The tumor suppressor, PTEN is key to the regulation of diverse cellular processes, making it a prime candidate to be tightly regulated. The PTEN level is controlled in a major way by E3 ligase-mediated degradation through the Ubiquitin-Proteasome System (UPS). Nedd 4-1, XIAP, and WWP2 have been shown to maintain PTEN turnover. Here, we report that CHIP, the chaperone-associated E3 ligase, induces ubiquitination and regulates the proteolytic turnover of PTEN. It was apparent from our findings that PTEN transiently associates with the molecular chaperones and thereby gets diverted to the degradation pathway through its interaction with CHIP. The TPR domain of CHIP and parts of the N-terminal domain of PTEN are required for their interaction. Overexpression of CHIP leads to elevated ubiquitination and a shortened half-life of endogenous PTEN. On the other hand, depletion of endogenous CHIP stabilizes PTEN. CHIP is also shown to regulate PTEN-dependent transcription presumably through its down-regulation. PTEN shared an inverse correlation with CHIP in human prostate cancer patient samples, thereby triggering the prospects of a more complex mode of PTEN regulation in cancer.

PTEN (phosphatase and tensin homologue deleted on chromosome TEN) is a tumor suppressor with somatic mutations

**Background:** PTEN is targeted by multiple E3 ligases but that does not clearly decipher the rigid control of its level and activity. **Results:** CHIP interacts with PTEN and promotes its proteasomal degradation. **Conclusion:** CHIP acts as a bridge between the chaperone system and the degradation machinery for PTEN. **Significance:** Stabilization of PTEN by targeting CHIP can be a novel therapeutic approach in cancer regulation.
ment with the fact that multiple degradation pathways may co-exist in different cellular contexts to maintain optimal levels of a crucial player like PTEN in normal cells. A similar regulatory mechanism has been observed in case of the tumor suppressor p53, where besides the central E3 ubiquitin ligase Mdm2 (21, 22), p300, Pirh2, COP1 form part of the destruction machinery for p53 (23–25).

Recently, a new cellular pathway has been identified in connection with protein degradation that links the chaperone system to the UPS where E3 ubiquitin ligase activity is played primarily by the co-chaperone, C terminus of Hsc70-interacting protein (CHIP). CHIP, previously identified as a negative regulator of the chaperone ATPase activity, has a Ubox-dependent E3 ligase activity (26–28). It also contains three tandem tetra-ricopeptide repeat (TPR) motifs, through which it interacts with the chaperones Hsp70 and Hsp90 and ubiquitinates chaperone-bound substrates (29). Some of the victims include glucocorticoid hormone receptor (GR), misfolded cystic fibrosis transmembrane conductance regulator (CFTR), hyperphosphorylated tau (30–35). A number of oncogenic proteins including receptor-tyrosine kinase ErbB2, hypoxia-inducible factor 1a (HIF-1α), estrogen receptor-α (ER α), and hTERT have been shown to be regulated by CHIP (36–39).

However, CHIP has also been implicated in the modulation of tumor suppressor proteins with well known roles in the regulation of apoptosis-like p53, apoptosis-inducing factor (AIF), and interferon regulatory factor1 (IRF-1) (40–42). This is in connection with the report that mice lacking CHIP develop apoptosis in multiple organs after environmental challenge (43). Therefore, the possibility of regulation of PTEN stability by CHIP was explored. In this study, we established CHIP as a novel chaperone-assisted E3 ligase of PTEN that maintains its physiological level by diverting the transient association between the chaperones and PTEN to the degradation machinery.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Recombinant Proteins**—pGZ21dx-GFP-PTEN-WT (GFP-PTEN), pGZ21dx-GFP-PTEN-G129E (G129E), and pGZ21dx-GFP-PTEN-C124A (C124A) were kind gifts from Dr. K. M. Yamada. PTEN was subsequently subcloned into pGEX4T1-GST and pcDNA3.1-myc-his. The deletion mutants of PTEN D1, D2, D3, and D4 as described under “Results” were amplified from HEK293 cDNA while CHIP-K30A (K30A) mutants. CHIP WT (GFP-PTEN), pGZ21dx-GFP-PTEN-G129E (G129E), and pCI-HA-Nedd4-1 were procured from Addgene. The full-length coding sequence and three deletion mutants of human CHIP (NM_005861) viz., CHIP-TPR (1–134), CHIP-ΔTPR (135–303), CHIP-ΔUbox (1–189), and CHIP-Ubox (216–303) were PCR amplified from HEK293 cDNA while CHIP-ΔACC (Δ128–229) was generated following a previous protocol (44) and inserted into pRES-hrGFP-1a-Flag. The QuickChange XL Site-directed Mutagenesis kit (Stratagene) was used to generate the CHIP-H260Q (H260Q) and CHIP-K30A (K30A) mutants. CHIP WT was further subcloned into pET20b (+), pcDNA3.1-myc-his, and pGEX4T1-GST. The primer sequences used include CHIP: F, 5’-AATGGAATTCATGCGGGATGAGGCGAGGAGG-3’ and R, 5’-AACCTCGAGGTAGTCTCTCCACCCAGC-3’; SDM of CHIPK30A: F, 5’-GGGCGGACGAGCTGGAGGAGGAGG-3’ and R, 5’-GATGCGGTGCTGTCGAGCTCCTGCG-3’; SDM of CHIPH260Q: F, 5’-ACATCGAGGAGCCAGCGATGCGTGTCG-3’; SDM of CHIPK124A: F, 5’-ACATTGCGGAGCTGGAGGAGGAGG-3’.

