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ORIGINAL ARTICLE

RFP-mediated ubiquitination of PTEN modulates its effect on AKT activation

James T Lee[1], Jing Shan[1], Jiayun Zhong[1], Muyang Li[1], Brenda Zhou[1], Amanda Zhou[1], Ramon Parsons[1], Wei Gu[1]

[1]*Institute for Cancer Genetics, and Department of Pathology and Cell Biology, College of Physicians and Surgeons, Columbia University, 1130 St Nicholas Avenue, New York, NY 10032, USA*

The PTEN tumor suppressor is a lipid phosphatase that has a central role in regulating the phosphatidylinositol-3-kinase (PI3K) signal transduction cascade. Nevertheless, the mechanism by which the PTEN activity is regulated in cells needs further elucidation. Although previous studies have shown that ubiquitination of PTEN can modulate its stability and subcellular localization, the role of ubiquitination in the most critical aspect of PTEN function, its phosphatase activity, has not been fully addressed. Here, we identify a novel E3 ubiquitin ligase of PTEN, Ret finger protein (RFP), that is able to promote atypical polyubiquitinations of PTEN. These ubiquitinations do not lead to PTEN instability or relocalization, but rather significantly inhibit PTEN phosphatase activity and therefore modulate its ability to regulate the PI3K signal transduction cascade. Indeed, RFP overexpression relieves PTEN-mediated inhibitory effects on AKT activation; in contrast, RNAi-mediated knockdown of endogenous RFP enhances the ability of PTEN to suppress AKT activation. Moreover, RFP-mediated ubiquitination of PTEN inhibits PTEN-dependent activation of TRAIL expression and also suppresses its ability to induce apoptosis. Our findings demonstrate a crucial role of RFP-mediated ubiquitination in controlling PTEN activity.

***Keywords*:** PTEN; RFP; ubiquitination; AKT; phosphorylation
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Introduction

PTEN was originally identified as a tumor suppressor gene on chromosome 10, which is frequently lost in late-stage human cancers, especially those of the brain, prostate and breast [1]. Mutations, deletions and transcriptional silencing of PTEN have been found in a variety of human cancers, representing one of the most important tumor suppressor genes in the human genome. PTEN is a lipid phosphatase that catalyzes PtdIns(3,4,5)P3 (PIP3) to PtdIns(4,5)P2 (PIP2), thus antagonizing the activity of phosphoinositide 3-kinase (PI3K), which phosphorylates PIP2 to PIP3 [2]. Through their plekstrin homology domains, PDK1 and AKT are recruited by PIP3 to the plasma membrane, which facilitates PDK1-mediated AKT phosphorylation at threonine 308 (T308) [3]. After subsequent phosphorylation at serine 473 (S473) by mTORC2, AKT becomes fully activated and phosphorylates a wide range of substrates that inhibit apoptosis and promote cell survival and proliferation. Specifically, activated AKT can phosphorylate FOXO proteins, which prevents FOXO proteins from entering the nucleus to promote transactivation of genes that regulate apoptosis, such as TRAIL [4, 5]. Primary tumors and tumor cell lines that contain mutated or deleted PTEN all contain hyperactive AKT [6-9]. In fact, dysfunction of the AKT signaling pathway is present in a wide range of tumors, distinguishing PTEN as an essential component in tumor suppression [10-13].

Many mouse models have demonstrated the important role of PTEN in the AKT signaling pathway. In PTEN−/− tissues, AKT was found to have enhanced levels of phosphorylation at both T308 and S473 sites [14, 15]. AKT activity was elevated in almost every PTEN-deficient tissue or organ, and AKT1 deletion was sufficient to rescue PTEN+/– mice from developing tumors in many susceptible tissues, suggesting that PTEN is a critical regulator of AKT signaling [16]. The ability of PTEN to tightly

Correspondence: Wei Gu
Tel: 212-851-5282; Fax: 212-851-5284
E-mail: wg8@columbia.edu
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regulate AKT signaling has been proposed to be one of the major mechanisms of PTEN tumor suppression.

PTEN regulation of AKT signaling has been actively pursued by examining various posttranslational modifications of PTEN. For instance, phosphorylation, acetylation and oxidation have been demonstrated to regulate PTEN phosphatase activity [17-20]. In addition, a HECT domain-containing E3 ligase, Nedd4-1, was identified to be able to mediate PTEN ubiquitination. Wang *et al.* [21]. reported that Nedd4-1 polyubiquitinates and promotes proteasome-dependent degradation of PTEN. Conversely, Trotman *et al*. [22] demonstrated that Nedd4-1 mediates PTEN monoubiquitination, which regulates PTEN nuclear import. However, genetic ablation of Nedd4-1 did not result in the enhanced expression of PTEN or the decreased localization of PTEN to the nucleus, suggesting that there may be other E3 ligases involved in the ubiquitination of PTEN [23]. Recently, WWP2 was also shown to polyubiquitinate PTEN and target PTEN for proteasomal degradation [24]. Moreover, HAUSP, a deubiquitinating enzyme, was identified to mediate the removal of ubiquitin from PTEN [25]. Although Nedd4-1 and WWP2 may be involved in regulating PTEN localization and stability through ubiquitination, it remains to be determined whether ubiquitination can affect PTEN phosphatase activity directly.

We sought to identify additional novel E3 ligases that may also be able to ubiquitinate PTEN. In a screen for E3 ligases of PTEN, we discovered a potential E3 ligase Ret finger protein (RFP), or TRIM27. RFP, a ~58 kDa protein, is a member of the tripartite motif (TRIM) family, consisting of a conserved motif collectively called RBCC, which includes a RING finger (R), a B box zinc finger (B) and a coiled-coiled (CC) domain, and a specific carboxyl-terminal region known as the RFP domain. Although RFP has been found to regulate IKK function and serve as a transcription repressor, it is unknown whether RFP can mediate ubiquitination in order to execute its cellular functions [26, 27]. We demonstrate here that RFP is a novel E3 ubiquitin ligase and interacts with PTEN to catalyze a non-canonical form of PTEN ubiquitination. This novel form of ubiquitination diminishes the effect of PTEN on AKT signaling, without affecting PTEN stability or localization.

Results

RFP was identified as a novel binding partner of PTEN

Previously, we showed that HAUSP and E3 ligase Mdm2, which deubiquitinates and ubiquitinates p53, respectively, were found in the same complex [28]. As HAUSP is also a deubiquitinase for PTEN, we sought to identify whether a novel E3 ligase for PTEN exists in the HAUSP protein complex. Utilizing a Flag-HA-HAUSP stable U2OS cell line, we were able to purify interacting proteins of Flag-HA-HAUSP after sequential immunoprecipitations using M2 and HA pull-downs. Through mass spectrometry of isolated bands from immunoprecipitates resolved on SDS-PAGE, we identified 21 peptides corresponding to RFP/TRIM27, a novel HAUSP-binding partner. Although PTEN is a HAUSP substrate, the interaction between PTEN and HAUSP may be transient. Consequently, we were unable to purify a substantial amount of PTEN, although a small portion of PTEN may still form a complex with HAUSP (Supplementary information, Figure S1).

To demonstrate that RFP interacts with PTEN, we performed co-immunoprecipitation experiments using 293 cells cotransfected with untagged PTEN and Flag-RFP or empty vector (Figure 1A). Immunoprecipitation of transfected cell lysates with M2 beads revealed that PTEN was specifically co-immunoprecipitated in cells transfected with Flag-RFP (lane 3), but not with empty vector (lane 4). In addition, we performed co-immunoprecipitation analysis with either anti-PTEN or anti-RFP antibody to examine the interaction between endogenous PTEN and RFP in U2OS cells (Figure 1B). We found that PTEN was present in the immunoprecipitation obtained with anti-RFP antibody (lane 6); conversely, RFP was observed to be co-immunoprecipitated with anti-PTEN antibody (lane 3).

Next, to determine the PTEN-binding site on RFP, we first generated RFP truncation mutants as diagrammed in Figure 1C, and then coexpressed truncated, full-length RFP or empty vector with PTEN-EGFP in 293 cells (Figure 1C). Immunoprecipitation of transfected cell lysates with M2 beads revealed that PTEN did not interact with the truncation mutant that lacks the C-terminal RFP domain (RBC) (lane 3), but was still able to bind to the mutant with intact RFP domain (F) (lane 4), suggesting that the RFP domain is the critical PTEN-binding domain.

The RFP-binding domain on PTEN was identified in a similar fashion by utilizing PTEN truncation mutants as diagrammed in Figure 1D [29]. PTEN truncation mutants or empty vector were coexpressed with RFP in 293 cells (Figure 1D), and immunoprecipitation of transfected cell lysates with M2 beads revealed that RFP did not interact with the truncation mutant that lacks the Tail domain (NC2) (lane 2), whereas the PDZ-binding domain (bd) is dispensible for this interaction (lanes 3 and 4), suggesting that the Tail domain is the critical RFP-binding domain.

