Enzyme-catalyzed expressed protein ligation

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Expressed protein ligation is a valuable method for protein semisynthesis that involves the reaction of recombinant protein C-terminal thioesters with N-terminal cysteine (N-Cys)-containing peptides, but the requirement of a Cys residue at the ligation junction can limit the utility of this method. Here we employ subtiligase variants to efficiently ligate Cys-free peptides to protein thioesters. Using this method, we have more accurately determined the effect of C-terminal phosphorylation on the tumor suppressor protein PTEN.

The ability to install post-translational modifications, biophysical probes, unnatural amino acids, isotopic labels, and drug-like small molecules in a site-specific manner into proteins of any size offers enormous potential for both fundamental and applied biomedical research1–4. Semisynthesis using expressed protein ligation (EPL) has been exploited frequently in the construction of proteins containing diverse chemical modifications1,3. An N-Cys-containing synthetic peptide is reacted with a thiol to generate the isolated recombinant protein C-terminal thioester5–8. An N-Cys-containing synthetic peptide is then added to the protein thioester, which undergoes transthioesterification followed by rearrangement to a conventional amide bond5,6 (Fig. 1a). While it is a powerful method, the scope of EPL is narrowed by the requirement of a Cys at unnatural locations, sometimes perturbing protein function. Methods to overcome the Cys requirement8,9 in EPL are under development but have not yet proved robust.

Subtiligase is an engineered peptide ligase derived from the protease subtilisin that contains mutations (S221C, P225A) that alter the mechanism to favor aminolysis over peptidase activity, facilitating the ligation of a peptide containing a donor C-terminal ester to an acceptor peptide containing an α-amine10–12. Subtiligase has been used in a variety of settings but has not previously been applied to ligations with recombinant protein thioester fragments, a method we investigate here (Fig. 1a).

We initially examined subtiligase-catalyzed ligation with ubiquitin (Ub) C-terminal thioester, produced from an intein fusion protein after reaction with sodium mercaptoethylsulfonate (MESNA; Supplementary Fig. 1a). The extreme C-terminal residues of Ub are LRGG, and it is noteworthy that the reported preferences of subtiligase include a hydrophobic residue at the P4 position10–13 (Fig. 1b), which is Leu in Ub. A synthetic decapeptide (GLSGRGKGGK (biotin), 1 mM) was reacted with 100 µM Ub thioester, 7.5 µM subtiligase, 100 mM bicine at pH 8, and 5 mM CaCl2 at room temperature; and the reaction was monitored by SDS–PAGE. As shown in Figure 1c, the ligation appeared to plateau by 60 min at ~50% ligated product with half-maximal conversion at 5 min. Mass spectrometry confirmed the correct structure of the product (Supplementary Fig. 2). As controls, it was shown that subtiligase was required for the transformation, and standard Ub lacking a thioester functionality was unreactive under these conditions (Supplementary Fig. 1). Interestingly, recovered Ub showed that hydrolysis of the thioester was accelerated by subtiligase (Supplementary Fig. 3), which could account for the incomplete conversion at longer reaction times.

Prior data obtained with subtiligase and synthetic peptide oxyester ligations indicate that the P4, P1, P1′, and P2′ positions are the most influential for ligation efficiency10–13 (Fig. 1b). We studied Ub variants that contained a range of residues at the P4 (L, A, E) and P1 (Y, L, I, V, F, A, W, H, G) positions and a set of synthetic acceptor peptides with diversity introduced at the P1′ (G, A, R, F, M, S, Y, P, E) and P2′ (L, T, I, G) positions. As shown in Supplementary Figure 1 and Supplementary Table 1, the majority of the conversion efficiencies were in the range of 50–70%. Notable exceptions to these relatively good subtiligase-catalyzed Ub ligation efficiencies were when P4 or P1′ was Asp/Glu or when P1′ was Pro, previously identified as poor substrates for oxyester peptide ligations10–15.

