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Published in:
Journal of Biological Chemistry

DOI:
10.1074/jbc.M116.727750

Publication date:
2016

Document Version
Publisher's PDF, also known as Version of record

Link to publication in Heriot-Watt University Research Portal

Citation for published version (APA):
Controlling PTEN (Phosphatase and Tensin Homolog) Stability: a Dominant Role for Lysine 66

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Running title- Controlling PTEN stability: a dominant role for Lysine 66

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Keywords: PTEN, Ubiquitination, PTEN stability, Cancer, PI 3-Kinase, Phosphatase

ABSTRACT

PTEN is a phosphoinositide lipid phosphatase and one of the most frequently disrupted tumour suppressors in many forms of cancer, with even small reductions in the expression levels of PTEN promoting cancer development. Although the post-translational ubiquitination of PTEN can control its stability, activity and localisation, a detailed understanding of how PTEN ubiquitination integrates with other cellular regulatory processes and may be dysregulated in cancer has been hampered by a poor understanding of the significance of ubiquitination at individual sites. Here we show that Lys66 is not required for cellular activity, yet dominates over other PTEN ubiquitination sites in the regulation of protein stability. Notably, combined mutation of other sites (Lys13, 80 and 289) has relatively little effect on protein expression, protein stability or PTEN polyubiquitination. The present work identifies a key role for Lys66 in the regulation of PTEN expression and provides both an opportunity to improve the stability of PTEN as a protein therapy and a mechanistic basis for efforts to stabilise endogenous PTEN.

INTRODUCTION

The function of the PTEN tumour suppressor is disrupted in many forms of cancer by diverse mechanisms (1,2). PTEN is a PtdInsP3 lipid phosphatase which acts to oppose the class I phosphoinositide 3-kinases and therefore loss of PTEN function leads to aberrant accumulation of PtdInsP3 and activation of downstream oncogenic PI3K-dependent signalling as well as potentially other PI3K independent changes (2,3). PTEN is currently known to exist in two forms, a 403 amino acid cytosolic and nuclear protein usually simply called PTEN, and a longer 576 amino acid protein, PTEN-L, which due to its ability to cross cell membranes and inhibit tumour formation when injected into in mice, has been proposed as a protein therapy (4-6).

Although in clinical data from some tumour types, notably breast cancer, reduced PTEN mRNA levels correlate well with reduced PTEN protein (7), in others including prostate cancer, reduced PTEN protein levels are frequently observed in tumours apparently retaining normal levels of PTEN mRNA (8). Among the mechanisms potentially responsible for this selective loss of PTEN protein expression, accelerated degradation by post-translational ubiquitination seems likely to contribute. This conclusion is supported by the paradigm established for the p53 protein and the ubiquitin E3 ligase MDM2 that destabilisation of tumour suppressor proteins can be a significant oncogenic driver and also accumulating evidence for the dysregulation of ubiquitin ligases and proteases that control PTEN stability in several cancer types (9-12).

In addition to its stability, the activity and localisation of PTEN can also be controlled by its ubiquitination, notably with PTEN mono-ubiquitination being linked to its nuclear accumulation (13-15). Several E3 ubiquitin ligases have been proposed to contribute to PTEN ubiquitination, including NEDD4 and the related HECT-domain E3 ligase WWP2, as well as XIAP,
CHIP, RFP, SPOP and MKRN1 (9,10,16-20) and ubiquitin proteases have been identified which can deubiquitinate PTEN, including HAUSP/USP7, OTUD3 and USP13 (11,12,21). Although many of these studies have identified important effects of manipulating these ligases and proteases on PTEN function and often PTEN stability, clear pictures are yet to emerge of the molecular details of these regulatory events, in particular the sites at which PTEN becomes ubiquitinated, the form of the ubiquitin chains generated and, beyond proteasomal degradation, the mechanisms by which ubiquitination influences PTEN function, such as ubiquitin-dependent binding partners. To date, only two sites of PTEN ubiquitination have been described, Lysine 13 (K13) and Lysine 289 (K289), although evidence supports the existence of several more (15). Here we show that a novel site of PTEN ubiquitination identified in an unbiased proteomic screen, Lysine 66 (K66), is polyubiquitinated in cells and dominates in the regulation of PTEN stability.

