Published in final edited form as:

Methods Enzymol. 2018 ; 607: 405–422. doi:10.1016/bs.mie.2018.05.007.

Protein chemical approaches to understanding PTEN lipid phosphatase regulation

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Abstract

Since the discovery of C-tail phosphorylation of PTEN almost 20 years ago, much progress has been made in understanding its regulatory influences on the cellular function of PTEN. Phosphorylation of Ser380, Thr382, Thr383, and Ser385 drives a PTEN conformational change from an open to closed state where catalytic function is impaired, plasma membrane binding is reduced and cellular stability is enhanced. Despite these advances, a detailed structural and mechanistic model of how these phosphorylations impact PTEN function is lacking. We discuss here several recent approaches to analyzing PTEN phosphorylation and highlight several insights that have come from this work. We also discuss remaining challenges for the PTEN regulation field and potential directions for future research.

Keywords

phosphorylation; semi-synthesis; signaling; enzymology; PIP3

1. Introduction

Phosphatase and Tensin Homolog (PTEN) functions as a tumor suppressor by negatively regulating the PI3-kinase/AKT kinase signaling pathway (Figure 1). It is frequently mutated in many cancers resulting in a loss of function leading to unregulated cell growth, proliferation, and protein synthesis. Originally, PTEN was thought to be a protein tyrosine phosphatase based on its conserved catalytic motif of HCxxGxxR (Li & Sun, 1997; Li, Yen, Liaw, Podsypanina, Bose, Wang, 1997; Myers, Stolarov, Eng, Li, Wang, Wigler, 1997); however, in 1998 Maehama and Dixon elegantly demonstrated that PTEN is a lipid phosphatase that dephosphorylates the 3'-phosphate of phosphatidylinositol (3,4,5)-trisphosphate, providing the molecular basis for antagonism to PI3-kinase (Maehama & Dixon, 1998; Myers, Pass, Batty, Van der Kaay, Stolarov, Hemmings, 1998; Sun, Lesche, Li, Liliental, Zhang, Gao, 1999; Worby & Dixon, 2014). [Insert Figure 1]

PTEN is a 403-amino acid protein that is localized to the cytosol, plasma membrane, and nucleus. The architecture of PTEN consists of an N-terminal phosphatase domain followed by a C2 domain and a flexible C-terminal tail (Figure 2) (Lee, Yang, Georgescu, Di Cristofano, Maehama, Shi, 1999). These domains enable and regulate the catalytic function of PTEN to dephosphorylate PIP3 at the plasma membrane. The N-terminal phosphatase

