Title

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Abstract

The tumor suppressor PTEN is frequently lost in human cancers. In addition to gene mutations and deletions, recent studies have revealed the importance of post-translational modifications, such as ubiquitination, in the regulation of PTEN stability, activity and localization. However, the deubiquitinase that regulates PTEN poly-ubiquitination and protein stability remains unknown. Here we screened a total of 30 deubiquitinating enzymes (DUBs) and identified five DUBs that physically associate with PTEN. One of them, USP13, stabilizes PTEN protein via direct binding and deubiquitination of PTEN. Loss of USP13 in breast cancer cells promotes AKT phosphorylation, cell proliferation, anchorage-independent growth, glycolysis and tumor growth through downregulation of PTEN. Conversely, overexpression of USP13 suppresses tumorigenesis and glycolysis in PTEN-positive but not PTEN-null breast cancer cells. Importantly, USP13 protein is downregulated in human breast tumors and correlates with PTEN

protein levels. These findings identify USP13 as a tumor-suppressing protein that functions through deubiquitination and stabilization of PTEN.

The lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10) catalyzes the conversion of phosphatidylinositol-3,4,5-trisphosphate to phosphatidylinositol-4,5-bisphosphate[1, 2]. By antagonizing PI3K-AKT signaling, PTEN plays crucial roles in many cellular processes[3-5]. This protein is encoded by a tumor suppressor gene located at 10q23[6], which is one of the most frequently mutated genes in human cancer[7, 8]. Germline *PTEN* mutations occur in several inherited syndromes (such as Cowden syndrome) characterized by hamartomatous growth and predisposition to breast, thyroid and endometrial cancers, and somatic mutations of *PTEN* are observed in a wide cancer spectrum, including breast, prostate, kidney and brain tumors[7-9].

Despite frequent genetic alterations of *PTEN* in human tumors, only 25% of cancer patients show a correlation between loss of PTEN protein and loss of its mRNA[10], which underscores the importance of PTEN regulation at post-transcriptional and post-translational levels. Indeed, mono- or poly-ubiquitination, phosphorylation, sumoylation, acetylation and regulation by non-coding RNAs can control PTEN expression, activity or localization[5, 11, 12]. While recent studies have revealed the role of ubiquitination in modulating PTEN protein[5, 11, 12], the regulation of PTEN deubiquitination remains poorly understood. Several ubiquitin ligases of PTEN, including NEDD4-1[13, 14], WWP2[15], XIAP[16] and CHIP[17], have been found to target PTEN for proteasomal degradation. On the other hand, reversal of the mono-ubiquitination of PTEN by USP7 (also known as HAUSP) regulates PTEN subcellular localization without affecting its protein level[18]. However, the deubiquitinase that regulates PTEN poly-ubiquitination and protein stability has not been reported.

In this study, we identified USP13 as the first deubiquitinase that reverses PTEN poly-ubiquitination and stabilizes PTEN protein, and found that USP13 suppresses tumorigenesis and glycolysis through PTEN. In human breast cancer, loss of USP13 is highly associated with loss of PTEN.

RESULTS

USP13 regulates PTEN protein level and AKT signaling

Deubiquitinating enzymes (DUBs) are a group of proteases that regulate ubiquitin-dependent pathways by cleaving ubiquitin-protein bonds[19]. In order to identify PTEN-interacting deubiquitinases, we screened a panel of DUBs, in which a total of 30 deubiquitinase ORFs were fused with a triple-epitope tag, SFB (S-protein, FLAG tag and streptavidin-binding peptide), and then co-transfected with MYC-tagged PTEN into 293T cells. Immunoblotting assays showed that MYC-PTEN could be detected on S-protein beads conjugated with five DUBs, USP7, USP8, USP10, USP13 or USP39 (Fig. 1a). Moreover, MYC-PTEN transfected into HeLa cells could also be pulled down by each of these five SFB-tagged DUBs (Fig. 1b), further corroborating a physical association.

To examine the effects of these five PTEN-associated DUBs on PTEN expression and the growth of tumor cells, we stably expressed them individually in the MCF7 human breast cancer cell line. Although each of these five DUBs could interact with endogenous PTEN (Supplementary Fig. S1a), only one of them, USP13, significantly increased endogenous PTEN protein expression (Supplementary Fig. S1a). Compared with the control MCF7 cells, cells overexpressing USP7, USP10 or USP13 displayed a pronounced reduction in both proliferation (Supplementary Fig. S1b) and anchorage-independent growth (Supplementary Fig. S1c, d). Therefore, USP13 stood out as the top candidate for a possible PTEN deubiquitinase and a putative tumor suppressor.

As an alternative approach to identify PTEN-associated DUBs, we isolated PTEN-containing protein complexes using SFB-tagged PTEN. Tandem affinity purification using streptavidinsepharose beads and S-protein-agarose beads followed by mass spectrometric analysis identified six DUBs, USP10, USP13, USP7, USP8, USP39 and USP4, as PTEN interactors (Supplementary Table S1). Conversely, purification of SFB-tagged USP13 complexes identified PTEN as a USP13-interacting protein (Supplementary Table S2).

