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WWP2 is an E3 ubiquitin ligase for PTEN

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Abstract

PTEN, a lipid phosphatase, is one of the most frequently mutated tumour suppressors in human cancer. Several recent studies have highlighted the importance of ubiquitylation in regulating PTEN tumour-suppressor function, but the enzymatic machinery required for PTEN ubiquitylation is not clear. In this study, by using a tandem affinity-purification approach, we have identified WWP2 (also known as atrophin-1-interacting protein 2, AIP-2) as a PTEN-interacting protein. WWP2 is an E3 ubiquitin ligase that belongs to the NEDD4-like protein family, which is involved in regulating transcription, embryonic stem-cell fate, cellular transport and T-cell activation processes. We show that WWP2 physically interacts with PTEN and mediates its degradation through a ubiquitylation-dependent pathway. Functionally, we show that WWP2 controls cellular apoptosis and is required for tumorigenicity of cells. Collectively, our results reveal a functional E3 ubiquitin ligase for PTEN that plays a vital role in tumour-cell survival.

PTEN (phosphatase and tensin homologue deleted on chromosome 10) is a well-defined tumour suppressor that plays a critical role in cell survival and cell death[1-3]. *PTEN* is either mutated or deleted with high frequency in various types of human cancer to promote tumorigenesis[3-7]. Homozygous deletion of *Pten* in mice leads to embryonic lethality, whereas *Pten*-heterozygous mice develop spontaneous tumours in multiple tissues[8-10]. The importance of PTEN as a tumour suppressor was also supported by the occurrence of *PTEN* germline mutations in a group of autosomal dominant syndromes such as Cowden syndrome, Bannayan–Riley–Ruvalcaba syndrome and Lhermitte–Duclos diseases, which are characterized by hamartomatous overgrowth of various tissues and predisposition to the development of breast, thyroid and endometrial cancers[11-13].

Functionally, PTEN is a lipid phosphatase[14,15], which antagonizes the cellular phosphatidylinositol 3-kinase (PI3K) signalling pathway. Activation of membrane receptor tyrosine kinases by external growth factors initiates the PI3K signalling pathway[16-18], which

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AUTHOR CONTRIBUTIONS

S.M., S.K., N.R. and V.R.P. carried out most of the experiments. J.L.P. and J.N.S. carried out the *in vivo* xenograft experiments. S.M. and J.C. designed the experiments, analysed the data and wrote the manuscript.

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leads to downstream activation of lipid kinase PI3K. Once activated, PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) and converts it to phosphatidylinositol 3,4,5-triphosphate (PtdIns(3,4,5)P_3). In turn, PtdIns(3,4,5)P_3 accumulation at the cellular membrane results in recruitment of PDK1 (phosphoinositide-dependent kinase 1) and AKT (also known as protein kinase B; PKB), leading to AKT activation. Activated AKT controls several cellular functions such as cell survival and death by modulating the function of numerous downstream substrates. PTEN negatively regulates PI3K signalling by dephosphorylating PtdIns(3,4,5)P_3 to PtdIns(4,5)P_2 and thus mediates its tumour-suppressor function by inactivating downstream oncogenic AKT-mediated signalling[19].

In addition to its tumour-suppressor activity, PTEN was recently assigned new functions such as the maintenance of the haematopoietic stem-cell population and ovarian follicle activation[20,21]. The crucial function of PTEN in multiple cellular processes and its involvement in human diseases indicate that the enzyme needs to be tightly regulated *in vivo*. Previous studies indicated that *PTEN* is indeed regulated by multiple mechanisms at either the transcriptional or post-translational level[22,23]. At the post-translational level, PTEN function is regulated by various modifications such as phosphorylation, oxidation, *S*-nitrosylation and acetylation[23]. Ubiquitylation was also shown to regulate PTEN function, but the identity of the E3 ligase that mediates PTEN ubiquitylation is controversial. Whereas NEDD4-1 (neural precursor cell expressed, developmentally down-regulated 4) was reported as an E3 ligase for PTEN in ref. 24, this was later disputed by others[25]. In an attempt to identify the E3 ligases for PTEN, we established a 293T derivative cell line stably expressing a triple-epitope (S-protein, Flag and streptavidin-binding peptide, SBP)-tagged version of PTEN (SFB–PTEN). Tandem affinity purification using streptavidin–agarose beads and S-protein–agarose beads followed by mass spectrometry analysis enabled us to identify WWP2 as one among several PTEN-interacting proteins (Supplementary Table S1). WWP2 is an E3 ubiquitin ligase that belongs to the NEDD4-like protein family[26-29]. So far, a very limited number of substrates have been reported for WWP2, such as Oct-4 (octamer-binding transcription factor 4), RNA polymerase subunit Rpb1, the epithelial sodium channel and EGR-2, which are important for regulating transcription, embryonic stem-cell fate, cellular transport and T-cell activation processes[26-29].

To validate our tandem affinity purification results, we further tested the interaction of endogenous PTEN and WWP2 in cells. PTEN interacted specifically with WWP2 (Fig. 1a) but not EDD, another member of the HECT (homologous to E6AP carboxy terminus) family of E3 ligases. Although NEDD4-1 was discovered recently as an E3 ligase for PTEN (ref. 24), we did not identify NEDD4-1 in our purification (Supplementary Table S1), nor did we detect an interaction between NEDD4-1 and PTEN (Fig. 1a), which agrees with the recent report that NEDD4-1 might not be the main physiologically relevant E3 ligase for PTEN (ref. 25). We further confirmed the existence of PTEN–WWP2 complex *in vivo* by demonstrating that WWP2 co-immunoprecipitated with exogenously expressed PTEN in 293T cells (Fig. 1b). In contrast, NEDD4-1 was not seen in Flag (PTEN) immunoprecipitates (Fig. 1b). In addition, bacterially expressed glutathione *S*-transferase (GST)-PTEN pulled down WWP2 (Fig. 1c), but not NEDD4-1 (data not shown) from cell extracts, again indicating that PTEN forms a distinct complex with WWP2. PTEN has several domains that are critically important for its function. We generated expression constructs for Flag-tagged PTEN and a series of amino-terminal or carboxy-terminal deletion mutants that lack different domains (Fig. 1d). To map the WWP2-binding region on PTEN, we co-expressed these constructs along with full-length Myc-tagged WWP2. The immunoprecipitation results indicate that WWP2 interacts with the phosphatase domain of PTEN, probably within a region comprising residues 100–187 (Fig. 1e).

