

The deubiquitinase USP10 restores PTEN activity and inhibits non-small cell lung cancer cell proliferation

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The phosphatase and tensin homolog deleted on chromosome 10 (PTEN) protein is a key player in tumorigenesis of non-small cell lung cancer (NSCLC) and was recently found to be inactivated by tripartite motif containing 25 (TRIM25)-mediated K63-linked polyubiquitination. However, the deubiquitinase (Dub) coordinate TRIM25 in PTEN ubiquitination is still elusive. In the present study, we found that this K63-linked polyubiquitination could be ablated by the ubiquitin-specific protease 10 (USP10) in a screen against a panel of Dubs. We found using coimmunoprecipitation/immunoblotting that USP10 interacted with PTEN and reduced the K63-linked polyubiquitination of PTEN mediated by TRIM25 in NSCLC cells. Moreover, USP10, but not its inactive C424A deubiquitinating mutant or other Dubs, abolished PTEN from K63-linked polyubiquitination mediated by TRIM25. In contrast to TRIM25, USP10 restored PTEN phosphatase activity and reduced the production of the secondary messenger phosphatidylinositol-3,4,5-trisphosphate, thereby inhibiting AKT/mammalian target of rapamycin progrowth signaling transduction in NSCLC cells. Moreover, USP10 was downregulated in NSCLC cell lines and primary tissues, whereas TRIM25 was upregulated. Consistent with its molecular activity, re-expression of USP10 suppressed NSCLC cell proliferation and migration, whereas knockout of USP10 promoted NSCLC cell proliferation and migration. In conclusion, the present study demonstrates that USP10 coordinates TRIM25 to modulate PTEN activity. Specifically, USP10 activates PTEN by preventing its K63-linked polyubiquitination mediated by TRIM25 and suppresses the AKT/mammalian target of rapamycin signaling pathway, thereby inhibiting NSCLC proliferation, indicating that it may be a potential drug target for cancer treatment.

The phosphatidylinositol-3-kinase (PI3K)/AKT/mTOR (mammalian target of rapamycin) signaling pathway is one of the central nodes in regulating multiple cellular functions, including cell growth, differentiation, proliferation, survival, motility, invasion, and intracellular trafficking (1, 2). After being activated by diverse upstream-activating signals, such as epidermal growth factor receptors and other receptor tyrosine kinases, PI3K catalyzes the production of phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P₃), which triggers activation of the AKT/mTOR signaling cascade (1, 2). Notably, PI(3,4,5)P₃ could be reverted to phosphatidylinositol-4,5-trisphosphate by the phosphatase and tensin homolog deleted from chromosome 10 (PTEN) (3). Therefore, PTEN is a tumor suppressor and a critical player in antagonizing PI3K/AKT/mTOR signaling transduction and regulates various biological activities as well as the pathological processes of various diseases, especially in cancers, including non-small cell lung cancer (NSCLC) (4). It is well known that maintaining PTEN stability in both expressional and functional levels is essential in controlling lung cancer cell biological behavior (4). However, PTEN expression is reduced in 74% in NSCLC because of mutations, deletion, hypermethylation of the PTEN promoter, and loss of heterozygosity at microsatellites in chromosome 10q23 (5).

Moreover, the phosphatase activity of PTEN can be modulated by the post-translational modifications, including methylation, acetylation, SUMOylation, and ubiquitination. Ubiquitination is a biological process where ubiquitin is covalently attached to a substrate protein under the direction of three sequential enzymes: ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s) (6). Ubiquitination usually results in the degradation of substrates, affecting their activity or altering their subcellular localization depending on the ubiquitination types (1, 7, 8). Ubiquitin is a polypeptide composed of 76 amino acids containing seven lysine residues of which each can be

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acted as a ubiquitin acceptor. Therefore, ubiquitination could be classified as monoubiquitination, multi-monoubiquitination, polyubiquitination (K6, K11, K27, K29, K33, K48, and K63 linked), and each type of ubiquitination leads to specific modulation on target proteins. It is well known that K48-linked polyubiquitination leads to protein degradation, whereas K63-linked modulates protein subcellular localization and function. It has been demonstrated that the ubiquitin ligase NEDD4-1 induces K48-linked polyubiquitination that leads to PTEN degradation (7). NEDD4-1 also mediates PTEN for monoubiquitination that leads to PTEN translocation to nucleus (9). WWP1, another ubiquitin ligase, specifically triggers nondegradative K27-linked polyubiquitination of PTEN to suppress its membrane recruitment and tumor-suppressive functions (1). Our recent study found that tripartite motif containing 25 (TRIM25), an E3 ubiquitin ligase, induces K63-linked polyubiquitination of PTEN and inhibits its phosphatase activity (10).

Notably, protein ubiquitination is a dynamic process, and the ubiquitin-specific proteases (USPs) or deubiquitinases (Dubs) can hydrolyze the attached ubiquitin molecules from their substrates (11). Several Dubs have been assigned to modulate PTEN stability, including Otud3 (12), USP7 (13), USP11 (14), and USP13 (15), but all these Dubs stabilize PTEN protein but do not modulate PTEN K63-linked polyubiquitination. In the present study, we identified that USP10 acts as a tumor suppressor Dub of PTEN, and it restores the phosphatase activity of PTEN by preventing TRIM25-mediated K63-linked polyubiquitination in NSCLC.

Results

USP10 prevents PTEN from K63-linked polyubiquitination

Our recent study demonstrated that TRIM25 mediates PTEN with K63-linked polyubiquitination and inhibits its phosphatase activity, thereby activating the AKT/mTOR signaling in NSCLC cell lines (10). Because protein ubiquitination is a dynamic process, we wondered which Dub might be responsible for removing PTEN K63-linked ubiquitination. To this end, we transfected a panel of 48 deubiquitinating enzymes into human embryonic kidney 293T (HEK293T) cells, followed by measurement of AKT phosphorylation. This primary screen revealed that USP10, USP11, USP13, USP39, and USP46 might suppress AKT activation in terms of phosphorylation (Fig. 1, A and B), of which USP11 (14) and USP13 (15) have been reported to inhibit AKT by stabilizing PTEN protein stability. To find out which Dub might be responsible for the K63-linked polyubiquitination of PTEN, HEK293T cells were cotransfected with K63-Ub and individual Dubs, followed by immunoprecipitation (IP)/immunoblotting (IB) assays. As shown in Figure 1C, although all these candidate Dubs downregulated p-AKT, USP10 was the most potent one to suppress PTEN in terms of K63-linked polyubiquitination (Fig. 1C). Subsequently, USP10 was chosen for further studies for PTEN K63-linked polyubiquitination. In line with our hypothesis, USP10 decreased PTEN polyubiquitination in a concentration-dependent manner as evidenced by

overexpression (Fig. 1D) or siRNA knockdown (Fig. 1E). We further confirmed this finding by knocking out USP10 by its specific single-guide RNA (sgRNA). As shown in Figure 1F, sgUSP10 led to increased K63-linked polyubiquitination by using an anti-K63 Ub-specific antibody. Next, we measured the effects of USP10 on PTEN Ub levels in the cell-free setting. The results showed that the addition of USP10 significantly reduced the ubiquitination levels of PTEN *in vitro* (Fig. 1G), further suggesting USP10 was a Dub of PTEN. Moreover, it is reported that C424 is an essential residue in maintaining USP10 activity (16). To find out the role of C424 on PTEN ubiquitination, we constructed a C424A USP10 mutant and transfected it into NSCLC cells, followed by IP/IB assays. The results showed that the WT but not the C424A mutant USP10 strikingly prevented PTEN from K63-linked polyubiquitination (Fig. 1H). Therefore, all these results concluded that USP10 is a Dub of PTEN, and it specifically decreases the K63-linked polyubiquitination of PTEN.

