Cdh1, a Substrate-recruiting Component of Anaphase-promoting Complex/Cyclosome (APC/C) Ubiquitin E3 Ligase, Specifically Interacts with Phosphatase and Tensin Homolog (PTEN) and Promotes Its Removal from Chromatin*

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Background: PTEN exhibits nuclear localization. However, molecular regulation of nuclear PTEN remains unclear.

Results: Cdh1 specifically interacts with PTEN, negatively regulating chromatin PTEN via polyubiquitination during mitotic exit.

Conclusion: Cdh1 plays an important role in the removal of chromatin PTEN during the cell cycle.

Significance: Our studies underscore the importance of PTEN in regulating mitosis.

A pool of PTEN localizes to the nucleus. However, the exact mechanism by which nuclear PTEN is regulated remains unclear. We have recently reported that Plk1 specifically phosphorylates PTEN on Ser-380 during mitosis. Here we report that PTEN also localized to chromatin and that chromatin PTEN was removed by a proteasome-dependent process during mitotic exit. Pull-down analysis revealed that Cdh1, but not Cdc20, was significantly associated with PTEN. Cdh1 interacted with PTEN via two separate domains, and their interaction was enhanced by MG132, a proteasome inhibitor. Cdh1 negatively controlled the stability of chromatin PTEN by polyubiquitination. Phosphorylation of PTEN on Ser-380 impaired its interaction with Cdh1, thus positively regulating PTEN stability on chromatin. Significantly, the PTEN interaction with Cdh1 was phosphatase-independent, and Cdh1 knockdown via RNAi led to significant accumulation of chromatin PTEN, delaying mitotic exit. Combined, our studies identify Cdh1 as an important regulator of nuclear/chromatin PTEN during mitosis.

Phosphatase and tensin homolog (PTEN) is a well known tumor suppressor as it is mutated at a high frequency in a variety of human malignancies, 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 and its activity profoundly influence cell growth, survival, and tumor susceptibility (5, 6). A number of studies in the past few years show that PTEN also has nuclear functions (7, 8). However, molecular regulation of nuclear PTEN remains poorly understood despite the reports that it is involved in the maintenance of genomic stability through modulating DNA repair, chromosomal segregation, and cell cycle arrest (9, 10).

PTEN is subjected to modification by several types of the post-translational mechanisms including phosphorylation, oxidation, ubiquitination, and acetylation (11). Phosphorylation of several serine/threonine residues (e.g. Ser-370, Thr-382, Thr-383, and Ser-385) in the C-terminal tail region of PTEN by casein kinase 2 and Plk3 is essential for the tail-dependent regulation of stability as phospho-defective mutant proteins exhibit decreased stability in comparison with the wild type PTEN (12–14). Glycogen synthase kinase-3β phosphorylates PTEN at Ser-362 and Thr-366 (12). PTEN is also phosphorylated on tyrosine residues by Rak, and this phosphorylation stabilizes PTEN as well (15). PTEN can be modified by mono- or polyubiquitination, which appears to regulate its nuclear localization and stability, respectively (11). NEDD4-1, WWP2, and RFP are three reported ubiquitin E3 ligases that mediate PTEN ubiquitination (16–18). NEDD4-1 appears to be dispensable for regulation of PTEN subcellular localization and stability (19) whereas RFP functions to regulate its enzymatic activity (18). Cdh1 is a substrate-specific activator of anaphase-promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase that targets specific substrates for degradation by the 26S proteasome during the cell cycle. Extensive research reveals that APC/C-Cdh1 is tightly regulated for the timely degradation of its targets including cyclin B and Plk1 during the cell cycle. APC/C-Cdh1 activity is increased in late anaphase and persists through the G1 phase of the cycle (20). A recent study shows that nuclear PTEN associates with APC/C, promoting its physical interaction with Cdh1 (10).
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Given that nuclear PTEN and Cdh1 are involved in chromosomal stability (10, 20), we investigated whether PTEN was a potential downstream target of Cdh1 during mitosis. We observed that Cdh1 physically interacted with chromatin-bound PTEN and that the interaction was greatly enhanced in the presence of MG132 but impaired after PTEN phosphorylation in the tail region. Significantly, Cdh1 promoted polyubiquitination of PTEN and its removal from chromatin during mitotic exit. Our combined studies strongly suggest that Cdh1 may play a crucial role for the stability and function of chromatin-bound PTEN during the cell cycle.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HeLa and HEK293T 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. Transfection of HeLa cells was achieved with either FuGENE HD (Roche Diagnostics) or Lipofectamine 2000 (Invitrogen) following the manufacturers’ protocols. 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, p-PTEN5380, cyclin B1, α-tubulin, PARP, HA, and FLAG were purchased from Cell Signaling Technology. Antibody to GFP was purchased from Santa Cruz Biotechnology. Antibodies to GST, APC3, SKP1, and GFP were purchased from Santa Cruz Biotechnology. Cdh1 antibody was purchased from Invitrogen.

