Plk1 Protein Phosphorylates Phosphatase and Tensin Homolog (PTEN) and Regulates Its Mitotic Activity during the Cell Cycle*

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PTEN is a well known tumor suppressor through the negative regulation of the PI3K signaling pathway. Here we report that PTEN plays an important role in regulating mitotic timing, which is associated with increased PTEN phosphorylation in the C-terminal tail and its localization to chromatin. Pulldown analysis revealed that Plk1 physically interacted with PTEN. Biochemical studies showed that Plk1 phosphorylates PTEN in vitro in a concentration-dependent manner and that the phosphorylation was inhibited by Bi2635, a Plk1-specific inhibitor. Deletional and mutational analyses identified that Plk1 phosphorylated Ser-380, Thr-382, and Thr-383, but not Ser-385, a cluster of residues known to affect the PTEN stability. Interestingly, a combination of molecular and genetic analyses revealed that only Ser-380 was significantly phosphorylated in vivo and that Plk1 regulated the phosphorylation, which was associated with the accumulation of PTEN on chromatin. Moreover, expression of phospho-deficient mutant, but not wild-type PTEN, caused enhanced mitotic exit. Taken together, our studies identify Plk1 as an important regulator of PTEN during the cell cycle.

Phosphatase and tensin homolog (PTEN) functions as a tumor suppressor because it is frequently mutated in a variety of cancer cells, and inherited PTEN mutations cause cancer-susceptibility conditions including Cowden syndrome (1–4). Biochemically, PTEN dephosphorylates the lipid second-messenger phosphatidylinositol 3,4,5-trisphosphate to generate phosphatidylinositol 3,4-bisphosphate and, by doing so, antagonizes the PI3K/Akt signaling pathway. The PTEN level, as well as its activity, profoundly influences cell growth, survival, and tumor susceptibility (5, 6). Extensive research in the past has revealed that the PTEN tumor suppressor is a central negative regulator of the PI3K/PDK1/Akt signaling axis that controls multiple cellular functions including cell growth, survival, proliferation, and angiogenesis (5).

PTEN is subjected to modifications by several types of the post-translational mechanisms including phosphorylation, oxidation, ubiquitination, and acetylation (7). PTEN is extensively phosphorylated at the C-tail region by several protein kinases. Phosphorylation of several serine/threonine residues (e.g. Ser-370, Thr-382, Thr-383, and Ser-385) in the C-tail region of PTEN by casein kinase 2 (CK2) and Plk3 is essential for the tail-dependent regulation of stability as phospho-defective mutant proteins exhibit decreased stability compared with the wild-type PTEN (8–10). Glucose synthase kinase 3β phosphorylates PTEN at Ser-362 and Thr-366 (8). PTEN is also phosphorylated on tyrosine residues by Rak, and this phosphorylation stabilizes PTEN as well (11).

Plk1 is an important member of the POLO kinase family (12–14). Extensive investigations in the past have uncovered a variety of cellular processes and molecular pathways that are regulated by Plk1, including DNA-damage response and repair (15–19), tRNAs and ribosome RNA transcription (20), mitotic onset and progression (21–25), microtubule dynamics (26), and centrosome duplication and maturation (27–30). Although Plk1 expression is associated with the development of a wide spectrum of malignancies evidence also exists suggesting that Plk1 may possess additional functions that are not related to its growth promotion. For example, mutations in PLK1 have been detected in several types of tumor cells (31), and haploinsufficiency of PLK1 results in enhanced tumorigenesis (32). Moreover, Plk3 and Plk4, both members of the POLO kinase family bearing a close structural similarity to Plk1, function to suppress cell and tumor growth (33, 34). Therefore, to gain a full...
appreciation of Plk1 function in the regulation of cell division, it is necessary to further identify and characterize new molecular components that are targeted by Plk1.

Given that PTEN also has a nuclear function and is involved in regulating chromosomal stability (35, 36), we investigated whether Plk1 might directly target PTEN by phosphorylation. Our extensive biochemical and molecular analyses revealed that Plk1 phosphorylates PTEN in vitro and regulates its phosphorylation in vivo. We also obtained evidence that Plk1-mediated phosphorylation was important for PTEN binding to chromatin, as well as for normal mitotic progression.

