Genetic Screening for Synthetic Lethal Partners of Polynucleotide Kinase/Phosphatase: Potential for Targeting SHP-1–Depleted Cancers

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Abstract

A genetic screen using a library of 6,961 siRNAs led to the identification of SHP-1 (PTPN6), a tumor suppressor frequently mutated in malignant lymphomas, leukemias, and prostate cancer, as a potential synthetic lethal partner of the DNA repair protein polynucleotide kinase/phosphatase (PNKP). After confirming the partnership with SHP-1, we observed that codepletion of PNKP and SHP-1 induced apoptosis. A T-cell lymphoma cell line that is SHP-1 deficient (Karpas 299) was shown to be sensitive to a chemical inhibitor of PNKP, but resistance was restored by expression of wild-type SHP-1 in these cells. We determined that while SHP-1 depletion does not significantly impact DNA strand-break repair, it does amplify the level of reactive oxygen species (ROS) and elevate endogenous DNA damage. The ROS scavenger WR1065 afforded protection to SHP-1–depleted cells treated with the PNKP inhibitor. We propose that codisruption of SHP-1 and PNKP leads to an increase in DNA damage that escapes repair, resulting in the accumulation of cytotoxic double-strand breaks and induction of apoptosis. This supports an alternative paradigm for synthetic lethal partnerships that could be exploited therapeutically. Cancer Res; 72(22): 5934–44. ©2012 AACR.

Introduction

A recent exciting development in cancer treatment is the potential use of synthetic lethality as a patient and cancer-specific therapy. Synthetic lethality arises when the simultaneous disruption of 2 nonallelic, nonessential genes, or their proteins in the same cell induces lethality (1, 2). Recently, this phenomenon has been shown to occur between combinations of DNA repair genes and much attention has focused on the codisruption of the single-strand break repair (SSBR) protein PARP and the breast cancer–associated (BRCA) proteins (3–5), which are naturally lost or mutated in tumor cells of women affected with hereditary breast and ovarian cancer and have roles in DNA double-strand break repair (DSBR). One proposed explanation for this synthetic lethality is that chemical inhibition of PARP causes the generation of DSBs by preventing SSBR (6, 7). As cells progress through S-phase, naturally occurring SSBs collapse the replication fork to give rise to DSBs, which in BRCA−/− cancer cells accumulate, eventually leading to cancer cell death. Normal cells in BRCA patients retain BRCA heterozygosity and, therefore, possess the capacity to fully repair DSBs and so are not appreciably affected by PARP inhibitors during the treatment, and thus the deleterious side effects typically associated with cancer therapy are greatly reduced (8).

It has been estimated that there are approximately 10^4 SSBs formed per cell per day probably as a result of the generation of reactive oxygen species (ROS) during normal metabolism (9). Many of these breaks harbor unligatable termini such as 3′-phosphates and 5′-hydroxyls that must be processed for DNA repair to proceed. Polynucleotide kinase/phosphatase (PNKP) is a bifunctional enzyme whose role is to process these termini during SSBR and DSBR by catalyzing the dephosphorylation of 5′-phosphate termini and the phosphorylation of 5′-hydroxyl ends (10). PNKP is a versatile protein acting in many DNA repair pathways, including base excision repair, single-strand break repair, homologous recombination repair, and nonhomologous end-joining repair.

Given the potential to target PNKP by small molecule inhibitors (12), we sought to identify synthetic lethal relationships of PNKP to expand the repertoire of targeted therapy taking advantage of this approach. By screening approximately 7,000 genes targeting the "druggable" genome, we have identified a variety of proteins potentially synthetic lethal with PNKP including several that are either known or are implicated as tumor suppressors, such as the protein tyrosine...
phosphatase SHP-1 (PTPN6). We also show that SHP-1 is likely not directly involved in DNA repair and therefore cell death based on DSBR accumulation caused by inhibition of 2 distinct but interacting DNA repair pathways, that is, SSBR and DSBR as previously described, is not the only explanation for the occurrence of synthetic lethality involving a DNA repair protein partner (6, 7). Instead, the observation that SHP-1 depletion causes an increase in ROS production supports an alternative paradigm for synthetic lethality that combines increased DNA damage production with limited DNA repair capacity, as was previously shown for the interaction between PTEN-induced putative kinase 1 (PINK1) and the mismatch repair proteins MSH2, MLH1, and MSH6 (13). This suggests that we can broaden the potential for clinical application of synthetic lethality.

