The function of PTEN in the cytoplasm largely depends on its lipid-phosphatase activity, though which it antagonizes the PI3K–AKT oncogenic pathway. However, molecular mechanisms underlying the role of PTEN in the nucleus remain largely elusive. Here, we report that DNA double-strand breaks (DSB) promote PTEN interaction with MDC1 upon ATM-dependent phosphorylation of T/S398-PTEN. Importantly, DNA DSBs enhance NSD2 (MMSET/WHSC1)-mediated dimethylation of PTEN at K349, which is recognized by the tudor domain of 53BP1 to recruit PTEN to DNA-damage sites, governing efficient repair of DSBs partly through dephosphorylation of γH2AX. Of note, inhibiting NSD2-mediated methylation of PTEN, either through expressing methylation-deficient PTEN mutants or through inhibiting NSD2, sensitizes cancer cells to combinatorial treatment with a PI3K inhibitor and DNA-damaging agents in both cell culture and in vivo xenograft models. Therefore, our study provides a novel molecular mechanism for PTEN regulation of DSB repair in a methylation- and protein phosphatase–dependent manner.

SIGNIFICANCE: NSD2-mediated dimethylation of PTEN is recognized by the 53BP1 tudor domain to facilitate PTEN recruitment into DNA-damage sites, governing efficient repair of DNA DSBs. Importantly, inhibiting PTEN methylation sensitizes cancer cells to combinatorial treatment with a PI3K inhibitor combined with DNA-damaging agents in both cell culture and in vivo xenograft models.
INTRODUCTION

The gene encoding the lipid and protein dual-specificity phosphatase PTEN is a tumor suppressor frequently mutated, deleted, or epigenetically silenced in various types of human cancer (1, 2). In the cytoplasm and membrane, PTEN primarily governs key cellular processes including cell survival, proliferation, aging, angiogenesis, and metabolism through its lipid-phosphatase activity, which allows it to antagonize the PI3K–AKT oncogenic pathway (3–5). However, compared with its well-studied lipid-phosphatase activity, the protein-phosphatase function of PTEN remains largely undefined. There is increasing evidence demonstrating that PTEN also possesses multiple important functions in the nucleus independent of its lipid-phosphatase activity, such as controlling chromosomal integrity (6), chromatin structure (7, 8), DNA replication (9), and DNA damage repair (DDR; refs. 10, 11). In support of a critical role for PTEN in DNA double-strand break (DSB) repair, both large-scale proteomic analyses and in vitro specific biochemical kinase assays found that PTEN is phosphorylated at T398 (S398 in mouse PTEN, hereafter referred as T/S398) by the ataxia telangiectasia mutated (ATM) kinase in response to DNA damage (11, 12). However, the precise molecular mechanism by which ATM-dependent phosphorylation of PTEN T/S398 regulates its role in DDR remains largely elusive.

Protein methylation, especially histone methylation, is one of the key post-translational modifications in regulating chromatin properties and gene-expression profiles, which governs various cellular processes including DNA replication and cell-cycle progression (13, 14). Beyond histone methylation, recent studies have revealed that methylation of nonhistone proteins also plays a critical role in controlling cellular signaling and functions (15, 16). For example, methylation of the tumor suppressor p53 at K372 by the methyltransferase SET9 activates its transcriptional function, whereas the SMYD2-mediated methylation of p53 at K372 has an opposite role (17, 18). It also has been reported that the tumor suppressor RB is methylated by SMYD2 at K860, which is then recognized by the MBT domain of the transcriptional repressor L3MBTL1 (19). Moreover, the SET7/9 methyltransferase methylates RB at K810, which is subsequently recognized by the tudor domain of 53BP1 to maintain RB in its hypophosphorylated state...
state and response to the DNA damage signaling (20). However, whether and how the tumor suppressor PTEN may be regulated by methylation remains largely unexplored.

Here, we report that DNA DSBs promote NSD2 (also named MMSET/WHSC1)-mediated dimethylation of PTEN at K349, which is read by the tudor domain of 53BP1 to recruit PTEN into DNA-damage sites to govern the timely repair of DSBs in part through dephosphorylating γH2AX. More importantly, inhibiting NSD2-mediated methylation of PTEN sensitizes cancer cells to combinatorial treatment with PI3K inhibitor and DNA-damaging agents in both cell culture and in vivo xenograft models.

RESULTS

ATM-Mediated Phosphorylation of PTEN Is Required for Binding the BRCT Domain of MDC1 upon DNA-Damage Signaling

To further explore the role of PTEN in the nucleus governing DSB repair, we found that phosphorylation of PTEN could be readily detected in different cell lines using the phospho-(Ser/Thr) ATM/ataxia telangiectasia mutated and rad3-related protein (ATR) substrate antibody upon treatment with various DNA-damaging agents including etoposide, irradiation (IR), or N-methyl-N-nitro-N-nitrosoguanidine (Fig. 1A; Supplementary Fig. S1A–S1D). Moreover, cellular fractionation assays showed that etoposide-induced phosphorylated PTEN existed in both the cytoplasm and nucleus (Supplementary Fig. S1E). Furthermore, we found that inhibiting the upstream kinase ATM, but not ATR, using the pharmacologic inhibitors or short hairpin RNA (shRNA) could dramatically decrease etoposide-induced phosphorylation of PTEN in cells (Supplementary Fig. S1F and S1G), suggesting that the ATM kinase is the major physiologic kinase that phosphorylates PTEN at S398 in response to DNA damage (Supplementary Fig. S1H). However, the precise molecular mechanism governing ATM-dependent phosphorylation of PTEN at S398 to regulate its role in DDR remains largely elusive.

Previous reports have revealed that pS/pTQ events can be recognized by the BRCA1 carboxy-terminal (BRCT) or forkhead-associated (FHA) domains in DDR proteins to facilitate the DDR process (Supplementary Fig. S1I; refs. 21, 22). Despite the subtle difference in the 398pTQ399 phospho-motif in human PTEN versus the 398SO399 motif in mouse PTEN, they are both phospho-acceptors and likely function in a similar manner (Supplementary Fig. S1J; refs. 11, 12). In keeping with this notion, we found that etoposide treatment enhanced both human PTEN and mouse PTEN binding to the BRCT domain of MDC1, but not its FHA domain, nor the BRCT domain derived from 53BP1 or BRCA1 (Fig. 1B; Supplementary Fig. S1K and S1L). Moreover, the etoposide-induced interaction between PTEN and NSD2 was largely disrupted in Mdc1−/− mouse embryonic fibroblasts (MEF; Fig. 1F), which suggests that MDC1 likely plays a crucial role in mediating the interaction between PTEN and NSD2 upon DNA damage signaling. Although ATM also phosphorylates MDC1 at the T4 sites to promote the dimer formation of MDC1 (26), the MDC1-A1-143 mutant with deletion of the N-terminal region containing T4 and the FHA domain still interacted with PTEN after etoposide treatment (Supplementary Fig. S1R), indicating that ATM-mediated phosphorylation of MDC1 likely does not play an important role in mediating the PTEN–MDC1 interaction upon DNA damage. Altogether, these results support a model in which, in response to DSBs, PTEN, MDC1, and NSD2 could form a ternary complex in an ATM-mediated PTEN phosphorylation–dependent manner.