Furthermore, to demonstrate that PTEN and RFP indeed interact directly and not through an intermedi-

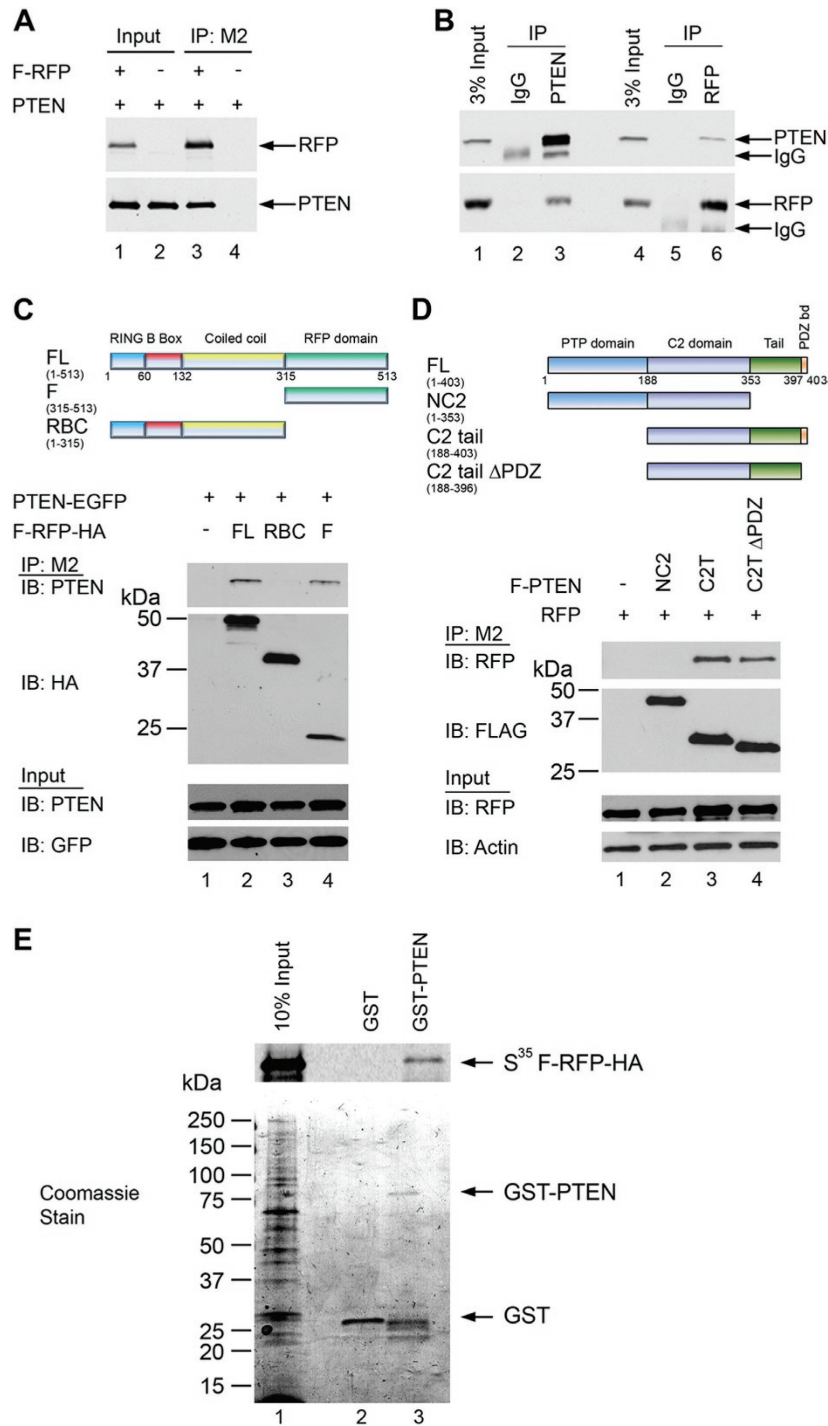

Figure 1 PTEN interacts with RFP. **(A)** PTEN co-immunoprecipitates with RFP in transfected 293 cells. In all, 293 cells were co-transfected with PTEN and F-RFP or empty vector, and the cell lysates were immunoprecipitated with M2 beads followed by immunoblotting with anti-PTEN and anti-RFP antibodies. **(B)** Endogenous interaction between PTEN and RFP in U2OS cells. U2OS cell lysates were immunoprecipitated with either anti-PTEN antibody, anti-RFP antibody or control IgG followed by immunoblotting for PTEN and RFP. The input represents 3% of total amount of lysate immunoprecipitated. **(C)** Mapping of PTEN interaction domain. In all, 293 cells were co-transfected with the indicated GFP, RFP and PTEN constructs. The lysates were immunoprecipitated with M2 beads, followed by immunoblotting with anti-HA and anti-GFP antibodies. **(D)** Mapping of RFP interaction domain. In all, 293 cells were co-transfected with the indicated RFP and PTEN constructs. The lysates were immunoprecipitated with M2 beads, followed by immunoblotting with anti-RFP, anti-FLAG and anti-actin antibodies. **(E)** PTEN interacts with RFP directly. GST or GST-PTEN was incubated with *in vitro*-translated S^{35} F-RFP-HA in a GST pull-down assay.

ate interaction, we performed a GST pull-down assay (Figure 1E). We incubated purified GST or GST-PTEN in the presence of *in vitro* translated S^{35} Flag-RFP-HA and demonstrated that GST-PTEN (lane 3), but not GST alone (lane 2), was able to specifically pull down S^{35} Flag-RFP-HA. Together, these data suggest that PTEN and RFP interact directly.

RFP is an E3 ligase for PTEN

As many members of the TRIM family harbor E3 ubiquitin ligase activity through the RING domain, we sought to determine whether RFP also functions as a *bona fide* E3 ligase. Indeed, using a cell-free ubiquitination assay, RFP was observed to self-ubiquitinate in a dose-dependent manner (Figure 2A). Moreover, the ability of RFP to self-ubiquitinate was confirmed through an in-cell ubiquitination assay in which 293 cells were transfected with RFP and His-ubiquitin or empty vector (Figure 2B). These data together suggest that RFP is able to function as a *bona fide* RING E3 ubiquitin ligase.

To test the possibility that PTEN was a substrate of RFP-mediated ubiquitination, we used a cell-free ubiquitination assay in which immunopurified Flag-PTEN protein was incubated with recombinant E1, E2 and either GST-RFP, ubiquitin or both (Figure 2C). Polyubiquitinated PTEN was detected when both GST-RFP and ubiquitin were present with Flag-PTEN (lane 2), but not when either component was absent (lanes 1 and 3), suggesting that RFP is responsible for the polyubiquitination of PTEN. In addition, we sought to confirm this ubiquitination in cells by transfecting PTEN-EGFP along with full-length (FL) or delta RING (ΔRING) Flag-RFP-HA, and found that in the absence of RFP, ubiquitinated PTEN was in very low abundance (Figure 2D, lane 2), whereas coexpression of FL RFP resulted in a significantly higher level of ubiquitinated PTEN (lane 4) than coexpressing RFP ΔRING (lane 3). Furthermore, we utilized an endogenous ubiquitination assay to examine the role of endogenous RFP in regulating endogenous PTEN ubiquitination. After transfection with RFP siRNA or control siRNA in the presence of HA-His-Ub, polyubiquitinated PTEN was detected by immunoblotting for PTEN (Figure 2E). When RFP expression was depleted by siRNA, endogenous PTEN ubiquitination levels were attenuated (lane 2) compared to mock depletion (lane 1). These data further suggest that RFP is an endogenous E3 ubiquitin ligase for PTEN polyubiquitination.

Polyubiquitination specificity is determined by the ubiquitin lysine linkage between each sequential ubiquitin conjugate. K48-linkage has been shown to target proteins for proteasomal degradation, whereas K63-linkage has been linked to trafficking of proteins to the lysosome, as well as other activities [30]. To elucidate the ubiquitin linkage associated with RFP-mediated PTEN ubiquitination, we transfected 293 cells with Flag-RFP-HA and PTEN-EGFP in the presence of various ubiquitins, including wild type (WT), a lysine mutant with all lysines mutated (K0), or lysine mutants that contain only one unaltered lysine site (Figure 3A). This experiment revealed that K27 ubiquitin was most readily incorporated by RFP-mediated PTEN ubiquitination (lane 7), but still exhibited a diminished ubiquitination signal compared with WT ubiquitin, suggesting that other ubiquitin linkages may also be catalyzed by RFP. This was also verified in a cell-free ubiquitination assay, demonstrating the ability of RFP to directly incorporate K27 ubiquitin onto PTEN (Supplementary information, Figure S2A). Next, by using lysine-to-arginine mutations of specific ubiquitin lysine sites, we determined that the K27-linkage, as well as other atypical linkages, were involved in RFP-mediated PTEN ubiquitination (Figure 3B). The usage of K27R ubiquitin mutant led to the most dramatic reduction of the RFP-mediated PTEN ubiquitination (lane 5), whereas exogenous expressions of K6R (lane 4), K29R (lane 6) and K63R (lane 8) also attenuated the ubiquitination level of PTEN, suggesting that other lysine linkages may also be present in RFP-mediated PTEN ubiquitination, albeit at lower levels. In addition, K48R ubiquitin-transfected cells (lane 7) exhibited similar ubiquitination levels as that of WT ubiquitin-transfected cells (lane 2), therefore ruling out K48-linkage as an important linkage for RFP-mediated PTEN ubiquitination.

