are common to diverse systems. Chemotaxis has been comprehensively studied in Dictyostelium, a model organism for eukaryotic development, that, like mammalian migratory cells, requires receptor/G protein coupled pathways (e.g. GPCRs, G proteins, ERKs, PKA, PI3K, TORC2, AKT, PLA2, PDK1, cyclases) for chemoattractant/chemokine response. Chemotactic response is highly dependent on cell density. We detected a loss in cell-cell Dictyostelium chemotaxis upon a minimal (e.g. 2x-fold) reduction in cell density. Remarkably, chemotactic response was fully restored to low density cells by the addition of extra-cellular media, derived from high density cells. Using chemotactic response of low density cells as an assay, we were

able to identify and purify a 150 kDa extra-cellular Chemotactic Factor (CF) protein to homogeneity, to obtain MS/MS micro-sequence data, and to determine the full gene sequence. CF is part of a large family of EGF repeat-containing proteins. Using native antibodies and expression of N- and C-terminal tagged CF variants, we can describe CF expression and topology. CF is synthesized as a 160 kDa precursor that is glycosylated and localized at the plasma membrane. Upon proteolytic, ecto-domain shedding, the active 150 kDa form is released to the extra-cellular media, leaving a C-terminal 10 kDa fragment at the cell surface, with a very short intracellular domain. Finally, to fully understand CF function, we created cell lines lacking the CF gene. Interestingly, CF-null cells failed to undergo cell-cell chemotactic response at low cell density and were severely compromised in directed chemotaxis assays, irrespective of cell density. Further studies are being carried out to investigate potential cell-surface, CF-interacting proteins. Our results, therefore, have led to the identification of a novel protein that plays a role in chemotaxis in Dictyostelium, thereby shedding new light on the mechanism of the key process in cells.

NIDDK
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Virology - RNA and Retroviruses
Hepatitis C virus infection down-regulates the intrinsic antiviral gene N-myc downstream regulated 1 for efficient viral replication
The hepatitis C virus (HCV) is a leading cause of hepatitis, cirrhosis, and hepatocellular carcinoma worldwide. Like all viruses, HCV relies on its target cell for replication and propagation, but the host cell contains a myriad of factors that can either aid or hinder the virus. Our lab recently conducted a whole genome siRNA screen and identified over 200 cellular factors, which were classified as either proviral or antiviral genes depending on the observed phenotypes on viral infection. N-myc downstream-regulated 1 (NDRG1) is a multifunctional protein that is not stimulated by interferon and may be an innate HCV restriction factor. We depleted NDRG1 expression in hepatocytes by siRNA and measured intracellular and extracellular viral RNA (vRNA) levels 48 hours post infection. Our data indicates that siRNA-mediated silencing of NDRG1 (>80%) in Huh7.5.1 cells significantly increased HCV replication (>8-fold) compared to the non-targeting control siRNA treated cells. We confirmed an enhancement of HCV replication in primary human hepatocytes treated with NDRG1-specific siRNAs although the effect was less robust (~1.70-fold increase). To determine which phase of HCV replication was altered, we used the HCV replicon system, which mimics vRNA translation/replication and does not produce infectious virus. We did not detect any significant difference in cells depleted of NDRG1, suggesting that NDRG1 may act on the late stages of the viral lifecycle such as assembly or release. We next sought to determine if HCV infection could alter NDRG1 expression. During a time course of infection, we found that both NDRG1 mRNA and protein levels were reduced approximately 60% by 72 hours post infection. We also confirmed this phenotype using immunofluorescence microscopy of HCV infected cells and observed that only cells staining positive for HCV core exhibited reduced NDRG1 fluorescence. The addition of the proteasome inhibitor Bortezomib did not rescue NDRG1 protein levels after HCV infection. Taken together, these data indicate that HCV specifically down-regulates NDRG1 and this reduction occurs prior to protein translation, possibly through altered transcriptional regulation. Overall, NDRG1 represents a novel intrinsic antiviral gene that is specifically targeted by HCV during infection. Further studies are being conducted to elucidate a mechanism underlying the NDRG1-mediated antiviral effect and how HCV regulates NDRG1 expression in productive viral infection.

NIEHS
Thuy-Ai Nguyen

Postdoctoral Fellow
Biochemistry - Proteins
The p53 protein interactome is also a p53-regulated cistrome
The p53 tumor suppressor is a transcription factor that regulates the expression of cell cycle, DNA repair, apoptosis and many other genes in response to cellular stress. While activated p53 directly binds genes and alters expression, the effects of cancer drugs on promoter selectivity, transcription regulation, and protein-protein interactions with p53 are poorly understood. We are elucidating the human p53 network by examining p53 binding, induced gene expression and protein interactors under diverse stress conditions. Combining p53 immunoprecipitation, SILAC (stable isotope labeling with amino acids in cell culture) and mass spectrometry, we have identified 45 novel p53 protein interactors in response to the chemotherapeutic drug Doxorubicin. Functional annotation of this interactome reveals proteins that participate in RNA binding, mitochondria, transcription and cellular metabolism. Remarkably, when we compared the list of p53 interactors with our p53 ChIP-seq and microarray studies, we found that p53 bound the promoters of 97% of these interactors. Additionally, the interactors all have at least one p53 target response element near their transcription start sites. Furthermore, over 30% of the interactors were differentially expressed in response to p53 activating drugs based on microarray analyses. We are currently validating whether the p53 interactome is also a p53-regulated cistrome using ChIP-qPCR to examine binding and qPCR to examine induced gene expression. While p53 is known to participate in several autoregulatory feedback loops, most of which act through Mdm2, we have uncovered what appears to be 44 new loops in the p53 regulatory network. This unexpected finding could be due to our examination of the p53 interactome in response to stress, whereas previous p53 mass spec studies were restricted to p53 overexpression in p53 null cells in the absence of stress signals. In its role as guardian of the genome, p53 may have numerous feedback loops to connect it with various stress and signal transduction pathways in order for p53 to coordinate the signals for proliferation and development of the cell. Overall, the spectrum of novel p53 protein interactors and the possible transcriptional regulatory consequences revealed by our study provide further insights into the many dynamic functions ascribed to p53 in maintaining genomic integrity as well as cellular metabolism in response to a variety of stresses.

NIEHS
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Biochemistry - Proteins
Molecular Mechanism of the Aprataxin-linked Neurodegenerative Disorder - Ataxia with Oculomotor Apraxia Type 1 (AOA1)
Cellular DNA is constantly under assault from many sources, resulting in damage that can lead to mutations and cell death. Thus, efficient repair of damage is critical for cell survival. All DNA repair and replication processes are finalized in a nick-sealing reaction by DNA ligases, which seal nicks through a mechanism that proceeds through an intermediate where an AMP (adenylate) moiety is attached to the 5´-phosphate side of the break to be sealed. The presence of unrepaired damage in the vicinity of the break can cause the reaction to fail at this intermediate step, generating 5'-adenylated DNA termini as a form of compound damage. These must be resolved quickly to prevent persistence of DNA strand breaks. Aprataxin (Aptx) proofreads ligation by removing these 5'-adenylates from DNA. Mutations in Aptx are linked to AOA1, a rare but debilitating neurodegenerative disorder. Although the catalytic mechanism of Aptx has been illuminated by recent structural work, the link between biological substrates for Aptx and progression of AOA1 disease is unclear. Given that ribonucleotide misincorporation represents a major source of damage found in DNA, we investigated whether ribonuleotides can be a source of substrates for Aptx. Here we show that 5'-RNA/DNA junctions

generated during Ribonucleotide Excision Repair (RER) trigger abortive ligation, generating 5'-adenylated DNA/RNA intermediates that are efficiently processed by Aptx. We report high-resolution X-ray crystal structures of human Aptx in the context of processing adenylated RNA/DNA junctions that describe the molecular basis of processing these lesions by Aptx. To gain insights into how Aptx dysfunction contributes to AOA1, we generated, expressed, and characterized 17 AOA1 variants. Solubility tests demonstrate that many of the AOA1 mutations affect protein stability, while 8 soluble variants were impaired in their ability to deadenylate RNA/DNA junctions. We determined X-ray structures of 5 AOA1 variants. While in some cases the molecular basis for Aptx impairment is clear (i.e. distorts protein-DNA contacts) in other cases the mutations are located distal from the active site suggesting they impact a mechanism of allosteric regulation of Aptx. Our work represented here reveals protein instability, substrate binding pocket distortion, and allosteric regulation underlie the causes of AOA1.

NIEHS
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Developmental Biology
Gli-similar 3 is a master regulator of retrotransposon silencing in male fetal germ cells
Germ cells are a uniquely important cell type because they give rise to gametes, the cells responsible for transmitting genetic information to subsequent generations. In order to clear the parent-specific imprinted marks acquired at fertilization, genomes of mammalian germ cells undergo global demethylation early in fetal development. DNA methylation marks are reestablished de novo during late fetal development in male germ cells and are maintained by a testis-specific piRNA pathway. Defects in retrotransposon promoter methylation lead to permissive retrotransposon activation and compromised genomic integrity in the germline, ultimately resulting in germ cell death and male infertility. In this study, we set out to uncover novel players involved in germ cell development using mice as a model organism. We found that Gli-similar 3 (Glis3), a transcription factor critical for cell lineage specification in the pancreas, is essential for male germ cell survival. In the testis, Glis3 is expressed specifically in germ cells between embryonic day (E)12.5-13.5, just prior to de novo methylation in the male germline. To identify the functions of Glis3, we examined gonads of global and tissue-specific Glis3 knockout (KO) mice. We found that testes of global Glis3 KO embryos had reduced numbers of germ cells and decreased expression of germ cell markers beginning at E15.5. Defects in germ cell development were not the result of deficiencies in somatic cell differentiation, as somatic cell-specific KO of Glis3 did not affect germ cell development. Microarray analysis revealed that several members of the retrotransposon silencing pathway, including Dnmt3l and the piRNA-processing genes Mael, Miwi, Mili, and Miwi2 were reduced in global Glis3 KO testes at E15.5. In concordance with the reduced expression of retrotransposon silencing pathway members, we found that mRNAs for the retrotransposons IAP and Line-1 were also significantly elevated in global Glis3 KO testes at this time. Sequence analysis reveals that putative GLIS3 binding sites are located in the promoters of many differentially regulated genes. Future studies will validate these potential interactions in vivo. In summary, these findings reveal that Glis3 is essential for retrotransposon silencing and germ cell survival in the fetal testis. The timing of germ cell loss observed in our model suggests that retrotransposon silencing is crucial for earlier stages of germ cell development than previously realized.