DNA Damage Promotes NSD2-Mediated Dimethylation of PTEN at K349

In support of a specific interaction between NSD2 and PTEN, we found that only NSD2, but none of the other methyltransferases we examined, including SET8, EZH2, and NSD3, interacted with PTEN in cells under etoposide treatment condition (Supplementary Fig. S2A and S2B). We further found that NSD2 interacted with PTEN largely through its C-terminal domain (784–1365) in cells (Supplementary Fig. S2C). Moreover, compared with the methyltransferase SET8 or EZH2, NSD2 could dramatically promote the methylation of PTEN in cells, as revealed by binding of methyl-lysine motif antibodies (Supplementary Fig. S2D). Notably, we observed significant dimethylation of PTEN at endogenous levels after etoposide treatment, which largely correlated with an elevated interaction between NSD2 and PTEN (Fig. 2A). Furthermore, compared with Nsd2−/− MEFs, the dimethylation of PTEN was dramatically reduced in Nsd2−/− MEFs upon etoposide
PTEN Methylation Governs DNA Damage Response

**Figure 1.** ATM-mediated phosphorylation of PTEN is required for its binding with the BRCT domain of MDC1 upon DNA-damage signaling. **A,** Phosphorylation of PTEN was detected using the phospho-(Ser/Thr) ATM/ATR substrate antibody (pS/TQ) upon etoposide treatment. Immunoblot (IB) analysis of anti-PTEN immunoprecipitations (IP) and whole-cell lysates (WCL) derived from NIH3T3 cells treated with 30 μmol/L etoposide at indicated time points before harvesting. **B,** Etoposide treatment promoted PTEN interaction with the MDC1 BRCT domain, but neither the MDC1 FHA nor the 53BP1 BRCT domain. IB analysis of GST pulldown and WCL derived from U2OS cells transfected with indicated constructs and treatment with or without 30 μmol/L etoposide for 30 minutes before harvesting. **C,** Etoposide treatment promoted wild-type (WT), but not T398A mutant, PTEN interaction with MDC1’s BRCT domain. IB analysis of GST pulldown and WCL derived from U2OS cells cotransfected with indicated constructs. Thirty-six hours after transfection, cells were treated or not treated with 30 μmol/L etoposide for 30 minutes and harvested for IP assays. EV, empty vector. **D,** PTEN, MDC1, and NSD2 formed a ternary complex in the nucleus upon etoposide treatment. IB analysis of anti-PTEN IPs from cytoplasm or nucleus as well as WCLs derived from U2OS cells treated with 30 μmol/L etoposide at indicated time points before harvesting. **E,** Depletion of ATM disrupted PTEN interaction with MDC1 and NSD2 upon etoposide treatment. IB analysis of anti-PTEN IPs and WCLs derived from U2OS cell lines stably expressing shControl or shATM transfected with indicated constructs. Thirty-six hours after transfection, cells were treated or not treated with 30 μmol/L etoposide for 30 minutes before harvesting. **F,** Depletion of Mdc1 impaired the interaction between PTEN and NSD2. IB analysis of anti-PTEN IPs and WCLs derived from Mdc1+/− and Mdc1−/− MEFs treated with 30 μmol/L etoposide at indicated time points before harvesting.

Figure 1. ATM-mediated phosphorylation of PTEN is required for its binding with the BRCT domain of MDC1 upon DNA-damage signaling. A, Phosphorylation of PTEN was detected using the phospho-(Ser/Thr) ATM/ATR substrate antibody (pS/TQ) upon etoposide treatment. Immunoblot (IB) analysis of anti-PTEN immunoprecipitations (IP) and whole-cell lysates (WCL) derived from NIH3T3 cells treated with 30 μmol/L etoposide at indicated time points before harvesting. B, Etoposide treatment promoted PTEN interaction with the MDC1 BRCT domain, but neither the MDC1 FHA nor the 53BP1 BRCT domain. IB analysis of GST pulldown and WCL derived from U2OS cells transfected with indicated constructs and treatment with or without 30 μmol/L etoposide for 30 minutes before harvesting. C, Etoposide treatment promoted wild-type (WT), but not T398A mutant, PTEN interaction with MDC1’s BRCT domain. IB analysis of GST pulldown and WCL derived from U2OS cells cotransfected with indicated constructs. Thirty-six hours after transfection, cells were treated or not treated with 30 μmol/L etoposide for 30 minutes and harvested for IP assays. EV, empty vector. D, PTEN, MDC1, and NSD2 formed a ternary complex in the nucleus upon etoposide treatment. IB analysis of anti-PTEN IPs from cytoplasm or nucleus as well as WCLs derived from U2OS cells treated with 30 μmol/L etoposide at indicated time points before harvesting. E, Depletion of ATM disrupted PTEN interaction with MDC1 and NSD2 upon etoposide treatment. IB analysis of anti-PTEN IPs and WCLs derived from U2OS cell lines stably expressing shControl or shATM transfected with indicated constructs. Thirty-six hours after transfection, cells were treated or not treated with 30 μmol/L etoposide for 30 minutes before harvesting. F, Depletion of Mdc1 impaired the interaction between PTEN and NSD2. IB analysis of anti-PTEN IPs and WCLs derived from Mdc1+/− and Mdc1−/− MEFs treated with 30 μmol/L etoposide at indicated time points before harvesting.

Consistent with the finding that MDC1 plays a crucial role in mediating the interaction of PTEN and NSD2 (Fig. 1F), etoposide-induced dimethylation of PTEN was also markedly decreased in Mdc1−/− MEFs compared with Mdc1+/+ MEFs (Supplementary Fig. S2E). Moreover, compared with wild-type (WT) PTEN, the ATM phosphorylation-deficient T398A-PTEN mutant was impaired in associating with NSD2 in response to etoposide treatment (Supplementary Fig. S2F) and exhibited a marked reduction in NSD2-mediated dimethylation of PTEN in cells (Supplementary Fig. S2G).
Taken together, these results demonstrate that MDC1 plays a pivotal role in connecting the NSD2 methyltransferase to ATM-mediated phosphorylation of PTEN and subsequently promoting NSD2-mediated methylation of PTEN.

Next, we set out to identify NSD2-mediated methylation site(s) on PTEN. Consistent with our observation that PTEN interacted with NSD2 through its C-terminal domain (Supplementary Fig. S2H and S2I), major dimethylation sites were identified in the C-terminus of PTEN under etoposide treatment condition (Supplementary Fig. S2J). Using a mutagenesis approach to remove major lysine residues exposed at the PTEN protein surface (27), we found that the K349R mutant was largely deficient in undergoing NSD2-mediated methylation in cells, but not other lysine mutants we examined including K266R, K289R, K332R, or K402R (Fig. 2C). Moreover, the K349 residue on PTEN was conserved among different species and located on the surface of crystal structure of PTEN (Supplementary Fig. S2K and S2L), further indicating K349 as a potential NSD2-mediated dimethylation site.

To further investigate the physiologic role of dimethylation of PTEN-K349 during DSB repair, we generated anti-PTEN-K349me2 and anti-PTEN-K349me3 antibodies. Using dot blot assays with synthetic PTEN peptides on K349 (PTEN-K349me2) or trimethylated on K349 (PTEN-K349me3), we found that the K349R mutant existed in both cytoplasm and nucleus. IB analysis of anti-PTEN IPs from cytoplasm or nucleus as well as WCLs derived from NIH3T3 cells treatment with etoposide at indicated time points before harvesting. M, Etoposide (min)

DNA damage promotes NSD2-mediated dimethylation of PTEN at K349. A, Methylation of PTEN was detected using a dimethyl lysine motif antibody. IB analysis of anti-PTEN IPs and WCLs derived from U2OS cells treated with 30 μmol/L etoposide at indicated time points before harvesting. B, Depletion of Nsd2 impaired the dimethylation of PTEN. IB analysis of anti-PTEN IPs and WCLs derived from Nsd2−/− and Nsd2+/+ MEFs treated with 30 μmol/L etoposide for 30 minutes before harvesting. C, K349 was identified as the major dimethylation site on PTEN. IB analysis of anti-HA IPs and WCLs derived from 293T cells transfected with the indicated constructs and treated or not treated with 30 μmol/L etoposide for 30 minutes before harvesting.