Materials and Methods

Cells

A549 (human lung carcinoma) and MCF7 (human breast adenocarcinoma) cell lines were obtained from the American Type Culture Collection, who conducted short tandem repeat profiling of each cell line before shipment. The cells were cultured for 2 to 3 weeks to generate stocks that were then kept frozen until used for the current experiments. These cells and their transfected derivatives were cultured at 37°C and 5% CO2 in a humidified incubator in a 1:1 mixture of Dulbecco’s Modified Eagle’s Medium and F12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mmol/L), nonessential amino acids (0.1 mmol/L), and sodium pyruvate (1 mmol/L). All culture supplements were purchased from Invitrogen. For comet assays and apoptosis/necrosis detection penicillin (50 U/mL) and streptomycin (50 μg/mL) were added to the DMEM/F12 (complete DMEM/F12). SUPM2 (DCMZ) and Karpas 299 (obtained as a gift from Dr. M. Kadin, Beth Israel Deaconess Medical Center, Boston, MA) human anaplastic large cell lymphoma cell lines were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% FBS, 0.3 g/L L-glutamine, and 2 g/L NaHCO3. Both SUPM2 and Karpas 299 cells were recently confirmed to carry monoclonal T-cell rearrangements by polymerase chain reaction and express the NPM-ALK fusion protein by Western blots.

Stable transfection

All cell lines were generated by stable transfection of pSUPER.neo constructs (Oligoengine) into A549 or MCF7 cells yielding several distinct cell lines. A short hairpin RNA (shRNA) directed against nucleotides 1,391 to 1,410 of PNKP (11) was used to stably deplete PNKP in A549 and MCF7 cells (A549ΔPNKP and MCF7ΔPNKP, respectively), and another shRNA expression vector targeting nucleotides 1,313 to 1,335 of SHP-1 was used to generate A549ΔSHP-1 cells. A control cell line was also generated in which an shRNA to no known gene target (a scrambled shRNA, pSUPER.neo.MammX; Oligoengine) was expressed in A549 cells (A549-Scramble).

Approximately 20,000 A549 or MCF7 cells were plated and allowed to adhere overnight in a 24-well dish at 37°C and 5% CO2. Lipofectamine2000 (Invitrogen) was used according to the manufacturer’s instructions to transfect cells with the shRNA plasmid constructs. The cells were then trypsinized and replated into 6 × 100 mm plates in DMEM/F12 without antibiotics and incubated overnight at 37°C and 5% CO2. The following day, media was removed and replaced with complete DMEM/F12 containing 500 μg/mL G418. After single-clone colonies were formed (10–18 days) the colonies were picked and expanded before protein analysis.

Transient transfections

Approximately 4,000 A549ΔPNKP, A549-Scramble, MCF7ΔPNKP, or MCF7 cells were plated per well in a 96-well plate, and allowed 24 hours to adhere in a humidified incubator at 37°C and 5% CO2. All wells surrounding samples were filled with 100 μL distilled water to control for evaporation effects. For protocol optimization and initial verification of selected hits, 56 nmol/L final concentration of siRNA was added to 50 μL total reaction volume in Opti-MEM (Invitrogen). At the same time as siRNA-Opti-MEM incubation, a 1:25 dilution of Dharmafect Transfection Reagent 1 (Dharmacon) in Opti-MEM was allowed to incubate at room temperature for 5 minutes, to provide a final volume of 0.23 μL transfection reagent per well. The 2 transfection solutions were then combined and held at room temperature for 20 minutes. The media was then removed from the cells and 100 μL of the transfection mixture was added per well and the plate was incubated at 37°C and 5% CO2 for 72 hours. All siRNAs were purchased from Qiagen.