USP10 ablates PTEN from K63-linked polyubiquitination mediated by TRIM25

The aforementioned study demonstrated that USP10 is a Dub of PTEN K63-linked polyubiquitination, whereas TRIM25 is a ubiquitin ligase that mediates PTEN for K63-linked polyubiquitination, thus we wondered whether USP10 could prevent PTEN from TRIM25-mediated K63-linked polyubiquitination. To this end, we first evaluated TRIM25-mediated K63-linked polyubiquitination in A549 cells in the presence of USP10 and four other Dubs that downregulated AKT phosphorylation (Fig. 1, A and B). As shown in Figure 2A, USP10 but not other Dubs markedly reduced K63-linked polyubiquitination from PTEN mediated by TRIM25. To verify the action of USP10 on K63-linked polyubiquitination, ATXN3, a known Dub to remove K63-linked polyubiquitination (17), and USP10 were transfected into HEK293T cells along with other plasmids. The result showed that, as expected, TRIM25 strikingly increased the K63-linked ubiquitination of PTEN, but this ubiquitination was significantly reduced by USP10 but not by ATXN3 (Fig. 2B). Subsequently, we compared the effects of USP10 and its inactive C424A mutant on K63-linked monoubiquitination. These individual Dubs were cotransfected into HEK293T cells with TRIM25 and K63-Ub. The subsequent IP/IB assays showed that WT USP10, but not its inactive form or USP7, a proven Dub of PTEN, downregulated K63-linked polyubiquitination on PTEN (Fig. 2C). To further evaluate USP10 action, we measured the K63-linked polyubiquitination level of PTEN in the cell-free assay by incubating PTEN, USP10 and TRIM25 in a ubiquitination reaction. The result showed that TRIM25 mediated PTEN for polyubiquitination, but it was markedly reduced by USP10 (Fig. 2D). Finally, our previous study showed that K266 is a key ubiquitin acceptor for PTEN K63-linked polyubiquitination, we thus wondered whether USP10 removed PTEN ubiquitination from K266. To this end, USP10, K63-Ub, and WT or K266R PTEN were transfected into HEK293T cells, followed by IP/IB assays. As shown in

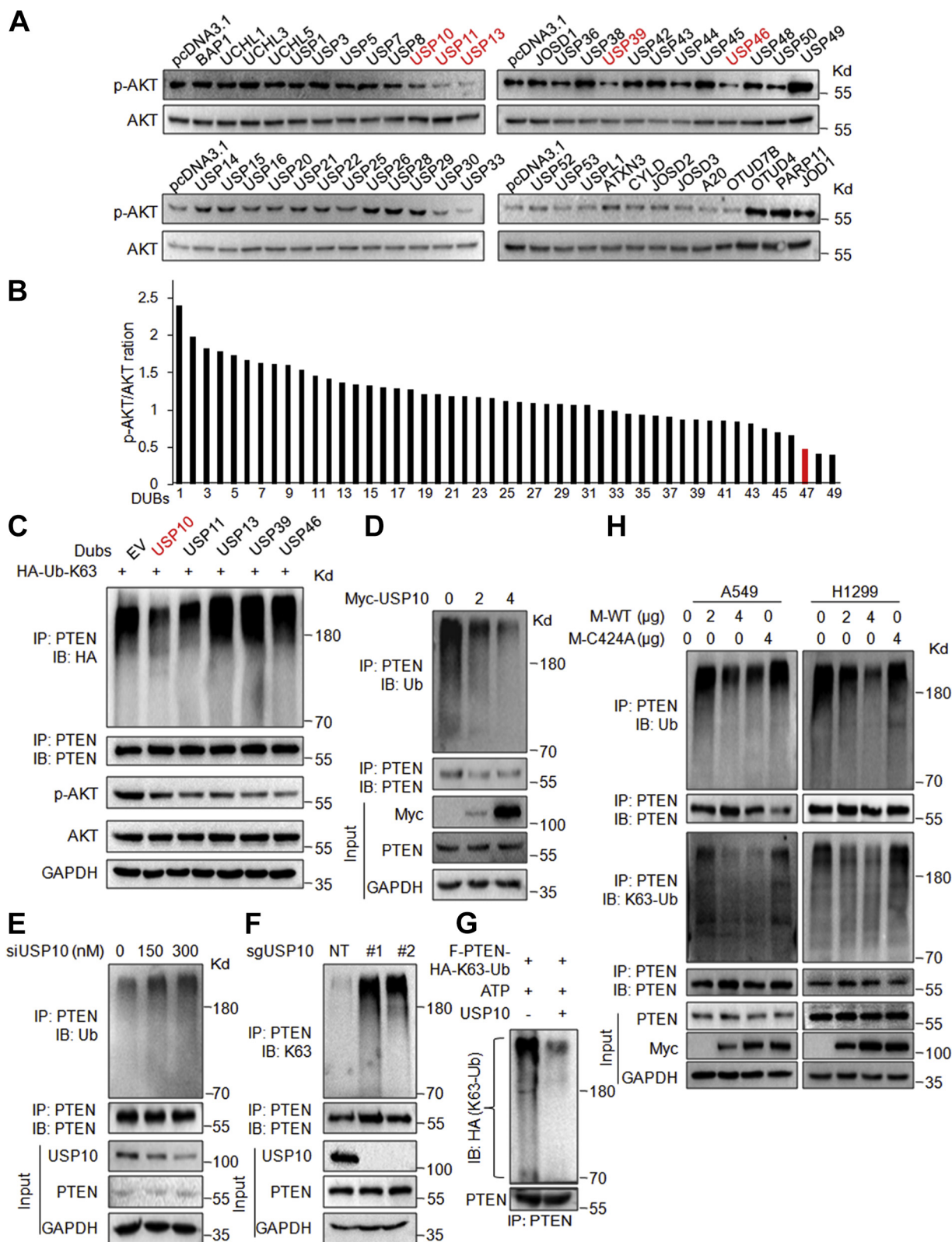


Figure 1. USP10 inhibits AKT phosphorylation and deubiquitinates PTEN with K63-linked polyubiquitination. A, HEK293T cells were transfected with a collection of 48 Dubs for 48 h. IB assays were performed to measure AKT phosphorylation level and total AKT expression. B, the ratio of p-AKT/AKT in the presence of each Dub (1, USP49; 2, USP28; 3, USP26; 4, USP15; 5, ATXN3; 6, USP14; 7, JQSD2; 8, USP29; 9, CYLD; 10, USP53; 11, JQSD3; 12, USP53; 13, USP25; 14, USP38; 15, USP52; 16, USP45; 17, OTUD4; 18, A20; 19, USP43; 20, USP22; 21, USP21; 22, USP16; 23, PARP11; 24, USP20; 25, OTUD7B; 26, JOD1; 27, USP42; 28, USP44; 29, JQSD1; 30, USP36; 31, BAP1; 32, empty vector; 33, UCHL1; 34, USP3; 35, USP30; 36, UCHL3; 37, USP48; 38, USP1; 39, USP7; 40, UCHL5; 41, USP5; 42, USP50; 43, USP39; 44, USP8; 45, USP46; 46, USP33; 47, USP10; 48, USP13; and 49, USP11). C, A549 cells were cotransfected with K63-Ub and selected Dubs for 48 h, followed by IP/IB assay as indicated. D, A549 cells were transfected with USP10 plasmids for 48 h, followed by IP/IB assays. E, USP10 was knockdown by siRNA from H1299 cells for 48 h, followed by IP/IB assays. F, USP10 was knocked out from H1299 cells by using its sgRNA species. Seventy-

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Figure 2E, compared with the wtPTEN control, of which the ubiquitination level was markedly reduced by USP10, but the ubiquitination on K266R PTEN could not be altered by USP10. These results thus collectively demonstrated that USP10 prevents PTEN from TRIM25-mediated K63-linked polyubiquitination.

USP10 disrupts the interaction between TRIM25 and PTEN

Our previous study demonstrated that TRIM25 interacts with PTEN, therefore mediating its ubiquitination (10), whereas USP10 counters TRIM25 in PTEN polyubiquitination, we thus wondered the interaction between USP10, TRIM25, and PTEN. To this end, we first evaluated the interaction between USP10 and PTEN by IP/IB assays in the tool cell line HEK293T and NSCLC cell lines A549 and H1299. The results showed that USP10 interacted with PTEN at both ectopic and endogenous contexts (Fig. 3, A and B). To confirm this physical interaction, we performed an immunofluorescent assay by staining cells with specific antibodies against PTEN or USP10. The results showed that USP10 was mainly found in cytoplasm, and it was colocalized with PTEN (Fig. 3C). Next, we measured the interaction between USP10 and TRIM25 by IP/IB assays. It showed that USP10 also interacted with TRIM25 as demonstrated in both HEK293T and H1299 cells in the reciprocal IP/IB assays (Fig. 3, D and E). Moreover, when USP10 was introduced, the binding of PTEN was significantly reduced from the TRIM25 immunoprecipitates (Fig. 3F), suggesting USP10 might interfere with the interaction between PTEN and TRIM25. All these results thus demonstrated that USP10 prevents PTEN from TRIM25-mediated K63-polyubiquitination by competing with TRIM25 to bind PTEN or by disrupting the interaction between TRIM25 and PTEN.