NIEHS
Margaret Adgent
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Epidemiology/Biostatistics - Etiology, Risk, and Prevention

Urinary triclosan and enterolactone: A cross sectional study of environmental influence on gut microbiome function

Emerging hypotheses contend that the gut microbiome plays an important role in the development of disease. The composition of the microbiome is influenced by external factors such as diet and antibiotic use, yet the influence of other environmental exposures is unknown. Triclosan (TCS) is an environmental exposure for which systemic microbiomic effects may be of particular interest. It is a phenolic broad-spectrum microbicide used primarily in soaps, dentifrices, and other personal care products, and exposure among humans is widespread. TCS is used primarily on the skin and in the mouth, but it can be detected in the urine, suggesting antimicrobial effects may occur beyond initial points of contact. As a preliminary investigation into the relationship between TCS and gut microflora, we assessed the association between TCS and enterolactone (ETL), an intestinal metabolite that is produced via bacterial transformation of dietary lignans (from seeds, fruits, nuts) and has known susceptibility to oral antibiotics. Methods: We examined urinary TCS and ETL for 2005-2008 United States (U.S.) National Health and Nutrition Examination Study subjects, aged = 20 years. We also examined the association between ETL and prescription antibiotic use to confirm its susceptibility to changes in bacterial composition of the body. Associations between natural log-transformed ETL and 1) any vs. no (<2.3 ng/mL) TCS, 2) TCS quantiles (Q1â€“Q5), and 3) any vs. no antibiotics were estimated with multiple linear regression, adjusting for sex, age, race, body mass index, poverty-to-income ratio, fiber intake, constipation, cotinine and creatinine (n = 2,441). Results: TCS was detected in 80% of subjects. After adjustment, ETL was not associated with TCS (any vs. none: beta = 0.09 (95% Confidence Interval (CI): -0.13, 0.31)), nor was there any suggestion of a dose response (e.g., Q5 (=104.5 ng/mL) vs. Q1 (none): beta = 0.10 (95% CI: -0.17, 0.37)). However, any antibiotic use (n = 112) was associated with significantly lower ETL (vs. none) (beta = -0.76 (95% CI: -1.21, -0.30)). Conclusions: Antibiotics, but not TCS, are negatively associated with urinary ETL. It is thought that antibiotics may reduce ETL by killing certain gut bacteria. At levels detected in the U.S., TCS does not appear to be acting similarly, despite broad antimicrobial properties. Further research is needed to better understand the influence of TCS and other environmental exposures on the gut microbiome.

NIEHS
Quaker Harmon
Postdoctoral Fellow
Epidemiology/Biostatistics - Prognosis and Response Predictions

Risk of fetal death with preeclampsia

Background: Accurate estimation of the risk of stillbirth with preeclampsia would be useful information for clinical decision-makers regarding pregnancy intervention, particularly in the preterm period. A fetuses-at-risk approach is required for unbiased estimation of stillbirth risk. However, the needed data on the timing of onset of preeclampsia has been lacking. Methods: We estimated gestational-age-specific risk of stillbirth for 554,333 singletons delivered in Norway during 1999-2008, using a life-table approach. Onset of preeclampsia was extrapolated from a subset of 1857 preeclamptic pregnancies for which prenatal records had been obtained. Weekly risks are expressed as absolute and relative risks. During three clinically important preterm intervals, we further compared expected mortality with immediate delivery (neonatal mortality) with expected mortality if delivery were delayed a week to allow the fetus to mature (fetal mortality in the intervening week plus lower neonatal mortality in following week). Results: Preeclampsia was recorded for 3.8% (n=21,020) of pregnancies. Risk of stillbirth was 3.6/1000 overall and 5.2/1000 among pregnancies with preeclampsia (relative risk (RR) =1.45, 95% confidence interval (CI) =1.19, 1.76). Using the data on timing of preeclampsia onset, we estimated that 8% of onset occurred by the end of week 28, 36% by the end of week 34, and 71% by the end of week 37. Relative risk of fetal death was markedly elevated with preeclampsia in early pregnancy.

In week 26 there were 11.6 fetal deaths per 1000 preeclamptic pregnancies at risk, compared with 0.1 fetal deaths per 1000 pregnancies without preeclampsia (RR=86, 95% CI=45, 122). Fetal risk with preeclampsia declined as pregnancy advanced, but remained consistently higher than in non-preeclamptic pregnancies. When comparing the mortality associated with immediate versus delayed delivery, in weeks 26-27 the mortality associated with immediate delivery exceeded that with delayed delivery (RR=1.65, 95% CI=1.2, 2.2). However, in weeks 28-33 (RR=1.06, 95% CI=0.8, 1.4) and 34-36 (RR=1.06, 95% CI=0.8, 1.4), the risks were nearly equal indicating that the risk of fetal death associated with delaying delivery was offset by the improved neonatal mortality. Conclusions: Preeclampsia in early pregnancy constitutes a particular hazard to the fetus. Fetal death should be included among the important risks clinicians must balance in the management of preterm preeclampsia.

NIEHS
Senthilkumar Cinghu
Visiting Fellow
Gene Expression
Nucleolin regulates the homeostatic balance between self-renewal and differentiation in embryonic stem cells
Cell identity is governed by a set of key regulators, which maintain the gene expression program characteristic of that cell state while restricting the induction of alternate programs that could lead to a new cell state. Identification of genes associated with specific biological phenotypes is critical to understanding the molecular basis underlying homeostasis, development, and pathogenesis. Although RNAi-based high-throughput screens are routinely used for this task, false discovery and sensitivity remain a challenge. We developed a computational framework for systematic integration of published gene expression data for identifying genes defining a phenotype of interest. We applied our approach to rank-order all mouse genes based on their likelihood of determining embryonic stem cell (ESC) identity. RNAi-mediated loss-of-function experiments on top-ranked genes unearthed many novel determinants of ESC identity including Nucleolin (Ncl), a highly conserved RNA-binding protein abundant in stem and cancer cells. We show a mechanistic role for Ncl in the maintenance of ESC homeostasis by shielding against differentiation-inducing redox imbalance-induced oxidative stress. Specifically, we show that Ncl knockdown (KD) elevates endogenous reactive oxygen species (ROS) levels and p53 activity, resulting in p53-mediated suppression of Nanog and subsequent ESC differentiation. The differentiation phenotype due to Ncl KD is largely restored upon p53 depletion or Nanog overexpression. RNA-IP experiments show that Nclâ€™s repression of p53 is direct via its RNA-binding activity, and luciferase-based reported assay show that p53, activated upon Ncl KD, binds to and suppresses Nanog. Mechanistically, Ncl KD elevates ROS levels due to loss of ROS-scavenging antioxidant proteins. Addition of antioxidants to the culture medium attenuates ROS level and p53 activation, and restores the ESC phenotype. Moreover, 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. Altogether, our 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. Our studies connect the dots on a previously unknown regulatory circuitry involving genes associated with traits in both ESCs and cancer, and have profound implications for understanding cell fate decisions in cancer stem cells.

NIEHS
Sivapriya Ramamoorthy
Research Fellow
Gene Expression
Glucocorticoid Receptor Isoform Knock-in Mice have Unique Responses to Glucocorticoids

Glucocorticoids are essential for life and have an important role in the regulation of metabolism, immune functions and response to physical and psychological stress. The biological responses to glucocorticoids are determined by glucocorticoid sensitivity, which is influenced by multiple factors and varies considerably among tissues. We have reported that multiple glucocorticoid receptor (GR) isoforms are generated from the single GR gene by alternative translation initiation. These translational isoforms of GR have distinct tissue distribution patterns and unique gene targets, thus the unique GR isoforms composition within a cell or tissue could determine the cell-specific response to glucocorticoids. The C3 isoform of GR has enhanced transcriptional activity compared to the other GR isoforms and the D3 isoform of GR is transcriptionally compromised. To determine the physiological consequence of the GR isoform-specific gene regulation in vivo, we have generated knock-in mice that exclusively express only the C3-GR isoform or the D3-GR isoform. Knock-in of C3-GR and D3-GR leads to post-natal lethality due to respiratory distress within hours after birth and genotyping at 21 days of age revealed that the number of total knock-in mice failed to meet the expected Mendalian ratio (25%). However, we demonstrate that C3-GR isoform alone is sufficient to support mouse postnatal development after embryonic rescue with maternal administration of dexamethasone on embryonic day 15.5 and 16.5. In contrast the D3-GR isoform alone cannot yield viable adult mice after comparable glucocorticoid administration. Genome-wide microarray analysis of glucocorticoid-regulated genes in Mouse Embryonic Fibroblasts (MEFs) derived from WT and GR-C3 knock-in mouse reveals profound differences in their transcriptomes. The GR-C3 isoform selectively regulates genes involved in several canonical pathways including Integrin-linked kinase signaling, cardiac hypertrophy signaling, circadian rhythm signaling and inflammation. These findings suggest that, GR isoform composition within cells can determine the glucocorticoid transcriptional regulatory profile, which in turn contribute to tissue specific physiological action of glucocorticoids.

