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A Semisynthetic Approach to the Analysis of Tumor Suppressor PTEN Ubiquitination

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Abstract

Phosphatase and tensin homolog (PTEN) tumor suppressor protein is a PIP3 lipid phosphatase that is subject to multifaceted posttranslational modifications. One such modification is the mono-ubiquitination of Lys13 which may alter its cellular localization but is also positioned in a manner that could influence several of its cellular functions. To explore the regulatory influence of ubiquitin on PTEN's biochemical properties and its interaction with ubiquitin ligases and a deubiquitinase, generation of a site-specifically and stoichiometrically ubiquitinated protein could be beneficial. Here we describe a semisynthetic method that relies upon sequential expressed protein ligation steps to install ubiquitin at a Lys13 mimic in near full-length PTEN. This approach permits concurrent installation of C-terminal modifications in PTEN facilitating an analysis of the interplay between N-terminal ubiquitination and C-terminal phosphorylation. We find that N-terminal ubiquitination of PTEN inhibits its enzymatic function, reduces its binding to lipid vesicles, modulates its processing by NEDD4–1 E3 ligase, and is efficiently cleaved by the deubiquitinase, USP7. Our ligation approach should motivate related efforts for uncovering the effects of ubiquitination of complex proteins.

Graphical Abstract

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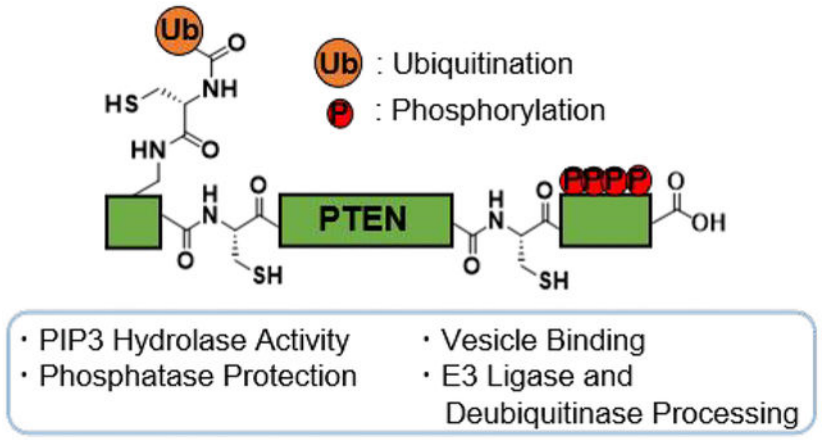
Supporting Information.

Supplemental information is available free of charge on the ACS Publications website:

- Additional experimental details, enzyme assay results, chromatograms, and mass spectra (PDF)
- Raw mass spectra and NMR spectra data files: https://doi.org/10.7910/DVN/HFFEUP



PTEN is a lipid phosphatase that cleaves the 3-phospho group from phosphatidyl-inositol-3,4,5-trisphosphate (PIP3), converting it to phosphatidyl-inositol-4,5-diphosphate (PIP2), opposing the growth stimulatory action of PI3-kinase in cell signaling.[1,2] Loss of function mutations are commonly observed in a variety of cancers, and PTEN is regarded as a major tumor suppressor gene.[3–5] As PTEN's physiological substrate PIP3 is embedded in the cell plasma membrane, PTEN's localization is critical to its function. PIP2 has been shown to be important in recruiting PTEN to the plasma membrane.[6–8]

PTEN is a 403 aa protein with architecture that includes an N-terminal segment that is suggested to be important in PIP2 binding, a catalytic phosphatase domain, a C2 membrane association domain, and a C-terminal regulatory tail.[2,9–11] PTEN is subject to a variety of post-translational modifications (PTMs), most notably C-terminal phosphorylation on a cluster of Ser/Thr residues (Ser380/Thr382/ Thr383/Ser385) and an N-terminal mono-ubiquitination (Ub) on Lys13 (Figure 1A).[12–14] It was shown previously that the C-terminal phospho-cluster can induce a closed conformation in PTEN, inhibiting its catalytic activity, reducing its membrane association, and diminishing its ubiquitination by WWP2 E3 ligase.[15–18] Ubiquitination of PTEN can have degradative and regulatory roles.[19–21] In this study, we focus on Lys13 mono-ubiquitination as it has been suggested to enhance its nuclear entry and Lys13 mutations have been noted to promote cancer.[14,22,23] However, inferences about the impact of PTEN ubiquitination have been based on cellular experiments using a ubiquitin resistant mutation, K13R, as Lys13Ub-PTEN has not been obtained previously as a purified entity.[14,22,24,25]