MATERIALS AND METHODS

Cell Culture and Transfection—HeLa and HEK-293T cell lines obtained from the American Type Culture Collection were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Invitrogen) and antibiotics (100 μg/ml penicillin and 50 μg/ml streptomycin sulfate; Invitrogen) at 37 °C under 5% CO₂. Murine embryonic fibroblasts (MEFs) were obtained from embryos through crossing wild-type and PLK1⁻/⁻ mice (a gift from Dr. Xiaochun Lu, Michigan University). Transfection of HeLa cells was achieved with either FuGENE HD (Roche Diagnostics) or Lipofectamine 2000 (Invitrogen) following the manufacturers’ protocol. Transfection efficiency was estimated to be between 80 and 100% in all cases through transfecting a GFP-expressing plasmid.

Antibodies and Reagents—Antibodies to PTEN, phospho-PTEN Ser-380/Thr-382/Thr-383 (p-PTEN S/T/T), p-PTEN Ser-380, cyclin E, cyclin B1, α-tubulin, PARP, HA, and phospho-histone H3 were purchased from Cell Signaling Technology. Antibody to GFP was purchased from Santa Cruz Biotechnology. Anti-β-actin antibody was purchased from Sigma-Aldrich. Plk1 antibody was obtained from Millipore. Antibodies to GST and GFP were purchased from Sigma-Aldrich. Plk1 inhibitor (Bi2536) was purchased from Selleck Chemicals.

RNA Interference—Human ON-TARGETplus SMARTpool siRNA oligonucleotides that specifically target PTEN (L-003023-00-0010) and PLK1 (L-003290-00-0010) were purchased from Dharmacon. Each set of siRNA oligonucleotides contains four sequences as follows: PTEN (5’-GAUCAGCAUA-CACAAAUUA-3’, 5’-GACUUAAGACUUGACUAAU-3’, 5’-GCAUGUGACUUGCCUA-3’, and 5’-CGAUGUAGUGGU-UGCAUAUA-3’). Individual deletion fragments of GST-PTEN were purchased from Epitomics. Plk1 antibody was obtained from Millipore. Antibodies to GST and GFP were purchased from Santa Cruz Biotechnology. CK2 inhibitor (4,5,6,7-tetramethylbenzimidazole abbreviated as TBZ) was purchased from Sigma-Aldrich. Plk1 inhibitor (Bi2536) was purchased from Selleck Chemicals.

Preparation of Protein Extracts and Immunoblotting—Total cell lysates were prepared in a buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% IGEPAL, 0.1% SDS, and 0.5% sodium deoxycholate) supplemented with a mixture of protease and phosphatase inhibitors. Chromatin and cytosolic/soluble extracts were obtained as described previously (37). In brief, cell extracts were prepared in the harvest buffer (10 mM HEPES (pH 8.0), 50 mM NaCl, 0.5 mM sucrose, 0.1 mM EDTA, 0.5% Triton X-100) containing both protease inhibitors (1 mM dithiothreitol (DTT), 2 mg/ml pepstatin, 4 mg/ml aprotinin, 100 mM PMSF) and phosphatase inhibitors (10 mM tetrasodium pyrophosphate, 100 mM NaF, 17.5 mM β-glycerophosphate). The low speed supernatant (500 × g) containing cytoplasmic proteins was collected, and nuclear extracts were made by vortexing the nuclei for 15 min at 4 °C in a buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% IGEPAL CA-630, and protease inhibitors. Protein concentrations were measured using the bicinchoninic acid protein protein assay reagent kit (Pierce Chemical Co). Equal amounts of protein lysates from various samples were used for SDS-PAGE analysis followed by immunoblotting. Specific signals on immunoblots (polyvinylidene difluoride) were visualized using enhanced chemiluminescence (Super-Signal; Pierce Chemical Co.).