NIEHS
Natacha Steinckwich-Besancon
Visiting Fellow
Immunology - Innate and Cell-mediated Host Defenses
Role of the calcium sensor protein, STIM1, in neutrophil chemotaxis and infiltration into psoriatic inflamed skin
Plaque-type psoriasis is a chronic inflammatory skin disorder involving a gradient of chemoattractants driving neutrophils into the epidermis. STIM1 is a calcium sensor protein resident in intracellular endoplasmic reticulum calcium stores. STIM1 detects the depletion of calcium stores and communicates with plasma membrane Orai1 calcium channels to activate store operated calcium entry. STIM1 is known to be involved in the signaling pathway for the G-protein-coupled chemoattractant receptor FPR1 in cell lines in vitro, but little is known about its role in vivo in pathological conditions such as psoriasis. In this study, we investigated STIM1 involvement in neutrophil infiltration into psoriasis inflamed skin in mice lacking STIM1 in myeloid lineage cells (primarily macrophages and neutrophils; LysM-cre STIM1fl/fl). Chemotaxis of mouse neutrophils was evaluated in vitro using a transwell assay, and in vivo using an imiquimod-induced psoriasis-like skin model. Using the LysM-cre STIM1fl/fl mouse model, we demonstrate that STIM1 is required for fMIVIL, WKYMVM, KC and MIP2-induced chemotaxis inducing receptors FPR1, FPR2, CXCR1 and CXCR2. We show that LysM-cre STIM1fl/fl mice had significantly less infiltrated neutrophils in the epidermis than STIM1fl/fl control mice, while macrophage migration is unaffected. Consistent with this observation, LysM-cre STIM1fl/fl mice display a more rapid reversal of psoriatic plaques. We have also carried out studies aimed at understanding how STIM1 functions during the process of chemotaxis. In the neutrophil-like HL-60 cell line, we find knockdown of STIM1 substantially inhibits fMLF-induced chemotaxis while eYFP-tagged STIM1 overexpression augments it. By imaging eYFP-tagged STIM1 during chemotaxis toward a micro-pipette releasing fMLF,

we show that STIM1 cellular localization was not homogeneous, but rather STIM1 accumulated in the rear of migrating cells, the uropode. In summary, our results suggest that STIM1 is important for neutrophil chemotaxis in vivo and that STIM1 knock out reduces neutrophil contribution to psoriatic inflammation. Furthermore, STIM1 appears to play a role in the spatiotemporal dynamics of the uropode. Our data provide new insights to our understanding of the role of STIM1 and store-operated calcium entry in chemotaxis.

NIEHS
Pengyi Yang
Research Fellow
Informatics/Computational Biology
Master transcription factors establish cell type-specific transcription attenuators for rheostat control of gene expression
Developmental progression is driven by spatiotemporal patterns of gene expression, which require precise orchestration of a complex set of interactions among a myriad of proteins and cis-regulatory elements including core promoters, enhancers, and insulators. It was widely assumed that RNA Polymerase II (PolII) recruitment to the promoters during transcription initiation is the rate-limiting step in transcription, but recent findings have revealed that most genes thought to be transcriptionally inactive experience transcription initiation. Although transcriptional regulation occurs well after transcription initiation via promoter-proximal pausing and regulated release of PolII into productive elongation (RNA synthesis), the factors and mechanisms that regulate PolII progression during productive elongation remains elusive. In an effort to elucidate mechanisms regulating transcription elongation, we developed an algorithm to mine PolII ChIP-Seq data and identified PolII peaks, within genes, with >10-fold PolII enrichment compared to the average gene body PolII density in mouse embryonic stem cells (ESCs). PolII peaks within 1kb of transcription start sites were classified as promoter-proximal PolII pause sites (PPPSs), and those within the gene bodies (>1kb away from TSSs) were classified as intragenic PolII pause sites (IPPSs). Analysis of IPPSs and PPPSs using published ESC ChIP-Seq data for various histone modifications and transcription factors (TFs) revealed that, unlike PPPSs, IPPSs are enriched for chromatin features reminiscent of enhancers and master ESC TF occupancy. Surprisingly, analysis of Hi-C data revealed that, unlike enhancers which are known to interact with promoters via intra- and inter-chromosomal interactions, IPPSs are not involved in chromatin interactions, suggesting that IPPSs could be novel regulatory elements. These observations led us to hypothesize that master ESC TFs bind IPPSs en masse to impede PolII progression, attenuate RNA synthesis, and thus regulate transcription. Indeed, luciferase-based reporter assay confirmed reduced expression for constructs containing IPPSs but not control regions. Moreover, our analyses in five different cell types reveal that while PPPSs are largely cell type-invariant, IPPSs are cell type-specific bound primarily by master TFs. Currently, we are conducting experiments to determine whether master TFs establish IPPSs for rheostat-like control of IPPS-containing host gene expression.

NIEHS
Georgia Alexander
Research Fellow
Neuroscience - Integrative, Functional, and Cognitive
Neuronal Activity in Hippocampal Area CA2 During Spatial Processing
GM Alexander, NW Plummer, CB Pantazis, P Jensen, SM Dudek The hippocampus is widely appreciated to contribute to the processing and storage of information about the environment, particularly that relating to spatial locations. The specific role of hippocampal area CA2 in brain function, however, is unknown, although experiments in mutant mice suggest that it too, plays a role in spatial memory

processing. Therefore, whether CA2 pyramidal cells display firing properties similar to neurons in its primary output region, area CA1, is critical to our understanding of the computations taking place in the hippocampus. In addition, the relationship between CA2 pyramidal cell firing and network oscillatory activity patterns seen during spatial exploration is unknown. Using electrophysiological recordings from awake, behaving rats and mice, we asked whether CA2 pyramidal neurons fire in spatially-restricted areas (known as place fields) and how the firing of these neurons relates to network oscillatory activity. In addition, we have generated a mouse strain that expresses cre recombinase predominantly in CA2, enabling us to express cre-dependent excitatory Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) in CA2. Using this approach, we have activated CA2 neurons and asked how network oscillatory activity patterns are affected. We found that, like CA1 neurons, CA2 pyramidal cells tended to fire in spatially-restricted areas, although they often showed more of these place fields than neurons in CA1. In addition, CA2 pyramidal cell firing was more highly correlated with network theta oscillations, which along with gamma oscillations, are prominent oscillatory patterns seen during spatial exploration. Finally, in mice, we found that selectively activating CA2 pyramidal neurons with DREADDs was sufficient to increase gamma oscillatory activity, which has been proposed to support the formation and retrieval of memory. These findings support the idea that neurons in hippocampal area CA2 function in spatial processing. In addition, the higher correlation of CA2 neuronal firing with theta oscillatory activity suggests a possible role for CA2 as a novelty detector, as theta is commonly seen with any interesting or novel stimulus in addition to during active exploration. This finding, paired with our observation of increased gamma oscillatory activity seen upon CA2 activation, is highly suggestive of a role for CA2 in spatial memory formation.

NIEHS
Qing Cheng
Research Fellow
Neurotransmission and Ion Channels
Activation of a7 nicotinic acetylcholine receptors increased intracellular cAMP levels in cultured hippocampal neurons
The activation of a7 nicotinic acetylcholine receptors (nAChRs) has been shown to improve hippocampal-dependent learning and memory. However, the molecular mechanism of a7 nAChRsâ€™ action remains elusive. We previously reported that activation of a7 nAChRs induced a prolonged enhancement of glutamatergic synaptic transmission in a protein kinase A (PKA)-dependent manner. Here, we investigated if there is a direct link between the activation of the a7 nAChR and cyclic adenosine monophosphate (cAMP) signaling in hippocampal neurons. To address this question, we employed a Förster- Resonance Energy Transfer (FRET)-based biosensor (mTurquoise-Epac(CD, ?DEP)-cp173Venus-Venus) to measure the intracellular cAMP levels directly via live cell imaging. We found that application of the a7 nAChR-selective agonist choline (2 mM; in the presence of the a7 nAChR positive allosteric modulator PNU-120596 (5 uM)) induced a significant change in the YFP/CFP ratio, which indicated an increase in intracellular cAMP levels. This choline-induced increase was abolished by the a7 nAChR antagonist MLA (40 nM) and the calcium chelator BAPTA (10mM), suggesting that the cAMP increase depends on the a7 nAChR activation and subsequent intracellular calcium rise. The soluble adenylyl cyclase (AC) inhibitor KH7 (25 uM) also blocked the choline-induced cAMP increase, suggesting that calcium dependent ACs are required for cholineâ€™s action. To determine the involvement of AC1, we tested the effect of siRNA against AC1. We found that this treatment reduced significantly the choline-induced FRET ratio change. This suggested that AC1 is the main mediator for the choline-induced cAMP rise. Our findings provide the first direct evidence to link activation of a7 nAChRs to a cAMP rise, which defines a new signaling pathway employed by a7 nAChRs. Our study sheds light into

the molecular mechanisms of the positive cognitive actions of a7 nAChR agonists and development of therapeutic treatments for cognitive impairments.

NIEHS
Deirdre Robinson
Doctoral Candidate
Pharmacology and Toxicology/Environmental Health
Assessing early developmental and pubertal effects in CD-1 mice following in utero exposure to bisphenol (BP) analogs
Growing public concern over the use of Bisphenol A in many consumer products has resulted in the implementation of several replacement analogs including the fluorinated and sulfonated derivatives, BPAF and BPS. However, both analogs have been found to have estrogenic properties that were either enhanced or comparable to BPA, indicating that they may be just as capable of inducing adverse effects on endocrine related tissues. Our goal was to evaluate the effects of these BP analogs on pubertal end points. We conducted single dose disposition studies, analyzed by HPLC-MS/MS, and confirmed the presence of each analog in the amniotic fluid. Urinary half-lives were calculated as 4.7, 5.5 and 8.2 hours for BPAF, BPA and BPS, respectively. The maternal and peri-pubertal effects of BPA, BPAF or BPS following gestational exposure in CD-1 mice were examined. Timed pregnant CD-1 mice were administered a twice daily oral gavage of vehicle, BPAF or BPS (0.05, 0.5 or 5 mg/kg/day) or BPA (0.5, 5 or 50 mg/kg/day) from gestational days 10-17. Dams were assessed daily for weight and behavioral changes. Female offspring were sacrificed on postnatal days 20, 28, 35 and 56 and evaluated for pubertal hallmarks which included mammary development, and timing to vaginal opening and 1st estrus. Carmine stained mammary whole mounts were assigned a developmental score of 1(poor)-4(best). Gestational maternal weight gain remained similar among all dose groups compared to controls, and resulted in full term pups with no observed litter size differences. Similarly, body weights of PND 1 offspring were unchanged. Body weight assessment of female offspring revealed no treatment related effects at PND 20 or 28, however, weights for 0.5 mg BPAF at PND 35 and 5 mg BPS at PND 56 were significantly reduced. Mammary gland development was accelerated at PND 35 (3.3 ±0.2) and 56 (3.5 ± 0.2) in the 50 mg BPA group. Similarly, the 0.05mg BPAF (3.6± 0.4) and BPS (3.1 ± 0.2) were significantly advanced compared to controls at PND 35. Vaginal opening and 1st estrus in all groups were comparable to control. This study is the first to confirm transplacental transfer and report half-lives in the pregnant CD-1 dam for these analogs. Although other pubertal events were unaltered, the mammary gland may be the most sensitive target for these chemicals. Because early BPA exposures are thought to contribute to the onset of adult diseases, it may be necessary to assess later time points.