**Chemicals and Antibodies**—The inhibitors used include 17-AAG, MG132, and cycloheximide (Calbiochem). Cycloheximide was dissolved in water and treated at a final concentration of 50 μM at time periods indicated in the figures. MG132 and 17AAG were dissolved in DMSO. All MG132 treatments were at 50 μM for 4 h unless otherwise stated, while 17AAG was used as stated in the figure for 24 h. Antibodies against CHIP, XIAP, GST, GFP, ubiquitin (Ub), Hsp70, Hsp90, α-catenin, AKT, pAKT, Ki67 were purchased from Cell Signaling Technology (CST), while those against PTEN, WWP2, Nedd4-1, mouse IgG, β-Actin, Lamin-B, α-tubulin were obtained from Santa Cruz Biotechnology (SZ). The source of antibodies against HA, His, and Flag tags was Sigma Aldrich. COX IV antibody was purchased from Abcam. All secondary antibodies except goat (Sigma Aldrich) were purchased from CST. AlexaFluor488 and AlexaFluor594 secondary antibodies were purchased from Invitrogen.

**Cell Culture, Lysate Preparation, and Co-immunoprecipitation**—The human cell lines HEK293 (human embryonic kidney), DU145, PC3 (prostate cancer), HeLa (cervical cancer), and DBTRG-05MG (glioma) were procured from ATCC and cultured in DMEM supplemented with 10% FBS, 2000 units/liter penicillin, 2 mg/liter streptomycin, and 3 mg/liter gentamycin reagent solution (Invitrogen). The cells were grown at 37 °C with 5% CO2. The whole cell lysates (WCL) for Western blotting were prepared in Tris lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10% glycerol, and Protease Inhibitor Mixture Set V, Calbiochem), as described previously. The same protocol was followed for the preparation of lysates for immunoprecipitation with the exception of the IP lysis buffer (50 mM HEPES pH 7.2, 250 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1.0 mM EDTA, 0.5 mM DTT, 10 mM PMSF, and Protease Inhibitor Mixture Set V). All immunoprecipitation experiments were performed following established protocol(s) (45) with protein A-Sepharose beads (Amersham Biosciences), using 1 mg of total protein unless otherwise mentioned.

**RNA Interference and Transfections**—siRNA for scramble, WWP2, Nedd4-1, and CHIP (human) were obtained from Dharmacon. The following target sequence: 5’-AACCAAGCAGCAGCAGTACAT-3’ was amplified by PCR, annealed, and inserted into pPMKO.1 to produce the human shRNA against CHIP. Non-targeting control shRNA was similarly constructed. Sequencing was done to confirm all the constructs. DNA transfections were carried out following the calcium phosphate method as per the previous protocol (47) or using Attractene method as per the previous protocol (47) or using Attractene. Transfection efficiency was determined using Lipofectamine-2000 (Invitrogen).
CHIP Promotes Degradation of PTEN

Subcellular Fractionation—The cells were lysed on ice using subcellular fractionation buffer (250 mM sucrose, 20 mM HEPES pH 7.4, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and Protease Inhibitor Mixture Set V) per standard protocols. The nuclear pellet was centrifuged at 3000 rpm for 5 min. The supernatant was further centrifuged at 8000 rpm for 10 min and 40,000 rpm for 1 h to get the mitochondrial and cell membrane pellet, respectively. The supernatant was kept separately as the cytoplasmic fraction. The lysates were extracted from the pellets into standard lysis buffer (IP lysis buffer following the same protocol used in co-immunoprecipitation. The cell-free method of analyzing the ubiquitin ligase activity involved overexpression of the proteins separately in HEK293 cells. 250 µl of buffer TN2 (50 mM Tris-HCl, pH 8.0, 400 mM NaCl, 50 mM reduced glutathione, 1 mM EDTA, 1 mM DTT) or 2× SDS loading buffer and subjected to Western blot analysis.