USP10 has no effects on PTEN stability

Given that TRIM25-mediated K63-linked ubiquitination does not alter PTEN stability (10), and USP10 prevents PTEN from K63-linked polyubiquitination mediated by TRIM25, we wondered whether PTEN stability was modulated by USP10. To this end, USP10 was overexpressed in HEK293T cells in a time-dependent and concentration-dependent manner. The subsequent IB analysis revealed that the PTEN protein level was not markedly altered by USP10 either at increased concentrations (Fig. 4A) or in extended incubation time (Fig. 4B). Furthermore, USP10 had no effects on PTEN at the transcription level (Fig. 4C). Moreover, the cycloheximide (CHX) chase assay showed that the half-life of PTEN protein remained unchanged in the context of ectopic expression (Fig. 4, E and F) or knockout of USP10 (Fig. 4, G and H). Together, these results demonstrated that USP10 decreased the K63-linked polyubiquitination levels on PTEN but has no

effects on its protein or mRNA stability, which was consistent with the effects of TRIM25 on PTEN ubiquitination and stability (10). These results further demonstrated that USP10 antagonizes TRIM25 in PTEN ubiquitination modification without affecting its stability.

USP10 is lowly expressed in NSCLC cells and suppresses the activation of the AKT/mTOR signaling pathway

The aforementioned studies have demonstrated that USP10 counters TRIM25 in terms of PTEN K63-linked polyubiquitination, we subsequently wondered whether the expression and activity of USP10 also contradicts TRIM25. To find out this relevance, we first evaluated the expression pattern of USP10 in a panel of cancer cell lines and primary NSCLC tissues by IB assay. As shown in Figure 5A, USP10 was highly expressed in NSCLC cell lines such as myeloma and prostate cancers, but it was downregulated in NSCLC cell lines including A549 and H1299. Moreover, it was not detected in H460, another typical NSCLC cell line. In contrast, USP10 was highly detected in normal human bronchial epithelial cells (Fig. 5A), suggesting that USP10 might be downregulated in NSCLC cells. We next examined the expression profile of PTEN and TRIM25 in NSCLC tumor tissues and paired normal tissues. The results showed that USP10 expression was markedly reduced, whereas TRIM25 was highly expressed in primary NSCLC tissues compared with their paired normal counterparts (Fig. 5, B and C).

Because TRIM25 mediates the K63-linked ubiquitination on PTEN therefore suppressing PTEN and activating the AKT/mTOR pathway, we wondered whether USP10 inhibits this pathway as an activator of PTEN. To this end, A549 and H1299 cells were transfected with USP10 followed by IB assay. The results showed that overexpression of USP10 downregulated the phosphorylation levels of both AKT and mTOR but showed no effects on their total protein levels in both NSCLC cell lines (Fig. 5D). In agreement with this finding, depletion of USP10 by sgRNA triggered the activation of AKT/mTOR signaling (Fig. 5E). To confirm this effect, NSCLC cells were treated with spautin-1, a small molecule inhibitor of USP10 (18), followed by the evaluation of the AKT/mTOR signaling. As showed in Figure 5F, spautin-1 significantly upregulated the phosphorylation of both AKT and mTOR without affecting PTEN stability. Moreover, when PTEN were depleted from these cells by shRNA, overexpression of USP10 failed to activate the AKT/mTOR signaling compared with the shRNA-treated cells (Fig. 5G). These data thus demonstrated that USP10 might activate PTEN, thus inhibiting the AKT/mTOR signaling pathway. Furthermore, given that PTEN is a phosphatase of PI(3,4,5)P3 (19, 20), we next measured the production of PI(3,4,5)P3 in the presence of USP10 in NSCLC cells. As expected, ectopic expression of USP10 significantly

two hours later, cells were subjected to IP/IB assays against K63-linked polyubiquitination. G, Flag-PTEN (F-PTEN) purified from K63-Ub-expressing cells were incubated with ATP and purified USP10 in tube for 30 min. After termination, the reaction was subjected to IP/IB assays to measure PTEN ubiquitination. H, A549 and H1299 cells were transfected with the Myc-tagged WT (M-WT) or its C424A mutant USP10 for 48 h, followed by IP/IB assays as indicated. Dub, deubiquitinase; HEK293T, human embryonic kidney 293T; IB, immunoblotting; IP, immunoprecipitation; PTEN, phosphatase and tensin homolog deleted on chromosome 10; sgRNA, single-guide RNA; USP10, ubiquitin-specific protease 10.

