Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** The Fox Chase Cancer Center

2. **Reporting Period (start and end date of grant award period):** 1/1/2009 – 12/31/2011

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Maria Minko Gill

4. **Grant Contact Person's Telephone Number:** 215-728-2659

5. **Grant SAP Number:** 4100047634

6. **Project Number and Title of Research Project:** 5 – Regulation of Human Somatic Wee1 by Cyclin A/Cdk2 Complexes

7. **Start and End Date of Research Project:** 7/1/2010 – 12/31/2011

8. **Name of Principal Investigator for the Research Project:** Greg H. Enders, M.D., Ph.D.

9. **Research Project Expenses**.

   9(A) Please provide the amount of health research grant funds spent on this project for the entire duration of the grant, including any interest earned that was spent:

   $580,832.22

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project	Cost
Enders	PI	50% YR01-02	$166,985.16

Boquoi	Postdoctoral Associate	45% YR01;	$40,680.26
Chen	Scientific Technician II	70% YR01	$40,055.09

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____ No___X_____

If yes, please indicate the source and amount of other funds:

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes_____ No____X_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
	☐NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)		$	$
	☐NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)		$	$
	☐NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)		$	$

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes____X_____ No_____

If yes, please describe your plans:

We expect to be in a good position within a year to apply for NIH R01 funding of the project. Our position would be strengthened by further evidence that nuclear export of Wee1 is important for efficient cell cycle progression under at least some conditions and evidence that we can generate additional knockin mutations of the NES regulatory domain with reasonable efficiency.

12. Future of Research Project. What are the future plans for this research project?

We are testing further whether nuclear export of Wee1 is important for efficient cell cycle progression, under at least some conditions, and working to generate additional knockin mutations of the NES regulatory domain. This work may position us well for application for NIH R01 funding.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes____X_____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male				
Female				1
Unknown				
Total				**1**

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic				1
Unknown				
Total				**1**

	Undergraduate	Masters	Pre-doc	Post-doc
White				1
Black				
Asian				
Other				
Unknown				
Total				**1**

14. Recruitment of Out-of–State Researchers. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No____X_____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. Impact on Research Capacity and Quality. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes____X_____ No_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

The funding allowed significant advances in the project, putting us in better position to garner federal funding.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes_____ No____X_____

If yes, please describe the collaborations:

16(B) Did the research project result in commercial development of any research products?

Yes_____ No____X_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No____X_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant application's strategic plan). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

This report contains confidential information highlighted below and should be redacted before releasing this information to the public.

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

a) Determine whether the Wee1 [redacted] mutant demonstrates reduced Crm1 binding.

b) Generate and validate antibody specific to [redacted]–phosphorylated Wee1.

c) Define the temporal pattern of [redacted] phosphorylation through the cell cycle.

d) Determine whether mutation of RxL1 and/or inhibition of cyclin A/Cdk2 inhibition ablates [redacted] phosphorylation.

e) [redacted]

Aim 2) Elucidate the functional role of Wee1 nuclear export

a) Test the hypothesis that the nuclear export signal (NES) mutant and [redacted] mutant will show greater inhibition of mitotic entry than Wee1-wt in cells with reduced Cdk activity and/or DNA damage.
b) Generate a 'knockin' mouse with replacement of the somatic Wee1 gene with an NES mutant.

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

Studies on Aim 1 focused initially on generating and validating rabbit polyclonal antibodies (Ab) specific to the designated phosphorylated form of human Wee1 (hWee1), which we will term here 'SITE1' (Aim 1b). This site is near the RxL1 binding site of cyclin A/Cdk2 complexes on Wee1 and the NES and was chosen based on several criteria, including our molecular modeling of potential interactions with the Crm1 nuclear exporter (Li et al, Molecular and Cellular Biology 30; 116-130, 2010). Mutation of the phospho-acceptor residue in this site to an alanine residue abolished nuclear export of hWee1 whereas mutation to a phospho-mimetic aspartate residue preserved export. These findings provided strong evidence that hWee1 export was dependent on phosphorylation of SITE1. 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 binds to 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 (completing Aim 1b).

