UCSF
UC San Francisco Previously Published Works

Title

Synthetic Lethal Targeting of PTEN-Deficient Cancer Cells Using Selective Disruption of Polynucleotide Kinase/Phosphatase

Permalink

https://escholarship.org/uc/item/24k1j128

Journal

Molecular Cancer Therapeutics, 12(10)

ISSN

1535-7163

Authors

Mereniuk, Todd R
Gendy, Mohamed AM El
Mendes-Pereira, Ana M
et al.

Publication Date

2013-10-01

DOI

10.1158/1535-7163.mct-12-1093

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

Europe PMC Funders Group
Author Manuscript
Mol Cancer Ther. Author manuscript; available in PMC 2014 April 01.

Published in final edited form as:
Mol Cancer Ther. 2013 October ; 12(10): 2135–2144. doi:10.1158/1535-7163.MCT-12-1093.

Synthetic lethal targeting of PTEN-deficient cancer cells using selective disruption of polynucleotide kinase/phosphatase

Todd R. Mereniuk[1], **Mohamed A.M. El Gendy**[1,2], **Ana M. Mendes-Pereira**[3], **Christopher J. Lord**[3], **Sunita Ghosh**[1], **Edan Foley**[4], **Alan Ashworth**[3,5], and **Michael Weinfeld**[1]

[1]Experimental Oncology, Department of Oncology, University of Alberta, Edmonton, AB, Canada T6G 1Z2

[2]Department of Pharmacognosy, Pharmaceutical Sciences Division, National Research Centre, Dokki, Giza, Egypt 12622

[3]The Breakthrough Breast Cancer Research Center, The Institute of Cancer Research, London, UK

[4]Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, AB, Canada T6G 2S2

[5]CRUK Gene Function Laboratory, The Institute of Cancer Research, London, UK

Abstract

A recent screen of 6961 siRNAs to discover possible synthetic lethal partners of the DNA repair protein polynucleotide kinase/phosphatase (PNKP) led to the identification of the potent tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*). Here we have confirmed the PNKP/PTEN synthetic lethal partnership in a variety of different cell lines including the PC3 prostate cancer cell line, which is naturally deficient in PTEN. We provide evidence that co-depletion of PTEN and PNKP induces apoptosis. In HCT116 colon cancer cells the loss of PTEN is accompanied by an increased background level of DNA double strand breaks, which accumulate in the presence of an inhibitor of PNKP DNA 3′-phosphatase activity. Complementation of PC3 cells with several well-characterized mutated *PTEN* cDNAs indicated that the critical function of PTEN required to prevent toxicity induced by an inhibitor of PNKP is most likely associated with its cytoplasmic lipid phosphatase activity. Finally, we show that modest inhibition of PNKP in a PTEN knockout background enhances cellular radiosensitivity, suggesting that such a "synthetic sickness" approach involving the combination of PNKP inhibition with radiotherapy may be applicable to PTEN-deficient tumors.

Keywords

Synthetic lethality; polynucleotide kinase/phosphatase; PTEN; targeted therapy; synthetic sickness

INTRODUCTION

Synthetic lethality provides a means to target loss-of-function mutations commonly associated with the formation of neoplastic malignancies because it takes advantage of a

Correspondence to: Michael Weinfeld Cross Cancer Institute University of Alberta 11560 University Ave Edmonton, AB, Canada, T6G 1Z2 Tel: 780 432 8438 Fax: 780 432 8428 michael.weinfeld@albertahealthservices.ca..

Potential conflict of interest: T.R. Mereniuk, E. Foley and M. Weinfeld have submitted a Provisional US patent application - UNAB 017 Synthetic lethality in cancer.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

cell's propensity to lose tumor suppressor function during its stepwise progression to cancer cell formation by targeting a second, distinct protein not essential for cell survival. Co-disruption of both of these non-essential proteins, or the genes encoding them, in the same cell causes lethality, whereas each corresponding single disruption is compatible with survival (1-4). In this way it is possible to selectively kill only those cells in which both of these proteins are disrupted, i.e. cancer cells, while the effect on normal cells is considerably less detrimental. Therapeutic advantage can also be gained through the related concept of "synthetic sickness", in which co-disruption of the genes/proteins severely weakens cells and increases their sensitivity to radiation or cytotoxic drugs (5-7).

To date, most of the focus on synthetic lethality has centered on the use of inhibitors of poly(ADP-ribose) polymerase (PARP) and cancers that have lost function of the breast cancer susceptibility loci, BRCA1 and BRCA2 (8-11). However, increasing evidence shows that PARP inhibitors may benefit not only BRCA-deficient cancers but also cancers lacking non-canonical DNA repair proteins such as the important tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (12). PTEN, is inactive in a broad spectrum of hereditary and sporadic human cancers, and is the second most frequently lost tumor suppressor behind only p53 (13, 14). It participates in regulating cell growth and the cell cycle and plays a critical role in anti-apoptotic pathways (13).

We have recently broadened the scope of synthetic lethality to include polynucleotide kinase/phosphatase (PNKP) as another viable therapeutic target (15). Like PARP, PNKP is an enzyme involved in the repair of DNA strand breaks. It possesses two activities, a 3′-DNA phosphatase and 5′-DNA kinase, which are required to restore the chemical composition of strand break termini to forms suitable for the subsequent action of DNA polymerases and ligases, i.e. 3′-hydroxyl and 5′-phosphate termini (16, 17). PNKP participates in several repair pathways including base excision repair, single and double-strand break repair (18, 19). Depletion of PNKP activity by shRNA, siRNA or a small molecule inhibitor of its phosphatase activity sensitizes cells to ionizing radiation and the topoisomerase I poison camptothecin (20, 21). Importantly, reduction of PNKP increases the spontaneous mutation frequency, indicating that it is required for the repair of endogenous DNA damage induced by reactive oxygen species (20).

To identify potential synthetic lethal partners of PNKP we previously performed an siRNA-library based screen of the "druggable" genome and discussed our findings regarding the partnership with the tumor suppressor SHP-1 (15). Among the other potential synthetic lethal partners of PNKP we identified *PTEN*. Here we report the validation of our initial findings confirming that indeed PNKP and PTEN act in a synthetic lethal partnership and describe initial experiments to uncover the potential mechanism. We also show that loss of *PTEN* coupled with partial inhibition of PNKP significantly sensitizes cells to ionizing radiation. Our data suggest that the clinical usefulness of PNKP disruption may be extended to include $PTEN^{-/-}$ cancers.

MATERIAL AND METHODS

Cell Lines

A549 cells were purchased from the American Type Culture Collection (Manassas, VA). A549δPNKP (A549 cells stably depleted of PNKP using shRNA) and A549-SC (A549 cells stably expressing a scrambled shRNA) have been previously described (15). The PC3 human prostate cancer parental cell line and its variants were previously described (12). PC3 cells were sourced from ATCC and maintained according to the supplier's instructions in a mycoplasma free environment. To confirm the identity of PC3 cells (both in their original and modified forms), we used STR typing of DNA derived from PC3 cultures as described

in Dirks and Drexler (22). (The correct identity of PC3 cells was last confirmed on Feb. 8, 2013). The HCT116 human colon cancer parental cell line and its PTEN knockout variants and G418-resistant control (23) were generously provided by Dr. Todd Waldman (Georgetown University, Washington, DC). The presence or absence of PTEN expression was confirmed by Western blot but no further authentication was done by the authors.

Cell lines were cultured at 37°C and 5% CO_2 in a humidified incubator in a 1:1 mixture of Dulbecco's Modified Eagle's Medium and F12 (DMEM/F12) supplemented with 10% FBS, 50 U/mL penicillin, 50 μg/mL streptomycin, 2 mM L-glutamine, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate. All culture supplements were purchased from Invitrogen (Carlsbad, CA).

Vectors and siRNA

pSUPER.neo vectors (Oligoengine, Seattle, WA) contained either an shRNA directed against nucleotides 1391-1410 of PNKP (20) to stably deplete PNKP in A549 cells or an shRNA to no known gene target (scrambled shRNA) to generate the control cell line A549-SC.

The preparation of pBABE.puro (Addgene, Cambridge, MA) vectors containing wild type RAD51 cDNA or wild type or mutated PTEN cDNA used to generate the PC3 reconstituted cell lines: WT PTEN (full length, wild-type PTEN), p.K289E (PTEN mutant with reduced nuclear shuttling), p.R55fs*1 (truncation mutant found in PC3), p.C124S (a phosphatase inactive PTEN mutant) was reported previously (12).