It was previously identified that both K13 and K289 in PTEN were sites of monoubiquitination by Nedd4-1 [22]. We sought to determine whether K13 and K289 were targets of RFP-mediated PTEN ubiquitination. As demonstrated in an ubiquitination assay in Figure 3C, the mutations of both K13 and K289 to glutamic acids (K13, K298E) did not affect RFP-mediated ubiquitination (lanes 6 and 8) or the binding to RFP (Supplementary information, Figure S2B), suggesting that RFP-mediated PTEN ubiquitination occurs at novel lysine sites. In addition, RFP was also able to mediate K27-linked ubiquitination on PTEN K13, 289E (Supplementary information, Figure S2C). Furthermore, in an effort to identify the essential lysine site(s) ubiquitinated by RFP on PTEN, we screened 21 other lysine residues and concluded that not one, but many lysine residues, may be ubiquitinated by RFP (Figure 3D). In lanes 8-11, those lysine mutations did not prevent RFP-mediated PTEN ubiquitination, but instead, attenuated PTEN ubiquitination levels compared with the WT control. These mutated lysine residues seem to be clustered in the C2 domain, suggesting that ubiquitination may cause conformational changes similar to the

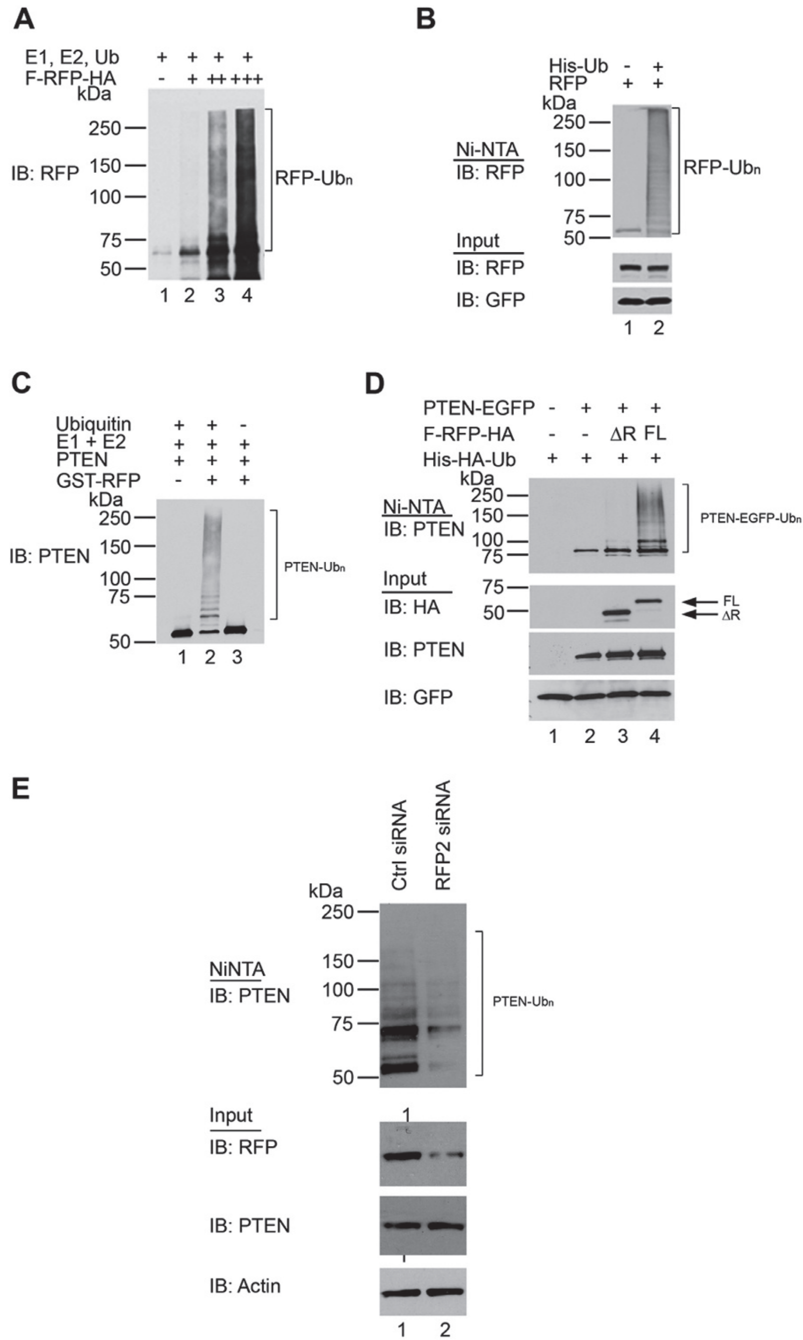

Figure 2 RFP is a *bona fide* E3 ubiquitin ligase for PTEN. **(A)** RFP self-ubiquitinates in cell-free conditions. Increasing amounts of immunopurified Flag-HA-RFP was added to recombinant E1, E2 and ubiquitin, and the mixture was incubated at 37 °C for 2 h, followed by subsequent immunoblotting by RFP antibody. **(B)** RFP self-ubiquitinates in cells. 293 cells were co-transfected with GFP, RFP and His-ubiquitin or empty vector. The input was immunoblotted for RFP and GFP (middle and bottom panel). Ni-NTA-purified lysate is immunoblotted for anti-RFP (top panel). **(C)** PTEN was ubiquitinated by RFP in cell-free conditions. Immunopurified PTEN was added to recombinant GST-RFP, E1, E2 and ubiquitin, and the mixture was incubated at 37 °C for 2 h, followed by subsequent immunoblotting by anti-PTEN antibody. **(D)** PTEN is ubiquitinated by RFP FL, but not ΔRING. In all, 293 cells were co-transfected with GFP, PTEN-EGFP and F-RFP-HA FL or ΔRING. The input was immunoblotted for RFP with anti-HA antibody, PTEN-EGFP with anti-PTEN antibody and GFP transfection control with anti-GFP antibody. Ni-NTA-purified lysate was immunoblotted with anti-PTEN antibody. **(E)** Endogenous RFP mediates endogenous PTEN ubiquitination. In all, 293 cells were transfected with either control or RFP 2 siRNA for 48 h then subsequently transfected with His-HA-Ub for an additional 24 h. The input was immunoblotted with anti-RFP, anti-PTEN and anti-actin antibodies. Ni-NTA-purified lysate was immunoblotted with anti-PTEN antibody.