As WWP2 is a known HECT-domain-containing E3 ligase that regulates ubiquitin-dependent degradation of its substrates, we further assessed the significance of the PTEN–WWP2 interaction using ubiquitylation assays. HeLa cells were transiently transfected with either wild-type WWP2 or catalytically inactive $WWP2^{C838A}$ along with haemagglutinin (HA)-tagged ubiquitin. The level of PTEN ubiquitylation detected by immunoblotting after immunoprecipitation of PTEN shows that PTEN was readily polyubiquitylated by wild-type but not catalytically inactive WWP2 (Fig. 2a). To further support the idea that WWP2 mediates PTEN ubiquitylation, we carried out *in vitro* ubiquitylation assays using GST–PTEN as substrate in the presence of wild-type or mutant WWP2 along with the E2 ubiquitin-conjugating enzyme UbcH5b. Wild-type WWP2 but not the catalytically inactive mutant resulted in robust PTEN polyubiquitylation (Supplementary Fig. S1a).

Recently, Rak kinase was shown to regulate PTEN polyubiquitylation through tyrosine phosphorylation[30]. By modulating Rak protein levels in cells, we did not observe any significant changes in PTEN–WWP2 interaction or the PTEN protein levels (data not shown), indicating that Rak-mediated tyrosine phosphorylation might not play a role in regulating WWP2-mediated PTEN ubiquitylation. Nevertheless, several patient-derived tyrosine mutations in the PTEN phosphatase domain were reported to affect the stability of PTEN protein[31-33]. As WWP2 interacts with the PTEN phosphatase domain, we further examined these patient-derived tyrosine mutations within the WWP2-binding region. Interestingly, we found that mutation of the PTEN Tyr 155 residue significantly increased the association of WWP2 with PTEN, followed by enhanced polyubiquitylation and reduced PTEN protein levels (Fig. 2b), indicating that some yet-to-be-identified tyrosine kinases may be involved in the regulation of the WWP2–PTEN interaction.

We further evaluated endogenous PTEN ubiquitylation in cells transfected with either control short interfering RNA (siRNA) or siRNAs specific for WWP2, EDD1 or NEDD4-1 in the presence of MG132, a proteosomal inhibitor. PTEN was polyubiquitylated in the presence of intact WWP2, but its ubiquitylation was significantly reduced by the depletion of WWP2 (Fig. 2c and Supplementary Fig. S1b,c). In contrast, PTEN polyubiquitylation was unaffected in cells transfected with siRNAs against EDD1 or NEDD4-1, again indicating that WWP2 might be the predominant E3 ligase for PTEN in cells. Polyubiquitylation of PTEN by WWP2 is likely to be required for PTEN degradation, as the knockdown of WWP2 but not EDD1 or NEDD4-1 increased the steady-state levels of PTEN protein (Fig. 2d). Similar results were observed with different sets of *WWP2* siRNAs (Supplementary Fig. S1d). Moreover, in a cyclohexamide chase experiment, co-expression of Myc-tagged wild-type WWP2, but not the catalytically inactive mutant, with Flag-tagged PTEN led to diminished PTEN protein half-life (Fig. 2e). On the other hand, short hairpin RNA (shRNA)-mediated knockdown of WWP2 stabilized PTEN (Supplementary Fig. S1e). Together, these data indicate that PTEN is a substrate of WWP2.

PTEN ubiquitylation was also shown to be essential for PTEN nuclear import in addition to the regulation of its protein stability[34]. Thus, we next tested whether WWP2-mediated PTEN ubiquitylation plays a role in regulating PTEN cellular localization. Endogenous PTEN localized in both nucleus and cytoplasm, and siRNA-mediated depletion of WWP2 did not significantly affect PTEN localization (Supplementary Fig. S2a). However, in contrast to wild-type PTEN, a Cowden syndrome-associated lysine mutant of PTEN, K289R, which is also defective in ubiquitylation[34], was mainly localized in the cytoplasm (Supplementary Fig. S2b). Together, these data indicate that WWP2-mediated polyubiquitylation is mainly involved in the regulation of PTEN protein stability, whereas other E3 ligases may be responsible for PTEN subcellular localization in the cell.

As PTEN is a potent negative regulator of the PI3K–AKT pathway, we next tested whether WWP2 can regulate AKT signalling through PTEN, by using a prostate cell-line model. Indeed, knockdown of WWP2 by siRNA in DU145 cells resulted in increased endogenous PTEN protein levels and a simultaneous decrease in AKT phosphorylation with no significant effect on total AKT levels (Fig. 3a). WWP2 regulates AKT activation in a PTEN-dependent manner, because simultaneous depletion of WWP2 and PTEN by siRNA rescued AKT phosphorylation. It is well known that PTEN positively regulates stress-induced apoptosis[35,36]. As WWP2 acts as a negative regulator of PTEN, we hypothesized that loss of WWP2 might sensitize cells towards stress-induced cell death. To test this hypothesis, we depleted WWP2 by siRNA and further treated the cells with doxorubicin. Indeed, knockdown of WWP2 by siRNA sensitized DU145 cells to doxorubicin-induced apoptosis (Fig. 3b and Supplementary Fig. S3a). Further, a simultaneous depletion of WWP2 and PTEN by siRNA partially rescued the cell sensitivity towards doxorubicin-induced cell death when compared with WWP2 depletion alone (Fig. 3b). In contrast, overexpression of wild-type but not catalytically inactive WWP2 in cells derived from normal prostate epithelium (BPH1) showed increased AKT phosphorylation (Fig. 3c), followed by increased resistance to stress-induced cell death (Fig. 3d). Taken together, these results indicate that WWP2 negatively regulates stress-induced cell death, in a manner at least partly dependent on PTEN.

