Fox Chase Cancer Center

Annual Progress Report: 2008 Formula Grant

Reporting Period

July 1, 2011 – December 31, 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

Summary of Research Completed

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

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 research 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

Summary of Research Completed

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

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.

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

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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 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

Overall Progress in Goals:
The broad long term objective of the proposed research was to foster the development of a new rationale for cancer therapy, that of inhibiting autophagy. Autophagy is a cancer-critical survival
pathway that is an Achilles Heel for cancer cells, and we posit that inhibiting autophagy will lead to superior therapeutic outcomes for the majority of cancers.

There were two goals of the proposed work. The first was to better understand the role of the ARF tumor suppressor in autophagy; this goal focused on identifying the domain(s) of ARF required for autophagy. The second was to understand and develop a novel HSP70 inhibitor, which we found inhibits autophagy, for cancer therapy. This compound, which we call PAS (phenylacetylenesulfonamide), is an exciting new weapon in the battle against cancer because of the reliance of cancer cells on the survival pathways mediated by HSP70 and autophagy.

Aim 1: To define the domains of ARF necessary for mitochondrial localization, autophagy induction, and interaction with HSP70.

Progress: This aim is close to being completed. It was published in a manuscript by Pimkina and Murphy in Cancer Biology and Therapy in 2011, and will be published in a manuscript in preparation by Budina and Murphy.

Presently there is dissent in the literature about the role of ARF in autophagy. Specifically, the group of Kimchi indicates that only a small variant of ARF, that they call smARF, can induce autophagy in cells. In contrast, both our group and that of Wei Gu published that full-length ARF can likewise induce autophagy. Additionally, unlike the group of Kimchi, who reported that ARF-mediated autophagy was cytotoxic, we found that ARF and autophagy were beneficial to human tumors. We set out to resolve this controversy by creating stable cell lines with tetracycline-inducible versions of ARF (amino acids 1-169), smARF (amino acids 50-169), and a mutant of ARF that we find is impaired for autophagy induction (1-100).

Since the Kimchi report, a number of more reliable assays for autophagy have been developed; using these cutting edge assays, we were able to unequivocally demonstrate that the small variant of ARF, termed smARF, is unable to induce autophagy. In fact, this variant is an inhibitor of autophagy. In all likelihood the lack of sophistication of autophagy assays at the time of the Kimchi report resulted in this mis-interpretation by this group. In addition, we were able to demonstrate that amino acids 120-169 of ARF were required for autophagy induction by this protein. We were also able to identify amino acids 1-50 as being required for ARF’s mitochondrial localization (data not shown).

Interestingly, in performing the current cutting edge assays for autophagy, we discovered that ARF was not only able to induce autophagy, but in fact was also able to induce mitophagy. Mitophagy is the selective degradation of mitochondria, which frequently occurs when mitochondria are old and/or damaged. More recently, we have developed several novel assays for mitophagy in order to probe this question in more detail. Specifically, we have stably transfected our ARF-inducible cells with a GFP-linked marker for LC3; during autophagy LC3 becomes localized to autophagic vacuoles. We have likewise transfected these cells with an RFP fluorescent protein that is linked to a mitochondrial leader peptide; this protein allows mitochondria to be visualized as red. We have found that expression of ARF in these cells causes LC3 (tagged by green fluorescent protein) to co-localize with mitochondria (tagged with a red fluorochrome). In this work we have also developed 3 other assays that can be used to detect
mitophagy; these include the loss of mitochondrial trans-membrane potential, the degradation of mitochondrial proteins, and the co-purification of p62, an autophagy-specific adaptor protein, with purified mitochondria. We expect this manuscript, which is in preparation, to be a foundation for analyzing mitophagy, which is a field that is still in its infancy.

This manuscript also identified amino acids 120-169 of ARF, which are highly conserved across species, as being critical for ARF’s autophagy function. This is the first function of ARF that maps to this novel, highly conserved domain.