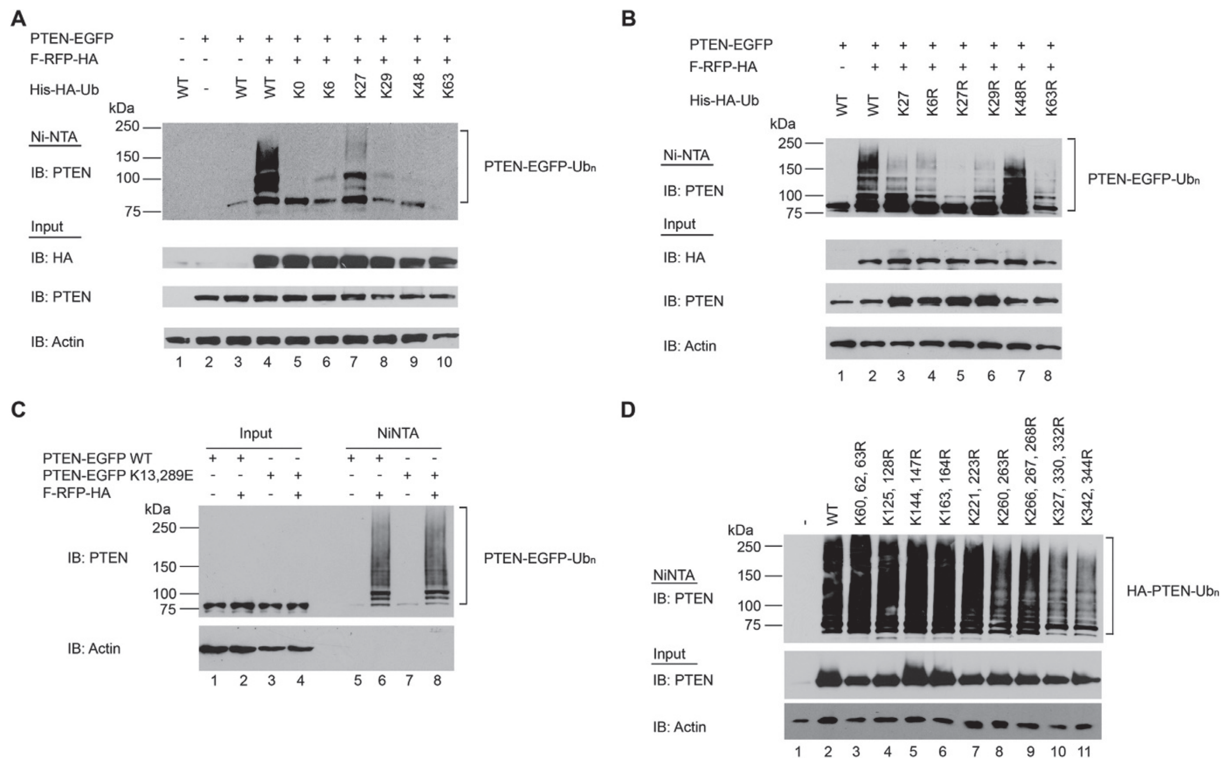

Figure 3 RFP mediates atypical ubiquitin linkages on PTEN C2 domain lysine residues. **(A, B)** RFP mediates K48-independent forms of poly-ubiquitination on PTEN. In all, 293 cells were co-transfected with His-Ubiquitin WT or lysine mutants, PTEN-EGFP, and Flag-RFP-HA. The input was immunoblotted for either HA, PTEN or actin. Ni-NTA-purified lysate was immunoblotted with anti-PTEN antibody. **(C)** RFP does not ubiquitinate K13 and K289 sites. 293 cells were co-transfected with His-Ubiquitin WT, PTEN-EGFP WT or K13, 289E mutant and Flag-RFP-HA. The inputs and Ni-NTA-purified lysates were immunoblotted for PTEN and actin. **(D)** Screen of RFP-mediated PTEN ubiquitination sites. In all, 293 cells were co-transfected with His-Ubiquitin WT, HA-PTEN WT or KR mutants, and Flag-RFP-HA. The input was immunoblotted for PTEN and actin. Ni-NTA-purified lysate was immunoblotted with anti-PTEN antibody.

effect that phosphorylation has on the C2 domain and the C-terminal tail [20, 31].

RFP-mediated PTEN ubiquitination does not affect PTEN stability but affects PTEN activity

Ubiquitination has been linked to the degradation of proteins, depending on the form of ubiquitination conjugated to the substrate protein [30]. To determine whether RFP affects PTEN stability, we transfected different amounts of Flag-RFP-HA along with PTEN in 293 cells and were unable to detect a change in PTEN stability as a result of RFP overexpression (Figure 4A, lanes 2 and 3). This suggests that atypical PTEN ubiquitination does not target it for degradation. These results were further validated by an experiment where RFP was depleted from 293 cells and subsequently treated with cyclohexamide (CHX) to block protein translation. We found that when endogenous RFP was depleted by siRNA, endogenous PTEN levels did not accumulate or decrease (Figure 4B, lanes 4-6), suggesting that RFP does not regulate PTEN stability. Moreover, we could not detect a change in the localization of PTEN after RFP overexpression (Figure 4C).

As RFP-mediated PTEN ubiquitination did not affect the stability or localization of PTEN, we aimed to identify the function of PTEN ubiquitination. Various posttranslational modifications of PTEN, such as phosphorylation and acetylation, can change its conformation and affect its function [20]. In order to analyze whether PTEN ubiquitination leads to altered PTEN phosphatase activity, we immunopurified ubiquitinated PTEN and non-ubiquitinated PTEN from 293 cells overexpressing PTEN (Figure 4D) and examined their enzymatic activity by measuring the accumulation of PIP2 in the presence of increased dose of PTEN (Figure 4E). Ubiquitinated PTEN exhibits significant reduction in phosphatase activity as measured by the level of PIP2 accumulation (Figure 4E), suggesting that ubiquitination may directly

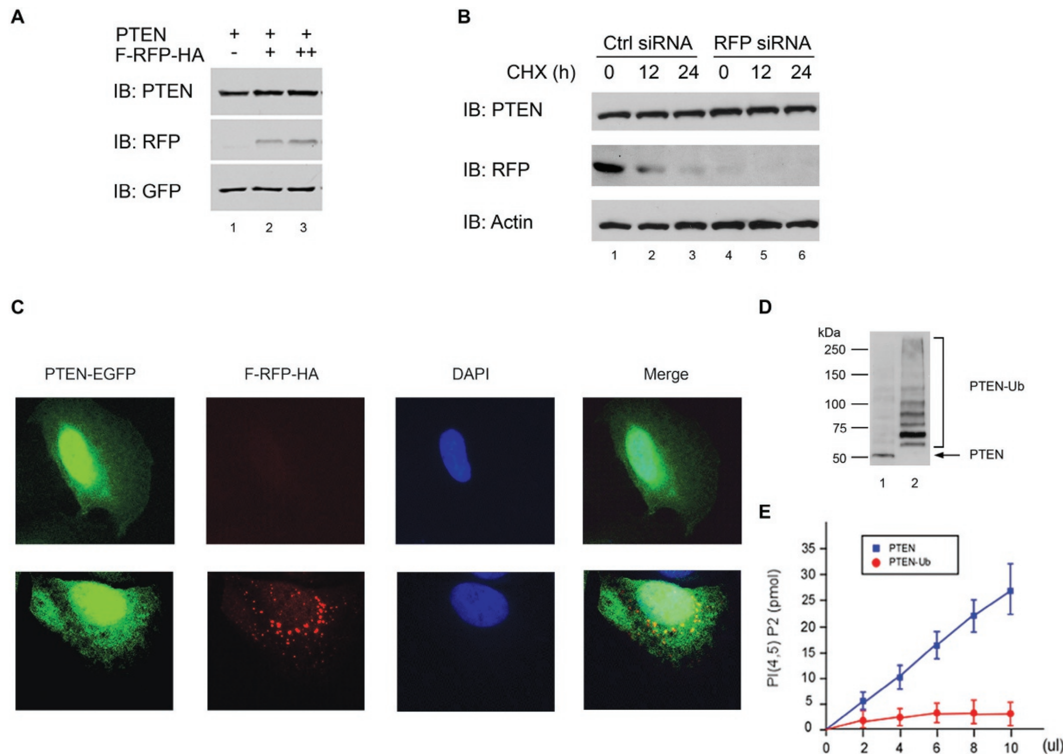

Figure 4 RFP-mediate PTEN ubiquitination does not affect PTEN stability, but affects PTEN phosphatase activity. **(A)** RFP overexpression does not affect PTEN stability. In all, 293 cells were co-transfected with GFP, PTEN and Flag-HA-RFP or empty vector. Lysate was immunoblotted for PTEN (top panel), RFP (middle panel), and GFP (bottom panel). **(B)** RFP expression knockdown does not affect endogenous PTEN stability. In all, 293 cells were transfected with RFP 2 siRNA or Ctrl siRNA for 48 h, and subsequently treated with 20 µg/ml CHX for 12 and 24 h. Cell lysates were separated on SDS-PAGE and immunoblotted with PTEN, RFP and actin antibodies. **(C)** RFP overexpression does not change PTEN localization. Top row, U2OS cells co-transfected with PTEN-EGFP (green) and empty vector. Bottom row, U2OS cells co-transfected with PTEN-EGFP (green) and F-RFP-HA. U2OS cells were immunostained with HA antibody (red) and stained with DAPI (blue). (**D**, **E**) PTEN was immunopurified from cells with PTEN antibody, while PTEN-Ub was double-immunopurified from cells with PTEN antibody and subsequently with HA-antibody (for HA-ubiquitin conjugated PTEN purification). Increasing amounts of PTEN and PTEN-Ub were used for ELISA assay. Colorimetric readings were measured to quantify the amount of PI(4,5)P2 converted from PI(3,4,5)P3 by PTEN phosphatase activity by comparing to a standard curve (n = 3). The data represent mean ± SEM.

affect PTEN activity without altering PTEN stability or localization.

RFP reverses PTEN-mediated AKT inhibition

PTEN phosphatase activity is critical for the regulation of AKT phosphorylation and its downstream signaling. Tumor suppressor function of PTEN is partially mediated through its regulation of AKT signaling. As we have determined that PTEN is ubiquitinated by RFP and ubiquitinated PTEN leads to a decrease in PTEN phosphatase activity, we sought to examine the effect that overexpressed RFP may have on AKT phosphorylation. To examine this, we overexpressed PTEN-Flag in the presence of Flag-RFP-HA FL, ΔRING, and the binding domain-deletion mutant RBC, in U87 cells, a PTEN-null cell line and observed that RFP FL overexpression was able to rescue PTEN-mediated inhibition of phosphorylated threonine 308 (P-T308) AKT levels, but not phosphorylated serine 473 (P-S473) levels (Figure 5A, lane 4). In addition, RFP enzymatic mutant (ΔRING) (lane 5) and PTEN-binding mutant (RBC) (lane 6) were both unable to rescue PTEN-mediated inhibition of AKT phosphorylation. Interestingly, this result was not due to changes in PDK1 level, suggesting that RFP acts directly on PTEN to ameliorate the inhibition of AKT signaling.