PTEN acts as a tumour suppressor by negatively regulating the PI3K–AKT pathway. Hence WWP2, being an E3 ligase and a negative regulator of PTEN, might function as a proto-oncogene. To test this possibility, we established DU145 cell lines with stable depletion of WWP2 using retroviral-based shRNA vectors. Consistent with our previous results, DU145-WWP2 knockdown stable clones derived from two independent shRNAs showed increased PTEN levels and decreased phosphorylated AKT when compared with control shRNA-expressing cells (Fig. 4a). Further, *WWP2* shRNA-expressing cells showed a decreased rate of cell proliferation when compared with control shRNA cells (Fig. 4b). In addition, we analysed the cell-transforming ability of WWP2 by carrying out soft-agar colony-formation assays. As shown in Fig. 4c, depletion of WWP2 markedly reduced the oncogenic capability of DU145 prostate cancer cells. We also tested the oncogenic potential of WWP2 by overexpressing WWP2 in a non-tumorigenic prostate epithelial cell line (BPH1). Stable overexpression of wild-type WWP2 resulted in reduced PTEN levels (Fig. 4d) followed by increased cell proliferation (Fig. 4e). Further, the expression of WWP2 promoted the transforming capability of normal prostate epithelial cells evident in soft-agar colony-formation assays (Fig. 4f). The transforming capability of WWP2 is dependent on its E3 ligase activity because the catalytically inactive mutant of WWP2 does not support proliferation or anchorage-independent cell growth (Fig. 4e,f). The reduced rate of proliferation and the transforming ability of *WWP2* shRNA cells were partially rescued by a simultaneous knockdown of PTEN (Fig. 4g,h; Supplementary Fig. S2d), indicating that WWP2 oncogenic potential is at least partly dependent on PTEN. Further, the tumorigenic potential of WWP2 was supported by our *in vivo* xenograft experiments. Nude mice injected with *WWP2* shRNA-expressing DU145 cells showed reduced tumour growth when compared with the control shRNA-expressing cells (Fig. 4i and Supplementary Fig. S3b). Collectively, these results indicate that WWP2 might be a potential oncogene.

Several studies have indicated that PTEN function is tightly regulated by various post-translational modifications such as phosphorylation, oxidation, *S*-nitrosylation and acetylation[23]. Recent reports have also indicated that ubiquitylation plays a critical role in regulating PTEN functions[24]. However, the mechanisms and the enzymatic machinery involved in PTEN ubiquitylation are controversial and far from completely understood[25]. In this study, we have identified WWP2 as a E3 ubiquitin ligase for PTEN. We have shown that WWP2 interacts with and ubiquitylates PTEN, promoting its degradation. Interestingly,

we found that the PTEN Tyr 155 residue plays a critical role in WWP2-mediated polyubiquitylation as indicated by increased association of WWP2 with PTEN, followed by enhanced polyubiquitylation and reduced PTEN protein levels with the $PTEN^{Y155F}$ mutant when compared with wild-type PTEN. As the PTEN Tyr 155 residue has been shown to be mutated in several cancers and the mutation leads to reduced protein stability[37,38], we speculate that under normal conditions PTEN is phosphorylated at the Tyr 155 residue by an as-yet-unknown kinase and thus prevents PTEN interaction with WWP2. However, in cancer cells harbouring the $PTEN_{Y155F}$ mutation, this residue can no longer be phosphorylated, which enables the interaction with WWP2 and destabilizes PTEN protein levels. In addition, treatment of cells with doxorubicin reduced the WWP2-PTEN interaction, leading to enhanced PTEN stability (Supplementary Fig. S2c). Thus, under certain stress stimuli it is also possible that PTEN tyrosine phosphorylation could be enhanced, which may negatively affect the PTEN-WWP2 interaction and help to stabilize PTEN.

Our functional studies further indicate that WWP2 plays an important role in regulating cell death, partially in a PTEN-dependent manner by modulating the PI3K–AKT pathway. In contrast to a previous report[24], we failed to detect the interaction of PTEN with NEDD4-1 or a PTEN-associated E3 ligase activity. Although our *NEDD4-1* siRNA-mediated knockdown studies (data not shown) showed a reduced rate of cell proliferation, this might not be dependent on PTEN because we observed a similar effect of NEDD4-1 knockdown on cell proliferation in PTEN-deficient cells.

We have also uncovered a function of WWP2 as a potential oncogene. Genetic and functional studies indicated that several closely associated members of WWP2 in the HECT family of E3 ligases, such as WWP1, Itch and Smurf1/2, play crucial roles in tumorigenic processes[39]. However, so far no studies have attributed human cancers to aberrant activation of WWP2. Based on our results, WWP2 deserves further detailed investigation to fully unravel its potential in cancer development and progression. Although we identified PTEN as an important substrate of WWP2, it is certain that WWP2 has additional substrates that might also be involved in tumour progression. Our current studies are focused on identifying other functional substrates of WWP2 in tumorigenesis and extending the roles of WWP2 in other cellular processes.