RNA Interference—Human ON-TARGETplus SMARTpool siRNA oligonucleotides that specifically target CDH1 (FZR1, L-015377-00-0010) were purchased from Dharmacon. The siRNA oligonucleotide set contains four sequences as follows: CDH1 (5'-CCACAGGAUUAACGAGAAU-3', 5'-GGAACAACGCUAGACAGGA-3', 5'-GCAACGAUGUGUGUCUCGCCUA-3', and 5'-GAAGAGGGUCUCGCAG-3'). Individual sets of siRNAs were transfected into HeLa cells with Dharmafect I according to the protocol provided by the supplier. Briefly, cells seeded at 50% confluence in an antibiotic-free culture medium without 5% FBS; Invitrogen) were transfected with siRNA duplexes at a final concentration of 50 nM for 24 h. Small interfering RNAs targeting firefly (5'-UUCCTACGCTGAGTACTTCGA-3') luciferase (5'-CCACAGGAUUAACGAGAAU-3', 5'-GGAACAACGCUAGACAGGA-3', 5'-GCAACGAUGUGUGUCUCGCCUA-3', and 5'-GAAGAGGGUCUCGCAG-3') luciferase (5'-UUCCTACGCTGAGTACTTCGA-3') were used as negative control for transfection.

Plasmids—Plasmids encoding HA-tagged PTEN (pSG5L-HA-PTEN) and its phosphorylation mutants including T366A, S370A, S380A, T382A, and T383A were obtained from Addgene. The plasmid construct expressing EGFP-F3-N3 was kindly provided by Dr. Xiaoqi Liu (Purdue University). To make plasmids encoding GST-PTEN fusion proteins, PCR products encoding human PTEN residues of 1–403, 1–347, 1–186–347, 186–403, or 347–403 were inserted into the BamHI and EcoRI site of pGEX-3X (GE Healthcare). Individual GST-PTEN mutants were obtained using QuikChange Site-directed Mutagenesis kit (Agilent Technologies). PTEN cDNA and its deletion fragments were subcloned into pGEX-3X plasmid vector to create GST-PTEN fusion constructs. GST-PTEN mutants were generated using the QuikChange Lightning Multi Site-directed Mutagenesis kit (Agilent Technologies) according to the manufacturer’s instructions. Individual deletion fragments of GST-PTEN cDNA, as well as wild-type GST-PTEN cDNA, were also subcloned into pcDNA3 plasmid, a mammalian expression vector. FLAG-cdh1, FLAG-cdc20, FLAG-Fbxw1, FLAG-Fbxw7α, FLAG-Fbxw7α3x, and Fbxw7γ were kind gifts from Dr. Michele Pagano. All deletion and mutation constructs were confirmed by DNA sequencing.