Figure 2. DNA damage promotes NSD2-mediated dimethylation of PTEN at K349. A, Methylation of PTEN was detected using a dimethyl lysine motif antibody. IB analysis of anti-PTEN IPs and WCLs derived from U2OS cells treated with 30 μmol/L etoposide at indicated time points before harvesting. B, Depletion of Nsd2 impaired the dimethylation of PTEN. IB analysis of anti-PTEN IPs and WCLs derived from Nsd2−/− and Nsd2+/+ MEFs treated with 30 μmol/L etoposide for 30 minutes before harvesting. C, PTEN was detected by a specific K349 dimethylation (K349me2) antibody. IB analysis of anti-HA IPs and WCLs derived from U2OS cells stably expressing shNsd2 (F) or Nsd2+/+ MEFs (G) that were treated with IR (5 Gy) at indicated time points before harvesting.
with K349 non-, mono-, di-, and trimethylation (Supplementary Fig. S2M), we validated that the PTEN-K349me2 antibody specifically recognized the dimethylated-K349-PTEN synthetic peptides, whereas the PTEN-K349me3 antibody specifically recognized the trimethylated-K349-PTEN synthetic peptides (Supplementary Fig. S2N). However, upon etoposide treatment, we detected only dimethylation, but not trimethylation, of PTEN at K349 in cells (Fig. 2D). More importantly, dimethylation of K349-PTEN was observed at endogenous levels upon etoposide or γ-irradiation treatment, a process that could be largely abolished following depletion of endogenous NSD2 (ref. 28; Fig. 2E–G; Supplementary Fig. S2O). Consistent with the results that ATM-mediated phosphorylation of PTEN is critical for PTEN methylation (Supplementary Fig. S2F and S2G), an ATM inhibitor also suppressed the dimethylation of PTEN at K349 after etoposide treatment (Supplementary Fig. S2P). Moreover, 3-deazaneplanocin A (DZNep), a pan-inhibitor of S-adenosylmethionine-dependent methyltransferases, including NSD2 (29, 30), could also dramatically suppress K349 dimethylation of PTEN in cells in a dose-dependent manner (Supplementary Fig. S2Q). Taken together, these results reveal NSD2 as a major physiologic upstream methyltransferase that predominantly promotes K349 dimethylation of PTEN in response to DNA-damage signals.

**NSD2-Mediated Dimethylation of PTEN Is Recognized by the Tudor Domain of 53BP1**

Given that methylation signals at DNA damage sites can be recognized by tudor domain–containing proteins, such as 53BP1 or histone demethylases, to facilitate the recruitment of DDR effector proteins including p53 and RB (Supplementary Fig. S3A; refs. 20, 31, 32), we explored the physiologic reader that can recognize NSD2-mediated K349 dimethylation of PTEN. To this end, we found that etoposide treatment could dramatically enhance PTEN interaction with 53BP1 largely through its tudor domain, but not other tudor domains we examined, including SETDB1 or KDM4B (Fig. 3A and B; Supplementary Fig. S3B and S3C), indicating a possible role for 53BP1 in mediating the recruitment of PTEN to DNA-damage sites. We also observed that DNA-damaging agents can lead to reduced protein abundance of 53BP1, a process that can be partially blocked by treatment with the 26S proteasome inhibitor MG132 (Supplementary Fig. S3D). This indicates that the ubiquitin proteasome pathway is involved in DNA-damage–induced 53BP1 destruction, but the underlying mechanism remains to be determined. Notably, the methyltransferase NSD2 promoted PTEN-WT, but not PTEN-K349R, interaction with the tudor domain of 53BP1, indicating the involvement of methyl-K349-PTEN and its subsequent recognition by the 53BP1 tudor domain (Fig. 3C; Supplementary Fig. S3E). However, although the PTEN-K349R mutant failed to interact with 53BP1 tudor domain, PTEN-K349R still bound to NSD2 (Supplementary Fig. S3F). Moreover, the ATM-mediated phosphorylation-deficient 53BP1-S/T28A also interacted with PTEN (Supplementary Fig. S3G), suggesting that 53BP1 interacts with PTEN largely through its tudor domain (Fig. 3B).

In further support of a direct association between the 53BP1 tudor domain and methyl-K349-PTEN, using an in vitro pulldown assay with synthetic PTEN peptides that are either unmodified K349 (K349-me0), monomethylated (K349-me1), dimethylated (K349-me2), or trimethylated (K349-me3; Supplementary Fig. S2M), we found that the purified recombinant tudor domain of 53BP1 specifically interacted with methylated (K349-me1,2,3), but not with unmodified (K349-me0), synthetic PTEN peptides in vitro (Fig. 3D). Moreover, among all three methylated synthetic PTEN peptides, the dimethylated PTEN peptide (K349-me2) appeared to interact with the 53BP1 tudor domain with a relatively higher affinity (Fig. 3D). More importantly, depletion of NSD2 dramatically reduced endogenous PTEN binding with 53BP1 in cells, which was largely correlative to the reduced PTEN-K349 dimethylation in NSD2-depleted cells (Fig. 3E and F). Taken together, these results demonstrate that NSD2-mediated K349 dimethylation of PTEN is required for efficient recognition by the methyl-lysine reading tudor domain of 53BP1 upon DNA-damage signals (Supplementary Fig. S3H).

**PTEN K349 Dimethylation and Protein Phosphatase Activity Are Required for Efficient DSB Repair**

To explore the physiologic function of PTEN in the DDR process, we investigated the DNA damage response in PTEN+/− and PTEN−/− HCT116 cells (33) after exposure to γ-irradiation or etoposide. Notably, we found that the magnitude and duration of γH2AX were dramatically elevated, especially during late stages post γ-irradiation or etoposide treatment (4-, 8-, and 24-hour time points) in PTEN−/− cells, as compared with PTEN+/− cells (Fig. 4A and B; Supplementary Fig. S4A). Although the PTEN-K349R mutant still displayed lipid-phosphatase activity at levels comparable with WT-PTEN (Supplementary Fig. S4B), it was largely deficient in rescuing the prolonged γH2AX status in PTEN−/− HCT116 cells post γ-irradiation or etoposide treatment (Fig. 4C and D; Supplementary Fig. S4C), suggesting that the lipid-phosphatase activity of PTEN might not be involved in this process, at least in this experimental setting. Because PTEN also possesses protein phosphatase activity (34) to affect the biological functions of its protein substrates (35, 36), we went on to explore whether the duration of γH2AX after irradiation in PTEN−/− cells is relevant to PTEN protein phosphatase activity. To this end, we reintroduced PTEN-WT, C124S (lipid and protein-phosphatase dead mutant), G129E (lipid-phosphatase dead mutant), or Y138L (protein-phosphatase dead mutant) into PTEN−/− HCT116 cells at comparable levels (Supplementary Fig. S4D and S4E). Consistent with previous reports (34, 35), ectopic expression of PTEN-WT and PTEN-Y138L mutant, but not the lipid-phosphatase–deficient C124S nor G129E mutants, could reduce the levels of pS473-AKT in PTEN−/− HCT116 cells post γ-irradiation (Fig. 4E). Notably, we found that PTEN−/− HCT116 cells reintroduced WT or G129E, but not Y138L or C124S, were capable of rescuing the elevated and prolonged γH2AX status upon γ-irradiation (Fig. 4E and F). Although the protein-phosphatase–deficient PTEN-Y138L mutant failed to rescue the γH2AX levels compared with the PTEN-WT, the PTEN-Y138L mutant could be still modified via etoposide-induced phosphorylation and methylation, as well as interacted with MDC1 and NSD2 upon etoposide treatment (Supplementary Fig. S4F).
post cells expressing C124S and Y138L that are deficient in PTEN stably expressing PTEN-WT and PTEN-G129E, but not in mediated dimethylation (Fig. 4G; Supplementary Fig. S4J).

or S398A mutants that are deficient in undergoing NSD2-protein-phosphatase activity, or in the cells expressing K349R protein-phosphatase activity toward regulating PTEN in regulating DSB repair, we detected the DNA damage–late-stage event in the process of DDR.

To further examine the PTEN protein phosphatase activity in regulating γH2AX levels might be the late-stage event in the process of DDR.