METHODS

Plasmids

Full-length PTEN and $PTEN^{K289R}$ mutant were cloned into an S-protein/Flag/SBP triple-tagged destination vector using the Gateway cloning system (Invitrogen). Full-length WWP2 and $WWP2^{C838A}$ were also cloned into a Myc-tagged destination vector. GST-tagged PTEN, myelin basic protein (MBP)-tagged PTEN and HA-tagged ubiquitin vectors were generated by transferring their coding sequences into destination vectors using the Gateway system. The point mutants for WWP2 and PTEN were generated by PCR-based site-directed mutagenesis and verified by sequencing. Retroviral-based WWP2 wild type and C838A mutant were also generated by using the Gateway cloning system.

Antibodies

Rabbit anti-WWP2 antibodies were raised by immunizing rabbits with full-length GST–WWP2 fusion protein. Antisera were affinity-purified using an AminoLink Plus immobilization and purification kit (Pierce). Monoclonal anti-PTEN clone 6H2.1 (Cascade Biosciences), anti-WWP2 (1:250 dilution), anti-NEDD4-1 (1:1,000 dilution), anti-GST (1:2,000 dilution), anti-Myc (1:1,000 dilution), clone 9E10 (all from Santa Cruz

Biotechnologies), anti-EDD (1:10,000 dilution; Bethyl Laboratories), anti-pAKT, anti-AKT (both 1:1,000 dilution; Cell Signaling Technology), anti-Flag, anti-actin (both 1:10,000 dilution), anti-HA (1:1,000 dilution; all from Sigma) and anti-ubiquitin (1:2,000 dilution) (Millipore) antibodies were used in this study.

Tandem affinity purification

PTEN-associated proteins were isolated by using tandem affinitiy purification as described before[40]. Briefly, 293T cells were transfected with SFB–PTEN and then three weeks later puromycin-resistant colonies were selected and screened for PTEN expression. The PTEN-positive stable cells were then maintained in RPMI medium supplemented with fetal bovine serum and 2 $\mu g\ ml^{-1}$ puromycin. The SFB–PTEN stable cells were lysed with NETN buffer (20 mM Tris-HCl at pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) containing 50 mM β-glycerophosphate, 10 mM NaF and 1 $\mu g\ ml^{-1}$ of each of pepstatin A and aprotinin on ice for 30 min. After removal of cell debris by centrifugation, crude cell lysates were incubated with streptavidin–Sepharose beads (Amersham Biosciences) for 1 h at 4 °C. The bound proteins were washed three times with NETN and then eluted twice with 2 $mg\ ml^{-1}$ biotin (Sigma) for 60 min at 4 °C. The eluates were incubated with S-protein–agarose beads (Novagen) for 1 h at 4 °C and then washed three times with NETN. The proteins bound to S-protein–agarose beads were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and visualized by Coommasie Blue staining. The identities of eluted proteins were revealed by mass spectrometry analysis carried out by the Taplin Biological Mass Spectrometry Facility at Harvard.

Cell transfections, immunoprecipitation and immunoblotting

293T, HeLa, DU145 and BPH1 cells were transfected with various plasmids using Lipofectamine (Invitrogen) according to the manufacturer's protocol. For immunoprecipitation assays, cells were lysed with NETN buffer as described above. The whole-cell lysates obtained by centrifugation were incubated with 2 μg of specified antibody bound to either protein A or protein G–Sepharose beads (Amersham Biosciences) for 1 h at 4 °C. The immunocomplexes were then washed with NETN buffer four times and applied to SDS–PAGE. Immunoblotting was carried out following standard protocols.

Retrovirus production and infection

Full-length WWP2 wild type or C838A mutant was cloned into the pEF1A–HA–Flag retroviral vector using the Gateway system. Virus-containing supernatant was collected 48 and 72 h after co-transfection of pEF1A–HA–Flag WWP2 vectors and pcl-ampho into BOSC23 packaging cells, and was used to infect BPH1 cells in the presence of polybrene. Two days later, BPH1 cells were cultured in medium containing puromycin for the selection of stable clones. The clones stably expressing HA–Flag–tagged WWP2 were identified and verified by western blotting and immunostaining using anti-Flag antibodies. A similar protocol was used to generate DU145 stable cell lines that express either control shRNA or *WWP2* shRNA.

GST pulldown and *In vitro* binding assays

Bacterial-expressed GST–PTEN or control GST bound to glutathione–Sepharose beads (Amersham) was incubated with 293T cell lysates for 1 h at 4 °C, the washed complexes were eluted by boiling in SDS sample buffer and separated by SDS–PAGE, and the interactions were analysed by western blotting.

RNA interference

Control siRNA and the smart pool siRNAs against WWP2 (siRNA no. 1, 5′-UGACAAAGUUGGAAGGAAUU-3′; siRNA no. 2, 5′-GGGAGAAGAGACAGGACAAUU-3′; siRNA no. 3, 5′-CAGGAUGGGAGAUGAAAUAUU-3′; siRNA no. 4, 5′-ACAUGGAGAUACUGGGCAAUU-3′), and the on-target individual siRNAs against EDD (ref. 40), WWP2 (siRNA no. 1, 5′-UGACAAAGUUGGAAGGAAUU-3′; siRNA no. 3, 5′-CAGGAUGGGAGAUGAAAUAUU-3′) and NEDD4-1 (5′-GGGAAGAGAGGCAGGAUAU-3′) were purchased from Dharmacon. Prevalidated *PTEN* siRNA was purchased from Qiagen (catalogue no. SI00301504). The retroviral shRNA set for WWP2 (shRNA no. 1, 5′-AGCACAGAGUCAUUUAGAUUUA-3′; shRNA no. 2, 5′-ACCUAUGUAUUGUUUAUUUGAA-3′) was purchased from Open Biosystems. Transfection was carried out twice 30 h apart with 200 nM siRNA using Oligofectamine reagent according to the manufacturer's protocol (Invitrogen).