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. This result, thus, provides initial evidence that regulation of Wee1 export by phosphorylation is conserved in mammalian Wee1 proteins. We have a large supply of the affinity-purified Ab, ample for all anticipated need.

Contrary to our model, however, results from initial experiments showed that an RxL1 mutant as well 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-ß is a logical candidate kinase (Aim 1e). 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 near the NES.

We have now identified a second candidate cyclin A/Cdk2 phosphorylation site near RxL1, SITE2. This site 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.

To lay the groundwork for a through dissection of the NES/RxL1 regulatory region in mWee1, 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 on the phospho-mimetic substitution SITE1E, rather than SITE1D, because molecular modeling (see our publication Li et al, Molecular and Cellular Biology, Vol. 30, No1. Jan 2010, pgs. 116-30) suggested that the longer side chain of glutamate would provide a better fit with the nuclear exporter CRM1. Further, bioinformatics analysis of CRM11 substrates showed that glutamate residues were more common than aspartate residues in the corresponding NES location.

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 in its NES sequence (Fig 4). This observation demonstrates the conservation of the NES regulatory motif in the mouse. Further, the SITE1 phospho-mimetic mutant showed preserved and possibly accelerated export, consistent with our model (Fig 4). 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. Comparison to immunofluorescence for mWee1 will allow us to assess the subcellular distribution of the latter relative to mitotic position in single cells (Fig 5). We may also be able to assess the impact of the mWee1 mutations on progression to and through mitosis, using the GFP-H2B readout.

<u>Aim 2) Elucidate the functional role of Wee1 nuclear export</u>

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 (Aim 2a) we transfected these mutants together with GFP-H2B into RPE-2 cells, a non-transformed human epithelial cell line with lower levels of Cdk activity than U2-OS cells (data not shown). These initial experiments have been hampered by low transfection efficiency. Further experiments are planned using irradiation, Cdk inhibitor drug treatment, and additional cell lines.

The rate-limiting step in this project is generating the mouse knock-in mutant lines (Aim 2b). We have therefore prioritized this step. We are using a Zinc finger (ZnF) approach commercialized by Sigma, wherein specific cleavage with an engineered pair of ZnFs spurs 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 NESm fragment into pro-nuclei. We first examined 15 potential founder mice for cutting at the ZnF site by PCR amplification, denaturation, and re-annealing. DNA from most mice 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 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 or 2) recombination occurred but not within the 200 bp of homology beyond the NESm site, 3) the injected DNA was not sufficiently intact or concentrated. To address these possibilities, we repeated injections using new preparations of NESm and SITE1E 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.

From 40 potential founder mice, we have identified one mouse with an intact SITE1E mutation (Fig 6). PCR based genotyping of its progeny suggests that about half carry the mutation, to be confirmed by sequencing. Therefore, we appear to have successfully generated our first knock-in mutation of the mWee1 NEM regulatory region. We have a second candidate SITE1E knock-in line that we are still characterizing. This work validates the functionality of our overall approach, as well as the specific ZnF pair and the injected DNA.

In further injections of the NESm fragment, a single potential founder was identified by PCR from a total of 110 mice injected with NESm DNA. However, sequencing showed a rearrangement of the NES rather than our desired mutation. Therefore, we have not obtained potential founder mice with NESm knockin mutations. This result may reflect some inefficiency of the method as 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 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, with 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 knockin lines more efficiently and either obtain the NESm line or obtain 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 knock-in lines should allow us to breed these mice, to assess whether or not homozygous SITE1E mice are viable and what phenotypes might become manifest. We should also be able to isolate embryonic fibroblasts from these 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 and a firm foundation for applications for extramural funding.