NIEHS
Qingshan Wang
Visiting Fellow
Pharmacology and Toxicology/Environmental Health
Ultra-low dose of diphenyleneiodonium attenuates progressive dopaminergic neurodegeneration and motor deficits in multiple rodent Parkinsonâ€™s disease models
Parkinsonâ€™s disease (PD) is the second most common neurodegenerative disorder characterized by progressive nigral dopaminergic neurodegeneration and behavioral deficits. Current therapeutic regimens for PD only temporarily relieve symptoms but fail to halt disease progression. Substantial evidence indicates that microglia-mediated chronic neuroinflammation is critical in driving the progressive neurodegeneration in PD. We previous demonstrated that microglial NADPH oxidase (NOX2) is a key enzyme for maintaining chronic neuroinflammation. Here, we investigated whether inhibiting NOX2 is an effective therapeutic strategy to arrest PD progression. As a proof of principle,

diphenyleneiodonium (DPI), a widely used NOX2 inhibitor, was used as a proto-drug. Despite its potent inhibitory effect on NOX2, DPI has not been clinically tested due to high toxicity at regularly used doses (μM in cultures and mg/kg in animals). For this reason, ultra-low dose DPI was employed. Initial in vitro studies using primary midbrain cultures revealed that DPI at 10-13-10-14 M displayed great specificity towards NOX2 and importantly, protected dopaminergic neurons against lipopolysaccharide (LPS)- and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced damage without any observed toxicity. These results prompted us to further test the therapeutic potential of ultra-low dose DPI in vivo using a post-treatment regimen. DPI (10 ng/kg/day) was infused subcutaneously via mini-pump for two weeks after mice received prior injection of either LPS or MPTP. We discovered that DPI mitigated both LPS- and MPTP-elicited motor deficits measured by rotarod activity. Morphometric studies revealed that DPI significantly attenuated nigral dopaminergic neurodegeneration and a-synuclein aggregation (a widely used morphological marker for PD) in both LPS- and MPTP-treated mice. These data strongly pointed out the potent efficacy of ultra-low dose DPI in halting the progressive dopaminergic neurodegeneration. Mechanistically, DPI at ultra-low dose was shown capable of inhibiting NOX2 and exhibited great specificity in vivo. Moreover, NOX2 deficiency abolished DPI-afforded protection. A battery of pathological examinations revealed no toxicity in DPI-treated mice. Altogether, ultra-low dose DPI was effective in halting PD progression without obvious toxicity. Our findings suggest that a new class of NOX2-inhibiting anti-inflammatory drugs should be pursued as potential candidates for PD therapy.

NIEHS
Barbara NICOL
Visiting Fellow
Physiology
Uncovering New Paradigm in Testis Differentiation Using Mouse Genetic Models
Genetic defects leading to sex-reversal at birth or infertility in adulthood are often the result of differentiation impairment of gonads during fetal life. In mammals, testis and ovary arise from the same embryonic organ called bipotential gonad. In males (XY), the testis-determining gene Sry initiates testis differentiation by inducing the expression of Sox9. Loss of Sox9 leads to complete testis-to-ovary sex reversal in XY individuals. In females (XX), conversely, ß-catenin activates the ovarian pathway while suppresses the SOX9-activated testis pathway. Loss of ß-catenin leads to a partial ovary-to-testis reversal in XX individuals. These genetic evidences support the model that the fate of bipotential gonads hinges upon the balance between pro-testis SOX9 and pro-ovary ß-catenin. To better understand the antagonistic relationship between pro-testis and pro-ovary signaling, we generated a genetic mouse model where SOX9 and ß-catenin are ablated specifically in the somatic cells of bipotential gonads and compared the phenotype of Sox9/ß-catenin double knockout (DKO) to ß-catenin and Sox9 single knockout (KO) and wild type embryos. In the XX gonads, ablation of Sox9 in addition to ß-catenin led to partial ovary-to-testis sex reversal similar to the ß-catenin single KO gonads, indicating that Sox9 is not responsible for the partial sex reversal. This result also suggests that ß-catenin in the ovary represses pro-testis genes other than Sox9. In the XY gonads, ablation of ß-catenin in addition to Sox9 resulted in formation of ovotestis, a less severe phenotype than a complete testis-to-ovary sex reversal in the Sox9 single KO. This result, in accordance with the XX DKO phenotype, suggests that ß-catenin represses certain testis characteristics independently of Sox9. Intriguingly, when the XY DKO was compared with XX DKO gonad, we found that the XY DKO gonads developed more prominent testis characteristics than the XX DKO gonads. This leads to a provocative hypothesis that in addition to stimulating Sox9 expression, Sry may also induce other unknown pro-testis genes. Our novel findings shed new light on the complex antagonisms between pro-ovary and pro-testis pathways. While testis differentiation was

thought to be the result of a direct induction by SOX9, we show here that it actually requires both Sox9-dependent and -independent morphogenetic changes.

NIEHS
Matt Quinn
Postdoctoral Fellow
Physiology
Imbalance of endogenous glucocorticoids and estrogen leads to the development of autoimmune hepatitis like symptoms in mice
Autoimmune hepatitis (AIH) is a condition in which the body's immune system attacks the liver in the absence of pathogen, leading to a chronic inflammatory state. There is a sexually dimorphic presentation of AIH with approximately 70% of AIH patients being female. Moreover, the typical age of diagnosis is between 14-40 years, a time period in females when endogenous estrogens are at their highest, indicating estrogens may play a causal role in the pathogenesis and progression of AIH. Clinically the first line of defense for AIH is the use of synthetic glucocorticoids due to their anti-inflammatory and immunosuppressive effects, however, the role of endogenous glucocorticoids in the etiology of AIH has not been empirically examined. Therefore, the aims of this study were to characterize the effects of long-term glucocorticoid deficiency in male and female mouse liver. To reduce endogenous glucocorticoids male and female mice were adrenalectomized for four months. Post-adrenalectomy male mice have normal liver histology, however female mice show the presence of inflammatory cell foci as well as ductular reaction. Furthermore female mice have increased expression of IL-1b and IL-6 and activation of NF-kB. Immunofluorescent staining show female but not male mice have increased activation of Kuppfer cells and infiltration of CD8+ T-cells in the liver. The inflammatory miliue of the female adrenalectomized liver leads to cell death demonstrated by TUNEL staining and cleaved caspase-3. To test whether endogenous estrogens are responsible for the sexual dimorphism in hepatitis, female mice were adrenalectomized or ovariectomized alongside adrenalectomy to reduce both glucocorticoids and estrogens. Ovariectomized adrenalectomized females were protected from the inflammatory phenotype of the adrenalectomy and appear similar to the intact mouse. These data indicate that the onset of AIH could be initiated by dysregulation or loss of glucocorticoid signaling and the unopposed actions of estrogen. Very few animal models exist for the study of AIH and none to date have been reported to display a sexually dimorphic component to the disease onset like that observed in humans. We propose long-term adrenalectomy as a novel model for the study of AIH and to decipher the signaling pathways different between males and females to determine the underlying molecular mechanism for the onset of this disease.

NIEHS
Andrew Oldfield
Postdoctoral Fellow
Stem Cells - General
NF-Y specifies cell identity by promoting chromatin accessibility for master transcription factors at active enhancers
Cell type-specific master transcription factors (MTFs) play crucial roles in defining cell identity and function. However, the roles of ubiquitous factors in the specification of cell identity remain unclear. The heterotrimeric CCAAT-binding complex NF-Y is one such factor with established functions in cell cycle. NF-Y, composed of NF-YA, NF-YB, and NF-YC subunits, is evolutionarily conserved from plants to Humans, and is expressed in nearly all cell types. Here we show that all three subunits of the NF-Y complex are required for the maintenance of embryonic stem cell (ESC) identity, and establish NF-Y as a novel component of the core pluripotency network. Using ChIP-Seq studies, we show that NF-Y binding

requires all three subunits and positively correlates with gene expression. Furthermore, we show that, in addition to binding to proximal-promoters, NF-Y co-occupies enhancers with ESC-specific MTFs. Using gene expression analysis, we show that NF-Y is required for the expression of core ESC self-renewal and pluripotency genes. RNAi- mediated loss-of-function experiments show that the loss of even one of the three NF-Y subunits leads to the loss of the pluripotent state. Genome-wide occupancy and transcriptomic analyses in ESCs and neurons reveal that not only does NF-Y regulate genes with house-keeping functions through cell type-invariant promoter-proximal binding, but also genes required for cell identity by binding to cell type-specific enhancers with MTFs. Mechanistically, we show that NF-Y's distinctive DNA-binding mode promotes enhanced MTF binding by facilitating a permissive chromatin conformation. Specifically, we show that NF-Y promotes enhanced binding of Oct4/Sox2/Nanog/Prdm14 at their sites of colocalization, but not vice versa. ChIP using antibodies against Oct4 or Sox2 followed by qPCR revealed a significant loss of Oct4/Sox2 binding specifically at Oct4/Sox2 sites co-bound by NF-Y but not at sites where Oct4/Sox2 bind without NF-Y. Using immunoprecipitation experiments, we establish interaction between Oct4 and NF-Y. DNaseI hypersensitivity experiments show that NF-Y promotes enhanced accessibility for Oct4/Sox2 binding by facilitating a favorable chromatin conformation. Together, our studies unearth a novel function for NF-Y in promoting chromatin accessibility, and suggests that other proteins with analogous structural and DNA-binding properties may function in similar ways.