In vivo ubiquitylation assay

HeLa cells were transfected with various combinations of plasmids as indicated in Fig. 2a along with HA-tagged ubiquitin. At 24 h post-transfection, cells were treated with MG132 (10 μM) for 6 h and the whole-cell extracts prepared by NETN lysis were subjected to immunoprecipitation of the substrate protein. The analysis of ubiquitylation was carried out by immunoblotting with anti-HA antibodies.

In vitro reconstitution assay

The reactions were carried out at 30 °C for 15 min in 25 μl of ubiquitylation reaction buffer (40 mM Tris-HCl at pH 7.6, 2 mM DTT, 5 mM $MgCl_2$, 0.1 M NaCl, 2 mM ATP) containing the following components: 100 μM ubiquitin, 20 nM E1 (UBE1), 100 nM UbcH5b (all from Boston Biochem). Various combinations of WWP2 E3 ligase components as indicated were added to the reaction. MBP–PTEN bound to maltose–Sepharose beads was used as a substrate in the reaction. After ubiquitylation reaction, the Sepharose beads were washed five times with NETN buffer and boiled with SDS–PAGE loading buffer, and the ubiquitylation of PTEN was monitored by western blotting with anti-ubiquitin antibody.

Apoptosis assays

DU145 cells were transfected with control, WWP2, or PTEN and WWP2 siRNAs. 72 h later, transfected cells were treated with doxorubicin (1 μM, 24 h). BPH1 cells were transfected with either wild-type or mutant WWP2, and 24 h later transfected cells were treated with doxorubicin for 24 h. The apoptotic cells were then washed with PBS and stained with fluorescein isothiocyanate–Annexin V and propidium iodide according to the manufacture's protocol (BD Bioscience Annexin V Kit). Apoptotic cells (Annexin V positive, propidium iodide negative) were then determined by flow cytometry.

Immunoflouroscence staining

Cells grown on coverslips were fixed with 3% paraformaldehyde solution in PBS containing 50 mM sucrose at room temperature for 15 min. After permeabilization with 0.5% Triton X-100 buffer containing 20 mM HEPES at pH 7.4, 50 mM NaCl, 3 mM $MgCl_2$ and 300 mM sucrose at room temperature for 5 min, cells were incubated with a primary antibody at 37 °C for 20 min. After washing with PBS, cells were incubated with rhodamine- or fluorescein isothiocyanate-conjugated secondary antibody at 37 °C for 20 min. Nuclei were counterstained with 4,6-diamidino-2-phenylindole. After a final wash with PBS, coverslips were mounted with glycerine containing paraphenylenediamine.

Soft-agar colony assays

Cells were resuspended in RPMI containing 10% fetal bovine serum along with 0.5% low-melting agarose and seeded on a plate coated with 1% agarose in RPMI and 10% fetal bovine serum. Viable colonies were scored after 3 weeks of incubation and the quantified data were presented from three independent experiments.

In vivo xenografts

Animal studies were carried out with previous review and approval by the Mayo Institutional Animal Care and Use Committee. Six-week-old female athymic nude mice were subcutaneously injected with 5×10^6 cells suspended in 200 μl of PBS. Starting one week after injection, tumour volumes were measured three times per week. Each cell subline was evaluated in five different animals.

Statistical analysis

The data are expressed as means ± s.d. from an appropriate number of experiments as indicated in the figure legends. The statistical analysis was done by using Student's *t*-test and $P < 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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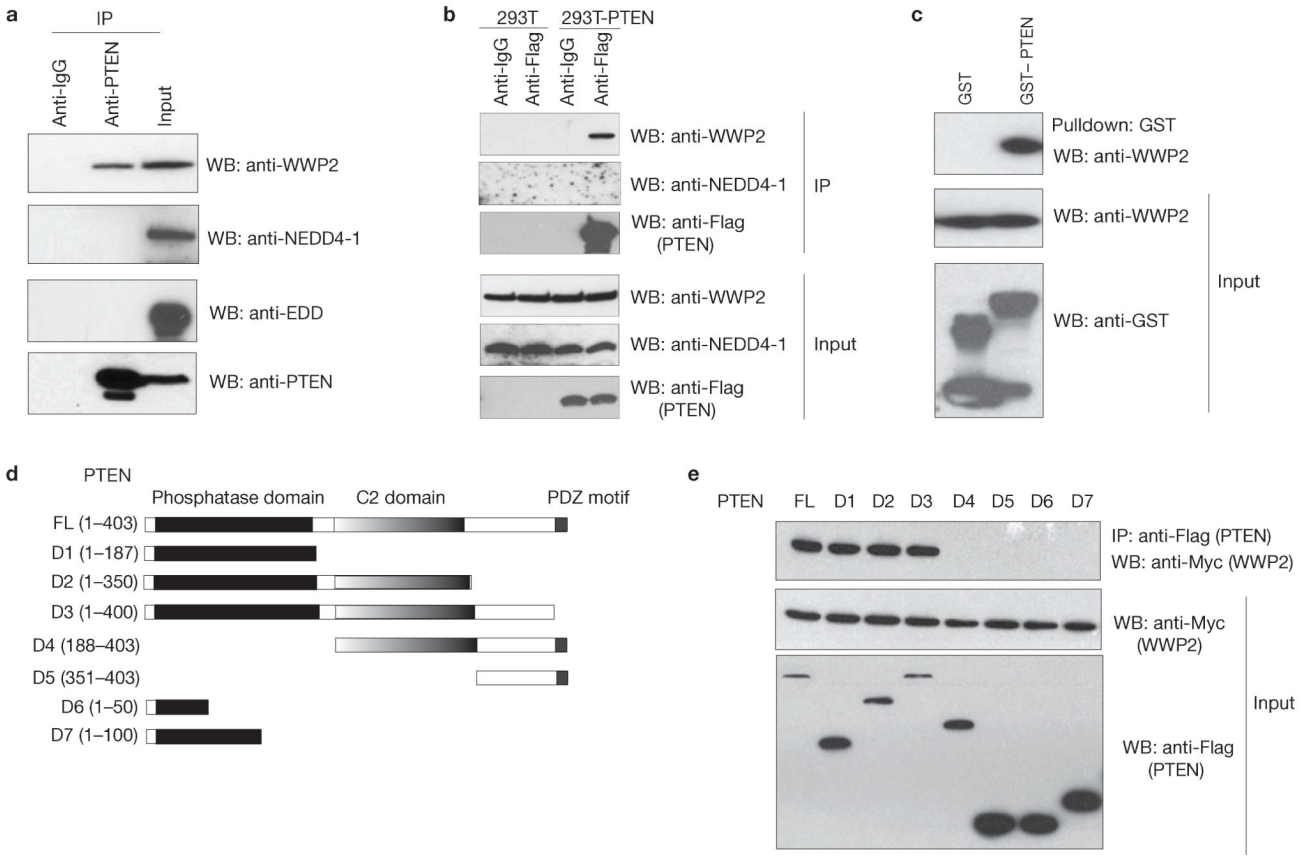