Protein Purification—GST-PTEN, GST-CHIP, and GST were expressed in BL21DE3 cells. The lysates were prepared following the manufacturer’s protocol (Qiagen) and were allowed to bind to a GSH column (bed volume, 2 ml; flow rate, 0.5 µl/s) for 2 h at 4 °C, washed three times with TN1, and eluted with 50 µl of buffer TN2 (50 mM Tris-HCl, pH 8.0, 400 mM NaCl, 50 mM reduced glutathione, 1 mM EDTA, 1 mM DTT) or 2× SDS loading buffer and subjected to Western blot analysis.

Ubiquitin Ligase Activity Assay—For the in vivo ubiquitination assay, 48 h post-transfected cells were treated with or without proteasome inhibitor MG132. WCLs were prepared in IP lysis buffer following the same protocol used in co-immunoprecipitation. The cell-free method of analyzing the ubiquitin ligase activity involved overexpression of the proteins separately in HEK293 cells. 250 µg of each protein were then incubated in combinations as described under “Results” at 4 °C. To ensure an equal protein load in each case, WCLs from HEK293 cells were added to the combinations. 50 µl (50% slurry) of protein A-Sepharose beads were mixed after 4 h of incubation followed by IP and immunoblotting. An in vitro ubiquitination assay was performed using 2 µg of purified rPTEN or GST in the presence or absence of GST-CHIP using the Ubiquitin Protein Conjugation kit (Calbiochem), following the manufacturer’s protocol in combinations as shown in the figure.

cDNA Preparation & Quantitative PCR (qPCR)—Cells were harvested and RNA isolated using TRizol (Invitrogen). Subsequently, cDNA was prepared with Revert Aid™ H Minus First Strand cDNA Synthesis kit (Fermentas) following the manufacturer’s protocol and used for qPCR analysis through Power SYBR Green Master Mix on 7500 Fast Real Time PCR system (Applied Biosystems). 18 S rRNA was used as an internal control. The primers used are: VEGF: F, 5'-AGGAGAGGGGCA-GAATCATCA-3' and R, 5'-CTCGGATGATGGCCGTGTA-GCT-3'; 18 S rRNA: F, 5'-GCTTTATTGACTCAACAG-GGC-3' and R, 5'-AGCTTACATCTGTAATCCTGTGCT-3'; Rbl2: F, 5'-CAAAACATCTTACATCAACAG-3' and R, 5'-GAGTTTCTCTTGCGTAGTAC-3'; Dre1: F, 5'-GAGTGCT-ACGATCTTAC-3' and R, 5'-GATACGCGTGTGTA-TCTTTC-3'; Rad51: F, 5'-GGTCTGATGTGTCTGTTG-TGA-3' and R, 5'-GGTGAAGGAAAGGCCATGTA-3'.

Immunocytochemistry (ICC) & Immunohistochemistry (IHC)—For ICC, cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton-X-100, and blocked with 2.5% BSA in PBS before incubation with primary antibodies overnight at 4 °C, followed by secondary antibodies for 1 h at room temperature under dark conditions according to established protocols (48). The images were captured on an Olympus BX61 motorized fluorescence microscope using Image ProPlus software and a Nikon A1 Laser scanning confocal microscope.

For IHC, tissue samples were fixed in formalin, and then processed for paraffin embedding as per standard procedures. Sections (5 µm) were obtained on poly-L-lysine (Sigma)-coated glass cover slides. Sections were then processed serially for IHC staining, which includes dewaxing, rehydration, heat, and pressure-induced antigen retrieval in citrate buffer (pH 6.0), serum blocking, overnight primary antibody incubation at 4 °C, peroxide blocking, HRP-linked secondary antibody incubation at room temperature for 1 h, DAB chromogen treatment, counterstaining using Mayer's Hematoxylin (Sigma), dehydration, and permanent mounting. For routine H&E staining, standard protocols were followed. Images were acquired at necessary magnifications as indicated under “Results” on BX61 (Olympus).