Summary of overall accomplishments

Some of the specific subaims of the proposal (Aims 1a, c, and e; Aim 2a) have not yet been completed. These aims were modified from a 4-year NIH R01 proposal and were probably a bit ambitious for the current funding period. We encountered some unexpected technical challenges in cloning the mWee1 region and the unexpected biological finding that disruption of the cyclin A/Cdk2 activity interaction with RxL1 were insufficient to abrogate phosphorylation of SITE1 (Aim 1d). The technical challenge slowed overall progress in generating knockin mice but did not prevent us from successfully generating a phospho-mimetic knock-in mutation of SITE1. Further, the provision by Sigma of new ZnFs closer to the regulatory region should speed further work and completion of Aim 2b. We have met the biological challenge by identifying a second good candidate cyclin A/Cdk2 phosphorylation site near the NES of mWee1 and have begun dissecting this site. We have correspondingly broadened Aim 1 to encompass candidate phophorylation SITE2 and have broadened Aim 2b to include generating SITE1E knockin lines.

In conclusion, we have confirmed that the NES in the Wee1 regulatory domain is functionally conserved in mammals. This fundamental finding validates this regulation as important in mammalian Wee1 proteins and validates the mouse as a model for understanding such regulation and its impact on cell cycle progression. We have determined that a phosphorylation event near the NES that is essential for export of hWee1 (on SITE1) is conserved in the mouse. We have generated sensitive and abundant rabbit polyclonal Abs that can recognize phosphorylation of this site on both hWee1 and mWee1 (Aim 1b). In initial studies, inhibiting the interaction of hWee1 with active cyclin A/Cdk2 complexes did not abrogate SITE1 phosphorylation, implying that a second kinase may (also) phosphorylate this site (Aim 1d). We have generated a battery of mutations in this regulatory region of mWee1 and cloned them into mammalian expression vectors that also express GFP-H2B. These constructs should allow determination of the timing of export of mWee1 (Aim 1c) and the effect of the SITE1A mutation on mitotic entry in cells with reduced Cdk activity (Aim 2a). Finally, we have generated the first mouse knockin mice with a

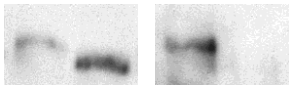

Fig 1. P-SITE1 Ab binding to hWee1 is phosphate-dependent. Extracts from asynchronous U2-OS cells were subjected to IP with a mouse anti-Wee1 Ab. Half the IP was treated with lambda phosphatase ('P'tase'). Each sample was divided in half again, followed by IB with rabbit anti-Wee1 Ab (left) or rabbit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

mutation in this regulatory region (SITE1E) (a modification of Aim 2b). This accomplishment paves the way for detailed study of effect of phosphorylation of this site under physiological conditions. Generation of this knockin line validates the feasibility of this approach, the functionality of our specific reagents, and our technical expertise in pursuing this line of work. We may know soon whether the NESm mutation is, as we suspect, embryonic lethal (thus completing a broader Aim 2b). We have laid important foundations for a competitive NIH R01 application as well as a new manuscript. We expect this work will provide key insights into the regulation of mitotic entry in mammals and the potential utility of manipulating this regulation in cancer chemotherapy.

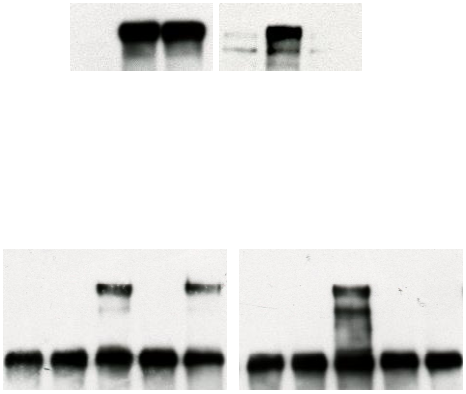

Fig 2. Reactivity of P-SITE1 Ab with hWee1 is dependent on SITE1. Asynchronous U2-OS cells were transfected with Wee1-wt or T173A. Cell lysates (above) and Wee1 immunoprecipitates (below, via the Myc tag) were subjected to IB with Ab directed against Wee1 (left) or P-SITE1 (right). P-SITE1 reacted strongly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