Figure 1.

WWP2 interacts with PTEN. (**a**) Immunoprecipitation (IP) using either control IgG or anti-PTEN antibody was carried out using extracts prepared from 293T cells. The endogenous interaction of WWP2, NEDD4-1 or EDD with PTEN was evaluated by immunoblotting (WB) with their respective antibodies. (**b**) Immunoprecipitation using control IgG or anti-Flag (PTEN) antibody was carried out using extracts prepared from either parental 293T cells or 293T derivative cells stably expressing Flag-tagged PTEN. The presence of WWP2 or NEDD4-1 in these immunoprecipitates was evaluated by immunoblotting with their respective antibodies. (**c**) GST pulldown assay was carried out using immobilized control GST or GST-PTEN fusion proteins on agarose beads followed by incubation with extracts prepared from 293T cells. The *in vitro* interaction of WWP2 with PTEN was assessed by immunoblotting with WWP2-specific antibodies. (**d**) Schematic representation of N-terminal Flag-tagged full-length PTEN (FL), along with its various deletion mutants (D1–D7). (**e**) 293T cells were co-transfected with the indicated Flag-tagged PTEN constructs along with those encoding Myc-tagged WWP2, and the interaction between PTEN and WWP2 was determined by immunoprecipitation and immunoblotting with the indicated antibodies. Uncropped images of blots are shown in Supplementary Fig. S4.

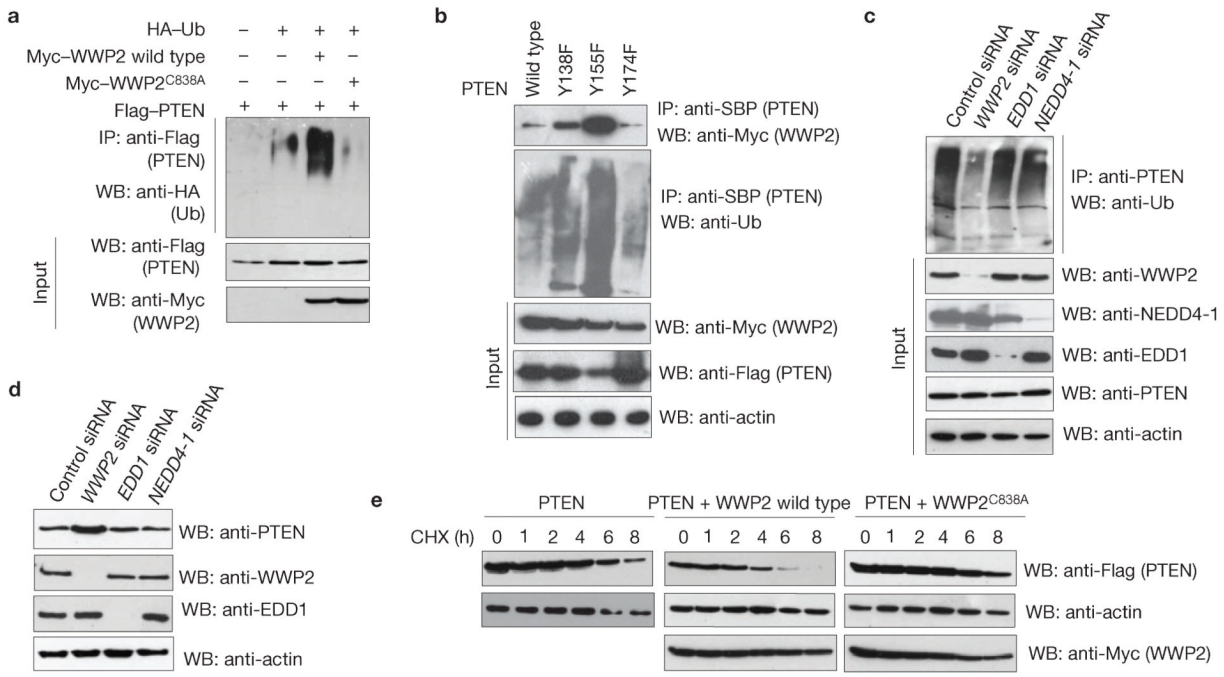

Figure 2.

