Fox Chase Cancer Center

Annual Progress Report: 2008 Formula Grant

Reporting Period

July 1, 2010 – June 30, 2011

Formula Grant Overview

The Fox Chase Cancer Center received $3,131,563 in formula funds for the grant award period January 1, 2009 through December 31, 2011. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

A Growth-Regulating Protein Tyrosine Phosphatase - We seek to understand how an enzyme called PTP1B is regulated and how it, in turn, regulates other proteins that control blood sugar, body weight, and, in women, the development of breast cancer. The knowledge we gain from these studies will help us better understand these processes and could also lead to the development of better drugs to treat diabetes, obesity, and breast cancer.

Duration of Project

1/1/2009 - 6/30/2011

Project Overview

The phosphorylation and dephosphorylation of proteins at tyrosine residues represents one of the most important means of regulating signal transduction in higher organisms. This process is governed by protein tyrosine kinases and protein tyrosine phosphatases. How the latter are regulated, and how they select their substrates, is not well understood. We have recently discovered that protein tyrosine phosphatase 1B (PTP-1B) - an enzyme that targets insulin, ErbB2, and leptin signaling pathways - is regulated by an unusual mechanism: enzymatic inhibition by sumoylation. This process occurs in an insulin-sensitive manner and is rapidly reversible, suggesting that sumoylation machinery is transiently engaged downstream of the insulin receptor to inhibit PTP-1B. We have also found that PTP1B regulates growth factor signals by at least two distinct mechanisms: PTP-1B targets receptor protein tyrosine kinases such as the insulin receptor by an N-terminal di-tyrosine binding motif; in contrast, it targets src-homology 3 containing substrates such as Src via a C-terminal proline-rich sequence. These putative targets for PTP-1B are key elements in growth factor-mediated signal transduction, and might account for PTP-1B's complex effects on cell proliferation and metabolism. Our overarching goals in this project are to identify the molecular mechanisms by which PTP-1B is regulated by growth factors, and to determine how this phosphatase, in turn, regulates signal transduction pathways relevant to cell metabolism and neoplastic transformation.

Specific Aim 1: Define the mechanism(s) by which SUMO (small ubiquitin-like modifier) modification regulates PTP1B activity.

Specific Aim 2: Determine the role(s) of PTP1B in ErbB2 signaling.

Principal Investigators

Jonathan Chernoff, MD, PhD
Senior Vice President and Chief Scientific Officer
Fox Chase Cancer Center
333 Cottman Avenue
Philadelphia, PA 19111-2497

Other Participating Researchers

Luis Arias-Romero, PhD, Jenny Yip, PhD, Olga Villamar, BS - employed by Fox Chase Cancer Center
Stevan Hubbard, PhD - employed by New York University

Expected Research Outcomes and Benefits

The enzyme PTP1B is a key regulator of the insulin receptor, the leptin receptor, and ErbB2, a protein that is frequently activated in human breast cancer. When the gene for PTP1B is knocked out in mice, these mice become resistant to diabetes, remain lean even when fed high-fat diets, and resist getting breast cancer in mouse models that are prone to this disease. For these reasons, there is intense interest in PTP1B as a therapeutic target in diabetes, obesity, and cancer. Our studies will help clarify how PTP1B regulates these processes, and, as such, could help guide the development of anti-PTP1B drugs. Such agents could impact a large number of people, as diabetes, obesity, and breast cancer are all important health problems in need of better treatments.

Summary of Research Completed

We completed the first specific aim, as detailed below:

Specific Aim 1: Define the mechanism(s) by which SUMO (small ubiquitin-like modifier) modification regulates PTP1B activity.

We had previously shown that PTP1B is sumoylated on at least two sites (K335 and K347) and that sumoylation inhibited PTP1B activity. We then used a variety of cell biology methods to determine the location of sumoylated PTP1B, which, in turn, has given clues as to its function.

As noted in our previous progress report, using both imaging and biochemical fractionation methods, we found that sumoylated PTP1B is found predominantly at an unexpected location, the inner nuclear membrane. This finding prompted us to examine if PTP1B interacts with phosphotyrosyl proteins in the inner nuclear membrane. We found that PTP1B interacts with the

inner nuclear membrane proteins emerin, a lamin-binding protein that is associated with the X-chromosome-linked Emery-Dreifuss muscular dystrophy (EDMD). Several tyrosine phosphorylation sites on emerin have been identified. One tyrosine residue, Y95, is of particular interest, as small deletions that include this tyrosine residue (Δ95-99) have been found in emerin in human EDMD. To ask if PTP1B interacts with emerin, PTP1B was immunoprecipitated from a HeLa cell lysate and probed with anti-emerin antibodies. Using MEFs, we found that emerin co-immunoprecipitates with PTP1B and with a known partner, lamin A/C, but not with control IgG (Fig. 1A). Interestingly, the same degree of binding was observed in pervanadate-treated cells and in cells transfected with catalytic inactive/ substrate trapping mutant of PTP1B (PTP1B-C215S mutant, data not shown). These results demonstrate that PTP1B and emerin interact and that this interaction requires neither phosphatase activity of PTP1B nor tyrosine phosphorylation of emerin.

To examine whether PTP1B regulates the tyrosine phosphorylation of emerin, we first asked if recombinant PTP1B could dephosphorylate emerin *in vitro*. We found that GFP-emerin, isolated from pervanadate treated cells, was readily dephosphorylated by recombinant PTP1B (data not shown). Next, we examined the levels of phosphotyrosyl emerin in cells expressing exogenous PTPT1B, PTP1B-CS or T7-SUMO-1. Expression of exogenous PTP1B reduced tyrosine phosphorylation of emerin, whereas expression of catalytic inactive mutant of PTP1B, PTP1B-CS, slightly elevated tyrosine phosphorylation of emerin (Fig. 1B). Interestingly, cells transfected with T7-SUMO-1 also showed increased emerin tyrosine phosphorylation. Given that sumoylation of PTP1B increases under such conditions, and that sumoylation inhibits PTP1B activity, these results suggest that PTP1B may dephosphorylate emerin at the INM in a sumoylation-regulated manner.

To further demonstrate the role of PTP1B in tyrosine dephosphorylation of emerin, HeLa cells were stably transfected with a scrambled shRNA or shRNAs designed specifically to knock down PTP1B, and measured the tyrosine phosphorylation level of emerin in these cells. Scrambled shRNA had no effect on PTP1B protein levels, while one out of the two PTP1B shRNAs significantly reduced the PTP1B expression (Fig. 1C). Lysates from the knockdown cells were subject to pTyr immunoprecipiatation and anti-emerin immunoblotting. We found that the tyrosine phosphorylation level of emerin was reciprocal to PTP1B expression level; reduced PTP1B correlated with increased PTyr-emerin (Fig. 1C).

Sumoylation of PTP1B is associated with elevated levels of phosphotyrosyl emerin.