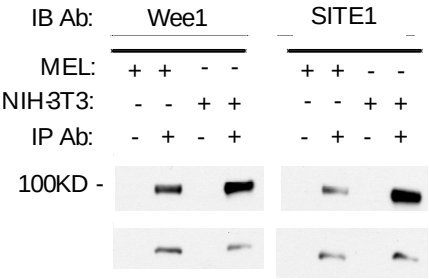

Fig 3. mWee1 is recognized by P-SITE1 Ab. MEL or NIH-3T3 lysates were subjected to IP, with (+) or without (-) mouse anti-Wee1 Ab, and IB with rabbit anti-Wee1 Ab (L) or P-SITE1 Ab (R).

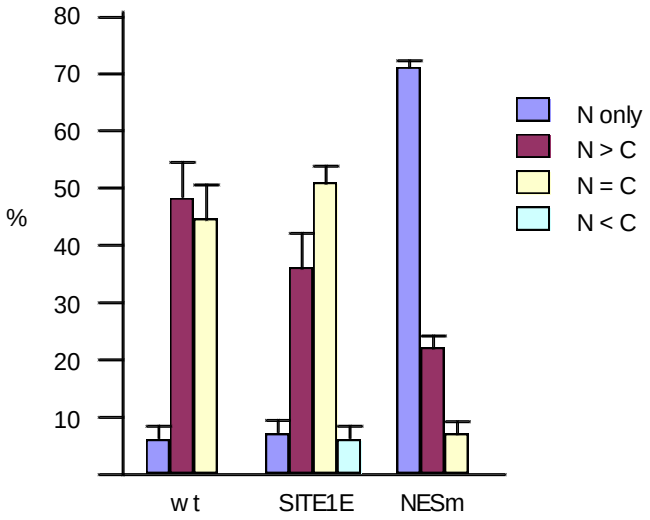

Fig 4. Confirmation of the functionality of the NES in mWee1 and evidence that SITE1 phosphorylation fosters export. U2-OS cells were transfected with the respective vectors. Anti-FLAG immunofluorescence staining was scored for nucleus (N) versus cytoplasmic (C) locations, as indicated. Note that the NESm mutation nearly abolished export whereas the phospho-mimetic SITE1E mutation does not and may foster export.

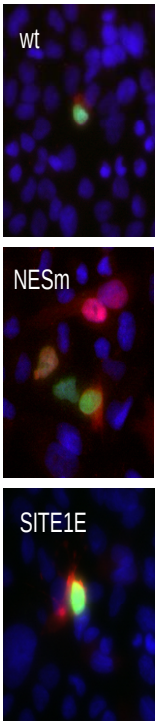

Fig 5. mWee1 distribution versus GFP-H2B. U2-OS cells were transfected with the indicated expression vectors and enriched for late S phase cells by thymidine block and release. FLAG IF (mWee1): red. Note the preferentially nuclear staining of NESm.

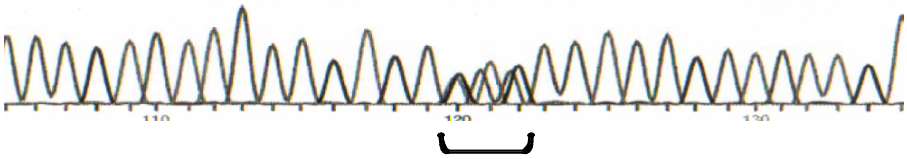

Fig 6. Sequence spanning mWee1 SITE1E in DNA amplified by PCR from the knockin mouse. Note the heterozygous sequences present exactly at the position of the desired SITE1 codon, reflecting wild type and knockin sequences. Surrounding sequences from each chromosome copy are identical, reflecting the absence of spurious mutations.