domain spans residues 1–185 and is responsible for plasma membrane binding and catalysis. It contains an N-terminal PIP2 binding motif believed to be important for PTEN activation and plasma membrane binding that works together with the phosphatase domain to achieve normal enzymatic function (Campbell, Liu, & Ross, 2003; McConnachie, Pass, Walker, & Downes, 2003; Redfern, Redfern, Furgason, Munson, Ross, & Gericke, 2008). The C2 domain is important for binding to the phospholipid bilayer in a Ca^{2+} independent manner. The C2 domain also makes extensive contacts with the phosphatase domain that stabilize the overall protein fold (Lee et al., 1999). The C-terminal tail is considered to be intrinsically disordered and is suggested to influence the stability, localization, protein-protein interactions, and enzymatic activity of PTEN. Scattered through these domains are numerous post-translational modifications (PTMs) including phosphorylation (Al-Khouri, Ma, Togo, Williams, & Mustelin, 2005; Cordier, Chaffotte, Terrien, Prehaud, Theillet, Delepierre, 2012; Miller, Lou, Seldin, Lane, & Neel, 2002; Torres & Pulido, 2001; Vazquez, Ramaswamy, Nakamura, & Sellers, 2000), acetylation (Ikenoue, Inoki, Zhao, & Guan, 2008; Meng, Jia, & Gan, 2016; Okumura, Mendoza, Bachoo, DePinho, Cavenee, & Furnari, 2006), sumoylation (Huang, Yan, Zhang, Zhu, Wang, Shi, 2012), ubiquitination (Wang, Trotman, Koppie, Alimonti, Chen, Gao, 2007), and oxidation (Lee, Yang, Kwon, Lee, Jeong, & Rhee, 2002) which are suggested to modulate PTEN in various ways. A cluster of phosphorylation sites on the C-terminal tail at positions Ser380, Thr382, Thr383, and Ser385 have been the focus of numerous studies. Casein kinase 2 or glycogen synthase kinase 3b have been reported to catalyze these PTEN phosphorylations (Al-Khouri et al., 2005; Torres & Pulido, 2001). Phosphorylation of these residues seems to convert PTEN to a more closed conformational state characterized by lower enzymatic activity, reduced plasma membrane binding, and increased stability (Bolduc, Rahdar, Tu-Sekine, Sivakumaren, Raben, Amzel, 2013; Das, Dixon, & Cho, 2003; Masson, Perisic, Burke, & Williams, 2016; Nguyen, Afkari, et al., 2014; Odriozola, Singh, Hoang, & Chan, 2007; Rahdar et al., 2009; Vazquez et al., 2000). Key to understanding the regulation of PTEN by these phosphorylations requires access to PTEN protein possessing stoichiometric and site-specific modifications for mechanistic studies. Recent efforts have led to a pair of semi-synthetic strategies to install phosphorylations at each site, classical expressed protein ligation (EPL) (Bolduc et al., 2013; Muir, Sondhi, & Cole, 1998), and a related technique enzyme-catalyzed expressed protein ligation (Henager, Chu, Chen, Bolduc, Dempsey, Hwang, 2016). In Sections 2 through 5 we discuss these methods and the analysis of modified semi-synthetic PTENs using biochemical and biophysical approaches. [Insert Figure 2]

2.0 Production of semi-synthetic PTENs

Semi-synthetic PTEN production has involved using insect-cell expression of the N-terminal recombinant fragment containing a C-terminal thioester for ligation to C-terminal synthetic peptides. PTEN constructs (aa 1–378 or aa 1–377) were sub-cloned into a pFastBac1 vector which also harbors a C-terminal mxgA intein fused to a chitin-binding domain (CBD). The plasmids were then transformed into competent DH10Bac *E. coli* cells for bacmid production. The bacmid was then transfected into SF21 insect cells using 1–2 ng/well in one 6-well plate containing 800,000 cells and incubated at 27 °C for 72–96 hours until viral infection was observed. The resulting P1 baculovirus was then used to infect additional

SF21 insect cells in a T75 flask at a density of 1 million/mL at 27 °C for 48–72 hours to generate a P2 virus with a higher viral titer. The resulting P2 virus was then used to infect HighFive insect cells in liquid suspension at a MOI = 1 and incubated at 27 °C for 48 hours. Typical expression yields of PTEN fusion proteins are 5–10 mg/L culture (Bolduc et al., 2013; Chen, Dempsey, Thomas, Hayward, Bolduc, & Cole, 2016).