Aim 2: To evaluate the efficacy of a novel HSP70 inhibitor (PAS) on human tumor cell lines

Progress: This aim has been completed. It was partly published in our Mol Cancer Res manuscript in 2011, along with a manuscript that is in preparation on the molecular modeling of PAS with HSP70, and the identification of a novel analogue of PAS that shows superior efficacy (Balaburski et al., manuscript in preparation).

PAS is an effective anti-cancer agent and autophagy inhibitor in cell lines, and in a mouse model of B cell lymphoma

We have now analyzed the IC50 for PAS in the entire NCI-60 cell line panel, using the MTT assay for cell viability. Our data indicate that all of the analyzed human tumor cell lines are sensitive to PAS, with an IC50 of 0.5-5 uM. In contrast, normal human fibroblasts, and the immortalized human breast epithelial line MCF-10A, are resistant to PAS (IC50>100 uM). We have performed cell cycle analysis of PAS-treated cells, and found that PAS induces a G2/M arrest in tumor cells. We have also shown that, in cells that are synchronized in G2/M using double-thymidine block, G2/M phase cells are markedly more sensitive to the cytotoxic effects of PAS.

Previously, we mapped the binding site for PAS to the C terminus of HSP70, to residues 386-641 (1). More recently, along with Dr. Roland Dunbrack, we used in silico docking programs to more accurately identify potential PAS binding sites in the C terminus of this protein. This analysis revealed three potential docking sites for PAS on HSP70. We next performed site-directed mutagenesis of key contact residues for each predicted docking site. Notably, for one of these docking sites, amino acids isoleucine 607, tyrosine 611 and asparagine 548 were deemed to be critical contacts, and mutagenesis of each of these three sites reduced the ability of PAS to interact, while retaining the overall structure of HSP70 (Figure 1). This analysis led to the identification of mutants of HSP70 that fail to bind, and be inhibited, by PAS.

Our finding that PAS causes G2/M arrest were presented at an internal seminar at Fox Chase Cancer Center, after which Dr. Tim Yen, an expert in mitotic checkpoints at Fox Chase, expressed an interest in using this compound in his in vitro assays of mitotic checkpoints. Notably, incubation with PAS, but not the HSP90 inhibitor Geldanamycin, was able to block the ability of the anaphase-promoting complex to catalyze the degradation of cyclin B1. This exciting finding will be explored further in a project to be submitted as part of a P01 in BRAF signaling in melanoma, to be submitted in September 2012.
PAS-Cl, a modified version of PAS, has 10-fold increased efficacy in cell lines, and in a mouse model of B cell lymphoma.

Our in silico docking work with Dunbrack revealed the possibility that a small halogen, Cl or Fl, might be placed in the meta position of PAS and confer enhanced binding to HSP70. Using the services of the Organic Synthesis Facility at Fox Chase Cancer Center, we outlined a synthesis protocol for PAS-Cl and were able to synthesize milligram quantities of this compound. Notably, PAS-Cl showed superior ability to ‘pull-down’ HSP70, along with 10-fold decreased IC50 in tumor cell lines. It also showed significantly increased efficacy in a mouse model of B cell lymphoma (Eu-myc; p<0.000006), compared to the parent compound, when administered to mice at 20 mg/kg every 7 days for 10 weeks. Notably, after this treatment all of the vehicle treated mice had succumbed to cancer, but over half of the PAS-Cl treated mice survived with no evidence for toxicity. This exciting finding is the basis for a pending Leukemia and Lymphoma Society application (letter of intent already accepted), due March 2012. It is also part of a manuscript that is in preparation (Balaburski and Murphy, in prep).

PAS-Cl is efficacious in drug-resistant melanoma lines. Primary melanocytes are resistant to PAS and PAS-Cl.

Along with the group of Meenhard Herlyn (Wistar Institute) we have analyzed PAS and PAS-Cl efficacy in melanoma lines, as well as primary melanocytes. We have found that the IC50 for PAS-Cl is between 100 and 500 nM in all melanoma lines tested to date, including those resistant to BRAF inhibitors. In contrast, the IC50 of PAS-Cl in primary melanocytes is greater than 100 uM (Figure 2).