RFP-mediated PTEN ubiquitination regulates insulin-AKT signaling pathway

Recently, insulin signaling was linked to the promotion of tumorigenesis [32]. Insulin signaling begins with the activation of the RTK insulin receptor by insulin. Subsequently, through multiple phosphorylation events,

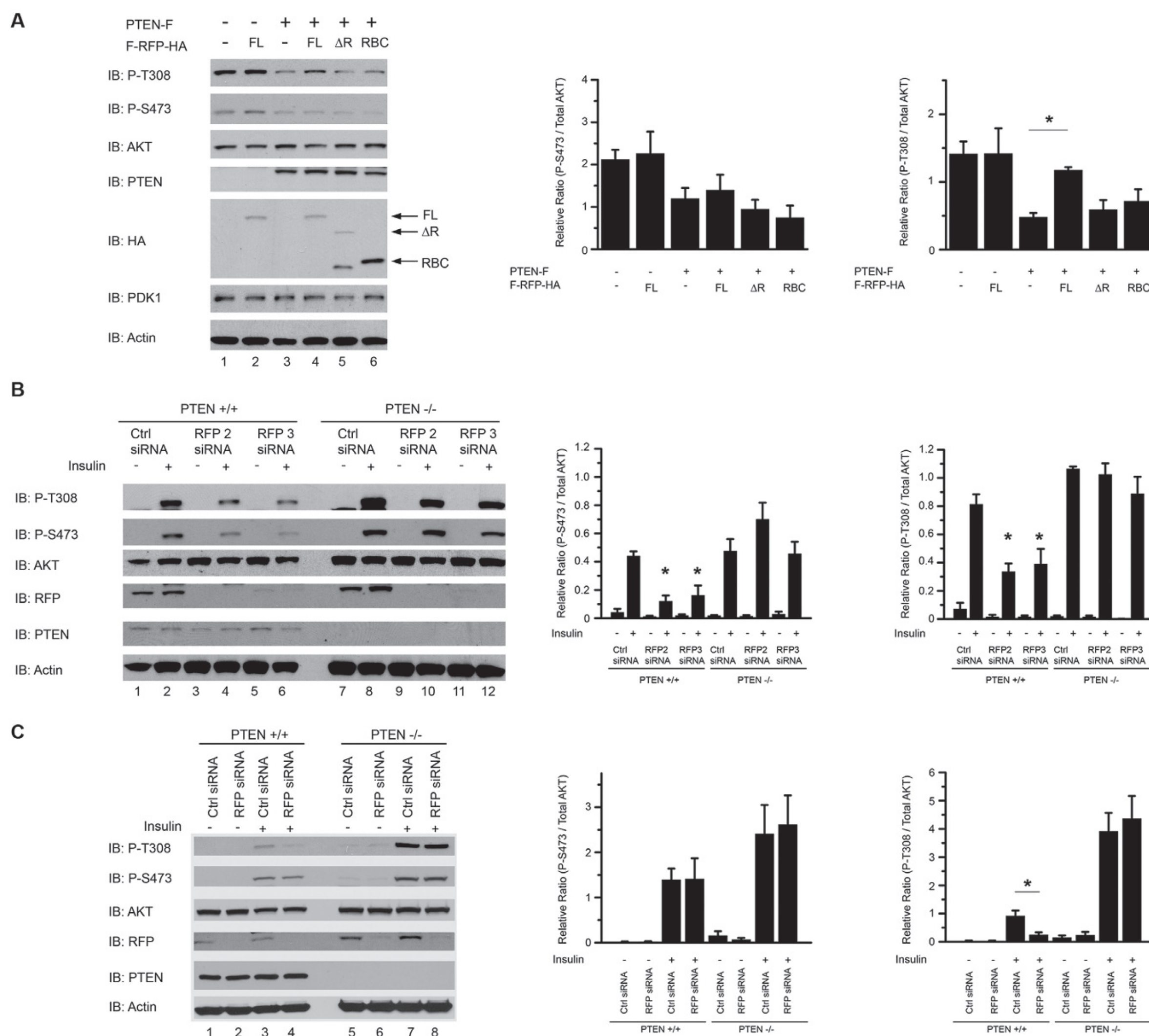

Figure 5 RFP-mediated PTEN ubiquitination regulates AKT phosphorylation. **(A)** RFP FL ameliorates PTEN inhibition of AKT phosphorylation, whereas RFP mutants cannot rescue PTEN inhibition. U87 cells were co-transfected with PTEN-Flag, Flag-RFP-HA FL or mutants. The lysates were immunoblotted by appropriate antibodies as stated. AKT phosphorylation level was quantified by comparing relative phosphorylated AKT to total AKT levels of three independent experiments. The data represent mean ± SEM from three independent experiments. * Significantly different from PTEN-F-transfected only cells ($P < 0.05$). **(B)** RFP-knockdown specifically attenuates AKT phosphorylation in PTEN+/+, not PTEN−/− MCF10A cells. MCF10A parental PTEN+/+ and PTEN−/− cells were transfected with either control or RFP siRNAs for 48 h. The cells were then serum-starved for 24 h and subsequently treated with 10 ng/ml insulin for 5 min. Lysate was immunoblotted by appropriate antibodies as stated. AKT phosphorylation level was quantified by comparing relative phosphorylated AKT to total AKT levels of three independent experiments. The data represent mean ± SEM from three independent experiments. * Significantly different from insulin-treated control siRNA-transfected cells ($P < 0.05$). **(C)** RFP knockdown specifically attenuate AKT phosphorylation in PTEN+/+, not PTEN−/− MEF cells. MEF PTEN+/+ and PTEN−/− cells were transfected with either control or RFP siRNAs for 48 h. The cells were then serum-starved for 24 h and subsequently treated with 10 ng/ml insulin for 5 min. The lysate was immunoblotted by appropriate antibodies as stated. AKT phosphorylation level was quantified by comparing relative phosphorylated AKT to total AKT levels of three independent experiments. The data represent mean ± SEM from three independent experiments. * Significantly different from insulin-treated control siRNA-transfected cells ($P < 0.05$). Student's *t*-test was performed for all the above experimental groups to determine statistical significance of samples.

PI3K phosphorylates PIP2 to PIP3, leading to the activation of the AKT signaling pathway [33]. This process is antagonized by PTEN, which leads to the attenuation of AKT signaling. We sought to determine whether RFP

was capable of regulating endogenous PTEN by performing siRNA-knockdown of RFP expression in two isogenic MCF10A cell lines that contained either homozygous null or WT PTEN alleles (Figure 5B). These cells were then serum-starved for 24 h and stimulated with insulin to induce AKT phosphorylation. Both siRNA oligos used to knockdown RFP expression significantly reduced RFP protein levels. With insulin stimulation, there were significant decreases in AKT phosphorylation levels at both phosphorylation sites (T308 and S473) after RFP knockdown (lanes 4 and 6) compared with that in mock knockdown (lane 2) in the MCF10A PTEN+/+ cells. In contrast, there were no changes in AKT phosphorylation levels in the PTEN−/− cells (lanes 8, 10, and 12). These data suggest that RFP affects insulin-induced AKT phosphorylation in a PTEN-dependent manner.

Next, we sought to confirm these results in PTEN−/− and PTEN+/+ MEFs by testing the response of these cells after siRNA-mediated knockdown of RFP, serum starvation and the subsequent insulin stimulation (Figure 5C). We observed a significant decrease in P-T308 AKT levels, but not in P-S473 AKT levels, after insulin treatment in RFP-depleted PTEN+/+ MEF cells (lane 4) compared with that in the control (lane 3). These results support the overexpression data in U87 cells, and demonstrate that RFP can affect the function of PTEN to regulate AKT phosphorylation at site T308.

