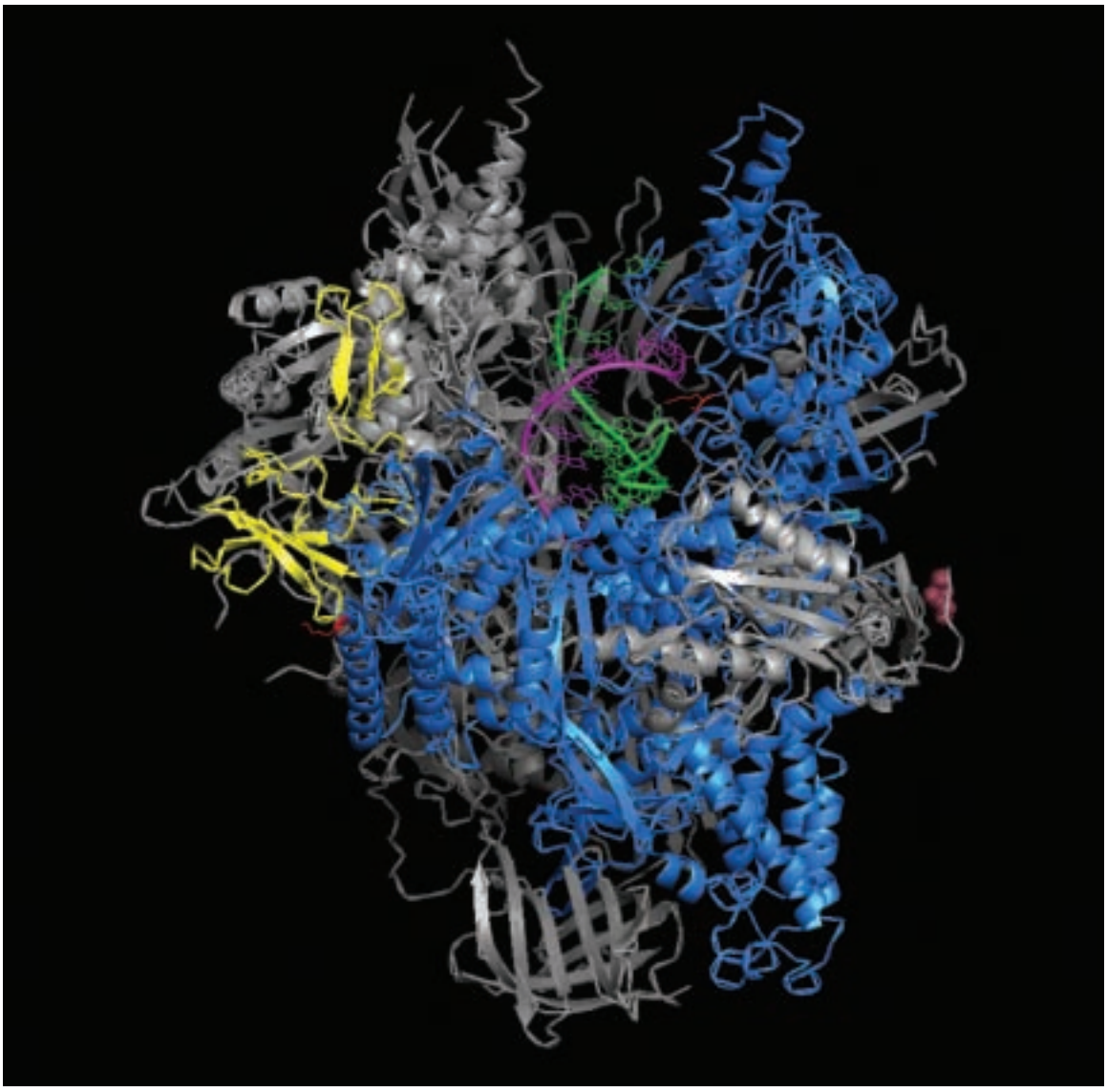
Summer Undergraduate Research Program



August 9, 2007

Gerstner Sloan-Kettering
Graduate School of Biomedical Sciences

Placement of the ubiquitylation sites in the yeast RNAPII structure (front view). Modified lysines (in red) are indicated with their side chains. Image courtesy of Paul Tempst and Jesper Q. Svejstrup.

Preface

The mission of Gerstner Sloan-Kettering Graduate School of Biomedical Sciences is to advance the frontiers of knowledge by providing to gifted and creative students in an interactive, innovative, and collegial environment the education and training they need to make new discoveries in the biological sciences.

PhD Program

The goal of the Gerstner Sloan-Kettering graduate program is to train a cadre of outstanding scientists who will exploit new advances and developing fields in biomedical sciences and apply their training directly to advancing the understanding of human disease. The School offers the next generation of basic scientists a program to study the biological sciences through the lens of cancer -- while giving students the tools they will need to put them in the vanguard of research that can be applied in any area of human disease.

Summer Undergraduate Research Program

The Gerstner Sloan-Kettering Graduate School sponsors a ten-week research program for outstanding undergraduate students who are interested in pursuing a career in biomedically related sciences. The explosion in knowledge that has driven recent progress in the diagnosis and treatment of cancer reflects the vitality of laboratory science at Sloan-Kettering. Students learn from scientists who are conducting research in areas such as developmental biology, genetics, structural biology, computational biology, cellular and molecular sciences, immunology, molecular pharmacology and chemistry, among others. Students have the opportunity to:

- Obtain hands-on research experience in cutting-edge laboratories
- Interact with faculty, postdoctoral fellows, and graduate students
- Attend a weekly seminar series, with presentations by Sloan-Kettering faculty
- Attend workshops such as presentation skills, interview skills, and others
- Attend and present at works-in-progress sessions with the cohort of summer students
- Present their research at a special poster session at the end of the program

Further information about the school and its programs can be found at www.sloankettering.edu or by contacting us at gradstudies@sloankettering.edu.