Fig. S4F-S4I), which suggests that the PTEN protein phosphatase activity toward PTEN interaction with 53BP1. IB analysis of anti-PTEN IPs and WCL derived from U2OS cells treated with 30 μmol/L etoposide at indicated time points before harvesting, B and C, PTEN-K349R mutant disrupted its binding with 53BP1 upon etoposide treatment. IB analysis of GST pulldown and WCLs derived from U2OS cells transfected with the indicated constructs. Thirty-six hours after transfection, cells were treated or not treated with 30 μmol/L etoposide for 30 minutes and harvested for GST pulldown assays. D, 53BP1 tudor domain had a high affinity for K349-me2-PTEN peptides. One microgram of indicated biotin-labeled synthetic PTEN peptides were incubated with 250 ng purified recombinant GST-tagged 53BP1 tudor domain, respectively. Streptavidin beads were added to perform pulldown assays and precipitations were analyzed by IB. Dot blot assays were performed to show that an equal amount of biotinylated peptides was used for the pulldown assay. E and F, Depletion of NSD2 disrupted PTEN interaction with 53BP1. IB analysis of anti-PTEN IPs and WCL derived from U2OS cells stably expressing shNSD2 or Nsd2−/− MEFs were treated or not treated with 30 μmol/L etoposide for 30 minutes before harvesting.

Consistently, cells expressing C124S, Y138L, K349R, and S398A, but not WT or G129E mutants of PTEN, also exhibited deficiencies in resolving γH2AX foci at 24 hours post γ-irradiation (Fig. 4H; Supplementary Fig. S4K). More importantly, we monitored the foci formation of the DSB markers 53BP1 and γH2AX in multiple cell lines including PTEN-deficient U87MG and PC3 reconstituted with WT-PTEN or K349R-PTEN to validate that cells deficient in undergoing K349R-PTEN methylation are defective in resolving DSB foci induced by IR (Supplementary Fig. S4L–S4O).

In addition, we performed the immunostaining for the homologous recombination (HR) markers RAD51 and BRCA1 after γ-irradiation in the PTEN−/− HCT116 cells reconstituted with PTEN-WT and PTEN-K349R, as well as empty vector (EV) as a negative control. Our results showed that, compared with putting back both PTEN-WT and PTEN-K349R, PTEN−/− HCT116 cells stably expressing EV exhibited a decreased number of RAD51 foci 4 hours after IR treatment (Supplementary Fig. S4P), which is consistent with previous reports that PTEN could recruit the RAD51 to the DNA.
PTEN Methylation Governs DNA Damage Response

Figure 4. PTEN K349 dimethylation and protein phosphatase activity are required for efficient DSB repair. A and B, PTEN deficiency elevated γH2AX 4 hours post-irradiation (IR) treatment. IB analysis of WCLs derived from PTEN−/− and PTEN+/− HCT116 cells after treatment with IR (5 Gy) at indicated time points (A). Quantification of protein intensity was performed using ImageJ software (B). γH2AX immunoblot bands were normalized to VINCULIN, and then normalized to the control (no IR treatment). Data are represented as mean ± SD, n = 3. *, P < 0.05; Student t test. C and D, PTEN-WT, but not K349R mutant, rescued PTEN deficiency-mediated high levels of γH2AX 4 hours post-IR treatment. IB analysis of WCLs derived from PTEN+/− HCT116 cells introducing PTEN-WT and PTEN-K349R as well as EV, which were treated with IR (5 Gy) at indicated time points before harvesting (C). Quantification of protein intensity was performed using ImageJ software (D). γH2AX immunoblot bands were normalized to VINCULIN and then normalized to the control (no IR treatment). Data are represented as mean ± SD, n = 3. *, P < 0.05; Student t test. E, F, PTEN protein-phosphatase activity, but not lipid phosphatase activity, was required for regulating γH2AX levels. IB analysis of WCLs derived from PTEN+/− HCT116 cells after reintroducing PTEN WT, C124S, G129E, and Y138L, as well as EV, which were treated with IR (5 Gy) at indicated time points before harvesting (E). Quantification of protein intensity was performed using ImageJ software (F). γH2AX immunoblot bands were normalized to VINCULIN, then normalized to the control (no IR treatment). Data are represented as mean ± SD, n = 3. *, P < 0.05; Student t test. G and H, PTEN+/− HCT116 cells reconstituted with the indicated PTEN constructs were treated with IR (5 Gy) and immunostained at the indicated times with anti-53BP1 or anti-γH2AX. Quantification of 53BP1 (G) or γH2AX (H) foci-positive cells (foci > 5 per cell) was performed by counting a total of 100 cells per sample, respectively. Data are represented as mean ± SD, n = 3. **, P < 0.01; Student t test. (continued on next page)

Consistent with a previous report (44), we found that PTEN colocalized with γH2AX in DNA-damage sites generated by laser microirradiation (Supplementary Fig. S4S). Moreover, the interaction between PTEN and H2AX appears to be dependent on NSD2-mediated methylation of PTEN at K349, as the interaction between H2AX and K349R-PTEN was dramatically decreased in comparison with WT-PTEN, after γ-irradiation (Fig. 4I). Moreover, Comet assays showed that compared with PTEN-WT cells, cells expressing PTEN-K349R exhibited significantly longer tail moment after etoposide treatment, which suggests the DDR process was dramatically suppressed in the context of K349R mutation (Fig. 4J and K). Furthermore, depletion of the Nsd2
PTEN could dephosphorylate the γH2AX or pS133-CREB in a dose-dependent manner (Fig. 4N). Although both our results and previous study (12) showed that PTEN could be recruited to DNA-damage sites, the factor(s) playing a critical role in recruiting PTEN into DNA damage sites remains elusive. On the basis of our results, we hypothesized that the DDR proteins MDC1, 53BP1, or γH2AX might be involved in this recruiting process upon DNA-damage signaling. To address this question, we used the Mdc1−/−, 53BP1−/−, or H2ax−/− as well as their counterpart WT cells to perform the cellular fractionation assays with or without etoposide treatment. Our results demonstrated that PTEN recruitment to DNA-damage sites might be largely dependent on MDC1 and 53BP1, but not H2AX, because upon etoposide treatment the accumulation of PTEN on chromatin was reduced in the Mdc1−/− or 53BP1−/− cells, but not in H2ax−/− cells, compared with their counterpart WT cells (Supplementary Fig. S4U–S4W). Moreover, dimethylation of PTEN at K349 was also critical for localization of PTEN on chromatin upon etoposide treatment because compared with the PTEN-WT, the PTEN-K349R mutant failed to localize on chromatin (Supplementary Fig. S4W). Notably, we also found that the ATM-mediated phosphorylation of 53BP1 at S25/29, two of 28 methyltransferase impaired irradiation-induced interaction of γH2AX with PTEN (Fig. 4L). Furthermore, we found that the purified recombinant GST-PTEN protein could pull down H2AX, but not H2A containing nucleosome core particles in vitro (Supplementary Fig. S4T). These results together indicate that upon DNA-damaging signals, a portion of PTEN could be recruited into the nucleus, where it interacts with H2AX and is involved in DDR.