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X__No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X__No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics

_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
___X__ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, the number of the publication and

an abbreviated research project title. For example, if you submit two publications for PI Smith for the "Cognition and MRI in Older Adults" research project (Project 1), and two publications for PI Zhang for the "Lung Cancer" research project (Project 3), the filenames should be:

Project 1 – Smith – Publication 1 – Cognition and MRI
Project 1 – Smith – Publication 2 – Cognition and MRI
Project 3 – Zhang – Publication 1 – Lung Cancer
Project 3 – Zhang – Publication 2 – Lung Cancer

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1. None				☐Submitted ☐Accepted ☐Published
2.				☐Submitted ☐Accepted ☐Published
3.				☐Submitted ☐Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes____X_____ No_____

If yes, please describe your plans:

The results in hand already are sufficient to justify submission of a manuscript in the future. We have demonstrated that the NES is functionally conserved from man to mouse. We have identified a phosphorylation near the NES that is needed for efficient function of the NES. We hope to be in a position to submit a fairly complete story in 2012.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis,

or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None.

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None.

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No___X_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No___X_____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*

Biosketches for Drs. Enders and Boquoi are attached.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Gregory H. Enders, M.D., Ph.D.	Associate Professor
eRA COMMONS USER NAME ENDERSGH	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Harvard College, Boston, MA	A.B.	1980	Biology
University of California, San Francisco, CA	Ph.D.	1980-1987	Genetics
University of California, San Francisco, CA	M.D.	1980-1988	Medicine
Brigham and Women's Hospital, Harvard Medical School, Boston, MA		1988-1990	Internal Medicine
Massachusetts General Hospital, Harvard Medical School, Boston, MA		1990-1993	Gastroenterology

PERSONAL STATEMENT

My research focuses on regulation of the mammalian cell cycle and its disruption in cancer.

POSITIONS

National Institutes of Health Medical Scientist Training Program, University of California, San Francisco 1980-1988

Ph.D. thesis, Dept. of Biochemistry and Biophysics, Dr. Harold Varmus, American Cancer Society Professor of Molecular Virology, Nobel Laureate in Medicine or Physiology 1989 (principal advisor) and Dr. Don Ganem, Professor of Medicine and Microbiology
Subject: transcription and replication of hepatitis B viruses 1983-1987

Resident in Medicine, Brigham and Women's Hospital; Harvard Medical School, Boston, MA 1989-1990

Clinical Fellow in Medicine, Massachusetts General Hospital andHarvard Medical School, Boston, MA 1990-1993

Research Fellow in Medicine, Massachusetts General Hospital and Harvard Medical School, Postdoctoral Fellow of Dr. Edward Harlow, American Cancer Society Professor of Genetics; Subject: Cloning and characterization of Cyclin dependent kinases (Cdks) 1991-1993

Instructor in Medicine, Harvard Medical School; Subject: Mechanism of action of the tumor suppressor and Cdk inhibitor $p16^{INK4a}$ 1993-1996

Assistant Professor of Medicine, University of Pennsylvania, Philadelphia, PA; Subjects: Mechanism of action $p16^{INK4a}$, function of Cdk2 1996-2006

Assistant Professor of Genetics, University of Pennsylvania; Subjects: Mechanism of action $p16^{INK4a}$, function of Cdk2 1998-2005

Director, Morphology Core Facility, NIH Center for Molecular Studies in Digestive and Liver Diseases, University of Pennsylvania 2003-2006

Associate Professor, Fox Chase Cancer Center, Philadelphia, PA, Department of Medicine, Epigenetics and Progenitor Cell Keystone Program; Subjects: tumor suppression by p16Ink4a, function of Cdk2, regulation of Wee1 2006-date