As for most proteins, enzymatic Lys ubiquitination of PTEN *in vitro* has been shown to be non-site selective and thus a chemical or semisynthetic approach to generate ubiquitinated proteins is desirable.[18,26–37] Here we have developed a new strategy to incorporate a Lys-Ub mimic on PTEN, and benchmarked its recognition by a deubiquitinase in comparison to several alternative synthetic mimics. The ubiquitination site at aa13 was modified using sequential sidechain and backbone expressed protein ligations (Figures 1B, 1C).[38] A PTEN N-terminal 16mer peptide was synthesized with an aminoAla-Cys isostere of Lys at aa13, and was then ligated to Ub thioester. The C-terminal aminoanilide of the Ub-peptide was then reacted with nitrite and MESNA to furnish a C-terminal thioester used for

chemoselective ligation to N-Cys containing PTEN (aa17–378; aa17–395) produced from insect cell expression.[39] The PTEN N-terminus is highly conserved, so the less conserved aa17 was chosen as the ligation junction.[6] The ligated PTEN fragments were themselves products of expressed protein ligation with either Cys to generate truncated PTEN (t-PTEN) or a N-Cys phosphorylated synthetic peptide (aa379–395; pS380/pT382/pT383/pS385).[15,16] t-PTEN was used as a previously validated surrogate for non-phosphorylated PTEN.[16,40] The final Ub-PTEN semisynthetic protein forms were purified in a monomeric state by size exclusion chromatography, appeared >90% pure by SDS-PAGE, and have the expected molecular weights by mass spectrometry (Figures 1D, S2–S3). By employing a soluble PIP3 substrate (C6-PIP3), we demonstrated that recombinant t-PTEN$_{Q17C}$ and semisynthetic t-PTEN$_{Q17C}$ control showed similar PIP3 hydrolytic activity to each other and were ~3-fold lower than recombinant t-PTEN$_{WT}$ (Figure 2A). These results show that semisynthesis on the N-terminus of PTEN and introduction of a Cys at aa17 are tolerable. The semisynthetic ubiquitinated PTEN forms, Ub-t-PTEN and Ub-4p-PTEN, lacked detectable PIP3 hydrolytic activity (at least 10-fold lower than the non-ubiquitinated forms) consistent with the sensitivity of PTEN's lipid phosphatase activity to Lys13 mutations.[6,11,41] As the decrease in catalytic activity of Ub-t-PTEN could be related to the unnatural Lys13 residue in this semisynthetic protein, we also prepared K13^{ac}-t-PTEN with an acetyl modification on the natural Lys13 sidechain (Figure S2). The ~5-fold reduced activity of K13^{ac}-t-PTEN (Figure 2A) indicates that Lys13 acylation itself diminishes PTEN's activity, regardless of the potential impact of the unnatural Lys sidechain.

The conformation of Ub-4p-PTEN was assessed by determining its susceptibility to C-terminal dephosphorylation by lambda phosphatase. Previous work showed that non-ubiquitinated 4p-PTEN adopts a conformation where the phosphates engage with the core of the protein to generate a "closed-state".[15,42] The propensity of PTEN to adopt the closed conformation can be assessed by a phosphatase sensitivity assay. In the closed-state, phosphates are shielded and resistant to dephosphorylation.[15] We observed that the rate of phosphate removal by lambda phosphatase from Ub-4p-PTEN (v/[E] 4.4±0.7 min^{-1}) closely matched that of 4p-PTEN (v/[E] 4.6±0.6 min^{-1}) (Figures 2B, S4). This similarity suggests that Lys13 ubiquitination of 4p-PTEN does not open the conformation and retains the engagement of the phosphorylated C-terminal tail with the core of PTEN. Moreover, the similar phosphatase sensitivities of Ub-4p-PTEN and 4p-PTEN provides evidence that the semisynthetic procedures do not disrupt PTEN's normal protein fold.