WWP2 regulates PTEN protein stability by polyubiquitylation. (**a**) Myc-tagged wild-type or a catalytically inactive C838A mutant of WWP2 were expressed in HeLa cells along with Flag–PTEN and HA–ubiquitin (Ub). 24 h post-transfection, cells were treated with MG132 (10 μM) for 6 h and the levels of PTEN ubiquitylation were evaluated by immunoprecipitation of PTEN using anti-Flag antibody followed by anti-HA immunoblotting. (**b**) A triple-tagged wild-type PTEN and the PTEN tyrosine mutants along with Myc–WWP2 were expressed in 293T cells and the level of PTEN–WWP2 interaction was detected by immunoprecipitation and immunoblotting with the indicated antibodies. The level of PTEN ubiquitylation was determined by immunoblotting with anti-ubiquitin antibodies. (**c**) HeLa cells were transfected with control siRNA or siRNAs against WWP2, EDD1 and NEDD4-1. Cell lysates prepared after 6 h MG132 (10 μM) treatment were subjected to immunoprecipitaton using anti-PTEN antibodies. The ubiquitylated PTEN was detected with anti-ubiquitin antibody. The protein expression and the specificity of different siRNAs were confirmed by immunoblotting of cell extracts using antibodies as indicated. (**d**) HeLa cells were transfected with control siRNA or siRNAs against WWP2, EDD1 and NEDD4-1. The protein levels of PTEN were assessed by immunoblotting using anti-PTEN antibody. (**e**) HeLa cells transiently expressing Flag-tagged PTEN were either transfected with plasmids encoding Myc-tagged WWP2 wild type or C838A mutant. Twenty-four hours post-transfection, cells were treated with cyclohexamide (CHX) and collected at the indicated times afterwards. The protein levels of PTEN were determined by anti-Flag immunoblotting. Uncropped images of blots are shown in Supplementary Fig. S4.

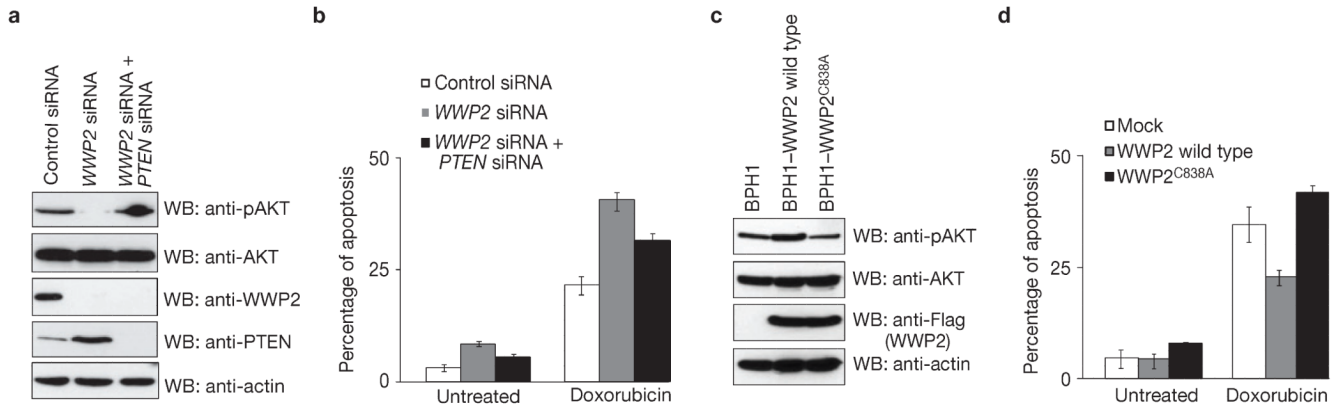

Figure 3.

WWP2 activates AKT signalling and regulates stress-induced cell death in a PTEN-dependent manner. (**a**) DU145 prostate cancer cells were transfected with either control siRNA, *WWP2* siRNA or a combination of *WWP2* siRNA and *PTEN* siRNA. 72 h after siRNA transfection, cells were lysed and cell lysates were blotted with the indicated antibodies. Activation of AKT was detected by western blotting with antibody specific to AKT phosphorylated at Ser 473 (anti-pAKT). (**b**) DU145 prostate cancer cells transfected with the indicated siRNA were either left untreated or treated with doxorubicin. The percentage of apoptotic cells was measured after 36 h of treatment by using Annexin-V staining. The data shown are derived from three independent experiments (±s.d., for doxorubicin treatment $P < 0.01$ for cells expressing *WWP2* siRNA, compared with cells expressing control siRNA or *WWP2* and *PTEN* siRNA; Student's *t*-test). (**c**) BPH1 (prostate epithelial-derived cell line) cells were transfected with either wild-type WWP2 or the C838A mutant. The cells were lysed and the activation of AKT was detected by western blotting with antibody specific to AKT phosphorylated at Ser 473 (anti-pAKT). Total AKT and expressed WWP2 proteins were detected by using AKT and Flag antibodies respectively. Actin was used as a loading control. (**d**) BPH1 cells transiently expressing wild-type or mutant WWP2 were either left untreated or treated with doxorubicin. After 36 h of treatment, apoptotic cells were determined by Annexin-V staining. The data shown are derived from three independent experiments (±s.d., for doxorubicin treatment $P < 0.05$ for cells expressing WWP2 wild-type, compared with mock treatment; Student's *t*-test). Uncropped images of blots are shown in Supplementary Fig. S4.