HONORS

NIH Clinical Investigator Award	1993-1997
American Cancer Society Research Scholar Award	1999-2003
Co-chair, Cell Cycle Checkpoints and Tumor Development mini-symposium, American Association for Cancer Research annual meeting, Atlanta, GA	2001
Miles and Shirley Fiterman Basic Research Award, American Gastroenterological Association	2001
Reviewer, Dutch National Cancer Society (ad hoc)	2002
Reviewer, NIH SPORE in Pancreatic Cancer	2003
Associate Editor, Cancer Research	2003-2009
Reviewer, NIH Cell Signaling and Dynamics Study Section (ad hoc)	2005-2006
Reviewer, Vanderbilt University Pilot Projects Program (ad hoc)	2007
Reviewer, NIH Challenge Grants, Cell Biology	2009
Reviewer, NIH Cell Signaling and Regulatory Systems review group (ad hoc)	2009
Reviewer, Medical Research Council (United Kingdom)	2010
AACR Annual Meeting Cell Cycle Program Committee	2011
Reviewer, NIH/NCI Barrett's Esophagus Translational Research Network Special Emphasis Panel	2011

SELECTED PEER-REVIEWED PUBLICATIONS

Meyerson, M., **Enders, G.H.**, Wu, C.-L., Su, L.-K., Gorka, C., Nelson, C., Harlow, E., Tsai, L.-H. A family of human cdc2-related protein kinases. *EMBO J.* **11**:2909-2917, 1992.

Koh, J., **Enders, G.H.**, Dynlacht, B.D., Harlow, E. Tumour-derived p16 alleles encoding proteins defective in cell-cycle inhibition. *Nature* **375**:506-510, 1995.

Mitra, J., Dai, C.Y., Somasundaram, K., El-Deiry, W.S., Satyamoorthy, K., Herlyn, M., **Enders, G.H.** Induction of $p21^{WAF1/CIP1}$ and inhibition of Cdk2 mediated by the tumor suppressor $p16^{INK4a}$. *Mol. Cell. Biol.* **19**:3916-3928, 1999.

Hu, B.,* Mitra, J.,* van den Heuvel, S., **Enders, G.H.** S and G2 phase roles for Cdk2 revealed by inducible expression of a dominant negative mutant in human cells. *Mol. Cell. Biol.* **21**:2755-2766, 2001. *equal contribution

Dews, M., Homayouni, A., Yu, D., Murphy, D., Sevignani, C., Wentzel, E., Furth, E.E., Lee, W.M., **Enders, G.H.**, Mendell, J.T., Thomas-Tikhonenko, A. Augmentation of tumor angiogenesis by a Myc-activated microRNA cluster. *Nat. Genet.* **38**:1060-1065, 2006.

Boquoi, A., Chen, T., **Enders, G.H**. Chemoprevention of mouse intestinal tumorigenesis by the cyclin-dependent kinase inhibitor SNS-032. *Cancer Prev. Res.* **2**(9):800-806, 2009.

Li, C., Andrake, M., Dunbrack, R., **Enders, G.H.** A bi-functional regulatory element in human somatic Wee1 mediates cyclin A/Cdk2 binding and Crm1-dependent nuclear export. *Mol. Cell. Biol.* **30**:116-130, 2010.

Enders, G.H. (Editor). Cell Cycle De-Regulation in Cancer. Springer Science and Business Media, 2010.

Kennedy, A.L., Morton, J.P., Manoharan, I., Nelson, D.M., Pawlikowski, J., Jamieson, N.B., McBryan, T., Doyle, B., Oien, K.A., **Enders, G.H**., Zhang, R., Sansom, O.J., Adams, P.D. Activation of the PIK3CA/AKT pathway suppresses senescence induced by an activated RAS oncogene to promote tumorigenesis. Molecular Cell **42**:36-49, 2011.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Amelie Boquoi, M.D., Ph.D.	Postdoctoral Associate
eRA COMMONS USER NAME	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
Humboldt-University Berlin, Germany	M.D.	11/02	Medicine
Charité – Universitätsmedizin Berlin, Germany	Ph.D.	04/05	Medicine
Charité – Universitätsmedizin Berlin, Germany	Resident Physician	10/04	Gastroenterology and Emergency
The University of Pennsylvania, Philadelphia, PA	Postdoctoral Fellow	11/06	Molecular Biology of Cancer
Fox Chase Cancer Center, Philadelphia, PA	Postdoctoral Fellow	11/06 - present	Molecular Biology of Cancer