GST Pulldown Assay—To investigate the interaction between PTEN and Plk1, HeLa cells treated with nocodazole for 4 h were lysed in TBSN buffer (20 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.5% Nonidet P-40, 5 mM EGTA, 1.5 mM EDTA, 0.5 mM Na₃VO₄, and 20 mM β-glycerol phosphate). The cell lysates were clarified by centrifugation at 15,000 × g for 20 min at 4 °C.
Cleared lysates (1 mg) were added to either bead-bound GST (5 μg) or bead-bound GST-PTEN fusion protein (5 μg) followed by incubation in the TBSN buffer for 3 h at 4 °C. After incubation, proteins bound to each resin were washed extensively with the binding buffer, eluted in the SDS-PAGE sample buffer, and analyzed by SDS-PAGE followed by immunoblotting with anti-GST or Plk1 antibody.

Expression and Purification of Fusion Proteins—Overnight bacterial cultures (5 ml) of individual clones expressing GST-tagged PTEN protein or its mutants were inoculated into 100 ml of LB medium containing ampicillin (100 μg/ml) followed by incubation at 37 °C for 4 h in a shaking incubator. Protein expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (0.1 mM). After incubation at room temperature for 2 h, cells were harvested by centrifugation and resuspended in 2 ml of sonication buffer (50 mM Tris (pH 7.5) 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 200 mM His6-Plk1 protein (Sigma) was added to the kinase assay buffer partially using the protocol described (10). Briefly, recombinant protein was suspended in 50 mM Tris (pH 7.4), 2 mM EDTA, 10 mM MgCl2, 5 mM MnCl2, 1 mM DTT and 1 mM DTT) and then used as substrate for the kinase assay containing Plk1. After the reaction, the reaction mixtures were subjected to the immunoblotting analysis with antibodies to p-PTEN (Thr-366/Ser-370), p-PTEN(Ser-380/Thr-382/Thr-383), or total PTEN.

Genotyping of PLK1 Knockout Mice—MEFs were isolated from 13.5-day-old embryos. Genomic DNA was isolated from mouse tails mice and embryos, as well as from MEF cells. Genotyping was performed by polymerase chain reaction (PCR) using three primers including the sense primer for both wild-type (WT) and knock-out (KO) alleles (5′-TATTTCCGCAAT-TACATGAGTGAGC-3′), the antisense primer for WT allele (5′-TAGCAGATGAAAGGGCACCTGTC-3′), and the antisense primer for KO allele (5′-CTCTACATAGTG-GCAGTGTGG-3′). PCR products were analyzed on agarose gels.

RESULTS

PTEN Has a Role in Mitosis—Cyttoplasmic PTEN has a well-known function as a negative regulator of the PI3K/Akt signaling axis (5). Recent studies have shown that PTEN is also implicated in regulating APC/C activity (36) and maintaining chromosomal stability (4, 36). We first asked whether PTEN had a clear mitotic role. HeLa cells transfected with PTEN siRNAs, or control siRNAs, were arrested in mitosis after nocodazole treatment. Mitotic cells were then released into the cell cycle. Down-regulation of PTEN significantly accelerated the degradation of cyclin B, as well as the disappearance of phosphorylated histone H3 (p-H3) during mitotic release (Fig. 1A). The half-life of cyclin B was about 1.5 h in cells with PTEN down-regulation whereas it was close to 3 h in control cells (Fig. 1B). These observations strongly suggest that PTEN has an important function in regulating mitotic progression.

Plk1 Phosphorylates PTEN in Vitro—Given that PTEN is heavily phosphorylated at the C terminus (8, 38), we attempted to identify a mitotic kinase(s) that might play a role in the regulation of PTEN phosphorylation and its function during mitosis. When HeLa cells synchronized at the G1/S junction were released into the cell cycle, we observed that total PTEN and phosphorylated PTEN (epitope Ser-380/Thr-382/Thr-383, or total PTEN) were subjected to the kinase assay containing Plk1. His-tagged PTEN recombinant protein was first dephosphorylated using λ-phosphatase (New England Biolabs) according to the protocol provided by the supplier. Five hundred μl of immunoprecipitation buffer (20 mM HEPES (pH 7.4), 120 mM NaCl, 1% Triton X-100, 2 mM EGTA, 10% glycerol, 1 mM DTT, 500 μM PMSF, 2 μM pepstatin A, 1 μg/ml aprotonin) and 40 μl of nickel-nitrilotriacetic acid resin (50:50) was then added to the reaction. The mixture was incubated with rotation at 4 °C for 3 h. After incubation, the resin was washed three times with the immunoprecipitation buffer, three times with the kinase buffer (20 mM HEPES (pH 7.2), 2 mM EGTA, 10 mM MgCl2, 5 mM MnCl2, 1 mM DTT, 1 mM Na3VO4), and then resuspended in the kinase buffer as a 50:50 (v/v) slurry. The suspension was then used as substrate for the kinase assay containing Plk1. After the reaction, the reaction mixtures were subjected to the immunoblotting analysis with antibodies to p-PTEN(Thr-366/Ser-370), p-PTEN(Ser-380/Thr-382/Thr-383), or total PTEN.

