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CHARACTERIZATION OF THE ROLE OF THE PHOSPHATASE AND TENSIN
HOMOLOG, PTEN, IN DNA CROSSLINK REPAIR

BY

ELIZABETH ANN VUONO

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

CELL AND MOLECULAR BIOLOGY

UNIVERSITY OF RHODE ISLAND

2016

DOCTOR OF PHILOSOPHY DISSERTATION

OF

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2016

ABSTRACT

Fanconi Anemia (FA) is a rare autosomal and X-linked disease characterized by multiple congenital abnormalities, progressive bone marrow failure, and cancer predisposition. FA is caused by a biallelic mutation of any one of 21 genes; the proteins encoded by these genes regulate the removal of DNA interstrand crosslinks (ICLs) and are essential for maintaining chromosomal stability. This pathway is tightly regulated by posttranslational modifications (PTMs) such as ubiquitination and phosphorylation. Several kinases such as ATM, ATR and PLK1 have been linked to the pathway but no phosphatase has been firmly linked to the pathway. PTEN is a dual specificity phosphatase, capable of removing phosphate groups from phosphorylated lipids and also phosphorylated serine, threonine, and tyrosine residues, and it has recently been connected to genome stability. The goal of this research was to determine mechanistically PTEN's role in DNA repair. Through this research, we have established that PTEN is necessary for a functional FA pathway, and this is dependent on its protein phosphatase activity and its ability to be SUMOylated on residue K254.

ACKNOWLEDGMENTS

I first and foremost would like to thank my dissertation advisor, Dr. Niall G. Howlett for the opportunity to be involved with your research group. Your guidance throughout my graduate studies has been invaluable. Thank you to the faculty members for their feedback, time, and advice while serving on my thesis committee: Dr. Jay Sperry (Department of Cell and Molecular Biology), and Dr. Joanna Norris (Department of Biological Sciences). In addition, I would like to thank the chair of my defense committee, Dr. Steven Irvine (Department of Biological Sciences).

I would like to thank Dr. Ananda Mukherjee for all of his assistance and PTEN knowledge on uncovering the role of PTEN in ICL repair, Dr. Maurizio Mauro for teaching me clastogenic assays and the ability to prepare score metaphase spreads, David Vierra, Dr. Charlotte Hodson for their assistance in experiments for Manuscript II, and Dr. Andrew Deans for his guidance and collaborations on the phosphorylation of FANCM and for providing FANCM antibodies. I would like to thank Morganne Adroved for her hard work and dedication on both PTEN and ICL and PTEN SUMOylation projects.

I would also like to thank the past and present graduate students from the Howlett Lab, for your help, experimental insight, proofreading papers, abstracts, and this dissertation, but most importantly your friendship which has kept me sane through this process: Rebecca Boisvert, David Vierra, Karissa Paquin, Paul Azzinaro, and Meghan Rego.

To the Department of Cell and Molecular Biology at the University of Rhode Island, thank you for your continued guidance on my research and career advice throughout the years.

Lastly and most importantly, I would like to thank my friends and family. Your continuous love and support throughout this process has made this possible. Mom and Dad, thank you for your endless reassurance. I cannot put into words how much I appreciate and love you. Thank you to my brother Dan for always listening to my presentations and giving me feedback.

PREFACE

The following dissertation has been prepared in manuscript format. Chapter 1, “Mechanistic Insight into the function of Nuclear PTEN” was prepared for publication in *Cell Cycle* and is a review encapsulating our current understanding of the regulation and function of nuclear PTEN. Chapter 2, “The PTEN phosphatase functions cooperatively with the Fanconi anemia proteins in DNA crosslink repair” was prepared for publication in *Scientific Reports*. Chapter 3, “PTEN SUMOylation is important for DNA crosslink repair” was prepared for submission to *Oncotarget*.

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Manuscript-I

Prepared for submission to Cell Cycle

Mechanistic Insight into the role of Nuclear PTEN

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Mechanistic Insight of Nuclear PTEN

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Key Words: PTEN, DNA Repair, Replication, SUMOylation, Phosphorylation

Abstract:

PTEN is a tumor suppressor protein frequently mutated in cancers. Its main tumor suppressor function is localized in the cytoplasm, suppressing the PI3K/AKT signaling pathway by dephosphorylating phosphatidylinositol-3,4,5-triphosphate to phosphatidylinositol-4,5-bisphosphate. A separate nuclear function for PTEN has recently emerged, and there have been great efforts to understand how PTEN operates to preserve genome integrity. Here we have summarized the recent findings examining the role of nuclear PTEN.

Introduction:

Phosphatase and tensin homolog (*PTEN*) is a *bona fide* tumor suppressor gene located on chromosome 10, and it has been shown to regulate cellular proliferation and cell growth [1, 2]. *PTEN* encodes a dual specificity phosphatase capable of removing phosphate groups from both proteins and lipids [3, 4]. In 1997 Li et al. detected homozygous deletions, frameshifts, and nonsense mutations in *PTEN* in 63% of glioblastoma cell lines, 100% of prostate cancer cell lines, and 10% of breast cancer cell lines, leading to the categorization of *PTEN* as a tumor suppressor gene [1]. It was originally thought that the sole tumor suppressor function of PTEN was to dephosphorylate the lipid second messenger phosphatidylinositol-3,4,5-triphosphate (PIP₃), a potent activator of the AKT kinase [4]. PIP₃ is produced by the phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP₂) by phosphatidylinositol 3-kinase (PI3K) upon external or internal stimulation. The AKT kinase then interacts with PIP₃ for its activation, and targets many downstream signal transduction pathways preventing

apoptosis signaling, and thus promoting cell growth [5]. Loss of PTEN lipid phosphatase function leads to de-repression of the PI3K/AKT pathway and the stimulation of cell growth and survival [2, 6]. While plasma membrane localized PTEN function is central to tumor suppression, recent studies have established that PTEN has PI3K/AKT-independent nuclear tumor suppressive functions [7, 8]. Many of the mutations found in tumors are found in the phosphatase domain of PTEN, inactivating its phosphatase function, but mutations are also found outside of this domain, many of which result in truncated mutants lacking the carboxyl terminal of PTEN [1, 9, 10]. This indicates a tumor suppressor function independent of its lipid phosphatase activity and of its known role to antagonize the PI3K/AKT pathway. Recently there have been great efforts to acquire a better understanding of the role of PTEN outside its PI3K antagonist role, focusing on its nuclear function as a guardian of the genome.

Cells without functional PTEN display increased chromosome instability with a high frequency of DNA double strand breaks (DSBs) [10]. Important roles for PTEN in the regulation of cell cycle progression and the maintenance of chromosome stability have also recently been established [10-18]. This review will discuss the recent findings of PTEN nuclear function, independent of its lipid phosphatase activity. Here we will discuss PTEN's involvement in genome protection through its regulation of DNA replication, the cell cycle, stabilization of chromatin, and finally its own regulation via posttranslational modification.

PTEN:

PTEN encodes a 403 amino acid protein comprised of an N-terminal phosphatase domain and a C-terminal C2 domain (**Figure 1**). While the majority of the focus has been on the phosphatase domain, the C2 domain has also shown its importance with securing the genome through various protein-protein interactions [10, 13, 14, 16, 18]. C2 domains are known to interact and localize to cell membranes, thus promoting PTEN's lipid phosphatase activity [19]. Lastly PTEN C-terminus contains many phosphorylation sites that are important for its cellular localization and stability. Many of these phosphorylation sites are located in or around a PEST motif, which targets proteins with short intracellular half-life for proteolytic degradation [20].

PTEN and Chromatin Structure:

It was first shown in 2007 that PTEN has a nuclear tumor suppressor function independent of its canonical lipid phosphatase activity [10]. Mutations leading to truncation and loss of the C2 domain are known to cause Cowden syndrome, an autosomal dominant disease characterized by increased susceptibility to breast and thyroid cancers [9]. Additionally, *PTEN* mutations that retain intact lipid phosphatase activity have been found in multiple cancers resulting in intensive investigation of a novel tumor suppressor function. PTEN was shown to physically interact with the kinetochore subunit CENP-C, implicating it in centromere stability. Shen et al observed an increase in centromere like aberrations such as translocations in mouse embryonic fibroblast MEFs-PTEN^{-/-} compared to MEFs-PTEN^{+/+} [10]. Translocations can be easily visualized in a mouse model due to their chromosomes being acrocentric [21].

Figure 1. Domain Architecture and Structure of PTEN.

PTEN is comprised of an amino terminal phosphatase domain harboring the important catalytic residues C124 and G129. When the cystine is mutated to a serine PTEN is phosphatase dead while mutating the glycine to a glutamic acid, PTEN is only lipid phosphatase inactive. The carboxyl C2 domain contains the lysine that is SUMOylated and is shown to be important for DNA repair and the PEST (Proline, Glutamic acid, Serine, Threonine) motif [8, 11].

Interestingly, it has previously been shown that gene transcription profiles are altered in cells lacking PTEN [22]. This led the Shen group to investigate PTEN's role in gene expression and regulation by comparing chromatin architecture in PTEN^{-/-} MEFs to PTEN^{+/+} MEFs [14]. They discovered that PTEN interacts with the carboxyl terminus of histone 1 (H1) via PTEN's C2 domain [14]. H1 is a histone linker connecting nucleosome complexes thus regulating the higher order of chromatin architecture in the nucleus [23]. Nucleosomes are the core subunits of chromatin, around which DNA is wrapped and are comprised of 2 copies each of histones H2A, H2B, H3, and H4 [24, 25]. While the posttranslational modifications localized to the N- and C- terminals of the histones are important for gene accessibility or DNA repair, protein-protein interactions form complexes creating heterochromatin or gene silenced regions [26, 27]. An example of a protein known to interact with histones, Heterochromatin protein 1, HP1 α , is known to bind to methylated H3 and H1 thus condensing chromatin [27]. Chen et al. showed that PTEN stabilizes the H1-HP1 α protein complex, causing chromatin condensing into heterochromatin and genes silencing [14]. This new role of PTEN as a modulator of chromatin could be crucial for a better understanding of PTEN deficient cancers and their gene expression profiles. Also importantly, PTEN stabilization of centromeres through its interaction with the centromere specific binding protein, CENP-C, prevents chromosomal aberrations including radials and translocations that could lead to cancers and diseases [10]. CENP-C is an essential component of the kinetochore, stabilizing the centromere [28]. Through PTEN's C2 domain, PTEN interacts with CENP-C and without PTEN, the Yin lab observed an increase in chromosome aberrations [10].

PTEN in Cell Cycle Regulation:

It has been known that PTEN is involved in cell cycle regulation due to PTEN deficient cells show an arrest in G1 phase [29]. Recently Song et al discovered that PTEN directly interacts with the anaphase-promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase and its subunit CDH1. This promotes complex stability and has been shown to be independent of PTEN's phosphatase activity [13]. APC/C-CDH1 is active in late mitosis and early G1 phase of the cell cycle targeting its substrates, which includes mitotic cyclins, Cyclin A and B, and cell cycle kinases Polo-like kinase 1 (PLK1) and Aurora A kinase for proteasomal degradation [30, 31]. Cells lacking PTEN result in decreased activity of the APC/C-CDH1 complex, leading to prolonged expression of the cell cycle kinases previously mentioned and deregulated cell cycle.

In addition to PTEN regulating PLK1 protein levels by the stabilization of the APC/CDH1 complex, it also has been linked to PLK1 enzymatic regulation through its protein phosphatase activity. Zhang et al. have demonstrated that PTEN directly interacts with PLK1 and dephosphorylates it on residue T210, inactivating PLK1 kinase activity, causing a deregulation of mitosis and chromosome segregation. Interestingly, PLK1 phosphorylates PTEN on S380, leading to recruitment of PTEN to chromatin and normal mitosis progression, indicating a dynamic relationship between these two proteins [32]. The Dia lab also established that PTEN phosphorylated on S380 recruits the APC/C subunit CDH1 resulting in PTEN polyubiquitination and degradation [33]. PTEN-pS380 results in appropriate timing of mitotic entrance and exit transitioning into G1 [33]. The complex relationship between PTEN, PLK1, and APC/C-CDH1 exposes how important

the regulation of PTEN and PLK1 throughout the cell cycle is for normal cell progression.

PTEN and the Regulation of DNA Replication

PTEN has recently been connected to DNA replication and replication fork stability [15-17]. Various check points throughout the cell cycle determine genome quality, one occurring in S phase due to DNA lesions forming from faulty replication machinery [34, 35]. During their study of a PTEN carboxyl terminal deletion mutant found in individuals inflicted with Cowden syndrome, the Yin lab discovered defaults in replication [17]. This mutation not only showed that PTEN is important for DNA replication but that this function is phosphatase independent. A haplodeficient PTEN^{+/ Δ C} mouse model where one copy contained a C-terminus truncation was used to determine the cause of increased tumor development seen in Cowden syndrome patients [17]. Using a comparative genomic hybridization array to analyze genome-wide copy number changes, two well-known common fragile site (CFS) genes, *Wwox* and *Fhit*, were shown to be down regulated. CFSs are regions of DNA that display increased instability under replication stress, and fail to complete DNA synthesis [36]. Increased mutations and decreased gene expression of the genes at CFSs implies that PTEN is required for efficient replication.

This finding led the Shen lab to investigate PTEN's involvement in early S phase checkpoint regulation. PTEN null cells exhibit a lower percentage of cells in S phase arrest and an accumulation in G2/M arrest. This results in increased chromosome aberrations and anaphase bridges preventing chromosome segregation [16]. By dividing

S phase into three steps, early (S1), mid (S2), and late (S3), it was shown that, compared to wild type cells, PTEN^{-/-} cells have a reduced number of cells at all stages of S phase but more prominently in S1 indicating a role for PTEN in early S phase. This phenotype was corrected with over expression of RAD51 and CHK1. Using iPOND (isolation of proteins on nascent DNA), it was shown that both RAD51, a ssDNA binding protein essential for homologous recombination, and CHK1, a cell cycle kinase involved in DNA repair, are localized to replication forks [16]. Only loading of RAD51 was affected in PTEN^{-/-} cells, while CHK1 replication fork localization remained unaffected. Deregulation of RAD51 at DNA lesions leads to inadequate HR repair and increased DNA damage. PTEN not only stabilizes RAD51 at stalled replication forks, but also stabilizes the single-stranded DNA (ssDNA) binding protein RPA. Both RAD51 and RPA stabilization are independent of PTEN phosphatase activity. PTEN stabilizes RPA by recruiting OTUB1, the deubiquitinase (DUB) of RPA1 at stalled forks [18]. OTUB1 is a canonical OTU family DUB, targeting K48 linked ubiquitin chains [37, 38]. PTEN was shown to form a complex by interacting with RPA1 through its phosphatase domain and OTUB1 through its carboxyl terminal, allowing for the deubiquitination of RPA1, preventing its proteolytic degradation, and resulting in its stabilization of the RPA complex on ssDNA.