NIEHS
Mallikarjuna Metukuri
Research Fellow
Stress, Aging, and Oxidative Stress/Free Radical Research
Deletion of intestinal SIRT1 activates Paneth cells, enhances intestinal inflammation, and alters gut microbiota
Inflammatory bowel diseases (IBDs), including ulcerative colitis (UC) and Crohnâ€™s disease, are chronic inflammatory conditions of the gastrointestinal tract. Both genetic factors and environmental influences contribute to the pathogenesis of IBDs. However, the molecular mechanisms that integrate environmental signals with disease development remain poorly defined. Here we show that SIRT1, the most conserved mammalian NAD+-dependent protein deacetylase, is vital in modulation of intestinal tissue integrity. Using an intestinal-specific SIRT1 knockout (SIRT1 iKO) mouse model, we discovered that deletion of intestinal SIRT1 led to hyperacetylation of NF-?B, eliciting cellular NF-?B signaling and oxidative stress pathways and activating Paneth cells upon stress induced by high-fat diet, chemicals, and aging. Paneth cells are innate immune cells of intestinal origin that protect against enteric bacterial pathogens and maintain intestinal microbial homeostasis through the production of antimicrobial proteins. Paneth cell dysfunction is associated with alterations of intestinal microbiota and provides a site of origin for intestinal inflammation. Not surprisingly, hyperactivation of Paneth cells and oxidative stress response pathways in old SIRT1 deficient intestine triggered spontaneous gut inflammation, characterized with colon shortening, lymphocyte infiltration, and intestinal barrier impairment. Moreover, old SIRT1 iKO mice were specifically depleted of Bacilli, particularly Lactobacillus, a genus of anti-inflammatory probiotic bacteria that is diminished in human IBD patients. In line with these observations, transcriptomes of old SIRT1 deficient colons shared the highest similarity with those from colons of various colitis mouse models, and exhibited a significant overlap with colonic transcriptomes of human UC patients. Furthermore, the mRNA levels of SIRT1 were significantly reduced in colons of human UC patients, suggesting that SIRT1 may serve as a key genetic determinant of IBDs. In support of this notion, intestine-specific deletion of SIRT1 increased susceptibility to doxorubicin-induced intestinal mucositis and dextran sodium sulfate-induced colitis in young mice. Taken together, our findings identify a novel role of intestinal SIRT1 in the regulation of intestinal inflammation and homeostasis in response

to environmental influences, and suggest that pharmacological activation of SIRT1 may be beneficial for treatment of human IBDs.

NIMH
Vincent Costa
Postdoctoral Fellow
Neuropharmacology and Neurochemistry
Dopamine bias novelty seeking during probabilistic decision making
Novelty seeking refers to the tendency of humans and animals to explore unfamiliar stimuli and environments. Identifying the neural mechanisms mediating novelty seeking is an important question, as deviations in novelty seeking characterize various psychiatric and neurological disorders. Dopamine (DA) signaling seemingly contributes to the optimistic valuation of novel stimuli. Novel stimuli are known to excite DA neurons as well as activate brain regions receiving dopaminergic input. While these findings show that DA regulates attentional orienting, they fail to link novelty detection to novelty seekingâ€"particularly when framed as a decision making process. If DA does control novelty seeking, enhancing dopaminergic transmission should bias choice preferences in favor of exploring novel choice options. To determine if DA enhances novelty seeking, we manipulated DA by injecting either GBR-12909, a DA transporter (DAT) inhibitor, or saline, before the animals performed a probabilistic three-arm bandit task where novel choice options were randomly introduced. DAT is specific to the cell membrane of DA neurons and controls reuptake of extracelluar DA from the synapse. Blocking DAT therefore increases extracelluar DA levels. The task was structured to allow the monkeys the opportunity to explore novel options or to exploit familiar options that they had already sampled. We compared how often the monkeys chose novel vs. familiar options when DA levels were enhanced or not. Using a reinforcement learning (RL) model, we also examined if increased novelty seeking following DAT blockade was attributable to an increase in the value of novel options. We found that DAT blockade increased the monkeysâ€™ innate preference to select novel over familiar choice options. A RL model fit to the monkeysâ€™ choice data showed that increased novelty seeking following DAT blockade was driven by an increase in the initial value the monkeys assigned to novel options. However, blocking DAT did not affect the ability of the monkeys to discriminate high, medium, and low value options, their learning rates, or the total amount of reward they earned per block. These findings demonstrate that increases in extracellular DA levels underlie the positive valuation of novel stimuli in order to promote exploratory behavior. A clinical implication of the present findings is that alterations in DA reuptake may contribute to excessive novelty seeking and impulsivity.

NIMH
Lucas Glover
Doctoral Candidate
Neuroscience - General
A role for adult neurogenesis in the adaptation to an unpredictable, threatening environment
New neurons are born in the dentate gyrus throughout life. Previous work in our lab showed that new neurons diminish behavioral responses to stress. It is unclear, however, precisely how they do this and what contribution they make towards emotionality. One possibility is that these neurons affect the appraisal process or alter the perception towards an uncertain threat. We used mice that express the HSV-tk transgene (TK) under a GFAP promoter to selectively ablate adult-born neurons and to investigate responses to unpredictable, aversive experiences. In a fear conditioning task, a tone or light that always predicted an upcoming shock (â€˜perfect conditioningâ€™) produced similar freezing and startle behaviors in TK and wild type (WT) littermate controls. However, when additional cues were added so that only 50% of cues predicted shocks (â€˜imperfect conditioningâ€™), TKs froze and startled

less than WT mice. This same pattern of results was reflected in neural activation of the mature granule cells and CA3 pyramidal cells as measured by c-fos activation; TKs trained in the imperfect condition showed decreased activation relative to WTs and all mice in the perfect condition. Interestingly, cued fear conditioning has traditionally been seen as a hippocampus-independent task, but these findings show that the hippocampus is engaged when ambiguity about the cue is introduced. Because we see increased freezing during the cue in TKs even with imperfect conditioning, it appears that the cue-shock association is present, suggesting that these behavioral changes do not reflect a lack of learning. To look for lasting consequences of an unpredictable, aversive experience, mice were tested in the novelty-suppressed feeding task following perfect or imperfect fear conditioning. Following imperfect conditioning, WTs showed greater latency to eat food in a novel environment, while perfect conditioning had no effect on these mice. TKs, however, showed intermediate increases in latency regardless of the type of conditioning. Clamping stress hormones at low levels prevented the increased latency in WTs after imperfect conditioning but had no effect on TKs. These findings suggest that new neurons enhance protective stress-related behaviors in response to unpredictable threat and also regulate responses to future novel situations in a glucocorticoid-dependent manner. These changes could bias behavior to optimally adapt to adverse environments.

NIMH
Michael Gregory
Clinical Fellow
Neuroscience - General
Neuroimaging of myelination through development using a novel MRI sequence, mcDESPOT
Background: Myelination of the human brain occurs throughout childhood into early adulthood. Previous studies have sought to describe these changes using various structural imaging methods such as white matter density and diffusion imaging. Recently, it has become possible to directly estimate myelin content (MWF) using a novel MRI sequence, mcDESPOT. The one prior study of age-related changes in MWF using mcDESPOT only studied infants and children under six years old. Here, we use mcDESPOT to examine developmental changes in myelination through adolescence. Methods: Fifty-two typically developing children (mean age 13.1 years, range 7-18, 31 males) were imaged on a 3T MRI scanner. We obtained an ir-SPGR image and 8 flip angles of both SPGR and SSFP images at phase 0 and 180 degrees. Images were coregistered and MWF maps were calculated in each childâ€™s native space per the three-pool mcDESPOT protocol. A mean irSPGR template was created from all 52 children studied. Each childâ€™s irSPGR image was warped to this template. Resulting maps were spatially smoothed and a linear regression was computed to determine the effects of age on myelin content. Analyses were conducted on a voxel-wise basis across the entire brain and thresholded at p=0.05, corrected for multiple comparisons. Results: Age-related changes in MWF were seen in bilateral posterior limbs of the internal capsule and basal ganglia (putamen and globus palladus). Inferolaterally, these appeared to extend into the retrolenticular internal capsule, leading towards the temporal lobes. Additionally, clusters were seen bilaterally in anterior corona radiata (leading towards the frontal lobes), bilateral cerebral peduncles and anterior medullary pyramids (likely part of corticospinal tracts), bilateral anterior thalami and anterior corpus callosum. All changes were positively correlated with age; no regions showed a decrease in myelination with age. No significant sex or age by sex effects were observed. Conclusions: Our results suggest changes in myelination during adolescence occur preferentially in connections between basal ganglia, frontal and temporal lobes. This is consistent with past findings in this developmental period using white matter density and diffusion imaging. Furthermore, this highlights the critical changes occurring in the human brain during development and provides a framework for considering behavioral changes and neuropsychiatric diseases that emerge during adolescence.

NIMH
Rose-Marie Karlsson
Visiting Fellow
Psychiatry
Adult hippocampal neurogenesis affects motivation to obtain sucrose, but not food, reward in an operant task
Decreased hippocampal neurogenesis has been implicated in the pathogenesis of anxiety and depression. Diminished interest or pleasure, anhedonia, is one of the hallmark symptoms of major depression but is poorly understood. Previous findings in our laboratory have shown that mice lacking neurogenesis show decreased sucrose preference, which is a standard measure for studying anhedonia in rodents. The aim of the present study was to further investigate the role of adult hippocampal neurogenesis in motivation to obtain novel rewards. We inhibited adult neurogenesis using valganciclovir in transgenic mice and rats that express herpes simplex virus thymidine kinase (TK) under the control of the GFAP promoter. TK and wild-type (WT) littermate controls were mildly food restricted and trained to lever press for either chocolate flavored sucrose tablets or regular food tablets on fixed ratio (FR) and exponentially progressive ratio (PR) tasks. TK mice showed normal acquisition of lever press on a FR schedule and minimal pressing of inactive lever, suggesting normal learning of lever-reward association. However, when switched to a PR schedule, mice lacking adult neurogenesis showed significantly reduced responding compared to WT controls. When working for food tablets, there was no difference between WT and TK mice in either acquisition or PR responding suggesting normal motivation to obtain a food reward. As in the mouse study, transgenic TK rats showed normal FR and PR responding to food reward. However, TK rats respond significantly less to the sucrose reward even on an FR schedule, suggesting a more pronounced deficit in sucrose intake. In addition, when rats were given a fixed amount of respective reward for 1 hr in the home cage TK rats showed significantly decreased motivation to eat the chocolate sucrose tablets compared to WT controls, while TK rats readily ate all the food tablets suggesting the difference is not due to satiety but decreased motivation to consume sucrose. This is the first study to demonstrate that rodents lacking adult hippocampal neurogenesis have decreased motivation to work for a sucrose reward in an effort based task, consistent with the anhedonic phenotype seen in the sucrose preference test. Future studies will be needed to understand the role of hippocampal neurogenesis and the motivation to consume different types of rewards.