Next, we expressed USP13 in additional human breast cancer cells. This overexpression upregulated PTEN protein and downregulated AKT and FOXO1/3 phosphorylation in the MDAMB-231 cell line (Fig. 1c) which expresses moderate but detectable levels of endogenous PTEN (Supplementary Fig. S2a), while knockdown of PTEN in USP13-overexpressing MDA-MB-231 cells rescued the phosphorylation of both AKT and FOXO1/3 (Supplementary Fig. S2b). In contrast, expression of USP13 did not reduce phospho-AKT and phospho-FOXO levels in BT549 cells (Fig. 1c) which showed no PTEN protein expression (Supplementary Fig. S2a) due to a frameshift mutation[20, 21]. Overexpression of a catalytically inactive mutant of USP13, C345A[22], had no effect on PTEN protein levels and the phosphorylation of AKT and FOXO1/3 in these two cell lines (Fig. 1c).

To further validate regulation of PTEN protein by USP13, we performed loss-of-function analysis in multiple cell lines that express abundant USP13 and PTEN protein levels. Two independent USP13 shRNAs both decreased PTEN protein expression by 80% and increased phospho-AKT and phospho-FOXO1/3 levels by 3- to 5-fold in SUM159 breast cancer cells, while restoration of PTEN or expression of an RNAi-resistant 'silence mutant' (i.e., no amino acid change) of USP13 (USP13-RE) in USP13-depleted SUM159 cells completely reversed the effect of USP13 shRNA on upregulating the phosphorylation of AKT and FOXO (Fig. 1d and Supplementary Fig. S2c, d). Similarly, depletion of USP13 downregulated PTEN protein and upregulated AKT and FOXO1/3 phosphorylation in MCF10A (Fig. 1e) and MCF7 (Fig. 1f) mammary epithelial cells and in HCT116 colon cancer cells (Fig. 1g), but not in the isogenic PTEN-null HCT116 cells (Fig. 1g). In addition, USP13 shRNA potentiated insulin-induced AKT phosphorylation in SUM159 cells, which could be reversed by re-expression of PTEN (Supplementary Fig. S2e). We conclude from these data that USP13 inhibits AKT signaling through positive regulation of PTEN protein. It should be noted that neither knockdown nor overexpression of USP13 affected *PTEN* mRNA levels (Supplementary Fig. S3a, b). Thus, USP13 does not regulate PTEN expression at the transcriptional level.

In contrast to the knockdown effect of USP13, silencing of the other four PTEN-interacting DUBs, USP7, USP8, USP10 or USP39, did not affect PTEN protein levels (Supplementary Fig. S4a-d). USP7 (HAUSP), the only known PTEN deubiquitinase reported to date, regulates PTEN mono-ubiquitination and subcellular localization but not its protein stability[18]. In the present study, immunofluorescent staining (Fig. 2a, b) and fractionation assays (Fig. 2c, d) demonstrated that manipulating USP13 levels altered PTEN protein expression but not its localization; moreover, either overexpression or knockdown of USP13 had no effect on the levels of Cyclin A2 and PLK1 (Fig. 1c, d), the key substrates of the APC-CDH1 complex which has been shown to be regulated by nuclear PTEN in a phosphatase-independent manner[23]. In contrast, USP7 reduced nuclear localization of PTEN in PC3 cells without affecting total PTEN protein levels (Fig. 2c, d), consistent with previously reported findings[18].

USP13 deubiquitinates and stabilizes PTEN

We sought to determine whether USP13 directly interacts with PTEN and functions as a *bona fide* PTEN deubiquitinase. Consistent with the interaction observed in the initial screen, coimmunoprecipitation assays confirmed that ectopically expressed MYC-tagged PTEN could be detected in FLAG-tagged wild-type or the C345A mutant of USP13 immunoprecipitates (Fig. 3a), and that endogenous PTEN was present in endogenous USP13 immunoprecipitates (Fig. 3b). Moreover, purified GST-USP13, but not the GST-GFP control, was able to bind to FLAG-tagged PTEN under cell-free conditions (Fig. 3c), which demonstrated a direct interaction between USP13 and PTEN. PTEN consists of an N-terminal phosphatase domain, a C2 domain and a C-terminal PDZ motif[24]. To map the USP13-binding region on PTEN, we co-expressed MYC-tagged USP13 along with a series of deletion mutants of PTEN[15] (Fig. 3d). Co-immunoprecipitation assays demonstrated that the phosphatase domain of PTEN is essential for its physical interaction with USP13 (Fig. 3e).

We hypothesized that USP13 regulates PTEN through deubiquitination. Indeed, silencing USP13 expression by two independent shRNAs increased PTEN poly-ubiquitination by approximately 3-fold (Fig. 3f). On the other hand, ectopic expression of wild-type USP13, but not the C345A mutant which is still capable of interacting with PTEN (Fig. 3a), reduced the poly-ubiquitination of PTEN by 65% (Fig. 3g), suggesting that the enzymatic activity of USP13 is indispensable for USP13-dependent deubiquitination of PTEN. In order to determine whether PTEN is a direct substrate of USP13, we purified USP13 and ubiquitinated PTEN and then incubated them in a cell-free system. Wild-type USP13 purified from either bacteria or 293T cells, but not its catalytically inactive mutant C345A, decreased PTEN poly-ubiquitination by 64-70% *in vitro* (Fig. 3h, i). Therefore, USP13 can directly deubiquitinate PTEN.