As PTEN's cellular substrate PIP3 is embedded in the plasma membrane, the primarily cytoplasmic PTEN needs to associate with the cell membrane to convert PIP3 into PIP2. PTMs that loosen PTEN's interactions with phospholipid membranes, such as C-terminal phosphorylation, prevent its catalytic action *in vivo*.[12,15–18,43–45] To see if Lys13 ubiquitination of PTEN could influence its membrane engagement, we employed a previously developed assay in which PIP2-containing vesicles are sedimented by ultracentrifugation and the level of PTEN protein in the pellet is assessed by anti-PTEN Western blot (Figures 2C, S5).[15] In these experiments, non-phosphorylated t-PTEN was more tightly associated with vesicles than 4p-PTEN.[15] Interestingly, Ub-t-PTEN displayed diminished binding to vesicles relative to t-PTEN, and behaved similarly to 4p-PTEN. The weakening of PTEN's interactions with the vesicles by Lys13 ubiquitination supports a role

for this modification in reducing plasma membrane association. This could allow PTEN to localize at other cellular compartments including the nucleus.[14,22,46]

We also investigated how Lys13 PTEN ubiquitination would impact its processing by PTEN E3 ubiquitin ligases, WWP2 and NEDD4–1.[47–49] These E3 ligases contain an exosite for a non-substrate ubiquitin binding that can influence catalytic turnover, chain elongation, or substrate binding.[50–54] The affinities of free ubiquitin to these exosites are relatively low (K_D: 180–350 μM), so ubiquitinated substrates may occupy the exosite and influence catalysis.[51,54] The extent of ubiquitination of t-PTEN, Ub-t-PTEN, 4p-PTEN, Ub-4p-PTEN, was monitored by anti-PTEN Western blot. Concurrent monitoring of E3 ligase auto-ubiquitination was performed by a Coomassie stained SDS-PAGE (Figures 3A, S6–S9). Under the E3 ligase reaction conditions with purified full-length NEDD4–1 and WWP2, autoubiquitination was robust whereas PTEN ubiquitination only proceeded to a limited extent. Densitometry analysis of the ubiquitinated PTEN product bands showed increased (11 ± 3-fold) ubiquitination for Ub-t-PTEN by NEDD4–1 compared to t-PTEN. For WWP2, the levels of ubiquitination were similar, but with slight accumulation of the +1 Ub band (1.7 ± 0.5-fold) for Ub-tPTEN. Therefore, Lys13 ubiquitination may promote further ubiquitination of PTEN by NEDD4–1 but less so by WWP2. This behavior is interesting in light of how C-terminal phosphorylation reduces PTEN ubiquitination by WWP2 but has little effect with NEDD4–1.[18] Furthermore, Ub-4p-PTEN as a substrate showed no observable activity for either E3 ligase (Figures S8–S9), suggesting that the ubiquitination at residue 13 does not overcome the inhibitory effect of the closed conformation.

We next explored if our Ub-t-PTEN can be a deubiquitinase substrate, by assessing how well the Ub-aminoAla-Cys functionality serves as a faithful mimic of Lys-Ub for a deubiquitinase, and how the 16mer Ub-Lys13 peptide serves as a deubiquitinase substrate compared with the near full-length Ub-t-PTEN protein. To determine processing efficiency, we compared its substrate properties to two other Ub-Lys mimics and a natural isopeptide linkage in the PTEN N-terminal 16mer peptides (Figure 3B). The synthesis of the natural isopeptide linked Ub-peptide is complex, so we wanted to investigate how aminoAla-Cys and two other analogs compared as substrates. Ub-aminoAla-Ala mimic was generated by desulfurization of Ub-aminoAla-Cys.[55,56] A third Ub-Lys mimic investigated contained a hydrazide-linkage prepared using a Cys modification protocol.[57] The natural Ub-Lys isopeptide linkage was synthesized using a 2-aminooxy-ethane thiol auxiliary protocol (Figure S10).[58,59]