Expression and Purification of Fusion Proteins—Overnight bacterial cultures (5 ml) of individual clones expressing GST-tagged PTEN protein or its mutants were inoculated into 100 ml of LB medium containing ampicillin (100 μg/ml) followed by incubation at 37 °C for 4 h in a shaking incubator. Protein expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (0.1 mM). After incubation at room temperature for 2 h, cells were harvested by centrifugation and resuspended in 2 ml of sonication buffer (50 mM Tris (pH 7.5) 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT) and three times with wash buffer B (50 mM Tris (pH 7.5), 100 mM NaCl, and 1 mM DTT). Glutathione beads were resuspended in 50 μl of a buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM DTT and 10 mM reduced glutathione) for 30 min at room temperature to elute GST fusion proteins. The quantity of the bound GST fusion proteins was assessed by SDS-PAGE and Coomassie Blue staining.

Kinase Assays—Plk1 kinase assays were carried out essentially using the protocol described (10). Briefly, recombinant His6-Plk1 protein (Sigma) was added to the kinase assay buffer (20 mM HEPES (pH 7.4), 2 mM EGTA, 10 mM MgCl2, 5 mM MnCl2, 1 mM DTT). Substrates (PTEN or casein) were then added to each reaction mixture to achieve a total volume of 20 μl. Kinase reactions were initiated by the addition of 5 μl of either the radioactive ATP mix (50 mM MgCl2, 0.1 mM ATP, 2 μCi of [γ-32P]ATP) or the cold ATP mix (50 mM MgCl2, 5 mM ATP). After incubation for 30 min at 30 °C, SDS sample buffer was supplemented to each reaction. Kinase assay samples were fractionated on SDS-polyacrylamide gels, and the fractionated proteins were transferred to PVDF membranes followed by autoradiography or immunoblotting. To detect phosphorylation of PTEN (p-PTEN(T/S or p-PTEN(N/T/T) by Plk1, His-tagged PTEN recombinant protein was first dephosphorylated using λ-phosphatase (New England Biolabs) according to the protocol provided by the supplier. Five hundred μl of immunoprecipitation buffer (20 mM HEPES (pH 7.4), 120 mM NaCl, 1% Triton X-100, 2 mM EGTA, 10% glycerol, 1 mM DTT, 500 μM PMSF, 2 μM pepstatin A, 1 μg/ml aprotonin) and 40 μl of nickel-nitrilotriacetic acid resin (50:50) was then added to the reaction. The mixture was incubated with rotation at 4 °C for 3 h. After incubation, the resin was washed three times with the immunoprecipitation buffer, three times with the kinase buffer (20 mM HEPES (pH 7.2), 2 mM EGTA, 10 mM MgCl2, 5 mM MnCl2, 1 mM DTT, 1 mM Na3VO4), and then resuspended in the kinase buffer as a 50:50 (v/v) slurry. The suspension was
of separate protein kinases in regulating PTEN. Blotting with antibodies to PARP (primarily chromatin) and α-tubulin (primarily soluble) indicated that the cellular fractionation process was efficient.