In cases involving acidic residues at P1, QK subtiligase (E156Q, G166K) has been shown to improve oxyester peptide ligations10,13. We also designed Y217K subtiligase to complement acidic residues at the P1′ position (Supplementary Fig. 4a, b). In the case of Glu at P1, the conversion efficiency improved from 33% with standard subtiligase to ~50% with either QK or Y217K subtiligase (Supplementary Fig. 4c, d). In cases of Glu at P1′, the conversion efficiencies were markedly improved with Y217K subtiligase relative to the standard or QK forms (Supplementary Fig. 4c, d). These modified subtiligase forms thus broaden the scope of enzyme-catalyzed EPL.

To compare the efficiency of enzyme-catalyzed EPL with that of standard EPL, we performed the uncatalyzed reactions with an N-Cys-containing peptide substrate to a subtiligase-catalyzed
reaction where the N-terminal residue was Gly (Supplementary Fig. 5a,b). The initial subtiligase reaction rate was approximately three-fold faster than that of standard EPL (Supplementary Fig. 5c). These results underscore the rapid kinetics of enzyme-catalyzed EPL, although further work is needed to compare detailed rates across a range of substrates.

We assessed the generality of enzyme-catalyzed EPL with a series of glutathione S-transferase (GST) thioesters containing a range of C-terminal P4 (F,S,D) and P1 (Y,T,R,G,E) residues and a set of ligating decapetides containing varying P1’ and P2’ residues (MT, GL, SI) (Supplementary Fig. 6). In general, as shown in Supplementary Figure 6d and Supplementary Table 2, the efficiencies were similar to those of the Ub ligations. Interestingly, with Ser at P4, the reaction was favorable when P1 was Tyr (conversions close to 60%) but not when P1 was Gly (<5%). However, Gly at P1 was acceptable when P4 was Phe with conversions of ~30%. Given the Ub thioster ligations described above, perhaps the most surprising result was that Glu at P1 was well tolerated when P4 was Phe with conversions of ~60%. These results highlight how there can be an interplay among the C-terminal amino acids influencing ligation efficiency.

We next adopted enzyme-catalyzed EPL to determine the influence of C-terminal phosphorylation on the tumor suppressor lipid phosphatase PTEN. PTEN is phosphorylated on a cluster of four C-terminal S and T residues (S380, T382, T383, S385) in vivo by CK2 protein kinase, and these phosphorylations can inhibit the enzyme and induce an intramolecular conformational change in PTEN. Conventional EPL has previously been used to generate tetraphosphorylated PTEN. In this case, a Y379C mutation was introduced into PTEN since there was no nearby Cys for ligation. It has subsequently been discovered that Y379C PTEN behaves anomalously in cells. Thus, generating tetraphosphorylated PTEN without introducing a nearby Cys was desirable. The proximal natural sequence in this region, 374–PDHYRYS–380, suggested that positioning Y377 as the P1 residue and P374 as the P4 residue could be favorable for enzyme-catalyzed EPL. We thus generated the corresponding aa1–377 PTEN thioester (r-PTEN) and ligated it to a tetraphosphorylated (and unphosphorylated) peptide aa378–402 modified with a biotin at Lys402 (Fig. 1d,e and Supplementary Fig. 7), affording the desired semisynthetic tetraphosphorylated (Y379-4p-PTEN) and unphosphorylated (Y379-n-PTEN) products.

Western blot of Y379-4p-PTEN with an anti-phospho-PTEN Ab showed an ~4-fold more intense signal for the natural sequence compared with C379-4p-PTEN protein (Supplementary Fig. 7e), presumably because Y379 is important in the epitope. This enabled the use of the more natural 4p-PTEN to serve as a standard in determining the level of cellular C-terminal phosphorylation of endogenous PTEN isolated from mammalian cells. This revealed that the stoichiometry of tail phosphorylation of endogenous PTEN in mouse embryonic fibroblasts was ~72%, but the level of phosphorylation dropped after CK2 protein kinase inhibition (Fig. 2a), presumably because of the dynamic opposing actions of kinases and phosphatases.