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CD4+ CD25+ FoxP3+ Regulatory T Cell Reconstitution after T Cell Depleted HSCT

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Hematopoietic Stem Cell transplantation (HSCT) is a curative therapy for a variety of malignancies. Although HSCT can facilitate the treatment of certain illnesses, one of the major complications of this therapy is a lasting immunodeficiency of the recipient. Post-HSCT immunodeficiency is characterized by a significant delay in the reconstitution of T cells, both in number and function. T cells play an essential role in the eradication of tumors and infectious organisms. Studies have shown that a specific subset of T cells, Regulatory T cells (CD4+ CD25+ FoxP3+), inhibit the proliferation and function of conventional T cells. Therefore, targeting Regulatory T cells following HSCT could be an effective therapy for immunodeficient patients. The presence, functionality, and reconstitution patterns of regulatory T cells following HSCT have not been characterized. The purpose of this study is to investigate the reconstitution of Regulatory T cells in T cell depleted murine HSCT transplant models.

Diversity-Oriented Stereoselective Synthesis of Aromatic Spiroketals

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High range of bioreactivitiy, stereochemical diversity and frequent incidences of natural substructure occurrence of spiroketals make this group an attractive target for diversity-oriented synthesis into primary libraries for high-throughput screening. This work presents the progression of kinetically controlled stereoselective synthesis of two aromatic diastereomeric spiroketals with four stereogenic centers that increase stereochemical diversity and are part of an extensive primary library.

The multi-step synthesis of aromatic spiroketals began with single protection 1,3 propanediol with *tert*-butyldiphenylsilyl chloride, Swern oxidation of the product gave the corresponding aldehyde, which was subjected to a Diels-Alder 4+2 heterocycloaddition with Jacobsen catalyst and Danishefsky diene to produce the enantiomerical pureed pyrone. Stereoselective reduction of the pyrone with Noyori catalyst, followed triisopropylsilylchloride protection and then stanalytion afforded a glycol, which was subjected to Stille Cross-Coupling. The spiroketal precursor was prepared by the alcohol deprotection of the product from previous step.

At the time the abstract was written successful synthesis of 115 mg of the spiroketal precursor was completed with overall 53% product yield. Further synthesis and yield information for the aromatic spiroketals will be updated on the poster.

Investigating the Role of the Receptor Tyrosine Kinase PDGFRα in the Segregation of Primitive Endoderm in Mouse Embryonic Stem Cells

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Platelet-derived growth factor receptor α (PDGFRα) is a receptor tyrosine kinase that plays an essential role in mammalian development. Our laboratory has shown that PDGFRα expression initiates at preimplantation stages. PDGFR starts to be expressed dynamically within the inner cell mass of the early blastocyst stage embryo, and subsequently marks the primitive endoderm (PE) at the end of the preimplantation period. Genetic evidence has established the transcriptional factors GATA-4 and GATA-6 as key regulators of PE differentiation. To determine the role of PDGFRα in this genetic cascade we used mouse embryonic stem (ES) cells as an *in vivo* model for the formation of PE. We exploited gain and loss of function approaches to investigate the relationship between the GATA factors and PDGFRα. Our data supports a model whereby PDGFRα functions downstream of the GATA factors in the development of the PE.

The role of CD4+ and NK+ cells in regulating immunity to melanoma

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During tumor progression, tumors are infiltrated with multiple stromal cell types, notably immune and inflammatory cells. The field of cancer immunology has begun to focus on investigating the interplay between immune cells that infiltrate tumors, aiming to elucidate the role of each cell type. We are studying the relationship between these different stromal cell types in a mouse melanoma model, which we have adapted as subcutaneous lesions in mice. B16F10 is a spontaneous melanoma that arose in C57BL/6 mice. It is inherently poorly immunogenic and very aggressive, thus serving as a good model of human disease.

Previous experiments profiling the immune cells present in subcutaneous B16 tumors have demonstrated coordinated infiltration of myeloid cells, dendritic cells, NK cells, T cells (CD4+ and CD8+), and a unique NKT cell population. We are interested in gaining a clearer understanding of the cross-talk between these tumor infiltrating cell types as they interact within the draining lymph node and the tumor microenvironment.

To work towards this goal, we began conducting experiments to deplete different cell types and observe how the absence of a particular cell type affects infiltration by other cell types. Preliminary depletion experiments were conducted by using antibodies to deplete CD4+ cells (depleting tumor infiltrating CD4+ T cells) and NK1.1+ cells (depleting tumor infiltrating NK and NKT cells). Of note, early analysis shows increased myeloid and dendritic cell infiltration in late-stage tumors in the absence of CD4+ T cells. We have also identified a curious, previously undescribed population CD8+Foxp3+ T cells present in these tumors.

Our lab is currently developing and working with spontaneously-arising, transgenic mouse models of melanoma that express aberrant genes implicated in the human disease. We plan to extend these experiments to immune infiltrates present in endogenously arising melanoma.

The role of Ubiquitination and PCNA Interaction on the Function of p15PAF

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The oncogene, p15PAF (PCNA Associated Factor), is a 15 kD ubiquitously expressed protein that is over expressed in many tumors including esophageal, breast, uterine, cervix, brain, kidney, and lung. P15PAF was isolated through its ability to bind to proliferating cell nuclear antigen (PCNA), a well-characterized protein involved in DNA synthesis. Our lab is interested in determining the molecular function of p15PAF. To facilitate our analysis we generated a panel of monoclonal antibodies (mAbs) that specifically recognize p15PAF. Using these mAbs, our studies have shown that p15PAF is most highly expressed in T cells and B cells and that p15PAF protein levels are regulated during the cell cycle with highest levels observed in cycling cells. Biochemical analysis has shown that p15PAF exists in cells as three major forms; unmodified p15PAF, monoubiquitinated p15PAF and diubiqutinated p15PAF. Mass spectrometry analysis confirmed the ubiquitination of p15PAF and identified Lys 24 of as the site of ubiquitination. To dissect the role of p15PAF ubiquitination, site directed mutagenesis was used to mutate Lys 24 (to Arg). Additionally, to examine the importance of the interaction between p15PAF and PCNA, site directed mutagenesis was used to mutate both wild type and K24R templates of p15PAF. Mutant and control wild type constructs were transfected into HeLa cells to create stable cell lines. Studies are currently under way to determine the expression, subcellular localization, and ubiquitination status of the p15PAF mutants relative to wild type. Finally, studies are underway to map the epitopes recognized by the p15PAF specific mAbs. Collectively, these studies will help to elucidate the role of p15PAF in immune cells and tumors.