Protein analysis

Western blots were conducted using 50 μg of whole cell lysate. Monoclonal antibody toward PNKP (H101) was used as previously described (14) and was incubated at 1:1,000 in 5% PBSMT (PBS with 5% w/v skim milk and 0.1% Tween-20) overnight at 4°C. Polyclonal primary antibodies (SHP-1 and β-actin) were incubated (1:4,000 dilution) in 5% PBSMT for 1 h at room temperature (Santa Cruz Biotechnology). All secondary antibodies were incubated (1:5,000 dilution) for 45 minutes at room temperature (Jackson ImmunoResearch Laboratories, Inc.).

siRNA library screen

Qiagen’s druggable genome siRNA library comprises 4 sub-classifications: phosphatases, kinases, G-protein–coupled receptors, and uncategorized proteins consisting of 205, 696, 490, and 5,570 mRNA targets, respectively. The library was first distributed into 89 × 96-well plates at a total siRNA concentration of 1 μmol/L, each well containing a pool of 4 separate siRNAs to the same mRNA target. Also added to the plates were 3 additional control wells (C12, D12, and E12) of AllStars Negative (ASN) scrambled siRNA (Qiagen). Then, using a JANUS Automated Workstation (PerkinElmer), 4,000 A549ΔPNKP or A549-Scramble cells were seeded into each well of a 96-well plate in a final volume of 100 μL DMEM/F12 without penicillin/streptomycin and allowed to adhere overnight in a humidified incubator. The following day, transfection mixture was generated as described above (56 nmol/L siRNA and a total of 0.25 μL Dharmafect transfection reagent 1 per well) and distributed into each well. After 18 hours of incubation, cells were fixed with 100 μL 10% trichloroacetic acid (TCA) at room temperature for 20 minutes and washed 5 times with PBS. The fixed cells were then solubilized with 100 μL of 0.5% NP-40

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per well), media was aspirated from the wells, and 100 μL of the mixture was added to each well and allowed to incubate for 72 hours. Then 10% v/v of 440 μmol/L Alamar Blue (Sigma-Aldrich) was added to each well and the cells were incubated for 50 to 90 minutes, 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 (15). Each screen was conducted in duplicate. A result was deemed a potential synthetic lethal hit if survival under the simultaneous knockdown condition was 33% or less when compared with the internal plate controls average.

Transient transfections of siRNAs for synthetic lethal partners were used for confirmatory assays; however, each siRNA was used independently and at a concentration of 20 nmol/L. All other reagent concentrations remained constant. Each assay was conducted manually and the fluorescence was read with a FLUOStar Optima plate reader (BMG Labtec Inc.) using excitation and emission wavelengths of 563 and 587 nm, respectively.

**Statistical analysis**

$R^2$ values were generated in Microsoft Excel by plotting individual survival scores from the duplicate screen against one another. All p-values were generated using a 2-sided Student’s $t$ test. Z-scores were only generated for confirmatory data where an average from 23 to 96 individual wells of data per assay (conducted at least in triplicate) were measured, allowing us an appropriate number of replicates to achieve robust statistical data. A Z-score is a dimensionless quantity representing a measurement of 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
$\mu$ = population mean
$\sigma$ = standard deviation of the population

As such, Z-scores can be positive or negative depending on whether the sample is higher or lower than the mean of a control. For our results, we were interested in a negative Z-score as this showed that the survival of the experimental condition was lower than control (i.e., the condition was lethal). A sample with a $Z$-score of $-3$ or less is significantly different than control and is a threshold often used in synthetic lethal screens.

**Cell proliferation assay with anaplastic large cell lymphoma cell lines**

Karpas 299, SUPM2, or Karpas 299 (SHP-1–complemented) cells were plated in 96-well format at a density of 5,000 cells/100 μl in complete RPMI. Increasing concentrations of the PNKP inhibitor A12B4C3 was added to each well in a constant volume of dimethyl sulfoxide and left to incubate for 12 to 16 days. Eleven μL of 440 μmol/L Alamar Blue was then added to each well and left to incubate for 24 to 48 hours, after which fluorescence was determined as described above. At least 3 independent experiments were conducted with either 48 or 96 replicates for each experiment.

A pCl expression vector (Promega) was used to transiently reexpress SHP-1 in Karpas 299 cells (16). The Karpas 299 cells were grown in antibiotic-free RPMI, after which $10^7$ cells were harvested per transfection in 500 μL total volume of antibiotic-free RPMI. These cells were placed into a 4-mm electroporation cuvette (VWR) together with 10 μg of plasmid DNA. The cells were then electroporated using a BTX ECM 300 square electroporator (BTX Technologies Inc.) at 225 V for 3 pulses of 8.5 ms. The cells were then transferred to 20 mL of antibiotic-free RPMI and incubated for 24 hours before experimentation.