All siRNAs were purchased from Qiagen (Mississauga, ON) with the exception of PNKP siRNA (Ambion, Austin, TX).

Cell transfection

20,000 cells were plated and allowed to adhere overnight in a 24-well dish at 37°C and 5% CO_2. The transfection mixture was prepared from two separate solutions, one containing 1 μg of plasmid DNA dissolved in 50 μL total of Opti-MEM (Invitrogen) and the other 3 μL of Lipofectamine2000 (Invitrogen) in 50 μL total Opti-MEM. The solutions were incubated at room temperature for 5 min before combination, mixed and then held at room temperature for 20 min. The media from the pre-plated cells was removed and replaced with the transfection mixture, and the cells were incubated for 24 h at 37°C and 5% CO_2. The cells were then trypsinized and expanded as previously described (15). Transient transfections were performed using 4000 cells/well and a final concentration of 16 nM of siRNAs as previously described (15).

Cell proliferation assay

Assays were performed using the transient transfection technique described above, however, after incubation with siRNA for 72 h, 10% v/v of 440 μM Alamar Blue (Sigma-Aldrich, Oakville, ON) was added to each well and the cells were incubated for 50-90 min, after which the fluorescence in each well was determined using an EnVision 2104 Multilabel Reader (PerkinElmer) with an excitation wavelength of 563 nm and emission wavelength of 587 nm. HCT116 based cell lines were subjected to a 10.7% v/v 440 μM Alamar Blue solution per well.

Clonogenic survival assay

Cells, seeded in 60-mm dishes 24 h in advance, were treated with the PNKP inhibitor A12B4C3 (21, 24) (kindly provided by Dr. Dennis Hall, University of Alberta) for 9-14 consecutive days at 0, 0.1, 1, and 10 μM final concentration. (100 cells were plated for the 0,

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

0.1 and 1 μM concentration groups and 300 cells in the 10 μM concentration group.) Colonies were then stained with a crystal violet stain containing 20% methanol for one hour, after which the plates were washed in warm water and left to dry overnight. Colonies of 50 cells were counted using an automated colony counter (Oxford Optronix, Oxford, UK).

To determine the radiation response, cells were treated with 0, 1, 2, 4, 6, or 8 Gy γ-radiation (^{60}Co Gammacell, AECL, Ottawa, Canada) in the absence of PNKP inhibitor, or under continuous PNKP inhibition using 2 μM A12B4C3. Cells subjected to continuous PNKP inhibition were pre-treated with 2 μM A12B4C3 for 24 h before irradiation.

Statistical analysis

Reported p-values were generated using a two-sided Student's t-test. Z-scores were generated from an average of 24-96 individual wells of data per assay (performed at least in triplicate). The high number of replicates allowed us to achieve robust statistical data. A Z-score is a dimensionless quantity denoting the number of standard deviations a sample is above or below the mean of a control. It is defined as:

$$z = \frac{x - \mu}{\sigma}$$

z = Z-score

x = the raw score to be standardized

μ = population mean

σ = standard deviation of the population

Z-scores can therefore be positive or negative depending on whether the sample is greater or less than the mean of controls. We were interested in a negative Z-score as this showed that the experimental condition was lethal in comparison to control conditions. A Z-score of -3 is significantly different than control as it is at least three standard deviations less than the control average.

To analyze for additivity and synergy, we used the median effect principle as proposed by Chou (25, 26) to calculate the median effect and the combination index (CI) based on the multiple drug effect equation provided by Chou and Talalay (27, 28). The CI value of 1 was considered for additive effect, <1 was considered for synergism effect and >1 was considered for antagonism effect. The median effect and CI were calculated using CALCUSYN software version 1.2 (Biosoft).

Determination of mode of cell death

A549-SC or A549δPNKP cells were grown on coverslips in complete DMEM/F12 and transfected with ASN or PTEN siRNA. As a positive control, the cells were treated with 100 μM 5-(p-bromobenzylidine)-α-isopropyl-4-oxo-2-thioxo-3-thiozolidineacetic acid (BH3I-1, Sigma-Aldrich, Oakville, ON), which induces apoptosis. The cells were triple-stained after the indicated length of time with Hoechst 33342, Ethidium Homodimer III and Annexin V-FITC as previously described (15).

Monitoring double strand break repair

DSB were monitored based on the level of phosphorylated histone H2AX (γH2AX). Two experiments were performed. First, we monitored the level of γH2AX before and after γ-irradiation. Cells were seeded on cover slips (10^5 cells/well) in 6-well plates with 2 mL DMEM/F12 without antibiotics and left overnight to adhere. The cells were irradiated (5

Gy) and allowed to repair for the indicated time points, after which γH2AX was detected as previously described (29). Fluorescence was normalized against background fluorescence and quantified using MetaXpress software (version 5.0.0.21, Molecular Devices, CA). Second, we tested the effect of PNKP inhibition on the accumulation of DSBs in the absence of irradiation. Cells were incubated with 5 μM of A12B4C3 and γH2AX was detected and quantified at different time points.

RESULTS

Confirmation of PTEN as a possible synthetic lethal partner of PNKP

We previously sought to discover synthetic lethal partnerships of PNKP, as an alternative to PARP, without necessarily limiting our search to partner proteins directly involved in DNA repair (15). We performed a forward transfection screen with a library of siRNAs targeting 6961 genes using pooled samples of four distinct siRNAs targeting each gene. The screen was performed in duplicate using A549 lung cancer cells stably depleted of PNKP (A549δPNKP) and again using cells expressing a scrambled shRNA (A549-SC) under identical conditions. Cells were exposed to siRNA transfection complexes continuously for 72 h allowing for at least two cell cycles to occur at a concentration known to be effective at knocking down target proteins. Amongst the potential synthetic lethal partners of PNKP was the major tumor suppressor PTEN. Figure 1A shows the difference between five selected tumor suppressors, including the previously characterized PNKP synthetic lethal partner SHP-1 (15), compared to two proteins not synthetically lethal when co-disrupted with PNKP.

To confirm the synthetic lethal relationship between PNKP and PTEN, we repeated the transfections, but reduced the concentration of siRNA previously used in the screen by 3.5-fold. At this concentration, the siRNA still showed synthetic lethality in PNKP knockdown cells but not in A549-SC control cells (Fig. 1B). We then sought to use each of the four originally pooled siRNAs separately in order to further minimize the potential for off-target effects (Fig. 1C). When the distinct siRNAs directed against PTEN were assayed, two displayed selective killing of A549δPNKP cells and no toxicity in control cells, siRNA #6 Z-factor = −9.0, $p < 0.001$; siRNA #8 Z-factor = −9.1, $p < 0.001$). Since more than one siRNA showed synthetic lethality with PNKP, the effect was most likely attributable to the double knockdown of PNKP and PTEN and not due to off-target effects. Activation of the RNAi pathway using AllStars negative control siRNA (ASN) is also not responsible for the lethality seen, indicating this is a true synthetic lethal partnership. The greater cytotoxicity seen with the PTEN/PNKP double knockdown using PTEN #6 siRNA than when using PTEN #8 siRNA probably reflected the efficiency of the two siRNAs to knockdown PTEN expression (Fig. 1E). This would imply a dose response effect that may have implications regarding natural levels of active PTEN found in tumors.

To further substantiate that a synthetic lethal partnership exists between PTEN and PNKP, we carried out a similar analysis with the MCF7 breast cancer cell line and #6 siRNA. As seen with A549 cells, the combined disruption of both PTEN and PNKP was responsible for lethality, since the depletion of PNKP or PTEN individually was not lethal (Fig. 1D, Z-score for PTEN = −8.0, $p < 0.001$, Z-score for SHP-1 = −3.4, $p < 0.001$), nor is the activation of RNAi machinery responsible for lethality. These findings imply that a true synthetic lethal relationship exists between PTEN and PNKP.

Finally, isogenically matched HCT116 parental, Neo124 vector only ($PTEN^{+/+}$) and $PTEN^{-/-}$ (#22 and #35) cells (23) were subjected to increasing concentrations of the PNKP phosphatase inhibitor A12B4C3 (21). Figure 2 clearly shows that loss of PTEN sensitized

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

the cells to A12B4C3 and underscores the importance of the DNA 3′-phosphatase activity of PNKP to the synthetic lethal process.