Given that Plk1 is a major mitotic kinase whose increase correlated with PTEN accumulation in the chromatin fraction (Fig. 2B), we first determined the physical interaction between PTEN and Plk1. Pulldown analysis revealed that GST-PTEN, but not GST alone, was capable of binding to Plk1 from HeLa cell lysates (Fig. 3A). We next determined whether PTEN was a direct substrate of Plk1. In vitro protein kinase assays showed that Plk1 catalyzed a concentration-dependent incorporation of $^{32}$P into recombinant PTEN and α-casein (Fig. 3B), the latter being a well-known substrate for kinases of the POLO family. Inclusion of Plk1 in the kinase assay greatly increased p-PTEN/T/T signals (Fig. 3C), suggesting that Plk1 phosphorylates one or more residue(s) within the cluster. Moreover, incorporation of $^{32}$P into PTEN catalyzed by Plk1 was inhibited by Bi2536, a Plk1-specific inhibitor (39), and the inhibition was concentration-dependent (Fig. 3D). However, the incorporation of $^{32}$P into PTEN catalyzed by Plk3 was largely unaffected by Bi2536. Immunoblotting showed that Plk1 primarily phosphorylated the epitope of S/T/T and that its phosphorylation was inhibited by Bi2536 (Fig. 3E). Consistent with an earlier study (10), Plk3 primarily phosphorylated the T/S epitope, and its phosphorylation was not inhibited by Bi2536 (Fig. 3E).

**Plk1 Phosphorylates PTEN on Ser-380**—To systematically identify the site(s) of PTEN phosphorylation by Plk1, we made a series of PTEN deletion constructs (constructs A–C), as well as full-length PTEN constructs harboring individual clusters of mutations within the C-terminal tail (constructs C1–C5) (Fig. 4A). The individual deletion or mutation constructs of PTEN were expressed and purified as GST fusion proteins. Protein kinase assays revealed that Plk1 phosphorylated deletion constructs of D and E, as well as the full-length PTEN (Fig. 4B, Kinase Assay panel), indicating that the phosphorylation site resides in the C-tail of PTEN. Blotting the same gels with the anti-GST antibody revealed that similar amounts of recombinant GST-PTEN and its deletion mutant proteins were used for the assay (Fig. 4B, Anti-GST panel). Additional kinase assays showed that Plk1 significantly phosphorylated full-length GST-PTEN and various full-length mutant proteins except for C4 which harbored mutations of four residues (Fig. 4C, Kinase Assay panel). Given that equal amounts of recombinant GST-PTEN or its mutant proteins were used for the assay (Fig. 4C), this result strongly suggests that Plk1 directly phosphorylates...
Plk1 Regulates PTEN

**FIGURE 3. Plk1 specifically phosphorylates PTEN in vitro.** A, purified GST or GST-PTEN immobilized onto glutathione resin was incubated with HeLa cell lysates. Proteins specifically bound to the resin were eluted and blotted for GST. B, purified recombinant PTEN was incubated with various concentrations of Plk1 in the kinase reaction buffer containing \(\frac{[\gamma-32P]}{ATP}\). After the reaction, kinase assay samples were fractionated by SDS-PAGE followed by autoradiography. C, dephosphorylated recombinant PTEN obtained via pretreatment with a phosphatase was incubated with (or without) various concentrations of Plk1 in the kinase reaction containing cold ATP. After the reaction, the assay samples were fractionated by SDS-PAGE and blotted for PTEN phosphorylation, as well as for ectopically expressed total PTEN. Mutation of Ser-380 alone greatly reduced the signals detected by the phospho-specific antibody against the p-PTEN<sup>Ser-380</sup> epitope or against the S/T/T epitope, suggesting that Thr-382 and Thr-383 may not be major sites of phosphorylation. Intriguingly, mutation of either Thr-382 or Thr-383 did not significantly reduce the amount of signals detected by the phospho-antibody against the S/T/T epitope (Fig. 5B), indicating that Ser-380 is a major site of phosphorylation in vivo. Thr-382 or Thr-383 mutation also had a weak affect on reducing p-PTEN<sup>Ser-380</sup> on chromatin compared with WT control. Mutation of either Thr-366 or Ser-370 diminished the phospho-signals (the T/S epitope) compared with ectopically expressed wild-type PTEN; intriguingly, mutation of either residue, but not any other residues, essentially abolished p-PTEN<sup>T/T</sup> signals in the nuclear fraction (Fig. 5B), suggesting that their phosphorylation may regulate nuclear entry or retention of PTEN.