Cell Cycle Synchronization—HeLa cells were synchronized at the G1/S boundary by double-thymidine blocks. Briefly, cells were treated with 2 mM thymidine for 18 h followed by a 9-h release; these cells were then treated with 2 mM thymidine for another 18 h. At the end of the treatment, cells were released into the cell cycle for various times. In some experiments, cells released into the cell cycle for 10 h were supplemented with or without 5 μM MG132 (Sigma-Aldrich). Mitotic shake-off cells were obtained from gentle tapping of either exponentially growing rounded-up cells or cells treated with nocodazole (40 ng/ml; Sigma-Aldrich) for 14 h.

Lysate Preparation and Immunoblotting—Total cell lysates were prepared in a buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% IGEPAI, 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 (21). 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% IGEPAI, 0.630, and protease inhibitors. Protein concentrations were measured using the bicinchoninic acid protein assay reagent kit (Pierce). 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).

Co-immunoprecipitation—Equal amounts of protein lysates obtained from cells co-transfected with FLAG-Cdh1 expression plasmid and various GST-PTEN plasmid constructs were incubated with FLAG M2 antibody-conjugated agarose beads (Sigma). The agarose beads were washed five times with the washing buffer. After washing, the FLAG-beads were eluted at 95 °C for 5 min in the Laemmli buffer. Eluted proteins were subjected to SDS-PAGE analysis followed by immunoblotting. Specific signals on immunoblots (polyvinylidene difluoride) were visualized using enhanced chemiluminescence (Super-Signal).

Flow Cytometry—Flow cytometry was performed as described in our early studies (22). Briefly, cells were initially fixed in 75% ethanol and then suspended in a solution of PBS containing 100 μg/ml RNase A (Sigma) and 10 μg/ml propidium iodide.
(Molecular Probes) and kept at room temperature for 1 h. Cellular fluorescence was then measured using Beckman Coulter Epics XL-MCLTM flow cytometer. DNA content was deconvoluted using Muticycle software (Phoenix Flow System, San Diego, CA) to estimate percentage of cells in different phases of the cell cycle.

**Chromosome Spread and Immunostaining**—To obtain mitotic chromosome spreads, HeLa cells transfected with various GFP-PTEN constructs for 48 h were collected and incubated in 3.7 mM KCl at 37 °C for 15 min. Cells were finally suspended in a fixative solution (glacial acetic acid/methanol in a ratio of 1:3) prior to spreading onto microscope slides (Fisher Scientific). Chromosome spreads were counterstained with DAPI. Chromosomal images were captured with a Leica TCS SP5 confocal microscope or a Leica AF6000 fluorescence microscope. For each treatment, at least 50 metaphase spreads were examined.

**RESULTS**

We have previously demonstrated that Plk1 phosphorylates PTEN on Ser-380 (23), a residue located in a region known to control its stability (12, 13). To determine whether PTEN stability was affected by phosphorylation on Ser-380, HeLa cells transfected with a construct expressing HA-tagged PTEN\textsuperscript{S380A} mutant or HA-PTEN for 36 h were treated with nocodazole for 14 h to induce mitotic arrest. At various times after nocodazole removal, cell lysates were blotted for HA, as well as for GFP (used for co-transfection) and cyclin B. HA-PTEN\textsuperscript{S380A} mutant levels in asynchronously transfected cells were much lower than those of wild-type HA-PTEN although nocodazole treatment enhanced its accumulation (Fig. 1A). During the mitotic release, HA-PTEN\textsuperscript{S380A} rapidly declined with a decay half-life about 3 h, which was significantly shorter than that of the wild-type counterpart (Fig. 1B). These results strongly suggest that Ser-380 phosphorylation may stabilize PTEN during mitotic progression.