RESULTS

Ubiquitination site prediction software which successfully predicts PTEN K13 and K289 ubiquitination (22) also predicts sites at K66 and K147. More significantly, global tissue proteomic data (23) identifying ubiquitinated lysine residues reveals two new sites on PTEN in brain and liver at K66 and K80 (Figure 1A). Therefore, we studied the effects on PTEN function and stability of mutating these sites to similarly charged and sized arginine (R) residues which cannot be ubiquitinated. Wild-type PTEN (PTEN WT) and mutants, K13R, K66R, K80R and K289R were each expressed transiently and polyclonally using lentiviruses in PTEN null U87MG glioma cells alongside a phosphatase inactive mutant in which the active site nucleophilic cysteine is replaced (PTEN C124S) and a known unstable mutant (PTEN A3 which has 3 C-terminal phosphorylation sites mutated to alanine residues, S380A T382A T383A termed A3) as controls. Although levels of PTEN mRNA in each sample were not significantly different (Figure 1B, lower panel), levels of PTEN protein expression were significantly higher for PTEN K66R (Figure 1B, upper panel and Figure 1C). No significant changes in PTEN protein expression levels were observed for other ubiquitination site mutants (Figure 1C). Transfection experiments with independent plasmid expression vectors gave a similar result, showing elevated protein levels for PTEN K66R (Figure 1D). None of these mutations greatly affected PTEN activity as assessed by the ability to suppress cellular phosphorylation of the downstream PI3K/PIP3-regulated kinase AKT, except the known phosphatase dead PTEN C124S control (Figure 1B,D). To judge the consistency of this apparent effect, the experiment was repeated in several other PTEN null cell types: MDA-MB-468 breast cancer, PC3 and LNCaP prostate cancer and a PTEN-null clone of HCT116 colon cancer cells (24). In all cases, as in U87MG cells, expression of PTEN K66R was higher than wild-type and in most cases noticeably higher than the other mutants analysed (Figure 1E-H). To test whether the PTEN K66R mutation and the resultant higher levels of mutant protein cause a greater effect on cell signalling and proliferation, U87MG cells were transduced with increasing doses of multiple independently prepared viruses encoding PTEN WT or PTEN K66R. PTEN K66R viruses always led to a higher PTEN expression level (Figure 1A) and showed a concomitant greater inhibition of downstream cell signalling as seen by greater suppression of the phosphorylation of both AKT and ribosomal protein S6 (Figure 2A-C). PTEN K66R expression also had a greater effect on cell proliferation (Figure 2E). The observed effects on both signalling and proliferation appear to mirror PTEN expression level with no evidence for altered specific activity found (Figure 2D), suggesting that the K66R mutation increases PTEN protein expression without strong independent effects on protein function.

To dissect the consequences of ubiquitination at individual sites, a mutant was prepared with all the four lysine residues (K13, K66, K80 and K289) mutated to arginine, termed PTEN Kall4R (Figure 3A). When its expression in several different cell lines was investigated, this mutation of all the four lysine residues together resulted in similar or only marginally greater PTEN expression than K66R mutation alone in all the cell lines examined (Figure 3B-E), further implying a major role for K66 in regulating PTEN turnover. To test the effects of these mutations directly on protein stability, PTEN null cells expressing the mutants were treated with the...
protein synthesis inhibitor cycloheximide and the decay of extant protein observed. PTEN A3 was less stable than the wild-type enzyme, whereas PTEN K13R, K80R and K289R were all similar to, or modestly more stable than, PTEN WT. In contrast, both PTEN K66R and Kal4R with K66 mutated were significantly more stable (Figure 3F,G).