RFP-mediated PTEN ubiquitination reduces TRAIL activation and inhibits apoptosis

FOXO proteins are downstream targets of AKT, which has been demonstrated to regulate apoptosis by preventing FOXO from entering the nucleus after AKT-mediated FOXO phosphorylation [5]. In the presence of PTEN, AKT is not activated, therefore allowing FOXO proteins to enter the nucleus and subsequently activate gene transcription of many FOXO targets. One such target of FOXO1 that was identified to regulate apoptosis was TRAIL [4]. TRAIL protein levels were found to directly correlate with PTEN levels in prostate cancer cell lines [4]. Here, we sought to investigate the role of RFP-mediated PTEN ubiquitination in the transactivation of the TRAIL-luciferase (TRAIL-luc) reporter. We transfected 293 cells with TRAIL-luc and Flag-RFP-HA FL or the ΔRING mutant in the presence or absence of PTEN. In the presence of PTEN, TRAIL-luc activity was significantly enhanced, whereas RFP FL and ΔRING did not affect TRAIL-luc activity (Figure 6A). However, when RFP FL was coexpressed with PTEN, TRAIL-luc activity was significantly inhibited in a dose-dependent manner. This effect was regulated by RFP-mediated PTEN ubiquitination, as Flag-RFP-HA ΔRING was unable to significantly inhibit PTEN-activated TRAIL-luc activity. Moreover, we analyzed the ability of Flag-RFP-HA FL to rescue apoptotic cell death induced by PTEN overexpression in U2OS cells. We showed that overexpression of PTEN for 48 h induced PARP cleavage, which has been extensively used as an apoptosis marker (Figure 6B, lane 3). In contrast, the coexpression of Flag-RFP-HA reduced the total amount of cleaved PARP (lane 4). These data suggest that RFP-mediated PTEN ubiquitination can suppress TRAIL transactivation and apoptosis through the inhibition of PTEN phosphatase activity. Our proposed model is that RFP-mediated ubiquitination negatively regulates PTEN phosphatase activity, which results in the increase in AKT phosphorylation and inhibition of TRAIL-mediated apoptosis (Figure 6C).

Discussion

Accumulating evidence suggests that ubiquitination is an integral part of PTEN tumor suppression regulation. Previously, two E3 ubiquitin ligases were identified to regulate PTEN stability and localization, which resulted in the inhibition of PTEN-mediated dephosphorylation of PIP3 [21, 22, 24]. PTEN serves as a critical regulator of AKT by tightly controlling the response of AKT signaling triggered by insulin. Here, we have demonstrated that RFP, a novel E3 ligase, is capable of mediating a new form of PTEN ubiquitination. This ubiquitination does not affect PTEN stability, but negatively regulates PTEN phosphatase activity, which relieves the inhibition of AKT phosphorylation in both overexpression systems and endogenous insulin signaling.

RFP belongs to the TRIM (tripartite motif)/RBCC family proteins, as they comprise a RING domain, one or two B-box motifs and a Coiled-Coil region. We determined that RFP is a *bona fide* E3 ubiquitin ligase. Like other members in the TRIM family [34], such as TRIM5a, TRIM8, TRIM11, TRIM18, TRIM25 and TRIM33, RFP functions as an E3 ligase through its RING domain as demonstrated by our current study. Moreover, not only RFP is capable of self-ubiquitination, but also can mediate ubiquitination of PTEN. The major form of ubiquitination that RFP mediates on PTEN appears to be K27-linked ubiquitination, although other ubiquitin linkages also seem to be present. K27-linked ubiquitin chain disassembly by the 26S proteasome is as inefficient as that of K63-linked ubiquitins, suggesting that K27-linked ubiquitination may execute a novel function [35]. Not surprisingly, RFP-induced PTEN ubiquitination has no effect on PTEN stability as K48-linkage is not present in RFP-mediated PTEN ubiquitination. We also demonstrate that RFP-mediated PTEN ubiquitination

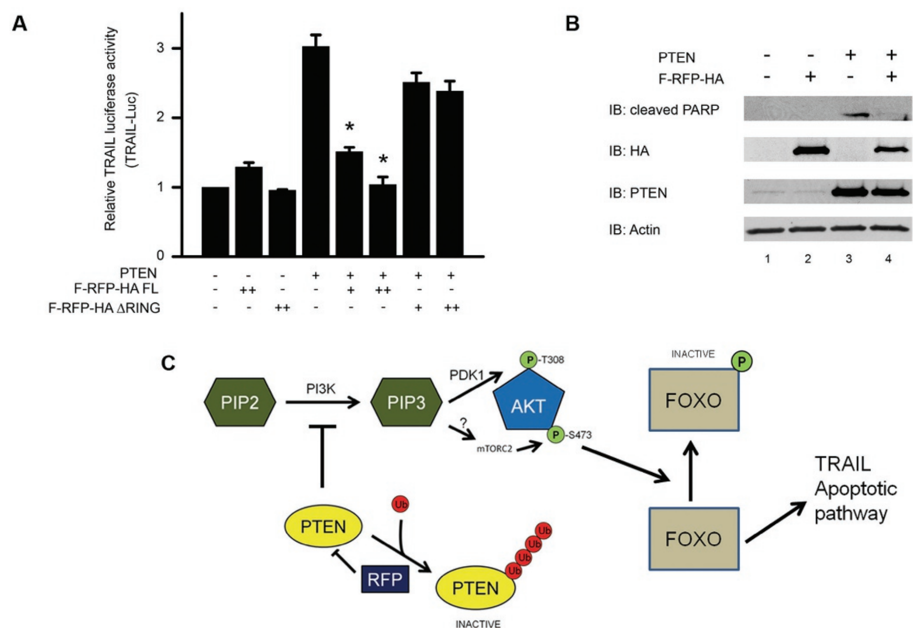

Figure 6 RFP-mediated PTEN ubiquitination inhibits TRAIL transactivation and apoptosis **(A)** RFP co-expression rescues PTEN-mediated activation of TRAIL-luc in a RING-dependent manner. In all, 293 cells were transfected with TRAIL-luciferase promoter construct and PTEN, F-RFP-HA FL or ΔRING as indicated. The lysates were assayed for the dual-luciferase activity. The data represent mean ± SEM from three independent experiments. * Significantly different from PTEN-transfected only cells ($P < 0.05$). Student's *t*-test was performed for all experimental groups above to determine statistical significance of samples. **(B)** RFP overexpression rescues cells from PTEN-induced apoptosis. U2OS cells were transfected with PTEN and F-RFP-HA as indicated for 48 h. The lysates were immunoblotted by appropriate antibodies as stated. **(C)** Model of PTEN activity regulation by RFP-mediated ubiquitination.

does not utilize the same sites as Nedd4-1 nor does it relocalize PTEN to the nucleus, but instead, RFP inhibits PTEN phosphatase activity, which relieves the negative regulation of PTEN on AKT activation. Indeed, RFP overexpression relieves inhibition of AKT signaling and reduces TRAIL-mediated apoptosis, in the presence of PTEN, suggesting that RFP has a critical role in regulating PTEN phosphatase activity through ubiquitination.

Insulin receptor antagonists have been demonstrated to be important in reducing tumorigenesis [32]. Here, we examined the effect of RFP on PTEN regulation of insulin signaling. Insulin binds to insulin receptors and induces a phosphorylation cascade where PI3K phosphorylates PIP2 to PIP3, leading to the activation of the AKT signaling pathway. PTEN actively antagonizes this activity to prevent sustained AKT phosphorylation. Knockdown of RFP in both MCF10A and WT MEF cells was sufficient to reduce insulin-induced AKT phosphorylation, which supports the role of RFP in regulating PTEN activity. We also determined that in MEF and U87 cells, RFP regulation of PTEN affected AKT phosphorylation at T308, but not at S473, whereas the levels of PDK1, the kinase for T308, remained unchanged. The experiments in MCF10A and MEF cells, when treated with insulin, demonstrate a potential endogenous role of RFP-mediated PTEN ubiquitination. These results support the notion that PTEN regulates T308 phosphorylation more readily through the reduction of PIP3 levels, by dephosphorylating PIP3 to PIP2 in the cells, which then inhibits PDK1-mediated AKT phosphorylation at T308 endogenously (Figure 6C). However, it is unknown how PTEN affects S473 phosphorylation, which is mediated by mTORC2. This may be regulated by PTEN through indirect mechanisms that could account for the results of the siRNA-mediated knockdown of RFP in MCF10A cells [6-9, 14, 15, 36]. Moreover, PDK1 knockout tissues were shown to have a complete loss of phosphorylation of T308, whereas S473 phosphorylation levels were unaffected [37]. This further supports the idea that PTEN regulation of S473 phosphorylation may not be through the recruitment of PDK1 by PIP3. However, AKT phosphorylation at both sites are required for robust AKT kinase activity, suggesting that diminishing phosphorylation levels at one site is sufficient to alter the function of its downstream effectors, such as FOXO proteins, which regulate TRAIL transactivation [4, 38]. Therefore, we conclude that RFP

is capable of fine-tuning PTEN to regulate insulin signaling through the modulation of AKT phosphorylation.

PTEN phosphatase activity has long been implicated as its major tumor suppressor function. Together with our current findings that RFP-induced PTEN ubiquitination inhibits PTEN phosphatase activity, we propose a model in which RFP may function as an oncogene by inhibiting PTEN tumor suppression. Interestingly, several lines of evidence have also implicated RFP to be involved in tumorigenesis. RFP mRNA is highly expressed in many human and mouse cancer cell lines [39], and high levels of RFP are observed in the solitary plasmacytoma and multiple myeloma [40]. In addition, positive RFP expression usually predicts a poor clinical outcome in patients with endometrial cancer and colon carcinoma [41, 42], and RFP expression is found in 41.4% of invasive breast carcinomas and in none of the non-neoplastic breast tissues [43]. Lastly, like two other members of the TRIM family, PML and TIF1a, RFP can become oncogenic by chromosomal rearrangement, in which its tripartite domain is fused with the tyrosine kinase domain of the Ret protein [39]. Thus, our current observation that RFP inhibits PTEN activity by mediating PTEN ubiquitination may provide an explanation for the positive correlation between RFP expression and oncogenesis. In addition, its effect on insulin signaling may further link RFP to a role in both regulating cancer growth and diabetes.