To determine whether a proteasome-dependent process was responsible for the decline of PTEN during or after mitotic exit, HeLa cells synchronized at the G1/S junction were released into the cell cycle. At about 10 h after release (roughly the mitotic stage), synchronized cells were treated with or without MG132 for an additional 3 or 5 h (see Fig. 1C for the treatment scheme). Immunoblotting analysis revealed that chromatin-bound PTEN was stabilized by MG132 at 13 h, but not 15 h, after release as compared with the vehicle-treated control (Fig. 1D). Significantly, chromatin-bound p-PTEN\textsuperscript{S380} signals declined at a much slower rate than the total PTEN at 13 h of the release, and MG132 treatment largely restored its level to the pretreatment level (Fig. 1D). Soluble PTEN, however, was not significantly impacted by the treatment of MG132. This study suggests one possibility that chromatin-bound PTEN may be subjected to the degradation by the proteasome during mitotic exit, which is attenuated by phosphorylation at Ser-380.

To further demonstrate that PTEN association with chromatin was phosphorylation-dependent, we transfected HeLa cells with plasmid constructs expressing GFP-PTEN or phospho-mimetic mutant (GFP-PTEN\textsuperscript{S380D}) with Ser-380, Thr-382, and Thr-383 replaced with either alanines (A) or aspartic acids (D). Transfected cells were then processed for chromosome spread analysis. We observed that significant GFP-PTEN signals were detected on chromosomes whereas few signals of GFP-PTEN\textsuperscript{A} were present (Fig. 2, A and B). However, PTEN phospho-mimetic mutant (GFP-PTEN\textsuperscript{D}) was easily detectable on chromosomes. Combined, these results strongly suggest that PTEN does have chromatin localization and that phosphorylation in the C-tail domain positively regulates the localization.

As the first step to determine whether chromatin-bound PTEN was subjected to proteolysis, we attempted to identify the potential ubiquitin E3 ligase(s) that might regulate PTEN during mitotic exit. HeLa cells were transfected with a series of plasmids expressing FLAG-tagged F-box proteins, Cdh1, and Cdc20 (substrate recognition subunits of ubiquitin E3 ligases (20, 24, 25)), after which cell lysates were immunoprecipitated with an anti-FLAG antibody. Immunoblotting revealed that FLAG-Cdh1 precipitates contained a significant amount of endogenous PTEN (Fig. 2C). Immunoprecipitations were effi-
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FIGURE 2. PTEN chromosomal localization and its specific interaction with Cdh1. A, HeLa cells were transfected with plasmid constructs expressing GFP-PTEN and its phospho-mutants (GFP-PTEN3A and GFP-PTEN3D) for 2 days. PTEN3A and PTEN3D were derived from replacing Ser-370, Thr-382, and Thr-383 with either alanines (A) or aspartic acid (D), respectively. Transfected cells were collected and processed for chromosome spread analysis. Mitotic chromosomes were examined under a fluorescence microscope. Representative chromosomes from each transfection are shown. B, mitotic cells positive for PTEN staining/signals on chromosomes as shown in A were counted. The data are summarized and plotted. C, HeLa cells were transfected with plasmid constructs expressing FLAG-tagged ubiquitin E3 ligases including Cdh1, Cdc20, and several F-box-containing proteins as indicated for 48 h. Equal amounts of cell lysates from various transfection samples were immunoprecipitated with the antibody to FLAG. Immunoprecipitates, along with lysate inputs, were then blotted for PTEN, APC3, and SKP1. Part of the PTEN blot after longer exposure is also shown. D, HeLa cells were transfected with HA-PTEN expression construct or vector alone for 24 h, after which equal amounts of cell lysates were immunoprecipitated (IP) with the antibody to HA tag. Immunoprecipitates, along with lysate inputs, were blotted for HA and Cdh1. E, HeLa cells were co-transfected with plasmids expressing HA-PTEN and FLAG-Cdh1 or HA-PTEN and FLAG-Cdc20 for 48 h. Equal amounts of cell lysates from various transfection samples were immunoprecipitated with the antibody to FLAG. FLAG immunoprecipitates, along with lysate inputs, were then blotted with antibodies to APC3, FLAG (Cdh1 or Cdc20), and HA (PTEN). Both short and long exposure blots are shown.