A. PERSONAL STATEMENT

After completing M.D. training, I pursued a Ph.D. through the Charite Medical University in Berlin, with research at M.D. Anderson Cancer Center in Houston. Dr. Enders recruited me to his laboratory in 2005 at the University of Pennsylvania and moved his research program to Fox Chase Cancer Center the next year. My dual training in clinical gastroenterology and basic science has been valuable in three inter-related projects. I examined intestinal epithelial cell proliferation and tumorigenesis in a transgenic mouse model expressing VEGF selectively in the intestinal epithelium. I pioneered studies of chemoprevention of intestinal tumorigenesis in mouse models using a small molecule Cdk inhibitor. Results from both projects were published in 2009. In unpublished work, we have now developed evidence for p16 function in intestinal stem cells and for pre-mature aging phenotypes in p16-transgenic mice. I have substantial experience and a proven track record of using animal models to study p16 function in the gastrointestinal tract.

B. POSITIONS AND HONORS

Positions – see above

Awardee: Research fellowship of the German Academic Exchange Service (DAAD) 2001
Awardee: The Edward David Lustbader Award, Fox Chase Cancer Center, 2nd place 2009
Awardee: The Edward David Lustbader Award, Fox Chase Cancer Center, 1st place 2010
(to the best poster presentation at the annual Fox Chase Cancer Center Postdoctoral Symposium)

C. PEER-REVIEWED PUBLICATIONS

1. Ramesh, R., Mhashilkar, A.M., Tanaka, F., Saito, Y., Branch, C.D., Sieger, K., Mumm, J.B., Stewart, A.L., **Boquoi, A.**, Dumoutier, L., Grimm, E.A., Renauld, J.C., Kotenko, S., Chada, S. Melanoma differentiation-associated gene 7/interleukin (IL)-24 is a novel ligand that regulates angiogenesis via the IL-22 receptor. *Cancer Res.* **63**(16):5105-5113, 2003.
2. Gibson, S.L., **Boquoi, A.**, Chen, T., Sharpless, N.E., Brensinger, C., Enders, G.H. p16(Ink4a) inhibits histologic progression and angiogenic signaling in min colon tumors. *Cancer Biol. Ther.* **4**(12):1389-1394, 2005.
3. **Boquoi, A.**, Enders, G.H. Ubiquitination of Myc – Flipping the switch. *Cancer Biol. Ther.* **5**(8):907-908, 2006.
4. **Boquoi, A.**, Enders, G.H. Adenomatous Polyposis Coli mutations in colon cancer - slipping off the brake and onto the gas pedal. *Cancer Biol. Ther*. **7**(4):485-487, 2008.
5. **Boquoi, A.**, Jover, R., Chen, T., Pennings, M., Enders, GH. Transgenic expression of VEGF in intestinal epithelium drives mesenchymal cell interactions and epithelial neoplasia. *Gastroenterology* **136(**2):596-606.e4, 2009. PMC2824597
6. **Boquoi, A.**, Chen, T., Enders, G.H. Chemoprevention of mouse intestinal tumorigenesis by the cyclin-dependent kinase inhibitor SNS-032. *Cancer Prev. Res.* **2**(9):800-806, 2009.
7. von Holzen, Q., Chen, T., **Boquoi, A.**, Richter, J., Falk, G.W., Klein-Szanto, A., Cooper, H., Litwin S., Weinberg, D., Enders G.H. Evidence for DNA damage checkpoint activation in Barrett's esophagus. *Transl. Oncol.* **3**(1):33-42, 2010.