Materials and Methods

Plasmids, antibodies and cell culture

Antibodies used in this study include: anti-PTEN 6H2.1 (Cascade Biosciences); anti-PTEN 138G6, anti-P-T308 244F9, anti-P-S473 9271, anti-PDK1, anti-AKT (Cell Signaling); anti-β-actin AC-15, anti-PML (PG-M3); PARP (F-2) (Santa Cruz), anti-RFP (American Research Product), anti-HA 3F10 (Roche), anti-FLAG M2 (Sigma) and anti-GFP JL-8 (Clontech). U2OS, U87, 293 and MEF cells were maintained in DMEM (Cellgro) media and supplemented with 10% fetal bovine serum. MCF10A cells were maintained in 50/50 DMEM/F12 (Cellgro) media supplemented with 5% horse serum, insulin, EGF, cholera toxin and hydrocortisone. Transfection with plasmid DNA was performed using the calcium phosphate method and Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Complex purification from human cells

The epitope-tagging strategy to isolate HAUSP-containing protein complexes from human cells was performed essentially as described previously [44]. In brief, a Flag-HA-HAUSP-expressing U2OS stable cell line was harvested near the confluence. The cell pellet was resuspended in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), and protease inhibitors). The cells were left on ice for 15 min, after which 10% Nonidet P40 (Fluka) was added to a final concentration of 0.5%. The tube was vigorously vortex-mixed for 1 min. The homogenate was centrifuged for 10 min at 1 000× *g*. The nuclear pellet was resuspended in ice-cold buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT and protease inhibitors) and the tube was rocked vigorously at 4 °C for 45 min. The nuclear extract was diluted with buffer D (20 mM HEPES, pH 7.9, 1 mM EDTA) to a final NaCl concentration of 100 mM, ultracentrifuged at 69 300× *g* for 2 h at 4 °C. After filtration with 0.45-µm syringe filters (Nalgene), the supernatants were used as nuclear extracts for M2 immunoprecipitations by anti-Flag-antibody-conjugated agarose (Sigma). The bound polypeptides were eluted with the Flag peptide and were further affinity-purified by anti-HA-antibody-conjugated agarose (Sigma). The final eluates from the HA beads with HA peptides were resolved by SDS-PAGE on a 4%-20% gradient gel (Novex) for silver staining or staining analysis with colloidal blue. Specific bands were cut out from the gel and subjected to mass-spectrometric peptide sequencing.

GST pull-down assay

This assay was performed as described previously [45]. In brief, GST and GST-PTEN were induced in Rosetta (DE3) pLys cells (Novagen) at room temperature (25 °C), extracted with buffer BC500 (20 mM Tris-HCl, pH 7.3, 0.2 mM EDTA, 500 mM NaCl, 10% glycerol, 1 mM DTT, 0.5 mM PMSF) containing 1% Nonidet P40, and purified on glutathione-Sepharose (Pharmacia). F-RFP-HA expression vector was incubated with ^{35}S-methionine during *in vitro* translation (TNT Coupled Reticulocyte Lysate System; Promega Corporation). ^{35}S-labeled protein (5 ml) was incubated overnight with the purified GST or GST-PTEN, as indicated, in the presence of 0.2% BSA in BC100 on a rotator at 4 °C. The proteins were pulled down with GST beads; the beads were washed three times with BC200 and twice with BC100. The beads were added to 40 ml of SDS sample buffer and boiled for 5 min. The presence of ^{35}S-labeled protein was detected by autoradiography.

Co-immunoprecipitation assay

Co-immunoprecipitation assays were performed as described previously [46]. In brief, to detect the endogenous protein interaction between PTEN and RFP, U2OS cells were lysed in BC100 buffer (20 mM Tris-HCl pH 7.3, 100 mM NaCl, 10% glycerol, 0.2 mM EDTA, 0.2% Triton X-100 and protease inhibitors). Ten per cent of the cell extracts was kept for input, and the rest was incubated with mouse IgG, anti-PTEN antibody, anti-RFP antibody or rabbit IgG for 1 h at 4 °C. A/G Plus-Agarose beads (Santa Cruz Biotechnology) were then added for overnight incubation at 4 °C. After the beads were washed stringently, the bound proteins were eluted by boiling in SDS sample buffer, and detected by western blotting.

siRNA knockdown

RFP siRNA oligos are from Ambion for human (RFP siRNA 2 s11960, and RFP siRNA 3 s11961) and Dharmacon On-target smartpool for mouse. The cells were transfected with these siRNA oligos using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, harvested or serum-starved after 48 h. The samples were resolved on SDS-PAGE for western blot analysis.

Cell-free ubiquitination assay

To perform the cell-free RFP self-ubiquitination assay, the purified GST-RFP protein was incubated with E1, UbcH5a and ubiqui-

tin under the proper buffer condition for 2 h at 37 °C as described before [47], and the reaction mixtures were then subjected to western blot analysis using anti-RFP antibody (American Research Product, Inc.). To perform the cell-free PTEN-Ub assay, immunopurified F-RFP-HA protein was incubated with E1, UbcH5a, ubiquitin WT, K27, or K48 and immunopurified PTEN or PTEN-EGFP under the proper buffer condition for 2 h at 37 °C, and the reaction mixtures were then subjected to western blot analysis using anti-PTEN antibody.

In-cell ubiquitination assay

To perform the RFP self-ubiquitination assay in cells, 293 cells were transfected with His-Ub and RFP. Twenty-four hours later, 10% of the cells were lysed with FLAG lysis buffer (50 mM Tris-HCl, pH 7.3,137 mM NaCl, 10 mM NaF, 1 mM $NaVO_4$, 10% glycerol, 0.5 mM EDTA, 1% Triton X-100 and 0.2% Sarkosyl) and subjected to western blot analysis; the rest of the cells were lysed with guanidine buffer and subjected to Ni-NTA pull-down followed by western blot using anti-RFP antibody. To perform the PTEN ubiquitination assay in cells, 293 cells were transfected with PTEN, His-Ub and FH-RFP constructs. The rest of procedure was the same as described above for RFP ubiquitination assay, except the use of anti-PTEN antibody.

For endogenous ubiquitination assay, 293 cells were transfected with control siRNA or RFP 2 siRNA for 48 h as described above. The cells were then transfected once again with His-Ub and lysed 24 h later as described above.

Protein purification and PTEN phosphatase activity assay

Protein purification was performed in phosphate-free buffers as previously described [29]. Briefly, 293 cells expressing Flag-PTEN alone or with RFP and HA-ubiquitin were lysed in BC100 buffer supplemented with protease inhibitors. The lysates were vortexed vigorously and centrifuged at 100 000× *g* for 2 h. The supernatant was pre-incubated with protein A/G beads for 1 h. The supernatant was then incubated with M2 beads for 4 h at 4 °C. The bound beads are then washed four times with phosphatase buffer PB (100 mM NaCl, 25 mM Tris, pH7.4). The protein was eluted with Flag peptide for 2 h at 4 °C. For PTEN-Ub protein purifications, additional binding of eluate with HA beads and HA peptide elutions were performed. The eluates were quantified both by spectrophotometer and Coomassie staining before use in PTEN activity assay.

The ELISA kit was purchased from Echelon Biosciences Inc., and the assay was performed according to the manufacturer's instructions. Briefly, PTEN and PTEN-Ub were diluted to equal concentrations and then an increasing amount of PTEN and PTEN-Ub was added to the plate and incubated for 1 h. Next, the PI (4,5) P2 detector and HRP conjugate were added step-wise. Finally, colorimetric readings were measured to quantify the amount of PI (4,5) P2 converted from PI (3,4,5) P3 by PTEN phosphatase activity and compared with a standard curve.

Luciferase reporter gene assay

In all, 293 cells were transfected at 40% confluence in 24-well plates with plasmid DNA as indicated in the relevant figure. After 48 h of incubation, the cells were then harvested and the luciferase activity was measured using the Dual Luciferase Reporter Assay System Kit from Promega according to the manufacturer's protocol. Activity was assayed in three separate experiments and shown as the average mean ± standard error (SE) and Student's *t*-test was performed to identify the significant differences between samples (n = 3).

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References

1 Li J, Yen C, Liaw D, *et al.* PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* 1997; **275**:1943-1947.

2 Leslie NR, Downes CP. PTEN function: how normal cells control it and tumour cells lose it. *Biochem J* 2004; **382(Pt 1)**:1-11.