Mode of cell death

To identify the mode of cell death responsible for synthetic lethality following co-disruption of PTEN and PNKP, A549-SC and A549δPNKP cells were grown on glass coverslips and transiently transfected with either PTEN or ASN siRNA. As a positive control, the known apoptosis inducer, BH3I-1 was added to the medium at a concentration of 100 μM. After the indicated lengths of time, cells were triple stained with Hoechst 33342, Ethidium Homodimer III and Annexin V-FITC to distinguish between those cells undergoing apoptosis, necrosis or neither. Figure 3A shows that a small population of both apoptotic and necrotic cells was present at every time point, however, treatment of cells with BH3I-1 dramatically increased the proportion of apoptotic cells relative to necrotic cells beginning at 24 h post-treatment in both A549-SC and A549δPNKP (Fig. 3B). Similarly, when both PTEN and PNKP were disrupted in the same cells, there was a substantial increase in apoptosis (Fig 3C). Conversely, when PTEN was knocked down in A549-SC cells, there was no increase in the proportion of either apoptotic or necrotic cells (Fig. 3C). Therefore, cells co-dysfunctional for PTEN and PNKP undergo cell death through apoptotic mechanisms, similar to cells in which both SHP-1 and PNKP are doubly disrupted (15).

DNA damage and repair in PTEN-null cells

To begin to identify a potential mechanism for the PTEN/PNKP synthetic lethal partnership, we examined the effect of loss of PTEN on DNA double strand break (DSB) repair, as determined by the level of phosphorylation of the histone variant H2AX in the HCT116 cell lines following exposure to ionizing radiation. As shown in Figure 4A, PTEN−/− HCT116 cells display (a) a higher background level of γH2AX in unirradiated cells compared to the control PTEN+/+ cells, and (b) slower repair kinetics, especially during the initial four hour period post-irradiation. The slower repair kinetics may reflect decreased homologous recombination seen previously in PTEN−/− HCT116 cells (12).

We then examined the influence of PNKP inhibition on the background level of DSBs and observed that exposure of the PTEN−/− HCT116 cells to A12B4C3 led to a modest but constant increase in γH2AX fluorescence while the control HCT116 cells showed no response to A12B4C3 (Fig. 4B).

Because the elevated background level of DSBs was reminiscent of our earlier observation with SHP-1 depleted cells (15), albeit to a less marked extent, we measured the production of reactive oxygen species (ROS) in PTEN-deficient cells. However, examination of the isogenically matched PTEN+/+ and PTEN−/− HCT116 cells revealed that these cells produced almost identical levels of peroxynitrite and hydroxyl radicals, indicating that knocking out PTEN, unlike SHP-1 depletion (15, 30), does not cause a significant increase in the production of these ROS (Supplementary Fig. S1).

Survival of naturally occurring PTEN negative cells in response to PNKP inhibition

The ability to effectively and efficiently target one member of a synthetic lethal partnership will determine synthetic lethality's clinical usefulness. We therefore investigated whether we could take advantage of the newly identified partnership between PTEN and PNKP. We subjected the prostate cancer cell line, PC3 (naturally PTEN$^{-/-}$), to an increasing concentration of the PNKP inhibitor A12B4C3 over a period of 12-16 days. The dose response curve (Fig. 5C) indicates that at A12B4C3 doses 10 μM there was a marked decrease in survival of the PC3 parental cell line. We then made use of ectopic expression of wild type and various mutant PTEN cDNAs to further analyze the role of key components of

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

PTEN (Fig. 5C). As anticipated, expression of the empty vector, p.BABE.puro, and a vector coding for the *PTEN* deletion-frameshift mutation found in PC3 cells, p.R55fs*1, did not alter the result seen with the parental PC3 cells. Similarly, expression of the catalytically inactive mutant of *PTEN*, coding for the C124S altered protein, also failed to elicit an increase in the survival of A12B4C3 treated cells. In contrast, PC3 cells reconstituted with either wild type PTEN (WT) protein or the K289E modified PTEN, which is phosphatase proficient but undergoes reduced shuttling to the nucleus, restored resistance to PNKP-inhibition. Finally, because of a potential association between PTEN and Rad51 (12, 31-33), we examined PC3 cells ectopically expressing *RAD51* cDNA, but found no increase in survival in response to treatment with A12B4C3 (Fig. 5D). From these data we infer that the critical function of PTEN lies in its phosphatase activity and its localization in the cytoplasm.

A cell proliferation assay (Supplementary Fig. 2) indicated that the natural differences in cell growth rate could not account for the observed responses of the PC3-derived cells to A12B4C3.

Radiosensitization by combined disruption of PNKP and PTEN

We have previously observed that depletion or inhibition of PNKP sensitizes cells to ionizing radiation (20, 21). We therefore examined if disruption of PNKP in PTEN negative cells would hypersensitize cells to ionizing radiation (Fig. 6). Wild type and the two $PTEN^{-/-}$ HCT116 cell lines were incubated with a non-toxic dose (2 μM) of A12B4C3 (or just the DMSO vehicle) for 24 h prior to irradiation with doses up to 8 Gy and then maintained in the presence of the PNKP inhibitor until colonies were counted. As expected A12B4C3 sensitized the control $PTEN^{+/+}$ HCT116 cells to radiation in a similar manner to that previously seen with A549 cells (21). We also observed that the $PTEN^{-/-}$ cell lines were modestly radiosensitive in comparison to the control cell line in accordance with published data (23). However, when PTEN and PNKP were simultaneously disrupted, there was a significant enhancement of radiosensitization. Applying the method of Chou and Talalay to analyze for additivity and synergy (27, 28), we found a synergistic radiation hypersensitization at 8 Gy ($PTEN^{-/-}$ #22 cells) and 6 Gy ($PTEN^{-/-}$ #35 cell line).

DISCUSSION

We have confirmed a synthetic lethal relationship between PTEN and the DNA repair protein PNKP following our initial screen (15). PTEN is recognized as the second most frequently compromised tumor suppressor. Its down regulation or complete loss is implicated in the development and/or progression of many sporadic human cancers (13, 14). For example, PTEN functional mutations or complete protein loss was found to occur frequently in glioblastoma, endometrial cancer, melanoma and prostate cancer (28.8%, 34.6%, 12.1% and 11.8% respectively) (14). PTEN-deficient tumors thus represent an excellent target for synthetic lethal approaches to treatment. Other synthetic lethal partners of PTEN have been discovered including PARP (12), and more recently the TTK protein tyrosine kinase (34).

PTEN plays a critical role as an antagonist of the phosphoinositide 3-kinase (PI3K) pathway in the cytoplasm through its lipid phosphatase function by dephosphorylating the 3 position of the second messenger phosphatidylinositol 3,4,5-triphosphate (PIP3) to form inactive phosphatidylinositol 4,5-bisphosphate (PIP2), thereby suppressing downstream signaling events, including those involving phosphoinositide-dependent kinase-1 (PDK1) and the serine/threonine kinase Akt (35-41). When PTEN is deficient, there is an accumulation of PIP3, which activates downstream signaling molecules such as the Akt and mTOR complex 1. In addition to its cytoplasmic roles, PTEN also localizes to the nucleus in a cell cycle

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

dependent manner with higher levels seen in G0/G1 (42). In the nucleus, the protein phosphatase activity of PTEN regulates MAPK phosphorylation and cyclin D1 (43, 44) and progression of the cell cycle. Several studies have also linked PTEN to genomic stability and homologous recombination primarily via expression of *RAD51* (12, 31-33), although this is an issue of some debate (45, 46).

The synthetic lethality manifested by the treatment of PTEN-deficient tumor cells with PARP inhibitors has been ascribed to a reduction in RAD51 levels and homologous recombination coupled with incomplete single-strand break repair (12). In contrast, the synthetic lethality we observed between PTEN and PNKP could not be alleviated by ectopic expression of *RAD51* (Fig. 5D). Furthermore, unlike the response to PARP inhibition, resistance to PNKP inhibition was restored by expression of the phosphatase active and cytoplasmically-enriched PTEN K289E protein, but not the catalytically inactive C124S protein that can enter the nucleus. Therefore, the function of PTEN that is critical for survival under PNKP disruption most likely lies in its cytoplasmic function as a lipid phosphatase in signal transduction pathways. Interestingly, in our original screen we identified MAGI3 and MAST2 as potential synthetic lethal partners of PNKP (15), although this will need further confirmation. Since it has been shown that PTEN stability is enhanced through interaction with the PDZ domains of both of these cytoplasmic proteins (47-49), it is conceivable that a synthetic lethal partnership with PNKP would be mediated by reduced stability and activity of PTEN located in the cytoplasm.