To determine whether USP13 regulates the stability of PTEN protein, we examined ectopically expressed or endogenous PTEN protein levels in the presence of cycloheximide (CHX), an inhibitor of protein translation. Notably, overexpression of USP13, but not the enzyme-dead mutant, led to a prominent increase in the stability of endogenous or overexpressed PTEN protein, whereas the stability of HSP90 or co-transfected GFP control

was not affected (Fig. 4a, b). Conversely, knockdown of USP13 resulted in destabilization of PTEN protein (Fig. 4c, d). Collectively, these results suggest that USP13 is a PTEN deubiquitinase that stabilizes PTEN.

Loss of USP13 promotes tumorigenesis through downregulation of PTEN

We asked whether USP13 functions as a tumor-suppressing protein by regulating PTEN. Two independent USP13 shRNAs (Fig. 1d) both markedly increased the proliferation (Fig. 5a) and anchorage-independent growth (Fig. 5b, c) of SUM159 breast cancer cells, while restoration of PTEN (Fig. 1d) or expression of an RNAi-resistant USP13 mutant (Supplementary Fig. S2c, d) completely reversed the effect of USP13 shRNA (Fig. 5a-d). Moreover, knockdown of USP13 promoted the proliferation of HCT116 colon cancer cells but not the isogenic PTEN-null HCT116 cells (Fig. 5e).

The effect of USP13 shRNA on cell proliferation is AKT-dependent, as treatment with the AKT inhibitor MK-2206 abolished this effect (Fig. 5f). Furthermore, since USP13 regulates AKT phosphorylation through PTEN (Fig. 1c-g), and since AKT plays a critical role in regulating the Warburg effect whereby cancer cells exhibit a high rate of glucose uptake and glycolysis[25-27], we speculated that USP13 might regulate the Warburg effect through PTEN. Indeed, knockdown of USP13 increased glucose uptake and glycolysis, which could be fully reversed by restoration of PTEN, as gauged by lactate production and glucose incorporation assays (Fig. 5g, h).

To investigate the biological function of USP13 in breast cancer cells *in vivo*, we subcutaneously implanted USP13-depleted SUM159 cells into nude mice and monitored tumor growth for more than nine weeks. Mice bearing USP13 shRNA-expressing SUM159 cells showed increased tumor growth throughout the experiment compared with mice implanted with control shRNA-infected cells (Fig. 5i). At 65 days after tumor cell implantation, we observed a 2.5-fold increase in tumor volume (Fig. 5i) and a 3.5-fold increase in the weight of the tumors formed by USP13-depleted SUM159 cells (Fig. 5j, k). Notably, restoring PTEN expression fully reversed the tumor-promoting effect of USP13 shRNA (Fig. 5i-k). Western blot analysis of tumor lysates confirmed that the effect of USP13 shRNA on PTEN and phospho-AKT was retained in these tumors (Fig. 5l). Therefore, loss of USP13 promotes tumorigenesis through downregulation of PTEN.

The anti-tumor function of USP13 depends on PTEN status

To further determine the dependence of the USP13 function on PTEN status, we compared the PTEN-positive cell line MDA-MB-231 and the PTEN-null cell line BT549 (Fig. 1c). Expression of USP13 (but not the C345A mutant) in MDA-MB-231 cells, which led to upregulation of PTEN and downregulation of phospho-AKT and phospho-FOXO1/3 (Fig. 1c), significantly inhibited cell proliferation (Fig. 6a), colony formation on soft agar (Fig. 6b, c), lactate production (Fig. 6d), glucose uptake (Fig. 6e) and tumor growth (Fig. 6f-h), while knockdown of PTEN (Supplementary Fig. S2b) rescued the proliferation of USP13-overexpressing MDA-MB-231 cells (Fig. 6i). In stark contrast, none of these effects were observed in USP13-overexpressing BT549 cells (Fig. 6a-e and 6j-l), which showed no substantial difference in AKT phosphorylation compared with mock-infected cells (Fig. 1c).

Western blot analysis of tumor lysates confirmed that the effect of USP13 on PTEN and phospho-AKT was retained in tumors formed by USP13-overexpressing MDA-MB-231 or BT549 cells (Fig. 6m). Taken together, USP13 has a PTEN-dependent tumor-suppressing function.

USP13 is downregulated in human breast tumors and correlates with PTEN protein levels