As our results showed that PTEN protein phosphatase activity plays a critical role in resolving the γH2AX foci, we wanted to explore whether PTEN could directly dephosphorylate γH2AX. To this end, we performed in vitro dephosphorylation assays using bacterially purified recombinant PTEN-WT, C124S, G129E, and Y138L proteins incubated with biotin-labeled H2AX peptides with or without phospholysis of Ser139. Notably, PTEN-WT and PTEN-G129E, but not the protein-phoshaphosphase–dead mutants C124S and Y138L, could dramatically dephosphorylate biotin-labeled γH2AX in vitro (Fig. 4M). To further mimic the reaction condition in cells, we purified the γH2AX from HCT116 cells after etoposide treatment and the positive control pS133-CREB (43) through using the anti-γH2AX or anti-pS133-CREB antibody, respectively. The in vitro dephosphorylation assays showed that
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PTEN Methylation Governs DNA Damage Response

B). Furthermore, 
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 γ-irradiation (IR) treatment compared with 

More importantly, to dissect the critical physiologic role of the protein-phosphatase activity of PTEN in DSB repair, we isolated 
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 MEFs and found that in comparison with WT MEFs, heterozygous 
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 MEFs also displayed relatively higher levels of γH2AX at 8- and 24-hour time points post-γ-irradiation (Fig. S5E; Supplementary Fig. S5B). Although our previous study showed that C124S or G129E mutations in PTEN affect their dimerization to regulate PTEN lipid phosphatase activity (46), the protein-phosphatase-deficient Y138L or methylation-deficient K349R mutant did not exert significant effects on PTEN dimerization in cells (Supplementary Fig. SSC and SSD). Taken together, these results suggest that the protein-phosphatase activity, but not lipid-phosphatase activity, of PTEN appears to play a crucial role in regulation of γH2AX status in response to DNA damage in vivo.

The Protein-Phosphatase Activity of PTEN Is Required for DSB Repair In Vivo

To study the physiologic role of PTEN protein-phosphatase activity in regulating γH2AX levels in vivo, we used Pten knock-in mice harboring 
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 or 
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 mutants (46). Compared with 
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 MEFs, the levels of γH2AX were elevated in 
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 MEFs at late stages of DSB repair post-γ-irradiation (8- and 24-hour time points; Fig. 5A and B). Furthermore, 
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 mice also displayed high levels of γH2AX at 24 hours post-γ-irradiation compared with 
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 and 
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 mice (Fig. 5C and D; Supplementary Fig. S5A).

NSD2-Mediated Methylation of PTEN at K349 Dictates Cellular Sensitivity to DNA-Damaging Agents

Given the critical role of DSB repair in conferring cell survival and apoptosis signaling, we further explored whether the dimethylation status or protein-phosphatase activity of PTEN plays an important role in regulating cell survival upon treatment with DNA-damaging agents. As PTEN has both lipid- and amino-terminal Ser/Thr-Gln (S/T-Q) sites that are required for recruiting DSB-responsive factors including PTIP and RIF1 (45), was dramatically reduced in the nucleus of PTEN knockout cells compared with PTEN WT cells (Supplementary Fig. S4Y and S4Z). This result suggested that PTEN might also function as a scaffold protein for amplifying the ATM-mediated phosphorylation signaling on 53BP1 to help recruit other DDR response factors participating in the DDR process. Taken together, these results support our model that upon DNA damage a portion of methylated PTEN species are recruited on DNA-damage sites along with other factors to help DSB repair largely through its protein-phosphatase activity in the nucleus.

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More importantly, to dissect the critical physiologic role of the protein-phosphatase activity of PTEN in DSB repair, we isolated 
Pten
 MEFs and found that in comparison with WT MEFs, heterozygous 
Pten
 MEFs also displayed relatively higher levels of γH2AX at 8- and 24-hour time points post-γ-irradiation (Fig. 5E; Supplementary Fig. S5B). Although our previous study showed that C124S or G129E mutations in PTEN affect their dimerization to regulate PTEN lipid phosphatase activity (46), the protein-phosphatase-deficient Y138L or methylation-deficient K349R mutant did not exert significant effects on PTEN dimerization in cells (Supplementary Fig. S5C and S5D). Taken together, these results suggest that the protein-phosphatase activity, but not lipid-phosphatase activity, of PTEN appears to play a crucial role in regulation of γH2AX status in response to DNA damage in vivo.

Figure 5. The protein-phosphatase activity of PTEN is required for DSBs repair in vivo. A and B, 
Pten
 MEFs displayed higher levels of γH2AX at 24 hours post-γ-irradiation (IR) treatment compared with 
Pten
, 
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, and 
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 MEFs, which were treated with IR (5 Gy) at indicated time points before harvesting (A). Quantification of protein intensity was performed using ImageJ software (B). γH2AX immunoblot bands were normalized to VINCULIN and then normalized to the control (no IR treatment). C and D, Representative IHC analysis of spleen tissues derived from 
Pten
, 
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, and 
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 mice (1st group), which were treated with IR (5 Gy) and sacrificed at 24 hours after irradiation (C). Scale bar, 50 μm. IB analysis of the sample was performed using indicated antibodies (D). Four mice each group. E, 
Pten
 MEFs displayed higher levels of γH2AX at 8 or 24 hours post-IR treatment compared with 
Pten
 MEFs. IB analysis of WCLs derived from 
Pten
 and 
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 MEFs, which were treated with IR (5 Gy) at indicated time points before harvesting.
protein-phosphatase activities, to study PTEN protein-phosphatase activity on DNA damage-induced cell survival and apoptosis, we utilized BKM120 (47), a pan-PI3K inhibitor, to block the PTEN lipid-phosphatase deacyvity pathway (Fig. 6A). To identify the lowest dose for treatment with BKM120 in combination with etoposide or IR, we used a gradient dose for BKM120, etoposide, or IR treatment for HCT116 with PTEN−/− and PTEN+/− cells. Compared with PTEN+/− cells, although PTEN−/− cells were relatively more sensitive to the PI3K inhibitor BKM120 at high doses (3 and 6 μmol/L) for 72-hour treatment, we did not observe significant apoptotic differences between PTEN+/− and PTEN−/− HCT116 cells upon low-dose BKM120 (1 μmol/L) single-reagent treatment (Fig. 6B). Moreover, there was no significant difference in the sensitivity to the low dose of etoposide (10 and 20 μmol/L) or IR (2 Gy) treatment between PTEN+/− and PTEN−/− HCT116 cells (Fig. 6C; Supplementary Fig. S6A). However, compared with PTEN+/− HCT116 cells, PTEN−/− HCT116 cells became more resistant to the high dose of etoposide (40 or 60 μmol/L) or IR treatment (5 or 10 Gy; Fig. 6C; Supplementary Fig. S6A), which might be due to elevated AKT oncogenic activity in PTEN-deficient cells.

In keeping with this notion, we found that although low-concentration BKM120 (1 μmol/L), etoposide (20 μmol/L), or IR (2 Gy) single-reagent treatment for 72 hours did not cause significant difference in apoptosis between PTEN+/− and PTEN−/− HCT116 cells, PTEN−/− HCT116 cells displayed an elevated sensitivity to low-dose treatment with BKM120 (1 μmol/L) in combination with etoposide (20 μmol/L) or IR (2 Gy) as shown in both apoptosis and cell-viability assays (Fig. 6D; Supplementary Fig. S6B and S6C). However, the low concentration of BKM120 (1 μmol/L) did not affect the etoposide-induced phosphorylation and methylation of PTEN or PTEN interactions with MDC1 or NSD2 in cells (Supplementary Fig. S6D–S6G). Moreover, BKM120 treatment did not affect the PTEN protein-phosphatase activity toward γH2AX in vitro (Supplementary Fig. S6H). However, BKM120 (1 μmol/L) could slightly induce the nuclear localization of PTEN (Supplementary Fig. S6I and S6J). Hence, in the remainder of the experiments we chose a low concentration of BKM120 (1 μmol/L) in combination with etoposide (20 μmol/L) or IR (2 Gy) treatment.

To further pinpoint the methylation status of PTEN as well as the PTEN protein-phosphatase activity involvement in this process, we monitored the chemosensitivity or radiosensitivity of PTEN−/− HCT116 cells stably expressing WT, C124S, G129E, Y138L, K349R, or T398A, cells expressing the protein-phosphatase–dead mutant C124S or Y138L, or methylation-deficient mutant K349R or T398A, were more sensitive to combined treatment with BKM120 in combination with DNA-damaging agents including etoposide or IR in different cancer cell lines including HCT116, U87MG, and PC3 (Fig. 6E; Supplementary Fig. S6K–S6P).