**Determination of mode of cell death**

A549-Scramble or A549ΔPNKP cells were grown on cover slips in complete DMEM/F12 and transfected with either ASN or SHP-1 siRNA. As a positive control for apoptosis the cell lines were treated with 100 μmol/L 5-(p-bromobenzylidine)-1-isopropyl-4-oxo-2-thioxo-3-thiozolidineacetic acid (BH3I-1, Sigma-Aldrich), which is a potent apoptosis inducer. The cells were grown under each condition for the indicated length of time before being subjected to a triple stain of Hoechst 33342, Ethidium Homodimer III, and Annexin V-FITC as described by the kit manufacturer (Biotium). Each experiment was conducted in duplicate, with 10 random microscope fields chosen per duplicate. The data were combined to generate a higher total number of cells counted. Between 250 and 1,000 cells for each time point were counted.

**Detection of γH2AX**

To monitor the level of H2AX phosphorylation before and after γ-radiation, $1 \times 10^5$ cells (A549-Scramble or A549ΔPNKP-1) were seeded on cover slips in 35 mm dishes with 2 mL DMEM/F12 without antibiotics and left overnight to adhere in a humidified incubator. The cells were then irradiated (5 Gy) and left to repair for the indicated time points, after which γH2AX was measured as previously described (17). Fluorescence was normalized to background fluorescence and quantified using ImageJ (http://rsbweb.nih.gov/ij/). For accumulation of DSBs under PNKP inhibition in the absence of irradiation, 5 μmol/L A12B4C3 (or 100 μmol/L BH3I-1 as control for apoptosis-induced H2AX phosphorylation) was added to the media at the time of plating, and left for 48 hours. Fluorescence quantification was then conducted as described above.

**Detection of reactive oxygen species**

The presence of hydroxyl radicals and peroxynitrite was detected using a commercial kit (Cell Technology). Cells were grown in 96-well format and transfected with either ASN or SHP-1 siRNA for 24 hours before ROS detection. Cells were rinsed twice with modified Hanks balanced salt solution (HBSS) supplemented with 10 mmol/L HEPES, 1 mmol/L MgCl$_2$, 2 mmol/L CaCl$_2$, and 2.7 mmol/L glucose, after which 100 μl of aminophenyl fluorescein or hydroxyphenyl fluorescein, diluted to 10 μmol/L in the same modified HBSS, was...
applied to the cells for 45 min at 37°C in the dark. The plates were then read using a FLUOstar Optima plate reader at an excitation wavelength of 488 nm and an emission wavelength of 515 nm.

Colony-forming assay
AS49/SHP-1 and A549-Scramble cells were plated in the presence of increasing concentrations of A12B4C3 alone, or with or without the ROS scavenger WR1065. Cells were subjected to these conditions continuously for 10 to 14 days, after which plates were stained with crystal violet and counted (11). Colonies containing fewer than 30 cells were omitted.

Results
siRNA screen for the synthetic lethal partners of PNKP
We 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. We conducted an unbiased forward transfection screen using an extensive library of siRNAs targeting 6,961 genes, in which a pool of 4 distinct siRNAs targets each gene. Two duplicate screens were conducted; the first used A549 lung cancer cells stably depleted of PNKP (A549PNKP), and the second A549 cells expressing a scrambled siRNA (A549-Scramble) under identical conditions. (The level of PNKP in the knockdown and control cells is shown in Supplementary Fig. S1.A). Cells were exposed to siRNA continuously for 72 hours (allowing for at least 2 cell cycles to occur) at a concentration known to be effective at knocking down target proteins (data not shown). Cell survival was then determined by an Alamar Blue-based fluorescence assay (15).