NINDS
Paul Lee
Clinical Fellow
Clinical and Translational Research
Protease-activated Receptor-1 (PAR-1) and Interleukin-1Beta Act Synergistically in Granzyme b Mediated Neurotoxicity
Objective To better understand how granzyme b acting at PAR-1 is neurotoxic. Background Protease Activated Receptor-1 (PAR-1) is a G-protein-coupled receptor that is activated by serine proteases. PAR-1 is expressed on 20% of neurons and has a known role in long-term potentiation. Previous research has shown that granzyme b, a serine protease released by T-cells, causes neuron death by activating PAR-1. PAR-1 surface expression is modulated by interleukin-1Beta (IL-1Beta.) In neuroinflammatory diseases such as multiple sclerosis, there are elevated levels of granzyme b and IL-1Beta within the cerebrospinal fluid, creating a milieu in which neurons chronically are exposed to a potent cytotoxic combination. Design/Methods Neurotoxicty assays utilized a commercial preparation of induced pluripotent stem cell-derived human cortical neurons. After maturing, neurons were exposed to granzyme b with or without IL-1Beta for 3-5 days. Inhibitors directed against PAR-1 mRNA and several second messengers were

screened for effects. Receptor antagonists for PAR-1 and IL-1Beta were also examined. Immunohistochemistry was used to examine PAR-1 and IL-1Beta in human post-mortem tissue samples obtained from controls or patients with multiple sclerosis. Results Granzyme b alone was toxic only to cortical neurons expressing PAR-1. The addition of IL-1Beta led to increased numbers of neurons with PAR-1 expression. Daily replenishment of granzyme b and IL-Beta eventually was lethal to all neurons in culture. Inhibition of PAR-1 mRNA or components of the phospholipase c pathway could prevent granzyme b neurotoxicity. Antagonism of PAR-1 and Il-1Beta significantly reduced granzyme b neurotoxicity. In human post-mortem tissues, PAR-1 expression was evident on neurons adjacent to inflammatory lesions. Conclusions These studies demonstrate that PAR-1 and IL-1Beta can act together to promote neurotoxicity. PAR-1 is expressed on neurons adjacent to inflammatory lesions in human brain tissue conferring in vivo risk. PAR-1 and IL-1Beta are potentially significant factors and may contribute to progressive neuronal loss in chronic neuroinflammatory diseases such as multiple sclerosis.

NINDS
Alessandra De Paula Alves Sousa
Visiting Fellow
Immunology - Autoimmune
Deep sequencing of T-cell receptor repertoire reveals enrichment of highly expanded clonotypes in cerebrospinal fluid from patients with multiple sclerosis
Immunological studies have demonstrated that inflammatory T cells in the central nervous system play a fundamental role in the development of multiple sclerosis (MS). However, in-depth clonal composition profiling of these T cells, that may reflect perturbations of the adaptive immune response, has been poorly understood due to technological limitations. Advances in high-throughput sequencing of T-cell receptors have provided a powerful tool to explore the depth and complexity of the immune response and may also serve to evaluate the frequency of specific T-cells in compartments such as peripheral blood (PB) and cerebrospinal fluid (CSF). We have used an unbiased molecular approach called 5â€™rapid amplification of cDNA ends to amplify the V-D-J genes of unsorted T-cells obtained from PB and CSF of 5 patients with MS and 5 individuals classified as non-inflammatory neurological disease (NIND), and evaluated the TCR repertoire using high-throughput DNA-sequencing technology. A total of 80 million short sequence in-frame reads covering the TCR hypervariable regions were generated through HiSeq2500 Illumina system platform. The data was analyzed using a bioinformatics program that align and match the human TCR beta-chain nucleotide sequences through IMGT database. We observed that number of distinct TCR based on V-D-J sequences rearrangement (known as richness) and the diversity of T-cell clonotypes of the PB was much higher compared to CSF compartment for both groups of individuals (p=0.01, paired parametric T-test). Surprisingly, the diversity of T-cell repertoire of CSF compartment in patients with MS was not significantly different from NIND controls (p=0.527, unpaired parametric T-test). However, when we looked at the frequency of expanded clonotypes within the CSF compartment, MS patients showed a statistically increased number of highly expanded clones compared to NIND controls. As expected, the majority of MS patients also showed a significantly expansion of clones in the CSF compared to the PB compartment. Importantly, analysis of the most shared expanded clones in both compartments revealed that very few T-cell clones from the circulating PB infiltrate the CSF compartment. Our findings strongly demonstrate a biased T-cell repertoire in the CSF compartment of MS patients and the feasibility of in-depth high throughput deep sequencing to comprehensively study the T-cell repertoire in patients with neurological immune-mediated disease.

NINDS
Richa Lomash

Visiting Fellow
Intracellular Trafficking
Role of neurolastin, a novel brain-specific GTPase, in regulating excitatory synapses
Glutamate receptors mediate the majority of excitatory neurotransmission in the mammalian nervous system. The precise regulation of the levels of these receptors at synapses is the underlying mechanism mediating synaptic plasticity and is controlled by processes such as membrane trafficking and post-translational modifications. Key players regulating membrane trafficking events include GTPases of the dynamin superfamily that hydrolyze GTP to steer membrane remodeling. A novel brain-specific GTPase (RNF112) of this superfamily was recently identified in the Roche lab. This protein, named neurolastin, has closest homology with atlastin, another dynamin family GTPase. These GTPases attach to membranes and are characterized by their ability to oligomerize and hydrolyze GTP. In accordance, we demonstrate that neurolastin can self-associate and hydrolyze GTP to mono-phosphate (GMP) using an in vitro GTPase assay. The ability to hydrolyze GTP to GMP is unique to atlastin or Guanylate-binding proteins (GBP). To better understand the protein topology, we used various extraction conditions (Tx-114 and high pH) and found that neurolastin is not an integral membrane protein distinguishing it from atlastin. Surprisingly, the analogous hydrophobic patches are not true transmembrane domains. Thus, neurolastin has a GTPase domain closest to atlastin, but also shares properties exclusive to GBPs or dynamin, such as being a peripheral, and not integral membrane protein. Using neurolastin knockout mice that we generated, we observe reduced levels of glutamate receptors demonstrating their regulation by neurolastin. Electrophysiology recordings of neurons from knockout mice show a reduction in the mEPSC frequency consistent with fewer functional synapses. Furthermore, we find that GTP hydrolysis is required for maintaining synapse number since expression of neurolastin and not its GTPase mutant (R340Q) leads to an increase in spine density both in wild type and knockout neurons. Neurolastin localizes to endosomes, and using mass spectrometry, we have now identified neurolastin interacting partners that include various endocytic proteins like Munc-18 and Adaptor protein (AP2). We tested these interactions using co-immunoprecipitation and pull down assays from brain and see specific binding. These results demonstrate that neurolastin is an endosomal GTPase that associates with an endocytic complex and regulates glutamate receptor dynamics and excitatory neurotransmission.

NINDS
Catherine Nezich
Doctoral Candidate
Molecular Biology - Eukaryotic
Abstract & Title removed at request of author

NINDS
Ellen Flannery
Postdoctoral Fellow
Neuroscience - Cellular and Molecular
VEGFR1: a developmental link between neuronal migration and vasculogenesis
Neuronal migration is essential during development for appropriate circuitry to form. Disruption of neuronal migration causes neurological disease states, leading to a number of devastating cognitive and reproductive problems. As such, it is critical to understand the mechanisms underlying normal neuronal migration to begin to address/treat the event(s) associated with a disease. In vertebrates, appropriate migration of gonadotropin-releasing hormone-1 (GnRH) neurons to their final location within the brain is necessary for hypothalamic control of sexual maturation and reproductive function. Failure of this process leads to hypogonadotropic hypogonadism (HH), resulting in delayed puberty and infertility. To

discover ligand/receptor signaling impacting this migration, we compared the transcriptomes of migratory vs. post-migratory GnRH neurons. Microarray analysis revealed that the receptor tyrosine kinase Fms-like tyrosine kinase 1 (also known as vascular endothelial growth factor receptor-1, VEGFR1) was upregulated in migrating neurons as compared to post-migratory neurons. VEGFR1 is most commonly associated with the developing vasculature and has been shown to function in vasculogenesis by sequestering VEGFA from another receptor, VEGFR2. Notably, both VEGFA and a related VEGFR1 ligand, VEGFB, were expressed along the GnRH migratory route and in GnRH cells, respectively, lending credence to their potential role in regulating GnRH neuronal migration. Single cell PCR and immunocytochemistry confirmed expression of VEGFR1 in migrating GnRH neurons. Functional assays revealed chronic treatment with a blocking antibody specific for VEGFR1 decreased the distance GnRH cells migrated compared to controls. In addition, acute in situ assays using the blocking antibody revealed an immediate decrease in cell migration rate. Unexpectedly, blocking the ligand VEGFA revealed a significant increase in migration rate, suggesting a dynamic balance between VEGFA and VEGFB in modulating VEGFR1-mediated GnRH migration. These data demonstrate a novel role for this receptor tyrosine kinase and its ligands in neuronal migration and may provide new candidates for genetic screening in HH patients. Furthermore, this work sheds light on the complex, yet well-known players in vasculogenesis and may provide an unexplored link between two critical developmental processesâ€"vasculogenesis and neuronal migration.