The clear differences between the responses to RAD51 and PTEN isoforms indicate that distinct mechanisms underlie the synthetic lethal pathways between PTEN and PARP and between PTEN and PNKP despite the fact that both PARP and PNKP play significant roles in DNA strand break repair. Our observation that PTEN-deficient cells show an increase in the baseline level of DSB and slower DSB repair (Fig. 4) suggests that loss of PTEN leads to increased production of DNA strand breaks with 3′-phosphate termini requiring PNKP for their repair. However, given the relatively modest differences between wild type and PTEN −/− cells, coupled with our failure to observe increased ROS production in PTEN−/− cells, this issue requires further investigation.

In addition to using the PTEN/PNKP relationship under purely synthetic lethal conditions, we also examined the possibility of taking advantage of synthetic sickness, i.e. weakening the cell to other therapeutic agents. From a clinical standpoint the use of a repair protein inhibitor in a synthetic sickness approach offers two advantages - either augmenting cell killing for a given dose of the primary genotoxic anticancer agent, or allowing the use of a lower dose of the primary agent to achieve the same level of cancer cell killing but reducing the likelihood of normal tissue damage. The potential of such an approach was shown by the increased radiosensitization afforded by co-treatment with the PNKP inhibitor. This provides a possible therapeutic modality in which PTEN depleted tumors would first be sensitized by inhibition of PNKP and then targeted by focused radiation. Since PNKP disruption is well tolerated by PTEN proficient normal cells, there would be little damage to normal tissues, and thus side effects should be minimized.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Todd Waldman (Georgetown University, Washington, DC) for providing the wild type and *PTEN* knockout HCT116 cell lines, Dr. Dennis Hall (University of Alberta, Edmonton, AB) for supplying the PNKP inhibitor.

FINANCIAL SUPPORT This work was supported by the Canadian Institutes of Health Research (grant numbers MOP 15385 and MOP 115069 to M. Weinfeld; MOP 77746 to E. Foley); the Breakthrough Breast Cancer and Cancer Research UK; NHS funding to the NIHR Royal Marsden Biomedical Research Centre to A. Ashworth. T.R. Mereniuk was the recipient of graduate studentships from the Alberta Cancer Foundation and Alberta Innovates - Health Solutions. M.A.M. El Gendy is a recipient of a postgraduate fellowship from Alberta Innovates - Health Solutions. E. Foley is a scholar of Alberta Innovates – Health Solutions and holds a Canada Research Chair in Innate Immunity.

REFERENCES

1. Hartwell LH, Szankasi P, Roberts CJ, Murray AW, Friend SH. Integrating genetic approaches into the discovery of anticancer drugs. Science. 1997; 278:1064–8. [PubMed: 9353181]
2. Reinhardt HC, Jiang H, Hemann MT, Yaffe MB. Exploiting synthetic lethal interactions for targeted cancer therapy. Cell Cycle. 2009; 8:3112–9. [PubMed: 19755856]
3. Brough R, Frankum JR, Costa-Cabral S, Lord CJ, Ashworth A. Searching for synthetic lethality in cancer. Curr Opin Genet Dev. 2011; 21:34–41. [PubMed: 21255997]
4. Chan DA, Giaccia AJ. Harnessing synthetic lethal interactions in anticancer drug discovery. Nat Rev Drug Discov. 2011; 10:351–64. [PubMed: 21532565]
5. Kaelin WG Jr. The concept of synthetic lethality in the context of anticancer therapy. Nat Rev Cancer. 2005; 5:689–98. [PubMed: 16110319]
6. Chalmers AJ, Lakshman M, Chan N, Bristow RG. Poly(ADP-ribose) polymerase inhibition as a model for synthetic lethality in developing radiation oncology targets. Semin Radiat Oncol. 2010; 20:274–81. [PubMed: 20832020]
7. Martin SA, McCabe N, Mullarkey M, Cummins R, Burgess DJ, Nakabeppu Y, et al. DNA polymerases as potential therapeutic targets for cancers deficient in the DNA mismatch repair proteins MSH2 or MLH1. Cancer Cell. 2010; 17:235–48. [PubMed: 20227038]
8. Bryant HE, Schultz N, Thomas HD, Parker KM, Flower D, Lopez E, et al. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. Nature. 2005; 434:913–7. [PubMed: 15829966]
9. Farmer H, McCabe N, Lord CJ, Tutt AN, Johnson DA, Richardson TB, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature. 2005; 434:917–21. [PubMed: 15829967]
10. Comen EA, Robson M. Poly(ADP-ribose) polymerase inhibitors in triple-negative breast cancer. Cancer J. 2010; 16:48–52. [PubMed: 20164690]
11. Bryant HE, Helleday T. Inhibition of poly (ADP-ribose) polymerase activates ATM which is required for subsequent homologous recombination repair. Nucleic Acids Res. 2006; 34:1685–91. [PubMed: 16556909]
12. Mendes-Pereira AM, Martin SA, Brough R, McCarthy A, Taylor JR, Kim JS, et al. Synthetic lethal targeting of PTEN mutant cells with PARP inhibitors. EMBO Mol Med. 2009; 1:315–22. [PubMed: 20049735]
13. Simpson L, Parsons R. PTEN: life as a tumor suppressor. Exp Cell Res. 2001; 264:29–41. [PubMed: 11237521]
14. Yin Y, Shen WH. PTEN: a new guardian of the genome. Oncogene. 2008; 27:5443–53. [PubMed: 18794879]
15. Mereniuk TR, Maranchuk RA, Schindler A, Penner-Chea J, Freschauf GK, Hegazy S, et al. Genetic screening for synthetic lethal partners of polynucleotide kinase/phosphatase: potential for targeting SHP-1-depleted cancers. Cancer Res. 2012; 72:5934–44. [PubMed: 22962271]
16. Jilani A, Ramotar D, Slack C, Ong C, Yang XM, Scherer SW, et al. Molecular cloning of the human gene, PNKP, encoding a polynucleotide kinase 3′-phosphatase and evidence for its role in repair of DNA strand breaks caused by oxidative damage. J Biol Chem. 1999; 274:24176–86. [PubMed: 10446192]
17. Karimi-Busheri F, Daly G, Robins P, Canas B, Pappin DJ, Sgouros J, et al. Molecular characterization of a human DNA kinase. J Biol Chem. 1999; 274:24187–94. [PubMed: 10446193]