To test the hypothesis that the observed effects of lysine mutations on PTEN stability were mediated directly through changes in ubiquitination, we applied a previously established assay of cellular ubiquitination using Flag epitope-tagged ubiquitin (13,25). These experiments showed strong ubiquitination of wild-type PTEN which was dramatically lower (~95%) in PTEN Kal4R (Figure 4), suggesting that these four lysines do represent the major sites of ubiquitination incorporation into PTEN, or at least are required for incorporation at other sites. Although PTEN K13R, K80R and K289R appeared to display somewhat lower ubiquitination than PTEN WT, PTEN K66R had very low levels of ubiquitination, similar to Kal4R (Figure 4). These results strongly indicate that K66 specifically is required for the normal levels of PTEN polyubiquitination.

To further test the contributions of individual ubiquitination sites and the potential specific significance of K66, PTEN expression vectors were made in which one of the four mutated ubiquitination sites in PTEN Kal4R (K13R K66R K80R K289R) was mutated back to lysine, so that each construct expresses PTEN with only one of these lysine residues available for ubiquitination. For brevity of labelling, these constructs are referred to as shown in Figure 5A as, e.g. K13K which is K66R K80R K289R with only K13 residue available for ubiquitination out of these four studied ubiquitination sites. Notably, the higher PTEN expression seen in PTEN Kal4R is reduced to WT PTEN levels if Lysine 66 is retained in its wild-type form (i.e. K13R K80R K289R labelled PTEN K66K). In contrast individually retaining K13, K80 or K289 in their wild-type form (mutants PTEN K13K, K80K and K289K, respectively) had no effect on the PTEN expression, with these mutants still showing similar enhanced PTEN expression as seen with PTEN Kal4R (Figure 5B,C). Direct analysis of PTEN protein stability showed that these effects on expression levels correlated with observed protein half-life, with PTEN proteins retaining K66 showing significantly lower stability than mutants which include the K66R change (i.e. PTEN Kal4R, K13K, K80K and K289K) (Figure 5D,E). The low level of ubiquitination seen in PTEN Kal4R is also reverted to WT PTEN levels if K66 is retained in unmutated form (i.e. PTEN K66K). In contrast, other PTEN Kal4R derivatives with K66R change (i.e. PTEN K13K, K80K or K289K) despite the presence of K13, K80 or K289, respectively, show low levels of ubiquitination as seen in PTEN Kal4R (Figure 5F). These results provide further compelling evidence for a dominant role of K66 in regulating PTEN expression, stability and its polyubiquitination. Additionally, experiments using antibodies which recognise poly-ubiquitin chains with specific linkage topologies suggested that the poly-ubiquitination occurring on K66 of PTEN may be linked through K48 of ubiquitin (a poly-ubiquitin chain topology which is commonly involved in proteasomal degradation of target proteins), as a stronger signal was apparent with a K48-linkage specific ubiquitin antibody with immunopurified PTEN K66K (Figure 5F). Further methods would need to be used to provide conclusive evidence of chain topology specific ubiquitination.

Unstable PTEN mutant proteins have been identified encoded in the germ line of patients with diverse pathologies which appear to be linked to the biological activity of these mutant enzymes (26-29). Treatment of a subset of unstable PTEN mutants found in PHTS (PTEN hamartoma tumour syndrome) patients with proteasome inhibitors could restore mutant PTEN protein levels (30) highlighting an important role for the ubiquitin proteasome pathway in controlling their stability, irrespective of the mechanism(s) by which the relevant PTEN mutations diminish protein stability. Therefore, we investigated the effects of the K66R mutation on the expression of unstable PTEN mutant proteins identified in patients with tumour and autism-related syndromes. Additional mutation of K66R increased the protein expression level of the unstable PTEN mutant proteins D252G and H118P which have been identified in patients with autism (29) and also appeared to have a similar but more modest effect on PTEN
L108P, an unstable PTEN mutant protein which has been identified in patients with severe tumour phenotypes (29,30) (Figure 6A,B). In further study of the stabilisation of the PTEN D252G disease-associated unstable protein, the K66 ubiquitination site seemed dominant over other ubiquitination sites, as all mutant PTEN proteins containing the K66R mutation showed significant stabilisation of D252G mutant enzyme and other K to R mutations had little or no effect even when combined (termed the D252G-K66K mutant, with only K66 retained in its wild-type form) (Figure 6C,D). It was also noted that the treatment with proteasome inhibitor MG132 lead to a modest increase in the protein stability of the unstable PTEN D252G mutant. Of note, the same MG132 treatment in PTEN D252G mutant with the additional K66R mutation does not show any further significant increase in the protein levels (Figure 6E). Finally, the PTEN K66R mutant was also found to be resistant to the small but significant NO-mediated proteasomal degradation of PTEN that has been proposed as the mechanism for PTEN inhibition in neurodegeneration (31) (Figure 6F). Taken together, these results strongly point to a dominant role of K66 ubiquitination site in controlling ubiquitin proteasome-mediated degradation of PTEN and thus in regulating PTEN stability. We found that the PTEN K66R mutant could equally well bind to PTEN ubiquitin ligase NEDD4 as PTEN WT (Figure 6G).