PTEN plays a pivotal role in human breast cancer suppression and is dose-dependent. Female patients with Cowden syndrome have partial loss of PTEN due to heterozygous germline *PTEN* mutations and are estimated to have a 25-50% risk of developing breast cancer[28]. Genetic analysis of mouse models has revealed *Pten* haploinsufficiency and dose dependence in breast tumor suppression[29]. Moreover, while approximately 5% of sporadic breast tumors harbor *PTEN* mutations[9], loss of PTEN immunoreactivity is found in nearly 40%[30], which indicates that post-transcriptional and post-translational regulation of PTEN may contribute substantially to the development of human breast cancer. To determine the relevance of regulation of PTEN by USP13 in patients, we performed immunohistochemical staining of PTEN and USP13 (Fig. 7a) on the breast cancer progression tissue microarrays (TMAs) from the National Cancer Institute[31], with antibodies validated for immunohistochemistry (Supplementary Fig. S5). Notably, downregulation of PTEN and USP13 was observed in 73.8% (152 of 206) and 41.3% (83 of 201) of breast tumors, whereas only 31.8% (14 of 44) and 13.2% (5 of 38) of normal mammary tissues exhibited low expression of PTEN and USP13 (Fig. 7b, c), respectively, suggesting that both PTEN and USP13 are downregulated in human breast tumors. Moreover, a significant positive correlation ($R = 0.25$, $P = 4 \times 10^{-4}$) between PTEN and USP13 protein levels was observed in these breast carcinomas, in which 88% (73 of 83) of the tumors with low USP13 expression also displayed low PTEN expression (Fig. 7d). However, it should be noted that 38.8% (78 of 201) of total tumor specimens had low PTEN expression but high USP13 expression (Fig. 7d). Collectively, these data suggest that loss of USP13 may contribute to loss of PTEN in a substantial fraction of human tumors, whereas in other tumors PTEN can be inactivated by different mechanisms, including genetic alterations and upregulation of PTEN ubiquitin ligases (such as NEDD4-1[14] and WWP2[15]).

DISCUSSION

The current study identified USP13 as a PTEN deubiquitinase and a tumor-suppressing protein. Besides USP13, another two PTEN-interacting DUBs, USP7 (HAUSP) and USP10, also exhibited a growth-inhibitory effect (Supplementary Fig. S1b-d), which might be explained by USP7-mediated delocalization of PTEN[18] and USP10-mediated stabilization of p53[32], respectively. However, neither USP7 nor USP10 regulates PTEN protein levels (Supplementary Fig. S1a and S4a, b). In contrast, here we report USP13 as the first PTEN deubiquitinase that reverses the poly-ubiquitination of PTEN, leading to PTEN stabilization and tumor suppression. Whereas the majority of USP7 (HAUSP) protein is present in the nucleus[18], which is consistent with its role in reversing the mono-ubiquitination of nuclear PTEN and promoting PTEN export from the nucleus, USP13 is predominantly cytoplasmic or membrane-bound (Fig. 2a, b and 7a; Supplementary Fig. S5), which is consistent with its role in reversing the poly-ubiquitination of cytoplasmic or membrane-bound PTEN protein.

In contrast to the fast turnover of another major tumor suppressor protein, p53 (half-life: 5–20 minutes[33]), PTEN has a relatively long half-life (3–6 hours; Fig. 4a-d). We propose that unlike p53, PTEN is by default a relatively stable protein but its degradation is accelerated upon upregulation of PTEN ubiquitin ligases or downregulation of PTEN deubiquitinases. Because USP13 is downregulated in human breast tumors and correlates with PTEN expression, and because a large fraction of human cancers exhibit loss of only one *PTEN* allele[34], we propose that loss of USP13 may drive breast tumorigenesis in mammary tissues with heterozygous inactivation of *PTEN*. Future studies are needed to determine the physiological functions of USP13 and how USP13 expression is lost in human cancer.

METHODS

Methods and any associated references are available in the online version of the paper.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

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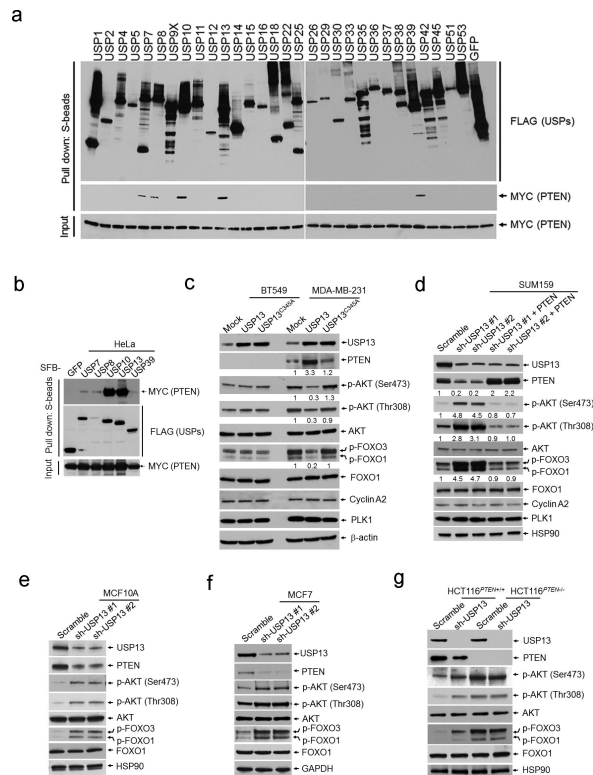
Figure 1. USP13 is a PTEN-interacting deubiquitinase that regulates PTEN and AKT signaling

(**a**) Five of 30 DUBs physically associate with PTEN. SFB-tagged DUBs were co-transfected with MYC-PTEN into 293T cells, followed by pull-down with S-protein beads and immunoblotting with antibodies to FLAG and MYC.

(**b**) Five SFB-tagged DUBs were co-transfected with MYC-PTEN into HeLa cells, followed by pull-down with S-protein beads and immunoblotting with antibodies to MYC and FLAG.

(**c**) Immunoblotting of USP13, PTEN, p-AKT, AKT, p-FOXO1/3, FOXO1, Cyclin A2, PLK1 and β-actin in BT549 and MDA-MB-231 cells transduced with wild-type USP13 or the USP13^{C345A} mutant.