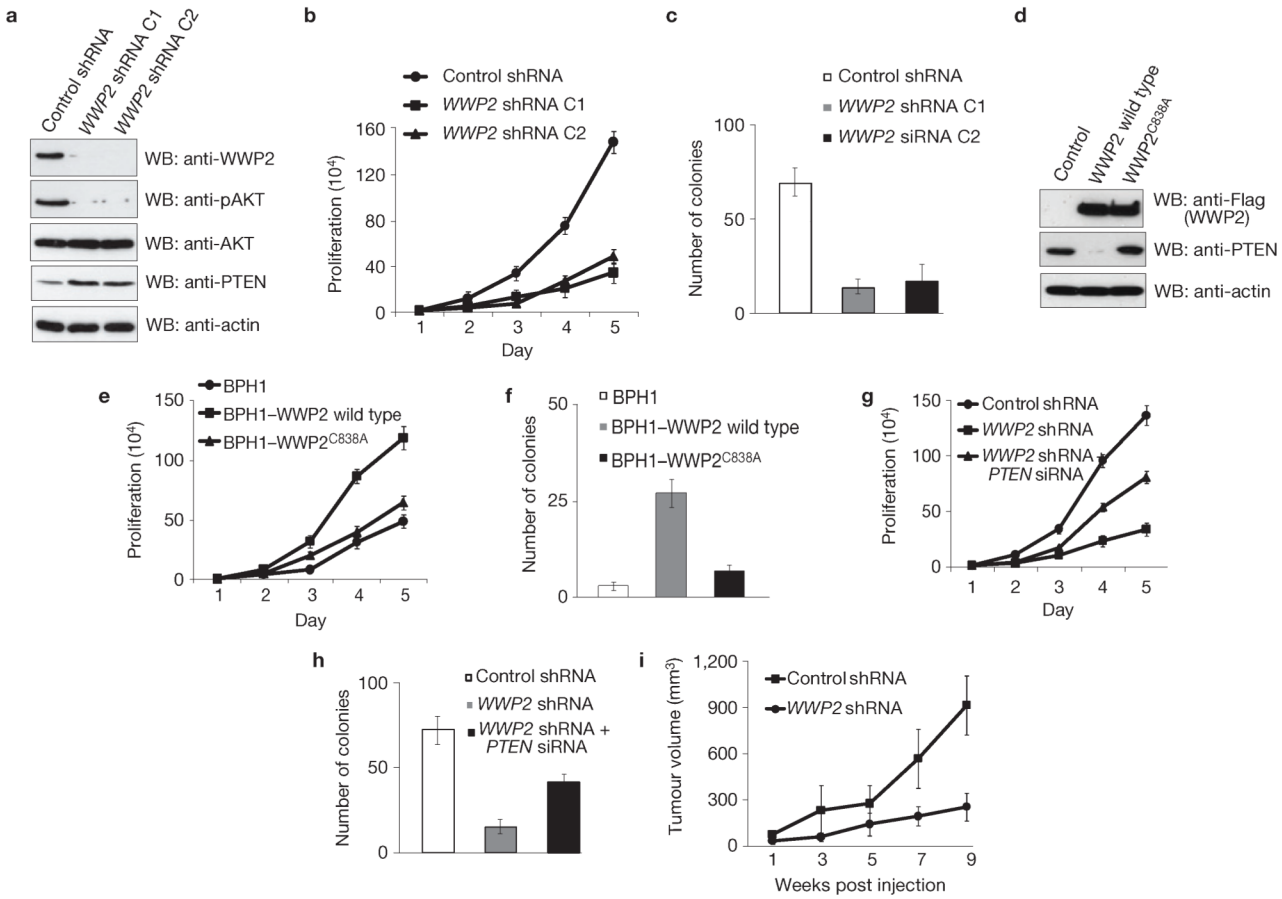

Figure 4.

WWP2 is required for tumorigenicity of cells. (**a**) DU145 cells were stably transfected with either retroviral-based control shRNA or two different *WWP2* shRNAs. The expression levels of various proteins were analysed by immunoblotting with their respective antibodies. Actin was used as a loading control. (**b**) DU145 clones stably expressing control shRNA or WWP2 shRNAs were seeded and analysed for proliferation. The data shown are derived from four independent experiments (±s.d., $P < 0.01$, compared with cells expressing control shRNA; Student's *t*-test). (**c**) DU145 cell lines stably expressing control shRNA or *WWP2* shRNA were tested for anchorage-independent growth in a soft-agar colony assay. Viable colonies after 3 weeks were counted and the data (±s.d.) from three independent experiments were presented ($P < 0.01$, compared with cells expressing control shRNA; Student's *t*-test). (**d**) Puromycin-resistant BPH1 prostate epithelial cells stably expressing either WWP2 wild type or the C838A mutant were established and the expression levels of PTEN and WWP2 were detected by the indicated antibodies. (**e**) A BPH1 parental cell line and BPH1-WWP2 wild-type or C838A mutant cells were analysed for proliferation in a similar way to that described in **b**. The data shown are derived from four independent experiments (±s.d., $P < 0.05$, compared with BPH1 parental cell line; Student's *t*-test). (**f**) A non-transformed BPH1 cell line along with WWP2 wild-type- or C838A-mutant-expressing BPH1 cells were tested for anchorage-independent growth in a similar way to that described in **c**, and the data (±s.d.) were presented as summary of three independent experiments ($P < 0.05$, compared with BPH1 parental cell line; Student's *t*-test). (**g**) DU145 stable clones expressing control shRNA or *WWP2* shRNA alone or in combination with *PTEN* siRNA were analysed for proliferation in a similar way to that described in **b**. The data shown are derived from four

independent experiments (±s.d., $P < 0.01$, compared with cells expressing control shRNA; Student's *t*-test). (**h**) DU145 stable cell lines expressing control shRNA or *WWP2* shRNA alone or in combination with *PTEN* siRNA were tested for anchorage-independent growth in a similar way to that described in **c** and the data (±s.d.) were presented as a summary of three independent experiments ($P < 0.05$, compared with cells expressing control shRNA; Student's *t*-test). (**i**) Control shRNA- or *WWP2* shRNA-expressing DU145 stable cells (5×10^6) were subcutaneously injected into nude mice and the tumour volumes were measured three times per week (±s.d., $n = 5$, $P < 0.05$, compared with cells expressing control shRNA; Student's *t*-test). Uncropped images of blots are shown in Supplementary Fig. S4.