These four Ub-PTEN peptide conjugates and Ub-t-PTEN protein were subjected to USP7 deubiquitinase, which was previously reported to deubiquitinate PTEN.[60,61] Deubiquitination was monitored by Coomassie stained SDS-PAGE for the Ub-peptides and anti-PTEN Western blot for Ub-t-PTEN protein (Figures 3B, S11–S12). For Ub-aminoAla-Cys, we analyzed both the time and substrate concentration dependence. The deubiquitination time course was fit to an exponential curve as initial rate could not be assessed. The Ub-aminoAla-Cys peptide substrate displayed a $K_{M(app)}$ of ~23 μM, and we found that the v/[E][S] was 10.6 ± 0.4 $\mu M^{-1}min^{-1}$ with 5 μM substrate (Figure S13). The other three Ub-Lys mimics were also deubiquitinated with similar v/[E][S] (Ub-aminoAla-Ala: 16±2 $\mu M^{-1}min^{-1}$, Ub-hydrazide: 9.9±0.5 $\mu M^{-1}min^{-1}$, Ubnatural isopeptide: 11 ± 1

$\mu M^{-1}min^{-1}$). This is consistent with a previous study that showed no significant reduction in deubiquitinase activity with alkylated thiolysine linkages.[62] The insensitivity of USP7 to linkage properties, such as the lowered pKa of the hydrazide linkage, or the potential for the Cys linkage to form a hydrogen bond to the departing nitrogen, indicates that these alternative linkages can be a reliable surrogate for understanding Ub-t-PTEN processing by USP7.

We found that the deubiquitination of Ub-t-PTEN by USP7 was 4-fold faster than the corresponding Ub-aminoAla-Cys peptide (v/[E][S] 40 ± 3 $\mu M^{-1}min^{-1}$). Thus, USP7 may recognize more than the local amino acids surrounding Lys13 for efficient processing, perhaps making distal interactions with the folded PTEN substrate.

In summary, we have described a semisynthetic method for installing a site-specific ubiquitin mimic in a challenging to produce signaling protein. We have shown that the Ub-aminoAla-Cys functionality can serve as a reliable mimic of the natural Ub-Lys isopeptide linkage with respect to USP7-mediated deubiquitination. Our results suggest that Lys13 ubiquitination of PTEN diminishes its catalytic activity and reduces its membrane association, liberating PTEN to localize to other subcellular compartments. In this regard, it has been proposed that nuclear PTEN can have non-enzymatic functions in gene regulation.[63–65] Crosstalk between phosphorylation and ubiquitination have been described previously[66] and can also be studied further for PTEN.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

PTEN	phosphatase and tensin homolog
Ub	ubiquitin
PIP3	phosphatidylinositol 3,4,5-trisphosphate
PIP2	phosphatidylinositol 4,5-diphosphate
t-	truncated
4p-	tetraphosphorylated