(**d**) Immunoblotting of USP13, PTEN, p-AKT, AKT, p-FOXO1/3, FOXO1, Cyclin A2, PLK1 and HSP90 in USP13 shRNA-transduced SUM159 cells with or without ectopic expression of PTEN.

(**e-g**) Immunoblotting of USP13, PTEN, p-AKT, AKT, p-FOXO1/3, FOXO1 and HSP90 (or GAPDH) in USP13 shRNA-transduced MCF10A (**e**), MCF7 (**f**), HCT116$^{PTEN+/+}$ and HCT116$^{PTEN-/-}$ (**g**) cells.

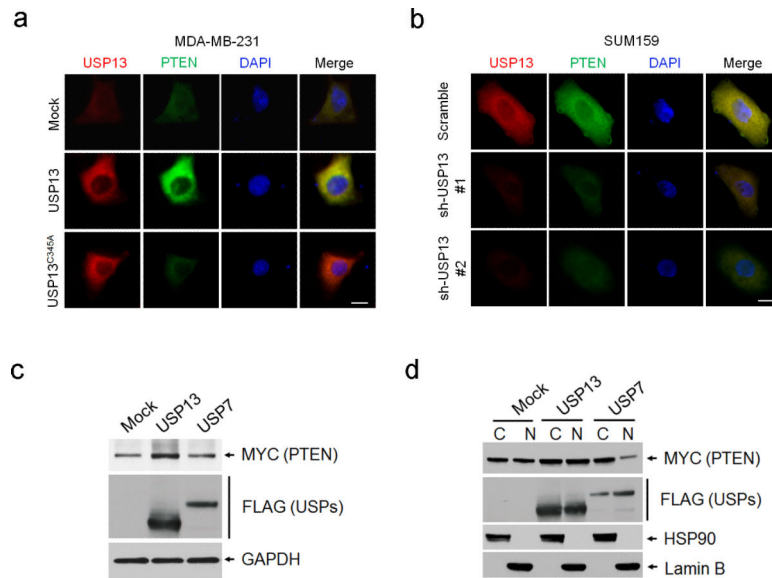Uncropped images of blots are shown in Supplementary Fig. S6.

Figure 2. USP13 regulates PTEN protein level but not its subcellular localization

(**a**) Immunofluorescent staining of USP13 (red) and PTEN (green) in MDA-MB-231 cells transduced with wild-type USP13 or the USP13^{C345A} mutant. Right panels are the overlay of USP13, PTEN and nuclear 4',6-diamidino-2-phenylindole (DAPI; blue) staining of the same field. The GFP and RFP sequences in the pLOC vector were mutated to silence GFP and RFP expression from this vector. Scale bar: 10 μm.

(**b**) Immunofluorescent staining of USP13 (red) and PTEN (green) in SUM159 cells infected with USP13 shRNA or the pGIPZ vector with a scrambled sequence. Right panels are the overlay of USP13, PTEN and nuclear DAPI (blue) staining of the same field. The GFP sequence in the pGIPZ vector was mutated to silence GFP expression from this vector. Scale bar: 10 μm.

(**c**) Immunoblotting of MYC-PTEN, FLAG-USP and GAPDH in whole-cell lysates of PC3 cells co-transfected with MYC-PTEN and FLAG-tagged USP13 or USP7.

(**d**) Immunoblotting of MYC-PTEN, FLAG-USP, HSP90 (cytoplasmic marker) and Lamin B (nuclear marker) in cytoplasmic (C) and nuclear (N) fractions of PC3 cells co-transfected with MYC-PTEN and FLAG-tagged USP13 or USP7.

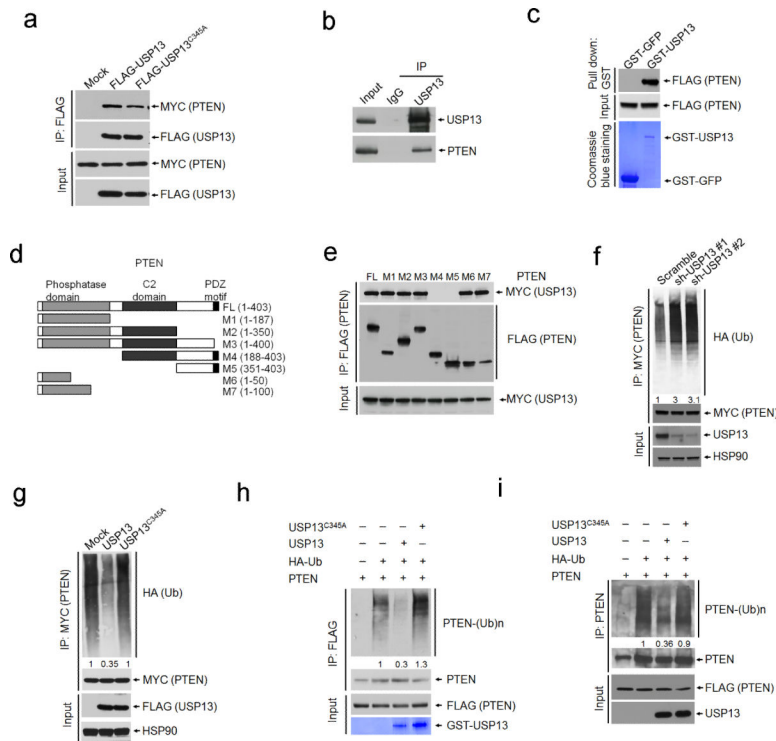Uncropped images of blots are shown in Supplementary Fig. S6.