Aim 3. To test the hypothesis that ARF and HSP70 silencing are inhibitory to human tumor development in a xenograft model.

Progress: This aim has evolved into an analysis of PAS efficacy in two human tumor types, melanoma and acute lymphocytic leukemia (ALL). It has also evolved to encompass an analysis of pharmacokinetics and pharmacodynamics of PES and PES-Cl in the mouse. This evolution was necessitated by a change in the scientific acceptance of xenograft models for cancer, and the current favor in the scientific community for performing drug efficacy studies using patient-derived primary tumors in the mouse (patient-derived xenografts, or PDX). Therefore, instead of performing xenograft assays with tumors from established cell lines, we have partnered with Dr. Meenhard Herlyn at the Wistar Institute to perform PDX with primary melanoma samples, as well as with Drs. Gwenn Danet-Desnoyers and Jeffrey Barrett to perform PDX with primary adult and pediatric ALL. This aim is ongoing.
Figure 1. Molecular model of PAS bound to the C-terminus of HSP70. Highlighted are critical amino acids contacts at residues 607, 611 and 548; mutation of these residues disrupts the ability of PAS to bind, and inhibit, HSP70.

Figure 2. PAS is cytotoxic to two melanoma cell lines (A375 and 1205(Lu)), but not to primary human melanocytes (FOM134sc). The concentrations of PAS used are depicted on the x axis, and the percent survival, as determined by MTT assay, is depicted on the y axis.
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.

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 Cdk complexes 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.

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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

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

Contrary to our model, results from initial experiments showed that an RxL1 mutant as well as hWee1 isolated from cells treated with a Cdk1/2 inhibitor drug still bound P SITE1 Ab (data not shown). These results imply that cyclin A/Cdk2 might not be the sole kinase responsible for this phosphorylation event. Based on studies of cyclin D, GSK3-β is a logical candidate kinase. Alternatively, mutation of RxL1 and the conditions of drug treatment may have been insufficient to block SITE1 phosphorylation by cyclin A/Cdk2. Further work will be required to distinguish these possibilities. Nonetheless, these findings spurred us to search for other potential sites of Wee1 phosphorylation by cyclin A/Cdk2. We have now identified a second candidate cyclin A/Cdk2 phosphorylation site near RxL1, SITE2. This site in hWee1 has been shown to be phosphorylated in proteomic screens and fits with consensus Cdk phosphorylation sites. We hypothesize that this site may also foster nuclear export and show greater dependence than SITE1 on phosphorylation by cyclin A/Cdk2 complexes. We generated the following mutations in mWee1: RxL1m, the putative Wee1 box phosphorylation site mutant T286A (alanine substitution), SITE1A, SITE1D, SITE1E, and SITE2A. We are focusing initial work on the phospho-mimetic substitution SITE1E, rather than SITE1D, because our molecular modeling suggested that the longer side chain of glutamate might provide a better fit with the CRM1 cargo-binding surface. Further, bioinformatics analysis of CRM1 substrates showed that glutamate residues were more common than aspartate residues in corresponding NES locations of exported proteins.

We cloned the wild type (wt), NESm, and SITE1E mWee1 proteins, respectively, into a 3xFLAG mammalian expression vector and compared their subcellular localization by anti-FLAG Ab immunofluorescence. The results show that export of mWee1, like that of hWee1, is dependent on its NES sequence (Fig 1). This observation demonstrates conservation of the NES regulatory motif in mammalian Wee1 proteins. Further, the SITE1 phospho-mimetic mutant showed preserved and possibly accelerated export, consistent with our model (Fig 1). We will now clone each of the other mWee1 mutants into the 3xFLAG vector and examine their subcellular distribution in the same way.