BrdU Incorporation Assay—HEK293 cells were seeded in 60-mm culture dishes at ~30–40% confluency and transfected with GFP-PTEN and Flag-CHIP. Post 36 h, cells were pulsed with 10 µM BrdU (Invitrogen) for 4 h in fresh growth media. Cells were then trypsinized for harvest and fixed using 70% chilled ethanol. Fixation was carried out at ~20 °C for 18 h. On the next day, cells were treated appropriately and stained for BrdU, which includes acid denaturation of DNA with 2 M HCl for 30 min at 37 °C, neutralization of the acid with 1 M Borax solution, blocking with 0.5% BSA, and final incubation with anti-BrdU-FITC antibody (Invitrogen) for 1 h at room temperature under mild rocking. PI was used as counterstain to label DNA. The level of BrdU incorporation was analyzed in a fluorescence-activated cell sorter (FACS) using CellQuestPro (BD) software under standard parameters.

RESULTS

Identification of Potential E3 Ligase for PTEN—It has been established that Nedd4-1 is rather dispensable for the regulation of PTEN stability, and a substantial amount of ubiquitinated forms of PTEN was present even after the knock-out of Nedd4-1. With this background, we assumed that PTEN might be regulated in a more robust way through multiple ubiquitin ligases. To search for a novel E3 ligase for PTEN regulation, we speculated whether the co-chaperone, CHIP could interact
with PTEN. To verify this possibility, a pull-down experiment was performed using lysates from HEK293 cells overexpressing His-PTEN with Ni-NTA resin and found endogenous CHIP with PTEN. The assay also confirmed the presence of XIAP and WWP2 with PTEN, but Nedd4-1 could not be detected by the same (Fig. 1A). However, Nedd4-1 under overexpressed conditions could interact well with PTEN (supplemental Fig. S1). A GST pull-down assay was carried out for further confirmation of the interaction between PTEN and CHIP, where His-CHIP was incubated with or without GST-PTEN and GST (all separately expressed in bacteria). The incubated lysates were allowed to bind to the GSH-Sepharose resin. The observation that immobilized GST-PTEN was able to pull-down CHIP but not GST solidified our claim that CHIP is a PTEN-interacting E3 ligase (Fig. 1B).

To further support this finding, we expressed GFP-PTEN with or without Flag-CHIP in HEK293 cells. The lysates were subjected to immunoprecipitation separately with antibodies against either PTEN or Flag. IP results showed co-immunoprecipitation of CHIP with PTEN in either case (Fig. 1, C and D). PTEN when overexpressed alone can also interact with endogenous CHIP (Fig. 1E). The interaction between CHIP and PTEN was also supported by their colocalization in cancer cells HeLa and DBTRG-05MG (Fig. 1F). In summary, it can be concluded that PTEN might be a potential substrate of CHIP and co-localizes for its post-translational regulation.

Mapping the Interacting Domains for PTEN and CHIP—Based on the observation that CHIP co-localizes and interacts with PTEN, we wanted to define the structural requirements for their interaction. To test this possibility, we generated and transfected full-length CHIP and its truncated mutants /H9004 TPR, /H9004 Ubox (all Flag-tagged) (Fig. 2A) along with GFP-PTEN in HEK293 cells. The lysates were prepared and subjected to IP using anti-Flag and IB with anti-PTEN antibodies. The full-length CHIP and /H9004 Ubox could interact with PTEN, whereas the interaction between /H9004 TPR and PTEN was severely hampered. Interestingly, the TPR domain of CHIP could not only interact with PTEN but does so with a stronger affinity. So it is the TPR domain of CHIP that is essential for this interaction with PTEN (Fig. 2B). It may also suggest that the interaction may be chaperone dependent as the TPR domain of CHIP is also required for its interaction with the chaperones. Yet based on the fact that the bacterially expressed PTEN and CHIP could interact with each other and the presence of some residual interaction between PTEN and /H9004 TPR compelled us to think of some other way of interaction between the two proteins. Accordingly, we co-transfected GFP-PTEN with CHIP or either of its deletion mutants /H9004 CC, Ubox (all Flag-tagged) (Fig. 2A). WCLs were immunoprecipitated with Flag antibody and checked for PTEN. To our surprise, it was found that the interaction between PTEN and /H9004 CC was quite poor when compared with the wild type forms while that between PTEN and Ubox showed no interaction.
whatsoever (Fig. 2C). This suggests that the CC domain of CHIP might well be involved in its interaction with PTEN.