Characterization of a Mouse Limb Patterning Mutant

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The development of the vertebrate limb is a complex process involving regulated outgrowth and patterning. The patterning of the proximal-distal axis of the limb is controlled by interactions between the apical ectodermal ridge (AER), located at the distal apex of the limb, and the mesenchyme beneath it called the progress zone (PZ). During the course of an ENU-based forward genetic screen in the mouse, we have identified a mutant with striking limb deformities. This recessive mutation, known as HV1, was discovered at e12.5, but the phenotype is apparent as early as e10.5. Cartilage staining of e12.5 embryos revealed that in HV1 mutants there are severe deformities in the distal limb elements. In these mutants the autopod is absent and there is a single bone zeugopod. By using DNA polymorphisms (Simple Sequence Length Polymorphisms and Single Nucloetide Polymorphisms) as genetic markers we have mapped HV1 to a locus on Chromosome 4 in the interval between 43.0Mb and 54.2Mb. While several mouse and human genes have been implicated in the proximal-distal patterning of limbs, none of these genes are in this interval. HV1 appears to define a novel gene important in the growth and patterning of the vertebrate limb on the proximal-distal axis.

Muscle size and shape: genetic screens reveal genes directing muscle morphogenesis

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The mechanisms by which cells achieve their specific size, shape and orientation are not well understood. To gain insight to this process, we have focused on the musculature of the model organism *Drosophila melanogaster*. There are thirty body wall muscles per hemisegment in the *Drosophila* embryo, each a single multinucleated cell with a specific size, shape and orientation. The final muscle morphology is acquired rapidly over a period of only six to seven hours. To identify genes that regulate muscle morphogenesis, we have undertaken two types of genetic screens, both of which rely on the same assay: a fluorescently tagged marker that labels the nuclei of a subset of embryonic body wall muscles. This assay allows us to screen "live," giving us the ability to analyze large numbers of mutations quickly. Our first screen examines the effects of microRNA (miRNA) misexpression. miRNAs help direct development by post-transcriptionally downregulating target genes. We found that overexpression of some miRNAs severely disrupted the labeled muscles, leading to mesoderm patterning defects, strong muscle morphology defects, and missing or undersized muscles. Several miRNAs have been selected for further study, including electronic prediction of their targets. In addition, we are conducting a forward genetic EMS screen to identify new genes that affect muscle development. Through this screen we have isolated many mutants and have selected several showing similar phenotypes to the miRNA misexpression for mapping and further investigation. The results of these studies will provide additional insight to the complex process of morphogenesis.

Study of the effects of single nucleotide polymorphisms on human CTLA-4 regulation

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T cell activation is regulated by a complex balance between positive and negative signals including receptor/coreceptor interactions and soluble molecules. The temporal and spatial separation of expression of positive and negative regulatory molecules is key for the full activation of the T cells and for controlling the magnitude of the immune response. In humans, another level of complexity results from inherited genetic variants within the population that have the potential to affect gene expression and function. Recently, single nucleotide polymorphisms (SNPs) in a key negative regulator, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), have been linked to a higher risk for Graves' disease and Hashimoto thyroiditis, both autoimmune disorders. The human CTLA-4 gene consists of four exons, with SNPs found in the promoter region, the +49 position of exon-1, and the 3'-UTR. It is possible that the polymorphisms located at the promoter region may be causing a defect in CTLA-4's transcriptional efficiency. In addition, the +49 SNP involves an amino acid sequence change in the leader sequence, and thus has the potential to affect ER translocation efficiency. Thus, we propose to explore the potential effects of the promoter and +49 SNPs on CTLA-4 regulation. We will use transient transcription assays of a luciferase reporter gene and track subcellular localization of CTLA-4 for the different allelic variants. This will be compared to our results from monitoring the levels of expression and subcellular localization of mouse CTLA-4, where no polymorphisms have been described. The functional implications of CTLA-4 polymorphisms at the mechanistic level must be understood in order to determine their putative connection with these autoimmune diseases.

The Functional Significance of the IRF9-GRIP1 interaction during GC-induced disruption of the Jak-STAT Pathway of α/β Type-I IFN Signaling

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Glucocorticoid hormones (GC) possess potent anti-inflammatory properties, proving highly effective for treating immune and inflammatory diseases such as lupus and rheumatoid arthritis. They function through the ligand-dependent transcription factor, glucocorticoid receptor (GR). It has been well-established that GR inhibits the production of many cytokines. In contrast, cytokine signaling appears to be unaffected or even stimulated by GCs. Earlier studies in the lab unexpectedly found that their action specifically counters the pro-inflammatory Janus activated kinase/ Signal Transducer and Activator of Transcription (Jak-STAT) signaling pathway of α/β Type I Interferons (IFNs). However, the exact steps and mechanism(s) of repressive action of GCs on the Jak-STAT pathway remain unknown. The IFN-induced transcriptional complex that becomes localized to the nucleus and is utilized in this pathway is the heterotrimer ISGF3, composed of STAT1, STAT2, and Interferon Regulatory Factor 9 (IRF9). Previous studies suggest that GR inhibits IRF3-dependent gene expression by competing for a common co-factor, GR-Interacting Protein-1 (GRIP1). Our recent evidence indicates that GRIP1 can directly bind to recombinant GST-IRF9 *in vitro*. Therefore, we seek to determine the functional significance of the IRF9-GRIP1 interaction. We first established and mapped the interface, utilizing *in vitro* binding assays and will use chromatin immunoprecipitation (ChIP), to determine whether IRF9 and GRIP1 co-occupy promoters of genes inducible by the Type I IFN pathway and whether GRIP1 recruitment is GC-sensitive. Further loss-of-function experiments with GRIP1 knockout mice will help to establish whether GRIP1 is required for ISGF3-mediated transcription of target genes, and whether loss of GRIP1 from the ISFG3 complex accounts for the observed inhibition of IFN signaling in response to GCs.