NINDS
Christopher Harris
Visiting Fellow
Neuroscience - Cellular and Molecular
Studying the dynamics and connectivity of neural circuits underlying goal-directed behavior
Understanding how brains generate goal-directed behavior is a central aim of neuroscience. Complex goal-directed behaviors such as predation depend on multiple interacting brain regions and involve several neural processes including perception, recognition, motivation, action selection and motor control. To better understand the neural basis of goal-directed behavior we are developing a set of techniques that integrate information about behavior, neural circuit dynamics and synaptic connectivity. First we developed a virtual reality assay to elicit prey-tracking behavior in larval zebrafish. We carefully restrained one week-old zebrafish by the neck in agarose, leaving the eyes and tail free to move. The restrained fish were presented with a prey-like target on a LED screen moving on the left or right side of the visual field. Prey-tracking behavior in the form of target-directed swims and a characteristic pattern of sustained eye convergence was detected in real-time by machine vision algorithms and used to update the movement of the virtual prey in a way that simulates approach. Using this closed-loop behavioral assay we were able to evoke sequences of up to five target-directed swims with sustained eye convergence. Having confirmed that our behavioral assay reliably induces goal-directed behavior we are now working to monitor the brain during these episodes of predation by means of two-photon light-sheet microscopy in fish expressing calcium indicators in the brain. In preliminary experiments we have been able to image the whole brain of agarose-embedded live fish at cellular resolution and with a frame rate of about 1 Hz. Once we have gathered detailed information about the activity and location of neurons whose activity is associated with prey-tracking we will use 3D electron microscopy (EM) to study the synaptic connectivity of those neurons. We are particularly interested to see how known features of the behavior, such as the fine tuning of motor output to target trajectory, is reflected in the underlying circuit structure. We are currently able to generate even EM staining throughout the brain and can identify individual synaptic connections. In combination with methods for serial sectioning and dense circuit reconstruction, also available in our lab, this work will help answer fundamental questions about the neural circuit basis of goal-oriented behavior in the vertebrate brain.

NINDS
Alicia Pickrell
Postdoctoral Fellow
Neuroscience - Cellular and Molecular
The Loss of Endogenous Parkin Causes Dopaminergic Neurodegeneration in a Mouse Model of Mitochondrial Aging
Parkinsonâ€™s disease is the most common motor deteriorating neurodegenerative disease caused by the loss of a subpopulation of dopaminergic (DA) neurons in the substantia nigra (SN). Early-onset familial cases have identified recessive PARK2 gene mutations encoding the E3 ubiquitin ligase Parkin. Parkin selectively translocates to dysfunctional mitochondria promoting their removal by autophagy (mitophagy) by targeting mitochondria with low mitochondrial membrane potential and/or pathogenic mitochondrial DNA (mtDNA) mutations. Patients with PARK2 mutations often confer a loss of mitochondrial function, postulated as a consequence due to a defect in the surveillance of mitochondrial quality. To study the role of Parkin in vivo in relation to its role in mitochondrial quality control, we utilized an aging mouse model that accumulates dysfunctional mitochondria caused by an accelerated generation of mtDNA mutations (Mutator mice) crossed to a Parkin knockout (KO) mouse. We hypothesized that the loss of endogenous Parkin would exacerbate phenotypes of a mouse model harboring deleterious mtDNA mutations. Mutator Parkin KO mice performed behavioral testing revealing the appearance of L-DOPA (a pharamcological drug used to alleivate PD symptoms) reversible motor coordination defects at 52 weeks-of-age. At this time point, we found 40% of DA neurons in the SN degenerated and a significant reduction in the amount of DA axons projecting into the striatum of Mutator Parkin KO mice. This specific neuronal loss caused a depletion of DA in the striatum, which led to the motor deficits displayed in these mice. Age-matched wildtype, Parkin KO, and Mutator mice exhibited none of these abnormal behaviors or had any DA neurodegeneration present. The gross neuroanatomy and brain weight appeared normal in Mutator Parkin KO mice suggeting that DA neurons are particularly sensitive to mitochondrial defects. Our study provides the first evidence that endogenous Parkin protects DA neurons haboring high levels of deleterious mtDNA mutations implying an important role for Parkin in mitochondrial quality control in vivo.

NINDS
Marta Pallotto
Postdoctoral Fellow
Neuroscience - General
Unravelling the olfactory bulb circuit: comparing the roles of peri-natale and adult born granule cells.
In the olfactory bulb (OB) of mammals, inhibitory GABAergic interneurons, granule cells (GCs), regulate the activity of OB principal cells, specifically mitral (MCs) and tufted cells (TCs). MCs and TCs are functionally distinct and process different aspects of olfactory information, forming two different sub-circuits. In the mouse brain, GCs are produced from birth to adulthood. The role of GCs in the OB has been extensively investigated. However, whether the time of birth of these interneurons contributes differently to the inhibition of MC and TC output neurons is unknown. The aim of this work is to investigate whether adult-born and perinatal-born GCs have different functions in MCs â€“TCs OB sub-circuits. This question is key to understanding the intra-bulbar circuits and the specific role of adult-born cells. We used injections of adeno-associated viral vectors (AAV) at different time points, to visualize simultaneously both perinatal and adult-born GCs. The injections were performed at post-natal day 3 (p3) using a GFP-encoding AAV and at p50-60 using an RFP-AAV. Morphological analysis confirmed that adult-born GCs are located mainly in the inner GC layer, whereas p3 GCs are located mainly in the external part of the GC layer. Next, we used injection of viral vectors encoding calcium indicators

(GCaMP6m and RCamP) and a two-photon microscope to monitor the excitability of perinatal and adult-born GCs. We found that both perinatal and adult-born GCs show calcium transients and respond to glutamate stimulation. Ongoing experiments use double-transgenic mice in which MCs and TCs express the light- activatable molecule ChR2. Using a custom two-photon microscope fitted with a DLP-based visual stimulator, we selectively optically stimulate TCs or MCs somata and record calcium responses in adult-born and peri-natal born GCs labeled with the calcium indicators. These experiments will help us to investigate the specificity of connectivity between MCs, TCs and adult-born and perinatal born GCs. Lastly, we will collect large 3D electron microscope volumes from the same tissue to explore the connectivity amongst these and additional neuron types in the OB.

NINDS
Kristina McLinden
Postdoctoral Fellow
Neuroscience - Neurodegeneration and Neurological disorders
A neurodegenerative mutation increases the intrinsic rate of aging in Drosophila
Aging is the single greatest risk factor for neurodegenerative diseases (NDDs) including Alzheimerâ€™s and Parkinsonâ€™s Disease, yet the reasons for this remain unknown. Gain or loss of Cdk5 function, a kinase activated only in neurons, has been implicated in a variety of human NDDs. We now find that when we inactivate Cdk5 in Drosophila, by deletion of its activating subunit, p35, we observe the early emergence of phenotypes that are typically not observed in adult flies until old age. These include increased cell death, impaired autophagy, and accumulations of protein aggregates, as well as traits common in human NDD such as axonal swellings. To interrogate whether the phenotypes observed represent a true acceleration of the normal rate of aging, we performed microarray analysis of the head and thorax tissues of 10 day old wild-type and mutant flies and compared these profiles to wild-type flies at 3 and 30 days of age. First, we identified genes that are changing between wild-type and mutant flies of the same age. These included changes in mitochondrial function, proteolysis, and stress response. Next, using RNA from wild-type flies, we derived a set of â€œaging-related genesâ€ and developed a â€œstandard curveâ€ of aging to ask whether flies with p35 loss of function exhibited the expression profiles of wild-type flies that were younger, older, or the same age. upon comparing the RNA profile of young, pre-symptomatic p35 mutant flies with the standard curve, we found that gene expression in young mutants correlated most closely with the profile of wild-type flies of the oldest age. To confirm these findings, we compared our expression data with a publically available dataset that profiled flies at 7 different timepoints, and these results corroborated our original finding that young mutants show expression patterns of much older flies. Finally, we used gene ontology groups to compare the gene categories changing in p35 mutants with â€œaging-related genesâ€ and found an almost complete overlap. These data provide strong evidence that flies that are just entering the progression to neurodegeneration, due to loss of Cdk5 activity, undergo a dramatic acceleration in their intrinsic rate of aging. These findings suggest a novel relationship of aging to neurodegeneration, and have profound implications for the selection of potential therapeutic targets for this class of diseases.

NINDS
Avner Meoded
Research Fellow
Neuroscience - Neurodegeneration and Neurological disorders
Cerebro-cerebellar functional and structural networks in Primary Lateral Sclerosis
Introduction: Primary lateral sclerosis (PLS) is a form of motor neuron disease characterized by selective corticospinal degeneration. Resting state functional magnetic resonance imaging (RS-fMRI) and diffusion tensor imaging (DTI) are methods for assessing functional and structural brain connectivity. The aim of

the study was to assess the functional and structural PLS connectome. Materials and Methods: PLS group consisted of successive clinic patients seen during 2012-2013. Age-gender matched controls were also included. Functional-connectivity was performed using the data driven approach. White matter integrity was evaluated with tract-based-spatial-statistics (TBSS) Structural connectome analysis with topology and network-basedâ€“statistics (NBS) evaluation was carried out. Results: Sixteen PLS patients (59.7 ± 8.6 y) and 14 healthy-controls (51.6 ± 10.3y) participated in the study. PLS patients had increased functional connectivity in both motor and extra-motor brain regions compared to healthy controls. The strength of connectivity between these regions and the right cerebellum was inversely correlated with the ALSFRS-R within the PLS patient group. There was widespread decrease of FA of the cortical white matter in the PLS patient group compared to controls in the TBSS analysis. NBS did not detect any significantly increased or decreased structural networks between groups. The topology analysis showed that PLS patients had higher global efficiency and a higher small-world index compared to healthy-controls. Three common hubs were identified in the precuneus region bilaterally and the right lingual gyrus. PLS patients had an additional hub in the right cerebellum. Four modules were identified in controls and seven modules were identified in the PLS group. Conclusion: In PLS patients, changes in functional/structural connectivity occur among brain regions outside the motor cortex, including the cerebellum that are often overlooked in motor neuron disease. Increased connectivity in these regions may suggest plasticity in which other motor regions are now accounting for functions previously attributed to the primary motor cortex. Alternatively, the increased connectivity may be due to widespread reduced intra/inter-hemispheric inhibition due to the degeneration of inhibitory interneurons. The PLS network demonstrated greater modularity, which may be a compensatory reorganization that economizes the cost.