We next tried to map the domain of PTEN required for its interaction with CHIP. To execute it, a number of GFP-fusion constructs containing full-length PTEN, and its mutants D1 (1–399): without the PDZ-interacting domain, D2 (1–351): the N-terminal region of PTEN spanning the phosphatase and C2 domains, D3 (187–403): C2 and CT domains, D4 (352–403): the C-terminal regulatory region were generated (Fig. 2D). The full-length PTEN and its deletion mutants as well as His-CHIP were expressed separately in HEK293 cells. To investigate the interaction in a cell-free system, His-CHIP-overexpressed lysate was separately incubated with lysates expressing PTEN and its mutants followed by IP with anti-GFP antibody. Contrary to the previous reports on interaction between PTEN and other E3 ligases, the C2 domain of PTEN could interact with CHIP as effectively as the full-length N-terminal domain of PTEN. But we did not find any interaction between the C-terminal regulatory regions of PTEN with CHIP (Fig. 2E). Therefore, these observations strongly suggest that both the TPR and CC domains of CHIP interact with parts of the N-terminal domain of PTEN.

CHIP Induces Ubiquitination of PTEN—CHIP could interact with PTEN; it was now a matter of interest to check whether CHIP can be yet another ubiquitin ligase for PTEN. To confirm this possibility, we performed ubiquitination assays, with CHIP as an E3 ubiquitin ligase. Under in vivo conditions, we overexpressed GFP-PTEN and HA-ubiquitin together with or without Flag-CHIP in HEK293 cells. WCLs prepared from 48 h post-transfected cells were immunoprecipitated using either anti-Ub or anti-PTEN antibodies. The Western blot showed the presence of both mono- and polyubiquitinated PTEN adducts. The polyubiquitination of PTEN was markedly enhanced in the presence of CHIP and was stabilized upon treatment with the proteasome inhibitor, MG132 (Fig. 3, A and B). A similar pattern was followed when only exogenous PTEN was checked (Fig. 3C). To substantiate the role of CHIP in ubiquitinating PTEN, we tried to find out whether this function of CHIP existed under endogenous conditions as well. Accordingly, CHIP was knocked down in HEK293 cells, and the lysates were subjected to IP using anti-PTEN antibody. IB analysis revealed that depletion of endogenous CHIP significantly reduced the PTEN ubiquitination level (supplemental Fig. S2).
nous ubiquitination levels of PTEN when we depleted the cellular levels of either CHIP, Nedd4-1, or WWP2 with siRNA. The detection of Ub in PTEN-immunoprecipitated samples established CHIP to be as potent an E3 ligase for PTEN as are its existing ligases (Fig. 3D).

To further explore the ubiquitination efficiency of CHIP, we carried out an ubiquitination assay in a cell-free system. Here, GFP-PTEN, Flag-CHIP, HA-ubiquitin, and Flag-H260Q (the ubiquitin ligase mutant of CHIP) were expressed separately in HEK293 cells. GFP-PTEN was then incubated with or without HA-Ub and in the presence or absence of either Flag-CHIP or Flag-H260Q and each of the incubated mixtures was then pulled down with PTEN and IB with Ub. F, GST-PTEN (rPTEN), GST-CHIP (rCHIP), and GST were purified using a GST column. The purified proteins were then checked by SDS-PAGE and stained with Coomassie Blue. GST or rPTEN were incubated for 4 h at 37 °C with or without rCHIP, F1, and F2 in the presence of Ub and ATP. The incubated mixtures were then immunoprecipitated with anti-Ub antibody and IB with PTEN.

An in vitro ubiquitination assay was performed to rule out the involvement of any other E3 ligase(s) in the ubiquitination of PTEN and to establish CHIP as a novel E3 ligase for PTEN and for that purpose GST-PTEN (rPTEN), GST-CHIP (rCHIP), and GST were purified using a GSH-Sepharose column. With either rPTEN or GST as the substrate, an ubiquitination mixture was prepared in the presence or absence of either rCHIP or F1’ and F2’ (containing E1 and E2) alone or in combination, incubated at 37 °C for 4 h along with ATP and Ub. The incubated samples were then pulled down with anti-Ub antibody and IB with PTEN. This result quite clearly highlights the capacity of CHIP to independently ubiquitinate PTEN (Fig. 3F). Collectively, these results indicate that CHIP ubiquitinates PTEN under both in vivo and in vitro conditions.