DISCUSSION

In this study, we present the first studies of a novel PTEN ubiquitination site, Lysine 66, and show that its mutation causes a large increase in PTEN stability. Our data show that Lysine 66 specifically is required for the normal levels of PTEN polyubiquitination and imply that Lysine 66 is the site of much of the polyubiquitination on PTEN, promoting the proteasome-mediated degradation of PTEN. Importantly, we found in multiple cell types that PTEN Lysine 66 dominates over other known ubiquitination sites (Lysine 13, 80 and 289) in regulating PTEN expression, stability and PTEN polyubiquitination. Two alternate sites of PTEN ubiquitination, K13 and K289 have been previously identified as major monoubiquitination sites essential for cytoplasmic-nuclear shuttling of PTEN (15). Our data indicate that they can influence PTEN stability but play weaker roles than K66. It has been previously observed that PTEN ubiquitination can suppress phosphatase activity (13). Expression of PTEN K66R did have greater effects than the wild-type enzyme on downstream AKT-mediated signalling, however, this correlates with its higher cellular concentration (Figure 2). Along with the location of Lys66 on the surface of the PTEN phosphatase domain facing away from the active site and membrane-interacting surface, this implies that direct effects of PTEN ubiquitination on phosphatase activity are more likely to be mediated through other sites of ubiquitination.

Notably, in the context of unstable mutant PTEN proteins which have been identified in sporadic tumours and germline PTEN mutation carriers, we observed that additional mutation of the K66 ubiquitin site could significantly improve PTEN stability.

The upregulation of PTEN ubiquitin ligases or downregulation of PTEN deubiquitinases has been implicated in accelerated PTEN degradation and thereby in driving tumourigenesis and has identified these E3 ligases and deubiquitinases as potential drug targets (9-12). Future studies firmly identifying the E3 ligases or deubiquitinases which specifically modify PTEN K66 would be highly relevant for the development of therapies aiming to indirectly enhance PTEN expression. The potential of PTEN as a protein therapy has been raised by the discovery of a cell-penetrant form of PTEN (PTEN-Long or PTEN-L) which can cause regression of tumour xenografts when purified protein is injected into mice (4). Additionally, mutations have been identified which improve the cellular activity of PTEN through increased membrane association and which could be used in the context of PTEN-L (32). Particularly since increased membrane association of PTEN also leads to poly-ubiquitination and destabilisation (13), it seems an appealing strategy to engineer PTEN-L protein in this way with mutations leading both to enhanced activity and stability.

EXPERIMENTAL PROCEDURES

Cell Culture and Lentivirus Transduction– U87MG glioblastoma, MDA-MB-468 breast cancer, PC3 and LNCaP prostate cancer and
HEK293T embryonic kidney cell lines were procured from the European Collection of Animal Cell Cultures (Public Health England, Salisbury, UK) and maintained in the recommended medium supplemented with 10% fetal bovine serum (FBS). PTEN-null clone of HCT116 colon cancer cells were kindly provided by Todd Waldman (Georgetown University) and cultured in McCoy’s 5A medium with 10% FBS. Standard cell culture medium, additives, and sera were from Invitrogen. Lentiviral particles were prepared as described previously (33). Cells were transduced with lentviruses in medium supplemented with polybrene (16 μg/ml; Sigma). The medium was changed 24 hours after transduction, and polyclonal cell populations were processed without selection 48 hours after transduction.