Figure 3. USP13 directly interacts with and deubiquitinates PTEN

(**a**) 293T cells were transfected with MYC-PTEN alone or in combination with FLAG-tagged USP13 or the USP13^{C345A} mutant, immunoprecipitated with FLAG beads and immunoblotted with antibodies to MYC and FLAG.

(**b**) Endogenous USP13 was immunoprecipitated from SUM159 cells and immunoblotted with antibodies to USP13 and PTEN.

(**c**) Top: GST-GFP or GST-USP13 was retained on glutathione-sepharose beads, incubated with extracts of FLAG-PTEN-transfected 293T cells and then immunoblotted with the antibody to FLAG. Bottom: recombinant GST-GFP and GST-USP13 were purified from bacteria and analyzed by SDS-PAGE and Coomassie blue staining.

(**d**) Schematic representation of FLAG-tagged full-length PTEN (FL) and its various deletion mutants (M1-M7).

(**e**) 293T cells were co-transfected with MYC-USP13 and FLAG-tagged full-length PTEN or its deletion mutants, immunoprecipitated with FLAG beads and immunoblotted with antibodies to MYC and FLAG.

(**f**) 293T cells were co-transfected with MYC-PTEN, USP13 shRNA and HA-ubiquitin (Ub), immunoprecipitated with MYC beads and immunoblotted with antibodies to HA and MYC. Cells were treated with MG132 (10 μM) for 6 hours before harvest.

(**g**) 293T cells were co-transfected with MYC-PTEN, HA-ubiquitin (Ub) and FLAG-tagged USP13 or the USP13^{C345A} mutant, immunoprecipitated with MYC beads and immunoblotted with antibodies to HA and MYC. Cells were treated with MG132 (10 μM) for 6 hours before harvest.

(**h**) Top: unubiquitinated or ubiquitinated SFB-PTEN was incubated with GST-tagged USP13 or the USP13^{C345A} mutant purified from bacteria with glutathione-sepharose beads.

After reaction, SFB-PTEN was immunoprecipitated with FLAG beads and immunoblotted with the antibody to PTEN. Bottom (input for the *in vitro* assay): SFB-PTEN was purified with streptavidinsepharose beads and immunoblotted with the antibody to FLAG. Recombinant GST-USP13 was purified from bacteria and analyzed by SDS-PAGE and Coomassie blue staining.

(**i**) Top: unubiquitinated or ubiquitinated SFB-PTEN was incubated with SFB-tagged USP13 or the USP13^{C345A} mutant purified from 293T cells with streptavidin-sepharose beads. After reaction, PTEN was immunoprecipitated with the antibody to PTEN and immunoblotted with the antibody to PTEN. Bottom (input for the *in vitro* assay): SFB-USP13 and SFB-PTEN were purified with streptavidin-sepharose beads and immunoblotted with antibodies to USP13 and FLAG, respectively.

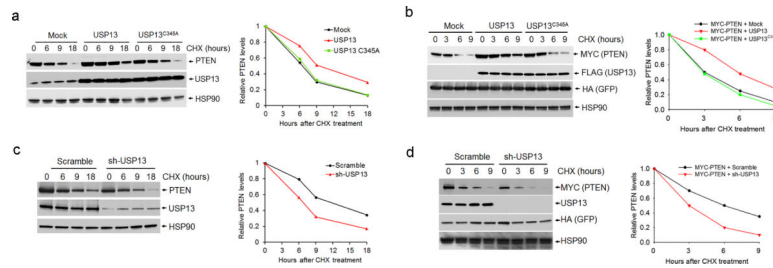Uncropped images of blots are shown in Supplementary Fig. S6.

Figure 4. USP13 stabilizes PTEN protein

(**a**) Left: MDA-MB-231 cells were transduced with USP13 or USP13[C345A], treated with 100 μg/ml cycloheximide (CHX), harvested at different time points and then immunoblotted with antibodies to USP13, PTEN and HSP90. Right: quantification of PTEN protein levels (normalized to HSP90).

(**b**) Left: 293T cells were co-transfected with MYC-PTEN, HA-GFP and FLAG-tagged USP13 or USP13[C345A], treated with 100 μg/ml cycloheximide (CHX), harvested at different time points and then immunoblotted with antibodies to MYC, FLAG, HA and HSP90. HA-GFP serves as the control for transfection. Right: quantification of PTEN protein levels (normalized to HSP90).

(**c**) Left: SUM159 cells were transduced with USP13 shRNA, treated with 100 μg/ml cycloheximide (CHX), harvested at different time points and then immunoblotted with antibodies to USP13, PTEN and HSP90. Right: quantification of PTEN protein levels (normalized to HSP90).