**FIGURE 4. Plk1 phosphorylates PTEN in the C-tail region.** A, schematic presentations show various deletion and mutation constructs of PTEN. B, full-length PTEN and various deletion counterparts were expressed and purified as GST fusion proteins. Purified proteins, along with purified GST, were incubated with Plk1 in the assay reaction containing \(\frac{[\gamma-32P]}{ATP}\). After the reaction, the assay samples were fractionated by SDS-PAGE followed by autoradiography (lower panel). The same blot was also probed with the antibody to GST (upper panel). NS denotes a nonspecific band. C, full-length PTEN and various mutational counterparts in the C-tail domain as described in A were expressed and purified as GST fusion proteins. Purified proteins, along with purified GST, were incubated with Plk1 in the kinase reaction containing \(\frac{[\gamma-32P]}{ATP}\). After the reaction, the assay samples were fractionated by SDS-PAGE followed by autoradiography (upper panel). The same blot was also stained with Coomassie Blue (lower panel). NS denotes a nonspecific band.

one or more residues with the C4 cluster (Ser-380/Thr-382/Thr-383/Ser-385).

We then made and purified additional GST-PTEN fusion proteins with mutations in one or more sites within the C4 cluster. Mutations of Ser-380, Thr-382, or Thr-383, but not Ser-385, greatly reduced the incorporation of \(\frac{[\gamma-32P]}{ATP}\) (Fig. 5A), strongly suggesting that Plk1 may target these three sites in vitro. Consistent with early studies (10), mutations in all four sites (4A) also greatly reduced the Plk1-mediated phosphorylation in vitro.

**PTEN Ser-380 Is Phosphorylated in Vivo**—To determine which residue(s) within the C4 cluster was the major site of phosphorylation in vivo, we made and transfected HA-tagged constructs expressing full-length PTEN or individual mutants with single amino acid substitutions into HeLa cells (which express wild-type Plk1). Transfected cells were fractionated into soluble and chromatin fractions, which were subsequently blotted for PTEN phosphorylation, as well as for ectopically expressed total PTEN. Mutation of Ser-380 alone greatly reduced the signals detected by the phospho-specific antibody against the p-PTEN<sup>Ser-380</sup> epitope or against the S/T/T epitope (Fig. 5B), indicating that Ser-380 is a major site of phosphorylation in vivo. Intriguingly, mutations of either Thr-382 or Thr-383 did not significantly reduce the amount of signals detected by the phospho-antibody against the S/T/T epitope, suggesting that Thr-382 and Thr-383 may not be major sites of phosphorylation in vivo. Thr-382 or Thr-383 mutation also had a weak affect on reducing p-PTEN<sup>Ser-380</sup> on chromatin compared with WT control. Mutation of either Thr-366 or Ser-370 diminished the phospho-signals (the T/S epitope) compared with ectopically expressed wild-type PTEN; intriguingly, mutation of either residue, but not any other residues, essentially abolished p-PTEN<sup>T/T</sup> signals in the nuclear fraction (Fig. 5B), suggesting that their phosphorylation may regulate nuclear entry or retention of PTEN.

**Plk1 Regulates PTEN Phosphorylation and Chromatin Association**—To determine whether Plk1 regulated PTEN phosphorylation on Ser-380 in vivo, we determined PTEN phosphorylation in cells with altered Plk1 activities. We first treated cells with Bi2536, a Plk1-specific inhibitor, at two different concentrations. As a control, we also treated cells with a CK2 inhibitor,
Plk1 Regulates PTEN

**DISCUSSION**

Our current study reveals a previously unrecognized role of Plk1 in the negative regulation of cell proliferation through phosphorylation of PTEN. To date, Plk1 has been largely considered as an oncogene, promoting cell proliferation and malignant transformation (13). However, there are several reports in the literature that are consistent with a potential inhibitory role of Plk1 in cell growth. Plk3 and Plk4, members of the **POLO** family sharing significant sequence homology with Plk1, function both as negative regulators for cell proliferation and as suppressors for tumor development (33, 34). Moreover, mutations in **PLK1** are reported in HepG2, A431, MKN74, and A549 (31), all of which are tumor-derived cell lines, suggesting that mutations confer certain advantages for the transformed phenotype. Furthermore, haploinsufficiency of **PLK1** leads to enhanced development of tumors in mice (32). Therefore, the biological activities of Plk1 are likely to be more complicated than originally thought.