Preparation of Whole Cell Extracts, SDS-PAGE, and Western Blotting – Cells were washed twice with ice-cold phosphate-buffered saline and lysed in ice-cold lysis buffer (25 mM Tris-HCl, pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1 mM EGTA, 1 mM EDTA, 5 mM sodium pyrophosphate, 10 mM β-glycerophosphate, and 50 mM sodium fluoride) containing 0.1% 2-mercaptoethanol and protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Equal amounts of proteins were separated by SDS-PAGE using precast 4–12% gradient gels (Invitrogen) and blotted onto polyvinylidene difluoride membranes (Polyscreen, PerkinElmer Life Sciences). Most reagents for electrophoresis and blotting were purchased from Invitrogen, and standard manufacturers’ protocols were followed. Mouse anti-PTEN monoclonal antibody (clone A2B1) was purchased from Santa Cruz Biotechnology. Antibodies against phospho-AKT (Ser473), phospho-AKT (Thr308), total AKT, phospho-S6K (Thr389), total S6K, phospho-S6 (Ser240/244), total S6, K48-linkage specific polyubiquitin, K63-linkage specific polyubiquitin and NEDD4 were obtained from Cell Signalling Technologies. Anti-GAPDH antibody was from Merck Millipore. Anti-FLAG antibody M2 was purchased from Sigma.

In cell PTEN ubiquitination assay – Cellular assays to measure PTEN polyubiquitination were performed as described previously (13,25). Briefly, where indicated, cells were transfected with vectors encoding PTEN and flag-epitope tagged Ubiquitin. Whole cell extracts were prepared using lysis buffer containing fresh protease and phosphatase inhibitors and 40 mM N-ethylmaleimide (Sigma). PTEN was immunoprecipitated from an equal volume of the diluted cell lysates containing 1 mg of soluble protein using monoclonal antibody A2B1 pre-coupled with protein G sepharose beads. The proteins bound to beads were released by boiling in SDS-PAGE sample buffer (Invitrogen) for 10 min. The samples were then resolved by 4–12% SDS-PAGE, followed by immunoblot analysis using anti-FLAG antibody M2 or mouse monoclonal A2B1 anti-PTEN antibody.

Acknowledgments
We would like to thank Todd Waldman (Georgetown University) for providing us PTEN-null clone of HCT116 colon cancer cells and the members of the NRL for their support and constructive discussions. This work was funded by the Medical Research Council (grant code G0801865/2) and by SU2P.

Conflict of Interest
The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions
Conceived and designed the experiments: AG, NRL. Performed the experiments: AG. Analyzed the data: AG, NRL. Wrote the paper: AG, NRL.
REFERENCES


The abbreviations used are: PTEN, phosphatase and tensin homolog; PtdInsP3, phosphatidylinositol 3,4,5-trisphosphate; WT, wild-type; K, lysine; R, arginine; NEM, N-ethylmaleimide; Ub, ubiquitin; PHTS, PTEN hamartoma tumour syndrome.