2.1 Classical expressed protein ligation

Classical EPL takes advantage of a heterozygously expressed protein with a truncated C-terminus fused to an intein and chitin binding domain (CBD) (Muir et al., 1998; Schwarzer & Cole, 2005; Vila-Perello & Muir, 2010). The CBD is used to immobilize the protein-intein fusion to chitin resin for purification purposes. The intein facilitates a reversible N→S acyl shift that is released after treatment with a small molecule thiol (typically MESNA) resulting in the free protein thioester fragment (Figure 3). This protein thioester is then treated with peptide containing an N-terminal cysteine which undergoes a transthioesterification followed by intramolecular rearrangement to generate a native amide bond with a cysteine at the ligation point. This method is particularly useful for proteins that harbor a cysteine near the C-terminus; however, PTEN has no such cysteine. Therefore, Tyr379 was replaced with a Cys for the ligation step (Bolduc et al., 2013). This Y379C PTEN mutation appeared to be tolerated as it had similar catalytic activity as wild-type (wt) PTEN in hydrolyzing diC6-PIP3, a six-carbon fatty-acid containing PIP3 analog diC6-PIP3 commonly used in biochemical studies as a model substrate because it is soluble and easy to work with unlike natural PIP3 which contains longer fatty acids and forms vesicles. This PTEN hydrolysis of diC6-PIP3 was assayed by measuring the release of inorganic phosphate using the Malachite green assay (Van Veldhoven & Mannaerts, 1987). The tail peptides used for ligation were synthesized by Fmoc solid-phase synthesis strategies. The synthesis of the tetraphosphorylated 25mer peptide (aa379–403) proved challenging, presumably because the four phosphorylated residues clustered in a hexa-amino acid segment were mono-protected but still negatively charged and bulky. As peptides are typically synthesized from the C-terminus to the N-terminus, the amino acid couplings starting at pSer385 were performed manually with six equivalents of Fmoc residues for extended times using a 1:1 ratio of dichloromethane: dimethylformamide to maximize yields by semi-quantitative ninhydrin tests. Furthermore, characterizing the phospho-peptide products using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was also challenging because of the suppressing effects of clustered phosphates; in some cases, negative ion mode with α -cyano-4-hydroxycinnamic acid as the matrix in 50% acetonitrile and 5 mM ammonium citrate enhanced detection. [Insert Figure 3]

As described in Section 2.0, the recombinant PTEN (aa1–378) fusion protein was produced in HighFive insect cells. Insect cells express the enzyme chitinase that can interfere with the standard chitin-resin affinity purification. To remove chitinase, the cell lysates are pre-incubated with fibrous cellulose prior to immobilizing the extracts on chitin resin. Ligations were carried out using a “onepot” strategy where 400 mM MESNA and 2 mM N-Cys containing peptide in aqueous buffer (pH 7.2) are added to the immobilized PTEN-intein-CBD and incubated at room temperature for 48–72 hours until > 80% conversion monitored by Coomassie-stained SDS-PAGE (Bolduc et al., 2013). The semi-synthetic PTEN was

further purified using anion exchange chromatography (MonoQ) with a 240 mL linear gradient from 50 mM Hepes pH 8.0, 5 mM NaCl, 10 mM DTT to 50 mM HEPES pH 8.0, 500 mM NaCl, 10 mM DTT. Initial studies afforded semi-synthetic tetraphosphorylated (Ser380, Thr382, Thr383, and Ser385) and unphosphorylated PTEN. Remarkably, the elution pattern of these proteins on an anion exchange resin was reversed compared to the predicted outcome. The unphosphorylated PTEN eluted at higher ionic strength relative to tetraphosphorylated PTEN, despite the fact that the four phosphates were expected to add eight negative charges. This paradox was ultimately rationalized as resulting from a conformational closure of tetraphosphorylated PTEN driven by an intramolecular interaction between the tail and core of PTEN that effectively masks the anionic phosphates from contributing to interaction with the positively charged quaternary ammonium resin. It should be noted that even the unphosphorylated PTEN C-tail is anionic and is presumably more available for binding the positively charged resin. Following anion exchange chromatography, the semi-synthetic PTEN proteins were further purified by size-exclusion chromatography with either a Superdex 200 or Superdex 75 column with a mobile phase of 50 mM Tris pH 8.0, 150 mM NaCl, 10 mM DTT. Purified fractions were combined and concentrated to 1–5 mg/mL and purity assayed to be > 95% based on Coomassie-stained SDS-PAGE.