Research in the past has identified several protein kinases that phosphorylate PTEN in the C-tail region (2, 7). Glucose synthase kinase 3β phosphorylates Ser-362 and Thr-366 whereas CK2 phosphorylates Ser-370 and Ser-385 (8). Although one study reported that CK2 also phosphorylates Ser-380 (9) the data that support this claim are rather weak in that study. We have directly compared the effect of Plk1 and CK2 on with the amount of chromatin PTEN that peaked at mitosis as revealed by p-H3 signals. Significantly, levels of chromatin-bound PTEN and its Ser-380 phosphorylation were closely correlated with the **PLK1** status. Combined, these results strongly suggest that Plk1 specifically phosphorylates PTEN on Ser-380 in vivo and that the phosphorylation may positively regulate chromatin association of PTEN.

**PTEN Phosphorylation on Ser-380 Is Important for Its Role in Mitosis**—To determine whether p-PTEN<sup>Ser-380</sup> alone plays a major role in regulating mitotic progression, cells were co-transfected with PTEN siRNAs (3′-UTR) and an HA-PTEN (WT) or HA-PTEN<sup>Ser380A</sup> expression construct and treated with nocodazole. Mitotic cells were collected and then released into the cell cycle. Flow cytometric analysis revealed that PTEN<sup>Ser380A</sup> expression led to significant reduction of mitotic cells, starting at approximately 30 min after release, which was correlated with an increase in G<sub>2</sub> population (Fig. 7A and B). This observation thus suggests that Ser-380 phosphorylation is important for the PTEN role in normal mitotic progression.

We further analyzed mitotic progression of cells with depletion of endogenous PTEN but expressing transfected HA-PTEN or HA-PTEN<sup>Ser-380</sup> via time lapse confocal microscopy. We observed that cells expressing PTEN<sup>Ser380A</sup> underwent rapid mitotic progression and exit (Fig. 7C). The average mitotic time for cells expressing the mutant was 35 min whereas cells expressing PTEN spent approximately 55 min in mitosis (Fig. 7D). Blotting analysis revealed that knocking down of endogenous PTEN and expression of transfected PTEN plasmid constructs were efficient (Fig. 7E). We consistently observed that PTEN<sup>Ser380A</sup> was expressed at a lower level than that of WT PTEN, suggesting that Ser-380 phosphorylation may play a role in stabilizing PTEN during mitosis.
phosphorylating Ser-380 of PTEN and found that Plk1 is the primary protein kinase that phosphorylates Ser-380 residue and regulates chromatin-association of PTEN because Plk1 inhibitor, but not the CK2 inhibitor, compromises PTEN association with chromatin (Fig. 6A). Plk3 also phosphorylates Ser-380, and its phosphorylation stabilizes PTEN in vivo (10). Intriguingly, Plk1 and Plk3 exhibit rather mutually exclusive substrate specificity even through both kinases target PTEN. Plk1 does not significantly phosphorylate Ser-370; similarly, Plk3 catalyzes little phosphorylation of Ser-380 (Fig. 3E). Several previous reports show that the C-terminal domain of PTEN is important for its stability as well as the phosphatase activity (9, 10, 40). Deletion of C-terminal region or mutations of phosphorylation sites within the region including Thr-366, Ser-370, and 4A cluster (Ser-380, Thr-382, Thr-383, Ser-385) all lead to reduced stability of PTEN (9, 10, 40). Our current study reveals that Ser-380 is a key residue, phosphorylation of which stabilizes chromatin PTEN. During the cell cycle, Plk1 is regulated primarily at the protein level although it is subjected to regulation by phosphorylation (14, 22). During mitosis, Plk1 undergoes dramatic subcellular translocation. Plk1 associates with centrosomes in prophase and then is enriched at kinetochores from prometaphase to metaphase. Subsequently, Plk1 translocates to the central spindle and then accumulates in the