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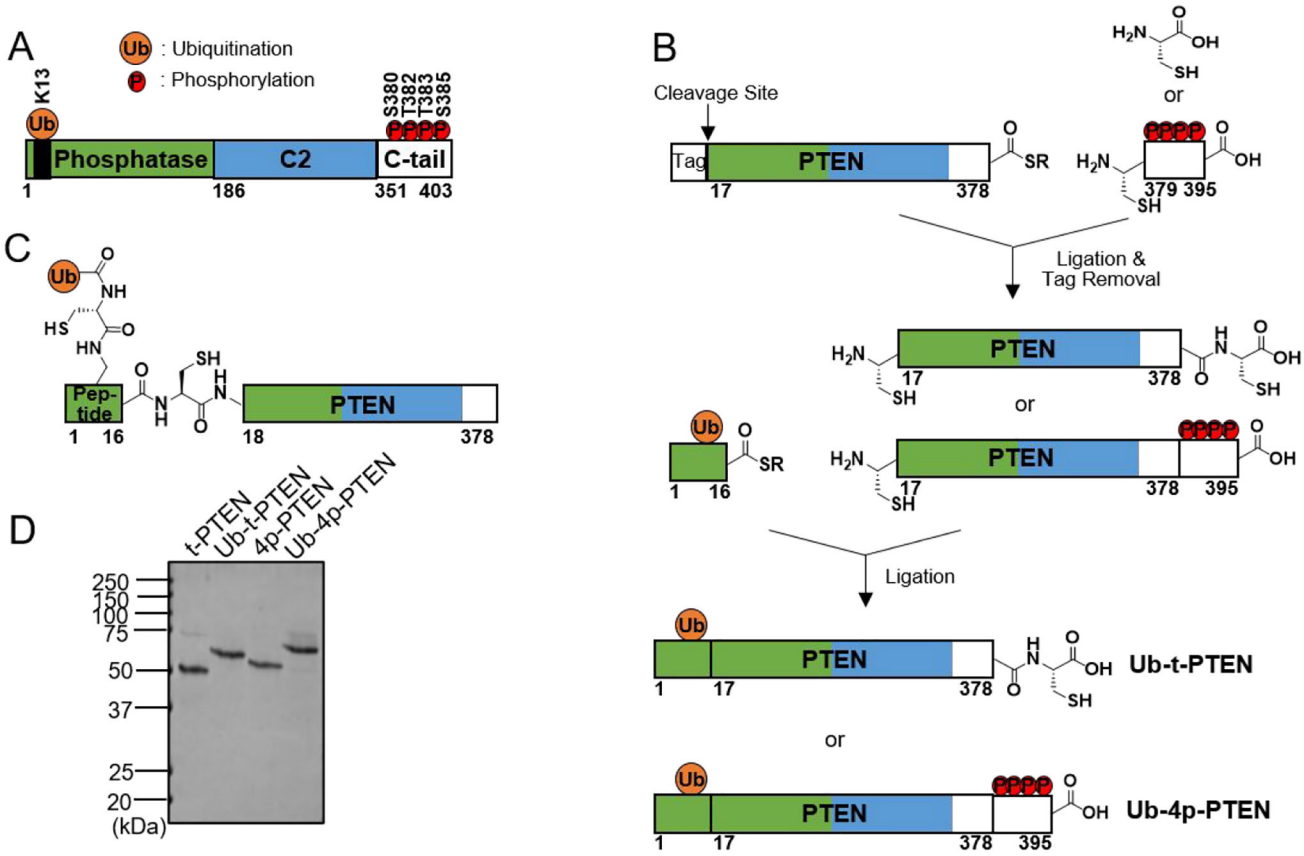

Figure 1.

Semisynthesis of site-specifically modified PTEN (A) Domain structure of PTEN depicting relevant PTMs, with the PIP2 binding motif (aa 6–14) shaded in black. (B) Semisynthetic strategy to generate ubiquitinated and/or phosphorylated PTEN. (C) Illustration of Ub-t-PTEN linkage structure. (D) Coomassie stained SDS-PAGE of recombinant and semisynthetic PTEN proteins.