Since sumoylation is known to inhibit PTP1B activity, we asked if sumoylation of PTP1B affects emerin tyrosine phosphorylation. HeLa cells were transfected with HA-PTP1B-WT or HA-PTP1B-K4R alone or in combination with T7-SUMO-1, followed by measurement of the relative tyrosine phosphorylation level of emerin in these cells. As expected, we found that PTP1B-WT, but not the PTP-K4R mutant, was sumoylated when the cells were co-transfected with T7-SUMO-1 **(**Fig. 2, top panel). To measure the tyrosine phosphorylation level of emerin, cell lysates were subjected to anti-pTyr (PY20) immunoprecipitation followed by anti-emerin immunoblot. Cells expressing PTP1B-WT plus T7-SUMO showed markedly increased tyrosine phosphorylation of emerin (Fig. 2, second panel, lane 4), whereas cells expressing a non-sumoylatable form of PTP1B (PTP1B-KR), showed much lower levels of emerin tyrosine

phosphorylation (Fig. 2, second panel, lane 5), suggesting that PTP1B regulates the tyrosine phosphorylation of emerin in a sumoylation-dependant manner.

*<u>Specific Aim 2</u>**:** Determine the role(s) of PTP1B in ErbB2 signaling.*

This aim was also completed, as detailed in prior progress reports.

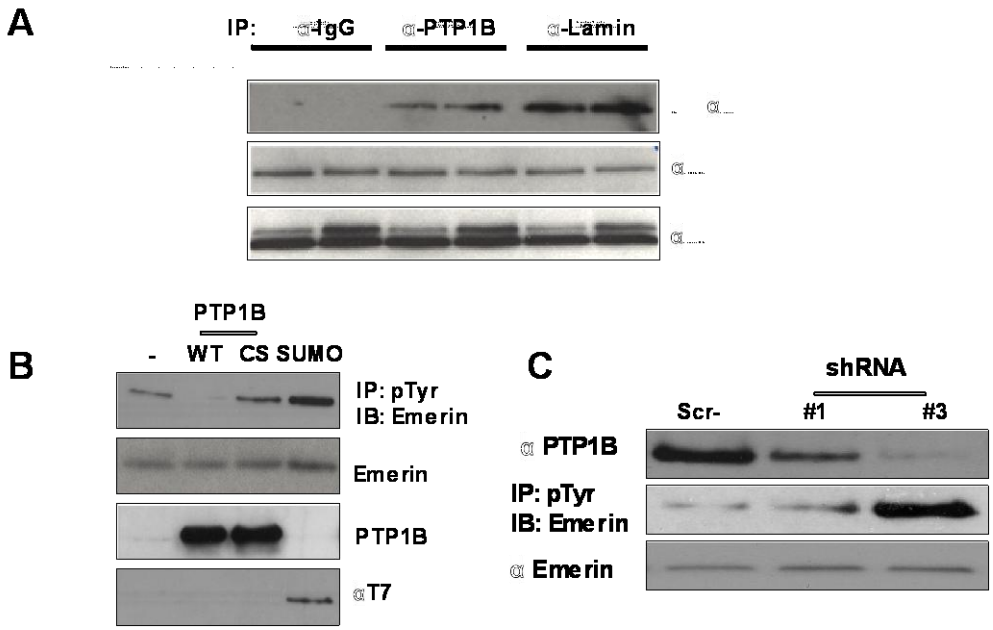

Fig. 1. PTP1B binds to emerin and regulates its tyrosine phosphorylation. (A) Endogenous lamin A/C and PTP1B were immunoprecipitated from HeLa cells and the immunoprecipitates were examined by immunoblot for the presence of emerin. To ensure equal loading, the bottom panels show the whole cell lysate protein expression of PTP1B and emerin from HeLa cells (bottom panels). **(B)** HeLa cells were transfected with WT or CS-PTP1B or T7-SUMO-1. The lysates were subjected to anti-emerin immunoprecipitation and tested for phosphotyrosine levels by immunoblot. **(C)** Emerin tyrosine phosphorylation was determined in cells stably transfected with either of two shRNAs against PTP1B: one that is effective (#3) and one that is ineffective (#1).

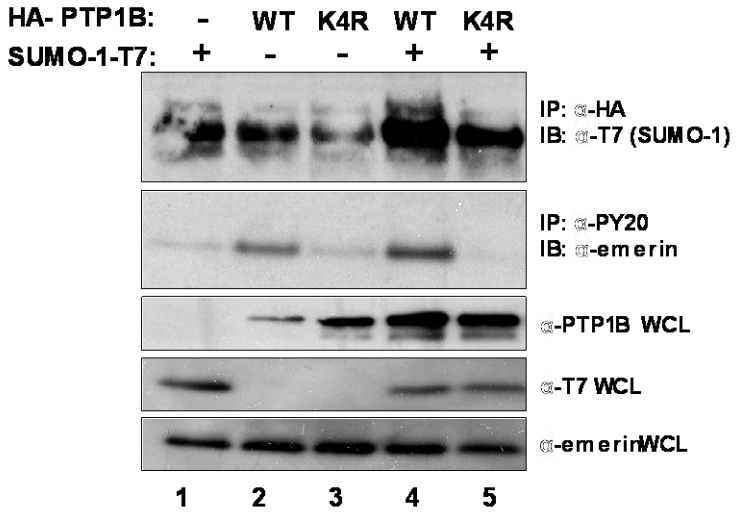

Fig. 2. Effects of PTP1B sumoylation on tyrosine phosphorylation of emerin. HeLa cells were transfected with HA-PTP1B-WT, or HA-PTP1B-K4R alone, or together with T7-SUMO-1. The cells were lysed and immunoprecipitated with anti-HA or anti-pTyr (PY20) antibodies to measure PTP1B sumoylation **(Top panel)** and tyrosine phosphorylation level of emerin**.** The whole cell lysates were probed with anti-PTP1B, anti-T7 to check the expression level of exogenous genes. Anti-emerin whole cell lysate immunoblot indicated equal loading of the immunoprecipitated samples of PTP1B and emerin from Hela cells. (B) *PTP1B*-WT or *PTP1B*–null MEFs were transfected with GFP-emerin together with catalytic inactive mutants of PTP1B (PTP1B-C215S or PTP1B-D181A). or GFP-Emerin was transfected together with siRNA against PTP1B in *PTP1B*-WT MEFs. Tyrosine phosphorylation status of Emerin was examined.

Research Project 2: Project Title and Purpose

Characterization of the Role of MTAP Gene in Tumorigenesis - Methylthioadenosine phosphorylase is a highly evolutionarily conserved metabolic enzyme involved in methionine and adenine metabolism that is expressed in all tissues throughout the body. Deletion of the gene for methylthioadenosine phosphorylase (*MTAP*) is one of the most frequent genetic changes observed in many different types of human tumors. Because *MTAP* is located near the *CDKN2A/ARF* tumor suppressor gene, it is unclear if loss of *MTAP* plays a primary role in tumorigenesis or if it is lost simply due to proximity to *CDKN2A/ARF*. The purpose of this project is to confirm that loss of *MTAP* directly affects tumorigenesis and to identify potential oncogenic mechanisms affected by loss of *MTAP* expression.