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

18. Allinson SL. DNA end-processing enzyme polynucleotide kinase as a potential target in the treatment of cancer. Future Oncol. 2010; 6:1031–42. [PubMed: 20528239]
19. Weinfeld M, Mani RS, Abdou I, Aceytuno RD, Glover JN. Tidying up loose ends: the role of polynucleotide kinase/phosphatase in DNA strand break repair. Trends Biochem Sci. 2011; 36:262–71. [PubMed: 21353781]
20. Rasouli-Nia A, Karimi-Busheri F, Weinfeld M. Stable down-regulation of human polynucleotide kinase enhances spontaneous mutation frequency and sensitizes cells to genotoxic agents. Proc Natl Acad Sci U S A. 2004; 101:6905–10. [PubMed: 15100409]
21. Freschauf GK, Karimi-Busheri F, Ulaczyk-Lesanko A, Mereniuk TR, Ahrens A, Koshy JM, et al. Identification of a small molecule inhibitor of the human DNA repair enzyme polynucleotide kinase/phosphatase. Cancer Res. 2009; 69:7739–46. [PubMed: 19773431]
22. Dirks WG, Drexler HG. STR DNA typing of human cell lines: detection of intra- and interspecies cross-contamination. Methods Mol Biol. 2013; 946:27–38. [PubMed: 23179824]
23. Lee C, Kim JS, Waldman T. PTEN gene targeting reveals a radiation-induced size checkpoint in human cancer cells. Cancer Res. 2004; 64:6906–14. [PubMed: 15466180]
24. Freschauf GK, Mani RS, Mereniuk TR, Fanta M, Virgen CA, Dianov GL, et al. Mechanism of action of an imidopiperidine inhibitor of human polynucleotide kinase/phosphatase. J Biol Chem. 2010; 285:2351–60. [PubMed: 19940137]
25. Chou T. Relationships between inhibition constants and fractional inhibition in enzyme-catalyzed reactions with different numbers of reactants, different reaction mechanisms, and different types and mechanisms of inhibition. Mol Pharmacol. 1974; 10:235–47. [PubMed: 4212316]
26. Chou TC. Derivation and properties of Michaelis-Menten type and Hill type equations for reference ligands. J Theor Biol. 1976; 59:253–76. [PubMed: 957690]
27. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul. 1984; 22:27–55. [PubMed: 6382953]
28. Chou, TC. The median effect principle and the combination index for quantitation of synergism and antagonism. In: Chou, TC.; Rideout, DC., editors. Synergism and antagonism in chemotherapy. Academic Press; San Diego: 1991. p. 61-102.
29. Ismail IH, Andrin C, McDonald D, Hendzel MJ. BMI1-mediated histone ubiquitylation promotes DNA double-strand break repair. J Cell Biol. 2010; 191:45–60. [PubMed: 20921134]
30. Krotz F, Engelbrecht B, Buerkle MA, Bassermann F, Bridell H, Gloe T, et al. The tyrosine phosphatase, SHP-1, is a negative regulator of endothelial superoxide formation. J Am Coll Cardiol. 2005; 45:1700–6. [PubMed: 15893190]
31. Shen WH, Balajee AS, Wang J, Wu H, Eng C, Pandolfi PP, et al. Essential role for nuclear PTEN in maintaining chromosomal integrity. Cell. 2007; 128:157–70. [PubMed: 17218262]
32. McEllin B, Camacho CV, Mukherjee B, Hahm B, Tomimatsu N, Bachoo RM, et al. PTEN loss compromises homologous recombination repair in astrocytes: implications for glioblastoma therapy with temozolomide or poly(ADP-ribose) polymerase inhibitors. Cancer Res. 2010; 70:5457–64. [PubMed: 20530668]
33. Mukherjee A, Karmakar P. Attenuation of PTEN perturbs genomic stability via activation of Akt and down-regulation of Rad51 in human embryonic kidney cells. Mol Carcinog. Apr 4.2012
34. Brough R, Frankum JR, Sims D, Mackay A, Mendes-Pereira AM, Bajrami I, et al. Functional Viability Profiles of Breast Cancer. Cancer Discov. 2011; 1:260–73. [PubMed: 21984977]
35. Zhang S, Yu D. PI(3)king apart PTEN's role in cancer. Clin Cancer Res. 2010; 16:4325–30. [PubMed: 20622047]
36. Myers MP, Stolarov JP, Eng C, Li J, Wang SI, Wigler MH, et al. P-TEN, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase. Proc Natl Acad Sci U S A. 1997; 94:9052–7. [PubMed: 9256433]
37. Tamura M, Gu J, Matsumoto K, Aota S, Parsons R, Yamada KM. Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. Science. 1998; 280:1614–7. [PubMed: 9616126]
38. Mounir Z, Krishnamoorthy JL, Robertson GP, Scheuner D, Kaufman RJ, Georgescu MM, et al. Tumor suppression by PTEN requires the activation of the PKR-eIF2alpha phosphorylation pathway. Sci Signal. 2009; 2:ra85. [PubMed: 20029030]

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

39. Raftopoulou M, Etienne-Manneville S, Self A, Nicholls S, Hall A. Regulation of cell migration by the C2 domain of the tumor suppressor PTEN. Science. 2004; 303:1179–81. [PubMed: 14976311]
40. Carracedo A, Alimonti A, Pandolfi PP. PTEN level in tumor suppression: how much is too little? Cancer Res. 2011; 71:629–33. [PubMed: 21266353]
41. Wong KK, Engelman JA, Cantley LC. Targeting the PI3K signaling pathway in cancer. Curr Opin Genet Dev. 2010; 20:87–90. [PubMed: 20006486]
42. Ginn-Pease ME, Eng C. Increased nuclear phosphatase and tensin homologue deleted on chromosome 10 is associated with G0-G1 in MCF-7 cells. Cancer Res. 2003; 63:282–6. [PubMed: 12543774]
43. Weng LP, Brown JL, Baker KM, Ostrowski MC, Eng C. PTEN blocks insulin-mediated ETS-2 phosphorylation through MAP kinase, independently of the phosphoinositide 3-kinase pathway. Hum Mol Genet. 2002; 11:1687–96. [PubMed: 12095911]
44. Weng LP, Brown JL, Eng C. PTEN coordinates G(1) arrest by down-regulating cyclin D1 via its protein phosphatase activity and up-regulating p27 via its lipid phosphatase activity in a breast cancer model. Hum Mol Genet. 2001; 10:599–604. [PubMed: 11230179]
45. Fraser M, Zhao H, Luoto KR, Lundin C, Coackley C, Chan N, et al. PTEN deletion in prostate cancer cells does not associate with loss of RAD51 function: implications for radiotherapy and chemotherapy. Clin Cancer Res. 2012; 18:1015–27. [PubMed: 22114138]
46. Hunt CR, Gupta A, Horikoshi N, Pandita TK. Does PTEN loss impair DNA double-strand break repair by homologous recombination? Clin Cancer Res. 2012; 18:920–2. [PubMed: 22179663]
47. Wu Y, Dowbenko D, Spencer S, Laura R, Lee J, Gu Q, et al. Interaction of the tumor suppressor PTEN/MMAC with a PDZ domain of MAGI3, a novel membrane-associated guanylate kinase. J Biol Chem. 2000; 275:21477–85. [PubMed: 10748157]
48. Valiente M, Andres-Pons A, Gomar B, Torres J, Gil A, Tapparel C, et al. Binding of PTEN to specific PDZ domains contributes to PTEN protein stability and phosphorylation by microtubule-associated serine/threonine kinases. J Biol Chem. 2005; 280:28936–43. [PubMed: 15951562]
49. Terrien E, Chaffotte A, Lafage M, Khan Z, Prehaud C, Cordier F, et al. Interference with the PTEN-MAST2 interaction by a viral protein leads to cellular relocalization of PTEN. Sci Signal. 2012; 5:ra58. [PubMed: 22894835]
50. Harris BZ, Lim WA. Mechanism and role of PDZ domains in signaling complex assembly. J Cell Sci. 2001; 114:3219–31. [PubMed: 11591811]

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

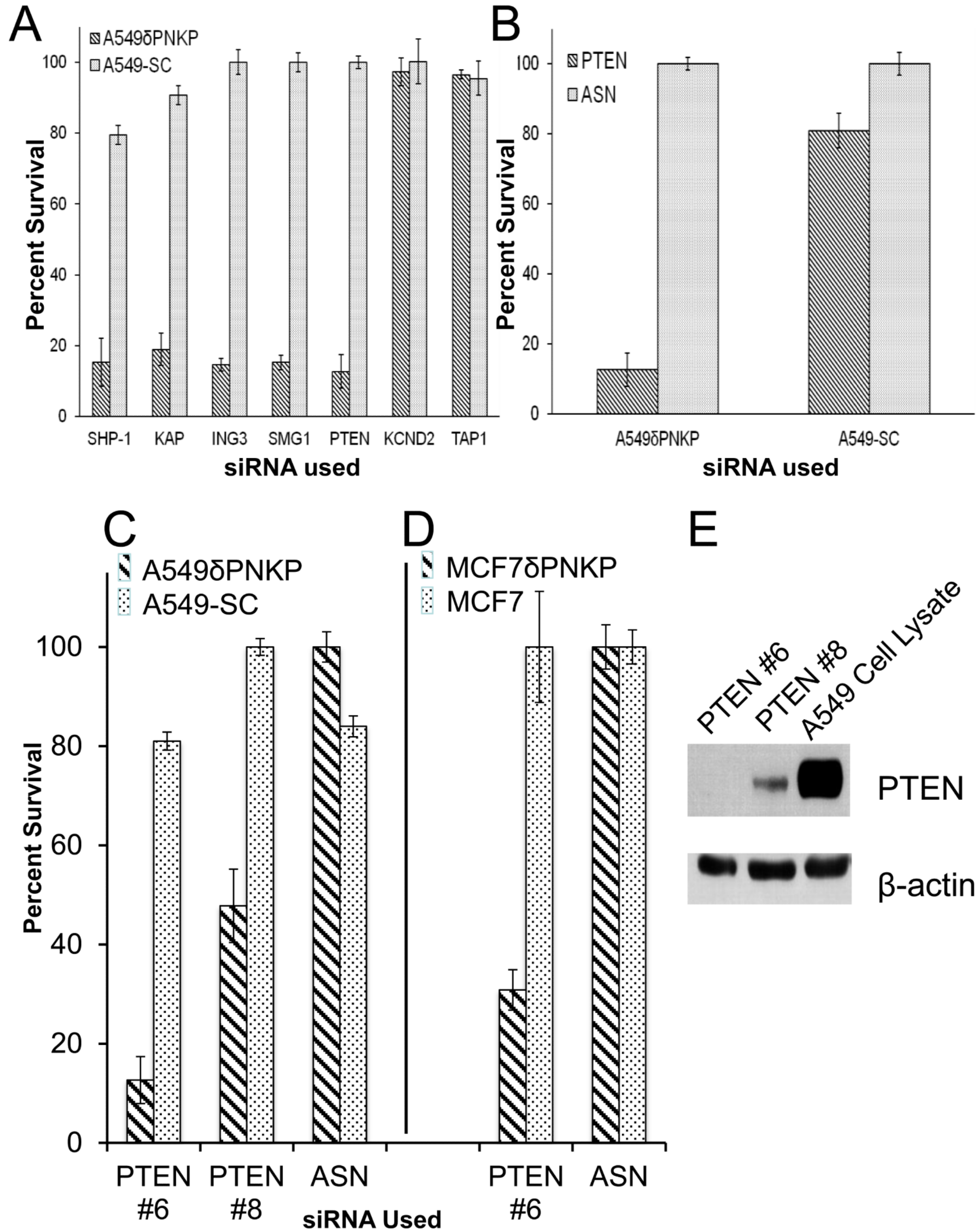


Figure 1.