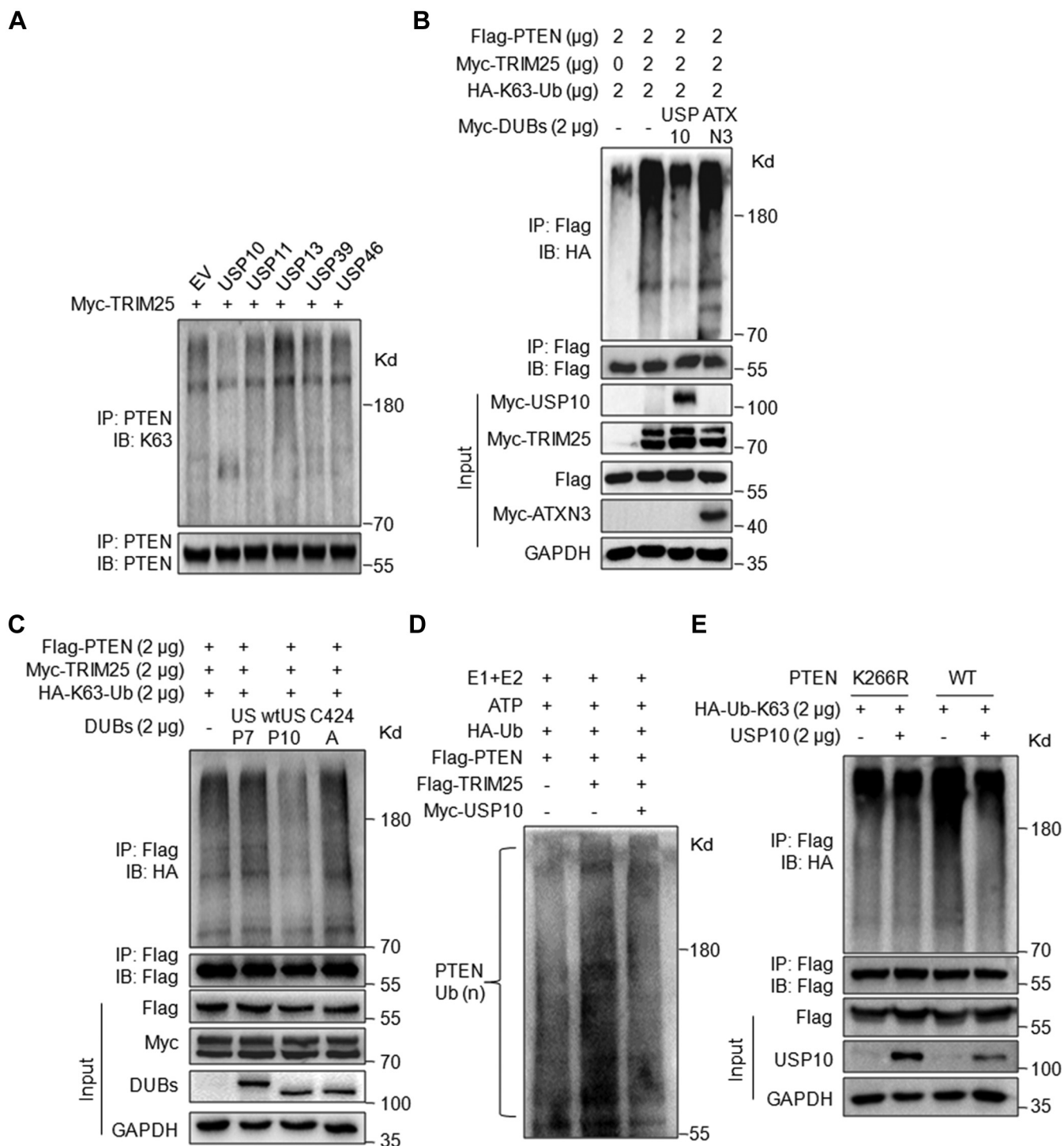


Figure 2. USP10 deubiquitinates PTEN in K63-linked polyubiquitination mediated by TRIM25. *A*, A549 cells were cotransfected with TRIM25 and selected deubiquitinases based on Figure 1 and literatures for 48 h, followed by IP/IB assay as indicated. *B*, HEK293T cells were cotransfected with PTEN, TRIM25, K63-Ub, and USP10 or ATXN3 for 48 h, followed by IP/IB assays against specific antibodies as indicated. *C*, HEK293T cells were cotransfected with plasmids of PTEN, TRIM25, K63-Ub, and wtUSP10 or C424A mutant or USP7 (a negative control) for 48 h, followed by IP/IB assays. *D*, purified E1, E2, ATP, Ub, PTEN as well as TRIM25 and USP10 were incubated in tube for 30 min. After termination, the reaction was subjected to IB assay for PTEN ubiquitination assay. *E*, WT PTEN or its K266R mutant was cotransfected with K63-Ub and USP10 plasmids for 48 h, followed by IP/IB assays as indicated. HEK293T, human embryonic kidney 293T; IB, immunoblotting; IP, immunoprecipitation; PTEN, phosphatase and tensin homolog deleted on chromosome 10; TRIM25, tripartite motif containing 25; USP10, ubiquitin-specific protease 10.

decreased the production of PI(3,4,5)P3 in both NSCLC cell lines (Fig. 5H), indicating that USP10 enhanced the phosphatase activity of PTEN.

Previous studies have demonstrated that plasma membrane localization of PTEN is critical for its activity to hydrolyze PI(3,4,5)P3 (21, 22). Our previous study has

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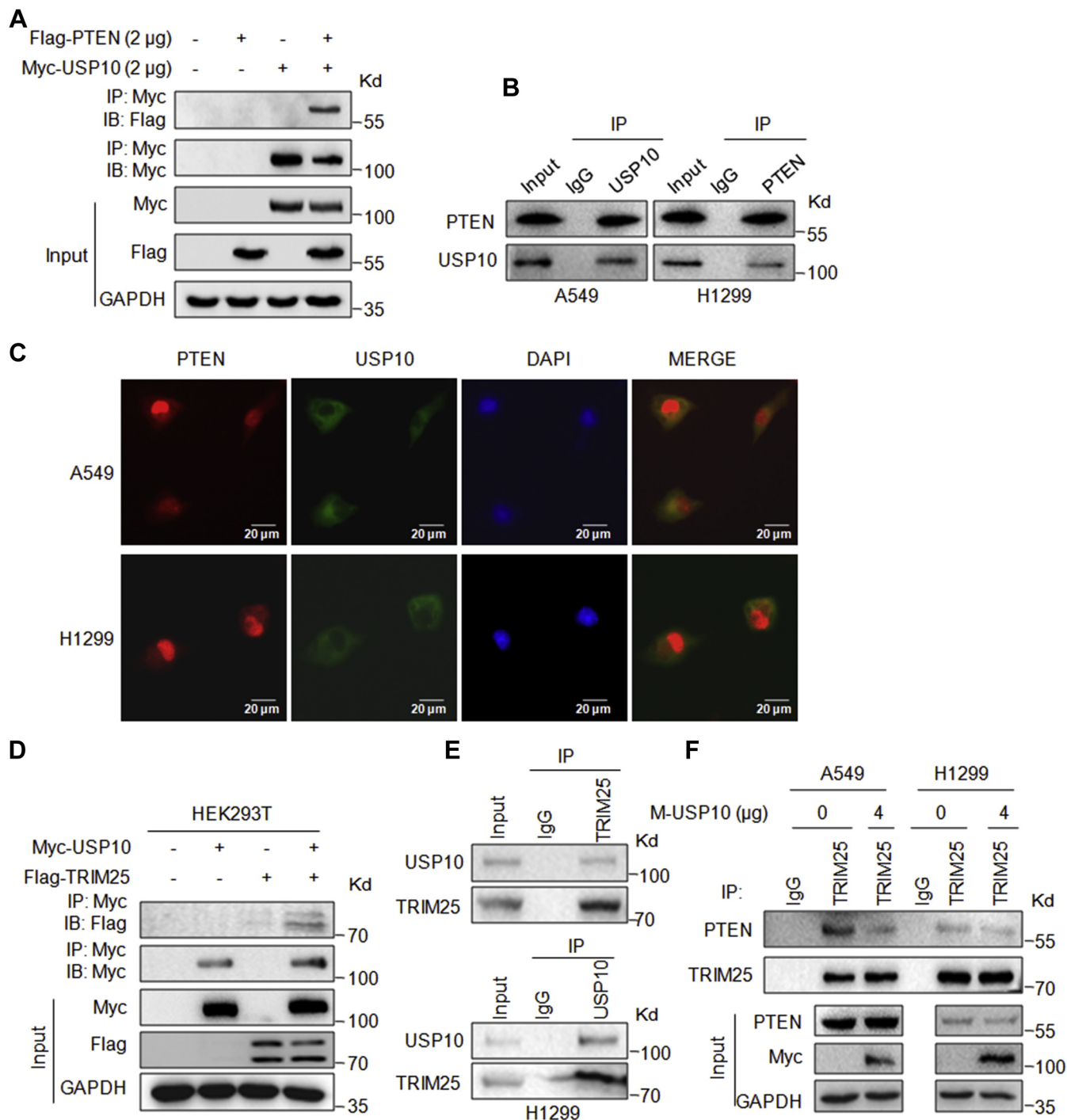


Figure 3. USP10 competitively binds to PTEN against TRIM25. A, HEK293T cells were cotransfected with Flag-PTEN and Myc-USP10 plasmids for 48 h, followed by IP/IB assays. B, A549 and H1299 cell lysates were subjected to IP and IB as indicated. C, A549 and H1299 cells were subjected to immunofluorescence assay as indicated. D, HEK293T cells were transfected with USP10 and TRIM25 plasmids for 48 h, followed by IP/IB assays as indicated. E, H1299 cell lysates were subjected to reciprocal IP/IB assays with specific antibodies as indicated. F, A549 and H1299 cells were transfected with a Myc-USP10 (M-USP10) plasmid for 48 h, followed by IP/IB assays as indicated. HEK293T, human embryonic kidney 293T; IB, immunoblotting; IP, immunoprecipitation; PTEN, phosphatase and tensin homolog deleted on chromosome 10; TRIM25, tripartite motif containing 25; USP10, ubiquitin-specific protease 10.

demonstrated that TRIM25-mediated PTEN for K63-linked polyubiquitination and reduced its localization to plasma membrane (10); therefore, we next evaluated the plasma membrane distribution of PTEN in the presence of USP10. The IB assays showed that introduction of USP10 markedly increased but TRIM25 reduced the localization

of PTEN to plasma membrane (Fig. 5I), which is consistent with the previous finding (10). Moreover, the K266R mutant PTEN was less found in plasma membrane fraction (Fig. 5J). Altogether, the aforementioned results concluded that USP10 is downregulated in NSCLC, but its restoration suppresses the AKT/mTOR signaling pathway