NINDS
Mary Anne Hutchison
Postdoctoral Fellow
Neurotransmission and Ion Channels
The role of glutamatergic synaptic transmission onto midbrain dopamine neurons in reward-related behaviors
The release of dopamine (DA) from midbrain neurons is critically involved in motivated and reward-related behavior. These DA neurons show a variety of firing patterns which are regulated by the activation of glutamate receptors. While burst firing in DA neurons have been shown to code for novelty and salience of rewarding stimuli, the relative contribution of glutamatergic input as a whole to DA cell firing and to specific reward-related behaviors remains largely unclear. A better understanding of these processes will help to identify the biological mechanisms underlying disorders such as drug addiction. To this end we combined genetic approaches with immunohistochemistry, electrophysiology and behavioral assays to determine the role of glutamatergic input onto DA neurons in reward-related behaviors. For this, we developed a novel quadruple conditional knockout mouse line in which three genes encoding AMPAR subunits (GluA1, A2 and A3) plus the gene encoding GluN1, the obligatory NMDAR subunit, are all conditional alleles (GRIA1-3fl/flGRIN1fl/fl). We then crossed a well-characterized DAT-Cre knock-in mouse line that expresses Cre specifically in DA neurons with these mice to generate GRIA1-3fl/flGRIN1fl/fl/DAT-Cre mice. We used immunohistochemistry and electrophysiology to confirm the loss of functional NMDAR and AMPAR subunits. To study the consequence of how these synaptic changes might affect behavior, we tested a battery of basic and reward-related behaviors. We found that neither gross movement nor performance on a rotating beam test was altered in the mutant mice. In addition, no differences were seen in a sucrose preference test or with operant responding for food. Surprisingly, the mutant mice performed as well as controls in an associative-learning test with sweetened food pellets. Therefore, although DA release has long been associated with reward, we have

shown that DA activation via glutamate receptors is not crucial for the response to natural reinforcers. Further studies are ongoing to determine the role of glutamate receptors in the behavioral response to cocaine, a drug that acts through on dopamine system, in Pavlovian conditioning and other behavioral tests related to dopamine signaling. Together, the results of these studies will clarify the biological underpinnings of reward-related behaviors such as drug addiction, and will hopefully provide novel targets for therapeutic interventions.

NINDS
Michael Ferenczy
Postdoctoral Fellow
Virology - DNA
A cell culture model of developing human neural cells reveals early transcriptional and epigenomic events in JC virus infection
JC Virus (JCV), the etiological agent of progressive multifocal leukoencephalopathy (PML), is a ubiquitous polyomavirus that causes significant morbidity and mortality in AIDS patients and people on certain immunosuppressive therapies. There are no therapies for PML, and no animal models for JCV infection. Productive infection by JCV is limited to glial cells of human origin. The few available cell culture systems are mostly SV40 T antigen-immortalized or transformed cells. SV40 T antigen is able to initiate late gene transcription and replication of JCV, thus complicating investigation of events in early infection. In response to these challenges, we have developed a cell culture system in which we differentiate primary human neural progenitor cells into progenitor-derived neurons or astrocytes. JCV lytically infects human astrocytes, and can nonproductively infect neurons. The outcome of infection is determined in the nucleus, so infection with JCV is a marker of the host cell transcriptional environment during differentiation into distinct types of neural cells. Encapsidated JCV is nucleosomal, indicating that histone modifications may play a role in the JCV life cycle. Progenitor-derived neurons and astrocytes were infected with JCV, and analyzed during early infection by qPCR, RT-PCR and chromatin immunoprecipitation. DNA replication, T antigen (early) and VP1 (late) RNA expression were significantly greater in astrocytes than neurons. This correlated with chromatin structure. Histone H3 levels and acetylation of histone H3, which is a mark of active transcription, were similar on the JCV genome in both cell types. Levels of histone H3 trimethyl lysine 9 and heterochromatin protein 1 gamma (HP1 gamma), marks of repressed and condensed chromatin, were greater on the viral genome in neurons than in astrocytes. HP1 gamma binding correlated with binding to the JCV promoter of known JCV-repressive transcription factors NFIA, c-jun, and c-fos. NFIA and c-fos RNA were also highly expressed in neurons in comparison to astrocytes. Positive acting factors showed limited difference between cell types. Thus, negative regulators of JCV transcription and replication, including chromatin modifications and transcription factors, are likely determinants of repression of JCV in non-permissive cell types. Further analysis of JCV transcription may yield insight into neural development, JCV pathogenesis, and markers for PML susceptibility.

NLM
Gang Fu
Postdoctoral Fellow
Informatics/Computational Biology
Meta Path-based Semantic Network Analysis for Link Predictions between Compounds and Genes
A semantically linked data set integrating domain knowledge across chemical and biological space can promote large-scale data mining in drug discovery. Link predictions based on the observed topological structure have emerged as an interesting research topic in the semantic network analysis. However, most of the similarity-based link prediction algorithms designed for homogeneous networks cannot take

into account the heterogeneous types and relations defined in the semantic networks. Therefore, we proposed the meta path-based topological features for link predictions. A meta path defines a composite relation between the starting and ending objects. The numbers of instance paths belonging to a given meta path can be considered as an important topological feature, which can be studied by machine learning models. We have studied an integrated chemical and biological network consisting of nine semantic types, including compounds, ChEBI types, substructures, genes, adverse side effects, gene ontology termss, tissues, biological pathways, and diseases, as well as twelve semantic links between instances of different semantic types. We focused on the link predictions between compounds and genes, which are most rewarding in drug repositioning and polypharmacology research. 12,971 positively labeled links and 26,682 negatively labeled links were collected, and the positively labeled links were removed from the semantic network when we calculated the meta path-based topological features. Those topological features were encoded in commuting matrixes, which were calculated by multiplying a sequence of adjacent matrixes. We have calculated a total of 49 commuting matrixes for all of the possible meta paths from compounds to genes. Random forest (RF) was used to build the binary classification models. The labeled links were randomly split into training and test set by a ratio of 2:1. RF model produced high predictive performance on both training set (accuracy 0.986 and F-score 0.979) and test set (accuracy 0.979 and F-score 0.967) without over-fitting. RF models have intrinsic feature ranking algorithm. According to the significant scores assigned by the RF model, six most important meta paths were selected. The RF model built upon this limited feature space produced almost identical predictive performance (accuracy 0.976 and F-score 0.962) for test set. The proposed framework can be used for other types of semantic link predictions as well.

NLM
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Cataloging Marketed Indications for Human Drugs
Drug-disease treatment relationships, i.e. drugs and their indications, are among the top information needs of clinicians and researchers. Such information is very useful in controlling medication errors in EMRs, and training systems to predict novel indications. There have been many key efforts to create a repository on drug indications. However, most existing resources are unstructured and do not support computer processing or interoperability. Also, the performances of automatic methods to extract structured indications from drug descriptions are limited due to the presence of disease mentions that are not indications. To this end, we propose a text-mining pipeline to create a structured and normalized gold standard of drug indications using a hybrid approach combining human judgments and automatic tools. As the data source, we used the DailyMed which houses the up-to-date drug labels submitted to FDA by drug companies. A drug label provides detailed information on indications in free-text format and is normalized to precise drug concepts. Our pipeline comprises a clustering method to identify representative drug labels from a given corpus, a state-of-the-art tool to recognize and normalize the indication candidates from drug labels, and an annotation workflow for experts to accept/reject the candidates and finalize the gold standard. For this study, we started with 8,151 drug labels corresponding to 250 most searched ingredients on PubMedHealth and used the pipeline to extract accurate indications. The results include a text corpus of 500 representative labels double annotated with 88% agreement, and a dataset of 7,805 drug-disease relationships for 1,513 drugs where a drug is defined as: IN(Ingredient),DF(DoseForm),ST(Strength). To our best knowledge, this is the first large-scale study on using human annotations for drug indication mining. This is a non-trivial problem as we find that the experts rejected 45% of the machine-proposed indication candidates. A comparison of our results with an existing machine-curated resource revealed several discrepancies and reinforced our

motivation to have human involvement. In addition, different from existing studies that represent a drug simply by its ingredient, this study regards indication as a function of all key properties (IN,DF,ST) and also identifies the candidate drugs to further study this assumption. Future plans include publicly releasing the gold and scaling with machine learning and crowdsourcing.

NLM
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Identifying Bacterial Pathogens and Viruses in the Human Intestinal Tract
While some bacteria have an innate ability to cause disease, others are instead recruited as pathogens from commensal populations that exist in the normal, healthy human microbiome. Several examples of conversion of previously benign bacteria to a pathogenic form by bacteriophage-mediated transduction of virulence factors are widely known, including cases involving the diphtheria and Shiga toxins, genes necessary for cholera epidemics carried by Vibriophages, and Neisseria meningitides that crosses the bloodâ€“brain barrier and kill a previously healthy individual within hours. Microbiome studies have yielded many insights into the bacterial populations associated with the human body, but far less is known about viruses in these environments, or more importantly, how they contribute to human diseases. In order to gain a deeper insight into host-viral interactions in the human gut, we are currently developing several tools and approaches that will enable metagenomics studies to identify, quantify, and distinguish between different types of viruses in a sample, which we demonstrate also aids in probing the nature of conversion to pathogenicity. One major focus is the identification of signature genes that act as sensitive and precise diagnostic indicators of the presence and quantitative abundance of a given type of virus in a metagenomic sample. These were derived from an updated dataset of orthologous genes in phages (Phage Orthologous Groups, POGs), now containing genes from >1,000 complete genomes. Strikingly, taxon-specific, virus-specific, single-copy signature POGs could be found for two-thirds of the 57 taxa that were tested. Another major focus is the identification of phage-derived regions within bacterial chromosomes (including partially-degraded remnants of integrated prophages that may contain virulence factors), which may provide the ability to design similar markers to distinguish between a pathogenic and benign form of a given bacteria. In a large dataset of over a hundred complete bacterial genomes from four diverse genera, we identified phage-derived regions as clusters of homologs to POGs, and observed that several properties of pathogens differ significantly from their closely-related (at the genus, species, and strain level) benign cousins. We are continuing to develop this approach to work on an even larger scale, as well as investigating common metabolic pathways along the route to pathogenicity.