The enzymatic activity of Y379-4p-PTEN was decreased compared with that of C379-4p-PTEN, and both levels of enzyme activity were lower than the those of the unphosphorylated and truncated (t-PTEN) forms (Fig. 2b), indicating that the Y379 residue may stabilize the closed, autoinhibited phosphorylated PTEN conformation. Such autoinhibition was most marked at a higher NaCl concentration (200 mM), suggesting that Y379-4p-PTEN is more resistant to conformational opening at high ionic strength compared with C379-4p-PTEN. Alkaline phosphatase has previously been employed to probe the conformation of C379-4p-PTEN. We thus compared alkaline-phosphatase-mediated dephosphorylation of natively folded Y379-4p-PTEN to that of C379-4p-PTEN, and this showed that the former had about a
ten-fold slower rate of phosphate removal (Fig. 2c,d). These data suggest that Y379-4p-PTEN does indeed reside in a more tightly closed conformation relative to C379-4p-PTEN\textsuperscript{16}, and can account for the anomalous cellular behavior of Y379 PTEN mutations\textsuperscript{18}.

PTEN exemplifies the benefits of studying post-translationally modified proteins with their precise native primary sequence; it would have been difficult to generate Y379-4p-PTEN without relying on enzyme-catalyzed EPL. The relatively broad flexibility of amino acid sequences around the ligation junction, the simplicity of the protocol, and the speed of the process combine to make enzyme-catalyzed EPL an attractive technique for protein semisynthesis.

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

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
S.H.H., N.C., J.W., and P.A.C. designed experiments. S.H.H., N.C., Z.C., D.B., D.R.D., and Y.H. analyzed data. S.H.H., N.C., and P.A.C. drafted the manuscript, and all authors contributed out experiments. S.H.H., N.C., Z.C., D.B., D.R.D., and Y.H. analyzed data. S.H.H., N.C., and P.A.C. designed experiments. S.H.H., N.C., and Z.C. carried author contributions

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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Figure 2 | Y379-4p-PTEN biochemical properties compared to those of C379-4p-PTEN. (a) Representative western blots showing a decrease in PTEN phosphorylation in cells following treatment with a CK2 inhibitor. (b) Activity of t-, Y379-n-, C379-4p-, and Y379-4p-PTEN toward 160 μM soluble di-C6 PIP2 with 60 mM or 200 mM NaCl. Values are the averages of two replicates, and error bars represent the span of measurements. Conc., concentration. (c) Illustration of the open and closed conformations of PTEN and the change in accessibility of the phosphorylation sites to alkaline phosphatase. Red circles indicate phosphorylated residues. Cat., catalytic domain; C2, C2 domain; AP, alkaline phosphatase. (d) Western blot analysis of phosphorylation levels of Y379C-4p-PTEN compared with those of C379-4p-PTEN after alkaline phosphatase treatment.
ONLINE METHODS
A step-by-step protocol for EPL with subtiligase is available as Supplementary Protocol (and see ref. 20).

B. subtilis transformation with subtiligase. This was carried out analogously to the previously described methods10–13. E. coli–B. subtilis shuttle plasmid pPW 04 containing the preprosubtiligase sequence was purified from E. coli K12 ER1821 (NEB) and transformed into B. subtilis BG2864 (ΔaprE AnprE ΔlfaA::kan, ATCC). B. subtilis was grown on LB agar + 25 µg/ml kanamycin. 2x YT media (5 ml) was inoculated with a single colony and grown overnight at 37 °C in a spinning incubator. Cells were pelleted and resuspended in 5 ml bacillus medium A (80 mM K2HPO4, 45 mM KH2PO4, 15 mM (NH4)2SO4, 4 mM C6H12O6-Na3, 5 mM MgCl2, 50 µg/ml tryptophan, 0.5% glucose, 0.02% ampicilase). 1 ml of this suspension was added to 50 ml medium A and grown at 37 °C until 90 min after the culture had exited log-phase growth. Then, 0.5 ml of this culture was added to 5 ml medium B (80 mM K2HPO4, 45 mM KH2PO4, 15 mM (NH4)2SO4, 4 mM C6H12O6-Na3, 5 mM MgCl2, 2.5 µg/ml tryptophan, 0.5% glucose, 0.005% ampicilase). Next, 300 µl of this culture was added to tubes containing 2 µg of plasmid pPW 04 and grown at 37 °C. After 2 h, 1 ml 2x YT medium containing 0.5 µg/ml chloramphenicol was added, and the culture was grown for an additional 1 h. Culture was spun down (4,000 × g, 2 min), resuspended in 150 µl 2x YT, and plated on LB agar containing 5 µg/ml chloramphenicol.