2.2 Enzyme-catalyzed expressed protein ligation

It was subsequently learned that Y379C was not a silent mutation in tetraphosphorylated PTEN (see Section 4.0) (Nguyen, Afkari, et al., 2014). We thus adopted a newly developed semi-synthetic method termed enzyme-catalyzed expressed protein ligation to produce PTEN containing a natural Tyr379. This enzyme-catalyzed method avoids the Cys requirement by use of an engineered protease called subtiligase in which the catalytic S221C along with a P225A are mutated to promote peptide ligations over proteolysis (Abrahmsen, Tom, Burnier, Butcher, Kossiakoff, & Wells, 1991; Braisted, Judice, & Wells, 1997; Chang, Jackson, Burnier, & Wells, 1994; Henager et al., 2016). It was shown that enzyme-catalyzed EPL can accommodate a diverse array of amino acids near the ligation junction, obviating the specific need for Cys, which is poorly represented in proteomes. For this alternative ligation strategy, the expression and generation of the protein thioester fragment is the same as classical EPL; however, instead of a one-pot reaction to ligate the peptide, the protein-thioester is isolated after treating the resin overnight with 300 mM MESNA in HEPES pH 7.5 containing buffer at room temperature (Figure 3) (Henager et al., 2016). Subtiligase is expressed and secreted from *B. subtilis* and purified by successive ammonium sulfate precipitation, ethanol precipitation, cation-exchange (Mono S) chromatography and finally stored in 100 mM BICINE pH 8.0, 5mM DTT (Abrahmsen et al., 1991; Braisted et al., 1997; Chang et al., 1994; Jackson, Burnier, Quan, Stanley, Tom, Wells, 1994). Ligation reactions are typically carried out in 100 mM BICINE, 5 mM CaCl₂, pH 8.0, 0.55 μ M subtiligase, 40 μ M protein-thioester, with 3 mM peptide at 25 °C. Reaction conversions for enzyme-catalyzed EPL usually range from 50 – 70% depending on the P1, P4, P1', and P2' residues surrounding the ligation junction; however, a reduction in yield was observed for ligations that contain an acidic residue at either P4 or P1'. Furthermore, high concentrations of MESNA were noted to inhibit subtiligase-mediated ligations so that a buffer exchange is required to remove MESNA from the protein sample prior to ligations.

For PTEN, a ligation between Tyr377 and Arg378 was selected because the peptide was still manageable in size and is compatible with subtiligase preferences. Ligation of the C-tail peptide was carried out in 100 mM BICINE, 110 mM CaCl₂, pH 8.0, 25 μ M subtiligase, 20 μ M PTEN_{aa1-377}-thioester, 3 mM C-tail peptide_{aa378-402}-biotin at 25 °C for 4 hours (Henager et al., 2016). The especially high Ca concentration was used in case the phosphopeptide was chelating the metal. Ligation yields for the tetraphosphorylated and unphosphorylated peptides were 30% and 50%, respectively. A biotinylated lysine at amino acid 402 was appended to expedite purification by avidin affinity chromatography of the ligated semi-synthetic PTEN product. The lower ligation yield for the phosphorylated peptide may relate to the phosphorylated serine at P3' although P3' is normally a flexible position.

3. C-terminal phosphorylation impact on enzymatic function of PTEN.

In initial experiments, semi-synthetic PTEN prepared by classical expressed protein ligation containing a Y379C mutation was analyzed in enzyme assays. Using the physiologically relevant vesicle bound ³²P-PIP3 containing palmitoyl chains as substrate, PTEN forms were added and interfacial enzyme kinetic analysis was performed which considers both the bulk and surface concentrations of substrate when measuring kinetic constants (Bolduc et al., 2013; Deems, Eaton, & Dennis, 1975; Hendrickson & Dennis, 1984; McConnachie et al., 2003). These interfacial enzymatic assays, which quantify ³²P-inorganic phosphate release, do not follow classical Michaelis-Menten kinetics as two compartments are involved and the rate of reaction depends on both bulk and surface substrate concentrations in contrast to standard solution phase monodisperse substrate assays. To make interfacial enzyme kinetic measurements, the assays are performed by separately varying the bulk and surface substrate concentrations. The bulk dilution experiment varies both the carrier lipid and substrate bulk concentration proportionally whereas the substrate surface concentration is held constant. The surface dilution experiment holds the bulk concentration of substrate constant while varying the surface substrate by varying the carrier lipid concentration. In these assays, the ratio of vesicles to PTEN molecules was maintained at 4:1 to increase the chances that only one molecule of PTEN is bound to an individual vesicle at a time. These experiments revealed that the *iK_m* (interfacial *K_m*, a measure of the *K_m* in the context of two-dimensional diffusion on the vesicle surface) and *k_{cat}* were similar for non-phosphorylated (n-PTEN) and tetraphosphorylated PTEN (4p-PTEN) (Bolduc et al., 2013). In contrast, the *K_s* (effectively the *K_d* of the PTEN binding to the vesicles) was 3-fold higher for 4p-PTEN relative to n-PTEN. These results suggest that 4p-PTEN relative to n-PTEN has reduced affinity for lipid vesicles. The effect was most pronounced at low PIP3 concentrations (mol% < 1%) which is thought to be more biologically relevant as PIP3 concentrations in the cell are less than 0.001 mol%.