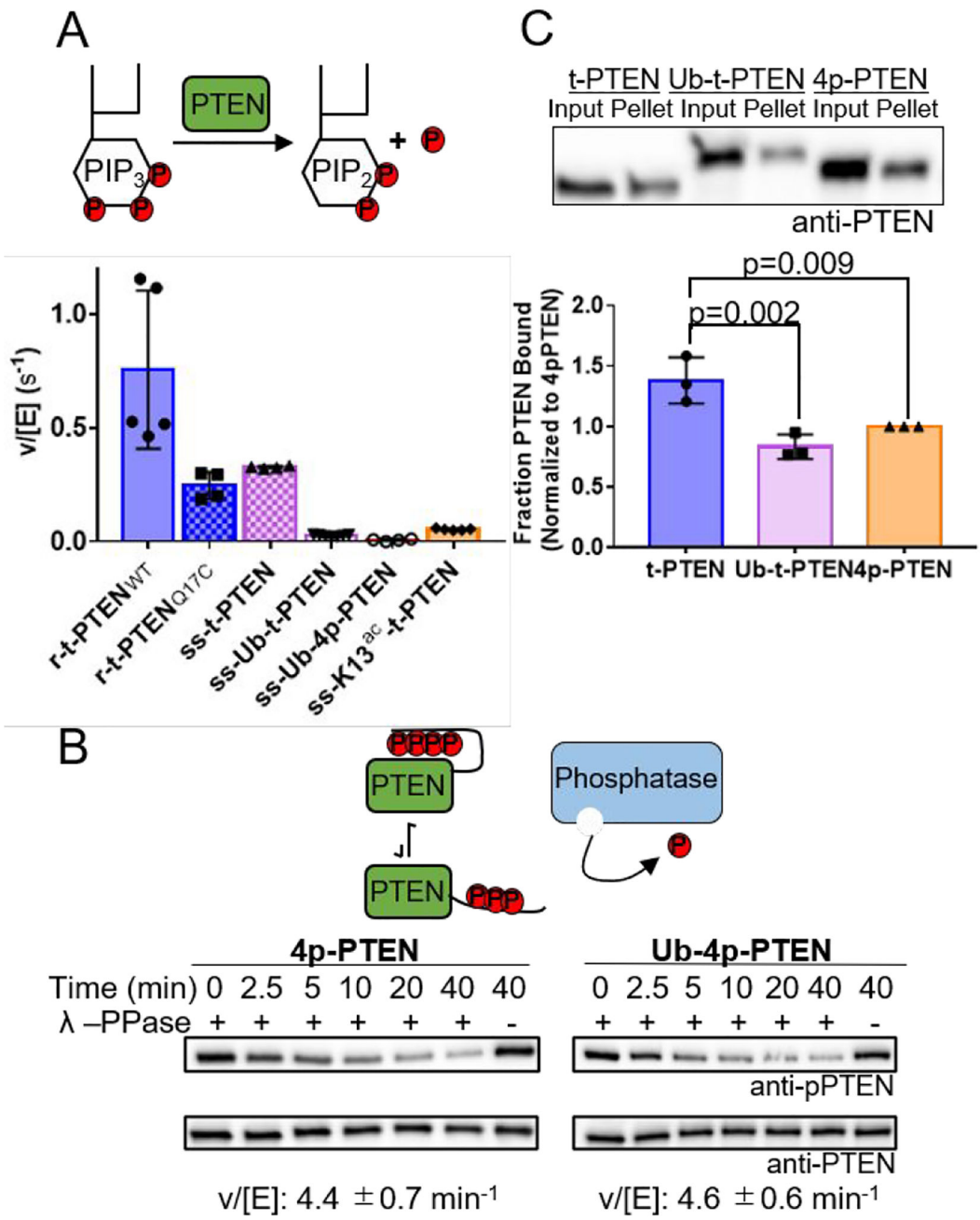

Figure 2.

Characterization of ubiquitinated PTEN. (A) PIP3 hydrolase assay of recombinant and semisynthetic PTEN with 160 μM soluble PIP3. (B) Phosphatase protection assay of PTEN with 25 nM λ-phosphatase. (C) Vesicle pulldown assay of PTEN with PIP2 containing vesicles.