Cell survival scores after targeting each of the 6,961 mRNAs were compared with an average internal plate control located on every plate screened, consisting of the average survival of 3 wells of the cells screened (A549PNKP or A549-Scramble) transiently transfected with ASN control siRNA under identical screen conditions. Genes and their corresponding proteins were classified as potential hits for synthetic lethality with PNKP if the survival under the simultaneous knockdown condition was 33% or less when compared with the internal plate controls average. A comparison of the duplicate screens showed that an overwhelming majority of the siRNAs yielded reproducible phenotypes (Supplementary Material Fig. S2) and a summary of the data derived from the mean values is outlined in Fig. 1. Most of the proteins identified as potential "hits" in the screen were lethal only in combination with PNKP disruption and were not singularly lethal (for comparison SHP-1 data is graphically represented alongside 2 randomly selected proteins deemed "nonhits," Supplementary Fig. S3).

A master list of potential synthetic lethal partners is shown in Supplementary Table S1. The positive hit rate was found to be 6.1% (425/6,961) including 8 phosphatases, 97 kinases, 117 G-protein–coupled receptors, and 203 unclassified proteins. Of note, 14 tumor suppressors were identified as potentially synthetically lethal with PNKP (Supplementary Table S2).

Confirmation of SHP-1 as a possible synthetic lethal partner of PNKP
One potential hit for synthetic lethality with PNKP identified in the screen was SHP-1, a protein tyrosine phosphatase that has been implicated as a tumor suppressor, functioning in the regulation of signaling transduction pathways (18) to counter growth-promoting and oncogenic signals through its phosphatase activity (19). To confirm the synthetic lethal relationship between PNKP and SHP-1, we repeated the analysis but reduced the concentration of siRNA previously used in the screen from 56 to 20 nmol/L and used each of the 4 originally pooled siRNAs separately to minimize the potential for off-target effects and limit toxicity in A549-Scramble cells transfected with the SHP-1 siRNA. When the distinct SHP-1 siRNAs were assayed, all 4 displayed selective killing of A549PNKP cells and no or limited toxicity in control cells (Fig. 2A, siRNA#5 Z-factor = −12.3, P < 0.001; siRNA#10 Z-factor = −17.2, P < 0.001, siRNA#11 Z-factor = −6.88, P < 0.001, siRNA#6 Z-factor = −6.44, P < 0.001). As all 4 siRNAs showed synthetic lethality with PNKP, as well as the capacity to deplete SHP-1 protein (Supplementary Fig. S1B), the effect was most likely attributable to the simultaneous depletion of PNKP and SHP-1, and not because of off-target effects. Furthermore,
the lack of toxicity seen using ASN control siRNA with A549δPNKP cells indicated that nonspecific activation of the RNAi pathway was not responsible for the observed lethality.

To further substantiate that a synthetic lethal partnership exists between SHP-1 and PNKP, we carried out a similar analysis with the MCF7 breast cancer cell line. We conducted the cell proliferation assay using 20 nmol/L of SHP-1 siRNA#5 with an MCF7 cell line stably depleted of PNKP (MCF7δPNKP). As seen with A549 cells, the combined disruption of both SHP-1 and PNKP was responsible for lethality, as the depletion of PNKP or SHP-1 individually was not lethal (Fig. 2B, Z-score = −3.4, P < 0.001), nor was the activation of RNAi machinery alone responsible for lethality (Fig. 2B). Similarly, in the reciprocal experiment, in which stable SHP-1–depleted A549 cells (A549δSHP-1) or A549-Scramble cells were transfected with siRNA targeting PNKP (Fig. 2C) or exposed to a small molecule inhibitor of PNKP 3′-phosphatase activity, A12B4C3 (12), lethality was only observed when both PNKP and SHP-1 were disrupted (Fig. 2D).

We also show that small molecule inhibition or siRNA-mediated knockdown of PARP1 is insufficient to cause a lethal effect in A549δSHP-1 cells (Supplementary Fig. S4), indicating that PARP1 cannot substitute for PNKP in synthetic lethal relationships with SHP-1, and therefore, for some tumors PNKP may serve as an alternative therapeutic target to PARP1.