In addition, a vesicle sedimentation assay was adapted to determine the affinity of semi-synthetic PTENs for PIP2 or phosphatidylserine containing vesicles. These data indicated that the anionic lipids stimulate binding of 4p-PTEN and n-PTEN; however, 4p-PTEN required a greater concentration of the anionic lipid to mimic the binding of n-PTEN, confirming that C-tail phosphorylation reduces membrane binding (Bolduc et al., 2013).

The enzymatic activities with semi-synthetic PTENs were also investigated using the soluble diC6-PIP3 substrate. Although the physiologic significance of this enzymatic assay is unclear, it has proven to be a reliable predictor of conformational state (Section 4.0). The diC6-PIP3 hydrolysis assay is much easier to perform compared to the interfacial enzymatic assay as it avoids the technically challenging synthesis of radiolabeled PIP3 substrate, impregnating vesicles with this substrate or isolation of released radiolabeled inorganic phosphate following the enzymatic assay for analysis. Furthermore, the half-life of ^{32}P is short so all interfacial enzyme assays must be complete within about two weeks of receiving the radiolabeled ATP used to make ^{32}P -PIP3 (Goodier & Pritchard, 1966). Enzymatic analysis using the soluble PIP3 assay showed that the 4p-PTEN decreases the catalytic efficiency (k_{cat}/K_m) approximately 10-fold relative to n-PTEN with a significant increase in the diC6-PIP K_m for 4p-PTEN (Bolduc et al., 2013; Chen, Dempsey, et al., 2016). Notably, it was shown that several of the enzymatic properties identified with 4p-PTEN were recapitulated with intermolecular experiments. In particular, C-tail truncated recombinant PTEN diC6-PIP3 phosphatase activity was potently inhibited by the synthetic tetraphosphorylated tail peptide which with an apparent K_d of 1 μM (Bolduc et al., 2013). The tetraphosphorylated peptide also inhibited binding of C-tail truncated recombinant PTEN to lipid vesicles. These data indicated that the mechanism of impact of the phospho-tail on PTEN involves an interaction between the phospho-C-tail and the core of PTEN.

4. Structural analysis of phospho-PTEN.

It was shown by co-immunoprecipitation from cell extracts that the core of PTEN could interact with a recombinant C-tail, however these experiments had uncertain phosphorylation stoichiometry and relied on impure mixtures which could complicate interpretation (Odriozola et al., 2007; Rahdar, Inoue, Meyer, Zhang, Vazquez, & Devreotes, 2009). Consistent with an intramolecular interaction, however, both trypsin resistance and small angle X-ray scattering support a more compact conformation of semi-synthetic 4p-PTEN (Bolduc et al., 2013). Semi-synthetic phospho-PTENs were subsequently prepared using classical expressed protein ligation to study the specific contributions of individual phospho-sites to intramolecular interactions. A commercially available phospho-C-tail specific antibody of PTEN (Cell Signaling Technology, Cat # 9549) was shown to be selective for phospho-Ser380 (Chen, Dempsey, et al., 2016). An assay with alkaline phosphatase was developed to examine the exposure of the phospho-C-tail with different PTEN forms (Figure 4). This alkaline phosphatase sensitivity assay probes the conformational state of PTEN by catalyzing the dephosphorylation of the C-terminal tail which is monitored by Western blot. If PTEN is in a conformationally open state, then the rate of dephosphorylation should be greater because the phosphate(s) are more accessible to alkaline phosphatase. If PTEN is in a conformationally closed state, then the rate of dephosphorylation should be slower as tail:core interactions will shield the C-tail phosphates from alkaline phosphatase. These experiments revealed that each phosphorylation partially contributes to conformational closure and no individual phosphorylation site of the four stood out as being dominant. [Insert Figure 4]