It was also discovered that PTEN is involved in the stabilization of the replisome *via* its phosphatase domain by dephosphorylating the MCM2 subunit of the MCM helicase on residue S41 during replication stress [15]. The MCM helicase contains 6 subunits MCM2- 7 forming a hexamer to unwind DNA for replication [39]. MCM2 is involved in all three stages of DNA replication: initiation, elongation, and termination.

DNA replication is a complex process and MCM2 has been shown to be an essential regulator of the replisome under stressful conditions, helping to prevent DNA damage. [40]. Feng et al. discovered that PTEN associated with MCM2 only under stressful replicative conditions, such as treating with the exogenous agent hydroxyurea, a chemical that prevents the production of new deoxyribonucleotides and depleting the free nucleotide pools [15]. Upon replication stress, PTEN associates with and dephosphorylates S41 of MCM2, pausing replication and preventing fork progression in order for high fidelity DNA repair to occur by the stabilization of RPA and the recruitment of RAD51.

PTEN and its Regulation by Posttranslational Modification:

Protein posttranslational modification (PTM) is an essential cellular process, which regulates complex protein pathways [41, 42]. PTMs signal proteins for localization, activation, deactivation, and for protein degradation and cellular compartmentalization [41]. PTEN is posttranslationally modified by various small molecules such as phospho and acetyl groups or by the addition of small proteins including ubiquitin and importantly, SUMO (small ubiquitin-like modifier) for regulation of its many cellular activities [8]. It has already been established that PTEN is monoubiquitinated for its nuclear localization and that phosphorylation of S380 leads to its chromatin stabilization and subsequent polyubiquitination and degradation for progression of mitosis. Recently the characterization of SUMOylation at residue K254 has been described as crucial for its nuclear tumor suppressor function [11]. The addition of SUMO to a target protein occurs in multiple biochemical steps. The first is cleavage at the C-terminal of SUMO, exposing

the di-glycine motif on the now functional SUMO protein allowing a covalent bond to a cysteine on an E1 enzyme, which creates a thioester linkage. The SUMO thioester bond can then be transferred to the sole E2 SUMO conjugating enzyme, UBC9, and with the help of a RING-domain E3 ligase, the SUMO will then be transferred from the E2 to a lysine on the target protein [43, 44]. This process can be repeated to form SUMO chains. DeSUMOylating enzymes, SENPs, can reverse this process [45].

Genome stability by DNA repair pathways are tightly regulated by posttranslational modification and recently, SUMOylation of many DNA damage response (DDR) proteins has been shown to be important for the fluency of DNA repair [46]. A DDR protein that has been shown to be SUMOylated is RPA. As previously stated, RPA binds and protects ssDNA from nucleolytic degradation. To allow for the loading of RAD51 onto the ssDNA creating the nucleofilaments for the essential strand invasion step of HR, RPA is polySUMOylated, then polyubiquitinated targeting its proteasomal degradation [46]. PTEN is SUMOylated on K254 for its removal from the nucleus upon ionizing radiation (IR) damage [11]. Cells expressing the PTEN SUMOylation incompetent mutant, K254R, display genome instability and increased error-prone repair [11]. PTEN harbors a conserved ATM (ataxia telangiectasia mutated) consensus sequence at S398, and when mutated to an alanine, PTEN is no longer able to be SUMOylated and subsequently cannot be removed from the nucleus. It has been shown that SUMOylation of PTEN is integral to its role in genome stability, but the mechanism behind PTEN SUMOylation has yet to be fully understood. PTEN SUMOylation could possibly prime PTEN for its polyubiquitination and subsequent degradation similar to the regulation of RPA, regulating its nuclear protein levels.

Conclusions

PTEN is one of the most widely mutated genes found in cancer due to its integral roles in many cellular processes. Not only is it the antagonist to the PI3K/AKT pro-survival pathway, but it also protects the genome at various levels. Here we have summarized how *PTEN* is involved in many stages of the cell cycle and gene regulation by modulation of chromatin architecture (**Figure 2**). Furthermore, *PTEN* is highly involved in DNA replication fork stability. *PTEN* is not only involved in the restart of stalled replication forks by stabilizing RPA and RAD51 for efficient DNA repair, but *PTEN* also dephosphorylates the regulatory subunit of the MCM helicase complex stabilizing the replisome and allowing efficient DNA repair and replication restart. Understanding the relationship between *PTEN* and RAD51 allows for new therapies strategies targeting cells defective in HR including PARP inhibitors. Further, understanding of how *PTEN* is involved in these cellular processes throughout the cell cycle, such as its relationship with PLK1, can establish new treatments targeting the many *PTEN* deficient tumors. PLK1 is over expressed in various tumors and chemical inhibitors against this kinase. For example, BI2536, is currently in clinical trials. Determining mechanistically how *PTEN* is involved in DNA replication stability and how specific mutations modify protein function can lead to new therapeutic approaches targeting *PTEN* deficient tumors and Cowden syndrome.

Figure 2. PTEN's DNA protection throughout the cell cycle

1. PTEN involvement in controlling chromatin architecture. First, throughout mitosis, PTEN stabilizes centromeres through the direct interaction of the kinetochore subunit CENP-C, preventing chromosome aberrations such as translocations. Second, PTEN regulates chromatin condensation through its interaction with histone H1 and HP1 α regulating gene expression.
2. PTEN and DNA replication fork stability. PTEN stabilizes the replisome when it comes in contact with a DNA lesion by dephosphorylating S41 on MCM2, a subunit of the MCM helicase. Once the replication fork is stabilized, PTEN recruits RPA's DUB, OTUB1, preventing RPA's degradation, protecting the ssDNA. PTEN then recruits and stabilizes RAD51 to the stalled replication fork, initiating HR.
3. PTEN and PLK1 relationship and the regulation of mitosis. PTEN regulates the activity of PLK1 through mitosis by two independent functions; first by dephosphorylation of PLK1 on T210, deactivating the kinase and allowing for efficient mitosis and chromosome segregation and second by stabilizing the APC/C-CDH1 E3 ubiquitin ligase, targeting PLK1 for proteolytic turnover. Interestingly, PLK1 phosphorylates PTEN on S380 in late G2, stabilizing PTEN onto chromatin and allowing cell cycle progression into M phase. PTEN pS380 signals its chromatin removal and degradation in late M phase by recruiting CDH1 and the APC/C complex for entrance into G1.

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Manuscript-II

Prepared for submission to Scientific Reports

The PTEN phosphatase functions cooperatively with the Fanconi anemia proteins in DNA crosslink repair

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Abstract

Fanconi anemia (FA) is a genetic disease characterized by bone marrow failure and increased cancer risk. The FA proteins function primarily in DNA interstrand crosslink (ICL) repair. Here, we have examined the role of the PTEN phosphatase in this process. We have established that PTEN-deficient cells, like FA cells, exhibit increased sensitivity to ICL cytotoxicity, increased levels of chromosome structural aberrations, and increased error-prone mutagenic DNA repair. The increased ICL sensitivity of PTEN-deficient cells is caused, in part, by elevated PLK1 kinase-mediated phosphorylation of FANCM, constitutive FANCM polyubiquitination and degradation, and the consequent inefficient assembly of the FA core complex, FANCD2, and FANCI into DNA repair foci. We also establish that PTEN function in ICL repair is dependent on its protein phosphatase activity, yet independent of its lipid phosphatase activity. Finally, *via* epistasis analysis, we demonstrate that PTEN and FANCD2 function cooperatively in ICL repair.

Introduction

Fanconi anemia (FA) is a rare autosomal and X-linked disease characterized by congenital abnormalities, progressive pediatric bone marrow failure, and increased cancer risk in early adulthood¹. FA is caused by mutation of any one of 21 genes (*FANCA*, *-B*, *-C*, *-D1/BRCA2*, *-D2*, *-E*, *-F*, *-G*, *-I*, *-J/BRIP1*, *-L*, *-M*, *-N/PALB2*, *-O/RAD51C*, *-P/SLX4*, *-Q/ERCC4*, *-R/RAD51*, *-S/BRCA1*, *-T/UBE2T*, *-U/XRCC2* and *-V/REV7*). The FA proteins function in the FA-BRCA pathway to facilitate the removal of DNA interstrand crosslinks (ICLs) and to maintain genome stability²⁻⁴. ICLs are highly toxic lesions that covalently link DNA strands, imposing a direct physical block to DNA replication and

transcription. The FA-BRCA pathway resolves ICLs by first converting these lesions into DNA double-strand breaks (DSBs)⁵. The DSB is then repaired by homologous recombination (HR), a conservative DNA repair mechanism that uses the homologous DNA sequence on a sister chromatid or homologous chromosome as a template to copy and repair the damaged DNA. The RAD51 protein is the major cellular HR protein and catalyzes the critical strand invasion step of HR^{6,7}.

Eight FA proteins, as well as several accessory proteins, form the FA core complex, a multisubunit E3 ubiquitin ligase that catalyzes the monoubiquitination of the FANCD2 and FANCI proteins^{2,8}. FANCD2/I monoubiquitination is an essential step in the activation of the FA-BRCA pathway^{8,9}. Monoubiquitinated FANCD2 co-localizes in discrete chromatin-associated nuclear foci with several DNA repair proteins, including RAD51/FANCR and BRCA2/FANCD1^{10,11}. FANCD2 and BRCA2/FANCD1 function together to promote RAD51/FANCR nucleoprotein filament formation and DNA strand exchange during HR^{10,12,13}.

In addition to being regulated by ubiquitination, the FA-BRCA pathway is also extensively regulated *via* phosphorylation. For example, FANCD2 and FANCI are phosphorylated by the two major DNA damage response kinases ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3-related)¹⁴⁻¹⁷. FANCI phosphorylation on six clustered SQ/TQ motifs is required for its monoubiquitination and nuclear foci formation¹⁶. In addition, FANCM is hyperphosphorylated by PLK1 during mitosis, promoting its ubiquitination and degradation by the proteasome¹⁸. Importantly, to date, no phosphatases have been directly linked to the FA-BRCA pathway.

PTEN encodes a dual specificity phosphatase capable of removing phosphates from both proteins and lipids^{19,20}. The principal catalytic function of PTEN is to dephosphorylate the lipid second messenger phosphatidylinositol-3,4,5-triphosphate (PIP₃), a potent activator of the AKT kinases²⁰. As a consequence, loss of PTEN catalytic function leads to de-repression of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway and stimulation of cell growth and survival pathways^{21,22}. While this plasma membrane localized PTEN function is central to tumor suppression, recent studies have established that PTEN has PI3K/AKT-independent nuclear tumor suppressive functions^{23,24}. Indeed, important roles for PTEN in the regulation of cell cycle progression and the maintenance of chromosome stability have recently been established²⁵⁻²⁸.

In this study, we have investigated the role of PTEN in ICL repair and in the regulation of the FA-BRCA pathway. We have established that PTEN plays an important role in ICL repair as PTEN-deficient cells, like FA patient cells, exhibit increased sensitivity to ICL-mediated cytotoxicity, and display increased levels of chromosome structural aberrations following ICL exposure. The increased ICL sensitivity of PTEN-deficient cells is caused, in part, by elevated PLK1 kinase-mediated phosphorylation of FANCM, constitutive FANCM polyubiquitination and degradation, and the consequent inefficient assembly of the FA core complex, FANCD2, and FANCI into DNA repair foci. We also show that PTEN function in ICL repair is independent of its lipid phosphatase activity yet dependent on its protein phosphatase activity. We also establish that PTEN deficiency leads to increased mutagenic ICL repair, exemplified by increased 53BP1 and DNA-PKcs-pS2056 nuclear foci formation, biomarkers of the error-prone nonhomologous DNA end joining (NHEJ) repair pathway. Finally, using an RNA

interference approach in FA-D2 patient cells and PTEN-deficient tumor lines, we demonstrate that PTEN and FANCD2 function epistatically during ICL repair. Our results uncover important mechanistic insight into the role of nuclear PTEN in ICL repair and establish the convergence of two critical tumor suppressor pathways.

Results

PTEN is required for chromosome stability and cellular survival following mitomycin C treatment

To investigate the role of PTEN in ICL repair we treated isogenic HCT116 PTEN^{+/+} and PTEN^{-/-} cells with mitomycin C (MMC) and examined cellular cytotoxicity and metaphase chromosome aberrations. Similar to FA patient cells that are characteristically sensitive to ICL-inducing agents^{29,30} two independently derived PTEN^{-/-} lines exhibited increased sensitivity to MMC. The calculated LD₅₀ values for PTEN^{+/+} cells were 2-fold greater than those for both PTEN^{-/-} lines (**Figure S1A**). PTEN^{-/-} cells also exhibited increased spontaneous and MMC-inducible chromosome gaps and breaks and complex aberrations, including radial formations (**Figures 1A-C**). For example, we observed a ~4-fold increase ($P < 0.05$) in spontaneous gaps and breaks in PTEN^{-/-} #22 cells compared with PTEN^{+/+} cells (**Figure 1B**). Importantly, the chromosome instability phenotype of PTEN^{-/-} cells could be rescued by re-expression of wild-type PTEN (see **Figure 6**). We next examined the role of PTEN in ICL repair in a non-transformed cell model using the isogenic mammary epithelial cells MCF10A PTEN^{+/+} and PTEN^{-/-}. Again PTEN^{-/-} cells exhibited increased sensitivity to the cytotoxic effects of MMC (**Figure S1B**). We also

Figure 1. PTEN^{-/-} cells are hypersensitive to the clastogenic effects of mitomycin C

HCT116 and MCF10A PTEN^{+/+} and PTEN^{-/-} cells were incubated in the absence or presence of mitomycin C (MMC) for 24 h and metaphase spreads were analyzed for numerical and structural chromosome aberrations.

(A) Representative images of the types of chromosome aberrations - including radial formations, telomere fusions, dicentrics, and complex aberrations - observed in PTEN^{-/-} cells following MMC treatment.

(B and C) Quantification of chromosome gaps and breaks (B) and total chromosome aberrations (C) observed in HCT116 PTEN^{+/+} and two independent clones of PTEN^{-/-} cells incubated in the absence or presence of 20 nM MMC for 24 h.