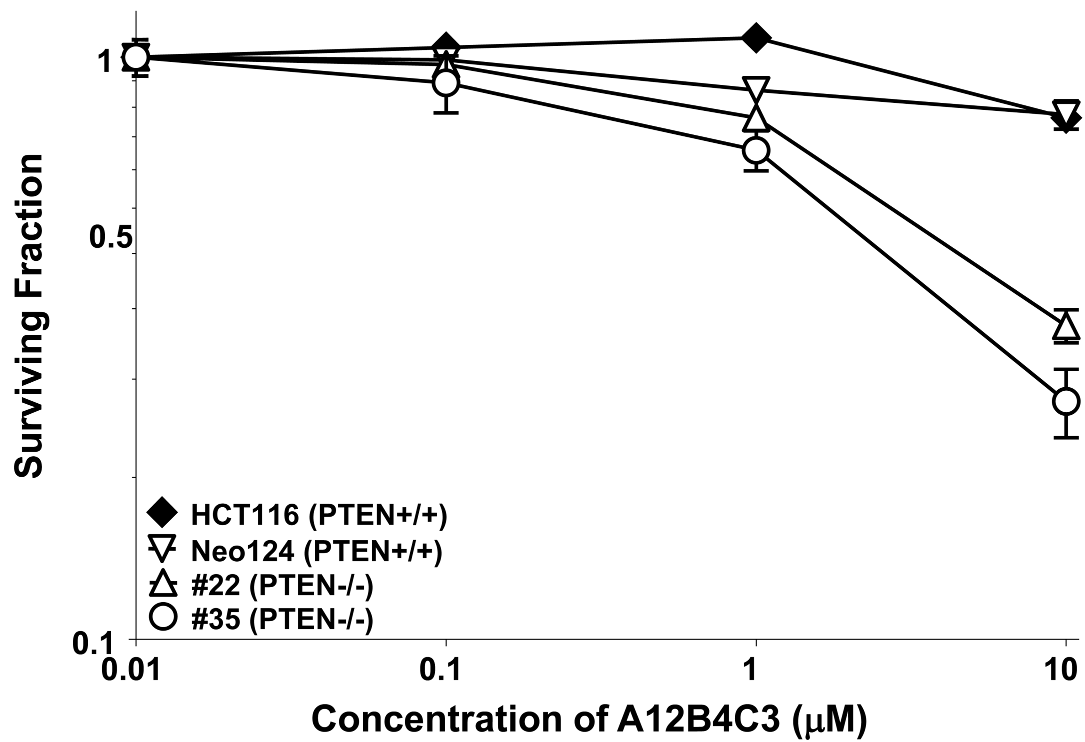
Tumor suppressors, including PTEN, identified by an siRNA-based screen of cell proliferation (15) as potentially synthetic lethal with PNKP. Two cell lines were used for the screen: A549 cells stably depleted of PNKP by shRNA (A549δPNKP) and A549 cells expressing a scrambled shRNA (A549-SC). In addition to the five tumor suppressor genes showing potential synthetic lethality, two randomly selected genes (*KCND2 and TAP1*) are shown to identify the difference between potential hits and non-hits. (B) Further confirmation of PTEN-PNKP synthetic lethality. To limit the off-target effects, 16 nM of siRNA (3.5-fold less than the concentrations employed for screening) was used to transiently transfect A549δPNKP and A549-SC cells with the PTEN siRNA pool and a

scrambled control siRNA (ASN). Only when PTEN was knocked down in combination with PNKP did we see significant lethality (Z-factor = −9.01, $p < 0.001$). Error bars represent ± SEM from at least three independent determinations. (C) Validation of the PTEN-PNKP synthetic lethal partnership. The four siRNAs that were pooled for screening purposes were tested independently to assess their effectiveness at killing A549δPNKP cells as determined by the cell proliferation assay. Only two of the four siRNAs caused lethality in combination with PNKP disruption, with PTEN #6 siRNA displaying greater cytotoxicity than #8 siRNA (PTEN #6 Z-factor = −9.01, $p < 0.001$, PTEN #8 Z-factor = −9.06, $p <0.001$). (D) Wild type MCF7 cells and MCF7 cells stably depleted of PNKP (MCF7δPNKP) were transiently transfected with PTEN #6 siRNA or control scramble siRNA (ASN). Error bars represent ± SEM from at least three independent determinations. (E) Western blot of A549 cells transiently transfected with PTEN #6 and #8 siRNAs.

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts



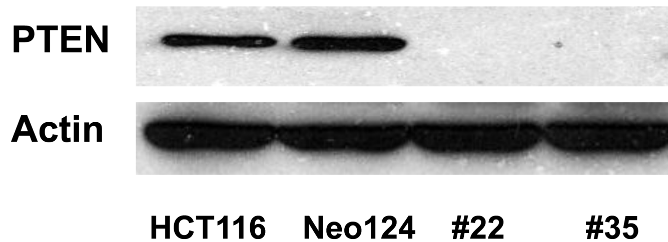


Figure 2.

Survival of PTEN null cells treated with a PNKP phosphatase inhibitor. Isogenically matched $PTEN^{+/+}$ cells (HCT116 parental and Neo124 vector control cells) and two $PTEN^{-/-}$ HCT116 strains #22 and #35 were subjected to increasing concentrations of the PNKP inhibitor A12B4C3 and survival was assessed by clonogenic survival assay. Error bars represent ± SEM from at least three independent determinations, each assay was performed in triplicate for a minimum of nine total assessed plates per point.