The 3x FLAG vector, in addition, expresses GFP-histone H2B. This fusion protein marks chromosomal DNA in living and fixed cells, allowing mitotic chromosome dynamics to be recognized. Comparison to immunofluorescence for mWee1 should allow us to semi-
quantitatively assess the subcellular distribution of Wee1 relative to mitotic position in single cells (Fig 2). We may also be able to assess the impact of the mWee1 mutations on progression to and through mitosis, using the GFP-H2B readout. In preliminary experiments, we have generated movies that follow the fate of individual GFP-H2B(+) cells expressing different mWee1 proteins (data not shown). This work provides a complementary approach to the mouse knockin work described in Aim 2 below for assessing the functional role of Wee1 nuclear export.

**Aim 2) Elucidate the functional role of Wee1 nuclear export.**

To begin to test the hypothesis that the nuclear export signal (NES) mutant and SITE1 mutant will show greater inhibition of mitotic entry than Wee1-wt in cells with reduced Cdk activity and/or DNA damage we transfected these mutants together with GFP-H2B into RPE-1 cells, a non-transformed human epithelial cell line with lower levels of Cdk activity than U2-OS cells (data not shown). Initial experiments were hampered by low transfection efficiency. Further experiments are planned using additional cell lines, irradiation, induction of a dominant negative mutant of Cdk2, and Cdk inhibitor drug treatment.

From 40 potential founder mice, we have thus far identified one mouse with an intact SITE1E mutation (Fig 3). PCR based genotyping of its progeny suggests that about half carry the mutation. Therefore, we appear to have successfully generated the first knockin mutation of the mWee1 NES regulatory region. We have a second candidate SITE1E knockin line that we are characterizing. This work validates our overall approach and the functionality for recombination of the ZnF pair and the injected DNA.

In further injections of the NESm fragment, a single potential founder was identified by PCR from 110 mice generated. However, sequencing showed a rearrangement of the NES region rather than our desired mutation. Therefore, we have not yet obtained potential founder mice with NESm knockin mutations. This result may reflect some inefficiency of the method, as currently configured and discussed, and/or lethality of this mutation, even in a heterozygote. Note that the NESm mutant is predicted to remain nuclear, potentially compromising mitosis in some cells.

To facilitate further knockin work, Sigma has now provided us with new ZnFs closer to the NESm. Narrowing the available region for recombination between the ZnF cut site and NESm should favor recombination distally, resulting in a higher yield of recombination events that integrate the mutation into genomic DNA. We are now testing these additional ZnFs. With higher recombination efficiency, we should be able to establish additional SITE1E knockin lines more efficiently and either obtain the NESm line or evidence with statistical power that the NESm mutation is lethal. If lethality prevents establishment of an NESm knockin mouse line, we will use the ZnFs to generate NESm cell lines in vitro.

Establishment of the SITE1E knockin mice should allow us to interbreed them, to assess whether or not homozygous SITE1E mice are viable and what phenotypes might become manifest. We are likely to be able to isolate embryonic fibroblasts from such mice and examine the impact of the mutation on cell cycle progression. Such cells should express SITE1E at physiologic levels,
in the absence of competing wild type protein, thereby providing an ideal setting for functional analysis. This work should form a strong foundation for applications for extramural funding.
**Fig 1.** Confirmation of the functionality of the NES in mWee1 and evidence that SITE1 phosphorylation fosters export. U2-OS cells were transfected with the respective vectors. Anti-FLAG immunofluorescence staining was scored for nucleus (N) versus cytoplasmic (C) locations, as indicated. Note that the NESm mutation nearly abolished export whereas the phosphomimetic SITE1E mutation does not and may foster export.

**Fig 2.** mWee1 distribution versus GFP-H2B. U2-OS cells were transfected with the indicated expression vectors and enriched for late S phase cells by thymidine block and release. FLAG IF (mWee1): red. Note the preferentially nuclear staining of NESm.

**Fig 3.** Sequence spanning mWee1 SITE1E in DNA amplified by PCR from the knockin mouse. Note the heterozygous sequences present exactly at the desired SITE1 codon, specifying wild type and knockin sequences. Surrounding sequences from each chromosome copy are identical (homozygous), reflecting the absence of spurious mutations in the knockin allele.