More importantly, compared with PTEN-WT–expressing cells, combined treatment with BKM120 and etoposide caused a greater inhibition of xenograft tumor growth in tumors bearing PTEN−/− HCT116 cells stably expressing the methylation-deficient mutant K349R, accompanied with elevated levels of γH2AX and cleaved caspase-3 in tumors with the PTEN-K349R (Fig. 6F–H). In further support of a critical role of NSD2-mediated PTEN dimethylation in dictating cellular sensitivity to DSBS and DNA-damaging agents including etoposide or IR, in different cancer cell lines including HCT116, U87MG, and PC3 (Fig. 6E; Supplementary Fig. S6K–S6P).
growth in an in vitro cell culture system as well as in an in vivo xenograft tumor growth following combined treatment of BKM120 with etoposide, coupled with increased γH2AX and caspase-3 cleavage (Fig. 6L–K; Supplementary Fig. S6Q–S6S). We further observed that inhibition of NSD2 using the pan-methyltransferase inhibitor DZNep (29, 30) also led to a marked elevation in cellular sensitivity to treatment with BKM120 together with etoposide or IR in PTEN+/−, but not in PTEN−/−, HCT116 cells (Fig. 6L–N; Supplementary Fig. S6T–S6X). Taken together, these results coherently demonstrate that NSD2-mediated PTEN methylation plays a critical role in dictating cellular sensitivity to combination treatment with DSB-inducing agents and PI3K inhibitors (Fig. 6O; Supplementary Fig. S6Y).

**DISCUSSION**

Beyond the well-studied function of PTEN in the cytoplasm, increasing evidence demonstrated that PTEN also plays an important role in the extracellular environment through secretion (48, 49) and regulation of DDR in the nucleus (10, 11). Specifically, DNA DSBs induced the phosphorylation of PTEN at T/S398 by ATM kinase (11). However, the physiologic consequence of this event remains largely elusive. In this study, we found that phosphorylation of PTEN at T/S398 is required for binding with MDC1's BRCT domain, which also recognized γH2AX upon DNA-damage signaling (50). Furthermore, we found that DNA-damaging agents could induce complex formation between PTEN and the NSD2 methyltransferase, which is largely dependent on MDC1. Subsequently, our results showed that NSD2 promotes PTEN dimethylation at K349, which is read by the tudor domain of 53BP1 to recruit PTEN into DNA damage sites.

Although it has been reported that PTEN has lipid and protein dual phosphatase activity (34, 51), previous studies have been predominantly focused on its lipid phosphatase activity toward phosphatidylinositol-3,4,5-trisphosphate (PIP3) to antagonize the PI3K–AKT oncogenic signaling pathway. The protein- phosphatase activity of PTEN has not been well recognized. However, genetic evidence revealed that mice with Pten deficiency and transgenic expression of activated AKT displayed development of different tumor types (52, 53). These reports indicate that in addition to the lipid phosphatase activity PTEN may have other critical biological functions, such as protein phosphatase activity. In support of this notion, we found that the protein phosphatase activity of PTEN is critical for DNA DSB repair. Compared with PTEN+/− cells, the magnitude and duration of γH2AX persistence in PTEN−/− cells are dramatically elevated, especially at a late stage post γ-irradiation, which can be largely rescued by reintroducing WT or lipid-phosphatase-deficient mutant (G129E), but not protein-phosphatase-deficient mutants (C124S and Y138L). Consistent with these findings, compared with Pten+/− and PtenG129E+/− mice, the levels of γH2AX were upregulated in PtenC124S+/− mice at 24 hours post γ-irradiation. More importantly, we also showed that PTEN could interact with and dephosphorylate γH2AX after treatment with DNA-damaging agents, which is required for NSD2-mediated dimethylation of PTEN at K349. As previous reports demonstrated the phosphatases PP2A and PP4 directly dephosphorylate γH2AX to facilitate DDR (54, 55), it warrants further in-depth study to explore how these different protein phosphatases are involved in dissolving γH2AX foci to control the process of DSB repair. Previous studies showed that PTEN could be modified by other post-translational modifications including the monoubiquitination of K289 and SUMOylation of K254 and K266 to control PTEN nuclear import or retention (11, 46, 56). Moreover, Bassi and colleagues found that SUMOylation of PTEN at K254 is critical for its nuclear retention and that SUMOylated PTEN was rapidly excluded from the nucleus upon activation of DNA damage signaling (11). On the other hand, studies from other two groups showed that the SUMOylation of PTEN at K254 and K266 is required for PTEN’s plasma membrane localization to suppress the PI3K–AKT signaling pathway (56, 57). Our results showed that the monoubiquitination-deficient PTEN-K289R mutant, but not the SUMOylation-deficient PTEN-K266R could slightly decrease the NSD2-mediated dimethylation of PTEN and subsequently reduce PTEN interaction with the 53BP1 tudor domain (Figs. 2C and 3C). In addition, recent reports demonstrated that WWP1-mediated K27-linked ubiquitination and PRMT6-mediated arginine methylation of PTEN regulate PTEN lipid-phosphatase activity toward the PI3K–AKT oncogenic pathway and tumorigenesis (5, 58), whereas FGFR2-mediated tyrosine phosphorylation of PTEN controls DDR and radiation sensitivity in glioblastoma in part via governing the chromatin localization of PTEN (59). However, the cross-talk between our finding that PTEN lysine methylation and other post-translational modifications including SUMOylation and tyrosine phosphorylation under DNA-damaging conditions remains elusive.

Given that NSD2 is frequently overexpressed or mutated to enhance its catalytic activity in many types of human cancer (25, 60), and overexpression of NSD2 facilitates tumorigenesis in mouse models, emerging evidence suggests NSD2 as a potential therapeutic target in cancer (61, 62). Moreover, previous studies demonstrated that NSD2 enhances DNA DSB repair, Ig class switch recombination, and resistance to DNA-damaging agents largely through NSD2 methyltransferase activity and its ability to recruit DNA-damaging factors to DNA-damage sites (23, 24, 32, 63), which indicates that pharmacologic suppression of NSD2 might synergize with DNA-damaging agents for cancer therapy. Our results also suggest that the development of NSD2-specific inhibitors, which if paired in combination with PI3K inhibitors, could efficiently sensitize cancer cells with WT PTEN genetic status to chemotherapeutics or radiotherapeutics (Supplementary Fig. S6Y). However, although two NSD2-specific inhibitors, LEM-06 and LEM-14, were developed for suppressing NSD2 activity in vitro (64, 65), we did not confirm the biological effects of either inhibitor in cell culture system as evidenced by there being no obvious changes in H3K36me2 upon treatment with either LEM-06 or LEM-14 in U2OS or HCT116 cells, which warrants further study to develop and optimize NSD2-selective inhibitors for cancer therapy.
Taken together, our study uncovers a critical role of NSD2-mediated PTEN methylation in regulating DNA DSB repair, which dictates cellular sensitivity to DNA-damaging agents including both chemotherapeutics and radiotherapeutics, partly by governing the DSB repair process via dephosphorylating γH2AX. DNA-damaging agents induce MDC1, NSD2, and PTEN, forming a ternary complex, and promote NSD2-mediated methylation of PTEN, which can be recruited to DNA damage sites by the 53BP1 tudor domain and help DNA DSB repair largely through dephosphorylating γH2AX (Fig. 6O, left). However, inhibiting NSD2 methyltransferase activity using shRNA or an inhibitor can decrease PTEN methylation at K349 and subsequently retard 53BP1-mediated recruitment to DNA damage sites, which causes DNA DSB repair deficiency and sensitizes cancer cells to DNA-damaging agents (Fig. 6O, right). This work therefore sheds new light into the precise molecular mechanism by which nuclear PTEN controls the DNA damage pathway in a methylation- and protein-phosphatase–dependent manner.