(D and E) Quantification of chromosome gaps and breaks (D) and total chromosome aberrations (E) observed in MCF10A PTEN^{+/+} and PTEN^{-/-} cells incubated in the absence or presence of 24 nM MMC for 24 h. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

observed an increased frequency of both spontaneous and MMC-inducible chromosome gaps and breaks and complex aberrations in the MCF10A PTEN^{-/-} cells compared to PTEN^{+/+} cells (**Figures 1A and D**). A ~4-fold ($P < 0.001$) increase in spontaneous complex chromosome aberrations and a ~2-fold ($P < 0.05$) increase in MMC-inducible complex aberrations were observed for MCF10A PTEN^{-/-} cells, compared to PTEN^{+/+} cells (**Figures 1A and E**). MCF10A PTEN^{-/-} cells also exhibited a striking increase in both spontaneous and ICL-inducible centromere aberrations, exemplified by de-condensed centromeres, similar to that previously described²⁷ (**Figures S1C and D**).

PTEN is required for efficient MMC-inducible FANCD2 and FANCI nuclear foci formation

To gain insight into the molecular basis of the increased ICL sensitivity of PTEN-deficient cells, we examined the activation of the FA-BRCA pathway in these cells, a pathway known to play a critical role in the cellular ICL response³. Activation of this pathway occurs *via* the site-specific monoubiquitination of the FANCD2 and FANCI proteins and their assembly into discrete nuclear foci^{9,31,32}. HCT116 PTEN^{+/+} and PTEN^{-/-} cells were treated with MMC for 16 h followed by a 24 h recovery period. Following MMC treatment, PTEN^{+/+} cells exhibited a strong increase in FANCD2 and FANCI nuclear foci formation with ~60% of cells displaying greater than 5 discrete nuclear foci (**Figures 2A and B**). In contrast, no appreciable induction of FANCD2 or FANCI nuclear foci formation was observed for PTEN^{-/-} cells following MMC exposure (**Figures 2A and B**). Re-expression of PTEN in PTEN^{-/-} cells restored efficient MMC-inducible FANCD2 or FANCI nuclear foci formation (see **Figures 6C and D**). No overt differences in MMC-inducible FANCD2 or FANCI monoubiquitination were observed

Figure 2. PTEN is required for efficient mitomycin C-inducible FANCD2 and FANCI nuclear foci formation

(A) HCT116 PTEN^{+/+} and PTEN^{-/-} cells were incubated in the absence or presence of 40 nM mitomycin C (MMC) for 18 h and FANCD2 and FANCI nuclear foci formation were analyzed by immunofluorescence microscopy. Representative images of FANCD2 and FANCI nuclear foci from cells exposed to MMC are shown.

(B) Quantification of the percentage of PTEN^{+/+} and PTEN^{-/-} nuclei displaying greater than five discrete FANCD2 or FANCI nuclear foci. Cells were incubated in the absence (NT) or presence of 40 nM MMC for 18 h and allowed to recover for up to 24 h. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(C) Immunoblotting for FANCD2, FANCI, and RAD51 reveals no appreciable differences in levels of MMC-inducible FANCD2 and FANCI monoubiquitination or RAD51 between PTEN^{+/+} and PTEN^{-/-} cells. Cells were incubated in the absence or presence of 200 nM MMC for 24 h and allowed to recover for up to 24 h. α -Tubulin was used as a loading control. To improve clarity and conciseness, the presented blots have been cropped. All gels were run under the same experimental conditions.

between PTEN^{+/+} and PTEN^{-/-} cells (**Figure 2C**). Furthermore, using a cellular fractionation approach, we did not observe appreciable differences in the chromatin enrichment of FANCD2 or FANCI in the absence of PTEN (**Figure S2A**). Very similar results were obtained with MCF10A PTEN^{-/-} cells, which exhibited markedly reduced levels of MMC-inducible FANCD2 and FANCI nuclear foci, compared with PTEN^{+/+} cells (**Figures S2B and C**), but no discernible differences in MMC-inducible FANCD2 or FANCI monoubiquitination or chromatin enrichment (**Figures S2D and E**).

Increased FANCM instability and defective chromatin recruitment of the FA core complex in PTEN-deficient cells

Previous studies have established that the FANCM protein is required for efficient recruitment of the FA core complex to chromatin and for the assembly of FANCD2 and FANCI nuclear foci^{33,34}. Therefore we examined FANCM expression in PTEN^{+/+} and PTEN^{-/-} cells. In the absence of PTEN, we observed reduced levels of FANCM (**Figures S3A and B**). Modest reductions in total cellular levels of FANCA were also observed (**Figures S3A and B**). A cycloheximide pulse-chase experiment revealed that reduced FANCM levels were a consequence of increased FANCM turnover: increased FANCM instability could be rescued by inhibition of the proteasome with MG132 (**Figures 3A and S3C**). p53 was used a positive control for these experiments because of its fast proteolytic turnover. Furthermore, we observed reduced chromatin-associated FANCM and ICL-inducible FANCM nuclear foci formation in the absence of PTEN (**Figures 3B, 3C, S3D, and S3G**). Consistent with an important role for FANCM in promoting the

Figure 3. Increased FANCM instability and defective chromatin recruitment of the FA core complex in PTEN-deficient cells

(A) HCT116 PTEN^{+/+} and PTEN^{-/-} cells were incubated in the absence or presence of 40 µg/mL cycloheximide (CHX) alone or 40 µg/mL CHX and 4 µM MG132 (CHX + MG) for the indicated times. Whole-cell lysates were prepared and immunoblotted with anti-FANCM, anti-p53, and anti-PTEN antibodies. RBI, Relative protein band intensity.

(B) Chromatin fractionation analysis of MCF10A PTEN^{+/+} and PTEN^{-/-} cells reveals a defect in the chromatin localization of FANCM and FANCA in the absence of PTEN. Cells were incubated in the absence (NT) or presence of 200 nM mitomycin C (MMC) for 24 h. W, unfractionated whole-cell lysate; S, soluble cytoplasmic and nuclear fraction; C, chromatin fraction. For (A) and (B), to improve clarity and conciseness, the presented blots have been cropped. All gels were run under the same experimental conditions. C:S, Ratio of protein in the chromatin versus soluble fraction.

(C) Quantification of FANCM nuclear foci formation in MCF10A PTEN^{+/+} and PTEN^{-/-} cells reveals a defect in MMC-inducible FANCM nuclear foci formation in PTEN^{-/-} cells. Cells were incubated in the absence (NT) or presence of 200 nM MMC for 18 h. ***, $P < 0.001$.

(D) Quantification of FANCA nuclear foci formation in MCF10A PTEN^{+/+} and PTEN^{-/-} cells reveals reduced FANCA nuclear foci formation in PTEN^{-/-} cells both in the absence (NT) and presence of MMC. Cells were treated as described for (C). ***, $P < 0.00$

chromatin localization of the FA core complex, we also observed defective ICL-inducible FANCA chromatin localization and nuclear foci formation in PTEN^{-/-} cells (**Figures 3B, 3D, S3E, S3F, and S3H**).

Inhibition of PLK1 rescues defective FANCD2 nuclear foci formation but is not sufficient to rescue the chromosome instability of PTEN^{-/-} cells

Previous studies have established that nuclear PTEN stabilizes the APC/C (anaphase-promoting complex/cyclosome) E3 ubiquitin ligase complex and, in the absence of PTEN, there is an increase in the activity of APC/C substrates as they escape ubiquitin-mediated proteolysis²⁸. One such APC/C target is the PLK1 mitotic kinase²⁸. PLK1 phosphorylates FANCM, a FA core complex member, and primes it for polyubiquitination and degradation during mitosis¹⁸. To determine if increased PLK1 activity contributes to the increased FANCM instability observed in PTEN^{-/-} cells, we incubated cells in the absence and presence of MMC and the small molecule PLK1 inhibitor BI2536^{35,36}. We observed a marked stabilization of FANCM in both PTEN^{+/+} and PTEN^{-/-} cells following BI2536 treatment, stabilization being more pronounced in PTEN^{-/-} cells (**Figure 4A**). Furthermore, higher molecular weight FANCM isoforms, which are consistently more evident in PTEN^{-/-} cells (**Figures 4A and B, compare lanes 1 and 5**), become reduced upon BI2536 treatment (**Figure 4A**). A λ -phosphatase assay indicates that these higher molecular weight FANCM isoforms most likely represent both phosphorylated and polyubiquitinated FANCM, consistent with previous findings¹⁸, and that FANCM is particularly unstable in PTEN^{-/-} cells following MMC exposure (**Figure**

Figure 4. Inhibition of PLK1 rescues defective FANCD2 nuclear foci formation but is not sufficient to rescue the chromosome instability of PTEN^{-/-} cells

(A) HCT116 PTEN^{+/+} and PTEN^{-/-} cells were incubated in the absence (-) and presence (+) of 100 nM BI2536 and 500 nM mitomycin C (MMC) for 18 h, and whole-cell lysates were immunoblotted with the indicated antibodies. L:S, Ratio of phosphorylated to unphosphorylated FANCM.

(B) PTEN^{+/+} and PTEN^{-/-} cells were incubated in the absence and presence of 200 nM MMC for 24 h. Whole-cell lysates were then incubated in the absence (-) or presence (+) of 10 U/μg λ-phosphatase for 4 h at 30 °C, followed by immunoblotting with the indicated antibodies. For (A) and (B), to improve clarity and conciseness, the presented blots have been cropped. All gels were run under the same experimental conditions. RBI, Relative unmodified FANCM protein band intensity.

(C) PTEN^{+/+} and PTEN^{-/-} cells were incubated in the absence or presence of 2 nM BI2536, 20 nM MMC, or both BI2536 and MMC for 24 h and metaphase spreads were analyzed for the presence of numerical and structural chromosome aberrations.

(D) PTEN^{-/-} cells stably expressing empty vector or wild-type PTEN were incubated in the absence or presence of 5 nM BI2536, 200 nM MMC, or both BI2536 and MMC for 24 h, and allowed to recover for 8 h, and FANCD2 nuclear foci formation were analyzed by immunofluorescence microscopy. ***, $P < 0.001$, in comparison to cells incubated without BI2536.

4B). To determine if PLK1 inhibition could rescue the ICL sensitivity of PTEN^{-/-} cells, we co-treated cells with MMC and BI2536 and examined metaphase spreads for chromosome aberrations. While BI2536 treatment resulted in a slight reduction in levels of ICL-inducible chromosome aberrations in PTEN^{-/-} cells, this decrease was not statistically significant at the concentrations tested (**Figure 4C**). Higher concentrations of BI2536 lead to M-phase arrest precluding metaphase chromosome analyses. BI2536-mediated PLK1 inhibition did, however, rescue the defective MMC-inducible FANCD2 nuclear foci formation in PTEN^{-/-} cells expressing empty vector (**Figure 4D**). Importantly, 8 h following exposure to MMC and BI2536, while the percentage of PTEN^{+/+} nuclei exhibiting FANCD2 nuclear foci had returned to pre-exposure levels, FANCD2 nuclear foci persisted in PTEN^{-/-} cells (**Figure 4D**). As increased FANCM phosphorylation in PTEN^{-/-} cells could be a consequence of increased PLK1 kinase activity or decreased PTEN phosphatase activity, we performed an *in vitro* kinase and phosphatase assay with purified FANCM, PLK1, and PTEN. While we observed robust PLK1-mediated FANCM phosphorylation, under the conditions tested, we did not observe any appreciable decrease in levels of phosphorylated FANCM upon incubation with PTEN (**Figure S4**). Taken together, these results indicate that, in the absence of PTEN, elevated PLK1 activity contributes to increased FANCM proteolysis leading to inefficient chromatin recruitment of the FA core complex and attenuated FANCD2 nuclear foci formation.

Figure 5. Increased γ H2AX, 53BP1, and DNA-PKcs pS2056 nuclear foci formation in PTEN-deficient cells

(A) Quantification of γ H2AX nuclear foci formation in HCT116 PTEN^{+/+} and PTEN^{-/-} cells reveals persistent elevated levels of γ H2AX nuclear foci in PTEN^{-/-} cells following mitomycin C (MMC) treatment. Cells were incubated in the absence (NT) or presence of 40 nM MMC for 18 h and allowed to recover for up to 24 h. ***, $P < 0.001$.

(B) Quantification of 53BP1 nuclear foci formation in PTEN^{+/+} and PTEN^{-/-} cells reveals persistently elevated levels of 53BP1 nuclear foci in PTEN^{-/-} cells following MMC treatment. Cells were treated as described for (A).

(C) Quantification of DNA-PKcs pS2056 nuclear foci formation in PTEN^{+/+} and PTEN^{-/-} cells reveals persistently elevated levels of DNA-PKcs pS2056 nuclear foci in PTEN^{-/-} cells. Cells were treated as described for (A and B).

(D) Quantification of FK2 nuclear foci formation reveals no overt differences in levels of mono-, multi-, or poly-ubiquitin conjugates between PTEN^{+/+} and PTEN^{-/-} cells. Cells were treated as described for (A-C). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Increased γ H2AX, 53BP1 and DNA-PKcs pS2056 nuclear foci formation in PTEN-deficient cells To gain further insight into the underlying mechanisms of chromosome instability in PTEN-deficient cells, we next examined the levels of several DNA damage response biomarkers in PTEN^{+/+} and PTEN^{-/-} cells using immunofluorescence microscopy. Cells were treated with MMC for one cell cycle and allowed to recover for up to 24 h following exposure. We observed a large increase in γ H2AX nuclear foci formation in both PTEN^{+/+} and PTEN^{-/-} cells following MMC exposure (**Figure 5A**). Following 24 h recovery, the percentage of PTEN^{+/+} nuclei exhibiting γ H2AX nuclear foci had returned to pre-exposure levels. However, in contrast, γ H2AX nuclear foci persisted in PTEN^{-/-} nuclei at this time point (**Figure 5A**). We also observed a large increase in 53BP1 nuclear foci formation in both PTEN^{+/+} and PTEN^{-/-} cells following MMC exposure (**Figure 5B**). However, persistently elevated levels of PTEN^{-/-} nuclei staining positive for 53BP1 nuclear foci formation were observed for up to 24 h following MMC treatment (**Figure 5B**). Similar findings of increased and/or persistent γ H2AX and 53BP1 nuclear foci were observed for MCF10A PTEN^{-/-} cells (**Figures S5A and B**). We also observed increased levels of chromatin-associated 53BP1 in PTEN^{-/-} cells in the absence and presence of MMC (**Figure S5C**). Next we examined DNA-PKcs pS2056 nuclear foci formation, a biomarker of NHEJ^{37,38}, in PTEN^{+/+} and PTEN^{-/-} cells. Similar to γ H2AX and 53BP1, persistently elevated levels of DNA-PKcs pS2056 nuclear foci formation were observed in PTEN^{-/-} nuclei at all time points following MMC treatment (**Figure 5C**). In contrast, no differences in overall levels of mono-, multi-, or poly-ubiquitin conjugates between PTEN^{+/+} and PTEN^{-/-} cells were observed (**Figure 5D**). Collectively, these results strongly suggest that PTEN-deficient cells have a defect

in efficient DSB repair, possibly as a consequence of aberrant DSB end processing, and preferentially use mutagenic DSB repair pathways such as NHEJ. Notably, we did not observe any significant differences in levels of RAD51 protein expression (**Figures 2C and S2D**), chromatin enrichment (**Figures S2A and E**), or nuclear foci formation (**Figures S5D and E**), between PTEN^{+/+} and PTEN^{-/-} cells for both HCT116 and MCF10A cell models.