Duration of Project

1/1/2009 - 12/31/2010

Project Overview

Methylthioadenosine phosphorylase (MTAP) is a key enzyme in the methionine salvage pathway whose function is to convert 5′-deoxy-5′-methylthioadenosine (MTA) into methionine. Inactivation of *MTAP*, generally by homozygous deletion, is found in both solid and hematologic malignancies at a high frequency. Because *MTAP* is located near the *CDKN2A/ARF* tumor suppressor gene, it is unclear if loss of *MTAP* plays a primary role in tumorigenesis or if it is lost simply due to proximity to *CDKN2A/ARF*. In published work and our preliminary data, we have shown that reintroduction of *MTAP* into *MTAP*-deleted tumor cell lines significantly suppresses tumorigenicity and affects processes involved in cell migration and invasion *in vitro*. We also found that mice heterozygous for an *MTAP* null allele ($Mtap^{lacZ}$) die prematurely of a lymphoproliferative disease resembling T-cell lymphoma. Taken together, these data suggest that *MTAP* loss plays a functional role in the development of cancer. The overall goal of this project is to determine how MTAP loss contributes directly to the development of cancer. There are three specific aims. In the first aim we will characterize MTAP-deficient tumor cells from two different systems, mouse lymphoma cells and human fibrosarcoma cells. In the second aim we will create a Tet-inducible MTAP expressing cell line to facilitate the study of how MTAP expression alters gene regulation. In the third aim we will use the Tet-inducible system created in Aim 2 to perform kinetic analysis of MTAP regulated transcripts in response to MTAP expression. These studies are significant because an understanding of the role that *MTAP* deletion plays in tumorigenesis may lead to novel therapeutic strategies for *MTAP*-deleted tumors. In addition, these studies will establish how a "housekeeping" metabolic enzyme can have a novel role as a tumor suppressor gene.

Principal Investigator

Warren D. Kruger, PhD
Professor
Fox Chase Cancer Center
333 Cottman Ave
Philadelphia, PA 19111-2497

Other Participating Researchers

Baiqing Tang, PhD, Yuwaraj Kadariya, MD, PhD, Yibai Chen, PhD, Harvey Hensley, PhD - employed by Fox Chase Cancer Center

Expected Research Outcomes and Benefits

Deletion of the gene for methylthioadenosine phosphorylase (*MTAP*) is one of the most frequent genetic changes observed in many different types of human tumors. This project is relevant to human health because the identification of *MTAP* as a tumor suppressor gene could identify individuals at increased risk of cancer. In addition, understanding of the mechanism by which *MTAP* deletion contributes to tumorigenesis may lead to novel strategies that can selectively target *MTAP*-deleted tumor cells for destruction. This would potentially have relevance to many different kinds of human cancer.

Summary of Research Completed

The summary of research below includes progress in the final six months of this project from 7/1/2010 – 12/31/2010.

Microarray studies of a Tet-inducible MTAP expressing cell line.

We performed global mRNA expression studies on the Tet-inducible MTAP expressing MCF-7 cell line which we described in our last report. A total of four plates were grown, two with doxycycline and two without. RNA was then isolated, labeled and hybridized to Agilent G4112A chips according to manufacturer's instructions. Data for each array was captured using an Agilent G2565 scanner. Data for each array was normalized using Agilent Feature Extraction software.

We identified over 283 genes whose expression levels changed at least 50% with a p-value <0.05. We then performed pathway analysis on these changed genes to identify pathways that were enriched in this subset of genes using the Gene Set Analysis Toolkit V 2.0. We observed significant enrichments for genes with functions in the extracellular matrix, genes involved in bone development, and genes encoding for oxidoreductases.

We next confirmed the microarray analysis on a series of regulated genes using quantitative reverse-transcription PCR (qRT-PCR). A total of ten genes were examined; five that were up-regulated by MTAP expression and five that were down regulated (Figure 1). For each of these genes we obtained probes from Applied Biosystems and measured mRNA levels by Taqman assay. Using this approach, we were able to confirm differential regulation in eight of the ten genes assessed.

In the microarray analysis we observed that the gene with the greatest level of induction was SEC16B, with a 61-fold induction after a 48 hour exposure to doxycycline. However, we were unable to confirm this induction by qPCR. To understand these findings, we carefully examined the structure of the SEC16B gene and found that the Taqman assay used for RT-qPCR was directed at sequences in exon boundary 12 and 13, while the probe used to detect SEC16B on the microarray was directed at the 3'untranslated exon (exon 26). Examination of the UCSC genome database indicated that there was an alternative transcript which initiated downstream of exon 12 and thus lacked the Taqman probe region. To determine if this transcript was responsible for the differential regulation we obtained a new Taqman probe designed to detect the exon 25 and 26 boundary region. Using this probe, we confirmed that SEC16B was upregulated at least 50-fold within four hours of MTAP induction by doxycycline (Figure 2). These findings suggest that MTAP expression results in the production of an alternate form of the SEC16B message.

Gene	Array M+/M-	qPCR M+/M-
AGPAT4	6.21	13.84
COL8A1	5.10	nd
FGF18	0.51	0.46
GALNTL1	2.61	3.26
IGFBP5	0.45	0.38
MGP	0.55	0.61
MUC20	1.55	1.69
SEC16B	61.17	nd
TIMP3	0.49	0.92
TFF1	0.64	0.43

Figure 1. MTAP regulated Genes. Number indicates fold-increase in mRNA in doxycycline treated (M+) versus non-treated (M-) cells. Column labeled Array is data form microarray while column labeled qPCR was assessed using Taqman RT-PCR.

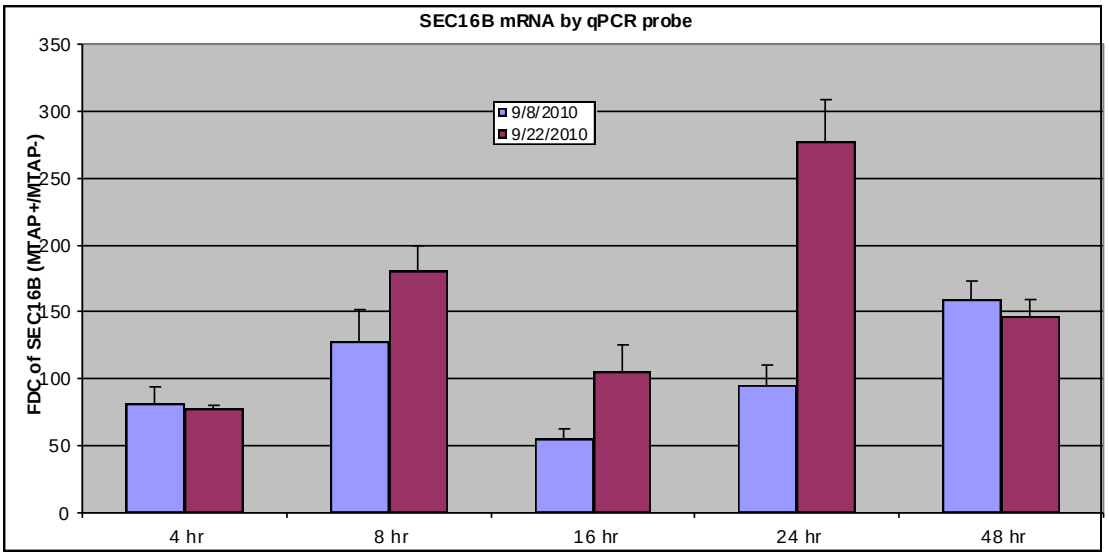

Figure 2. Induction of SEC16B mRNA assessed by Taqman. MCF-7$^{MTAP:Teton}$ cells were treated with doxycycline for the specified period of time and assessed for SEC16B message using a probe specific to the exon 25-26 junction. The experiment was performed on biological replicates grown on two separate occasions. Fold-increase was determined by comparing to mRNA levels present in non-doxycycline treated cells.