CHIP Promotes Proteasome-mediated Degradation of PTEN—With the finding that PTEN is ubiquitinated by CHIP, we examined the level of PTEN in HEK293 cells under the influence of CHIP. Our results indicate that overexpressed CHIP down-regulates PTEN while H260Q was ineffective (Fig. 4A). The two point mutants of PTEN, G129E (lipid phosphatase activity is impaired), and C124A (both the lipid and protein phosphatase activities are impaired) were transfected with the either HA-Ub or GFP-PTEN alone or in combination in the presence of or absence of Flag-CHIP as shown in the figure, followed by treatment with or without MG132. IP was performed with either PTEN or Ub and IB against Ub, PTEN, or GFP, respectively.
CHIP Promotes Degradation of PTEN

FIGURE 4. CHIP accounts for the proteasomal degradation of PTEN. HEK293 cells were transfected with: A, 2.5 μg of Flag-CHIP or Flag-H260Q, B, 2 μg of either PTEN mutants, C124A or G129E in the presence or absence of Flag-CHIP, IB was performed against anti-GFP. C, 30 nm of scrambled siRNA or CHIP siRNA, D and E, with or without similar amounts of Flag-CHIP or CHIP siRNA, followed by a treatment with 50 μM cycloheximide (CHX) for the time period indicated in the figure. The lysates were then checked for PTEN levels. F, EV or His-CHIP and treated with or without MG132. G, with 2 μg of GFP-PTEN, 1.5 μg of HA-Ub, 2 μg of Flag-CHIP, 1.5 μg of either HA-K48 or HA-K63 in combination as indicated in the figure. IP was performed with anti-PTEN antibody, and IB was carried out with anti-Ub antibody. All lysates were prepared post 48 h of transfection. EV: empty vector.

Hampered) showed similar effect upon CHIP overexpression (Fig. 4B). Knock-down of CHIP produced the anticipated antagonistic effect (Fig. 4C).

It has been established that CHIP negatively regulates the steady-state levels of its substrates. To investigate this function, endogenous levels of PTEN were observed in overexpressed and knocked-down CHIP in HEK293 cells, followed by treatment with cycloheximide. The PTEN half-life was either decreased or enhanced in the presence of CHIP or CHIP siRNA, respectively (Fig. 4, D and E). The half-life of PTEN is ~9 h although in the presence of CHIP it was reduced to ~4 h. To verify the involvement of the proteasome system in CHIP-mediated degradation of PTEN, we treated cells with MG132. The treatment stabilized PTEN and ablated the reduction of PTEN expression even in the presence of CHIP (Fig. 4F). This result indicates a possible involvement of the proteasome system in PTEN degradation by CHIP.

We probed further into the involvement of proteasome by checking the level of PTEN ubiquitination in the presence of Ub-WT and its mutants, Ub-K48R or Ub-K63R, with or without CHIP. The ubiquitination of PTEN was not that influenced by the ubiquitin mutant K63, but the ubiquitination level was dramatically reduced in the presence of K48 (Fig. 4G). This is probably because ubiquitin molecules, which are linked together in chains to a protein utilizing the lysine48 (K48) residue of each ubiquitin, are mainly recognized by the proteasome system and act as a degradation signal while the K63-linked polyubiquitin chains appear to play a role in non-proteolytic, cell signaling (49–50). Thus it was quite evident that CHIP-regulated degradation of PTEN is through the proteasome machinery.

CHIP-mediated PTEN Regulation Is Hsp70/Hsp90-dependent—As most of the proteins regulated by CHIP are presented to it via the chaperone system, we wanted to investigate for a similar possibility in the case of PTEN modulation. To test this, HEK293 cells were treated with the Hsp90 inhibitor 17AAG in a dose-dependent manner. 17AAG specifically inhibits the ATPase activity of Hsp90, leading to the degradation of chaperone-dependent client proteins and shifts the balance of the transient association between the chaperones, and its clients towards the degradation machinery. Here, it was shown that degradation of PTEN is elevated upon 17AAG treatment in a dose-dependent manner (Fig. 5A). The involvement of the chaperone complex in this process was also supported by a direct association of PTEN with both Hsp90 and Hsp70 (Fig. 5B) and consistent with the fact that CHIP and 17AAG together showed a positive correlation in down-regulating PTEN levels (Fig. 5C). 17AAG was used to treat the scrambled or CHIP siRNA-transfected cells, which could not produce the desired effect in CHIP-depleted conditions (Fig. 5D), confirming the fact that the down-regulation of PTEN by 17AAG is through the involvement of CHIP.