(**d**) Left: 293T cells were co-transfected with MYC-PTEN, HA-GFP and USP13 shRNA, treated with 100 μg/ml cycloheximide (CHX), harvested at different time points and then immunoblotted with antibodies to MYC, USP13, HA and HSP90. HA-GFP serves as the control for transfection. Right: quantification of PTEN protein levels (normalized to HSP90). Uncropped images of blots are shown in Supplementary Fig. S6.
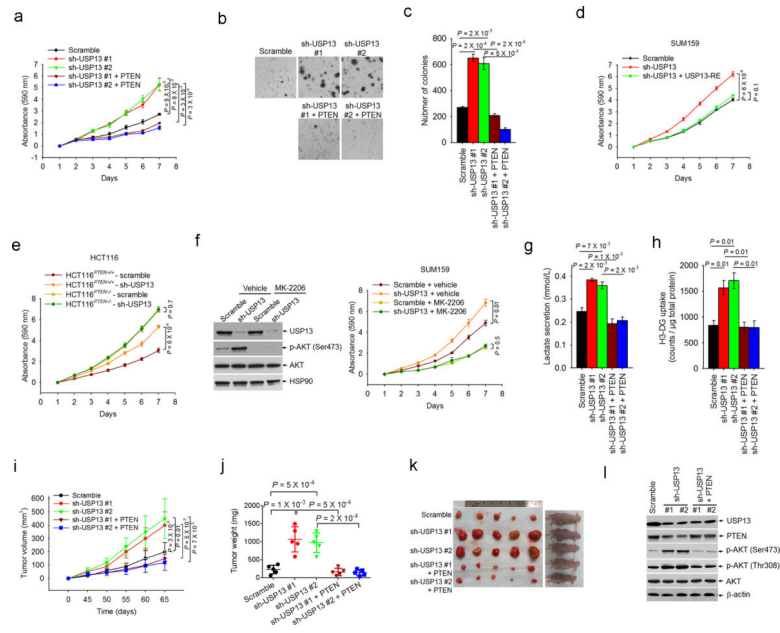
Figure 5. Loss of USP13 promotes tumor growth and glycolysis through downregulation of PTEN

(**a**) Growth curves of USP13 shRNA-transduced SUM159 cells with or without ectopic expression of PTEN.

(**b, c**) Images (**b**) and quantification (**c**) of anchorage-independent growth of USP13 shRNA-transduced SUM159 cells with or without ectopic expression of PTEN.

(**d**) Growth curves of USP13 shRNA-transduced SUM159 cells with or without ectopic expression of an RNAi-resistant mutant of USP13 (USP13-RE).

(**e**) Growth curves of USP13 shRNA-transduced HCT116$^{PTEN+/+}$ and HCT116$^{PTEN-/-}$ cells.

(**f**) Left: immunoblotting of USP13, p-AKT, AKT and HSP90 in USP13 shRNA-transduced SUM159 cells cultured in the presence or absence of the AKT inhibitor MK-2206 (1 μM). Right: growth curves of USP13 shRNA-transduced SUM159 cells cultured in the presence or absence of the AKT inhibitor MK-2206 (1 μM).

(**g, h**) Lactate secretion (**g**) and 2-deoxy-D-[^{3}H]glucose (H3-DG) uptake (**h**) by USP13 shRNA-transduced SUM159 cells with or without ectopic expression of PTEN.

(**i**) Tumor growth by 5×10^6 subcutaneously injected USP13 shRNA-transduced SUM159 cells with or without ectopic expression of PTEN.

(**j, k**) Tumor weight (**j**) and tumor images (**k**) of mice with subcutaneous injection of 5×10^6 USP13 shRNA-transduced SUM159 cells with or without ectopic expression of PTEN, at day 65 after implantation. Data in (**a**) and (**c**) – (**j**) are mean ± s.e.m. $n = 5$ mice per group in (**i**) and (**j**). Statistical significance was determined by two-tailed, unpaired Student's t test.

(**l**) Immunoblotting of USP13, PTEN, p-AKT, AKT and β-actin in tumor lysates from (**k**). Data in (**a**) and (**c**) – (**h**) are the mean of 3 wells per group and error bars indicate s.e.m. The experiments were repeated 3 times. The source data for (**a**) and (**c**) – (**h**) can be found in Supplementary Table S3. Uncropped images of blots are shown in Supplementary Fig. S6.
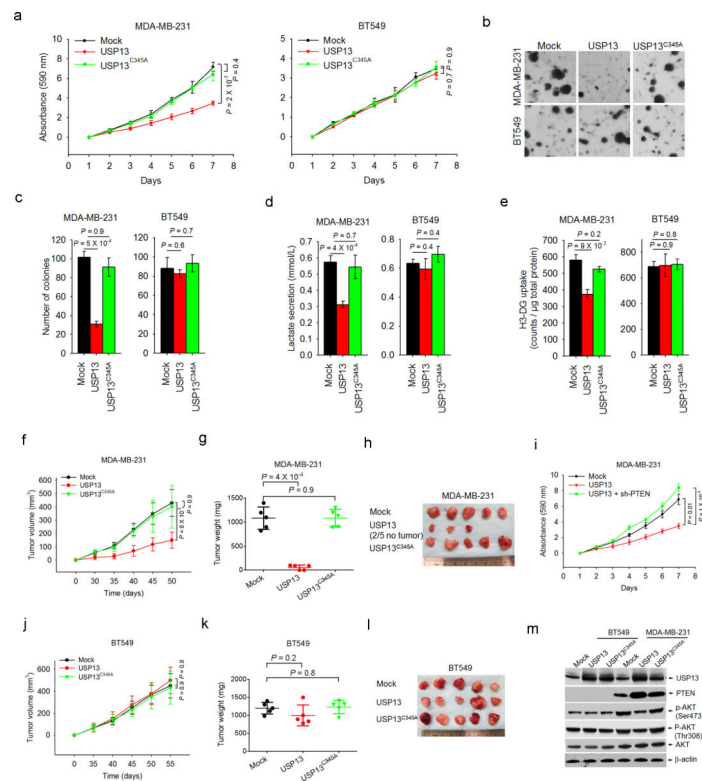
Figure 6. USP13 suppresses tumorigenesis and glycolysis in PTEN-positive but not PTEN-null breast cancer cells