FIGURE LEGENDS

FIGURE 1. Mutation of PTEN Lysine 66 increases PTEN expression. (A) A cartoon illustrating the 403 amino acid PTEN protein and the ubiquitinated lysine residues analysed in this study. (B, C) PTEN-null U87MG glioblastoma cells were transduced with similar units of lentivirus particles encoding for PTEN WT or the indicated PTEN mutants. Control cells were transduced with viruses encoding EGFP. PTEN expression and AKT phosphorylation were investigated by western blotting of total cell lysates using total and phospho-specific antibodies. GAPDH levels were used as a loading control. A representative blot from at least three independent experiments is shown. Parallel samples from U87MG cells transduced with PTEN WT or mutants were used to analyze the levels of PTEN mRNA by Q-PCR (B, lower panel). The PTEN transcript levels are expressed relative to GAPDH. Y-axis represents the average levels of transcript ± SEM from three experiments each performed in duplicate. No significant difference in the transcript levels was observed between PTEN WT and each PTEN mutant-expressing sample. (C) Bar representing the relative densitometric values of PTEN expression. The quantitation is derived from three independent experiments and values are mean ± SEM. PTEN K66R was significantly more stable compared to PTEN WT (P value < 0.001). No significant changes in PTEN levels were observed with mutation of other ubiquitin sites (n.s, non significant). (D) PTEN-null U87MG glioblastoma cells were transfected with plasmid expression vectors encoding for PTEN WT, PTEN mutants or EGFP as a control. Cells were lysed 48 hours post transfection and PTEN expression and AKT phosphorylation were investigated by western blotting of total cell lysates using total and phospho-specific antibodies. GAPDH levels were used as a loading control. A representative blot from three independent experiments is shown. These plasmid transfection experiments recapitulate the increase in PTEN expression with K66R mutation relative to PTEN WT (P value < 0.001) as observed with lentiviral transduction approach. (E-H) PTEN-null clone of HCT116 colon cancer cells (E), MDA-MB-468 breast cancer cells (F), PC3 (G) and LNCaP prostate cancer cells (H) were transduced with similar units of lentivirus particles encoding for PTEN WT or the indicated PTEN mutants. Control cells were transduced with viruses encoding EGFP. PTEN expression and AKT phosphorylation were investigated by western blotting of total cell lysates using total and phospho-specific antibodies. GAPDH levels were used as a loading control. In each case a representative blot from at least three independent experiments is shown. In all cases, as in U87MG cells, expression of PTEN K66R was significantly higher than wild-type (P value < 0.01).

FIGURE 2. Mutation of PTEN Lysine 66 increases PTEN expression and shows a concomitant greater effect on downstream cell signalling and proliferation. (A-D) U87MG cells were transduced with increasing doses of lentiviruses encoding EGFP, PTEN WT or PTEN K66R (A) PTEN expression and effect on downstream cell signaling was analyzed by immunoblotting with PTEN, phospho-AKT Ser473, phospho-AKT Thr308, AKT, phospho-S6K Thr429, S6K, phospho-S6 Ser235/236 and S6 antibodies. GAPDH levels were used as a loading control. (B, C) Graph representing the effect of increasing doses of PTEN WT or PTEN K66R lentiviruses on the suppression of (B) AKT Ser473 (C) S6 Ser235/236 cellular phosphorylation, relative to EGFP transduced cells. Y-axis shows the mean relative densitometric values of phosphorylation ± SEM from three independent experiments. X-axis shows the units of lentivirus used. PTEN K66R lentiviruses were significantly more effective in suppressing both AKT and S6 phosphorylation compared with similar dose of PTEN WT lentiviruses. (D) The graph representing the specific activity of PTEN-WT vs PTEN K66R. The average relative densitometric values of AKT Ser473 phosphorylation from three independent experiments for PTEN WT and PTEN K66R was plotted against the relative PTEN expression levels with increasing doses of PTEN WT or PTEN K66R lentiviruses. (E)
PTEN null U87MG cells were transduced with similar units of lentiviruses encoding EGFP, PTEN WT or PTEN K66R and viable cell number was quantified 5 days post transduction by MTT assay. X-axis shows the mean measured cellular metabolic activity relative to EGFP transduced cells ± SEM from three experiments each performed in triplicate. t-test ** P value < 0.01; * P value < 0.05; n.s, non significant.