Next we investigated whether the interaction between PTEN and CHIP is dependent upon the activity of CHIP or its ability to bind chaperones. We compared the interaction of PTEN with CHIP-WT, and its mutants, CHIP-K30A and CHIP-H260Q; the former being unable to interact with chaperones, and the latter is an E3 ligase-deficient mutant. The co-immunoprecipitation results showed that the interaction of CHIP-K30A with PTEN is reduced significantly (as was the case with the interaction between PTEN and ATPR domain of CHIP) while CHIP-H260Q demonstrates a strong interaction with PTEN (Fig. 5E), suggesting that the interaction of CHIP with PTEN is dependent on the K30 residue of CHIP in the TPR domain, which is critical for its chaperone binding activity. It was also observed that CHIP-K30A could not efficiently degrade PTEN (Fig. 5F). The ubiquitination of PTEN was also greatly hampered when CHIP was mutated at K30, the ubiquitination assay being performed in a cell-free system (Fig. 5G). We hereby reached to the conclusion that CHIP is a chaperone-associated E3 ligase of PTEN under physiological conditions that diverts PTEN from the chaperone to the ubiquitin proteasome system for its degradation.

CHIP Interferes with PTEN-mediated Functional Activities—The most important function of PTEN is to control AKT activation through PI3K-PDK1-mediated phosphorylation (pAKT-Ser473), which regulates diverse cellular processes. The level of pAKT-Ser473 was decreased during knock-down of CHIP in HEK293 cells without any detectable change in the total AKT level. We also examined the effect of CHIP overexpression on the pAKT level, and the result showed increased levels of AKT phosphorylation when CHIP is overexpressed (Fig. 6, A and B). A similar effect was observed in different subcellular fractions where pAKT was elevated to the maximum in the cytoplasmic pool (Fig. 6C).

Besides polyubiquitinating PTEN, CHIP could monoubiquitinate it as well (Fig. 3, A, B, and C), which encouraged us to
investigate whether CHIP had any role in the nuclear localization of PTEN. It was observed that upon CHIP siRNA transfection, PTEN was elevated in the cytoplasm while the phenomenon was reversed in the nuclear fraction. The ICC data in HeLa cells also corroborated this finding (supplemental Fig. S3, A and B), which indicates a role for CHIP in the compartmentalization of PTEN.

The transcript levels of PTEN and CHIP were compared in the prostate cancer cell lines DU145 and PC3 with respect to the levels in HEK293. The CHIP transcript level was more than PTEN in DU145 cells while the PTEN transcript was absent in PC3 cells. To confirm the role of CHIP in down-regulation of PTEN, we wanted to investigate further for any change in PTEN activities upon subsequent changes in CHIP levels while focusing on the transcriptional activity of PTEN. For this aspect, PTEN was expressed in prostate cancer cells, PC3, with PTEN-null background in the presence or absence of CHIP overexpression. cDNAs were prepared and subjected to quantitative real time PCR (qPCR). The genes of interest examined here were Rad51, Rbl2, Dre1 (up-regulated by PTEN), and VEGF (down-regulated by PTEN) (10, 52, 53) while 18 S rRNA was taken as an internal control. The regulation of all these genes by PTEN was reversed in the presence of CHIP while c-Myc showed no change (Fig. 6, D and E).

Quantitative cell cycle analysis of populations that have been stained for incorporated BrdU depicts an increase in proliferation upon CHIP overexpression in HEK293 cells. When compared with control (8.12%), BrdU-positive cells were decreased when PTEN was transfected (7.49%). CHIP transfection increased the proliferative capacity (12.19%). An anticipated compensatory effect was observed upon dual transfection of both PTEN and CHIP (9.77%) (Fig. 6F). An inverse correlation was also observed between PTEN and CHIP by immunohistochemistry in human prostate cancer patient samples (Fig. 6G). All these results indicate that CHIP is an E3 ubiquitin ligase of PTEN that can regulate its functional activities by down-regulating its cellular level.