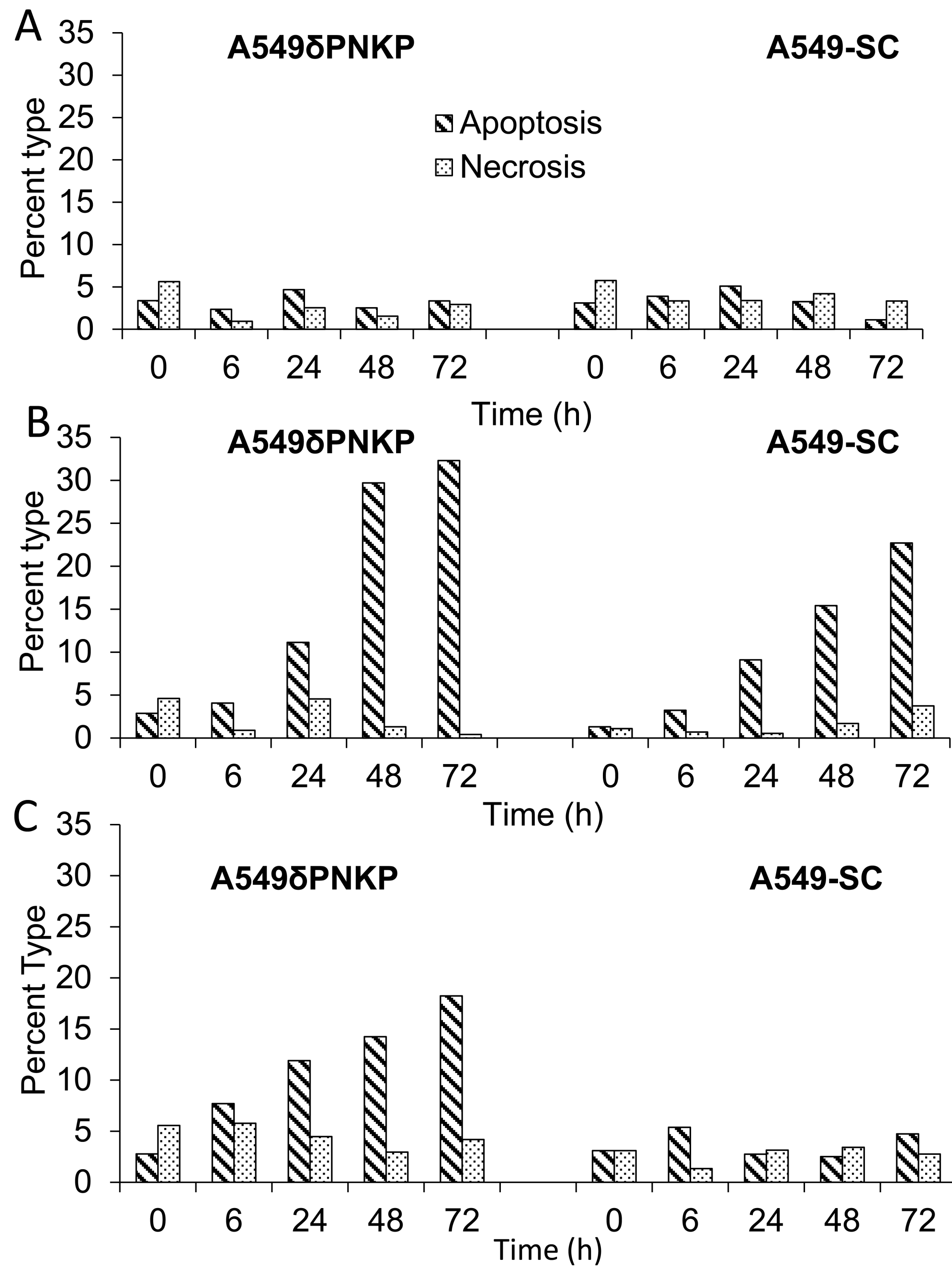


Figure 3.
Determination of the mode of cell death of cells undergoing synthetic lethality due to the concurrent ablation of PTEN and PNKP function. (A) A549δPNKP and A549-SC cells were transiently transfected with scrambled control siRNA (ASN) and the proportion of apoptotic versus necrotic cells was determined at times post-transfection as described in Materials and Methods. (B) ASN-treated cells were additionally treated with the apoptosis inducer BH3I-1. (C) Measurement of apoptotic and necrotic cells at the indicated time points following transient transfection with PTEN siRNA.

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

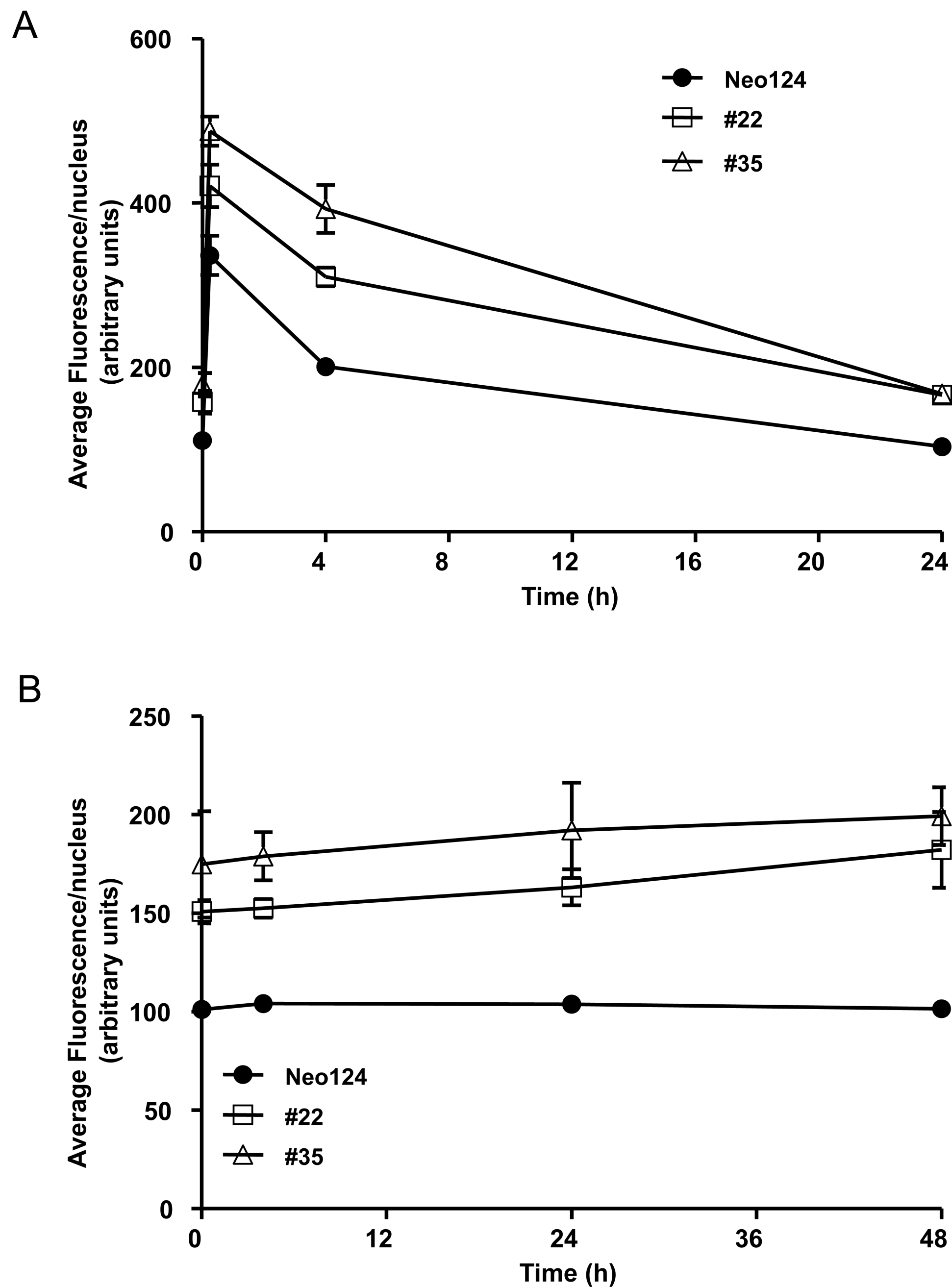


Figure 4.

Influence of PTEN depletion on DNA DSB induction and repair. (A) $PTEN^{+/+}$ HCT116 (Neo 124) and $PTEN^{-/-}$ (#22 and #35) cells were plated 24 h in advance, after which they were subjected to γ-radiation (5 Gy). The level of DSBs was monitored by quantification of the average integrated fluorescence intensity per nucleus due to phosphorylation of histone H2AX as a function of time after 5-Gy irradiation. (B) Accumulation of DSBs by exposure of the HCT116 cells to 5 μM A12B4C3. Error bars represent ± SEM.