Efficient as both Cdh1 and Cdc20 precipitated an APC/C component (APC3) and as various F-box proteins precipitated SKP1, as expected. Intriguingly, a slow migrating PTEN signal about 70 kDa was also co-precipitated with FLAG-Cdh1, and the band corresponded to some minor PTEN signals in total lysates (long exposure panel), suggesting that it may be a modified form of PTEN. To further determine the physical interaction between PTEN and Cdh1, we ectopically expressed HA-tagged PTEN in HeLa cells, after which cell lysates were subjected to immunoprecipitation using the anti-HA antibody. HA-PTEN precipitates contained a significant amount of endogenous Cdh1 whose signal was roughly correlated with the amount of precipitated PTEN (Fig. 2D). Because Cdc20 also regulates APC/C activity during mitosis, we asked whether it interacted with PTEN as well. Co-immunoprecipitation experiments revealed that Cdh1, but not Cdc20, interacted with PTEN although both pulled down APC3 with an equal efficiency (Fig. 2E).

We next delineated the PTEN domain that mediated the interaction with Cdh1. Various PTEN deletion constructs expressed as GST fusion proteins were co-transfected with the FLAG-Cdh1 construct into HEK293T cells. Wild-type GST-PTEN and various PTEN deletion mutant proteins were expressed after transfection although their levels of expression varied (Fig. 3A). FLAG-Cdh1 that were expressed at a similar level pulled down full-length PTEN and PTEN deletion mutants A, B, C, D but not mutant E (Fig. 3, A and B). Deletion of C-tail alone seemed to enhance the PTEN interaction with Cdh1 as mutant proteins A and B (lack of C-tail) that were expressed at a very low level also exhibited a strong interaction with Cdh1 (Fig. 3A). Moreover, GST-Cdh1 pulled down full-length PTEN3A mutant (S380A, T382A, and T383A) more efficiently than it did with wild-type PTEN and PTENT366A/S370A (T/S construct) (Fig. 3, A and B) even though it was expressed at a lower level than the other two, suggesting that the phosphorylation at the 3A cluster plays an inhibitory role in the interaction with Cdh1.
To study whether Cdh1 has a role in the removal of chromatin PTEN through degradation or translocation, we asked whether the interaction between Cdh1 and PTEN was sensitive to the inhibition of the proteasomal activity. Treatment of transfected cells with MG132 greatly enhanced the interaction between ectopically expressed Cdh1 and PTEN even though a similar amount of FLAG-Cdh1 was precipitated by the FLAG antibody (Fig. 4A). The increased association between PTEN with Cdh1 was comparable in both total lysates and the chromatin fraction, suggesting that MG132 mediates stabilization, rather than translocation, of PTEN. The increased interaction with Cdh1 after MG132 treatment was also observed with several key lysine mutants of PTEN (Fig. 4B). Again, the immunoprecipitation was specific as FLAG signals were only present in cells with transfection of FLAG-Cdh1 (Fig. 4B). Significantly, additional PTEN signals with a slow mobility were pulled down by FLAG-Cdh1 in both total lysates and the chromatin fraction in cells treated with MG132 (Fig. 4A), suggesting