Mode of cell death
To identify the mechanism by which cells undergo disrupted SHP-1/PNKP–mediated synthetic lethality, A549-Scramble, and A549δPNKP cells were grown on cover slips and transiently transfected with ASN or SHP-1 siRNA. As a...
positive control, cells were treated with the apoptosis inducer BH3I-1. Cells were then simultaneously stained with Hoechst 33342, Ethidium Homodimer III, and Annexin V-FITC. Hoechst 33342 stains the nuclei of healthy and unhealthy cells alike, whereas Ethidium Homodimer III identifies cells that are in late stage of apoptosis or are necrotic, and Annexin V identifies early apoptotic cells. Figure 3A shows there was a small population of apoptotic and necrotic cells following transfection of both cell lines with ASN. As expected, treatment with BH3I-1 induced a substantial increase in apoptosis in both cell lines (Fig. 3B). Codisruption of SHP-1 and PNKP by transient transfection of SHP-1 siRNA into the PNKP-depleted cell line also caused a substantial increase in the proportion of apoptotic cells, with only a small increase in the necrotic population (Fig. 3C). In contrast, no induction of apoptosis was observed following transient transfection of SHP-1 siRNA in the cell line expressing scramble shRNA. Thus, these data indicate that cells undergoing SHP-1/PNKP induced synthetic lethality do so by an apoptotic mechanism.

Survival of naturally occurring SHP-1–positive and –negative cells in response to PNKP inhibition

The utility of synthetic lethality will lie in the capacity to translate potential associations into targeted therapy, possibly using inhibitors of one of the partners as a single agent. To investigate the feasibility of taking advantage of the newly identified partnership between SHP-1 and PNKP, we subjected 2 anaplastic large cell lymphoma cell lines, Karpas 299 (naturally SHP-1 deficient), and SUPM2 (which expresses SHP-1), to an increasing concentration of the PNKP inhibitor, A12B4C3, over a period of 12 to 16 days. The dose response curves (Fig. 4) indicate that at A12B4C3 doses 10 μmol/L or more there was a marked decrease in survival of the Karpas 299 cells, whereas the SUPM2 cells remained viable. To confirm the central role of SHP-1 in the observed response we expressed wild-type SHP-1 in Karpas 299 cells (Western blot shown in Supplementary Fig. S1C), and these cells displayed reduced sensitivity to A12B4C3.

Underlying mechanism of the PNKP/SHP-1 synthetic lethal partnership

The mechanism for synthetic lethality involving PARP1 and BRCA1 or 2 is considered to be an interplay between 2 DNA repair pathways (3, 4, 7, 20), and thus to date, there has been considerable focus on the critical involvement of both proteins of a synthetic lethal partnership in DNA surveillance or repair (6, 8, 20–25). SHP-1 is a protein tyrosine phosphatase known to negatively regulate receptor tyrosine kinase signaling (18, 26). There is no evidence to date to indicate that SHP-1 is involved in DNA repair. We therefore sought to determine if SHP-1 plays a major role in regulating DSB or SSB repair. Accordingly, A549-Scramble and A549SHP-1 cells were irradiated and DSB repair was followed by visualizing the formation of γH2AX foci as well as by single-cell gel electrophoresis (comet

Figure 3. Mode of cell death of cells undergoing synthetic lethality because of the simultaneous disruption of SHP-1 and PNKP. A, A549iPNKP and A549-Scramble cells were transiently transfected with ASN control siRNA and apoptosis and necrosis were determined over a 72-hour period as described in Materials and Methods at times after transfection. B, additional treatment of the ASN-transfected cells with the potent apoptosis inducer BH3I-1. C, induction of apoptosis and necrosis in A549iPNKP and A549-Scramble cells transiently transfected with SHP-1 siRNA.