PTEN function in ICL repair is protein phosphatase dependent

To gain further mechanistic insight into the role of PTEN in ICL repair, we complemented HCT116 PTEN^{-/-} cells with wild type PTEN, lipid phosphatase-defective PTEN-G129E, and lipid and protein phosphatase-defective PTEN-C124S^{19,25,39}. The PTEN variants behaved as predicted with only wild type able to down-regulate the PI3K/AKT pathway, as demonstrated by loss of AKT-pS473 signal (**Figure 6A**). We exposed these cells to MMC and analyzed metaphase chromosomes for the presence of gaps, breaks, and radial formations. Both wild type PTEN and the PTEN-G129E mutant rescued the sensitivity of PTEN^{-/-} cells to MMC-induced chromosome damage. However, expression of the PTEN-C124S failed to rescue the ICL sensitivity of PTEN^{-/-} cells (**Figure 6B**). Cells stably expressing PTEN-C124S exhibited increased chromosome gaps and breaks and radial formations compared with cells expressing wild-type PTEN, following exposure to MMC ($P < 0.001$) (**Figure 6B**). Furthermore, analysis of FANCD2 and FANCI nuclear foci formation in these cells revealed inefficient MMC-inducible FANCD2 and FANCI nuclear foci formation in PTEN^{-/-} cells stably expressing PTEN-C124S, similar to cells expressing LacZ (**Figures 6C and D**). To further confirm the

Figure 6. PTEN function in ICL repair is protein phosphatase dependent

(A) HCT116 PTEN^{-/-} cells were stably transduced with pLenti6.2-LacZ, -PTEN-WT, -PTEN-C124S, and -PTEN-G129E. Whole-cell lysates were prepared and immunoblotted for PTEN, AKT, AKT pS473, RAD51, and α -tubulin. To improve clarity and conciseness, the presented blots have been cropped. All gels were run under the same experimental conditions.

(B) PTEN-C124S fail to rescue the sensitivity of PTEN^{-/-} cells to the clastogenic effects of mitomycin C (MMC), in contrast to PTEN-WT or PTEN-G129E. Cells were incubated in the absence (NT) or presence of 20 nM MMC for 24 h and metaphase spreads were analyzed for structural chromosome aberrations. ***, $P < 0.001$.

(C and D) Defective FANCD2 (C) and FANCI (D) nuclear foci formation in PTEN^{-/-} cells expressing LacZ, and PTEN-C124S. Cells were incubated in the absence (NT) or presence of 200 nM MMC for 18 h. ***, $P < 0.001$.

requirement for PTEN protein (but not lipid) phosphatase activity, we also show that PTEN^{-/-} cells stably expressing PTEN-Y138L are ICL sensitive and fail to properly form FANCD2 and FANCI nuclear foci formation (**Figures S6A-D**). This missense mutation was previously shown to abrogate PTEN protein phosphatase activity but not lipid phosphatase activity⁴⁰. Moreover, while wild type PTEN and the PTEN-G129E mutant rescued defective ICL-inducible FANCA and FANCM nuclear foci formation, the PTEN-C124S, and Y138L mutants did not (**Figures S6E and F**). Taken together, these results establish that PTEN functions in the activation of the FA pathway, and ICL repair is lipid phosphatase-independent.

PTEN and FANCD2 epistasis analysis

To determine if PTEN functions epistatically with the FA proteins in ICL repair, we used an RNA interference approach to deplete PTEN in FA-D2 (*FANCD2*^{-/-}) patient-derived cells stably expressing empty vector or pLenti6.2-FANCD2 (FA-D2 + FANCD2), and examined their response to MMC. Increased levels of AKT S473 phosphorylation confirmed the functional depletion of PTEN in FA-D2 and FA-D2 + FANCD2 cells (**Figure 7A**). Consistent with our earlier findings demonstrating an important role for PTEN in ICL repair, depletion of PTEN in FA-D2 + FANCD2 cells resulted in increased sensitivity to the cytotoxic effects of MMC (**Figure 7B**). However, no differences in sensitivity to MMC cytotoxicity were observed between FA-D2 cells transfected with control non-targeting siRNA or PTEN siRNA, or FA-D2 + FANCD2 cells transfected with PTEN siRNA (**Figure 7B**). Similarly, depletion of PTEN in FA-D2 cells did not lead to a further increase in the numbers of chromosome gaps, breaks or complex

Figure 7. PTEN functions epistatically with FANCD2 in ICL repair

(A) siRNA-mediated knockdown of PTEN in FA-D2 (*FANCD2*^{-/-}) and FA-D2 + FANCD2 cells. Increased levels of AKT S473 phosphorylation confirmed the functional depletion of PTEN. To improve clarity and conciseness, the presented blots have been cropped. All gels were run under the same experimental conditions.

(B) Knockdown of PTEN (siPTEN) in FA-D2 + FANCD2 cells leads to increased sensitivity to mitomycin C (MMC) cytotoxicity. In contrast, knockdown of PTEN in FA-D2 (*FANCD2*^{-/-}) cells does not lead to a further increase in sensitivity to MMC cytotoxicity. siCtrl, non-targeting siRNA.

(C and D) Metaphase chromosome analysis of FA-D2 (*FANCD2*^{-/-}) cells reveals that knockdown of PTEN does not lead to a further increase in the levels of chromosome gaps and breaks (C) or total chromosome aberrations (D), including radial formations, dicentrics, and complex aberrations.

aberrations (**Figures 7C, D and S7A**). We also performed the reciprocal analyses whereby we knocked down FANCD2 in the PTEN-deficient prostate carcinoma cell line PC-3 again using siRNA (**Figure S7B**). No increase in MMC-sensitivity was observed upon knockdown of FANCD2 in PC-3 cells (**Figure S7C**). Similarly, depletion of FANCD2 in PC-3 cells did not lead to an increase in chromosome gaps, breaks or complex aberrations (**Figures S7D-F**). Collectively, these results strongly suggest that FANCD2 and PTEN function epistatically in the cellular ICL response.

Discussion

PTEN is one of the most widely mutated genes in cancer⁴¹⁻⁴³. While a major tumor suppressive function of PTEN is the regulation of PI3K/AKT signaling, several recent studies have indicated an additional and important PI3K/AKT-independent function for PTEN in the maintenance of chromosome stability^{25,27,44}. However, this function remains poorly defined. Here, we have expanded our understanding of the function and regulation of PTEN in the maintenance of chromosome stability by specifically analyzing the role of PTEN in ICL repair. We demonstrate that cells lacking PTEN are phenotypically similar to cells from FA patients and exhibit increased ICL-sensitivity. We also establish a novel requirement for PTEN in the activation of the FA-BRCA pathway: PTEN is necessary for efficient ICL-inducible FANCD2 and FANCI nuclear foci formation. In contrast, ICL-inducible FANCD2 and FANCI monoubiquitination and chromatin enrichment are intact in the absence of PTEN. The uncoupling of monoubiquitination and chromatin enrichment from nuclear foci formation and effective ICL repair has previously been observed: *Usp1*^{-/-} murine embryonic fibroblasts display elevated levels of

monoubiquitinated Fancd2 and Fancd2 chromatin localization, but fail to support Fancd2 nuclear foci formation, and exhibit ICL-hypersensitivity⁴⁵. Similarly, Fancm or Fancs/Brca1 knockout mouse cells show Fanci and Fancd2 monoubiquitination after high doses of DNA damage, but do not properly localize these proteins into DNA damage foci^{46,47}. These findings emphasize that the assembly of FANCD2 and FANCI into nuclear foci is critical for efficient ICL repair. It is highly likely that the targeting of FANCD2 and FANCI to specific genomic loci, for example actively transcribed regions where co-transcriptional RNA-DNA hybrids (R-loops) arise, is essential for the promotion of efficient ICL repair^{48,49}. Indeed, ongoing studies in our laboratory indicate that targeting to specific histone methyl marks is key for the assembly of FANCD2 and FANCI nuclear foci and efficient ICL repair (Paquin, K.L. and Howlett, N.G., unpublished findings).

Here, we also provide novel mechanistic insight into the role of PTEN in the promotion of efficient ICL repair (**Figure S8**). We establish a requirement for PTEN protein phosphatase activity, but not its lipid phosphatase activity, in ICL repair. PTEN has recently been shown to dephosphorylate MCM2 S41 and PLK1 T210^{50,51}, and both of these mechanisms are likely to contribute to the observed phenotypes of PTEN^{-/-} cells. Dephosphorylation of MCM2 S41 prevents replication fork progression under conditions of replication stress⁵⁰. ICLs are well known to pose a potent block to replication fork progression⁵². PLK1 T210 dephosphorylation leads to its deactivation and destabilization⁵¹. As PLK1 phosphorylates FANCM during mitosis and promotes its polyubiquitination and degradation by the proteasome¹⁸, a failure to deactivate PLK1 results in constitutive FANCM polyubiquitination and degradation. Nuclear PTEN has

also been shown to promote the assembly of the APC/C-CDH1 ubiquitin ligase complex²⁸. In the absence of PTEN, APC/C-CDH1 substrates, such as PLK1 and Aurora A kinase escape ubiquitin-mediated proteolysis and remain active²⁸. In our study, we demonstrate that FANCM protein instability and FANCD2 nuclear foci formation can be rescued by inhibition of the proteasome and PLK1, respectively, supporting a model whereby, in the absence of PTEN, constitutive PLK1 activity leads to constitutive FANCM polyubiquitination and degradation, precluding efficient activation of the FA pathway. It is also important to note that the relationship between PTEN and PLK1 is complex; in addition to PTEN dephosphorylating PLK1⁵¹ and promoting the assembly of the APC/C-CDH1 complex²⁸, PLK1 directly phosphorylates PTEN on S380 promoting its accumulation in chromatin⁵³.

Finally, using FA-D2 patient cells, PTEN-deficient tumor lines, and siRNA targeting FANCD2 and PTEN, we have also established that PTEN and FANCD2 function largely epistatically during the process of ICL repair, as the combined loss of both proteins conferred no greater sensitivity to the cytotoxic or clastogenic effects of MMC. These findings are consistent with PTEN functioning upstream of FANCD2 and FANCI activation as discussed. Similar to FA patient cells, we have also established that error-prone DNA repair pathways are preferentially activated in the absence of PTEN^{37,61}. PTEN-deficient cells display elevated and persistent levels of 53BP1 and DNA-PKcs pS2056 nuclear foci following ICL exposure, indicative of increased usage of the typically mutagenic NHEJ DNA repair pathway. Persistent 53BP1 nuclear foci have also been observed in PTEN^{-/-} cells exposed to ionizing radiation²⁵. The defect in the timely dissolution of γ H2AX nuclear foci formation and the increased incidence of

chromosome structural aberrations in $PTEN^{-/-}$ cells observed in our study are also indicative of an increased reliance on NHEJ. The reasons for NHEJ bias in FA and $PTEN$ -deficient cells remain to be determined. A previous study had established that $PTEN$ and the E2F-1 transcription factor cooperate to regulate the transcription of $RAD51$ ²⁷. As DSBs can be repaired *via* HR or NHEJ, the increased NHEJ bias in $PTEN$ -deficient cells may be a direct consequence of compromised HR due to reduced $RAD51$ protein levels²⁷. However, consistent with the findings of others⁶², we did not observe appreciable differences in $RAD51$ expression, chromatin enrichment, or nuclear foci formation between $PTEN^{+/+}$ and $PTEN^{-/-}$ cells. Elegant studies on ICL repair using *Xenopus* egg extracts have recently established that $RAD51$ ICL binding occurs upstream of DSB formation and is not affected by the absence of FANCD2 or FANCI, indicating that they are most likely not required for $RAD51$ chromatin loading⁶³. FANCD2 is, however, necessary for the dual incisions flanking the ICL that occur prior to DSB formation⁶⁴. In the absence of $PTEN$, inefficient FANCD2 and FANCI nuclear foci formation most likely leads to a failure to generate these incisions resulting in the subversion of repair from error-free HR to error-prone NHEJ. Accordingly, several studies have established an important role for $PTEN$ in HR^{25,65}. As HR-deficient tumors are hypersensitive to inhibitors of poly (ADP-ribose)-polymerase-1 (PARP)^{66,67}, our studies, and those of others, continue to raise the prospect of tailored combination chemo- and radiation therapeutic approaches for $PTEN$ -deficient tumors.

Materials and Methods

Cell culture

HCT116 $PTEN^{+/+}$, $PTEN^{-/-}$ #22, and $PTEN^{-/-}$ #35⁶⁸ (a kind gift from Todd Waldman, Lombardi Cancer Center, Georgetown University) were grown in McCoy's medium supplemented with 12% v/v FBS, 1% v/v L-glutamine, and 1% v/v penicillin/streptomycin. Stably complemented HCT116 lines were generated by transduction with pLenti6.2/V5-DEST (Invitrogen) lentivirus harboring wild type or mutant *PTEN* cDNAs. Stable cell lines were grown in McCoy's medium supplemented with 5 μ g/ml blasticidin. FA-D2 (*FANCD2*^{hy/-}) cells were purchased from Coriell Cell Repositories (Catalog ID GM16633)⁶⁹. Corrected FA-D2 cells were generated by transduction with pLenti6.2-FANCD2 lentivirus, followed by selection in DMEM supplemented with 15% v/v FBS, 1% v/v L-glutamine, 1% v/v penicillin/streptomycin and 2 μ g/ml blasticidin. The PC-3 prostate adenocarcinoma line (a kind gift from David Mills, Department of Medicine, Brown University) was grown in DMEM supplemented with 12% v/v FBS, 1% v/v L-glutamine, and 1% v/v penicillin/streptomycin. MCF10A $PTEN^{+/+}$ and $PTEN^{-/-}$ cells (Horizon Discovery, U.K.) were grown in DMEM F12 supplemented with 5% v/v horse serum, 20 ng/ml epidermal growth factor, 0.5 mg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 μ g/ml insulin, 1% v/v L-glutamine, and 1% v/v penicillin/streptomycin. Detailed descriptions of plasmids, site-directed mutagenesis, siRNA transfections, and chromosome breakage, cytotoxicity, and cell proliferation assays are included in the Supplemental Experimental Procedures.