**METHODS**

**Cell Culture, Transfections, and Viral Infections**

HEK293T, HEK293, U2OS, U87MG, MEFs, and NIH3T3 were cultured in DMEM supplemented with 10% FBS (Gibco), 100 U/mL of penicillin, and 100 μg/mL streptomycin (Gibco), which were obtained from William G. Kaelin, Jr. (Dana-Farber Cancer Institute, Boston, MA), in June 2006. HCT116, HCT116 PTEN−/−, and HCT116 PTEN−/− cells were obtained from Todd Waldman (School of Medicine, Georgetown University, Washington, DC) in June 2014. MEF Mdc1+/− and Mec1−/− cells were obtained from Anyong Xie (The Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou, China) in July 2015. MEFs Nsd2+/+ and Nsd2−/− (also named Whsc1+/− and Whsc1−/−) cells were obtained from Keiko Ozato (National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD) in July 2016. DU145 cells, PC3 cells, and PTEN+/+, PTEN+/−, PTEN−/+, and PTEN−/− MEFs were obtained from the lab of P.P. Pandolfi in 2018. PTEN+/+ and PTEN+/− MEFs were obtained from the lab of Nicholas R. Leslie in October 2017. H2AX−/− cells were obtained from the lab of Yang Shi in November 2018. DR-GFP-U87MG cells were a gift from the lab of Vuk Stambolic at University of Toronto (Toronto, Ontario, Canada) in January 2015. Cell lines were obtained between 2006 and 2018, and were routinely tested negative for Mycoplasma. However, cell line authentication was not routinely performed. Cell line authentication was performed using Lipofectamine and plus reagents.

**Plasmids and NSD2 shRNAs**

Mouse and human HA-PTEN were generated by inserting the corresponding cDNAs into pCDNA3-HA vector. FLAG-NSD2, FLAG-EZH2, and FLAG-SET8 were cloned by constructing corresponding cDNAs into puromycin CMV vector. pCMV-GST-MDC1-BRCT, pCMV-GST-53BP1-BRCT, pCMV-GST-BRCA1-BRCT, and pCMV-GST-53BP1 Tudor domain were cloned into mammalian expression GST-fusion vectors. GST-PTEN was constructed by inserting the cDNA into pGEX-4T-1 vector. pBabe-Super-PTEN was constructed by subcloning the PTEN cDNA into pBabe-Super-HA-hygro vector. Various PTEN mutants were generated using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions. All mutants were generated using mutagenesis PCR and the sequences were verified by DNA sequencing. pcDNAs-FRT/T0-FLAG-53BP1 (52507), 3xFLAG-pcDNA5-FRT/T0-53BP1 28A (52505) and pSpCas9(2B)-T2A-Puro_h53BP1_gRNA_D were purchased from Addgene. FLAG-MDC1 WT and Δ1–143 mutants were gifts from the lab of Xingzhi Xu at the Shenzhen University (Guangdong Sheng, China). pLenti5x3-7-shRNA vectors to deplete endogenous NSD2 were kind gifts from Zhenkun Luo in the Department of Oncology at Mayo Clinic. The sequences for NSD2 shRNA are listed below: NSD2 shRNA 1: 5′-GACGCTTACACACAAGTTT-3′; NSD2 shRNA 2: 5′-GACGACGGTCTCCGGAGAAGAGACACAATCAG-3′; sgPTEN-F: CACCGGATATTTATTTACTG; sgPTEN-R: AACGATGAATATAATGGCC.

**Antibodies and Reagents**

All antibodies were used at a 1:1,000 dilution in TBST buffer with 5% nonfat milk for Western blotting. Anti-phospho-ATM/ATR Substrate (S5′Q) (9607), anti-phospho-ATM/ATR Substrate (6966), anti-pS15/pS3 (9284), anti-pT68-CHK2 (2661), anti-CHK2 antibody (3440), anti-Db-Methyl Lysine Motif (14117), anti-Tri-Methyl Lysine Motif (14680), anti-His tag (2366), anti-γH2AX (9718), anti-pS473-AKT (4060), anti-AKT1 antibody (2938), anti-PTEN (9188), anti-cleaved caspase-3 (9661), anti-MEK1/2 (8727), anti-pS25/29-53BP1 (2674), and anti-γH2AX (7631) antibodies were purchased from Cell Signaling Technology. Anti-PTEN (sc-7974), anti-MDC1 (sc-27737), anti-pS5 (sc-6243/126), anti-ATM (sc-23921), anti-ATR (sc-1887), anti-pCREB (sc-81486), anti-Lamin A/C (sc-7293), anti-GAPDH (sc-47724), anti-BRCA1 (sc-6954), anti-53BP1 (sc-22760), and anti-Rad51 (sc-398587) antibodies were purchased from Santa Cruz Biotechnology. Anti-FLAG (F-8034), anti-γH2AX (9718), anti-H2AX (9718), anti-53BP1 (sc-22760), anti-MDC1 (sc-2869), anti-ME20 (sc-398587), and anti-Rad51 (sc-398587) antibodies were purchased from Cell Signaling Technology. Anti-Flag (M2), anti-β-Actin (A-5316), anti-53BP1 (A-5316), and anti-β-Actin (A-5316) antibodies were purchased from Sigma. Anti-PTEN (C-18) was purchased from Santa Cruz Biotechnology. Anti-PTEN (C-18) was purchased from Santa Cruz Biotechnology. Anti-PTEN (C-18) was purchased from Santa Cruz Biotechnology.
antibodies. For immunoprecipitation analysis, 1,000 µg lysates were incubated with the indicated antibody-conjugated beads for 4 hours at 4°C. The recovered immunocomplexes were washed four times with NETN buffer (20 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, 1 mmol/L EDTA, and 0.5% NP-40) before being resolved by SDS-PAGE and immunoblotted with indicated antibodies. For the endogenous PTEN methylation assays, EBC and NETN buffer containing 300 mmol/L NaCl were used to disrupt the nonspecifically interacting proteins.

**In Vitro Dephosphorylation Assays**

For measuring the dephosphorylation of γH2AX by PTEN, the GST-fusion PTEN WT, C124S, G129E, and Y138L, as well as GST protein were purified from E.coli BL21 as described previously (34). γH2AX (catalog no. 12-0040), H2AX (catalog no. 12-0039) peptides from EpitCypher or full-length γH2AX purified from HCT116 cells after etoposide treatment as well as the positive control ps133-CREB from HCT116 cells obtained using the anti-γH2AX or anti-ps133-CREB antibody served as substrates. The dephosphorylation assays were performed in phosphatase assay buffer (20 mmol/L HEPES, pH 7.2, 100 mmol/L NaCl, and 3 mmol/L DTT). The reactions were incubated at 37°C for 30 minutes with or without the addition of recombinant GST-fusion PTEN WT, C124S, G129E, and Y138L, as well as GST protein as negative control, and were stopped by adding 3× SDS loading buffer for Western blot analysis.

**Peptide-Binding Assays**

PTEN peptides with/without methylation modifications were synthesized at Tufts Medical School (Boston, MA). Each contained an N-terminal biotin and free C-terminus and was synthesized at 0.1 mmol/L scale. Peptides were diluted to 1 mg/mL for further biochemical assays. The sequences are listed below:

- PTEN WT: Biotin-NFKVKLYFTKTVEEPSNPE
- PTEN K349 mono-methylation (K-me1): Biotin-NFKVKLYFTK(me1)TVEEPSNPE
- PTEN K349 Di-methylation (K-me2): Biotin-NFKVKLYFTK(me2)TVEEPSNPE
- PTEN K349 Tri-methylation (K-me3): Biotin-NFKVKLYFTK(me3)TVEEPSNPE

Peptides (2 µg) were incubated with 1 µg of whole-cell lysates in a total volume of 500 µL EBC buffer. After incubation for 4 hours at 4°C, 10 µL Streptavidin Agarose (Thermo Fisher Scientific 20353) was added in the sample for another 1 hour. The agaroase was washed four times with NETN buffer. Bound proteins were added to 2× SDS loading buffer and resolved by SDS-PAGE for immunoblot analysis.