Site-directed mutagenesis of GST, ubiquitin, subtiligase, and PTEN. GST was cloned out of the pGEX-6P-1 vector. 1 µl of template was incubated with 200 nM forward and reverse primers and 2 mM dNTP mix (0.5 mM each dNTP) in 50 µl reaction buffer (20 mM Tris–HCl, 2 mM MgSO4, 10 mM KCl, 10 mM (NH4)2SO4, 0.1% Triton X-100, 0.1 mg/ml BSA, pH 8.8) along with 1 µl Pfu Ultra II polymerase (Agilent). PCR for mutagenesis was carried out as follows: 95 °C for 30 s; then 18 cycles of 95 °C for 30 s, 55 °C for 1 min, 68 °C for 1 min; followed by a final 5 min at 68 °C; then 4 °C for 5 min. After PCR, the reaction was incubated with 0.4 units/µl DpnI at 37 °C for 1 h to digest methylated DNA. The mutated plasmid was transformed into chemically competent E. coli DH5α. Quikchange mutagenesis was also used to further mutate the C termini of GST and Ubiquitin, as well as to mutate subtiligase and create r-PTEN from t-PTEN. Supplementary Table 4 shows the primers used for cloning and mutagenesis.

Subtiligase expression and purification. Parent and mutant subtiligase were expressed and purified using the following methods adapted from previous procedures10–13. Transformed B. subtilis was streaked out on LB agar + 10 µg/ml chloramphenicol. 2x YT media (5 ml) containing 10 µg/ml chloramphenicol was inoculated with one colony and grown overnight. 1 ml of overnight culture was added to 1 2x YT media containing 10 µg/ml chloramphenicol and grown at 37 °C for 24 h. Cells were pelleted and discarded, and then the supernatant was slowly treated with 600 g (NH4)2SO4 while stirring on ice at 4 °C. The solution was stirred for an additional 1 h, during which a brown protein precipitate was observed. The mixture was centrifuged (10,000 × g, 30 min, 4 °C), and the saved pellet was then redissolved in 75 ml sodium acetate buffer (25 mM CH3CO2Na, 5 mM DTT, pH 4.5). To this solution was added 300 ml ethanol, resulting in a pale brown precipitate, and the mixture was stirred for 30 min at 4 °C. The mixture was then centrifuged (5,000 × g, 15 min, 4 °C), and the pellet was saved and redissolved in 50 ml sodium acetate buffer, then dialyzed thoroughly against the same buffer. After dialysis, the mixture was centrifuged (17,500 × g, 15 min, 4 °C) and the pellet discarded. The supernatant was loaded onto a Mono S cation-exchange column (GE Healthcare Life Sciences). Protein was eluted in a 0–400 mM NaCl gradient in sodium acetate buffer, pH 4.5. Fractions containing subtiligase were dialyzed overnight against 100 mM BICINE, 5mM DTT, pH 8.0, then concentrated to ~100 µM in an Amicon Ultra 10 kDa MWCO filter unit, flash frozen, and stored at ~80 °C. Protein was estimated to be greater than 95% by SDS–PAGE, and the stock concentration was determined by comparison to bovine serum albumin standards stained with Coomassie blue.