(**a**) Growth curves of USP13- or USP13^{C345A}-transduced MDA-MB-231 and BT549 cells.

(**b, c**) Images (**b**) and quantification (**c**) of anchorage-independent growth of USP13- or USP13^{C345A}-transduced MDA-MB-231 and BT549 cells.

(**d, e**) Lactate secretion (**d**) and 2-deoxy-D-[^{3}H]glucose (H3-DG) uptake (**e**) by USP13- or USP13^{C345A}-transduced MDA-MB-231 and BT549 cells.

(**f**) Tumor growth by 5×10^6 subcutaneously injected MDA-MB-231 cells transduced with USP13 or USP13^{C345A}.

(**g, h**) Tumor weight (**g**) and tumor images (**h**) of mice with subcutaneous injection of 5×10^6 MDA-MB-231 cells transduced with USP13 or USP13^{C345A}, at day 50 after implantation.

(**i**) Growth curves of USP13-overexpressing MDA-MB-231 cells with or without knockdown of PTEN.

(**j**) Tumor growth by 5×10^6 subcutaneously injected BT549 cells transduced with USP13 or USP13^{C345A}.

(**k, l**) Tumor weight (**k**) and tumor images (**l**) of mice with subcutaneous injection of 5×10^6 BT549 cells transduced with USP13 or USP13^{C345A}, at day 55 after implantation. Data in (**a**), (**c**) – (**g**) and (**i**) – (**k**) are mean ± s.e.m. $n = 5$ mice per group in (**f**), (**g**), (**j**) and (**k**). Statistical significance was determined by two-tailed, unpaired Student's t test.

(**m**) Immunoblotting of USP13, PTEN, p-AKT, AKT and β-actin in tumor lysates from (**h**) and (**l**).

Data in (**a**), (**c**) – (**e**) and (**i**) are the mean of 3 wells per group and error bars indicate s.e.m. The experiments were repeated 3 times. The source data for (**a**), (**c**) – (**e**) and (**i**) can be

found in Supplementary Table S3. Uncropped images of blots are shown in Supplementary Fig. S6.

a

Normal   Breast carcinoma

PTEN

USP13

b

	PTEN-low	PTEN-high	Total
Normal breast	14	30	44
Breast carcinoma	152	54	206
Total	166	84	250

$P = 1 \times 10^{-5}$, $R = -0.34$

c

	USP13-low	USP13-high	Total
Normal breast	5	33	38
Breast carcinoma	83	118	201
Total	88	151	239

$P = 8 \times 10^{-4}$, $R = -0.21$

d

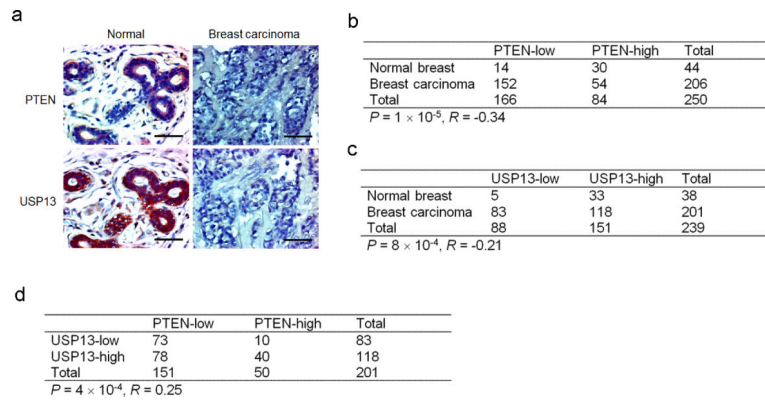|	PTEN-low	PTEN-high	Total
USP13-low	73	10	83
USP13-high	78	40	118
Total	151	50	201

$P = 4 \times 10^{-4}$, $R = 0.25$

Figure 7. USP13 protein is downregulated in human breast cancer and correlates with PTEN protein levels

(**a**) Immunohistochemical staining of PTEN and USP13 in representative normal breast and breast carcinoma specimens on the NCI progression TMAs. Brown staining indicates positive immunoreactivity. Scale bar: 50 μm.

(**b, c**) PTEN (**b**) and USP13 (**c**) protein expression status in normal breast and breast carcinoma specimens.

(**d**) Correlation between PTEN and USP13 protein levels in human breast tumors. Statistical significance in (**b**) – (**d**) was determined by χ^2 test. *R*: correlation coefficient.