Currently, there is no high-resolution structure that shows the position of the phospho-tail in the conformationally closed state. Mutagenesis of various regions in the core of PTEN have

been carried out to investigate potential contacts for phospho-tail interaction (Nguyen, Afkari, et al., 2014; Nguyen, Yang, Afkari, Park, Sesaki, Devreotes, & Iijima, 2014; Odriozola et al., 2007; Rahdar et al., 2009). A significant rescuing of 4p-PTEN catalytic function and greater sensitivity to tail dephosphorylation by alkaline phosphatase were observed when the basic CBRIII loop residues (Lys260/Lys263/Lys266/Lys267/Lys269) were all mutated to Asp, suggesting a role for the CBRIII loop in stabilizing the closed conformation (Bolduc et al., 2013). Notably, when these CBRIII Lys residues were replaced with Ala, the conformation of 4p-PTEN led to wt-like behavior. These data suggest that the role of the CBRIII loop may be indirect in driving conformational closure.

H/D exchange experiments on phosphorylated PTEN indicated that the Ca2 loop (aa319–342) might be important in stabilizing the closed conformation (Masson et al., 2016). Indeed, mutagenesis of the basic residues of the Ca2 loop (Lys327, Lys330, Lys332, and Arg335) revealed that both the aspartate and alanine replacements led to opening of 4p-PTEN assessed by alkaline phosphatase sensitivity and diC6-PIP3 processing efficiency (Chen, Dempsey, et al., 2016). These results are consistent with a model where the basic residues of the Ca2 loop directly mediate interactions with one or more C-tail phosphates in PTEN. However, these results cannot exclude an indirect role.

One technique that can establish spatial proximity between two protein surfaces involves photocrosslinking. To apply this method to phospho-PTEN, a photoactivatable crosslinking group was installed near the phospho-cluster in the C-tail using expressed protein ligation. The crosslinking residue selected was benzoyl L-phenylalanine (Bpa) in place of Phe392 in the synthetic tail peptide. Bpa is structurally somewhat similar to Phe and it can form stable crosslinks to a range of amino acids. Irradiation of the Bpa phospho-PTEN sample (also tagged in the C-terminus with a biotin) at 365 nm for 3 h at 4 °C was performed (Figure 5). It proved necessary to dephosphorylate the sample with alkaline phosphatase prior to trypsin digestion and avidin chromatography to enhance the LC/MS/MS analysis. Using this method, a crosslinked peptide was identified which corresponded to the C-terminus connected to the N-terminal region of PTEN within amino acids 42–50 (Chen, Dempsey, et al., 2016; Forne, Ludwigsen, Imhof, Becker, & Mueller-Planitz, 2012; Mueller-Planitz, 2015). Site-directed mutagenesis was performed to validate the relevance of this crosslink and the phosphorylated R41D/R47D/R74D PTEN variant was found to be modestly more open compared to native 4p-PTEN based on alkaline phosphatase sensitivity. [Insert Figure 5]