FIGURE 4. The interaction between PTEN and Cdh1 is greatly enhanced by MG132. A, HEK293T cells were co-transfected with plasmids expressing HA-PTEN and FLAG-Cdh1 for 48 h, after which cells were treated with MG132 for an additional 6 h. At the end of experiments, both the total cell lysates and the chromatin fraction were collected and subjected to immunoprecipitation (IP) with the anti-FLAG antibody. The immunoprecipitates, along with lysate inputs, were blotted for APC3, FLAG (Cdh1), and HA (PTEN). -Fold changes of PTEN signals (PTEN signals after MG132 treatment divided by control signals (set at 1.0) without MG132) are shown. Long exposure for HA-PTEN blot is also included to show slow mobility bands. Ubi denotes putative ubiquitinated signals of PTEN. B, HEK293T cells were co-transfected with plasmids expressing GFP, HA-PTEN (or HA-tagged mutants with individual lysine residues as indicated replaced with alanines), and FLAG-Cdh1 for 48 h. Transfected cells were then treated with or without MG132 for 6 h. At the end of experiments, cell lysates were collected and subjected immunoprecipitation with the anti-FLAG antibody. The immunoprecipitates were blotted for APC3, FLAG (Cdh1), and HA (PTEN). The total cell lysates were also blotted with antibodies to PTEN and GFP as control. C, HEK293T cells were co-transfected with plasmids expressing FLAG-Cdh1 and HA-PTEN (or its mutant) for 48 h, after which cells were treated with or without MG132 for 6 h. Mutant 3A denotes the construct expressing PTEN with Ser-380, Thr-382, and Thr-383 replaced with alanines. Immunoprecipitates brought down by the anti-FLAG antibody, along with lysate inputs, were blotted for FLAG (Cdh1) and HA (PTEN). -Fold changes of signals (signals of transfected PTEN and PTEN mutants with or without MG132 divided by signals of the wild-type control without MG132 (set at 1.0)) were included for direct comparison.
that these bands were likely to be ubiquitinated PTEN bound for degradation by the proteasome. Intriguingly, MG132 treatment also enhanced the interaction between Cdh1 and APC3.

It has been reported that the amino acid cluster (Ser-380, Thr-382, and Thr-383) is phosphorylated (12, 13). We have recently shown that Ser-380 is significantly phosphorylated in vivo and that Plk1 targets this residue by phosphorylation both in vivo and in vitro (23). To study whether Ser-380 phosphorylation or phosphorylation of Thr-382 and Thr-383 plays a role in mediating the interaction between PTEN and Cdh1, we co-transfected cells with plasmids expressing FLAG-Cdh1 and HA-PTEN$^{S380A}$ or HA-PTEN$^{S380A}$. We observed that HA-PTEN$^{S380A}$ mutant (S380A, T382A, T383A) was expressed at a level comparable with that of HA-PTEN$^{S380A}$ and interacted with Cdh1 with an efficiency equal to HA-PTEN$^{S380A}$ either in the presence or absence of MG132 (Fig. 4C). These results suggest that phosphorylation of Ser-380, but not Thr-382 or Thr-383, plays a major role in stabilizing PTEN.

The C-terminal domain is known to regulate the stability of PTEN. In fact, PEST motifs (sequences for targeting proteins with short intracellular half-life for proteolytic degradation) are present in the C-tail region (26). One PEST motif encompasses Ser-380 and is highly conserved in high eukaryotes (Fig. 5A).
Given that lysine (K) residues are essential for mediating ubiquitination, we transfected cells with plasmid constructs expressing HA-tagged PTEN mutants with a few Lys or Ser/Thr residues located in the C-terminal domain replaced with alanines and studied their interaction with Cdh1. Immunoprecipitation followed by blotting revealed that PTENS380Amutant, but not PTENT366A and PTENS370A mutants, interacted more efficiently with Cdh1, leading to a low steady-state level (Fig. 5B).

To further study the possibility that phosphorylation of Ser-380 and/or Thr-382 and Thr-383 was involved in mediating the interaction with Cdh1, we transfected cells with plasmid constructs expressing various phosphorylation-resistant mutants of PTEN, as well as wild-type PTEN. Blotting analysis revealed that mutation of Ser-380, Thr-382, and Thr-383, but not Thr-366 and Ser-370, of PTEN led to an increased association with Cdh1, as well as the presence of slow mobility signals (Fig. 5C).