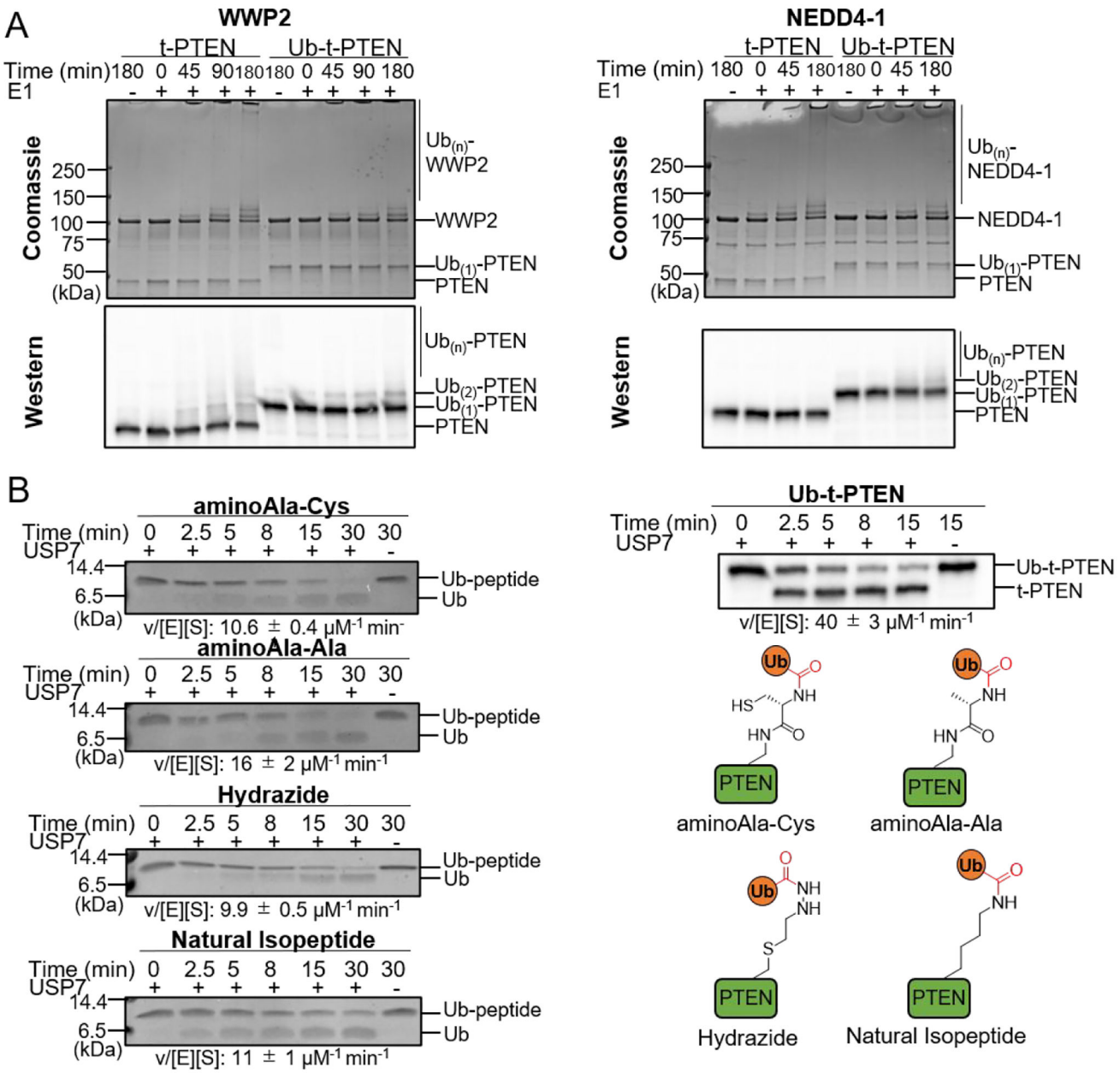

Figure 3.

Processing of ubiquitinated PTEN by E3 ligases and deubiquitinases. (A) In vitro ubiquitination assay for t-PTEN and Ub-t-PTEN by WWP2 and NEDD4–1 E3 ligases. E3 ligase autoubiquitination was monitored by SDS-PAGE and PTEN ubiquitination was monitored by Western blots. (B) USP7 deubiquitinase assay for various Ub-peptides linkages and Ub-t-PTEN. Illustration of aminoAla-Cys, aminoAla-Ala, hydrazide, and natural isopeptide linked Ub-peptides depicting their linkage structures.