3 Alessi DR, Deak M, Casamayor A, *et al.* 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the Drosophila DSTPK61 kinase. *Curr Biol* 1997; **7**:776-789.

4 Modur V, Nagarajan R, Evers BM, Milbrandt J. FOXO proteins regulate tumor necrosis factor-related apoptosis inducing ligand expression. Implications for PTEN mutation in prostate cancer. *J Biol Chem* 2002; **277**:47928-47937.

5 Zhang X, Tang N, Hadden TJ, Rishi AK. Akt, FoxO and regulation of apoptosis. *Biochim Biophys Acta* 2011; **1813**:1978-1986.

6 Haas-Kogan D, Shalev N, Wong M, *et al.* Protein kinase B (PKB/Akt) activity is elevated in glioblastoma cells due to mutation of the tumor suppressor PTEN/MMAC. *Curr Biol* 1998; **8**:1195-1198.

7 DeGraffenried LA, Fulcher L, Friedrichs WE, *et al.* Reduced PTEN expression in breast cancer cells confers susceptibility to inhibitors of the PI3 kinase/Akt pathway. *Ann Oncol* 2004; **15**:1510-1516.

8 Sircar K, Yoshimoto M, Monzon FA, *et al.* PTEN genomic deletion is associated with p-Akt and AR signalling in poorer outcome, hormone refractory prostate cancer. *J Pathol* 2009; **218**:505-513.

9 Kurose K, Zhou XP, Araki T, *et al.* Frequent loss of PTEN expression is linked to elevated phosphorylated Akt levels, but not associated with p27 and cyclin D1 expression, in primary epithelial ovarian carcinomas. *Am J Pathol* 2001; **158**:2097-2106.

10 Carpten JD, Faber AL, Horn C, *et al.* A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. *Nature* 2007; **448**:439-444.

11 Bleeker FE, Felicioni L, Buttitta F, *et al.* AKT1(E17K) in human solid tumours. *Oncogene* 2008; **27**:5648-5650.

12 LoPiccolo J, Blumenthal GM, Bernstein WB, Dennis PA. Targeting the PI3K/Akt/mTOR pathway: effective combinations and clinical considerations. *Drug Resist Updat* 2008; **11**:32-

50.
13 Brugge J, Hung MC, Mills GB. A new mutational AKTivation in the PI3K pathway. *Cancer Cell* 2007; **12**:104-107.
14 Stambolic V, Suzuki A, de la Pompa JL, *et al.* Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* 1998; **95**:29-39.
15 Chalhoub N, Zhu G, Zhu X, Baker SJ. Cell type specificity of PI3K signaling in Pdk1- and Pten-deficient brains. *Genes Dev* 2009; **23**:1619-1624.
16 Chen ML, Xu PZ, Peng XD, *et al.* The deficiency of Akt1 is sufficient to suppress tumor development in Pten+/− mice. *Genes Dev* 2006; **20**:1569-1574.
17 Odriozola L, Singh G, Hoang T, Chan AM. Regulation of PTEN activity by its carboxyl-terminal autoinhibitory domain. *J Biol Chem* 2007; **282**:23306-23315.
18 Okumura K, Mendoza M, Bachoo RM, *et al.* PCAF modulates PTEN activity. *J Biol Chem* 2006; **281**:26562-26568.
19 Seo JH, Ahn Y, Lee SR, Yeol Yeo C, Chung Hur K. The major target of the endogenously generated reactive oxygen species in response to insulin stimulation is phosphatase and tensin homolog and not phosphoinositide-3 kinase (PI-3 kinase) in the PI-3 kinase/Akt pathway. *Mol Biol Cell* 2005; **16**:348-357.
20 Tamguney T, Stokoe D. New insights into PTEN. *J Cell Sci* 2007; **120(Pt 23)**:4071-4079.
21 Wang X, Trotman LC, Koppie T, *et al.* NEDD4-1 is a proto-oncogenic ubiquitin ligase for PTEN. *Cell* 2007; **128**:129-139.
22 Trotman LC, Wang X, Alimonti A, *et al.* Ubiquitination regulates PTEN nuclear import and tumor suppression. *Cell* 2007; **128**:141-156.
23 Fouladkou F, Landry T, Kawabe H, *et al.* The ubiquitin ligase Nedd4-1 is dispensable for the regulation of PTEN stability and localization. *Proc Natl Acad Sci USA* 2008; **105**:8585-8590.
24 Maddika S, Kavela S, Rani N, *et al.* WWP2 is an E3 ubiquitin ligase for PTEN. *Nat Cell Biol*; **13**:728-733.
25 Song MS, Salmena L, Carracedo A, *et al.* The deubiquitinylation and localization of PTEN are regulated by a HAUSP-PML network. *Nature* 2008; **455**:813-817.
26 Zha J, Han KJ, Xu LG, *et al.* The Ret finger protein inhibits signaling mediated by the noncanonical and canonical IkappaB kinase family members. *J Immunol* 2006; **176**:1072-1080.
27 Shimono Y, Murakami H, Hasegawa Y, Takahashi M. RET finger protein is a transcriptional repressor and interacts with enhancer of polycomb that has dual transcriptional functions. *J Biol Chem* 2000; **275**:39411-39419.
28 Brooks CL, Li M, Hu M, Shi Y, Gu W. The p53--Mdm2--HAUSP complex is involved in p53 stabilization by HAUSP. *Oncogene* 2007; **26**:7262-7266.
29 Fine B, Hodakoski C, Koujak S, *et al.* Activation of the PI3K pathway in cancer through inhibition of PTEN by exchange factor P-REX2a. *Science* 2009; **325**:1261-1265.
30 Komander D. The emerging complexity of protein ubiquitination. *Biochem Soc Trans* 2009; **37 (Pt 5)**:937-953.
31 Wang X, Jiang X. Post-translational regulation of PTEN. *Oncogene* 2008; **27**:5454-5463.
32 Gallagher EJ, LeRoith D. Minireview: IGF, Insulin, and Cancer. *Endocrinology 2011*; **152**:2546-2551.
33 Taniguchi CM, Emanuelli B, Kahn CR. Critical nodes in signalling pathways: insights into insulin action. *Nat Rev Mol Cell Biol* 2006; **7**:85-96.
34 Meroni G, Diez-Roux G. TRIM/RBCC, a novel class of 'single protein RING finger' E3 ubiquitin ligases. *Bioessays* 2005; **27**:1147-1157.
35 Xu P, Duong DM, Seyfried NT, *et al.* Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation. *Cell* 2009; **137**:133-145.
36 Chen CH, Shaikenov T, Peterson TR, *et al.* ER stress inhibits mTORC2 and Akt signaling through GSK-3beta-mediated phosphorylation of rictor. *Sci Signal* 2011; **4**:ra10.
37 Nakamura K, Sakaue H, Nishizawa A, *et al.* PDK1 regulates cell proliferation and cell cycle progression through control of cyclin D1 and p27Kip1 expression. *J Biol Chem* 2008; **283**:17702-17711.
38 Downward J. Mechanisms and consequences of activation of protein kinase B/Akt. *Curr Opin Cell Biol* 1998; **10**:262-267.
39 Takahashi M, Inaguma Y, Hiai H, Hirose F. Developmentally regulated expression of a human "finger"-containing gene encoded by the 5′ half of the ret transforming gene. *Mol Cell Biol* 1988; **8**:1853-1856.
40 Tezel G, Nagasaka T, Iwahashi N, *et al.* Different nuclear/cytoplasmic distributions of RET finger protein in different cell types. *Pathol Int* 1999; **49**:881-886.
41 Kato T, Shimono Y, Hasegawa M, *et al.* Characterization of the HDAC1 complex that regulates the sensitivity of cancer cells to oxidative stress. *Cancer Res* 2009; **69**:3597-3604.
42 Tsukamoto H, Kato T, Enomoto A, *et al.* Expression of Ret finger protein correlates with outcomes in endometrial cancer. *Cancer Sci* 2009; **100**:1895-1901.
43 Tezel GG, Uner A, Yildiz I, Guler G, Takahashi M. RET finger protein expression in invasive breast carcinoma: relationship between RFP and ErbB2 expression. *Pathol Res Pract* 2009; **205**:403-408.
44 Chen D, Kon N, Li M, *et al.* ARF-BP1/Mule is a critical mediator of the ARF tumor suppressor. *Cell* 2005; **121**:1071-1083.
45 Chen D, Shan J, Zhu WG, Qin J, Gu W. Transcription-independent ARF regulation in oncogenic stress-mediated p53 responses. *Nature* 2011; **464**:624-627.
46 Shan J, Zhao W, Gu W. Suppression of cancer cell growth by promoting cyclin D1 degradation. *Mol Cell* 2009; **36**:469-476.
47 Li M, Brooks CL, Wu-Baer F, *et al.* Mono- versus polyubiquitination: differential control of p53 fate by Mdm2. *Science* 2003; **302**:1972-1975.

(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)