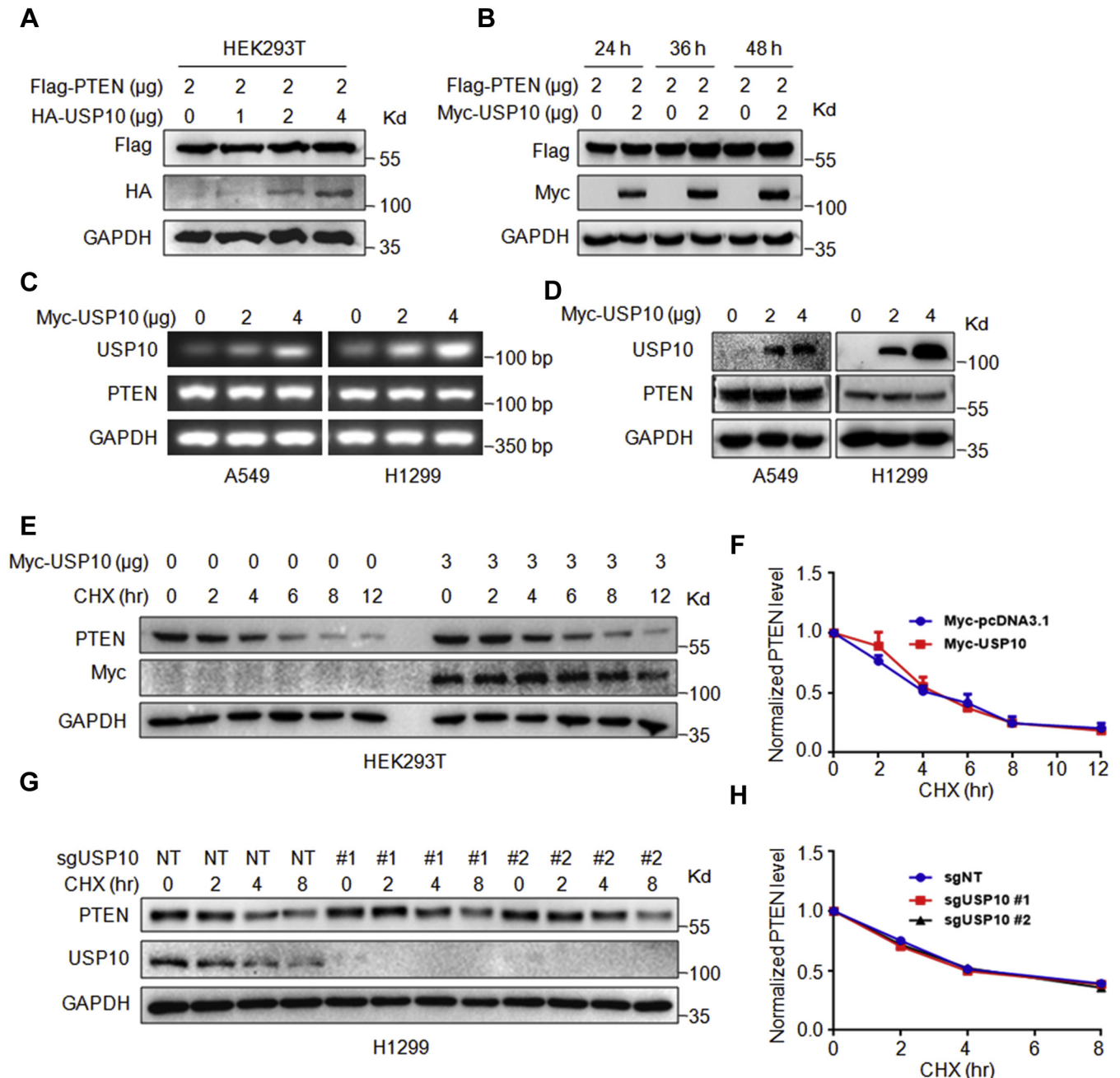


Figure 4. The stability of PTEN protein is not affected by USP10. A, HEK293T cells were cotransfected with Flag-PTEN and Myc-USP10 plasmids as indicated for 48 h, followed by IB assay. B, HEK293T cells were transfected with USP10 and PTEN plasmids for indicated periods, followed by IB assay. C and D, after being transfected with USP10 plasmids for 48 h, A549 and H1299 cells were applied for RT-PCR assay to measure PTEN mRNA level (C) and IB to measure its protein level (D). E, HEK293T cells were transfected with a USP10 plasmid for 24 h, followed by cycloheximide (CHX) treatment for indicated periods. Cell lysates were then prepared for IB assay. F, the statistical analysis of PTEN stability based on E. G, USP10 was knocked out from H1299 cells by its specific sgRNA species, 48 h later, cells were treated with CHX for indicated periods, followed by cell lysate preparation and IB assay. H, the statistical analysis of PTEN stability based on G. HEK293T, human embryonic kidney 293T; IB, immunoblotting; PTEN, phosphatase and tensin homolog deleted on chromosome 10; sgRNA, single-guide RNA; USP10, ubiquitin-specific protease 10.

by decreasing PTEN in terms of K63-linked polyubiquitination.

USP10 inhibits NSCLC cell viability, proliferation, and migration

It is well known that excessive activation of growth signals such as AKT/mTOR promotes NSCLC cell proliferation and survival

(1, 2). Our aforementioned studies have shown that USP10 restores the phosphatase activity of PTEN, thereby inhibiting the AKT/mTOR signaling. We thus wondered whether USP10 could modulate NSCLC cell behavior. To this end, we re-expressed USP10 in NSCLC cells and analyzed its effects on cell activity. As shown in Figure 6A, USP10 prominently decreased NSCLC cell viability as assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT). Moreover, PTEN has

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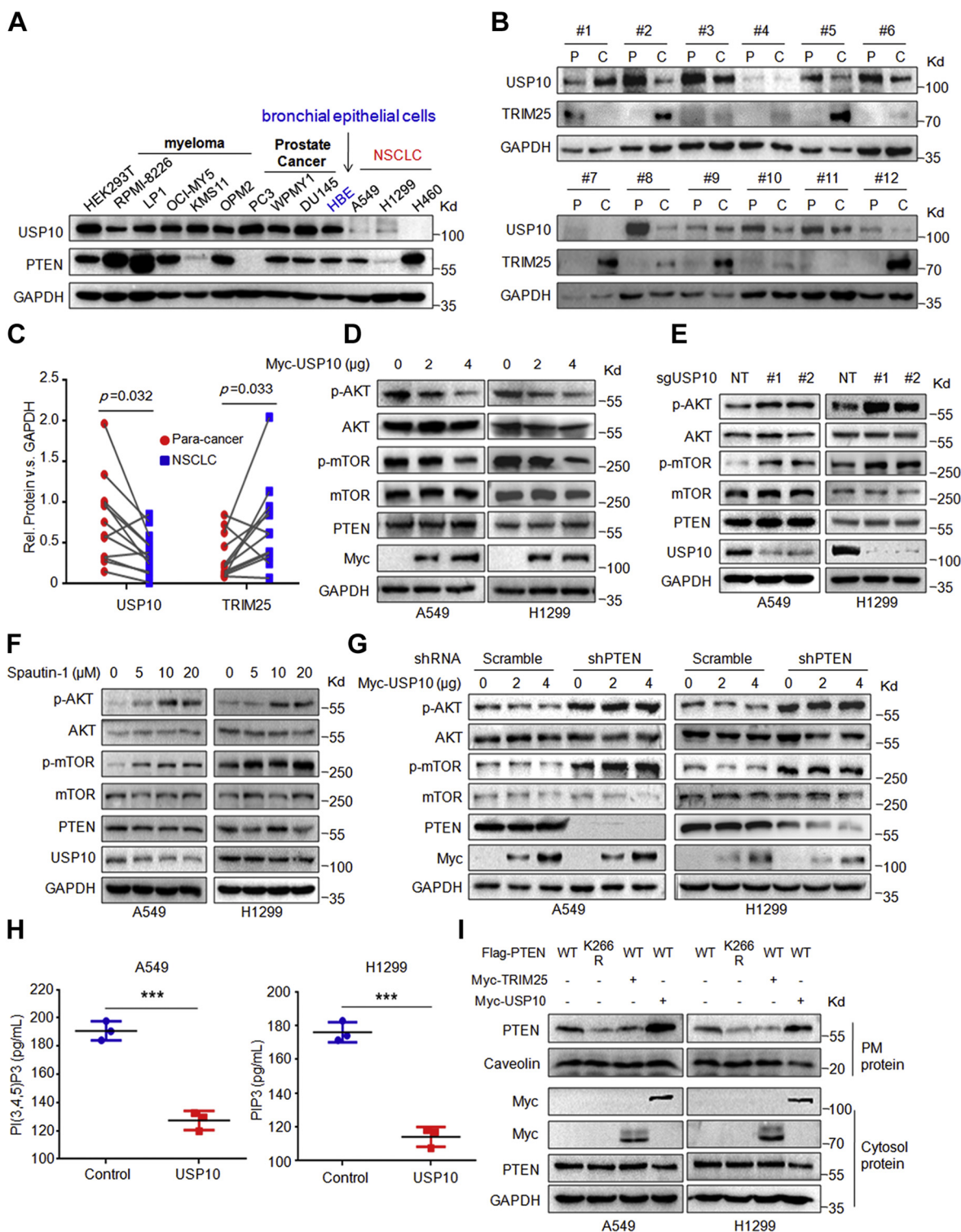