The specificity of Ser-380 mutation and enhanced association of other phospho-mutants (e.g. Thr-380 and Thr-383) were also confirmed with blotting with a phospho-specific antibody against Ser-380 (Fig. 5D).

To ascertain whether slow migrating PTEN signals were ubiquitinated forms, we co-transfected cells with plasmids expressing HA-PTEN, FLAG-ubiquitin, and/or FLAG-Cdh1 for 48 h, after which cell lysates were immunoprecipitated with the anti-FLAG antibody. Blotting analysis with the anti-HA antibody revealed that PTEN was modified by ubiquitin, forming typical ladders on the denaturing gel (Fig. 6A). Moreover, Cdh1 expression significantly enhanced the formation of polyubiquitinated PTEN in a concentration-dependent manner (Fig. 6B), which was associated with a low level of total PTEN in cell lysates compared with the cells without expressing Cdh1 (Fig. 6B, lysate input).

To further understand the role of Cdh1 in regulating PTEN stability during mitosis, mitotic cells transfected with Cdh1 siRNAs, or control siRNAs were released into the cell cycle. Chromatin and soluble fractions of the cell lysates collected at various stages of the release were blotted for PTEN as well as Cdh1 and cyclin B. Transfection of Cdh1 siRNAs greatly reduced the level of Cdh1 in the chromatin fraction although its effect was not as dramatic in reducing Cdh1 in the soluble fraction (Fig. 6, C and D). There was a noticeable increase in chromatin-bound PTEN during mitotic release, and Cdh1 down-regulation greatly enhanced its increase compared with the cells transfected with control siRNAs. Moreover, compared with the control, chromatin-bound cyclin B, as well as cyclin B in the soluble fraction, was significantly stabilized in cells transfected with Cdh1 siRNA. Taken together, these observations strongly suggest that Cdh1 plays a role in the negative regulation of the stability of chromatin-bound PTEN.

We further determined whether the phosphatase activity mediated the PTEN interaction with Cdh1. We co-transfected cells with FLAG-Cdh1 construct and an HA-PTEN mutant construct deficient in the phosphatase activity (HA-PTENC124S). Expression of HA-PTEN, but not HA-PTEN mutants, significantly suppressed Akt phosphorylation (Fig. 7A, lysate input). HA-PTENS380A, but not HA-PTENC124S, was
expressed at a much reduced level compared with the wild-type PTEN. Intriguingly, the lack of phosphatase activity suppressed the reduction of PTEN level induced by Ser-380 mutation (PTENC124S/S380A). Even though its level was much lower than that of HA-PTEN or HA-PTENC124S, HA-PTENS380A interacted with Cdh1 efficiently, which was correlated with an increased level of ubiquitinated HA-PTENS380A signals in Cdh1 precipitates (Fig. 7A, HA long exposure).

We have shown previously that PTEN plays a role in mediating mitotic progression (23). To further study whether the PTEN mitotic function was phosphatase-dependent, we measured cell cycle progression after depletion of endogenous PTEN via RNAi but expression of transfected PTEN or individual mutants. Flow cytometric analysis revealed that neither PTENC124S nor PTENS380A/C124S accelerated mitotic progression and exit compared with wild-type PTEN, which was in contrast to PTENS380A whose expression promoted mitotic exit (Fig. 7B). By 15 h after release, a majority of G2/M cells expressing PTENS380A exited from mitosis and entered G1 of the next cell cycle.

**DISCUSSION**

Our current study leads us to propose a model that explains molecular regulation of nuclear/chromatin PTEN during mitosis (Fig. 8): Phosphorylation of PTEN in the C-tail region by protein kinases including Plk1 regulates PTEN association with and/or stability on chromatin. PTEN is essential for regulating normal mitotic functions, which are independent of its phosphatase activity. At late mitosis, Cdh1 interacts with dephosphorylated, chromatin PTEN, leading to its polyubiquitination and degradation by the proteasome. Removal of chromatin PTEN is an important step for daughter cells to enter G1 of the next cell cycle.