GST expression and purification. GST was subcloned into a pTYB2 vector, which contains the VMA intein from S. cerevisiae, and subjected to insertional mutagenesis as described above, resulting in the appending of four residues (X-Ala-Ala-X) to the natural GST C terminus followed by the intein. The GST plasmids were expressed in E. coli BL21. LB media (5 ml) containing 100 µg/ml carbenicillin was inoculated with a single colony and grown overnight at 37 °C in a spinning incubator. 3.5 ml from overnight cultures was used to inoculate 1 L LB media containing 100 µg/ml carbenicillin, and the cultures were grown in shaker flasks at 37 °C until OD600 = 0.7, then 1 ml 1M IPTG was added to induce expression and the cultures were incubated overnight while shaking at 16 °C. Cells were pelleted and then resuspended in 40 ml lysis buffer (250 mM NaCl, 50 mM HEPES, 1 mM EDTA, 10% glycerol, pH = 7.5). Unused cell pellets were flash frozen and stored at -80 °C until needed. E. coli cells were lysed by French press, the lysate was pelleted (17,500 × g, 15 min, 4 °C), and the supernatant was loaded onto 5 ml of chitin resin (NEB). Resin was washed with 150 ml wash buffer (250 mM NaCl, 25 mM...
The standard ligation reaction conditions employed 40 µM protein thioester, 3 mM biotinylated peptide, 0.5 µM subtiligase in a buffer containing 100 mM Bicine, 5 mM CaCl$_2$, pH 8.0 for 90 min at 25 °C before quenching with SDS loading dye. A nonsubtiligase control was also included, as well as a zero timepoint where the reaction was quenched before adding subtiligase. Aliquots (3 µl) of quenched reaction mixtures were run on 12% SDS–PAGE and detected by Coomassie blue staining or western blot for ubiquitin or GST proteins, respectively.

**Ubiquitin and GST ligations.** The standard ligation reaction conditions employed 40 µM protein thioester, 3 mM biotinylated peptide, 0.5 µM subtiligase in a buffer containing 100 mM Bicine, 5 mM CaCl$_2$, pH 8.0 for 90 min at 25 °C before quenching with SDS loading dye. A nonsubtiligase control was also included, as well as a zero timepoint where the reaction was quenched before adding subtiligase. Aliquots (3 µl) of quenched reaction mixtures were run on 12% SDS–PAGE and detected by Coomassie blue staining or western blot for ubiquitin or GST proteins, respectively.

**r-PTEN expression and purification.** A pFastBac1 baculovirus vector containing the PTEN–intein–CBD fusion (PTEN aa1–377) was used to make bacmid and then baculovirus in SF-21 insect cells using standard methods$^{1+}$, and the baculovirus was then used to infect High Five insect cells with M.O.I. 1.0. After growing infected High Five cells in Express Five SFM Media (Gibco) for 48 h at 27 °C, they were pelleted (700 × g, 10 min, 4 °C) and then resuspended in 1/20 of the medium used for culture, pelleted again (discarding the supernatant), and then flash frozen and stored at −80 °C. Resuspended cells from 200 ml culture were lysed in a 40 ml homogenizer in 30 ml lysis buffer (150 mM NaCl, 50 mM HEPES, 1 mM EDTA, 10% glycerol, pH 7.5). The lysis was then centrifuged (17,500 × g, 40 min, 4 °C), and the supernatant was added to a 10 ml bed of powder cellulose (Sigma). Peptides were synthesized either on a Prelude peptide synthesizer or PS3 peptide synthesizer from Protein Technologies using standard Fmoc-based solid-phase peptide synthesis. Nonphosphorylated peptides were synthesized by double coupling every residue. Fmoc groups were deprotected for five times, 10 min each with 20% piperidine in DMF. Coupling times were 1.5 h.

**Peptide synthesis.** Peptides were synthesized either on a Prelude peptide synthesizer or PS3 peptide synthesizer from Protein Technologies using standard Fmoc-based solid-phase peptide synthesis. Nonphosphorylated peptides were synthesized by double coupling every residue. Fmoc groups were deprotected for five times, 10 min each with 20% piperidine in DMF. Coupling times were 1.5 h.