Figure 4. Survival of anaplastic large cell lymphoma cells under PNKP inhibition. Karpas 299 (naturally lacking functional SHP-1), SUPM2 cells (which express normal levels of SHP-1), Karpas 299 cells expressing SHP-1 (Karpas 299 + SHP-1), and vector-only controls (Karpas 299 + pcDNA3) were treated with increasing concentrations of the PNKP inhibitor A12B4C3 for 12 to 16 days. Survival was measured using an Alamar Blue-based fluorescence assay. Error bars represent standard error (± SE) from at least 3 independent determinations.
assay) under neutral conditions and SSBR by comet assay under alkaline conditions. A549-Scramble and A549SHP-1 cells showed reasonably similar kinetics (Fig. 5) for the formation and removal of \( \gamma \)H2AX foci over the course of 24 hours following irradiation, suggesting that loss of SHP-1 did not significantly affect the rate of repair of DSB, although a greater number of foci appeared to be generated in the first 15 minutes following irradiation of A549SHP-1 cells. Interestingly, there was also a notable presence of \( \gamma \)H2AX foci in the unirradiated A549SHP-1 cells and a proportionately higher level of foci at each time point after irradiation, including the 24-hour time point when almost all foci had disappeared in the A549-Scramble cells. The results of the \( \gamma \)H2AX assay were supported by the neutral comet assay (Supplementary Figs. S5 and S6). DSBR in irradiated A549-Scramble cells was almost complete by 24 hours (Supplementary Fig. 6A). In contrast, the loss of a recognized DNA repair enzyme such as PNKP (A549δPNKP cells) severely retarded the rate of repair (Supplementary Fig. S6B), in agreement with previous observations (27). The A549δSHP-1 cells showed a similar rate of DSBR as A549-Scramble cells (Supplementary Fig. S6C), again indicating that SHP-1 does not play a significant role in DSBR, but there was a noticeably elevated level of DSBR present in the untreated A549δSHP-1 cells as evidenced by a large proportion of cells showing type 2 comets or above (Supplementary Fig. S6C).

When A549-Scramble cells were subjected to the alkaline comet assay (Supplementary Fig. S6), we observed total repair of radiation-induced SSBs after 120 minutes (Supplementary Fig. S6D) in marked contrast to DNA repair deficient A549δPNKP cells (Supplementary Fig. S6E). SHP-1 knockdown cells showed a very similar response to radiation as A549-Scramble cells, indicating that SHP-1 is not significantly involved in the repair of SSBs (Supplementary Fig. S6F), but, as with the DSB data, we observed a modestly higher level of SSBs in the unirradiated SHP-1–depleted cells than in the controls.

The results above render it unlikely that the primary cause of the synthetic lethal partnership between PNKP and SHP-1 is because of an interaction between 2 DNA repair pathways akin to PARP and the BRCA proteins, and we therefore sought an alternative explanation. One clue provided by the repair assays was the higher level of strand breaks in the unirradiated SHP-1–depleted cells, which, together with reports in the literature about elevated levels of ROS in SHP-1–depleted cells (28), led us to an alternative hypothesis that reduced SHP-1 expression leads to the generation of ROS-induced DNA strand breaks, the repair of which are dependent on PNKP activity. To investigate this supposition, we examined the basal level of ROS (hydroxyl radicals and peroxynitrite together) produced in the wild-type and SHP-1–depleted cells that were used to establish the synthetic lethal partnership between PNKP and SHP-1. We found that when SHP-1 was depleted to approximately 15% of wild-type level, approximately 40% more ROS were produced in both A549 and MCF7-based cell lines (Figs. 6A and B).

To further corroborate a role for ROS in the synthetic lethal partnership between PNKP and SHP-1, we treated A549-Scramble and A549δSHP-1 cells with the PNKP inhibitor, A12B4C3, in the presence or absence of the ROS scavenger WR1065 (29, 30) to determine if a reduction in cellular ROS concentration would rescue the lethal phenotype conferred to cells upon codisruption of PNKP and SHP-1 (Fig. 6C). (Doses of the 2 chemical reagents were chosen so as to avoid toxicity in the control A549-Scramble cells.) In the absence of the ROS scavenger, treatment of the A549δSHP-1 cells with A12B4C3 resulted in approximately 30% cytotoxicity, which contrasts with the almost complete abrogation of cytotoxicity when A12B4C3 was codistributed with WR1065, implicating a critical role for ROS in the PNKP/SHP-1 synthetic lethal partnership.

Finally, an examination of the influence of PNKP inhibition on the ROS-induced DSBs, in which A549δSHP-1 and A549-Scramble cells were treated with a nontoxic dose of A12B4C3 and the level of H2AX phosphorylation was monitored over 48 hours, revealed that DSBs accumulate when SHP-1 and PNKP are simultaneously depleted (Fig. 7).