**DISCUSSION**

We establish here, a unique degradation pathway for the tumor suppressor, PTEN. The central role in this pathway is played by the chaperone-assisted E3 ubiquitin ligase CHIP. PTEN associates transiently with the molecular chaperones and is diverted towards the proteasomal degradation pathway by CHIP. Overexpressed PTEN could interact with endogenous CHIP. The two proteins also co-localized in multiple cell lines. However, there was no visible level of endogenous interaction between them, perhaps due to the context-dependent and dynamic nature of the interaction. A similar transient nature has been observed in case of the interaction between p53 and CHIP. Nevertheless, wild type PTEN was sensitive to an increase in the cellular levels of CHIP while knock-down of endogenous CHIP reduced the level of ubiquitination and stabilized PTEN. A comparative study involving the knock-down...
of CHIP, XIAP, and WWP2 placed the ubiquitinating potential of CHIP for PTEN at par with those of the existing E3 ligases. The ubiquitination of PTEN by bacterially expressed, purified CHIP under in vitro conditions cements its position as an independent E3 ligase of PTEN. The ablation of PTEN degradation upon treatment with MG132 and the involvement of the K48-linked polyubiquitin chain in PTEN down-regulation linked the phenomenon with the proteasome. In expected lines, CHIP-mediated PTEN ubiquitination and degradation involved the chaperones. We verified this hypothesis in HEK293 cells by 17AAG treatment. The interaction between Hsp90 and its clients is inhibited by 17AAG; thereby stimulating the CHIP-induced degradation of the Hsp70/Hsp90-interacting proteins. A synergistic effect of CHIP and 17AAG was also observed in this aspect. Interaction between overexpressed PTEN and Hsp70 and Hsp90 further supported this fact. A recent report on PTEN interaction with the chaperones supports this claim. But surprisingly enough, a chaperone-independent interaction between PTEN and CHIP was also observed. CHIP was able to ubiquitinate PTEN in the absence of the chaperones albeit with a lesser efficiency. In recent years, a number of substrates by-passing the requirement of the chaperones and binding directly to CHIP have been identified that include the death domain-associated protein, DAXX and Runx1, which bind CHIP under heat-stressed and normal cellular conditions, respectively (54, 55). This presents a more complex pattern of the functioning of CHIP, wherein it can interact with its partners in the presence or absence of chaperones and under different physiological contexts. Our study reflects this complexity as PTEN is able to interact with CHIP through the chaperones but at the same time bacterially expressed and purified PTEN and CHIP could also interact with each other. While delineating the structural requirements for this interaction, it was observed that the TPR and to some extent the CC domain of CHIP and portions of the N-terminal domain of PTEN were involved. In any case, it seems that PTEN
acts as a substrate of CHIP preferentially through the involvement of chaperone machinery with some chaperone-independent context borne role of CHIP.

Overexpressed CHIP was found to interfere with PTEN-mediated transcriptional activity. The phosphorylation of AKT was altered upon both CHIP overexpression and depletion. While the depletion of CHIP seemingly caused the nuclear exclusion of PTEN, it provides some evidence for its role in the nuclear localization of PTEN. CHIP increased the proliferative capacity and partially compensated the arrest of proliferation induced by PTEN. The flow cytometric BrdU incorporation assay suggested CHIP to be somewhat pro-proliferative. When compared with control, HEK293 cells had a reduction in proliferation upon PTEN transfection, which is well anticipated, considering the tumor suppressive property of PTEN. On the other hand, CHIP had the opposite effect of inducing proliferation, which could be explained by the fact that CHIP is acting as a major E3 ligase of PTEN leading to its degradation. An anti-apoptotic role of CHIP has been established in mice knock-out models that involve the heat shock factor1 (HSF1). This role was later linked to the ability of CHIP in degrading p53. But with our set of data, CHIP-mediated PTEN suppression may be an additional factor rendering CHIP an anti-apoptotic role.

The transcript levels of CHIP and PTEN in prostate cancer cell lines shared an inverse correlation. The same pattern was followed in human prostate cancer patient samples. Recently it was reported that increased CHIP expression was associated with histological grade of glioma, which involves higher levels of survivin (56). The established fact that PTEN can suppress survivin levels (57) leads to the speculation that CHIP may play this role through the regulation of PTEN levels, although it was reported that CHIP suppressed human breast cancer progression by inhibiting oncogenic pathways (51). This might suggest that CHIP plays multi-faceted roles in cancer depending upon its types and status of progression, presenting a complex scenario of the involvement of CHIP in cancer that demands broader introspection. The results presented in this study indicate an antagonistic role of CHIP towards PTEN, which might be critical in the control of PTEN-mediated cellular functions. Now, whether CHIP is directly involved in the progression of human cancer by regulating the level of PTEN needs further investigation.

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