FIGURE 3. Dominant role of PTEN Lysine 66 ubiquitination site in the regulation of PTEN expression and stability. (A) A cartoon illustrating the mutant PTEN Kall4R with all the four studied potential ubiquitination sites (Lysine13, K66, K80 and K289) mutated to arginine (R). (B-E) HEK293T cells (B), PTEN-null HCT116 colon cancer cells (C), PC3 prostate cancer cells (D) and MDA-MB-468 breast cancer cells (E) were transduced with similar units of lentivirus particles encoding for PTEN WT, indicated PTEN mutants or control EGFP. PTEN expression and AKT phosphorylation were investigated by western blotting of cell lysates using total and phospho-specific antibodies. GAPDH levels were used as a loading control. A representative blot from three independent experiments is shown. (F, G) The stability of PTEN ubiquitin-site mutants was determined using protein synthesis inhibitor cycloheximide (CHX). (F) PTEN-null U87MG cells transduced with viruses encoding PTEN WT or PTEN mutants were treated with 100μg/ml CHX for indicated times, followed by immunoblot analysis with anti-PTEN or anti-GAPDH antibodies. The blots shown are representative of three independent experiments. (G) Plot showing densitometric analysis of the CHX assay. Y-axis shows the relative PTEN levels (in percent) after normalizing with GAPDH levels. The quantitation is derived from three independent experiments and values are mean ± SEM. Both PTEN K66R and PTEN Kall4R, with K66 mutation, were significantly more stable compared to PTEN WT (P value < 0.01).

FIGURE 4. Dominant role of PTEN Lysine 66 ubiquitination site in the regulation of PTEN polyubiquitination. Cellular assay for PTEN ubiquitination. U87MG cells were transfected with an expression vector encoding FLAG-ubiquitin (Flag-Ub). 6 hours after transfection cells were transduced with viruses encoding PTEN WT or PTEN mutants. Lentiviruses were titrated prior to the experiment to ensure similar levels of PTEN protein in each sample. 48 hours post-transfection cells were treated with 10μM MG132 for 5 hours. Cells were lysed, and PTEN was immunoprecipitated (IP) before western blotting (WB) these immunoprecipitates with antibodies against the FLAG epitope and PTEN. In parallel, total cell lysates were immunoblotted with antibodies raised against the FLAG epitope, PTEN or GAPDH (left panel).

FIGURE 5. Contribution of individual ubiquitination sites in regulating PTEN expression, stability and PTEN polyubiquitination. (A) Cartoon illustrating PTEN mutants generated to assess the contribution of individual ubiquitination sites to expression and stability. (B, C) HEK293T cells (B) and PTEN-null U87MG glioblastoma cells (C) were transduced with similar units of lentivirus particles encoding for PTEN WT or PTEN mutants or EGFP. PTEN expression was investigated by western blotting GAPDH levels were used as a loading control. A representative blot from three independent experiments is shown. (D, E) The stability of these PTEN mutants was determined using protein synthesis inhibitor cycloheximide (CHX). (D) U87MG cells transduced with viruses encoding PTEN WT or PTEN mutants were treated with 100μg/ml CHX for indicated times, followed by immunoblot analysis with anti-PTEN or anti-GAPDH antibodies. The blots shown are representative of three independent experiments. (E) Plot showing densitometric analysis of the CHX assay. Y-axis shows the relative percentage PTEN levels after normalizing to GAPDH. The quantitation is derived from three independent experiments and values are mean ± SEM. PTEN K66K, with only K66 ubiquitination site available, showed significantly lower stability than PTEN Kall4R (P value < 0.01). PTEN mutants that included the K66R change (i.e. PTEN Kall4R, K13K, K80K and K289K) showed more or less similar stability with data points falling on top of each other. (F) Cellular assay for PTEN ubiquitination. U87MG cells were transfected to express FLAG-ubiquitin (Flag-Ub) and 6 hours later transduced with viruses encoding PTEN WT or the indicated PTEN mutants. Lentiviruses were titrated prior to the experiment to ensure similar levels of PTEN protein is expressed in each sample. 48 hours post-transfection, cells were treated
with 10μM MG132 for 5 hours. Cells were lysed, and PTEN was immunoprecipitated (IP) before western blotting (WB) these immunoprecipitates with antibodies raised against the FLAG epitope, K48-linkage specific polyubiquitin, K63-linkage specific polyubiquitin and PTEN. A smear of high molecular weight in FLAG western blot represents polyubiquitinated PTEN. A stronger signal is observed with a K48-linkage specific antibody in PTEN K66K mutant that has only K66 site available for ubiquitination out of the four studied ubiquitination sites. In parallel, total cell lysates were immunoblotted with antibodies raised against the FLAG epitope, PTEN or GAPDH (H, lower panel).