The development of enzyme-catalyzed expressed protein ligation allowed for the generation of 4p-PTEN possessing a natural Tyr379 instead of the Cys379 mutation introduced for classical expressed protein ligation. It was found that the Tyr379 4p-PTEN equilibrium between open and closed conformations favored the closed state by about 10-fold relative to the corresponding equilibrium for Cys379 4p-PTEN based both on alkaline phosphatase sensitivity and diC6-PIP3 PTEN processing (Henager et al., 2016). Notably, the natural Tyr379 4p-PTEN confers reduced conformational opening in the presence of high salt buffer. These experiments helped clarify the significance of the Tyr379 mutational sensitivity in cellular studies. Moreover, accessing natural Tyr379 4p-PTEN facilitated more accurate quantification of cellular 4p-PTEN stoichiometry on endogenous protein measured by

western blots because the Tyr379 also proved critical to phospho-PTEN antibody recognition (Henager et al., 2016). These experiments underscore that avoiding Cys mutation is a valuable attribute of enzyme-catalyzed expressed protein ligation.

5. Phospho-PTEN and protein ubiquitination

The protein stability of PTEN in the cell is a factor in its signaling properties and PIP3 control. When PTEN C-terminal phosphorylation was discovered, it was also found that there was a correlation between its phosphorylation state and protein stability (Vazquez et al., 2000). This behavior is somewhat paradoxical as the enzymatic activity of phospho-PTEN is impaired which means that the more stable PTEN form is also less functional in PIP3 hydrolysis. Furthermore, the molecular basis of PTEN protein destruction was not yet clear in this early work. Cellular protein clearance often involves Lys ubiquitination which then targets the protein for proteasome-mediated degradation. Ubiquitination is a three-step process involving E1, E2, and E3 ubiquitin transferring enzymes. Several ubiquitin E3 ligases for PTEN have been reported, and the two most extensively studied have been NEDD4-1 (Wang et al., 2007) and WWP2 (Maddika, Kavela, Rani, Palicharla, Pokorny, Sarkaria, & Chen, 2011). NEDD4-1 and WWP2 are both HECT domain E3 ligases and are composed of an N-terminal C2 domain followed by 4 WW domains and culminating in catalytic HECT domains.

In principle, phosphorylation of PTEN could inhibit its ubiquitination by the requisite E3 ligase as a mechanism to enhance PTEN protein stability. This is difficult to study in live cells. To address whether NEDD4-1 or WWP2 might be affected by PTEN phosphorylation, *in vitro* ubiquitination assays involving E1 and E2 enzymes along with NEDD4-1 and WWP2 were performed (Chen, Thomas, Solduc, Jiang, Zhang, Wolberger, & Cole, 2016). It was shown that WWP2 was much slower to ubiquitinate 4p-PTEN relative to n-PTEN. In contrast, NEDD4-1 did not distinguish between the modified and unmodified PTENs and ubiquitinated them with similar rates. These data suggest that WWP2 might be the more important PTEN ubiquitin ligase, at least in some cell types, as the *in vitro* selectivity of the ligase is consistent with the cellular behavior of PTEN stability.

These findings also suggest that factors that can block WWP2 catalytic activation might allow for enhanced PTEN stability. A recent study revealed that WWP2 is autoinhibited by a centrally located 30 aa peptide linker and that tyrosine phosphorylation of this peptide linker on either of two Tyr residues, aa369 and aa392, can relieve this ubiquitin ligase inhibition (Chen, Jiang, Xu, Li, Dempsey, Zhang, 2017). It was subsequently shown that mutation of these Tyr sites to Glu residues could activate WWP2 activity in cells and this led to reduced PTEN levels. These studies further confirm the WWP2-PTEN physiologic connection and suggest that protein tyrosine kinase inhibitors that block WWP2 phosphorylation could drive PTEN cellular stability even in the unphosphorylated state of PTEN.