Figure 5. USP10 restores PTEN activity and suppresses activation of the AKT/mTOR signaling pathway. *A*, the USP10 expression profile was evaluated by IB in various cell lines. *B*, primary NSCLC (*C*) and paracancerous (*P*) tissues were subjected to IB assay for USP10 and TRIM25 expressions. *C*, the statistical analysis of USP10 and TRIM25 in primary NSCLC tissues based on *B*. *D*, A549 and H1299 cells were transfected with increasing Myc-USP10 plasmids for 48 h, followed by IB assay against specific antibodies as indicated. *E*, USP10 was knocked out by its specific sgRNA, 72 h later, cells were subjected to IB assay. *F*, A549 and H1299 cells were treated with spautin-1 for 24 h, and cell lysates were subjected to IB assay. *G*, PTEN was knocked down by its specific shRNA from A549 and H1299 cells, followed by transfection of USP10 plasmids. Cells were then subjected to IB assay as indicated. *H*, A549 and H1299 cells were transfected with USP10 plasmids for 48 h, followed by the measurement of PI(3,4,5)P3. $***p < 0.01$. *I*, WT or K266R mutant PTEN was transfected into A549 and H1299 cells along with Myc-TRIM25 or Myc-USP10. About 24 h later, cells were subjected to isolation of plasma membrane (PM) from the cytosol fraction. The individual fractions were then subjected to IB for indicated proteins. IB, immunoblotting; mTOR, mammalian target of rapamycin; NSCLC, non-small cell lung cancer; PI(3,4,5)P3, phosphatidylinositol-3,4,5-trisphosphate; PTEN, phosphatase and tensin homolog deleted on chromosome 10; sgRNA, single-guide RNA; TRIM25, tripartite motif containing 25; USP10, ubiquitin-specific protease 10.

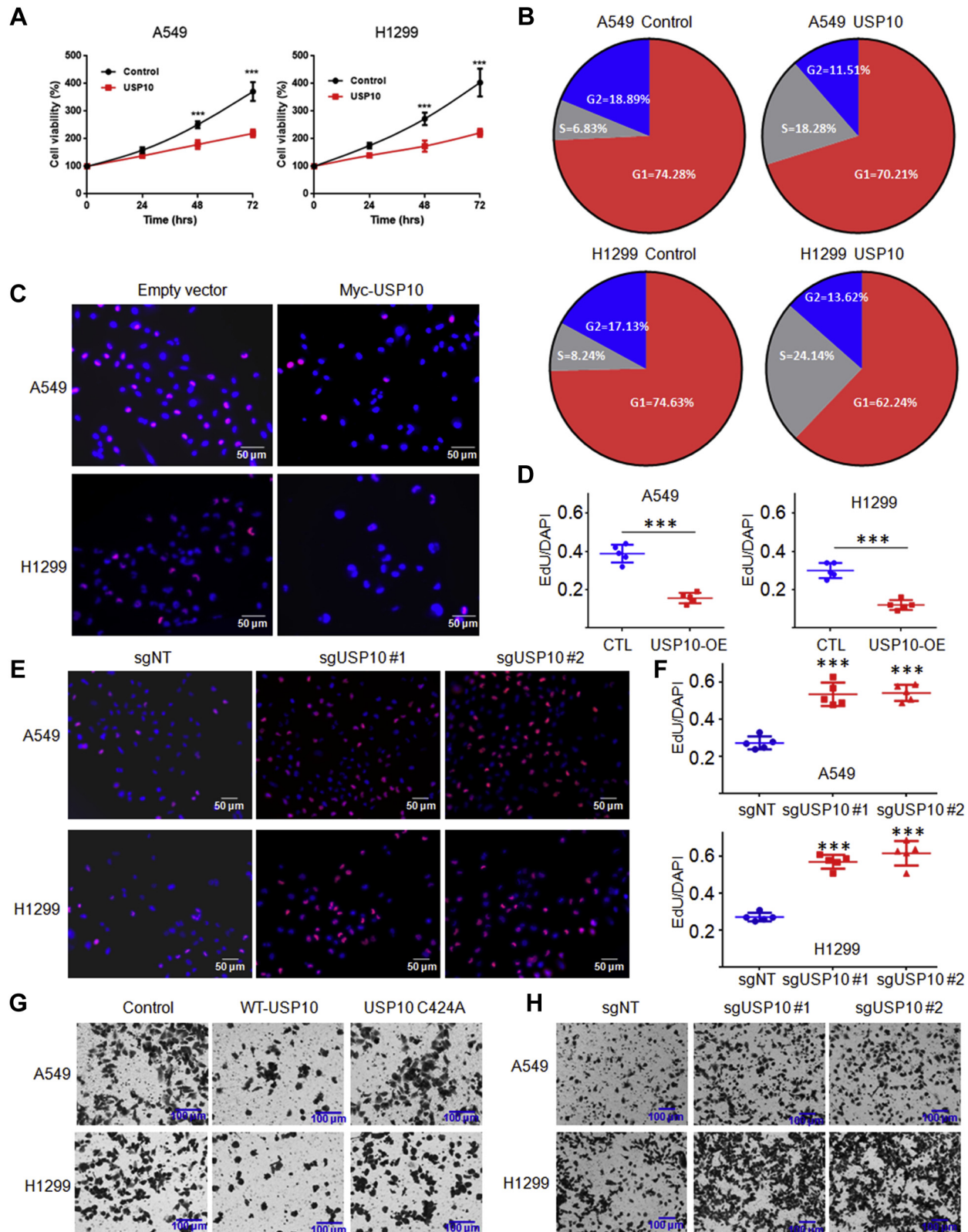


Figure 6. USP10 suppresses NSCLC cell proliferation and migration. *A*, A549 and H1299 cells transfected with a USP10 plasmid were replated in 96-well plates for MTT assay. *B*, A549 and H1299 cells were transfected with a USP10 plasmid for 48 h, followed by cell cycle analyses. *C*, A549 and H1299 cells were transfected with a USP10 plasmid for 48 h, followed by EdU incorporation and fluorescent microscopy assay. *D*, the statistical analysis of EdU-positive cells from *C*. *E*, USP10 was knocked out by its sgRNA from A549 and H1299 cells. Seventy-two hours later, cells were incorporated with EdU and analyzed by fluorescent microscopy assay. *F*, the statistical analysis of EdU-positive cells from *E*. *G*, A549 and H1299 cells were transfected with a WT or C424A mutant USP10 plasmid for 48 h, followed by transwell assay. *H*, A549 and H1299 cells were subjected to knock out USP10 using sgRNA, followed by transwell assay. EdU, 5-ethynyl-2'-deoxyuridine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; NSCLC, non-small cell lung cancer; sgRNA, single-guide RNA; USP10, ubiquitin-specific protease 10.

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been reported to arrest cancer cell cycle through its phosphatase activity (23). We thus further examined the effects of USP10 on NSCLC cell cycle progression by the flow cytometric assay. The results revealed that USP10 markedly increased the fraction of NSCLC cells at the S phase of the cell cycle (Fig. 6B), indicating it might suppress DNA synthesis, which was consistent with a previous study (24).