Mitochondrial morphology and dopaminergic neuron density in PINK1-deficient mice

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Parkinson's disease (PD) is the most common motor disease and the second most common neurodegenerative disease. Clinically characterized by bradykinesia, tremors, and muscle rigidity, PD develops from loss of dopaminergic neurons in the substantia nigra pars compacta. Although sporadic PD comprises 90% of PD, PINK1 (PTEN induced kinase 1) a disease gene for familial, recessive early-onset PD, may provide insight into many PD mechanisms. PINK1 contains a kinase domain and a mitochondrial targeting sequence. Furthemore, mitochondrial dysfunction has been implicated in PINK1, as shown in mutant drosophila with mitochondrial morphology defects. To further support this and in an attempt to localize PINK1 within a model, we have studied PINK1 knockout mice. We have examined the general morphology of wild-type and PINK1 knockout mice. Preliminary results suggest that there are no significant mitochondrial morphological abnormalities. Attempting to localize the PINK1 protein with an antibody revealed no differences and suggests an inefficient antibody. Dopaminergic cell densities using tyrosine hydroxylase (TH) labeling will be measured in the caudate and substantia nigra pars compacta.

Regulation of activation-induced cytidine deaminase (AID) by phosphorylation

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B cells undergo class-switch recombination and somatic hypermutation to achieve diversification of the antibody repertoire in response to antigen challenge. Both processes are initiated by activation-induced cytidine deaminase (AID), a B cell specific enzyme that deaminates cytidines specifically on single-stranded (ss) DNA. It has been proposed that the generation and deamination of ssDNA require transcription and interaction of AID with the ssDNA binding protein RPA. So far, it is known that RPA interacts only with AID that has been previously phosphorylated at Serine 38 (S38) by c-AMP dependent Protein Kinase A (PKA). However, the mechanism that activates PKA in B cells and the process that regulates the activity of phosphorylated AID are not known. One approach to address these issues is to develop monoclonal antibodies that can identify phosphorylated forms of AID in B cells. To this end, hamsters were injected with peptides containing the phosphorylated S38 and T27 epitopes of mouse AID protein. The obtained monoclonal antibodies were screened against phosphorylated mouse AID, which was expressed as a maltose binding protein-AID (MBP-AID) fusion protein bound to amylose beads. The S38A, T27A and S38A/T27A mutants were also expressed as MBP-fusion proteins, and were used to test the specificity of the antibodies against the residues of interest. Several antibodies appeared specific for the phosphorylated AID, and further studies will test whether they recognize the endogenous phosphorylated protein. To further explore additional regulation of AID activity, recombinant MBP-AID proteins were used in assays designed to identify potential AID phosphatases expressed in B cells. These studies on AID post-translational modifications will have significant impact on our understanding of the regulation of this essential B cell diversification enzyme.

Does p21 affect signaling through the PDGF pathway?

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The p21 protein is a cdk inhibitor in the Cip/Kip family that acts downstream of of p53 to block the cell cycle and inhibit proliferation. However, there is no evidence in human cancers of p21 being a tumor suppressor. Remarkably, evidence suggests that p21 may be growth-promoting by either stabilizing cyclinD/cdk4 or preventing apoptosis. p21 can also bind to various signaling molecules. Previous experiments from our lab demonstrated that PDGF-stimulated oligodendroglioma (ODG) formation was reduced in mice in the absence of p21. This correlated with impaired proliferation due to a reduction in cyclin D/cdk4 complex activity.

To determine if p21 affects PDGF signaling, we are comparing phosphorylation of PDGF pathway proteins in wild-type murine glial cells containing constitutive autocrine PDGF signaling with those in p21-deficient cells. We are employing three complementary approaches to studying the effects of p21. p21 knockout cells enable us to look at the effect of chronic p21 ablation on PDGF signaling in ODG. We have also initiated siRNA experiments to study the effect of acute p21 downregulation on the PDGF pathway in ODG. Furthermore, wild-type and p21-deficient mouse fibroblasts transiently stimulated with PDGF are being compared.

Results indicate that PDGF pathway protein phosphorylation is different between wild-type and p21-deficient cells. However, these differences are dependent on the specific experimental conditions and cell type examined. Having established that p21 can affect signaling, we will determine whether p21 is binding to these proteins in vivo through co-immunoprecipitation analysis. We will also attempt to recapitulate these interactions in vitro His-tagged affinity chromatograpy with recombinant protein. Completion of these studies will give us a greater understanding of how p21 expression affects receptor tyrosine kinase signals and provide insight towards important clinical progress.

Trp53 Tumor Suppressor Gene Loss of Heterozygosity in a Mouse Model of Mutant EGFR-induced Lung Adenocarcinoma

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Somatic mutations in exons encoding the tyrosine kinase (TK) domain of the epidermal growth factor receptor (*EGFR*) gene are found in 10% of human lung adenocarcinomas. Nearly 90% of the *EGFR* mutations are in-frame deletions in exon 19 that eliminate an LREA motif or a T-to-G base substitution in exon 21 that substitutes arginine for leucine at position 858 (*L858R*). Eighty percent of patients with tumors bearing these mutations respond to treatment with the TK inhibitors gefitinib and erlotinib. We used a tetracycline-inducible system to generate mice that conditionally express mutant *EGFR* in the lung epithelium. In this system, mice that express the reverse tetracycline transactivator in type II pneumocytes were crossed to *EGFR* transgenic mice carrying either of the common mutations ($EGFR^{\Delta L747\text{-}S752}$ or $EGFR^{L858R}$) downstream of a tetracycline-responsive element. Bitransgenic mice rapidly developed lung cancer after doxycycline induction. Removal of doxycycline or treatment with erlotinib resulted in rapid tumor regression, demonstrating that expression of mutant *EGFR* is required for lung tumor maintenance.

Human EGFR mutant lung tumors rarely regress completely upon treatment with erlotinib, suggesting that other genetic events, such as loss of tumor suppressor genes like *TRP53* may contribute to the tumorigenic process. To study the role of *Trp53* in the initiation and progression of lung adenocarcinomas induced by mutant *EGFR*, we crossed bitransgenic *EGFR* mice to *Trp*53-deficient mice. To examine whether *Trp53* loss of heterozygosity occurs in lung tumor nodules on a *Trp53 +/-* background, we are performing Southern analyses. Moreover, using quantitative reverse transcriptase PCR, we have observed loss of *Trp53* expression in these tumor nodules. We conclude that loss of *Trp53* may contribute to mutant *EGFR*-induced lung adenocarcinoma progression and that therapeutic strategies to treat this class of lung cancers could include restoration of *TRP53*.

Regulation of P-TEFb by T-loop Phosphorylation

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The expression of many eukaryotic genes is regulated by a pause at the elongation stage of transcription. The protein kinase activity of the positive transcription elongation factor b (P-TEFb) is thought to be essential for release of RNA Polymerase II (Pol II) from this block. P-TEFb antagonizes negative elongation factors, enhances the processivity of the Pol II holoenzyme via the phosphorylation of the Pol II carboxy-terminal domain (CTD), and couples pre-mRNA synthesis with splicing and polyadenylation. P-TEFb is composed of the cyclin dependent kinase Cdk9 and cyclin T. However, only a portion of P-TEFb is found in the active dimeric form whereas the rest is in a catalytically inactive small nuclear ribonucleoprotein (snRNP) complex with 7SK RNA and the HEXIM1 protein. A dynamic equilibrium exists between the active and snRNP bound forms of P-TEFb. Inhibition of global transcription by treatment of cells with 5,6-di-chloro-1-b-D-ribofuranosyl-benzimidazole or UV light disrupts the 7SK/HEXIM1/P-TEFb snRNP and shifts the equilibrium towards the active form, whereas inhibition of cell growth drives P-TEFb into the snRNP complex. T-loop phosphorylation of Cdk9 at threonine 186 is reported to be essential for both the protein kinase activity of P-TEFb and the assembly of the 7SK/HEXIM1/P-TEFb snRNP. However, the kinase responsible for this phosphorylation has not yet been characterized. Cdk7, the catalytic subunit of a CDK-activating kinase (CAK) responsible for T-loop phosphorylation of Cdk1, -2, -4, -5, -6 and -11, is a likely candidate to phosphorylate Thr186 of Cdk9. Preliminary data have shown that selective inhibition of Cdk7 causes the dissociation of the 7SK/HEXIM1/P-TEFb complex in HCT116 cells, but whether this occurs by a direct or indirect mechanism is not known. To address this question, we have reconstituted the 7SK/HEXIM1/P-TEFb interaction *in vitro* and will test the requirement for Cdk7 activity in the formation of the complex. An inhibitory affect of Cdk7 on Cdk9 might serve to ensure the proper order of CTD phosphorylation events during the transcription cycle.

Role of Frontoamygdala White Matter Tracts in Emotional Reactivity

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Adolescence is a time of emotional reactivity and increased prevalence of affective disorder onset. Increases in emotional reactivity and poor regulation of these emotions may put adolescents at greater risk for theses disorders. Studying limbic brain circuitry implicated in emotional reactivity and regulation during this period could help us to determine changes in the brain organization that may put adolescence at higher risk for developing affective disorders. In the present study, we examined how white matter tracts, as measured by diffusion tensor imaging (DTI), between the orbitofrontal cortex (OFC) and amygdala relates to developmental and individual differences in emotion regulation. DTI data were collected from 39 subjects between the ages of 7 and 32 years who completed an emotion regulation task. The task involved either pressing or avoiding a negative emotional expression (fearful face). To measure white matter fibers in limbic brain circuitry, fractional anisotropy, an indicator of axonal thickness and myelination, was calculated. The results showed that fractional anisotropy in the uncinate fasciculus, a fiber tract that connects OFC and amygdala regions and implicated in emotion, was negatively correlated with reaction time to press to a fearful face. In other words, the less fractional anisotropy (less integrity of white matter tracts) in this region, the slower the response. These results suggest decreased connectivity between the OFC and amygdala may lead to difficulty in regulating behavior in emotional contexts and put adolescents at greater risk for developing affective disorders.

Isolation of DNA hypercleavage *top2* mutants in *Saccharomyces cerevisiae*

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Topoisomerase II changes DNA topology by introducing transient DNA double-stranded breaks (DSBs) with covalently attached Top2 at the ends. Top2 then passes the other double-stranded DNA through the break and reseals the break. Top2 that fails to reseal the broken ends generates a DSB whose ends are masked by Top2. These Top2-attached DSBs (Top2-DSBs) can compromise cell viability if left unrepaired. Although it has been shown that homologous recombination is involved in repairing Top2-DSBs, it remains unknown how Top2 is removed from DSB ends.

Our long-term goal is to identify the repair pathways for Top2-DSBs, especially identifying factors that remove Top2 from DSB ends. To achieve this goal, we will first isolate DNA hypercleavage *top2* mutants that generate Top2-DSBs at a high frequency. We will then express this *top2* mutant in candidate mutant strains and examine their capabilities to repair Top2-DSBs.

In my summer research project, I seek to isolate DNA hypercleavage *top2* mutants. Here we hypothesized that *top2* mutants should require the homologous recombination pathway for viability. I will isolate these *top2* mutants by identifying cells that are dependent on a homologous recombination gene, RAD52, by using colony color variabilities of yeast. A yeast *ade2* mutant forms red colonies while an *ade2 ade3* double mutant forms white colonies. I will use the *ade2 ade3 rad52*Δ strain containing a plasmid with *ADE3* and *RAD52*. I will mutagenize *TOP2* by performing a mutagenic PCR with *Taq* polymerase in the presence of $MnCl_2$. I will cotransform into yeast cells this PCR product and gapped plasmid that contains sufficient sequence homology with the PCR product at each end but lacks the intervening sequence. Due to the ability of yeast to efficiently undergo homologous recombination, cells repair this gapped plasmid to generate a plasmid containing a mutagenized *top2*. I will screen for *top2* mutants that form nonsectored red colonies due to their requirement of *RAD52* plasmid.

Characterization of a Novel PLZF Mutant with Suppressed Biological Activity

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The transcription repressor promyelocytic leukemia zinc finger (PLZF), a member of the BTB/POZ family of evolutionarily conserved proteins, is widely known for its role in the molecular pathogenesis of a rare retinoic acid (RA) resistant subset of acute promyelocytic leukemias (APLs) and has recently been implicated as an important regulator of the development and lineage commitment of invariant natural killer T (iNKT) cells. We describe here the cloning and characterization of a fortuitously identified PLZF mutant protein with an aberrant C-terminal 17aa domain that confers significantly diminished biological activity *in vivo*. To study the mechanism by which the 17aa aberrant domain interferes with PLZF activity, two other groups of PLZF mutants were also amplified and characterized: the first with randomized 17aa extensions at the C-terminus, and the second with C-terminal truncations. Electrophoretic mobility shift assays and luciferase reporters were used to probe the ability of mutants to bind DNA and repress luciferase transcription. While the prospect of using the 17aa aberrant peptide as a free inhibitor of PLZF function remains nebulous, the results reported here shed light on the functional significance of domains near C-terminal PLZF residues and thus suggest a new framework for designing PLZF-specific inhibitors to interact with those domains. Such inhibitors of PLZF repressor activity may have widespread biological and therapeutic applications, not only for PLZF-associated RA intractable APLs, but also for other pathologies modulated by iNKT cells, like asthma.

Characterization of Dendritic Cell Interaction with Melanoma

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The presence of tumor-reactive T lymphocytes in the blood, draining lymph nodes, and the tumor of patients with progressive tumors indicates that tumor cells are capable of eliciting an immune response which is ineffective at controlling tumor. One explanation for this observation is ineffective presentation of tumor antigens to T cells, sufficient for priming but not full activation of T cells. Dendritic cells (DCs), the most potent type of antigen-presenting cells, can prime both $CD4^+$ and $CD8^+$ T lymphocytes by presenting antigens as peptides bound to MHC molecules and providing them with costimulatory signals and pro-inflammatory cytokines/chemokines. Many studies have investigated the biologic response of DCs upon encounter with pathogens, such as viruses and bacteria. However, very little is known thus far about how these cells interact with tumors.

In order to better characterize the DC-tumor cross-talk both in vivo and in vitro, we are setting up flow cytometry and immunofluorescence-based assays using the melanoma cell line B16F10 (B16), expressing the yellow fluorescent protein (YFP). For in vitro kinetic studies of tumor uptake, we co-cultured myeloid (mDC) and plasmacytoid (pDC) DCs with irradiated B16-YFP cells. The detection by flow cytometry of events positive for both YFP and DC-specific markers suggests uptake of B16 cells or cell fragments by DCs. Our preliminary results indicate that a peak of tumor cell-uptake occurs around 7 hours after co-incubation for both pDCs and mDCs. Co-localization will be confirmed in intact cells by immunofluorescence. Taking advantage of the same flow cytometric assay, we are currently studying tumor cell upake by DCs in vivo in tumor and tumor-draining lymph nodes of mice inoculated with B16-YFP.

Current and upcoming studies include the analysis by flow cytometry of the expression of DC surface activation markers CD80, CD86, CD40, and MHC I.

Functional analysis of microRNA *miR-124* in *Drosophila* embryonic nervous system development

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microRNAs (miRNA) belong to a class of small, non-coding RNAs known to regulate gene expression through the RNA interference pathway. After being transcribed, primary miRNAs adopt a hairpin structure that is then cleaved in the nucleus and transported to the cytoplasm to associate with the RISC complex. Subsequently, miRNAs post-transcriptionally downregulate the expression of target genes by interactions with evolutionary conserved sequences within the 3' UTR site of mRNAs. These miRNA:mRNA interactions lead to steric inhibition of translation or mRNA degradation. In this study, we performed a functional analysis of *miR-124* in *Drosophila melanogaster*. We demonstrate that *miR-124* is expressed exclusively within the central nervous system of the developing embryo. Using computational target gene prediction programs, we have created sensors to detect targeting interactions between *miR-124* and selected neuronal genes. Significant downregulation was observed when *Drosophila* S2 cells were transfected with *miR-124* and luciferase assay vectors containing corresponding 3' UTR sequences. Furthermore, misexpression of *miR-124* in the embryonic nervous system causes lethality due to defects that are currently being characterized. Thus, we believe *miR-124* plays a critical role in fine-tuning the development of both vertebrate and invertebrate nervous systems.

Enabling In Vitro Protein Synthesis Technologies for Single-Molecule Research

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High-spatial and -time resolution single-molecule fluorescence resonance energy transfer (smFRET) measurements have been used to probe the structural and kinetic parameters of transfer RNA (tRNA) movements within the aminoacyl (A) and peptidyl (P) sites of the ribosome. Such motions are basic component of the mechanism of fidelity in translation.

Previous investigation of tRNA motions on the ribosome reveals a dynamic exchange between three metastable tRNA configurations, whose equilibrium is determined in part by the length of the nascent peptide chain.

Here we discuss the global aims of our research and technologies that will enable future smFRET experiments that explore the basic mechanism of fidelity in translation. These technologies include altering the peptide length and composition carried by tRNA on the ribosome and the introduction of one or more fluorescent dye molecules site-specifically into elongation factor-Tu (EF-Tu), the enzyme that catalyzes tRNA incorporation into the ribosome.

MMN Responses to Speech Sounds Throughout Development

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Children have a greater ability to acquire a second language than adults. This suggests that a loss of brain plasticity happens throughout development. Furthermore, it is believed that expertise with native language limits the acquisition of a second language since the brain learns to attune the perceptual system to the phonemic categories present in the first language. We approached this theory by looking at auditory brain responses to different speech sounds in native English speaking children with ages ranging from 6 to 14 years old, focusing on possible differences in auditory evoked responses and mismatch negativity over development. During the experiment, subjects watched a silent movie of their own choosing and were told not to pay attention to a stream of sounds played over a loudspeaker. Using electroencephalography (EEG) we recorded auditory evoked responses to standard (frequent) stimuli and deviant (infrequent) stimuli. We tested four contrasting pairs: /la/-/ra/, /ra/-/wa/, /la/-/wa/, and /da/-/ga/. In a particular session a syllable would be the standard stimulus, whereas in other session the same syllable would be the deviant stimulus. There were eight sessions in total for each subject. The mismatch negativity was then computed by subtracting the standard response from the deviant response and averages were computed for the different contrasting speech sound pairs. This data was later compared with previous data collected on adult native English speakers. Preliminary data showed a mismatch response that was later than that seen in adults and positive instead of negative. These results are consistent with previous literature.

Identification and characterization of KIF17 Binding Partners

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Epithelial polarization is important for organ development and function. It has been shown that loss of epithelial polarity is associated with the development of several human diseases. Many cellular events contribute to polarization, such as cell adhesion, cytoskeletal reorganization and polarized membrane trafficking. The main goal of the lab is to understand the role of microtubules and microtubule-mediated transport in polarization. Research in our lab focuses on studying the role of kinesin family members (KIF's) in cell polarization. These Kifs are MT-associated motor proteins responsible for the intracellular trafficking of membrane and cytoplasmic proteins. My project during the summer focuses on determining and characterizing the cargoes of KIF17, which was found to regulate MT stabilization and potentially, early events in polarization. However, the cargoes transported by this protein remain unknown. We performed a yeast two hybrid (Y2H) analysis using KIF17 tail as bait. Twenty unique proteins were pulled out of this screen. Based on the sequence and blast analysis we were able to determine the identity of all the positive clones for the screen. Of these, we selected five for further analysis: estrogen related receptor alpha (ESRRA), actinin alpha 1, Huntingtin-interacting protein 1-related protein (HIP1R), PERQ and Vimentin. To confirm the Y2H results we decided to use biochemical and functional assays to test the interaction of KIF17 with these proteins. We cloned each protein into a GFP tagged expression vector. We then co-expressed each of the potential cargoes in HEK293 cells with Kif17 tail domain. Co-immunoprecipitation assays are being carried out to test their interaction. Furthermore, microinjections of GFP-KIF17 tail on MDCK cells have been performed to test its effect on the organization and distribution of endogenous proteins. Our preliminary results confirmed an interaction between the KIF17 tail and the PERQ, suggesting that these proteins may work together in the cell.

Overexpression of Topoisomerase IV Inhibits Cell Division by Preventing FtsZ Localization in *Escherichia coli*

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In *Escherichia coli* (*E. coli*) , Topoisomerase IV (Topo IV) plays a vital role in maintaining genomic integrity throughout the cell cycle. Topo IV activity is temporally restricted to the latter stages of the cell cycle, in which it serves to unlink replicated chromosomes. Unpublished research has shown that when Topo IV is overexpressed, cells filament. In this case, cell division is inhibited, yet DNA replication and chromosome segregation proceed normally. Our goal is to determine the mechanism by which Topo IV overexpression inhibits cell division in *E. coli*.

In order to divide, *E. coli* cells possess approximately fifteen proteins that localize at the division septum. These proteins arrange into a ring to constrict the cell envelope. Moreover, the proteins localize mid-cell in a hierarchical fashion as follows: FtsZ, FtsA/ZipA, FtsK, FtsQ, FtsL/B, FtsW, FtsI, and FtsN. The localization of each upstream protein in the hierarchy is required to recruit all downstream proteins to the septum.

The order of septal-ring assembly was exploited to determine the specific point at which cell division is inhibited during Topo IV overexpression. In our study, Topo IV was overexpressed in wild type cells, and immunofluorescence was conducted with anti-FtsZ antibodies. Immunofluorescence revealed a significant reduction of FtsZ localization, or “Z-ring” formation, at the division septum in wild type cells. This finding is further supported by evidence that shows no localization of the downstream protein FtsI during Topo IV overexpression. We conclude that cell division inhibition during Topo IV overexpression is due to the delocalization of FtsZ, leading to the lack of downstream proteins and constriction at the septum. Further studies of systems that oversee Z-ring assembly in *E. coli* will help to understand the mechanism by which Topo IV overexpression delocalizes FtsZ and will shed light on bacterial cell division.

Mechanism of Sonic Hedgehog Palmitoylation by Mart-2

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Sonic hedgehog (Shh) is a secreted signaling ligand that performs essential functions in vertebrate development. Aberrant expression of Shh has also been implicated in the growth of a number of cancers. Shh is post-translationally modified in the ER and/or Golgi with the addition of palmitate, a 16-carbon saturated fatty acid that appears to be essential for normal Shh signaling. We are studying the mechanims of palmitoylation of Shh by its putative palmitoyl acyltransferase, Mart-2, a protein with homology to the family of membrane bound O-acyltransferases (MBOAT). HA-tagged truncation mutants were generated for Mart-2, with various deletions from both the N- and C-terminus that eliminated transmembrane domains. Expression of the constructs in cultured mammalian cells was evaluated using western blotting and fluorescence microscopy, and the ability of each construct to catalyze palmitoylation *in vitro* was assayed using ^{125}I-labelled palmitate. Preliminary results indicate that the mutants are all expressed at comparable levels to the wild type, but that they are enzymatically inactive. Immunofluorescence staining suggests that several of the mutants may be associated with different subcellular structures than the wild type. In particular, some of the Mart-2 mutants appear to be retained in the Endoplasmic Reticulum, suggesting that Golgi localization is required for active palmitoylation. We have recently generated GFP fusion constructs containing various regions of Shh in order to identify the elements within Shh that are recognized for palmitoylation. A thorough understanding of the basic biochemistry behind Shh palmitoylation may ultimately lead to new treatments for Shh-related tumors.

Efforts Toward the Total Synthesis of Dehydrostemofoline

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Premature birth due to an early onset of labor is the leading cause of death in newborn infants. The hormone oxytocin plays a major role in the initiation and continuation of labor, and dehydrostemofoline was proven to act as an oxytocin receptor antogonist. Dehydrostemofoline was first isolated from the extracts of a perennial insecticidal plant, *Stemona collinsae*. Early clinical data revealed potent activity against the neonate larvae of *Spodoptera littoralis*, and therefore as a renewable insectiside. In addition to its structural complexity, dehydrostemofoline has shown anti-abortifacient activity, thus our group is currently attempting its total synthesis.

We have discovered a method for constructing pyrrolidine systems by using an azomethine ylide [3+2] cycloaddition. The application of this method toward the total synthesis of dehydrostemofoline can lead to a key intermediate, which contains the tricyclic aza-tricyclo[5.3.0.0] core. The azomethine ylide can be generated from a vinylogous amide and should undergo a cycloaddition to give the fused ring system with an activated dipolarophile such as methyl acrylate. Functional group interconversions can afford the α-diazoketone, which can participate in an intramolecular Rh-carbenoid C-H insertion process to form the core.

The vinyligous amide was prepared from 2-pyrrolidinone. This lactam was converted to a protected silyloxy thiolactam in four steps. An Eschenmoser sulfide contraction with 1-bromo-2-butanone afforded the vinylogous amide. Reaction of the vinyologous amide with trifluoromethanesulfonic anhydride generates a transient iminium triflate. The addition of tetrabutylammonium difluorotriphenylsilicate to the iminium triflate removes the trimethylsilyl group to form the azomethine ylide, which serves as a 1,3-dipole for cycloaddition. Studies to optimize the key cycloaddition are currently underway. Successful implementation of this strategy should lead to the total synthesis of dehydrostemofoline.

Localization of the AT_1 receptor and NADPH-oxidase subunits in Catecholaminergic neurons in the Rostral Ventrolateral Medulla in Male vs. Female rats

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Angiotensin (Ang II) is a central part of the renin-angiotensin system (RAS) which can act through AT_1 Receptors to activate NADPH–oxidase. This results in the formation of super oxides, a type of reactive oxygen species (ROS). A key region for these actions is the Rostral Ventrolateral Medulla (RVLM), which is a brainstem region that contains catecholaminergic neurons which project to spinal neurons and are involved in cardiovascular regulation. The current study is to determine if these AT_1 receptors and NADPH-oxidase subunits are on the dendrites of these TH-positive neurons and to see if their density and distribution depends on sex and estrogen levels. Sections from male, proestrus (high estrogen) and diestrus (low estrogen) female rat brain through the RVLM were examined. Immunogold particles labeling AT_1 receptors within tyrosine hydroxylase (TH) labeled catecholaminergic dendrites will be quantified for each group in terms of total number of membrane and cytoplasmic gold particles and the density of the TH- positive dendrites containing both the membrane and cytoplasmic gold particles. Based on our preliminary data, we expect that AT_1 receptor will be localized to both the cytoplasmic and plasma membrane sites with the TH- labeled dendrites and the number of AT_1 receptors will increase in the absence of estrogen in the TH- positive neurons.

The Role of Amnionless in Middle Primitive Streak Assembly in Mammalian Gastrulation

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Amnionless (Amn) is a transmembrane protein expressed in the visceral endoderm (VE) of mouse embryos. Mutations in *Amn* lead to the absence of non-axial trunk mesoderm, a derivative of the primitive streak located in the embryo proper. Evidence of the localization of Amn to the kidneys and intestine, in addition to the VE, implicates Amn as a protein required for secretion and resorption. Various studies connect Amn to Cubilin (Cubn), a multi-ligand scavenger receptor that binds Vitamin B12-Intrinsic Factor. In humans, mutations in both *CUBN* and *AMN* cause hereditary megaloblastic anemia (MGA) resulting from intestinal malabsorption of Vitamin B12. Because Cubn is a membrane-associated receptor protein, lacking transmembrane and cytoplasmic domains, it is believed that Amn interacts with Cubn through its extracellular domain. Evidence that Amn is actually required for proper Cubn localization to the apical membrane in both kidney proximal tubule cells and the VE, supports this hypothesis. Although Cubn probably does not interact with the cytoplasmic tail (CT) of Amn, this domain is highly likely to be required for Cubn-related activities, such as the endocytosis and/or transcytosis of Cubn bound cargo. The fact that mutations in the AMN CT in humans generate the MGA phenotype makes this hypothesis more probable. The discovery of what proteins bind to the cytoplasmic tail of Amn could further expand what is known about the role of the protein in VE function during gastrulation. A mouse E7.0 cDNA library was screened for interaction with the Amn CT by Yeast 2-Hybrid analysis. Amn-interacting proteins will be analyzed by *in situ* hybridization to confirm expression in Amn positive tissues. Immunoprecipitation and Mass Spec analysis will reinforce the protein-protein interactions. Finally the role of Amn, as well as which domains are of importance, will further be eludicated through endocytosis-transcytosis assays of various AMN CT deletions in MDCK cells.

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