6. Summary and future directions

This chapter has described how Ser/Thr phosphorylation of PTEN at residues 380, 382, 383, and 385 can influence PTEN structure and function. Phosphorylation of these residues

converts PTEN from an open to closed conformation resulting in a decrease in enzymatic function and plasma membrane binding, but also protects the protein from cellular destruction. This chapter also highlights the importance of modern chemical tools in investigating PTEN regulation by phosphorylation. Despite the progress, many gaps in our knowledge remain about PTEN regulation. A high-resolution picture revealing the position of the phosphorylated tail in the closed conformational is still lacking. It is unclear how C-tail phosphorylation renders PTEN more resistant to ubiquitination by WWP2. It will be important to define the molecular basis of PTEN recognition by WWP2 and whether there are surfaces that might be druggable. Moreover, it would be a great leap forward if allosteric modulators of phospho-PTEN itself could be identified that would restore its catalytic function (Qiao, Molina, Pandey, Zhang, & Cole, 2006). Such PTEN reactivation might also be achieved in a more traditional fashion by blocking CK2 protein kinase and/or the not yet identified tyrosine kinase that activates WWP2. In the longer term, a full inventory and analysis of the impact on the wide array of PTEN PTMs are needed. We believe that the next two decades of PTEN research promise to be at least as exciting as the first two.

Acknowledgments

We thank NIH CA74305 and NIH GM120855. We appreciate the helpful advice and collaboration of David Bolduc, Zan Chen, Sam Henager, Nam Chu, Stefani Thomas, Dawn Hayward, Sandra Gabelli, Peter Devreotes, Mario Amzel, and Miho Iijima.

Abbreviations

AP	alkaline phosphatase
Bpa	benzoyl-L-phenylalanine
<i>E. coli</i>	<i>Escherichia coli</i>
EPL	expressed protein ligation
PIP2	phosphatidylinositol (4,5)-trisphosphate
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PTEN	phosphatase and tensin homolog
PTMs	post-translational modifications
MESNA	sodium 2-mercaptoethanesulfonate
NCL	native chemical ligation
Ub	ubiquitin

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Figure 1.
Post-translational regulation of PTEN and its cellular influence.

Figure 2. PTEN architecture.

(A) Diagram showing the position of PTEN domains, the catalytic cysteine, and the C-tail phosphorylation sites. (B) PTEN X-ray crystal structure (1D5R) highlighting the location of the active site (green), CBRIII loop (blue), and Ca2 loop (red). The crystal structure displays the phosphatase domain (aa 14 –186) and C2 domain (aa 186 – 351) but structural information for the N-terminus (aa 1 – 13), D loop (aa 282 – 312), and C-terminal tail (aa 352 – 403) is missing. The CBRIII and Ca2 loops are highlighted because of their importance to membrane binding and the closed conformation of PTEN as discussed in Section 4.

Figure 3. Classical and enzyme-catalyzed expressed protein ligation (EPL).

Truncated PTEN (aa 1 – 377) is expressed with the GyrA intein and chitin binding domain (CBD) fused to its C-terminus. PTEN is then immobilized on to chitin resin for purification. The intein facilitates the reversible formation of a thioester which is treated with a small molecule thiol (MESNA) that undergoes a transthioesterification reaction to generate a PTEN-MESNA thioester. For classical EPL, the truncated PTEN-MESNA thioester is then incubated with a NCys containing synthetic peptide which undergoes another transthioesterification followed by intramolecular rearrangement to generate the full-length protein with a Cys at the ligation point and PTMs at their relevant positions. For enzyme-catalyzed EPL, a synthetic peptide is ligated to the isolated PTEN-thioester by subtiligase to generate the full-length wild-type protein with relevant PTMs.

Figure 4. Phosphate protection assay to sample conformational closure.

This assay probes the conformational state of PTEN by evaluating the accessibility of the C-tail phosphatase to alkaline phosphatase (AP). If PTEN is in the closed conformation, then the tail phosphates are masked from AP by interacting with the core of the protein which results in their slower dephosphorylation. If PTEN is in the open conformation, then the tail phosphates are more accessible to AP and their dephosphorylation is much faster. Red circles represent phosphorylation sites.

Figure 5. Photocrosslinking studies to map location of the C-tail in conformationally closed state. (A) Semi-synthetic PTEN generated for Bpa UV-crosslinking. (B) Diagram of experimental steps from UV-irradiation to analysis of Bpa crosslinks.