FIGURE 6. Mutating Lysine 66 counteracts destabilizing PTEN mutations associated with autism and tumour syndromes and NO-mediated proteasomal degradation. HEK293T cells (A) and PTEN-null U87MG cells (B) were transfected with vectors encoding PTEN WT or unstable PTEN mutants associated with autism spectrum disorder (ASD) and tumour syndromes (PHTS) or the same unstable mutants carrying an additional K66R mutation. Cells were lysed and PTEN expression investigated by western blotting. GAPDH levels were used as a loading control. Representative blots from three independent experiments is shown. Additional mutation of K66R significantly increased the protein expression level of the unstable PTEN mutant proteins D252G, H118P (P value < 0.001) and L108P (P value < 0.01). (C, D) Effects of PTEN ubiquitination site mutation in PTEN D252G mutant background. HEK293T cells (C) and U87MG cells (D) were transfected as indicated with expressing vectors encoding PTEN WT or PTEN mutants. Cells were lysed and PTEN expression was investigated by western blotting. GAPDH levels were used as a loading control. A Representative blot from three independent experiments is shown. All mutant PTEN proteins containing the K66R mutation showed significant stabilisation of D252G mutant enzyme (P value < 0.01). Mutant PTEN D252G-K66R retaining K66 in its wild-type form showed no significant effect on D252G mutant enzyme stability. (E) U87MG cells transfected with indicated expression vectors encoding PTEN WT or PTEN mutants were treated for 5 hours with 10μM MG132 proteasome inhibitor before PTEN expression was analyzed by western blotting for PTEN and GAPDH. (F) Mouse neuroblastoma Neuro-2a cells were transfected with quantities of indicated expression vectors encoding PTEN WT or PTEN K66R engineered to lead to similar expression of the two proteins. Cells were treated for 4 hours with 200μM freshly prepared S-nitrosocysteine (SNOC) or 200μM glutamate before PTEN expression was analyzed by western blotting for PTEN and GAPDH. A representative blot from three independent experiments is shown. (F, lower panel) Bar representing relative densitometric values of PTEN expression after SNOC and glutamate treatment in PTEN-WT and PTEN K66R transfected cells. The quantitation is derived from three independent experiments and values are mean ± SEM. SNOC acts as a physiological NO donor and glutamate is neurotoxic compound which induces NO generation. SNOC and glutamate mediated S-nitrosylation of PTEN has been shown to lead to enhanced PTEN ubiquitination leading to its proteasome-mediated degradation [32]. A modest but significant decrease in PTEN levels is observed with SNOC and glutamate treatment in PTEN WT overexpressing cells (two-tailed T-test, P value < 0.01). PTEN-K66R does not show this decrease in PTEN levels. (G) Co-immunoprecipitation of PTEN and NEDD4 in PTEN-WT and PTEN K66R transfected 293T cells. Quantities of expression vectors encoding PTEN WT or PTEN K66R were engineered to lead to similar expression of the two proteins. PTEN was immunoprecipitated (IP) and PTEN-bound endogenous NEDD4 was immunoblotted.
Figure 1
Figure 2
Figure 3

A

B

C

D

E

F

G

Relative PTEN levels (in percent)

0 16 24 36

CHX hrs

PTEN-WT

PTEN-K66R

PTEN-K80R

PTEN-K13R

PTEN-K289R

PTEN-Kall4R

PTEN-A3

PTEN-null HCT116 cells

PC3

WT

K66R

K80R

K13R

K289R

Kall4R

A3

**

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Figure 4
Figure 5
Figure 6