**IHC Assays**

Spleen tissues from 4-month-old mice (Pten+/+, PtenG1209R/+, and PtenG1209R/−) were dissected and fixed in 4% paraformaldehyde for IHC analysis. For staining, tissues were fixed in 4% paraformaldehyde overnight, paraffin embedded, and then sectioned at 5 µm. After deparaffinization and rehydration, antigen retrieval was performed in a pressure cooker with sodium citrate buffer at 95°C for 25 minutes. Sections were incubated in a 0.3% H2O2 solution in 1× PBS, and then a 10% serum solution in 1× PBS for 30 minutes each solution was used to block endogenous peroxidase and background from the secondary antibody, respectively. The sections were stained with γH2AX antibody (Cell Signaling Technology #9718, 1:500) in 1× PBS at 4°C overnight, and incubated in a biotinylated anti-rabbit secondary antibody in 1× PBS (1:1,000) at room temperature for 30 minutes. The Vectastain ABC Elite Kit was used to enhance specific staining, and the staining was visualized using a 3′-diaminobenzidine substrate. Stained sections were counterstained using hematoxylin and dehydrated before they were sealed with a coverslip with Richard-Allan Scientific Cytoseal XYL Mounting Medium. Stained slides were visualized with a brightfield microscope.

**γ-Irradiation Treatments**

Cells at 60% confluence were treated with irradiation (5 Gy). After IR treatment, cells were left to recover in the incubator for the indicated times. Total-body irradiation (3 Gy) of 4-month-old mice (Pten−/−, PtenG1209R/−, and PtenG1245R/−) was performed, after which the mice were caged separately before being sacrificed at 24 hours after treatment. Moreover, these mice were carefully monitored during the recovery period.

**Comet Assays**

The Comet assay was performed by following the manufacturer’s instructions (Comet SCGE Assay Kit ADI-900-166). Briefly, HCT116-PTEN-WT and K49R cells were treated with DMSO or etoposide for the indicated time points, respectively. Cells were then gently trypsinized and pelleted by centrifuge. Cells were resuspended at 1×10⁶ cells/mL in cold PBS, followed by combining cells with molten LMAgarose at a ratio of 1:10 (w/v) and immediately pipetting 75 µL onto a Comet slide. The slide was placed flat at 4°C in the dark for 10 minutes, immersed in prechilled lysis solution, and left on ice for 30 minutes. Excess buffer was tapped off from the slide. The slide was immersed in freshly prepared alkaline solution. The Comet slide was left in alkaline solution for 30 minutes at room temperature and then transferred to a horizontal electrophoresis apparatus to perform TBE electrophoresis. Samples were dried and placed in 100 µL of diluted stain onto each circle of dried agarose and stained for 30 minutes in the dark. Images were recorded by epifluorescence microscopy and data were analyzed by CometScore.

**Cellular Fractionation**

U2OS cells, NIH3T3 cells, or MEFs cultured in 6-cm dishes with 70% to 80% confluence were harvested. Cell pellets were resuspended in 8 volumes of the Buffer A (hypotonic: 10 mmol/L HEPES, pH 7.9, 0.2 mmol/L EDTA, 2 mmol/L NaCl, 1 mmol/L DTT). The 1× cell pellets were resuspended in 300 mmol/L NaCl were used to disrupt the nonspecifically interacting proteins. The Comet assay was performed by following the manufacturer’s instructions (Comet SCGE Assay Kit ADI-900-166). Briefly, HCT116-PTEN-WT and K49R cells were treated with DMSO or etoposide for the indicated time points, respectively. Cells were then gently trypsinized and pelleted by centrifuge. Cells were resuspended at 1×10⁶ cells/mL in cold PBS, followed by combining cells with molten LMAgarose at a ratio of 1:10 (w/v) and immediately pipetting 75 µL onto a Comet slide. The slide was placed flat at 4°C in the dark for 10 minutes, immersed in prechilled lysis solution, and left on ice for 30 minutes. Excess buffer was tapped off from the slide. The slide was immersed in freshly prepared alkaline solution. The Comet slide was left in alkaline solution for 30 minutes at room temperature and then transferred to a horizontal electrophoresis apparatus to perform TBE electrophoresis. Samples were dried and placed in 100 µL of diluted stain onto each circle of dried agarose and stained for 30 minutes in the dark. Images were recorded by epifluorescence microscopy and data were analyzed by CometScore.

**Cell Viability and Apoptosis Assays**

For cell viability assays, 2,000 cells per well were plated in 96-well plates and incubated with complete DMEM containing different treatments as indicated. Assays were performed with the Cell Titer-Glo Luminescent Cell Viability Assay Kit according to the manufacturer’s instructions (Promega). For detection of apoptosis, cells treated with indicated treatment were costained with Annexin-V-PE and 7-AAD (Annexin V-PE Apoptosis Detection Kit I, BD Biosciences) and analyzed using BD FACSCanto Flow cytometer according to the manufacturer’s instructions.

**Murine Models**

The Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee (IACUC) on Animal Research approved all animal experiments. The transgenic mice used in this study (Pten−/−, PtenG1209R/−, and PtenG1245R/−) were reported previously (46). Four mice per genotype were randomly chosen and analyzed at the indicated age.
PTEN Methylation Governs DNA Damage Response

Xenograft Tumor Growth

For the xenograft model, 6-week-old female nude mice housed in specific pathogen-free environments were injected subcutaneous-ly with 1.0 × 10^6 HCT116 derivatives mixed with DMEM and Matrigel (vol/vol, 1:1). Treatments were initiated when the mean tumor volume in each randomized group reached around 100 mm^3. NVP-BKM120 (MedChemExpress) was formulated in 0.5% methylcellulose/0.5% Tween 80 at 6 mg/mL. Etoposide (Selleckchem) was formulated in saline at 10 mg/mL. For BKM120, treatments were carried out orally, every other day, using an application volume of 25 mg/kg. For etoposide, treatments were carried out by intraperitoneal injection with 20 mg/kg every other day. DZNep treatments were carried out by intraperitoneal injection with 1 mg/kg body weight twice a week for 2 weeks. Animals were sacrificed when the tumor size in the vehicle control group reached 500 to 600 mm^3. Tumor volumes were determined using calipers. All experimental procedures strictly complied with the IACUC guidelines.

Statistical Analysis

Group data are presented as mean ± SD. The statistical signifi-
cance between experimental groups was determined by Student t test. P < 0.05 was considered to be significant. Analyses were performed using Microsoft Excel 2011 and GraphPad Prism V5.

Disclosure of Potential Conflicts of Interest

Y. Shi has ownership interest (including stock, patents, etc.) in Constellation Pharmaceuticals, Inc., Imago Biosciences, Inc., and Guangzhou BeBetter Medicine Technology Co., LTC, and is a con-
sultant/advisory board member for Active Motif, Inc. and Bioduro. P.P. Pandolfi has ownership interest (including stock, patents, etc.) in Rekindle Pharmaceuticals and is a consultant/advisory board mem-
ber for the same. W. Wei has ownership interest (including stock, patents, etc.) in Rekindle Therapeutics and is a consultant/advisory board member for the same. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: J. Zhang, Y.-R. Lee, W. Wei

Development of methodology: J. Zhang, Y.-R. Lee, W. Gan, C.-H. Hsu, J.M. Asara

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Zhang, Y.-R. Lee, W. Gan, C.-H. Hsu, J.M. Asara

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Zhang, Y.-R. Lee, W. Wei

Writing, review, and/or revision of the manuscript: J. Zhang, Y.-R. Lee, W. Wei

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y.-R. Lee, W. Gan, C.-H. Hsu, W. Wei

Study supervision: Y. Shi, P.P. Pandolfi, W. Wei

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PTEN Methylation by NSD2 Controls Cellular Sensitivity to DNA Damage

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