**Discussion**

Synthetic lethality is a promising avenue for cancer therapy and even in its early development appears to be clinically effective (31, 32). Our screen identified 425 possible synthetic...
lethal partners of PNKP, representing 6.1% of genes tested, which is typical for initial screens of this type (33–35). Of these proteins, 14 are currently considered to be tumor suppressors, including PTEN and SHP-1. From the standpoint of potential therapeutic benefit in cancer treatment, there is clearly considerable advantage to identifying partnerships of tumor suppressors. The participation of some tumor suppressors in synthetic lethal partnerships has been noted before. PTEN has a partnership with PARP (22), which has been attributed to reduced DSBR by homologous recombination (36), whereas p53 has been shown to have synthetic lethal partnerships with the protein kinases SGK2 and PAK3 (37). Loss-of-function mutants are considered notoriously hard to treat, as protein function is difficult to reestablish pharmacologically and reestablishment of tumor suppressor activity is technically challenging. However, through the use of the concept of synthetic lethality, such mutant cells become targetable (38, 39). This is possible because in principle synthetic lethality targets the tumor suppressor’s lethal partner, thereby only affecting the naturally protein deficient cancer cells to cause the cytotoxic double disruption, effectively leaving normal cells unharmed. Side effects are therefore theorized to be minor, and in practice, this can be seen. In the clinical trials using Olaparib in BRCA1- and BRCA2-mutated ovarian cancer, the only grade 3 toxicities observed were nausea (7%) and leukopenia (5%; ref. 40).

Of the tumor suppressors identified, we chose to further validate SHP-1 as it has been shown to be deficient or absent in a substantial number of human cancers (18, 26, 41). Tissue microarray analysis of the SHP-1 status of 207 paraffin-embedded samples of a diverse assortment of malignant lymphomas and leukemias revealed that 90% or more of diffuse large cell lymphoma, follicle center lymphoma, Hodgkin’s disease, mantle cell lymphoma, peripheral T cell lymphoma, adult T cell lymphoma/leukemia specimens and 100% of NK/T cell lymphoma specimens showed no detectable SHP-1 expression (41–43). Similarly, SHP-1 was expressed at reduced or...
that, similar to the control cells, there was a marked decline in foci in the A549SHP-1 cells by the 4-hour time point, indicative of efficient DSBR. This data coupled with our observation of efficient SSBR (Supplementary Fig. S6) led us to look for an alternative mechanism for SHP-1/PNKP synthetic lethality. It has been observed previously that SHP-1 depletion causes an increase in ROS production in human umbilical vein endothelial cells through its negative regulation of NAD(P)H-oxidase–dependent superoxide production (28). Furthermore, SHP-1, in common with many other protein-tyrosine phosphatases, is susceptible to oxidation of key cysteine residues in its catalytic domain by ROS, including those generated by ionizing radiation (47, 48). Our data (Fig. 6) indicate that SHP-1 depletion in A549 and MCF7 cells also causes an increase in ROS production, which in turn results in elevated DNA strand cleavage (Fig. 5 and Supplementary Fig. S6). (Radiation-induced inactivation of the residual SHP-1 present in the SHP-1 knockdown cells, and the resulting increase in ROS, could explain the higher production of γH2AX foci in these cells at 15 minutes postirradiation.) We inferred that, when coupled with PNKP-mediated disruption of DNA repair, increased ROS production causes a cytotoxic accumulation of DNA damage (Fig. 7). The elimination of cytotoxicity conferred by treatment with the free radical scavenger WR1065 (Fig. 6C) provided additional support for such a mechanism. As PNKP acts on SSBs, as well as DSBS, an increase in unrepared ROS-induced SSBS would lead to an increase in DSBR formation during S-phase, potentially saturating DSBR repair because these newly formed DSBS would also require the action of PNKP at their termini. Our findings further extend the observation by Martin and colleagues (13), who showed that depletion of PINK1 causes an elevation of ROS and toxicity in a mismatch-repair deficient background. Importantly, this mechanism may apply to other yet to be identified synthetic lethal partnerships between proteins involved in ROS regulation and oxidative DNA damage repair.

Disclosure of Potential Conflicts of Interest
T.R. Mereniuk, E. Foley, and M. Weinfeld have submitted a provisional US patent application: UNAB 017 Synthetic lethality in cancer. No potential conflicts of interest were disclosed by the other authors.

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References
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