Research Project 3: Project Title and Purpose

The ARF Tumor Suppressor and Autophagy - We have recently discovered that the ARF tumor suppressor (ARF) plays an integral role in a key survival pathway utilized by tumor cells called "autophagy". Autophagy is a self-catabolic process wherein cells exposed to metabolic or hypoxic stress use the lysosome to digest long-lived proteins and organelles in order to release free amino acids and thus survive. The importance of this pathway for cancer cell survival is epitomized by the fact that chloroquine, an autophagy inhibitor, can potently suppress the development of certain tumors. We propose to elucidate the role of ARF and an interacting protein, Hsp70, in autophagy. We will test the hypothesis that a novel autophagy inhibitor discovered in the laboratory of our collaborator, and found by our group to specifically target ARF-dependent autophagy, is an effective new chemotherapeutic agent.

Anticipated Duration of Project

1/1/2009 - 12/31/2011

Project Overview

The objective of this research is to improve our understanding of the role of the ARF tumor suppressor (ARF) in the pro-survival pathway of autophagy. We have recently found that ARF induces autophagy, and that it must traffic to mitochondria in order to do so. Further, we have found that ARF must interact with the cytosolic chaperone protein Hsp70 in order to traffic to mitochondria. Hsp70 is over-expressed in the majority of human tumors, and over-expression of this protein universally correlates with poor prognosis. A long-time collaborator recently identified a novel chemical inhibitor of Hsp70, which we term PAS. We have found that PAS selectively inhibits the ability of ARF to induce autophagy. Moreover, we find that PAS selectively kills cells that over-express ARF. ARF is not expressed in normal, non-transformed cells, and is transcriptionally repressed by the p53 tumor suppressor; therefore, tumors with mutant p53 tend to express high levels of ARF. We predict that PAS will be selectively toxic to tumor cell lines with mutant p53. We propose to test this hypothesis.

Aim 1 To define the domains of ARF necessary for mitochondrial localization, autophagy induction, and interaction with Hsp70
Aim 2 To evaluate the efficacy of a novel Hsp70 inhibitor (PAS) on human tumor cell lines
Aim 3 To test the hypothesis that ARF and Hsp70 silencing are inhibitory to human tumor development in a xenograft model

Principal Investigator

Maureen E. Murphy, PhD
Associate Professor
Fox Chase Cancer Center
333 Cottman Avenue
Philadelphia PA 19111-2434

Other Participating Researchers

Julia Pimkina, MD, PhD, Anna Budina, MD, Samuel Litwin, PhD - employed by Fox Chase Cancer Center

Expected Research Outcomes and Benefits

The overwhelming majority of chemotherapeutic drugs used in the clinic today preferentially kill tumor cells that contain wild type p53 gene. Sorely lacking in the clinic are drugs that target novel pathways (such as autophagy) and that preferentially target tumors with mutant p53 genes. The goal of this research is to use ARF and autophagy as biomarkers of efficacy of a novel compound, PAS.

The ARF tumor suppressor is not detectably expressed in normal, non-transformed cells. It is however highly expressed in tumors with mutant or inactive p53. We have recently found that tumor cells with mutant p53 and high levels of ARF have a survival advantage over tumor cells with no ARF expression. This is due to the integral role of ARF in the survival pathway known as autophagy. We have recently published that tumors with mutant p53 and high levels of ARF demonstrate increased survival in response to metabolic stress.

Additionally, we have found that silencing ARF in tumor cells with mutant p53 inhibits autophagy and impedes the progression of these tumors *in vivo*. We have found that a small molecule inhibitor of the chaperone protein Hsp70, termed PAS, inhibits the ability of ARF to traffic to mitochondria, and to induce autophagy. We have shown that this compound is toxic to tumor cells but not normal cells and that it is preferentially toxic to tumor cells with high levels of ARF (that is, those with mutant p53). We expect to learn from this research the mechanism of action and potential anti-cancer function of this unique small molecule. The identification and characterization of a novel small molecule (PAS) with a new mechanism of action (autophagy) are the two most important research benefits of this research.

Summary of Research Completed

In the past year three manuscripts were published that are relevant to the Aims listed above:

Leu JI, Pimkina J, Pandey P, Murphy ME, George DL. The Heat Shock Protein-70 (HSP70) Inhibitor 2-phenylethynesulfonamide (PES) Disrupts Protein Degradation Pathways and the HSP70/HSP90 Chaperone System in Tumor Cells. *Mol Cancer Res*, 9:936-47, 2011

Pimkina JS and Murphy ME. Interaction of the ARF tumor suppressor with cytosolic HSP70 contributes to its autophagy function. *Cancer Biol Ther*, in press.

Kung CP, Budina A, Balaburski G, Bergenstock M and Murphy ME. Autophagy in tumor suppression and cancer therapy. *Crit Rev Euk Gene Exp* 21: 71-100, 2011.

Our work is focused on autophagy, a critical cell survival program that is exploited by tumor cells. We believe that the need for autophagy is an Achilles heel for tumor cells. We have found that a novel HSP70 inhibitor (called phenylacetylsulfonamide, PAS; also called

phenylethynesulfonamide, or PES) kills tumor cells by virtue of its ability to inhibit autophagy, and targets tumors irrelevant of p53 or Bcl2 status.

PAS inhibits autophagy as well as proteasome function in cancer cells

The HSP70 chaperone is highly evolutionarily conserved, and plays a critical role in maintaining protein quality control. This protein is expressed at high levels in the majority of human tumor types, but is expressed at exceedingly low levels in normal, non-transformed cells. Moreover, in tumors that overexpress HSP70, this protein is a marker for poor prognosis. As such, there is considerable interest in HSP70 as an emerging target for cancer therapy.

We recently identified the molecule PAS as a specific HSP70 inhibitor, with preferential toxicity to tumor cells relative to normal, non-transformed cells. Specifically, we have found using MTT assays that the IC_{50} for PAS is 0.5 – 5 uM in all of the human tumor cell lines we have tested (>20 lines, of diverse histological type). In contrast, the IC_{50} in non-transformed human cells is >80 uM. Notably, the ability of PAS to inhibit autophagy is substantially increased in tumor cell lines. For example, we evaluated the ability of PAS to inhibit autophagy via western analysis for LC3 I and p62SQSTM1; these markers accumulate when autophagy is inhibited. We find that LC3 I and p62SQSTM1 accumulate substantially in the ovarian cancer line SKBr3 and the breast cancer line MCF7 upon treatment with PAS; in contrast, little accumulation of these autophagy markers is evident in immortalized, non-transformed human ovarian surface epithelial cells (HOI118) or in immortalized breast epithelial cells (MCF-10A) (Figure 1). These data suggest that transformation of cells renders them significantly more sensitive to PAS.

HSP70 is a chaperone that is required for the proper function of the chaperone HSP90; there are several HSP90 inhibitors used in the clinic for cancer therapy. Because of the requirement of HSP90 for HSP70 function, we next tested whether PAS also inhibits HSP90 function. Toward this end, we used immunoprecipitation followed by western blotting to assess the interaction of HSP90 with several of its key client proteins, including EGFR, HER2/Neu and AKT. We found that 24-hour treatment with 20 uM PAS was able to block the ability of HSP90 to interact with the client proteins EGFR, HER2/Neu, and AKT, as assessed by IP-western (Figure 2). These data support the premise that by inhibiting HSP70 function, PAS also renders compromised the ability of HSP90 to interact with client proteins.

Inhibition of HSP90 causes an accumulation of its client proteins into insoluble aggregates within the cell. In order to determine whether PAS causes HSP90 client proteins to accumulate in insoluble compartments within the cell, we treated the ovarian carcinoma cell line SKOV3 for 24 hours with 10 um or 20 uM PAS, and assessed the level of EGFR, HER2/Neu and AKT in detergent-soluble (1% NP-40) and detergent-insoluble fractions. These analyses revealed that treatment with PAS caused all three of these HSP90 client proteins to accumulate in the insoluble fraction of the cell (data not shown), consistent with the premise that PAS inhibits their function. The above data indicate that PAS can inhibit tumor cell growth by virtue of its ability to inhibit both HSP70, and HSP90, function. The above data were recently published in *Molecular Cancer Research*.

Molecular Modeling of the PAS-HSP70 interaction

In order to better understand the cytotoxic functions of PAS, we set out to map the HSP70-PAS interaction domain. Toward this goal we created several deletion mutants of HSP70, in an HA-tagged version, and transfected these into cells. These cells were then incubated with biotinylated PAS, and lysates were immunoprecipitated with streptavidin. In this way we were able to map the binding domain of PAS to the C-terminal region of HSP70, to amino acids 386-641 (Figure 3). More recently we were able to narrow this down to amino acids 543-641 (data not shown).

The human HSP70 C terminus (amino acids 543-641) has recently been crystallized. In collaboration with Roland Dunbrack (Fox Chase Cancer Center), we used the available crystal structure of human HSP70 (543-641) along with the *in silico* docking program AutoDock in order to identify energetically favorable binding sites for PAS on HSP70. This procedure revealed three highly energetically favorable binding sites. Dr. Dunbrack has predicted a series of twelve point mutations in HSP70, predicted to allow this protein to retain its structure but to potentially interrupt binding with PAS. We have recently created these twelve mutant versions of HSP70 protein, and are not testing them for the ability to bind to biotinylated PAS.

A novel derivative of PAS shows enhanced cytotoxicity and preclinical efficacy

The National Cancer Institute of the National Institutes of Health possesses an open chemical depository site called the "NCI/DTP Open Chemical Repository". We searched this site for analogies to PES, and identified five analogues of this compound; we ordered these from the NCI (these were provided by the NCI free-of-charge, plus the price of shipping). Using MTT assays for cell viability, we found that two of these analogues had comparable toxicity to PAS, but three were not cytotoxic; notably, in all cases the ability to inhibit autophagy tracked with cytotoxicity.

Based upon the chemical structure and cytotoxicity of these analogues, we were able to predict that substitution of a chloride residue for a hydrogen on the hydrocarbon ring of PAS might increase the cytotoxicity of this compound. This derivative of PAS, PAS-Cl, was synthesized in the Organic Synthesis Facility at Fox Chase Cancer Center. Using MTT and also apoptosis assays, we were able to show that PAS-Cl possesses an IC_{50} of 0.2 uM in all tumor cell lines tested, but like the parent compound PAS has limited toxicity to non-transformed cells. Based upon these data, we initiated pre-clinical trials of PAS-Cl, using the Eμ-myc mouse, which develops B cell lymphoma by six months of age due to transgenic expression of the myc gene, controlled by the immunoglobulin heavy chain enhancer.

Cohorts of 20 mice of six weeks of age were treated with dilution vehicle, PAS, or PAS-Cl (the latter two at 20 mg/kg) every five days for five weeks. Kaplan Meier survival analysis indicates that PAS-Cl show significant ability to inhibit lymphoma development and prolong the lifespan of Eμ-myc mice (Figure 4). The above research is in preparation for publication submission; it is also in preparation for submission as preliminary data in an R01 application to be submitted in October to the NCI.

PAS inhibits ARF-mediated autophagy

Using two-dimensional gels of purified mitochondria, we were able to identify HSP70 as a protein that interacts with ARF at the mitochondria. Using the technique of immunoprecipitation-western, we are able to detect HSP70-ARF complexes in all cell lines tested, using multiple antibodies, and in both directions (that is, IP ARF, western HSP70, or vice versa). Notably, we were also able to show that PAS inhibits the ability of ARF to localize to the mitochondria, and likewise inhibits ARF-mediated autophagy. Consistent with these data, we find that two different cell lines that express high levels of ARF are more sensitive than matched cell lines with ARF silenced. Notably, several groups now report that high ARF levels are characteristic of the most aggressive tumors (for example, Feldser et al, Nature 468:572-5, 2010). These data, combined with our findings that PAS is cytotoxic to tumor cells regardless of p53 or BCL2 status, argues that this compound shows great promise as a novel and highly effective avenue of cancer therapy. The above data were recently published in *Cancer Biology and Therapy*.

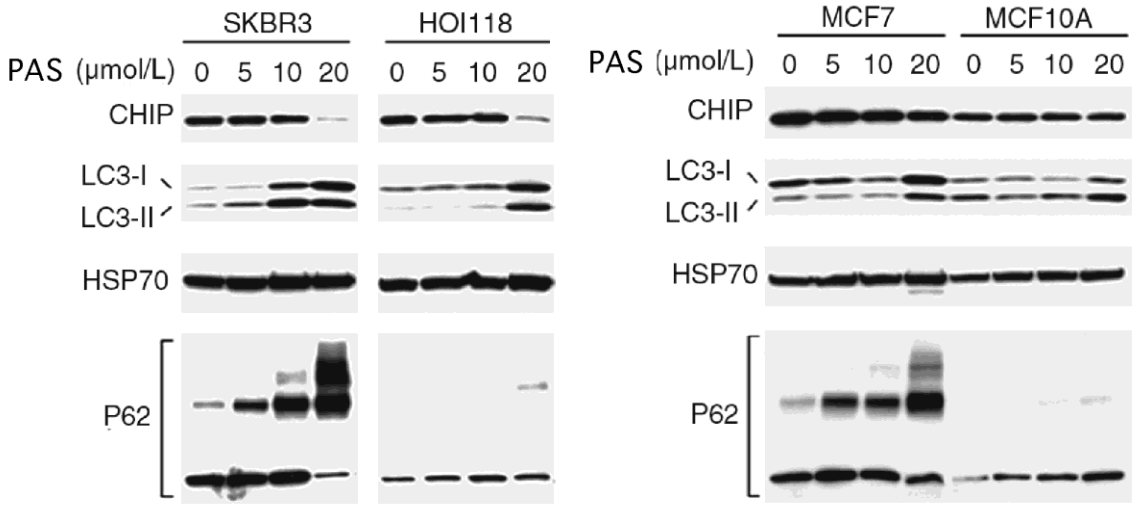

Figure 1. PAS inhibits autophagy to a greater extent in tumor cell lines, compared to non-transformed cells. The indicated cell lines (SKBR3, HOI118, MCF7, and MCF10A) were treated with the specified amount of PAS for 24 hours and examined for the proteins indicated by western blot. Accumulation of LC3-I, and oligomerization of p62, are indicators of inhibited autophagy.

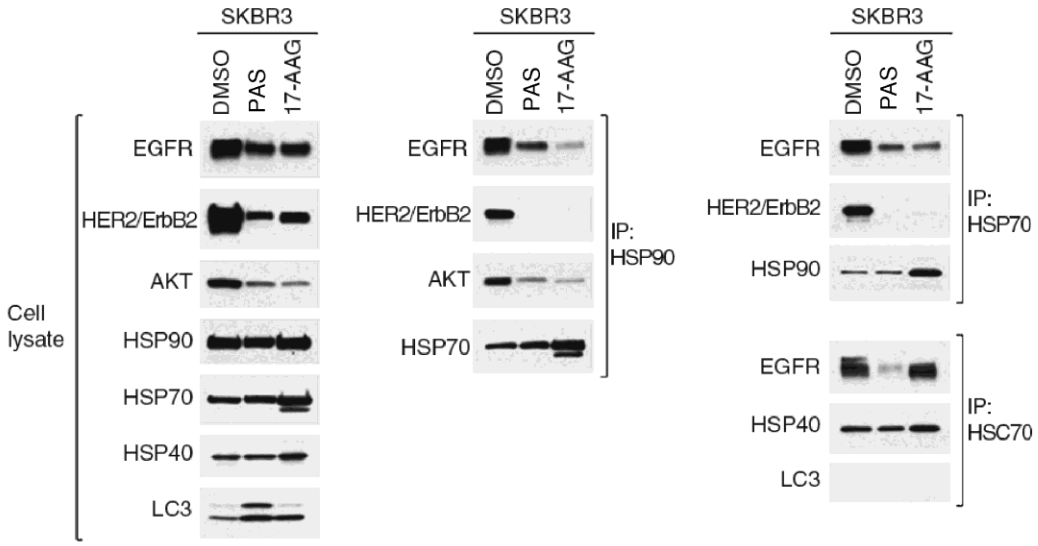

Figure 2. PAS attenuates HSP90 interaction with client proteins. SKBR3 breast carcinoma cells were treated with DMSO, 20 uM PES, or 1 uM 17-AAG (HSP90 inhibitor) for 24 hours. Western blot analyses assessed the relative protein abundance. Coimmunoprecipitation (IP) and Western blotting revealed a reduced degree of interaction between HSP90, HSC70, or HSP70 and the client proteins shown.

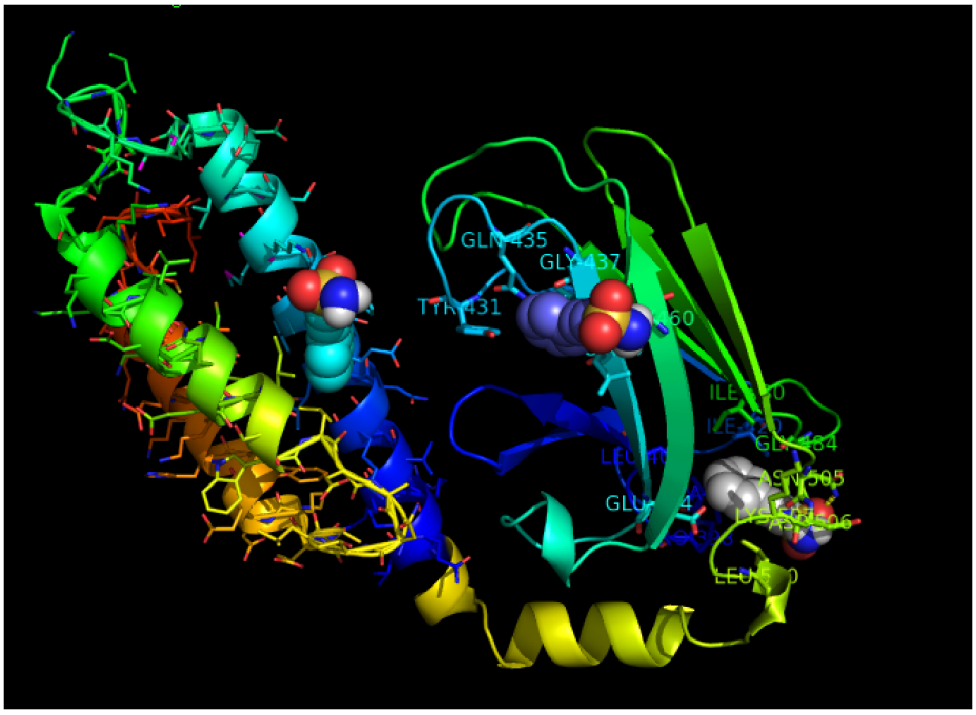

Figure 3. In silico docking of PAS, using the program AutoDock, reveals three energetically favorable binding pockets for PAS in the C-terminus of HSP70. PAS is shown as the red-white-blue-yellow compound. Amino acids that have been selected for mutation are depicted.

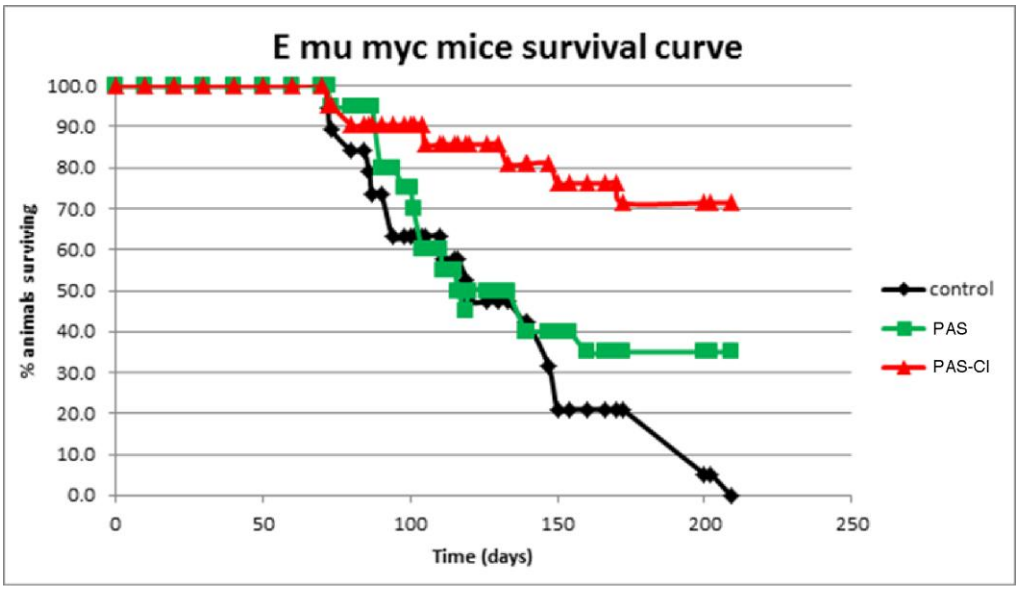

Figure 4. PAS-Cl shows significant efficacy in the treatment of B-cell lymphoma. Kaplan-Meier analysis of data; difference between PAS-Cl and control, $p<0.000005$ (Mantel-Cox test).

Research Project 4: Project Title and Purpose

Anti-Glucose Transporter-1 Antibodies as a Novel Treatment against Human Cancers - Glucose fuels the rapid growth and metabolism of tumors. Glucose uptake by cells is facilitated by a family of transporter proteins called "Gluts". Tumors frequently over-express the high affinity glucose transporter protein, called Glut-1. Normal cells, on the other hand, require a slow and steady supply of glucose and therefore use lower affinity glucose transporters, Glut-4 or Glut-12. In the studies outlined here we will use antibodies against Glut-1 to decrease the uptake of glucose by the tumor, while sparing glucose uptake by normal cells. This novel and new therapeutic strategy could be applicable to a wide variety of human cancers, including lung cancer, breast cancer, and essentially any cancer that shows increased glucose uptake on a PET scan.

Duration of Project

1/1/2009 - 1/31/2010

Summary of Research Completed

This project ended during a prior state fiscal year. For additional information, please refer to the Commonwealth Universal Research Enhancement C.U.R.E. Annual Reports on the Department's Tobacco Settlement/Act 77 web page at http://www.health.state.pa.us/cure.

Research Project 5: Project Title and Purpose

Regulation of Human Somatic Wee1 by Cyclin A/Cdk2 Complexes - Mitosis is the process by which a cell divides into two daughter cells. This step is pivotal in the life of the cell and, potentially, the organism as a whole. If mitosis is performed before cellular DNA is completely duplicated or mutations are repaired, the progeny cells may die or sustain cancer-causing changes. Furthermore, uncontrolled cell division is a hallmark of cancer. Drugs that can preferentially block cell division in cancer cells or can drive their division in the face of lethal amounts of unreplicated or severely damaged DNA may provide effective therapy. Wee1 is a protein that inhibits entry into mitosis in human cells. We have recently identified a specific site through which an enzyme complex called cyclin A/Cdk2 binds Wee1 and inactivates it. This regulation involves Wee1 phosphorylation and export from the cell nucleus. This project will study this regulation. The long-term goal is cancer drug development.

Anticipated Duration of Project

7/1/2010 - 12/31/2011

Project Overview

Wee1 inhibits entry into mitosis in all eukaryotes. Wee1 phosphorylates the ATP binding site in Cyclin dependent kinase 1(Cdk1), thereby inactivating Cdk1. Despite this key role, the regulation of Wee1 remains poorly understood. Wee1 harbors a well-conserved carboxy-terminal kinase domain and an equally large but less conserved amino terminal regulatory domain (NRD). Cyclin B/Cdk1 complexes, the major mitotic Cdks, have been thought to exert reciprocal regulation of Wee1 by phosphorylating the NRD and inactivating Wee1 kinase activity in a positive feedback loop. In the past 5 years, the first functional elements have been defined in the NRD. A short peptide called the 'Wee box' augments Wee1 kinase activity. Evidence from studies in Xenopus identified a Cdk1 phosphorylation site on T239 in the Wee box that inhibits Wee1 activity. We have recently uncovered a site at 180-2 that binds cyclin A/Cdk2 complexes, the major S and G2 phase Cdks, and directs T239 phosphorylation. These findings have delineated a specific molecular pathway through which cyclin A/Cdk2 complexes inhibit Wee1, implicating these Cdk complexes in licensing mitotic entry. We also found that residues 175-182 comprise a conserved binding site for Crm1 that mediates Wee1 export from the nucleus (nuclear export signal (NES)). Inhibition of cyclin A/Cdk2 activity by different means inhibited export, implicating these Cdks in the process. Surprisingly, a Wee1 NES mutant was not a more potent inhibitor of mitotic entry under the conditions tested. Therefore, the role of Wee1 export remains unclear.

We propose two Specific Aims to study the regulation and role of Wee1 export in greater depth: 1) We will test the hypothesis that phosphorylation by cyclin A/Cdk2 complexes fosters Crm1 binding and Wee1 export; and 2) We will elucidate the functional role of nuclear export of Wee1. These studies will substantially advance our understanding of the regulation of Wee1 and mitotic entry by Cdk complexes. In the long term, the results will inform efforts to improve cancer chemotherapy.

Principal Investigator

Greg H. Enders, MD, PhD
Associate Professor of Medicine
The Fox Chase Cancer Center
333 Cottman Ave, W225
Philadelphia, PA 19111

Other Participating Researchers

None

Expected Research Outcomes and Benefits

This is a basic science project with potential for translation to clinical care. The area of study is of crucial importance for understanding the regulation of cell proliferation. Drug inhibitors of the two types of enzyme being studied, Wee1 and Cyclin dependent kinases (Cdks), have been developed by the pharmaceutical industry, at great effort and expense. Some of these drugs are currently in clinical trials as cancer therapies. Some are showing promise. Others have been disappointing. The studies planned here will shed light on the fundamental roles of these enzymes in regulating cell proliferation and contexts in which their roles are substantially altered. Our studies are needed to understand the effects of inhibiting these enzymes and clarify which contexts may be most promising for use of the relevant drugs. In particular, we expect to demonstrate a specific site at which cyclin A/Cdk2 proteins modify (add a phosphate to) Wee1 and direct Wee1 out of the nucleus, preventing Wee1 from restraining cell division. We will also determine under what conditions this shift in Wee1 location within the cell is critical. We hypothesize that one such condition is the presence of DNA damage. Such a condition is routinely generated during cancer therapy. Thus, inactivation of Wee1 by cyclin A/Cdk2 complexes may be essential for cancer cells to continue to grow during conditions of standard cancer therapy.

Summary of Research Completed

<u>Aim 1) Test the hypothesis that cyclin A/Cdk2 complexes direct Wee1 nuclear export via phosphorylation of specific residues.</u>

Studies on Aim 1 focused initially on generating and validating an antibody (Ab) specific to a specific phosphorylated Wee1 which we will term here 'SITE1'. We have generated a specific Ab directed against P-SITE1 in hWee1. Given the sequence identity of SITE1/NES and most surrounding sequence in hWee1 and mWee1, we expected the P-SITE1 Ab to cross-react with mWee1 (see below). A peptide encompassing P-SITE A was used as immunogen. The Ab was purified on beads linked to the P-SITE1 peptide and counter-selected against beads linked to the unphosphorylated peptide. This Ab reacts with endogenous hWee1 (Fig 1) in a manner sensitive to phosphatase treatment. Reactivity with transfected hWee1 was eliminated by the SITE1A mutation (Fig 2), providing strong evidence that the Ab preferentially recognizes P-SITE1 hWee1. In initial studies, we obtained evidence that mutation of RxL1 or inhibition of cyclin

A/Cdk2 activity using small molecule Cdk2 inhibitors did not eliminate hWee1 reactivity with the anti-P-SITE1 Ab (Aim 1d, data not shown). Therefore, it appears that phosphorylation of SITE1 may not depend on cyclin A/Cdk2 activity and binding to RxL1. We therefore have scrutinized the primary sequence surrounding NES/RxL1 for other potential cyclin A/Cdk2 consensus phosphorylation sites. We noted that a site we term here 'SITE2' fits this consensus and was found to be phosphorylated in a large published proteomic screen. We therefore have made a SITE2 substitution mutation in mWee1.

Because we are moving to the mouse as a model for much of our analyses (Aim 2), a key goal was to determine whether or not the Ab effectively recognizes mouse Wee1. In initial experiments, we confirmed reactivity of the P-SITE1 Ab with IP'd endogenous mWee1 (Fig 3). This result provides evidence that we have effective reagents for study of mWee1 and that mWee1 is phosphorylated on SITE1. We have a large supply of the affinity-purified Ab, ample for all anticipated need. We have now made several mutations in a mouse Wee1 (mWee1) cDNA, in preparation for testing their biological properties: SITE1D, SITE1E, NESm, T239A, as well as SITE2A.

Aim 2) Elucidate the functional role of Wee1 nuclear export

The rate-limiting step in this project is generating the mouse knock-in mutant lines (Aim 2b). We have therefore invested substantial effort in this step up front. We are using a Zinc finger (ZnF) approach commercialized by Sigma, wherein specific cleavage with an engineered pair of ZnFs spurrs homologous DNA repair via recombination with co-injected DNA. We attempted to clone the mWee1 genomic DNA region but encountered difficulty. Inspection of its sequence reveals several G:C- rich and/or homopolymeric tracts, particularly in the region -200 to -400 relative to the NES. We therefore contracted with a commercial provider to synthesize a genomic clone that encompasses this region. They demurred from attempting to synthesize the -200 to -400 region, but were able to provide us with a clone from around -200 to +900, with the ZnF cutting site located around +480. Thus, our clone contains about 200 bp of homology upstream of the area that includes our mutations and about 400 bp of homology downstream of the ZnF cutting sites but about 500 bp of homology between the ZnF cutting site and our mutation region. We prepared mRNA from the two ZnFs and provided it to the Transgenic Animal Facility, which co-injected it with mWee1 genomic DNA NES1 fragment into pro-nuclei. We first examined 15 progeny for cutting at the ZnF site by PCR amplification, denaturation, and re-annealing. Most progeny DNA cut efficiently with the enzyme CEL-1, which cuts at areas of mismatched DNA, whereas negative control mouse-tail DNA did not. ZnF cuts that are not repaired by homologous recombination are repaired by error-prone mechanisms, leaving small deletions or duplications. Duplexes that form with the wild type allele therefore cut with CEL-1. Thus, the observed high fraction of positive results in the CEL-1 assay confirms efficient cutting by our ZnFs. However, PCR-based genotyping for the desired NES mutation showed was effective using the starting NESm plasmid DNA as positive control but not progeny DNA. Therefore, we did not obtain potential founder mice with knockin mutations. There are three main possibilities: 1) The NESm mutation is lethal during development or the immediate post-natal period, 2) Recombination occurred but not within the 200 bp of homology beyond the NESm site, or 3) The injected DNA was not sufficiently intact or concentrated. To address the second and third possibilities, we repeated injections using new preparations of NESm and SITE1 mutant mWee1 genomic DNA.

The SITE1E mutation is predicted to exert the opposite effect on mWee1 of NESm; It should either be inert or foster mWee1 export. Thus, it is unlikely that both mutations would be lethal. These experiments are in progress. To further address the first possibility, Sigma has promised to provide us with ZnFs closer to the NESm. Narrowing the available region for recombination between the ZnF cut site and NESm should favor recombination distally, with a higher yield of successful integration of our mutations.

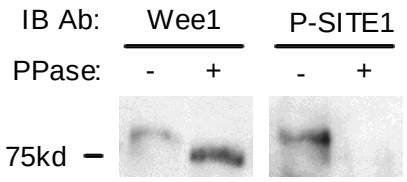

Fig 1. P-SITE1 Ab binding to hWee1 is phosphate-dependent. Extracts from asynchronous U2-OS cells were subjected to IP with a mouse anti-Wee1 Ab. Half the IP was treated with lambda phosphatase ('P'tase'). Each sample was divided in half again, followed by IB with rabbit anti-Wee1 Ab (left) or rabbit anti-P-SITE1 Ab (right). P'tase treatment increased Wee1 mobility (left), as described previously (1).

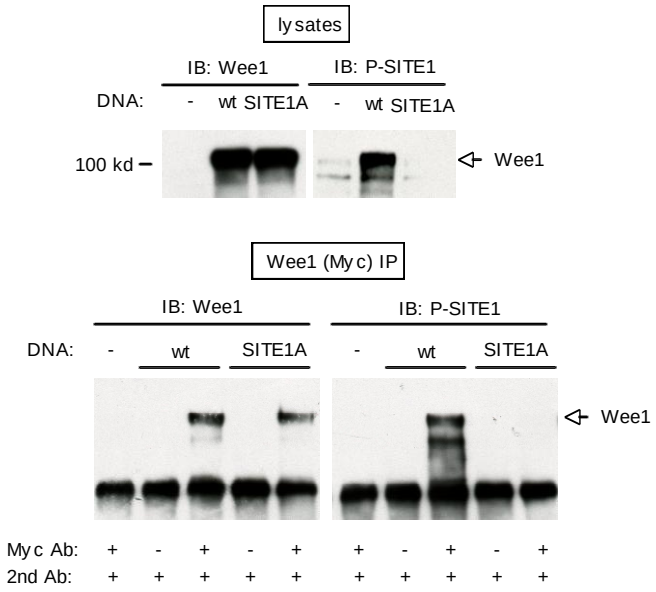

Fig 2. Reactivity of P-SITE1 Ab with hWee1 is dependent on SITE1. Asynchronous U2-OS cells were transfected with Wee1-wt or T173A. Cell lysates (above) and Wee1 immunoprecipitates (below, via the Myc tag) were subjected to IB with Ab directed against Wee1 (left) or P-SITE1 (right). P-SITE1 reacted strongly with wt but not SITE1A Wee1.

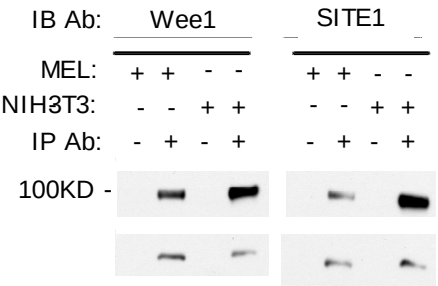

Fig 3. mWee1 is recognized by P-SITE1 Ab. MEL or NIH-3T3 lysates were subjected to IP, with (+) or without (-) mouse anti-Wee1 Ab, and IB with rabbit anti-Wee1 Ab (L) or P-SITE1 Ab (R).