![Figure 6](https://example.com/figure6.png)

**FIGURE 6. Plk1 regulates PTEN phosphorylation on Ser-380 in vivo.** A, HeLa cells synchronized at the G1/S junction by the double-thymidine (TT) treatment were released into the cell cycle. Cells were treated with or without CK2 or Plk1 inhibitor or vehicle for 10 h immediately after release. At the end of the treatment, cells were collected and fractionated into soluble (S) and chromatin (C) fractions. Equal amounts of lysates from both fractions were blotted for p-PTEN-Ser-380, total PTEN, PARP, and α-tubulin. B, HeLa cells transfected with GFP-Plk1 or GFP alone for 2 days were collected and fractionated into soluble and chromatin fractions. Equal amounts of proteins from both fractions were blotted for p-PTEN-Ser-380, α-tubulin, and PARP. The total cell lysates were also blotted for Plk1 (right panel). C, HeLa cells transfected with PLK1 siRNA (siPlk1) or luciferase siRNA (siLuc) for 2 days were collected and fractionated into soluble (Solub) and chromatin (Chrom) fractions. Equal amounts of proteins from both fractions were blotted for p-PTEN-Ser-380, α-tubulin, and PARP. The total cell lysates were also blotted for Plk1 (right panel). D, PLK1 genotyping was carried out using the PCR approach. Lanes 1 and 2 represent PLK1 heterozygous mouse genotyping. Samples in lanes 1 and 3 were performed with wild-type and knock-out sense primers and a wild-type antisense primer. Samples in lanes 2 and 4 were from PCRs using wild-type and knock-out sense primers and a knock-out antisense primer. Primer information is as described under "Materials and Methods." E, MEFs isolated from both wide-type (W) and PLK1 haploinsufficient (H) embryos were synchronized at the G1/S junction by thymidine-aphidicolin block. These cells were then released into the cell cycle by culturing in fresh medium. At various times after release, cells were collected and fractionated into cytoplasmic and chromatin fractions. Equal amounts of proteins from both fractions were blotted for Plk1, total PTEN, p-PTEN-Ser-380, phospho-H3, α-tubulin, and PARP.
midbody during late mitosis. Given that PTEN also exhibits
kinetochore/centromere localization (41), it is tempting to
speculate that Plk1 may directly interact and phosphorylate
PTEN on centromeres.

Amino acid residues of Ser-380, Thr-382, Thr-383, and Ser-
385 of PTEN consist of an epitope whose phosphorylation can
be detected by a specific antibody commercially available (Cell
Signaling; 9549). Mutational analysis followed by in vitro kinase
assays revealed that Plk1 displayed a weakened activity toward
PTENS380A compared with wild-type PTEN (Fig. 5A).
Intrigu-
antly, Plk1 also exhibited a compromised activity toward
PTENT382A and PTENT383A in in vitro kinase assays (Fig. 5A).
However, ectopically expressed PTEN382A and PTEN383A,
but not PTENS380A, in HeLa cells remained significantly phos-
phorylated as detected by the phospho-specific antibody to the
S/T/T epitope (Fig. 5B), suggesting that Thr-382 and Thr-383
may not be major sites of phosphorylation in vivo. Therefore, it
is possible that the compromised activity of Plk1 toward
PTEN382A and PTEN383A in vitro could be due to an altered
structure of these mutant proteins rather than the lack of tar-
gested sites. Obviously, future studies should focus on the func-
tion of Thr-382 and Thr-383 in regulating PTEN activity in vivo.

Our current study also confirms that PTEN has a significant
role in regulating mitotic progression and that Ser-380 phos-
phorylation by Plk1 mediates PTEN mitotic function. Although
the mechanism by which PTEN regulates mitotic progresses
remains unclear, several studies suggest that nuclear/chroma-
tin PTEN may be involved in modulating activities of genes
involved in homologous recombination (41, 42). Therefore, it
would be interesting to determine whether PTEN has a func-
tion in mediating mitotic recombination.

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Yu, University of Michigan, for PLK1 knock-out mice.

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