We also evaluated cell proliferation by the 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay to further confirm the role of USP10 on NSCLC cells. The results showed that ectopic USP10 led to significant decrease of EdU staining in both A549 and H1299 cells (Fig. 6, C and D). In contrast, knockout of USP10 increased EdU staining in both A549 and H1299 cells (Fig. 6, E and F). Because EdU incorporation is a biomarker for thymidine uptake in DNA synthesis and cell proliferation, this result firmly supported that USP10 suppresses NSCLC proliferation by inhibiting DNA synthesis. Given the PTEN/AKT/mTOR pathway also modulates cancer cell migration and epithelial–mesenchymal transition (25), we next measured the effects of USP10 on NSCLC cell migration by the transwell assay. As shown in Figure 6G, overexpression of USP10 significantly decreased A549 and H1299 cell number in transwells, but the C424A mutant failed to prevent NSCLC cell migration. Moreover, when USP10 was knocked out by its specific sgRNA, both cell lines displayed aggressive migration in the same assays (Fig. 6H). Therefore, USP10 suppressed NSCLC cell migration, which is consistent with the modulation of USP10 on the PTEN/AKT/mTOR signaling transduction. All in all, these results demonstrated that USP10 is downregulated in NSCLC cells, and restoration of USP10 inhibits NSCLC cell proliferation and migration by targeting the PTEN/AKT/mTOR pathway *via* preventing PTEN from K63-linked polyubiquitination.

Discussion

The aforementioned study found that USP10 is a specific Dub of PTEN by removing its K63-linked polyubiquitination. Moreover, although it is downregulated in NSCLC cells, restoration of USP10 can activate PTEN, therefore decreasing the production of PI(3,4,5)P₃ and suppressing the AKT/mTOR signaling transduction and inhibiting NSCLC cell proliferation and migration. To the best of our knowledge, this is the first study on a Dub that activates PTEN by preventing its K63-linked polyubiquitination.

Ubiquitination is a critical post-translational modification that regulates protein stability, subcellular localization, and activity. Several previous studies have demonstrated that PTEN could undergo various ubiquitination modifications, including monoubiquitination that affects PTEN shift between cytosol and nuclei (9), K48-linked polyubiquitination that leads to PTEN degradation (12–15). Our recent study found that PTEN could be modified by K63-linked polyubiquitination under the direction of the ubiquitin ligase TRIM25 (10). The ubiquitination is a dynamic process that specific Dubs are able to remove ubiquitin molecules from the substrate proteins. Although several Dubs have been

identified for PTEN deubiquitination, most of them including OTUD3 (12) and USP13 (15) stabilize PTEN by preventing its K48-linked polyubiquitination, whereas USP7 mediates PTEN in monoubiquitination and regulates its subcellular distribution (13). The present study identified USP10 as a putative Dub of PTEN, and it specifically decreases the K63-linked polyubiquitination from PTEN. Different from K48-linked polyubiquitination, K63-linked polyubiquitination regulates protein subcellular localization, trafficking, and signaling transduction. In the present study, we confirmed the feature of this type of polyubiquitination. As demonstrated in our previous study, TRIM25 redistributes PTEN by mediating its K63-linked polyubiquitination, thus inhibiting PTEN phosphatase activity (10). In contrast to TRIM25, the WT, but not the dead C424A mutant USP10, decreases the K63-linked polyubiquitin chain of PTEN and increases PTEN distribution in plasma membrane, thus restoring PTEN activity and suppressing the AKT/mTOR signaling pathway. The identification of USP10 as a Dub of PTEN for its K63-linked polyubiquitination is the last piece of the puzzle in the modulation network of PTEN ubiquitination, which is critical for understanding the regulation of PTEN phosphatase activity.

USP10 is one of the most important Dubs that have been associated with various substrates, therefore displaying both oncogenic and tumor suppressor activities depending on the contexts in the specific cancer types. For example, USP10 can promote tumorigenesis of prostate cancer as a cofactor of androgen receptor to stimulate the androgen response of target promoters, which is independent of the Dub activity (26). But in most cases, USP10 function is reportedly determined by its deubiquitinating activity. USP10 deubiquitinates the histone variant H2A.Z, thus activating androgen receptor-mediated gene activation in prostate cancer (27). In addition to prostate cancer, USP10 also promotes the proliferation of hepatocellular carcinoma as a Dub and a stabilizer of the oncogenic transcription factor Yes-associated protein/transcriptional coactivator with PDZ-binding motif (28). USP10 also confers to hepatocellular carcinoma metastasis by deubiquitinating and stabilizing Smad4 protein (29). However, USP10 also acts as a tumor suppressor gene especially in NSCLC. USP10 has been assigned as a Dub of several key tumor suppressor proteins including p53 (18), KLF4 (30), and p14ARF (31). To our attention, USP10 was also found to suppress the AKT/mTOR signaling pathway by inhibiting PTEN, but the underlying mechanisms are controversial. One study showed that USP10 stabilizes PTEN by deubiquitination, but it was performed in HEK293T cells but not NSCLC cells (32). And this result contradicts with the finding in NSCLC cells in which USP10 interacts with PTEN but has no effects on its protein stability (15). Another study showed that USP10 inhibits the AKT/mTOR signaling but does not provide sufficient data on PTEN stability in hepatocellular carcinoma cells (33). In the present study, we screened a panel of Dubs to identify novel modulators on PTEN/AKT/mTOR signaling and found that USP10 suppresses AKT activation. The subsequent experiments in various NSCLC cell lines and primary

tissues and *in vitro* ubiquitination assays provide robust evidence that demonstrate USP10 interacts with PTEN but has no effects on PTEN stability in NSCLC cells, which is consistent with the finding published in the *Journal of Nature Cell Biology* (15). Moreover, the present study demonstrates that USP10 deubiquitinates PTEN for its K63 polyubiquitination but not other ubiquitination forms under the direction of the ubiquitin ligase TRIM25 (10). By countering TRIM25, USP10 rescues PTEN phosphatase activity and reduces the production of PI(3,4,5)P₃ in NSCLC cells, therefore inactivating the AKT/mTOR signaling pathway in NSCLC cells (Fig. 5). Furthermore, either chemical or genetic inhibition of USP10 activates the AKT/mTOR signals (Fig. 5H); in contrast, restoration of wtUSP10 but not its dead mutant reactivates PTEN and suppresses NSCLC cell proliferation and migration (Fig. 6). Specifically, the underlying mechanism is probably that the K63-linked polyubiquitination of PTEN at K266 is critical for its plasma membrane localization. It has been well demonstrated that PTEN displays its phosphatase activity on plasma membrane, where it is easier to access PI(3,4,5)P₃, therefore hydrolyzing PI(3,4,5)P₃ to phosphatidylinositol-4,5-trisphosphate (21, 22). The present study further demonstrated this observation because IB assays showed that PTEN in plasma membrane is significantly modulated by TRIM25 and USP10 in that TRIM25 reduces but USP10 increases the distribution level of PTEN in the plasma membrane (Fig. 5 and Ref. (10)). This is further supported by the fact that K266 is a critical acceptor site for PTEN K63-linked polyubiquitination and K266R PTEN fails to be localized to plasma membrane (Fig. 5). All these findings are consistent with the PI(3,4,5)P₃ production and AKT/mTOR signaling activation by modulating by TRIM25 and USP10.

Overall, the present study is the first one to report USP10 as a Dub for PTEN K63-linked polyubiquitination mediated by TRIM25. Given that the PTEN/AKT/mTOR axis is a central signaling node in NSCLC proliferation and progression, the present study highlights a novel regulatory mechanism in the PTEN/AKT/mTOR signaling pathway. Given USP10 is frequently downregulated, restoration of USP10 may provide a new strategy for the treatment of patients with NSCLC.

Experimental procedures

Primary NSCLC tissues

Patients with NSCLC were diagnosed at the First Affiliated Hospital of Soochow University. All patients were provided with written notice for use of the tissue species for research purpose, and the informed consent form was signed by each patient before operation. Once the NSCLC tissues were obtained, the paracancerous tissues were isolated from the central tumors. The individual tissues were then dip frozen in liquid nitrogen for further use. This study was approved by the Review Board and Ethical Committee of Soochow University.