Immunoblotting and antibodies

For immunoblotting analysis, cell suspensions were washed in ice-cold PBS and lysed in 50 mM Tris.Cl pH 7.4, 1.0% v/v NP-40, 0.25% w/v Deoxycholic acid, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1 mM Na₃O₄V, 1 mM NaF, plus protease inhibitors cocktail (Roche). The cellular fractionation method is described in the Supplemental Experimental Procedures. Proteins were resolved on NuPAGE 3-8% w/v Tris-Acetate or 4–12% w/v Bis-Tris gels (Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membranes. The following antibodies were used: rabbit polyclonal anti-53BP1 (sc-22760; Santa Cruz Biotechnology), anti-FANCA (ABP6201; Cascade), anti-FANCD2 (NB100-182; Novus Biologicals), anti-FANCI (Dr. Patrick Sung, Yale University and A301-254A; Bethyl Laboratories), anti-FANCM (a kind gift from Dr. Ruhikanta Meetei, Cincinnati Children's Hospital), anti-FANCM (3821; Fanconi Anemia Research Fund), anti-H2A (07-146; Millipore), and anti-PTEN (9559;Cell Signaling), and mouse monoclonal anti-FANCM (CP3.2, CV11.1, and CV5.1 anti-γH2AX (05-636;Millipore), anti-PTEN (6H2.1;Cascade), anti-RAD51 (sc-8349; Santa Cruz), and anti-α-tubulin (MS-581-PO; Lab Vision). Band intensity was measured using ImageJ software.

***In vitro* phosphatase assays**

Recombinant FANCM and PTEN were purified as previously described^{70,71}. 20 μL reactions containing FANCM (0.2 μg) and/or PLK1 (Invitrogen, 0.2 μg) were incubated with 50 μM ³²P-ATP in reaction buffer (50 mM Tris, pH7.5, 10 mM MgCl₂, 2 mM EDTA, 1 mM TCEP) for 30 min. PTEN (0, 0.1, 0.3, 1 μg) was added together with PLK

inhibitor BI-2356 (to stop additional phosphorylation from occurring and ensure only dephosphorylation was being measured) followed by another 30 min at 30°C. Then sample-loading buffer (Invitrogen) was added to stop the reaction. After SDS-PAGE, the gel was stained with Instant BLUE (Expedeon), dried and exposed to radiographic film.

Immunofluorescence microscopy

For immunofluorescence (IF) analyses, cells were seeded in four-well tissue culture slides (BD Biosciences) or on cover slips (Corning) and treated with MMC for 18 h. Cells were fixed in 4% w/v paraformaldehyde in PBS for 15 min on ice, followed by permeabilization for 5 min in 0.3% v/v Triton X-100 in PBS. Fixed cells were incubated with primary antibodies in 5% v/v goat serum, 0.1% v/v NP40, in PBS for 1 h, washed three times with PBS and then incubated with Alexafluor 488-conjugated anti-mouse or anti-rabbit secondary antibodies (Invitrogen) for 45 min. Cells were then counterstained and mounted in vectashield plus 406-diamidine-2-phenylindole dihydrochloride (DAPI) (Vector Laboratories) and visualized using a Zeiss AxioImager.A1 upright epifluorescence microscope with AxioVision LE 4.6 image acquisition software. Primary antibodies used for IF were anti-53BP1 (H300; Santa Cruz Biotechnology), anti-DNA-PKcs pS2056 (ab18192; Abcam), anti-FANCA (ABP6201; Cascade), anti-FANCD2 (NB100-182; Novus Biologicals and sc-20022; Santa Cruz Biotechnology), anti-FANCI (A300-212A; Bethyl Laboratories), anti-FANCM (Meetei and Deans CE56.1 antibodies), anti-FK2 (sc-8017; Santa Cruz Biotechnology), and anti- γ H2AX (05-636; Millipore).

Statistical analysis

Error bars represent standard errors of the means from three independent experiments. *P* values were calculated using a two-tailed Student's *t*-test.

Acknowledgements

We thank members of the Howlett laboratory for critical reading of this manuscript and for helpful discussions. We thank Todd Waldman and David Mills for cells and Heung-Chin Cheng for recombinant PTEN protein. This work was supported by National Institutes of Health/National Heart, Lung and Blood Institute grant R01HL101977 (NGH); Rhode Island IDeA Network of Biomedical Research Excellence (RI-INBRE) grant P20GM103430 from the National Institute of General Medical Sciences; Cancer Council Victoria, Victorian Government IOS program, National Breast Cancer Foundation career development award (AJD), and Rhode Island Experimental Program to Stimulate Competitive Research (RI-EPSCoR) grant #1004057 from the National Science Foundation.

Additional Information

Supplemental information

Supplemental Information includes Extended Experimental Procedures and nine figures and can be found with this article online.

Author contributions

E.A.V. and A.M. performed the majority of the experiments. D.A.V. and M.M.A. assisted with revision experiments. C.H. and A.J.D. generated and provided FANCM antibodies and performed *in vitro* protein phosphatase assays. N.G.H and E.A.V. formulated the hypotheses, analyzed the data and wrote the paper.

Competing financial interests

The author(s) declare no competing financial interests.

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Manuscript-III

Prepared for submission to Oncotarget

PTEN SUMOylation is important for DNA crosslink repair

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Key Words: PTEN, SUMOylation, DNA repair, p97/VCP, RNF4

Abstract

Posttranslational modifications (PTMs) are integral for efficient and effective DNA repair pathway function. PTEN is a dual specificity phosphatase where its protein phosphatase activity has recently been shown to be necessary for replication dependent DNA repair. Not only does PTEN alter the PTM status of replication and repair proteins, but it itself is posttranslationally modified, which is essential for its correct localization and activity. Here we show that PTEN SUMOylation on K254 is imperative for its DNA repair function by signaling its removal from the nucleus in a RNF4 and p97/VCP dependent manner.

Introduction

PTEN is a tumor suppressor protein found to be mutated in many human cancers [1]. It is a well-characterized lipid and protein phosphatase involved in several cellular processes assaying the integrity of the genome [2]. The major phosphatase function for PTEN is dephosphorylation of the lipid second messenger phosphatidylinositol-3,4,5-triphosphate (PIP₃), opposing the cell proliferating PI3K/AKT pathway [2]. While this tumor suppressor function is localized in the cytoplasm, recent publications have shown many nuclear tumor suppressor functions that are either protein phosphatase independent or dependent [3-7]. Through protein-protein interactions PTEN stabilizes various complexes such as the anaphase-promoting complex/cyclosome (APC/C) E3 ubiquitin ligase and its subunit CDH1, regulating cell cycle progression [5]. PTEN also creates a complex

between RPA and OTUB1 deubiquitinase, resulting in a stabilization of RPA on single-stranded DNA (ssDNA) [5, 6]. PTEN not only stabilizes RPA at stalled replication forks but also recruits and stabilizes RAD51 promoting homologous recombination (HR) [4]. Two protein substrates for PTEN have recently been identified. First, PTEN targets the replisome under replication stress and dephosphorylates MCM2 on S41, stabilizing the replication machinery preventing DNA breaks [8]. Secondly, PTEN dephosphorylates polo-like kinase 1 (PLK1) on T210 resulting in deactivation of the mitotic kinase and destabilization of the protein [7]. Moreover, PTEN phosphorylation by ataxia telangiectasia mutated (ATM) on S398 leads to its subsequent SUMOylation on K254 and its removal from the nucleus upon DNA damage [3].

Recently PTEN has been connected to the Fanconi anemia (FA) DNA repair pathway [9]. FA is a DNA repair pathway comprised of 21 genes (*FANCA*, *-B*, *-C*, *-D1/BRCA2*, *-D2*, *-E*, *-F*, *-G*, *-I*, *-J/BRIP1*, *-L*, *-M*, *-N/PALB2*, *-O/RAD51C*, *-P/SLX4*, *-Q/ERCC4*, *-R/RAD51*, *-S/BRCA1*, *-T/UBE2T*, *-U/XRCC2* and *-V/REV7*) that code for proteins that specifically removes interstrand crosslinks (ICL) by HR [10-12]. *FANCA*, *-B*, *-C*, *-E*, *-F*, *-G*, *-L*, *-T* and FA associated proteins FAAP20, and -100 form the FA core complex. FANCM recruits the FA core complex to the ICL and the core complex acts as an ubiquitin E3 ligase that monoubiquitinates FANCD2 and FANCI on K561 and K523 respectively [10, 13]. Monoubiquitinated FANCD2 and I accumulates at sights of DNA damage and recruits nucleases to excises the ICL and promote HR by the downstream FA proteins, FANCD1, *-J*, *-N*, *-O*, *-R*, *-S*, *-U*, and *-V*.

DNA repair needs to be carefully orchestrated for proper protein recruitment and function at sites of DNA damage. Many DNA repair proteins are posttranslationally

modified throughout the DNA repair process. It is well known that protein phosphorylation, acetylation, methylation, and ubiquitination are involved in protein signaling and activation throughout the DNA repair process. Recently, it has been shown that proteins are polySUMOylated, forming SUMO2/3 chains signaling, for subsequent polyubiquitination and proteasomal degradation [14-16].

SUMOylation of proteins can stimulate protein-protein interactions through the contact of a SUMO-interacting motif (SIM) on the recruited protein. The SUMO-targeted E3 ubiquitin ligase (STubL), RNF4, contains multiple SIMs to interact with SUMO2/3 chains [17]. Through RNF4's RING domain and the assistance of an E2 ubiquitin-conjugating enzyme, RNF4 polyubiquitinates SUMOylated proteins for removal and subsequent degradation. Many DNA repair proteins are regulated by RNF4 mediated degradation to allow downstream DNA damage response (DDR) proteins access to the damage [14]. The protein complexes are removed from chromatin by chromatin-associated degradation [18]. Chromatin-associated degradation is a process in which the protein targets are SUMOylated followed by subsequent poly-ubiquitination targets the AAA+ ATPase p97/VCP [19]. Using ATP hydrolysis, p97/VCP extracts proteins from chromatin targeting the substrates to the 26S proteasome [20].

In this study, we have investigated the role of PTEN SUMOylation in response to DNA damage. We have established an increase in PTEN-SUMO upon DNA damage with various DNA damaging agents as well as a robust increase in PTEN-SUMO with inhibition of the proteasome. By observing the nuclear pool of PTEN, we see a stabilization of PTEN when either RNF4 is knocked down using siRNA or p97/VCP is chemically inhibited. When the SUMOylation incompetent missense mutant PTEN-

K254R is stably expressed in PTEN null cells, we observe an impairment of DNA repair by an increase in chromosome aberrations. Further investigation narrowed PTEN's genome instability to failure to activate the Fanconi anemia (FA) DNA repair pathway. We observe a defect in the accumulation of FANCA, -M, -D2, and -I at sites of DNA damage *via* immunofluorescence microscopy. Lastly, it was previously shown that PTEN SUMOylation signals its removal from the nucleus [3]. We observe stabilization in nuclear PTEN-K254R compared to wild type upon DNA damage. Our results uncover a novel mechanism of the regulation of PTEN during DNA repair, specifically by HR.

Results

PTEN SUMOylation targets PTEN for degradation

SUMOylation has recently been connected to DNA repair regulating protein turnover and the kinetics of proteins throughout the repair process [14]. We previously uncovered a connection between PTEN and the Fanconi anemia DNA repair pathway independent of its lipid phosphatase activity [9]. Furthermore, PTEN is SUMOylated on K254 and PTEN null cells expressing the SUMO incompetent mutant K254R have increased genome instability upon irradiation treatment [3]. To determine if PTEN SUMOylation is important for interstrand crosslink (ICL) repair we grew COS-7 cells in the absence or presence of mitomycin C (MMC) and observed PTEN SUMOylation. When treated with MMC, we see a strong induction of the higher molecular weight band, which Bassi et al has shown to be the SUMOylated form of PTEN (**Figure 1A**) [3]. By inhibiting the proteasome with MG132, we can see a strong increase in stability of PTEN-SUMO in COS-7, BJ-TERT and U2OS cell lines (**Figure 1B**) suggesting that PTEN-SUMO is

Figure 1. PTEN SUMOylation targets PTEN for degradation

(A) COS-7 cells were treated with or without 100 nM MMC for 24h. Whole cell lysates were prepared and immunoblotted for PTEN, and α -tubulin. To improve clarity and conciseness, the presented blots have been cropped. All gels were run under the same experimental conditions.

(B) COS-7, U2OS, and BJ-TERT cells were treated with 10 μ M of MG132 for 16h. Whole cell lysates were prepared and immunoblotted for PTEN and FANCD2 as labeled on the left. To improve clarity and conciseness, the presented blots have been cropped. All gels were run under the same experimental conditions.

(C and D) COS-7 cells were treated with either 1mM hydroxyurea (HU) (C) or 0.4 μ M VP16 (D) and with or without 2.5 μ M MG132 for 16h. Whole cell lysates were prepared and immunoblotted for PTEN, FANCD2 and α -tubulin.

A **B** **C** **D**

targeted for degradation. To determine if PTEN-SUMO is involved in not only ICL repair, but also double strand break (DSB) repair (**Figure 1C and D**), we treated COS-7 with a DNA damaging agent, hydroxyurea (HU), a proteasome inhibitor MG132, or cotreatment to prevent PTEN degradation. We see an increase in PTEN-SUMO when treated with HU and MG132 compared to MG132 alone (**Figure 1C**). This was also seen with COS-7 cells treated with the topoisomerase inhibitor VP16, causing double strand breaks, and MG132 (**Figure 1D**). The large induction of PTEN-SUMO with cotreatment of MG132 and DNA damaging agents suggests that PTEN-SUMO signals PTEN for its degradation.

SUMOylated PTEN is targeted for RNF4 and p97/VCP mediated degradation

RNF4 is a SUMO targeted E3 ubiquitin ligase that has been previously linked to DNA repair, helping to orchestrate the spatial coordination of the many proteins involved [14]. For example, RNF4 is targeted to polySUMO RPA through its SUMO interacting motifs (SIMs) for polyubiquitination and proteasomal degradation to allow for the efficient loading of RAD51 on the now free ssDNA for HR [14]. Since PTEN-SUMO increases in stability when the proteasome is inhibited, we wanted to see if SUMOylation of PTEN on K254 targets PTEN for proteasome degradation. To test this we knocked down RNF4 using targeted small interfering RNA (siRNA) and treated with HU, MG132, or cotreatment. Since PTEN is SUMOylated in order to remove it from the nucleus, we performed a cellular fractionation for a nuclear enrichment [9]. When looking at the nuclear pool of PTEN, depletion of RNF4 stabilized PTEN. There is also an increase in PTEN-SUMO upon depletion of RNF4 (**Figure 2A**). More importantly, we see a

Figure 2. Nuclear PTEN is targeted for degradation by RNF4 and p97/VCP

(A) HeLa cells were transfected with non-targeting siRNA (siCtrl.) or on target siRNA (siRNF4) for 96h and treated with 1 mM HU or 2.5 μ M MG132 for the last 16h. Cytoplasmic proteins were removed and nuclear enriched lysates were prepared and immunoblotted for PTEN, RNF4, and H2A.

(B) HeLa cells were treated with 2.5 μ M NMS-873 for 8 and 24h or 5 μ M NMS-873 for 8 h. Cytoplasmic proteins were removed and nuclear enriched lysates were prepared and immunoblotted for PTEN, and H2A.

large increase in PTEN stability with untreated siRNF4 compared to siCtrl. (lane 5 vs 1). PolySUMO and ubiquitin chains allow for binding of the Type II AAA+ ATPase, p97/VCP, and recruitment to the 26S proteasome [20]. By using the p97/VCP small molecule inhibitor NMS-873, we also see stabilization of nuclear PTEN (**Figure 2B**). These results suggest that the nuclear pool of PTEN is regulated by proteasomal degradation mediated by RNF4 and p97/VCP.

PTEN SUMOylation is important for DNA Repair

To gain further mechanistic insight into the role of PTEN in ICL repair, we complemented HCT116 PTEN^{-/-} cells with LacZ, wild type PTEN, and SUMOylation-incompetent PTEN-K254R [3, 21, 22]. The PTEN variants behaved as predicted with wild type and PTEN-K254R able to down-regulate the PI3K/AKT pathway, as demonstrated by loss of AKT-pS473 signal, while the phosphatase defective C124S and lipid phosphatase defective G129E mutants are unable to deactivate AKT (**Figure 3A**). We exposed the HCT116 PTEN^{-/-} + LacZ, -PTEN-WT, and -PTEN-K254R cells to MMC and analyzed metaphase chromosomes for the presence of gaps, breaks, and radial formations. Only wild type PTEN rescued the sensitivity of PTEN^{-/-} cells to MMC-induced chromosome damage, while PTEN-K254R failed to rescue the ICL sensitivity of PTEN^{-/-} cells (**Figure 3B and C**). Cells stably expressing PTEN-K254R exhibited increased chromosome gaps and breaks and radial formations, compared with cells expressing wild-type PTEN, following exposure to MMC ($P < 0.001$) (**Figure 3B**).

Figure 3. PTEN function in ICL repair is SUMOylation-dependent

(A) HCT116 PTEN^{-/-} cells were stably transduced with pLenti6.2-LacZ, -PTEN-WT, -PTEN-C124S, -PTEN-G129E, and -PTEN-K254R. Whole-cell lysates were prepared and immunoblotted for PTEN, AKT, AKT pS473, RAD51, and α -tubulin.

(B) PTEN-K254R fail to rescue the sensitivity of PTEN^{-/-} cells to the clastogenic effects of mitomycin C (MMC), in contrast to PTEN-WT. Cells were incubated in the absence (NT) or presence of 20 nM MMC for 24 h and metaphase spreads were analyzed for structural chromosome aberrations. *** $P < 0.001$.

(C) Images of PTEN-K254R chromosome aberrations observed in B compared to PTEN-WT.

A

B

C

Functional FANCD2 and FANCI is dependent on PTEN SUMOylation

To determine the status of the FA pathway in PTEN^{-/-} + LacZ, -PTEN-WT, and PTEN-K254R cells, we examined the activation of FANCD2 and its binding partner FANCI. FANCD2 and FANCI form a heterodimer called the ID2 complex. The monoubiquitination of FANCD2 and FANCI by the FA core complex is a key activation step of the pathway [23]. Activated FANCD2 and FANCI converge at sites of DNA damage, which can be visualized as nuclear foci via immunofluorescence [23]. Analysis of FANCD2 and FANCI nuclear foci formation in the HCT116 PTEN^{-/-} + LacZ, -PTEN-WT, and -PTEN-K254R cells revealed inefficient MMC-inducible FANCD2 and FANCI nuclear foci formation in PTEN^{-/-} cells stably expressing PTEN-K254R, similar to cells expressing LacZ (**Figures 4A and B**). Since PTEN-K254R is deficient in FANCD2 and FANCI nuclear localization, we next examined the nuclear localization of the FA core complex. We have previously determined that PTEN^{-/-} cells and PTEN^{-/-} cells expressing the protein phosphatase defective missense mutant C124S, are defective in the FA DNA repair pathway via deficient core complex recruitment and FANCD2 and FANCI activation [9]. From this we were interested in determining if PTEN SUMOylation is important for efficient FA core recruitment to sites of DNA damage. HCT116 PTEN^{-/-} + LacZ, -PTEN-WT, and -PTEN-K254R were incubated in the absence or presence of MMC and FANCA and FANCM nuclear foci formation were analyzed. While wild type PTEN rescued defective ICL-inducible FANCA and FANCM nuclear foci formation, the PTEN-K254R mutant did not (**Figures 4C and D**). This suggests that PTEN SUMOylation on K254 is important for ICL repair via activation of the FA DNA repair pathway.

Figure 4. FA pathway activation is dependent on PTEN SUMOylation

(A and B) Defective FANCD2 (A) and FANCI (B) nuclear foci formation in PTEN^{-/-} cells expressing LacZ, and PTEN-K254R. Cells were incubated in the absence (NT) or presence of 200 nM MMC for 18 h and let recover for 0, 4, and 8 h. *** $P < 0.001$.

(C and D) Defective FANCA (A) and FANCM (B) nuclear foci formation in PTEN^{-/-} cells expressing LacZ, and PTEN-K254R. Cells were incubated in the absence (NT) or presence of 200 nM MMC for 18 h. *** $P < 0.001$.

A **B** **C** **D**

SUMOylated PTEN signals for its Nuclear Exclusion

Posttranslational modifications such as monoubiquitination and SUMOylation can target a protein for cellular compartmentalization. An example of this is the monoubiquitination of PTEN, which has been shown to signal its nuclear localization [24]. Recent studies have suggested that PTEN SUMOylation promotes its nuclear export [3]. To determine if PTEN SUMOylation affects its cellular localization we treated HCT116 and MCF10A PTEN^{-/-} +PTEN wild type and +PTEN-K254R with MMC and performed a cellular fractionation. Consistent with the findings of Bassi et al, we observed increased PTEN-K254R stability and less efficient removal of PTEN-K254R from chromatin following ICL exposure, compared with wild type PTEN (**Figure 5**).

PTEN SUMOylation is dependent on FANCD2

Since PTEN^{-/-} cells have impairment in HR via the Fanconi anemia pathway, we assessed PTEN-SUMO in FA-D2 patient cells lacking functional FANCD2 protein. Increased PTEN-SUMO induction upon MG132 treatment was observed in FA-D2+FANCD2 cells compared to FA-D2 cells (**Figure 6A**). With further investigation, the analysis of FANCD2 ChIP-seq data showed that FANCD2 binds to DNA in the middle of the E3 SUMO ligase gene *PIAS1* [25]. Since we observe deficient PTEN SUMOylation in FA-D2 cells we then analyzed the protein expression of PIAS1. We observed a stark decrease in protein levels of PIAS1 in FA-D2 cells compared to FA-D2+FANCD2 and FA-FANCD2-K561R (monoubiquitination deficient mutant) (**Figure 6B**). These results indicate a complex relationship between FANCD2 and PTEN PTM. Interestingly, it also implies an FA DNA repair independent role for FANCD2 in the transcriptional regulation

Figure 5. SUMOylated PTEN signals for its Nuclear Exclusion

(A and B) HCT116-PTEN^{-/-} + PTEN or + PTEN-K254R (A) and MCF10A-PTEN^{-/-} +PTEN or +PTEN-K254R (B) were treated in the absence or presence of 200 nM MMC for 18h and a cellular fractionation was performed. W represents whole cell lysate, S is soluble cytoplasmic and nuclear proteins and C is chromatin bound proteins. Lysates were prepared and immunoblotted for PTEN, H2A and α -tubulin. The ratio of C:W was measured using ImageJ software.

of the *PIAS1* gene.

Discussion

PTEN is a ubiquitous protein throughout the cell that is involved in many cellular activities [26]. Because of this, PTEN is heavily posttranslationally modified [26]. These modifications regulate PTEN's activity and cellular localization. For example, PTEN monoubiquitination on two lysine residues 13 and 289 leads to PTEN's nuclear localization [24]. It has also been shown that PTEN is SUMOylated on residue K254 and this SUMOylation exports PTEN from the nucleus and SUMOylation of PTEN is integral to its role in DNA repair shown by prolonged γ H2AX nuclear foci after irradiation and decrease HR by a HR assay in PTEN-K254R cells compared to PTEN-WT U87MG cells [3]. It is still unclear the mechanism behind PTEN-SUMO and how it was involved in DNA repair. Here we show that PTEN SUMOylation increases after DNA damage and targets it for proteolytic degradation mediated by the SUMO targeted ubiquitin ligase, RNF4 and the AAA+ ATPase, p97/VCP.

By inhibiting the proteasome with MG132 we see a large induction of PTEN-SUMO compared to NT. But when treated with various DNA damaging agents (MMC, HU, and VP16) and MG132, we see a larger increase in PTEN-SUMO compared to solely MG132 treatment indicating PTEN is SUMOylated upon DNA damage and degraded since it is easily visualized by inhibiting the proteasome. We have established a requirement for PTEN SUMOylation on residue K254 in ICL repair. PTEN-K254R fails to restore efficient FANCD2 and FANCI nuclear foci formation through impaired recruitment of the FA core complex causing deficient ICL repair in PTEN^{-/-} cells. Accordingly, we observed increased PTEN-K254R stability and less efficient removal of

PTEN-K254R from chromatin following ICL exposure. This is due to the removal by p97/VCP targeting the polySUMO and ubiquitinated PTEN. Using small interfering RNA we can knock down protein expression of the ubiquitin E3 ligase RNF4, we see a stabilization of not only non SUMO PTEN but also SUMOylated PTEN. By chemically inhibiting p97/VCP, PTEN-SUMO is also stabilized.

The regulation of DDR proteins by SUMOylation has recently been connected to DSB repair for both HR and nonhomologous end joining (NHEJ) pathways [14]. During HR, the RPA complex binds to ssDNA protecting the DNA from nucleolytic degradation. To allow RAD51 to then bind to the ssDNA to form the nucleofilament for strand invasion, RPA1 of the complex is SUMOylated targeting it to be polyubiquitinated by RNF4. This results in its subsequent degradation and freeing up the ssDNA for RAD51 loading [14]. MDC1 is also removed from DNA by SUMOylation followed by ubiquitination by RNF4 to allow for the next steps in NHEJ. More recently, the regulation by SUMO was connected to the FA pathway [15]. Monoubiquitinated FANCD2 and FANCI (ID2) are recruited to DNA crosslinks. To regulate the dosage of activated ID2 at sites of DNA damage, SUMOylation by an E3 SUMO ligase PIAS1 or PIAS4 and polyubiquitination by RNF4 targets ID2 for removal via p97/VCP [15].

Taken together that PTEN SUMOylation is important for ICL repair, we hypothesize that PTEN dephosphorylates one or more key substrates. Following dephosphorylation, PTEN SUMOylation and its targeted degradation may be necessary to facilitate subsequent DNA repair steps or enable replication fork resumption following removal of the ICL block (**Figure S2**). These results establish that PTEN functions in the activation of the FA pathway, and that DNA repair is dependent on its protein

phosphatase activity and ability to undergo SUMOylation on K254. Lastly, uncovering decreased PTEN-SUMO in FA-D2 may be caused by decreased transcription of *PIAS1* require extensive research to determine the relationship.

Figure 6. PTEN SUMOylation is dependent on FANCD2

(A) PD20F (FA-D2) and PD20F+FANCD2 (FA-D2+FANCD2) were grown in the absence or presence of 5 μ M of MG132 for 8 or 16h. Whole-cell lysates were prepared and immunoblotted for PTEN, FANCD2, and α -tubulin. LE is light exposure and DE is a darker exposure of the same blot.

(B) PD20L-pMMP-empty, -FANCD2, and -FANCD2-K561R were grown in the absence or presence of 200 nM MMC for 18h. Whole-cell lysates were prepared and immunoblotted for PTEN, FANCD2, and α -tubulin.

A

B

Materials and Methods

Cell culture

HCT116 $PTEN^{+/+}$, $PTEN^{-/-}$ #22, and $PTEN^{-/-}$ #35[27] (a kind gift from Todd Waldman, Lombardi Cancer Center, Georgetown University) were grown in McCoy's medium supplemented with 12% v/v FBS, 1% v/v L-glutamine, and 1% v/v penicillin/streptomycin. MCF10A $PTEN^{+/+}$ and $PTEN^{-/-}$ cells (Horizon Discovery, U.K.) were grown in DMEM F12 supplemented with 5% v/v horse serum, 20 ng/ml epidermal growth factor, 0.5 mg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 μ g/ml insulin, 1% v/v L-glutamine, and 1% v/v penicillin/streptomycin. Stably complemented HCT116 and MCF10A lines were generated by transduction with pLenti6.2/V5-DEST (Invitrogen) lentivirus harboring wild type or mutant *PTEN* cDNAs. HCT116 and MCF10A stable cell lines were grown in McCoy's or DMEM F12 medium respectively supplemented with 5 μ g/ml blasticidin. FA-D2 (*FANCD2*^{hy/-}) cells were purchased from Coriell Cell Repositories (Catalog ID GM16633)[28]. Corrected FA-D2 cells were generated by transduction with pLenti6.2-FANCD2 lentivirus, followed by selection in DMEM supplemented with 15% v/v FBS, 1% v/v L-glutamine, 1% v/v penicillin/streptomycin and 2 μ g/ml blasticidin. PD20L cells were grown in RPMI media supplemented with 20% v/v FBS, 1% v/v L-glutamine, and 1% v/v penicillin/streptomycin and 1 μ g/ml puromycin. HeLa, BJ-TERT, COS-7, and U2OS cells were grown in DMEM supplemented with 12% v/v FBS, 1% v/v L-glutamine, and 1% v/v penicillin/streptomycin.

Plasmids and site-directed mutagenesis

The plasmids pSG5L-HA-PTEN wild type (Addgene) was used to amplify *PTEN* cDNA using the forward primer 5'-CACCATGACAGCCATCATCAAA-3' and the reverse primer 5'-TCAGACTTTTGTAAATTTGTGTATGCTG-3'. The cDNA was then recombined into pLenti6.2/V5-DEST (Invitrogen) using Invitrogen Gateway Cloning. The *PTEN-K254R* cDNA was generated by site-directed mutagenesis of the wild type *PTEN* cDNA using the Quikchange Site-directed Mutagenesis Kit (Stratagene). The forward and reverse oligonucleotide sequences used are as follows:

FP 5'-CGTTACCTGTGTGTGGTGATATCCGAGTAGAGTTCTTCCACAAAC-3'

RP 5'-GTTTGTGGAAGAACTCTACTCGGATATCACCACACACAGGTAACG-3'.

Cellular fractionation

Soluble proteins were removed by extraction in cytoskeletal buffer (CSK) (10 mM PIPES pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl₂, 1 mM EGTA, and 0.5% v/v Triton-X-100) for 10 minutes at 4°C. For nuclear enrichment, pellets were washed once with CSK buffer, lysed in SDS sample buffer (2% w/v SDS, 50 mM Tris-HCl pH 7.4, 10 mM EDTA), boiled for 15 min, followed by sonication for 10 s at 10% amplitude using a Fisher Scientific Model 500 Ultrasonic Dismembrator. For the second cellular fractionation assay, cell pellets were resuspended in Cytoplasmic buffer (10mM HEPES pH7.9, 10 mM KCl, 1.5m m MgCl₂, 340mM Sucrose, 10% glycerol, 1 mM DTT, 0.1 % v/v Triton-X-100, and Rouch Protease Inhibitor cocktail) and centrifuged at 1,000 x g for 5 min for soluble cytoplasmic proteins extraction. Pellets were then resuspended in chilled nuclear lysis buffer (10 mM HEPES pH7.9, 3 mM EDTA, 0.2 mM EGTA, and

Rough Protease Inhibitor cocktail) on ice for 15 minutes with occasional vortexing to remove nuclear soluble proteins. The remaining pellet containing chromatin bound proteins were lysed in SDS sample buffer (2% w/v SDS, 50 mM Tris-HCl pH 7.4, 10 mM EDTA), boiled for 15 min, followed by sonication for 10 s at 10% amplitude using a Fisher Scientific Model 500 Ultrasonic Dismembrator.

Chromosome breakage assay

For chromosome breakage assays, cells were incubated in the absence or presence of MMC for 18-24 h. Prior to harvesting, cells were treated with 0.1 μ g/ml Colcemid (Gibco/Invitrogen) for 2 h. Cell pellets were incubated in 0.075 M KCl at 37°C for 18 min, followed by fixation in Carnoy's fixative (3:1 methanol:glacial acetic acid) with multiple changes. Cells were dropped onto chilled slides and air-dried prior to staining with 2.5% w/v Giemsa solution (Sigma). Metaphases were analyzed using a Zeiss AxioImager.A1 upright epifluorescent microscope with AxioVision LE 4.6 image acquisition software.

Immunoblotting and antibodies

For immunoblotting analysis, cell suspensions were washed in ice-cold PBS and lysed in 50 mM Tris.Cl pH 7.4, 1.0% v/v NP-40, 0.25% w/v Deoxycholic acid, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1 mM $\text{Na}_3\text{O}_4\text{V}$, 1 mM NaF, plus protease inhibitors cocktail (Roche). Proteins were resolved on NuPAGE 3-8% w/v Tris-Acetate or 4–12% w/v Bis-Tris gels (Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membranes. The following antibodies were used: Rabbit anti-AKT (Cell Signaling), anti AKT-pS473

(Cell Signaling), anti-FANCA (ABP6201; Cascade), anti-FANCD2 (NB100-182; Novus Biologicals), anti-H2A (07-146; Millipore), anti-PIAS1 (Cell Signaling) and anti-PTEN (9559; Cell Signaling). Mouse monoclonal: anti-RAD51 (sc-8349; Santa Cruz), and anti- α -tubulin (MS-581-PO; Lab Vision).

Immunofluorescence microscopy

For immunofluorescence (IF) analyses, cells were seeded in four-well tissue culture slides (BD Biosciences) or on cover slips (Corning) and treated with MMC for 18 h. Cells were fixed in 4% w/v paraformaldehyde in PBS for 15 min on ice, followed by permeabilization for 5 min in 0.3% v/v Triton X-100 in PBS. Fixed cells were incubated with primary antibodies in 5% v/v goat serum, 0.1% v/v NP40, in PBS for 1 h, washed three times with PBS and then incubated with Alexafluor 488-conjugated anti-mouse or anti-rabbit secondary antibodies (Invitrogen) for 45 min. Cells were then counterstained and mounted in vectashield plus 406-diamidine-2-phenylindole dihydrochloride (DAPI) (Vector Laboratories) and visualized using a Zeiss AxioImager.A1 upright epifluorescence microscope with AxioVision LE 4.6 image acquisition software. Primary antibodies used for IF were anti-FANCA (ABP6201; Cascade), anti-FANCD2 (NB100-182; Novus Biologicals and sc-20022; Santa Cruz Biotechnology), anti-FANCI (A300-212A; Bethyl Laboratories), anti-FANCM (Meetei and Deans CE56.1 antibodies).

siRNA experiments

Cells were plated in six-well dishes at a density of 200,000 cells/well and transfected the following day with RNF4-specific (siRNF4), or control, non-targeting siRNA (siCtrl)

using Lipofectamine 2000 (Invitrogen). Seventy-two hours following transfection, cells were incubated in the absence or presence of MMC and harvested for nuclear enrichment as described above. The following sense and anti-sense sequences were used: siRNF4 (MWG Operon) 5'-GAAUGGACGUCUCAUCGUU-3' 5'-AACCAUGAGACGUCCAUUC-3' and for non-targeting siCtrl (Dharmacon) 5'-UAACGACGCGACGACGUAA-3' and 5'-UUACGUCGUCGCGUCGUUA-3'.

Statistical analysis

Error bars represent standard errors of the means from three independent experiments. *P* values were calculated using a two-tailed Student's t-test.

Acknowledgements

We thank members of the Howlett laboratory for critical reading of this manuscript and for helpful discussions. We thank Todd Waldman and Heung-Chin Cheng for recombinant PTEN protein. This work was supported by National Institutes of Health/National Heart, Lung and Blood Institute grant R01HL101977 (NGH); Rhode Island IDeA Network of Biomedical Research Excellence (RI-INBRE) grant [P20GM103430](#) from the National Institute of General Medical Sciences; Cancer Council Victoria, Victorian Government IOS program, National Breast Cancer Foundation career development award (AJD), and Rhode Island Experimental Program to Stimulate Competitive Research (RI-EPSCoR) grant #1004057 from the National Science Foundation.

Additional Information

Supplemental information

Supplemental Information two figures and can be found with this article online at

Author contributions

E.A.V performed the majority of the experiments and wrote the paper. M.M.A. assisted with experiments. N.G.H and E.A.V. formulated the hypotheses, analyzed the data.

Competing financial interests

The author(s) declare no competing financial interests.

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APPENDIX I

Conclusion

The mechanistic insight of the role of PTEN in genome stability is essential for efficient DNA repair and cell cycle progression. Recently, great efforts have uncovered several unique cellular processes in how PTEN functions as a tumor suppressor independent of its cytoplasmic lipid phosphatase activity. PTEN was first shown to have a separate nuclear function by interacting with the kinetochore, stabilizing chromosomes at the centromere, and regulating transcription of the essential homologous recombination protein, RAD51, mRNA with the transcription factor E2F1 [1]. The result of the transcriptional regulation of RAD51 has remained controversial and has led to an intense investigation to understand the mechanism behind nuclear PTEN [2, 3]. It has now been discovered that PTEN stabilizes replication forks when the replication machinery encounters DNA lesions [4, 5]. PTEN protects the ssDNA by stabilizing RPA through the recruitment of its deubiquitinating enzyme, OTUB1 [5]. Following the stabilization of ssDNA, PTEN then localizes RAD51 to the stalled replication fork, promoting error free DNA repair *via* homologous recombination [4].

Not only is PTEN involved in ensuring efficient DNA replication during S phase, PTEN posttranslational modification by phosphorylation on residue S380 by PLK1 allows for timely cell cycle entrance and exit of M phase [6, 7]. Improper regulation of PTEN through M phase leads to deregulation of chromosome segregation and increase aneuploidy in PTEN-deficient cells. Aneuploidy is a genetic phenotype and characterization of cancer cells. Several layers regulate the control of the proportion of PTEN phosphorylated on S380. PTEN dephosphorylates PLK1 on T210 and this

phosphorylation of T210 is necessary for PLK1 kinase activity [8]. PTEN then stabilizes the APC/C-CDH1 E3 ubiquitin ligase complex targeting many mitotic proteins for proteasomal degradation, such as PLK1 [9]. It was also shown that PTEN-pS380 is an APC/C-CDH1 target for its removal from chromatin for G1 entrance, indicating a complex and dynamic relationship between PTEN and PLK1 with ubiquitin mediated proteolytic degradation by the APC/C-CDH1 complex [7].

The main focus of my dissertation was identifying a phosphatase involved in the Fanconi anemia DNA repair pathway which specifically removes interstrand cross links (ICIs). *PTEN* became the best candidate since it recently was connected to DNA repair and is one of the ten familial breast and ovarian cancer susceptibility genes. The majority of these susceptibility genes are Fanconi anemia genes themselves or biochemically linked to the pathway [10]. The one outlier of the group was PTEN, which happens to be a bona fide phosphatase tumor suppressor. Using isogenic PTEN^{+/+} and PTEN^{-/-} non-transformed mammary epithelial cells (MCF10A) and transformed colorectal carcinoma cell line (HCT116), we determined PTEN^{-/-} cells have an FA patient cell like phenotype with increased sensitivity to DNA cross linking agents such as MMC with decreased cell survival and increased chromosome aberrations. We discovered that this was due to improper recruitment of the FA core complex and FANCD2 and FANCI to DNA damage, resulting in PTEN^{-/-} cells favoring error prone nonhomologous end joining over error free homologous recombination. This was caused by increased PLK1 activity since PTEN normally deactivates PLK1 by dephosphorylation and enhances its protein degradation by stabilizing the APC/C-CDH1 complex. Without PTEN, there is increased PLK1 activity, and it is known that PLK1 phosphorylates FANCM, priming FANCM for

its degradation leading to deficient FA core complex recruitment and a nonfunctional FA pathway [11]. We also determined that PTEN's role in ICL repair is dependent on its protein phosphatase activity by comparing the phosphatase deficient missense mutant C124S to the lipid phosphatase deficient G129E. This is interesting since PTEN targets PLK1. It is still unknown if PTEN dephosphorylates any FA proteins.

PTEN SUMOylation on residue K254 was shown to be important for protection from IR damage [2]. We also see a dependence of SUMOylation of K254 by characterizing the SUMO incompetent missense mutant K254R in ICL repair *via* the FA pathway see the same phenotype as PTEN^{-/-} cells. We next tried to understand the mechanism behind how PTEN SUMOylation on residue K254 protects cells from DNA damage. We determined PTEN SUMOylation targets its degradation and removal from the nucleus by RNF4 polyubiquitination, resulting in PTEN transportation to the 26S proteasome by p97/VCP. In preliminary data, we also discovered PTEN SUMOylation is dependant on FANCD2 by FANCD2 transcription regulation of the E3 SUMO ligase PIAS1. It is still not fully understood why PTEN is removed from the nucleus upon DNA damage while resulting in genome stability. Our working hypothesis is that PTEN dephosphorylates one or more proteins involved in DNA repair resulting in their deactivation. This would mean that PTEN removal would allow its protein phosphatase substrates to stay active only to be deactivated once PTEN has localized back in the nucleus.

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APPENDIX II

Supplemental information for manuscript II: “The PTEN phosphatase functions cooperatively with the Fanconi anemia proteins in DNA crosslink repair”

Supplementary information:

Materials and Methods

Antibody generation

FANCM monoclonal antibodies were generated by intra-peritoneal injection of recombinant purified FANCM peptide (His-tagged amino acids 1507-1647 purified from *E. coli* using Nickel-NTA resin under guanidine-HCl denaturing conditions). Standard hybridoma generation protocols were employed using Sp2/0-Ag14 murine myeloma cells, followed by single cell cloning of the highest titer lines. Independently derived monoclonal antibodies CE56.1, CP3.2, CV5.1, and CV11.1 were purified from hybridoma supernatants using protein A sepharose, and shown to detect a single band of 230 kDa by Western blotting, that is absent in FANCM siRNA treated cells (see **Figure S9**).

Plasmids and site-directed mutagenesis

The plasmids pSG5L-HA-PTEN wild type, -PTEN-C124S, -PTEN-G129E and pHR-SIN-PTEN-Y138L (Addgene) were used to amplify *PTEN* cDNA using the forward primer 5'-CACCATGACAGCCATCATCAAA-3' and the reverse primer 5'-

TCAGACTTTTGTAAATTTGTGTATGCTG-3'. The cDNAs were then recombined into pLenti6.2/V5-DEST (Invitrogen) using Invitrogen Gateway Cloning.

Cellular fractionation

Soluble proteins were removed by extraction in cytoskeletal buffer (CSK) (10 mM PIPES pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl₂, 1 mM EGTA, and 0.5% v/v Triton-X-100) for 10 minutes at 4°C. Pellets were washed once with CSK buffer, lysed in SDS sample buffer (2% w/v SDS, 50 mM Tris-HCl pH 7.4, 10 mM EDTA), boiled for 15 min, followed by sonication for 10 s at 10% amplitude using a Fisher Scientific Model 500 Ultrasonic Dismembrator.

Chromosome breakage and cytotoxicity assays

For chromosome breakage assays, cells were incubated in the absence or presence of MMC for 18-24 h. Prior to harvesting, cells were treated with 0.1 µg/ml Colcemid (Gibco/Invitrogen) for 2 h. Cell pellets were incubated in 0.075 M KCl at 37°C for 18 min, followed by fixation in Carnoy's fixative (3:1 methanol:glacial acetic acid) with multiple changes. Cells were dropped onto chilled slides and air-dried prior to staining with 2.5% w/v Giemsa solution (Sigma). Metaphases were analyzed using a Zeiss AxioImager.A1 upright epifluorescent microscope with AxioVision LE 4.6 image acquisition software. For the cell proliferation assay, cells were plated at 10,000 cells/well, treated in the absence or presence of various concentrations of MMC for 48 h and the CellTiter 96® AQueous One Solution Reagent (Promega) was added directly to cultured cells, incubated for 2 h and the absorbance at 490 nm was measured with a 96-

well Bio-Rad 680 microplate reader. For the MMC survival assay, cells were plated at a density of 10,000 cells/well in 6-well dishes and treated with MMC the following day for 7 days. Cells were then fixed with 10% v/v methanol and 10% v/v acetic acid, stained with 1% w/v crystal violet in methanol, extracted with 0.02% SDS in methanol, and the absorbance at 595 nm was measured with a 96-well Bio-Rad 680 microplate reader.

siRNA experiments

Cells were plated in six-well dishes at a density of 200,000 cells/well and transfected the following day with PTEN-specific (siPTEN), FANCD2-specific (siFANCD2), or control, non-targeting siRNA (siCtrl) using Lipofectamine 2000 (Invitrogen). Seventy-two hours following transfection, cells were incubated in the absence or presence of MMC and harvested for cytogenetic analysis as described above. The following sense and anti-sense sequences were used: siFANCD2 (Dharmacon) 5'-CAGCCUACCUGAGA UCCUA-3' and 5'-UAGGAUCUCAGGUAGGCUG-3' and for non-targeting siCtrl (Dharmacon) 5'-UAACGACGCGACGACGUAA-3' and 5'-UUACGUCGUCGCGUCGUUA-3'. PTEN siRNAs (siGENOME SMARTpool siRNA reagent D-001810-01-05) were obtained from Thermo Fisher Scientific.

Figure S1. PTEN^{-/-} cells are hypersensitive to mitomycin C

(A) HCT116 PTEN^{+/+} and two independent clones of PTEN^{-/-} cells were incubated in the absence or presence of the indicated concentrations of MMC for 7-10 days, and cell survival was determined using a crystal violet assay. (B) MCF10A PTEN^{+/+} and PTEN^{-/-} cells were incubated in the absence or presence of the indicated concentrations of MMC for 7-10 days and cell survival determined. (C and D) MCF10A PTEN^{+/+} and PTEN^{-/-} cells were incubated in the absence or presence of the indicated concentrations of MMC for 24 h and metaphase spreads were analyzed for chromosome aberrations. (C) Numbers of centromere aberrations observed in PTEN^{+/+} and PTEN^{-/-} cells and (D) representative images of centromere aberrations observed in PTEN^{-/-} cells. ***, $P < 0.001$.

Figure S2. PTEN is required for efficient mitomycin C-inducible FANCD2 and FANCI nuclear foci formation, but not for the localization of FANCD2 and FANCI to chromatin

(A) Chromatin fractionation analysis of lysates from HCT116 PTEN^{+/+} and PTEN^{-/-} cells reveals efficient localization of FANCD2, FANCI, and RAD51 to chromatin following mitomycin C (MMC) treatment. Cells were incubated in the absence (NT) or presence of 200 nM MMC for 24 h. W, unfractionated whole-cell lysate; S, soluble cytoplasmic and nuclear fraction; C, chromatin-enriched fraction. (B and C) MCF10A PTEN^{+/+} and PTEN^{-/-} cells were incubated in the absence or presence of 200 nM MMC for 18 h, allowed to recover for 8 h, and FANCD2 (B) and FANCI (C) nuclear foci formation was analyzed by immunofluorescence microscopy (IF). Quantification of the percentage of PTEN^{+/+} and PTEN^{-/-} nuclei displaying greater than five discrete FANCD2 or FANCI nuclear foci reveals defective MMC-inducible FANCD2 and FANCI foci formation in PTEN^{-/-} cells. ***, $P < 0.001$. (D) Immunoblotting for FANCD2, FANCI, and RAD51 reveals no appreciable differences in total cellular levels of MMC-inducible FANCD2 and FANCI monoubiquitination or RAD51 between PTEN^{+/+} and PTEN^{-/-} cells. Cells were incubated in the absence or presence of 200 nM MMC for 24 h and allowed to recover for 8 h. (E) Chromatin fractionation analysis of lysates from MCF10A PTEN^{+/+} and PTEN^{-/-} cells reveals efficient localization of FANCD2, FANCI, and RAD51 to chromatin following MMC treatment. Cells were incubated in the absence (NT) or presence of 200 nM MMC for 24 h. W, unfractionated whole-cell lysate; S, soluble cytoplasmic and nuclear fraction; C, chromatin-enriched fraction.

D **E**

Figure S3. Increased FANCM instability and defective chromatin recruitment of the FA core complex in PTEN-deficient cells

(A) Immunoblotting analyses of whole-cell lysates from HCT116 PTEN^{+/+} and PTEN^{-/-} cells reveal decreased levels of FANCM in PTEN^{-/-} cells. Cells were incubated in the absence (NT) or presence of 40 nM mitomycin C (MMC) for 18 h and allowed to recover for up to 24 h. (B) Immunoblotting analyses of whole-cell lysates from MCF10A PTEN^{+/+} and PTEN^{-/-} cells reveal decreased levels of FANCM in PTEN^{-/-} cells. Cells were treated as described for (A). RBI, Relative protein band intensity. (C) MCF10A PTEN^{+/+} and PTEN^{-/-} cells were incubated in the absence or presence of 40 µg/mL cycloheximide (CHX) alone or 40 µg/mL CHX and 4 µM MG132 (CHX + MG) for the indicated times. Whole-cell lysates were prepared and immunoblotted with anti-FANCM, anti-p53, and anti-PTEN antibodies. (D) Quantification of FANCM nuclear foci formation in HCT116 PTEN^{+/+} and PTEN^{-/-} cells reveals a defect in MMC-inducible FANCM nuclear foci formation in PTEN^{-/-} cells. Cells were incubated in the absence (NT) or presence of 40 nM MMC for 18 h. ***, $P < 0.001$. (E) Quantification of FANCA nuclear foci formation in HCT116 PTEN^{+/+} and PTEN^{-/-} cells reveals reduced MMC-inducible FANCA nuclear foci formation in PTEN^{-/-} cells. Cells were treated as described for (D). ***, $P < 0.001$. (F) Chromatin fractionation analysis of HCT116 PTEN^{+/+} and PTEN^{-/-} cells reveals a defect in the localization of FANCA to chromatin following MMC treatment. Cells were incubated in the absence (NT) or presence of 200 nM MMC for 24 h. W, unfractionated whole-cell lysate; S, soluble cytoplasmic and nuclear fraction; C, chromatin fraction. (G) Defective MMC-inducible FANCM nuclear foci formation in HCT116 PTEN^{-/-} cells. Cells were treated as described for (D). (H) Defective MMC-

inducible FANCA nuclear foci formation in HCT116 PTEN^{-/-} cells. Cells were treated as described for (D).

3G

HCT116

Figure S4. PTEN does not dephosphorylate PLK1-phosphorylated FANCM *in vitro*

FANCM was incubated in the presence of PLK1, ^{32}P -ATP and increasing concentrations of PTEN or PTEN-C124S (lipid and protein phosphatase defective). SDS-PAGE was used to separate the proteins and phosphorylation levels were detected by exposing the dried gel to radiographic film. Lanes 5-7 show the input proteins in isolation. *, Indicates some minor contaminants within protein preparations, in particular a minor band in PLK1 preparations runs at the same size as PTEN and is phosphorylated by PLK1 even in the absence of PTEN addition. Some PLK1 autophosphorylation also occurs.

Figure S5. Increased γ H2AX and 53BP1 nuclear foci formation in PTEN-deficient cells

(A) Quantification of γ H2AX nuclear foci formation in MCF10A PTEN^{+/+} and PTEN^{-/-} cells reveals constitutively elevated and persistent γ H2AX nuclear foci in PTEN^{-/-} cells. Cells were incubated in the absence (NT) or presence of 200 nM MMC for 18 h and allowed to recover for up to 24 h. ***, $P < 0.001$. (B) Quantification of 53BP1 nuclear foci formation in PTEN^{+/+} and PTEN^{-/-} cells reveals elevated levels of 53BP1 nuclear foci in PTEN^{-/-} cells with and without MMC treatment. Cells were treated as described for (A). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (C) Chromatin fractionation analysis of HCT116 PTEN^{+/+} and PTEN^{-/-} cells reveals increased 53BP1 chromatin localization in PTEN^{-/-} cells with and without MMC treatment. Cells were incubated in the absence (NT) or presence of 40 nM MMC for 18 h. W, unfractionated whole-cell lysate; S, soluble cytoplasmic and nuclear fraction; C, chromatin fraction. (D and E) Quantification of RAD51 nuclear foci formation in HCT116 (D) and MCF10A (E) PTEN^{+/+} and PTEN^{-/-} cells reveals no appreciable differences in the levels of RAD51 nuclear foci formation between PTEN^{+/+} and PTEN^{-/-} cells. Cells were incubated in the absence (NT) or presence of 40 nM (HCT116) or 200 nM (MCF10A) MMC for 18 h and allowed to recover for up to 24 h (E).

Figure S6. PTEN function in ICL repair is protein phosphatase dependent

(A) HCT116 PTEN^{-/-} cells were stably transduced with pLenti6.2-LacZ, -PTEN-WT, -PTEN-C124S, -PTEN-G129E, and -PTEN-Y138L. Whole-cell lysates were prepared and immunoblotted for PTEN, AKT, AKT pS473, RAD51, and α -tubulin. (B) PTEN-Y138L fails to rescue the sensitivity of PTEN^{-/-} cells to the clastogenic effects of mitomycin C (MMC), in contrast to PTEN-WT. Cells were incubated in the absence (NT) or presence of 20 nM MMC for 24 h and metaphase spreads were analyzed for structural chromosome aberrations. ***, $P < 0.001$. (C and D) Defective FANCD2 (C) and FANCI (D) nuclear foci formation in PTEN^{-/-} cells expressing PTEN-Y138L. Cells were incubated in the absence (NT) or presence of 200 nM MMC for 18 h. ***, $P < 0.001$. (E and F) Defective FANCA (E) and FANCM (F) nuclear foci formation in PTEN^{-/-} cells expressing LacZ, PTEN-C124S, and PTEN-Y138L. Cells were incubated in the absence (NT) or presence of 200 nM MMC for 18 h. ***, $P < 0.001$.

E **F**

Figure S7. FANCD2 and PTEN epistasis analysis

(A) Metaphase chromosome analysis of FA-D2 (*FANCD2*^{-/-}) cells reveals that knockdown of PTEN (siPTEN) does not lead to a further increase in the levels of complex chromosome aberrations. siCtrl, control, non-targeting siRNA. (B) siRNA-mediated knockdown of FANCD2 in the PTEN-deficient prostate adenocarcinoma cell line PC-3. (C) Knockdown of FANCD2 (siFANCD2) in PC-3 cells does not lead to a further increase in sensitivity to MMC cytotoxicity. siCtrl, control, non-targeting siRNA. (D-F) Metaphase chromosome analysis of PC-3 cells reveals that knockdown of FANCD2 does not lead to a further increase in the levels of chromosome gaps and breaks (E), complex chromosome aberrations (F), or total chromosome aberrations (G).

Figure S8. Proposed model of the role of PTEN in ICL repair

PTEN has previously been shown to be required for the assembly of APC/C-CDH1 and the degradation of PLK1¹. PLK1 directly phosphorylates PTEN, promoting its accumulation in chromatin². In turn, PTEN dephosphorylates PLK1, leading to its inactivation and destabilization³. When PTEN is present, upon DNA damage, it dephosphorylates PLK1, which allows FANCM to promote the chromatin recruitment of FANCA and the FA core complex, resulting in efficient FANCD2 and FANCI nuclear foci formation and error-free ICL repair. In the absence of PTEN, PLK1 fails to be dephosphorylated, remains constitutively active and phosphorylates FANCM. FANCM phosphorylation leads to its ubiquitination and subsequent degradation by the proteasome. The FANCM phosphatase remains to be identified. When FANCM levels are low, the chromatin recruitment of FANCA and the FA core complex is attenuated, leading to inefficient FANCD2 and FANCI nuclear foci formation and error-prone ICL repair. A, FANCA; D2, FANCD2; I, FANCI; M, FANCM; P, phosphorylation; Ub, ubiquitination. Black- and grey-filled arrows indicate functional and non-functional steps, respectively.

Figure S9. Validation of the FANCM mouse monoclonal antibodies used in these studies

293 cells were transfected with control non-targeting siRNA (lane 1) or siRNA targeting FANCM (lane 2). Whole-cell extracts were prepared in 10 mM Tris, 150 mM NaCl, 10%v/v glycerol, 0.1%w/v Triton X-100, 2 U/mL benzonase. Proteins were resolved on NuPAGE 3-8% w/v Tris-Acetate gels (Invitrogen), transferred to polyvinylidene difluoride (PVDF) membranes, and immunoblotted with the indicated anti-FANCM mouse monoclonal antibodies.

References

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APPENDIX III

Supplemental information for manuscript III: “PTEN regulation by SUMOylation is important for its DNA crosslink repair”

Supplemental Figures

Figure S1. PTEN-SUMO cellular localization

(A and B) Cellular fractionation analysis on HeLa cells showed a stabilization of PTEN-SUMO in the nucleus and bound to chromatin in the presence of MG132. This was visualized by two separate cell fractionation assays (A) separating cytoplasmic (CS), nuclear soluble (NS) and chromatin bound (CB) and (B) a nuclear enrichment.

A

W: Whole Cell
CS: Cytoplasmic
NS: Nuclear Soluble
CB: Chromatin Bound

B

Key
W, Whole-cell lysate
S, Soluble nuclear and cytoplasmic
C, Chromatin-associated

Figure S2. Proposed model of the role of PTEN SUMOylation in DNA repair

It has been shown that PTEN is SUMOylated on K254 and this SUMOylation is important for its role in DNA repair, but how this modification results in genome stability is still unclear [1]. We propose PTEN dephosphorylates one or more DNA repair proteins regulating their activity. To ensure the correct kinetics of DNA repair, PTEN SUMOylation and its subsequent ubiquitination by RNF4 regulate PTENs protein levels in the nucleus. p97/VCP then removes PTEN and shuttles it to the proteasome through the interaction of the SUMO/Ub chains and p97/VCP adapter proteins.

References

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