**Ubiquitin expression and purification.** Human ubiquitin was C-terminally fused with Mxe intein into a pTXB1 vector and subjected to mutagenesis as described above to generate L73X and G76X residues. The ubiquitin plasmids were expressed in E. coli Rosetta(DE3). LB media (15 ml) containing 100 µg/ml ampicillin and 10 µg/ml chloramphenicol was inoculated with a single colony and grown overnight at 37 °C in a shaking incubator. 10 ml from overnight cultures was used to inoculate 1 l LB media containing 100 µg/ml ampicillin and 10 µg/ml chloramphenicol, and the cultures were grown in shaker flasks at 37 °C until OD$_{600}$ = 0.7, then 1 ml 1M IPTG was added to induce expression, and the cultures were further incubated for 3 h at 37 °C. Cells were pelleted and then resuspended in 20 ml lysis buffer (250 mM NaCl, 50 mM HEPES, 1 mM EDTA, 10% glycerol, pH 7.5).

E. coli cells were lysed by French press, the lysate was pelleted (17,500 × g, 30 min, 4 °C), and the supernatant was loaded onto 5 ml of chitin resin (NEB). Resin was washed with 150 ml wash buffer (250 mM NaCl, 25 mM HEPES, 0.1% Triton X-100, pH 7.5) then incubated overnight in cleavage buffer (250 mM NaCl, 50 mM HEPES, 300 mM MESNA, pH 7.5). The cleavage buffer was eluted from the resin, and the buffer was exchanged with 150 mM NaCl, 50 mM MES, pH 6.0 using an Amicon 3.0 kDa MWCO filter unit. The ubiquitin thioester proteins were concentrated to 1 mM, flash frozen and stored at −80 °C; and they appeared to be >95% pure by Coomassie-stained SDS–PAGE. Protein concentration was determined by SDS–PAGE referenced to bovine serum albumin standards using Coomassie staining.

Peptide synthesis. Peptides were synthesized either on a Prelude peptide synthesizer or PS3 peptide synthesizer from Protein Technologies using standard Fmoc-based solid-phase peptide synthesis. Nonphosphorylated peptides were synthesized by double coupling every residue. Fmoc groups were deprotected for five times, 10 min each with 20% piperidine in DMF. Coupling times were 1.5 h.

Biotinylated peptides were synthesized using N-ε-biotin-lysine Wang resin (Iris Biotech). Phosphorylated peptides corresponding to residues 378–403 of PTEN were synthesized by double coupling residues 386–402 for 1.5 h each, triple coupling residues Asp384 and Asp381 for 1.5 h each, and double coupling phosphorylated residues for 3 h each. Phosphate groups were monoprotected by O-benzyl groups during the synthesis. All peptides were deprotected and cleaved from resin using reagent K (82.5:2.5:5:5:5–trifluoroacetic acid:ethane dithiol:water:thioglycolicphenol) then purified using reverse-phase C18 HPLC and lyophilized. Peptide structures were confirmed using MALDI mass spectrometry (see Supplementary Fig. 8), and peptide concentrations were determined by amino acid analysis.

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and incubated at 25 °C. After 4 h, 3 µl of the mixture was taken for SDS–PAGE analysis. Ligations with the tetraphosphorylated and nonphosphorylated tails proceeded to ~30% and ~50% completion, respectively, possibly indicating that pSer380 in the P3′ position may interfere with the ligation reaction. The remainder of the mixture was injected onto a Superdex 75 size-exclusion column (GE Healthcare Life Sciences). Size-exclusion chromatography was performed with a flowrate of 0.5 ml/min in a buffer containing 500 mM NaCl, 50 mM Na₂HPO₄, 5 mM DTT, pH 7.0; and 0.3 ml fractions were collected, and 5 µl of each fraction was analyzed by Coomassie-stained SDS–PAGE. Fractions containing ligated PTEN were combined and stored at 4 °C overnight and then loaded onto 500 µl preblocked (10 mM biotin followed by 0.1 M Glycine, pH 2.8, followed by 500 mM NaCl, 50 mM Na₂HPO₄, 5 mM DTT, pH 7.0) mono-avidin resin (Thermo) on ice. Resin was washed sequentially with 5 ml of 500 mM NaCl, 50 mM Tris, 10 mM DTT, pH 8.0 for 1 h followed by 5 ml of 1 M NaCl, 50 mM Tris, 10 mM DTT, pH 8.0 for 1 h. Next, the resin was equilibrated with 5 ml 150 mM NaCl, 50 mM Tris, 10 mM DTT, pH 8.0 and then eluted with 10 mM biotin in the same buffer. The resin was incubated with 100 µl of elution buffer for 15 min at room temperature before an additional 500 µl of elution buffer was added. The purified semisynthetic protein was shown to be >90% pure by Coomassie-stained SDS–PAGE.