Several lines of evidence suggest that the proteasome is involved in the removal of chromatin-bound PTEN because MG132 is capable of stabilizing it (Fig. 1D). Consistent with this notion, Cdh1 specifically interacts with PTEN, and the interaction is greatly enhanced by MG132. Moreover, the enhanced association between PTEN and Cdh1 by MG132 occurs in total cell lysates as well as on chromatin (Fig. 4A). There are two domains within PTEN that mediate its interaction with Cdh1. One domain lies within the phosphatase domain whereas the other is located in the C2 domain. The C-tail region of PTEN alone does not bind to Cdh1. On the contrary, it negatively regulates the interaction with Cdh1. The timing of removal of chromatin-associated PTEN coincides with mitotic exit when the activity of APC/Cdh1 is high. During early mitosis, Plk1 activity peaks and phosphorylates a series of proteins including PTEN. Plk1, as well as other protein kinases, protects nuclear/chromatin PTEN via phosphorylation in the C-tail region. It is tempting to speculate that heavily phosphorylated C-tail may directly interfere with binding with Cdh1. Our current study also strongly suggests that PTEN mitotic function is independent of its phosphatase activity.

To date, several PTEN ubiquitin E3 ligases, including NEDD4-1, WWP2, and RFP, have been reported and characterized (16–18) although NEDD4-1 appears to be dispensable for mediating PTEN ubiquitination and degradation in vivo (19). RFP-mediated ubiquitination affects neither PTEN stability nor subcellular localization but rather inhibits its phosphatase activity (18). WWP2 physically interacts with PTEN and mediates its degradation through the ubiquitination-depen-
dent process (17). It would be of interest to determine whether WWP2 may mediate the degradation/removal of chromatin-associated PTEN. It would be also tempting to study whether there is a functional interaction between APC/C<sub>Cdh1</sub> and WWP2 in the regulation of nuclear/chromatin PTEN during the cell cycle. It has been reported that nuclear PTEN physically interacts with APC/C components (10). Intriguingly, this study also implicates that nuclear PTEN regulates the activity of APC/C<sub>Cdh1</sub>, which is largely accomplished by <i>in vitro</i> ubiquitination assays and ectopic expression of nuclear exclusion mutants of PTEN (10). Given our observation that nuclear/chromatin PTEN specifically interacts with Cdh1 and APC components, it is conceivable that nuclear, but not cytoplasmic, PTEN immunoprecipitates could contain a significant amount of active APC/C<sub>Cdh1</sub>, which would enhance cyclin B ubiquitination as reported. Moreover, nuclear exclusion of PTEN by expressing PTEN<sub>K13E,K289E</sub> mutant, as described in this study, could impair mitotic functions of the tumor suppressor, thus compromising cell cycle progression. Given that APC/C<sub>Cdh1</sub> activity is tightly regulated, any perturbations of cell cycle progression can inevitably lead to an altered APC/C<sub>Cdh1</sub> activity. Our current study identifies PTEN as a substrate of APC/C<sub>Cdh1</sub>, and timely removal of chromatin-associated PTEN is an important step for daughter cells to exit from mitosis. Any perturbations in the regulatory pathway can conceivably contribute to chromosomal instability and enhanced malignant transformation.

Extensive studies in the past have indicated that phosphorylation plays a crucial role in stabilizing PTEN (12, 13). Our current study shows that protein kinases including Plk1 phosphorylate PTEN on Ser-380, and perhaps other residues, in the PEST sequence of the C-tail region during mitosis and that the phosphorylation appears to regulate PTEN association with and/or stability on chromatin. Thus, PEST sequences may function as an alternative “degron” that mediates the instability of PTEN. Further studies are necessary to refine the structural basis that mediates interaction between PTEN and its ubiquitin E3 ligase during the cell cycle.

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