Cell culture

HEK293T cells were kindly provided by Dr Michael F. Moran, University of Toronto. HEK293T cells were

maintained in Dulbecco's modified Eagle's medium (Corning). MM cell lines (RPMI8226, LP1, OCL-MY5, OPM2, and KMS11) were obtained from Dr Aaron Schimmer, University of Toronto, and cells were cultured in Iscove's modified Dulbecco's medium. Prostate cancer cells (PC3 and DU145) and NSCLC cell lines (H1299, H460, and A549) were purchased from American Type Culture Collection. Cells were cultured in RPMI1640 medium. Cell line WPMY1 was obtained from China Center for Type Culture Collection. All the media were supplemented with 10% of fetal bovine serum, glutamine, and antibiotics as required.

Plasmids and antibodies

TRIM25, PTEN, and Ub were cloned as described previously (23, 28, 34). USP10 was cloned from HEK293T cells by RT-PCR using primers 5'-ctagtctagaATGCCCTGGTTGCCCTCTCC-3' and 5'-ccgctcgagTTACAGCAGGTCCACTCGGC-3'. The full-length complementary DNA was then inserted into a pcDNA3.1 vector with a Myc tag. Monoclonal anti-HA, anti-Myc, and anti-Flag were obtained from MBL. Antibody against GAPDH was purchased from Proteintech. Antibodies against AKT, p-AKT, mTOR, p-mTOR, PTEN, TRIM25, and USP10 were purchased from Cell Signaling Technology, Inc. An antibody against Ub was purchased from Santa Cruz Biotechnology. Horseradish peroxidase-labeled goat antimouse and goat anti-rabbit immunoglobulin G (IgG) (H + L) antibodies were purchased from Beyotime Institute of Biotechnology.

siRNA and shRNA

Both siRNA and shRNA species were obtained from Ribobio. The specific siRNA sequences for USP10 were 5'-CCAAGGTTATACCACAAAA-3', 5'-TTACCAGCAGAGTTCACAAA-3', and 5'-CCACTCGGTCAATGAAGAA-3'. The shRNA sequences for PTEN were 5'-ACAGCTAGAAC TTATCAAA-3', 5'-ACAAATGAAGGGATATAAAA-3', and 5'-GCGCTATGTGTATTATTAT-3'. These siRNA and shRNA were transfected into HEK293T cells using polyethylenimine as the gene carrier (10). Forty-eight hours later, cells were prepared for IB assay to evaluate the knockdown efficacy.

CRISPR genome editing

To generate USP10-knockout A549 and H1299 cells, optimal guide RNA target sequences were designed using the Benchling CRISPR Genome Engineering tool (<https://www.benchling.com>). The sgRNA target sequences for USP10 were 5'-CACCGGACTCCTCGATCTTCAGTTG-3' (#1) and 5'-CACCGACGGGACAGAATGGCAGTGC-3' (#2). The sgRNA sequences were cloned into lentiCRISPRv2 as reported previously (35).

IB

After transfection with appropriate plasmids, cells were lysed on ice in a lysis buffer as described previously (36). After clarifying at high speed at 4 °C, protein concentrations were

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determined by bicinchoninic acid assay (Beyotime). Equal amounts of proteins (30 µg) were fractionated in SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore). The blots were subjected to analysis against appropriate antibodies as described previously (28).

IP

After transfection with appropriate plasmids for 48 h, cells were harvested to prepare whole cell lysates for the IP assay as described previously (28). The precipitated beads were then boiled with 2× SDS loading buffer for IB assay.

Immunofluorescence assay

A549 and H1299 cells were plated into 24-well plate. Cells were then fixed in 4% paraformaldehyde, permeabilized with 0.02% Triton X-100, and then blocked with PBS supplemented with 20% bovine serum. Cells were incubated with anti-PTEN and anti-USP10-specific antibodies, which were diluted at 1:100 in PBS with 0.3% Tween-20 and 1% bovine serum albumin and incubated overnight at 4 °C. Following three washes (5 min per wash) at room temperature with PBS, cells were incubated with secondary antibodies mixture containing goat antimouse IgG labeled with fluorescein isothiocyanate (1:200; Beyotime) and goat anti-rabbit IgG labeled with cyanine (Cy)3 (1:200; Beyotime) at room temperature for 3 h. Images were observed by laser scanning confocal microscope.

MTT assay

NSCLC cells were transfected with USP10 for 24 h before being subjected to MTT assay as described previously (36).

Cell proliferation EdU image assay

To measure cell proliferation activity, NSCLC cells were transfected with USP10 and stained with the Cell-Light EdU Apollo567 *In Vitro* Kit (Ribobio) according to the manufacturer's instruction and further analyzed on a fluorescence microscopy.

Cell cycle with flow cytometric analysis

NSCLC cells were transfected with USP10 for 48 h. Cells were collected for cell cycle reagents staining followed the instruction from the manufacturer (MultiSciences Biotech Co, Ltd) and subjected to analysis on a BD flow cytometer as described previously (36).

CHX chase assay

After transfected with plasmids of interest for 36 h, NSCLC cells were then treated with CHX (100 µg/ml) for 0 to 12 h before lysis. Proteins were then fractionated on SDS-PAGE and IB analyses with specific antibodies as described previously (37).

RT-PCR

Total RNA was extracted using Trizol (Sangon Biotech). RNA (1 µg) was reverse transcribed using an EasyScript

First-strand cDNA Synthesis (Vazyme) according to the manufacturer's instruction. PCR amplification was carried out using the following primers: for USP10, 5'-AATAAAGG-GAACTGGTGC-3' (Forward) and 5'-CTATCATGGGTGTTGACGT-3' (Reverse); for GAPDH, 5'-AATCCCATCACCA TCTTCC-3' (Forward) and 5'-CATCACGCCACAGTTTCC-3' (Reverse); for PTEN, 5'-ACCATAACCCACCACAGC-3' (Forward) and 5'-ACCAGTTCGTCCTTTCC-3' (Reverse). The PCR products were visualized by GoldView staining (TransGen), following electrophoresis on 1.5% agarose gels.

PI(3,4,5)P3 assay

PI(3,4,5)P3 concentration was determined by using the PI(3,4,5)P3 kit provided by Elabscience Biotechnology Co, Ltd. Specifically, PI(3,4,5)P3 was released by repeated freezing and thawing from 1×10^6 of A549 and H1299 cells transfected with USP10 or control, followed by high-speed centrifugation to remove cell debris. The concentration of PI(3,4,5)P3 was determined using the specific protocol as described in our previous study (10).

In vitro ubiquitination assay

This study was performed as described previously (38). In the study of USP10 removing TRIM25-mediated Ub chains from PTEN, purified Flag-PTEN, Flag-TRIM25, and Myc-USP10 were added to the reaction mixture containing ATP, HA-Ub, with or without E1 and E2 (Boston Biochem). The reaction was then terminated and subjected to IP with a PTEN antibody and subsequent IB assay with a Ub antibody. To determine the direct effects of USP10 on PTEN K63-linked polyubiquitination, Flag-PTEN and HA-K63-Ub plasmids were cotransfected into HEK293T cells for 48 h, followed by purification with anti-Flag antibodies. These purified Flag-PTEN proteins were then equally split into two tubes that were added with ATP, purified USP10, or its empty plasmid. After incubation at 30 °C for 2 h, the reaction was stopped with 2× SDS loading buffer and subjected to IB with anti-HA (for K63 Ub) antibody.

Densitometric analysis

Densitometric analysis of immunoblots in the protein stability were performed as described previously using ImageJ© software developed by the National Institutes of Health (39).

Statistics

Statistical difference between the control and the experimental groups was analyzed by Student's *t* test.

Data availability

All data are contained within the article.

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Abbreviations—The abbreviations used are: CHX, cycloheximide; Dub, deubiquitinase; EdU, 5-ethynyl-2'-deoxyuridine; HEK293T, human embryonic kidney 293T; IB, immunoblotting; IgG, immunoglobulin G; IP, immunoprecipitation; mTOR, mammalian target of rapamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; NSCLC, non-small cell lung cancer; PI(3,4,5)P3, phosphatidylinositol-3,4,5-trisphosphate; PI3K, phosphatidylinositol-3-kinase; PTEN, phosphatase and tensin homolog deleted on chromosome 10; sgRNA, single-guide RNA; TRIM25, tripartite motif containing 25; USP10, ubiquitin-specific protease 10.

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