MEFs and western blot analysis. Murine embryo fibroblasts (MEF) obtained from the Stivers lab at Johns Hopkins University or purchased from the ATCC (shown to be mycoplasma negative) were grown in DMEM, high glucose (Thermo Fisher) with 10% fetal bovine serum (FBS) at 37 °C, 5% CO₂. Once the cells had reached ~70% confluency, the media was exchanged with DMEM with 2% FBS. Cells were then either treated with 50 µg/ml 4,5,6,7-tetrabromobenzotriazole (TBB) or DMSO. After 12–15 h incubation at 37 °C, cells were washed with PBS then lysed with RIPA buffer plus 1 mM PMSF. The lysate was pelleted at 15,000 × g (10 min, 4 °C). The protein concentration in the supernatant was determined by BCA assay (Thermo Fisher). Supernatant with 50 µg of protein was run on a 10% SDS–PAGE gel along with Y379-4p-PTEN standards. Western blot membranes were incubated with either anti-phospho-PTEN antibody 44A7 (Cell Signaling no. 9549) or anti-PTEN antibody N-19 (Santa Cruz Biotech no. sc-6818) at 1:1,000 dilutions. Western blots were developed and analyzed as described above. Data are reported in Figure 2. Fraction phosphorylated: untreated, 0.72 ± 0.06; treated, 0.33 ± 0.09 (± standard error, n = 5 biological replicates, P = 0.0071, Student’s t-test). Note that it has previously been shown that phosphorylation can stabilize cellular PTEN21, explaining the reduction in total PTEN after CK2 inhibition.

Assay of phospho-PTEN’s sensitivity to alkaline phosphatase. This was adapted from previous methods16. 4p-PTEN (1 µg) was incubated with 5 µM calf intestine phosphatase (NEB) in 20 µl of reaction buffer (50 ng/µl PTEN) for a total of 4 h. Reaction buffer consisted of 50 mM Tris, 10 mM BME, pH 8.0. Samples were taken from the reaction at various timepoints, diluted ten-fold in SDS loading dye, and run on SDS–PAGE for western blot analysis. 10 µl were loaded for the Cys-PTEN and 2 µl were loaded for the Tyr-PTEN. Blots were analyzed using anti-phospho-PTEN antibody (Cell Signaling no. 9554) at 1:1,000 dilution. Images were analyzed using ImageJ software, and Prism 6 software (Graphpad) was used to determine phosphorylation half-life fit to a standard exponential decay. Replicates with different preps showed similar half lives (within 20%). Values reported in Figure 2 are plus or minus standard error. Data shown in Figure 2 are the average of two replicates for each PTEN construct. Representative western blots in Figure 2 show the decrease in phosphorylation with longer phosphatase treatment for both PTEN variants. C379-4p-PTEN half-life = 9.0 ± 2.2 min; Y379-4p-PTEN half-life = 112.1 ± 24.5 min (n = 2, P = 0.0273, Student’s t-test).

PTEN phosphatase activity assay. PTEN activity toward a water-soluble substrate (diC₆-PIP₃, from Avanti Polar Lipids) was determined by the evolution of inorganic phosphate as measured with a malachite green detection kit (R and D Biosystems). Assays were conducted as described previously16. Briefly, 0.5–1.5 µg PTEN was incubated with 160 µM diC₆ PIP₃ for 10 min in 25 µl reaction buffer (50 mM Tris, 10 mM BME, pH 8.0) at 30 °C. Samples were quenched with malachite green reagent (R and D Biosystems), and absorbance was measured at 620 nm.

Statistics. All experiments were performed at least two times. As the relevant biological or technical replicates in almost every case gave conversions or rate constants that were within 30% of each other, duplicate experiments were believed to be sufficient to ensure confidence in these findings.

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