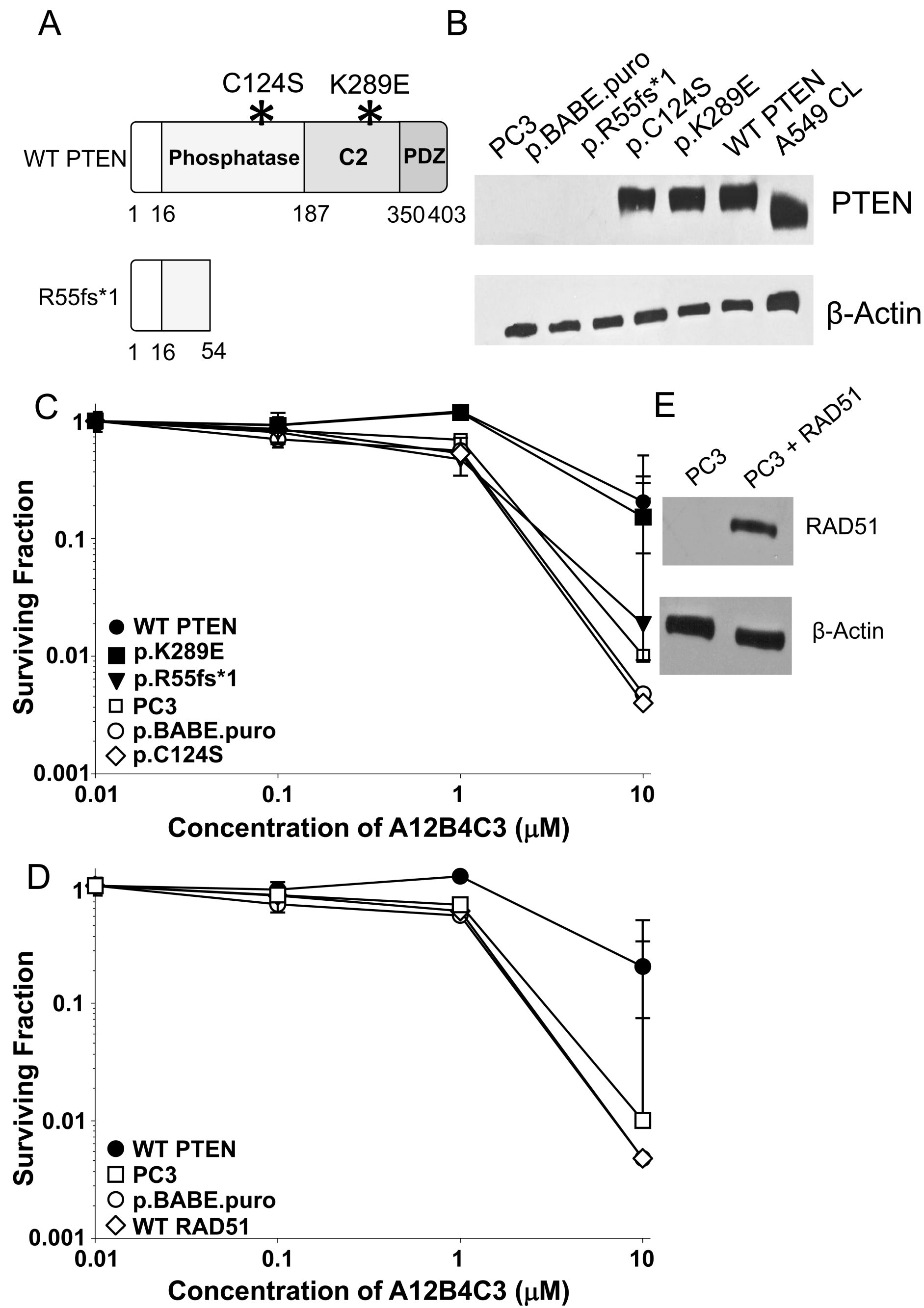


Figure 5.

Identification of the critical function of PTEN for survival under PNKP disruption. (A) Graphical representation of the forms of PTEN used to reconstitute PC3 cells, including the truncation mutation found in PC3 cells (R55fs*1), indicating the location of the other mutations in the phosphatase domain (C124S) and in the C2 structural domain involved in targeting proteins to cell membranes (K289E). The PDZ domain is a structural domain commonly found in signaling proteins, but was not mutated in these experiments (50). (B) Western blots of PC3 cells transfected with vector only (p.BABE.puro), cDNA for R55fs*1 or the different forms of full length PTEN. (C) Survival of PC3 cells transfected with expression vectors encoding various forms of PTEN and exposed to increasing

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

concentrations of A12B4C3: p.BABE.puro (vector only); WT PTEN (full length) - wild-type PTEN; p.K289E – PTEN mutant with reduced nuclear shuttling; p.R55fs*1 – truncation mutant; p.C124S – phosphatase inactive PTEN mutant. Error bars represent ± SEM from at least three independent determinations, each experiment carried out in triplicate for a minimum of nine total assessed plates per point. (D) Survival response of PC3 cells transfected with a vector expressing wild-type RAD51 cDNA (WT RAD51) and exposed to increasing concentrations of A12B4C3. Error bars represent ± SEM from at least three independent determinations, each experiment was performed in triplicate for a minimum of nine total assessed plates. (E) Western blot of RAD51 expression in PC3 cells.

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

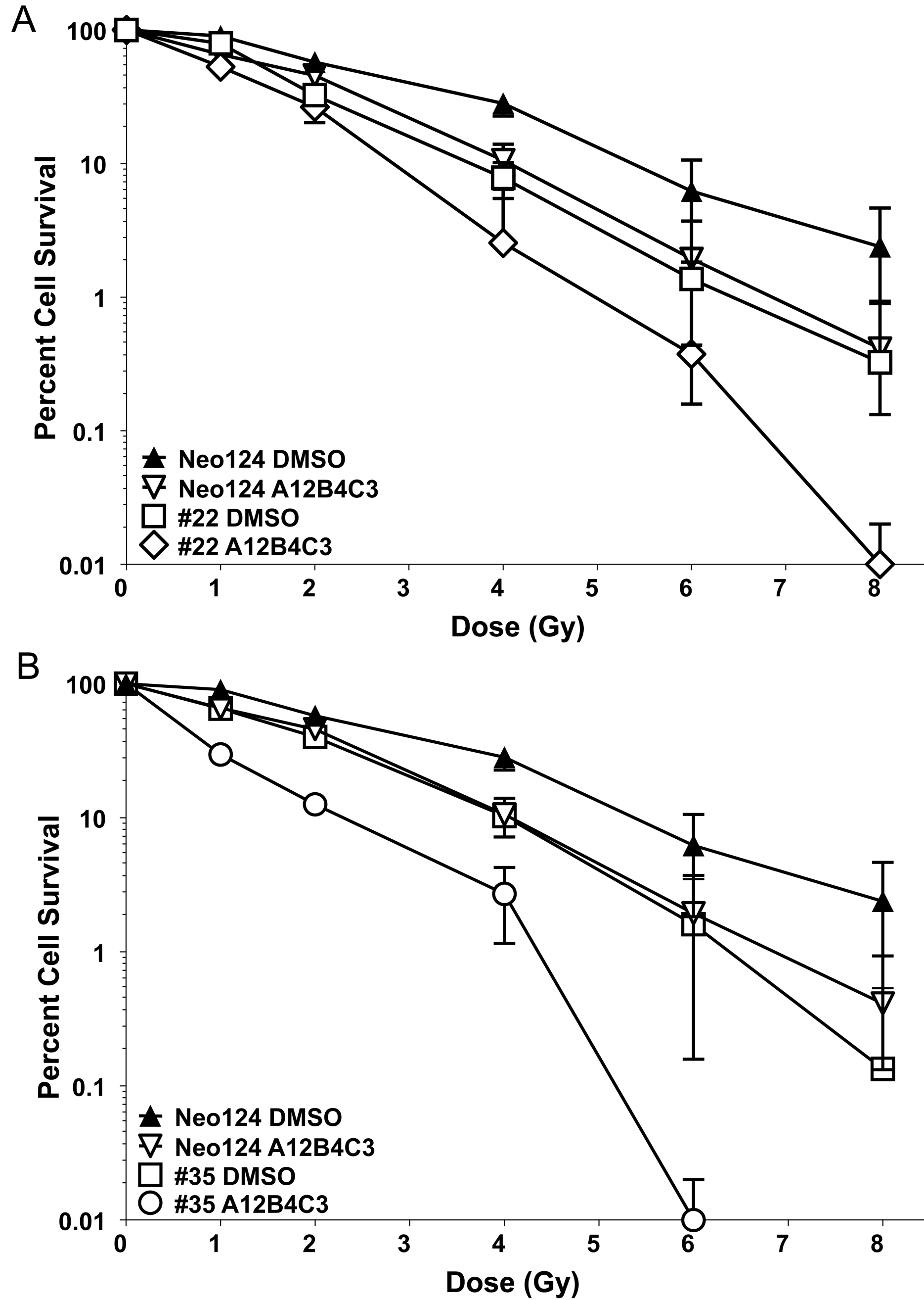


Figure 6.

Radiation sensitization by combined inactivation of PTEN and PNKP. (A) Vector only control HCT116 cells (Neo124), and HCT116 PTEN−/− strains #22 (A) and #35 (B) were grown in the presence of A12B4C3 (2 μM) or DMSO vehicle for 24 h and then subjected to increasing doses of γ-radiation and further incubated in the presence of A12B4C3. Survival was measured by clonogenic survival assay. Inhibition of PNKP and loss of PTEN alone modestly sensitized the cells to radiation, but a more marked radiosensitization was observed when the PTEN knockout cells were irradiated and further incubated in the presence of A12B4C3. Asterisks indicate synergy in radiosensitization due to combined inactivation of PTEN and PNKP. Error bars represent ± SEM from at least three

independent determinations, each experiment was performed in triplicate for a minimum of nine total assessed plates.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts