Published in final edited form as:

Biochemistry. 2015 February 24; 54(7): 1576–1582. doi:10.1021/acs.biochem.5b00028.

Conformational Stability and Catalytic Activity of PTEN Variants Linked to Cancers and Autism Spectrum Disorders

Sean B. Johnston[†] and Ronald T. Raines^{*†‡}

[†]Department of Biochemistry, University of Wisconsin–Madison, Madison, Wisconsin 53706, United States

[‡]Department of Chemistry, University of Wisconsin–Madison, Madison, Wisconsin 53706, United States

Abstract

Phosphoinositides are membrane components that play critical regulatory roles in mammalian cells. The enzyme PTEN, which catalyzes the dephosphorylation of the phosphoinositide PIP₃, is damaged in most sporadic tumors. Mutations in the *PTEN* gene have also been linked to autism spectrum disorders and other forms of delayed development. Here, human PTEN is shown to be on the cusp of unfolding under physiological conditions. Variants of human PTEN linked to somatic cancers and disorders on the autism spectrum are shown to be impaired in their conformational stability, catalytic activity, or both. Those variants linked only to autism have higher activity than those linked to cancers. PTEN-L, which is a secreted *trans*-active isoform, has greater conformational stability than does the wild-type enzyme. These data indicate that PTEN is a fragile enzyme cast in a crucial role in cellular metabolism, and suggest that PTEN-L is a repository for a critical catalytic activity.

Introduction

Phosphatase and tensin homolog on chromosome 10 (PTEN; EC 3.1.3.67) catalyzes the hydrolysis of phosphatidylinositol-(3,4,5)-trisphosphate (PIP₃) to form phosphatidylinositol-(4,5)-bisphosphate (PIP₂).¹ Its PIP₃ substrate activates the phosphatidylinositol-3-kinase (PI3K) Akt pathway that mediates cell proliferation. Hence, PTEN is a tumor suppressor whose recognized importance in human biology is increasing steadily.^{2–4} In addition to lipid phosphatase activity on the plasma membrane, which could alone contribute significantly to tumor suppression, PTEN has important roles in nuclear processes that include the promotion of chromosomal stability, repair of DNA damage,⁵ and regulation of the cell cycle.⁶

The *PTEN* gene is mutated in 50–80% of sporadic human cancers.⁷ Moreover, germline mutations in *PTEN* are associated with the molecularly defined PTEN hamartoma tumor syndromes (PHTSs). PHTSs include Cowden syndrome and Bannayan–Riley–Ruvalcaba

^{*}Corresponding Author: rtraines@wisc.edu.

Notes: The authors declare no competing financial interest.

Supporting Information: DSF raw data (Figure S1). This material is available free of charge via the Internet at <http://pubs.acs.org>.

syndrome, and are associated with increased cancer predisposition.² *PTEN* mutations are also strongly associated with macrocephaly.⁸

Some patients on the autism spectrum have germline mutations in the *PTEN* gene.⁹ Autism spectrum disorders (ASDs) are characterized by impaired social interactions, impaired verbal or nonverbal communication skills, and repetitive behaviors.¹⁰ ASDs are enigmatic: despite being heritable, defects in a single gene or set of genes do not seem to be present in all patients.¹¹ The biochemical link between *PTEN* dysfunction and ASDs is not known, though loss of *PTEN* function in developing mouse brain cells leads to their overgrowth, and deletion of the *PTEN* gene in neurons has large effects on neuronal morphology and circuitry.^{12,13}

Recently, alternative translation of the *PTEN* mRNA using an upstream CTG codon was discovered and found to result in the production of a secreted *trans*-active version of *PTEN*.¹⁴ Termed “*PTEN-L*”,¹⁵ this isoform is able to shrink *PTEN*-null tumors in mice. The same isoform has also been observed in the cytosol of cells and controls cell death upon interaction with mitochondria.¹⁶ Wild-type *PTEN* and *PTEN-L* have similar kinetic parameters for catalysis of PIP_3 hydrolysis.¹⁷

Conformational stability is critical to the biological function of enzymes and other proteins.¹⁸ Mutations that lead to unstable human proteins can lead to disease. For example, p53, like *PTEN*, is a tumor suppressor protein that is altered in a large fraction of human cancers. Extensive analyses have demonstrated that many cancer-associated p53 variants have compromised thermostability.^{19,20} That compromise also afflicts angiogenin, a human ribonuclease that has neuroprotective activity but is damaged in some patients with amyotrophic lateral sclerosis.²¹ To date, however, no data have been reported on the thermostability of *PTEN* or its disease-related variants.

In this work, we present a biochemical analysis of variants of *PTEN* that are linked to somatic cancer, PHTSs, and ASDs. First, we analyze the catalytic activity of these variants using a newly developed continuous assay.¹⁷ Then, we develop an assay to measure the conformational stability of *PTEN* and its variants, including *PTEN-L*, under a variety of conditions. The ensuing data provide the necessary biochemical insight on the relationship between *PTEN* dysfunction and human disease.

Experimental Procedures

Materials

Escherichia coli BL21(DE3) cells for protein production were from Novagen (Madison, WI). Expression plasmid pET30B-*PTEN* was a gift from Alonzo Ross (plasmid 20741 from Addgene, Cambridge, MA) and directs the expression of human *PTEN* with a C-terminal His₆ tag.¹⁷ Plasmids that direct the production of *PTEN* variants were created with the QuikChange site-directed mutagenesis kit from Agilent (Santa Clara, CA). Expression plasmid pET30B-*PTEN-L-S* was derived by Gibson assembly²² using a gBlocks® gene fragment from Integrated DNA Technologies (Coralville, IA). In pET30B-*PTEN-L-S*, the CTG encoding residue 1 was replaced with an ATG, the signal sequence (codons 2–21) was

removed, the ATG at the translation start site for wild-type PTEN was replaced with ATA (codon 174), and other codon optimizations for *E. coli* expression were implemented that did not affect the protein sequence in codons 2–173.^{14,17}

Terrific broth (TB) contained tryptone (12 g), yeast extract (24 g), K₂HPO₄ (72 mM), KH₂PO₄ (17 mM), and glycerol (4 mL). Columns of HisTrap HP, HiTrap Q HP, HiTrap Heparin HP, and Superdex G200 resins for protein purification were from GE Biosciences (Piscataway, NJ).

diC₈-Phosphatidylinositol-3,4,5-trisphosphate (PIP₃) was from Avanti Polar Lipids (Alabaster, AL) and Echelon Biosciences (Salt Lake City, UT). Bacterial nucleoside phosphorylase (PNPase) was product N2415 from Sigma–Aldrich (St. Louis, MO), dissolved in reaction buffer, and buffer-exchanged to remove residual phosphate. 7-Methyl-6-thioguanosine (MESG) was from Berry and Associates (Dexter, MI). Dithiobutylamine (DTBA)²³ was product 774405 from Sigma–Aldrich. A 5000× solution of SYPRO[®] Orange Protein Gel Stain was from Life Technologies (Grand Island, NY). Ficoll[®] PM 70 was product 257529A from Santa Cruz Biotechnology (Dallas, TX) and had a molecular mass of ~70 kDa. Phosphate-buffered saline (PBS) contained NaCl (137 mM), KCl (2.7 mM), Na₂HPO₄ (10 mM), and KH₂PO₄ (1.8 mM) at pH 7.4. All other chemicals and reagents were of commercial reagent grade or better, and were used without further purification.

Production and Purification of PTEN

Methods for the expression and purification of PTEN were based on those of Ross and coworkers.²⁴ PTEN expression plasmids were transformed into *E. coli* strain BL21(DE3), and grown in TB supplemented with kanamycin (30 μM). Expression was induced at OD = 0.5–0.6 at 600 nm by the addition of isopropyl β-D-1-thiogalactopyranoside to 0.10 mM, and cells were grown for 20–22 h at 18 °C. Cells were harvested by centrifugation and lysed in 20 mM sodium phosphate buffer, pH 7.4, containing NaCl (0.50 M), imidazole (20 mM), and β-mercaptoethanol (0.7% v/v) with a French pressure cell. Lysate was clarified by centrifugation at 20,000g, and the soluble fraction was applied to a HisTrap HP column. Protein was eluted with 0.50 M imidazole, and fractions containing PTEN were purified further by chromatography on a HiLoad 26/60 G200 Superdex gel-filtration column. As a final step, wild-type PTEN and variants were purified by chromatography on a HiTrap Q anion-exchange column at pH 7.4, and PTEN-L was purified by chromatography on a HiTrap Heparin HP affinity column using an ÄKTA system from Amersham–Pharmacia (Piscataway, NJ), and the results were analyzed with the UNICORN Control System. Aliquots of protein were supplemented with dithiothreitol (10 mM), glycerol (25% v/v), and ethylenediaminetetraacetic acid (EDTA) (2 mM); flash frozen in liquid nitrogen; and stored at –80 °C. Protein concentrations were measured with a Bradford assay.²⁵

Assays of Conformational Stability

The conformational stability of wild-type PTEN and its variants were assessed with differential scanning fluorimetry (DSF).²⁶ This technique relies on the increase in fluorescence of the dye SYPRO[®] Orange upon its binding to hydrophobic residues of a

protein that are exposed during thermal denaturation. Wild-type PTEN or a variant was dissolved at a concentration of 0.1 $\mu\text{g}/\mu\text{L}$ in 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid– (HEPES)–NaOH buffer, pH 7.4, containing NaCl (100 mM) and DTBA (10 mM). SYPRO[®] Orange, which is supplied at a concentration of 5000 $\times$, was added to a concentration of 15 $\times$. In experiments to probe molecular crowding, Ficoll[®] PM 70 or sucrose was present at a concentration of 50, 100, 150, 200, 250, or 300 mg/mL. Assay solutions were exposed to a temperature gradient from 10–95 $^{\circ}\text{C}$ with an increase of 1.0 $^{\circ}\text{C}$ per min. Upon heating, the fluorescence of solutions was monitored by using an excitation at (580 ± 10) nm and emission at (623 ± 14) nm. Heating and fluorescence-monitoring were performed with a ViiA[™] 7 Real-Time PCR System from Life Technologies (Grand Island, NY). Assays were also performed in PBS rather than HEPES buffer, in the presence of calcium ions (added from 0.1 μM –1.2 mM), or with a heating rate of 0.2 $^{\circ}\text{C}$ per min rather than 1.0 $^{\circ}\text{C}$ per min.

Thermal denaturation data were prepared for analysis with ViiA[™] 7 version 1.0 software, and analyzed with Protein Thermal Shift[™] version 1.2 software, both from Life Technologies (Grand Island, NY). Subsequent data analysis and plotting was performed with Prism version 6 software from Graphpad (San Diego, CA). The value of T_m was the temperature at the midpoint of the thermal transition between the low fluorescence of folded protein and the high fluorescence of unfolded protein after fitting to the Boltzmann equation.²⁶

Assays of Enzymatic Activity

Assays of the catalytic activity of wild-type PTEN and its variants were performed by using the continuous assay described previously.¹⁷ Briefly, PNPase concentration was measured with a Bradford assay²⁵ and added to a concentration of 57 $\mu\text{g}/\text{mL}$ (2 μM) in reaction buffer, which was 50 mM Tris–HCl buffer, pH 7.6, containing EDTA (2.0 mM), MESG (0.40–0.60 mM), and DTBA (40 mM). Known concentrations of PIP₃ substrate (0–320 μM) were added to the buffer, and reactions were initiated by the addition of PTEN. Wild-type PTEN was used at a concentration of 10 nM, PTEN-L at 20 nM, and PTEN variants at 100–300 nM. Measurements of absorbance at 360 nm were recorded at 25 $^{\circ}\text{C}$ with a Cary 60 UV–Vis spectrophotometer having a Varian Cary Single Cell Peltier temperature control accessory from Agilent Technologies (Santa Clara, CA). Data were analyzed with Prism version 6 software from Graphpad.

Results

Thermostability of Wild-type PTEN

We attempted to measure the conformational stability of wild-type PTEN with several methods, including thermal denaturation or chemical denaturation as monitored by ultraviolet spectroscopy or circular dichroism spectroscopy. In our hands, only DSF²⁶ provided a precise measure of conformational stability (Figure S1). With this assay, we found that wild-type PTEN has a T_m of (40.3 ± 0.1) $^{\circ}\text{C}$ (Table 1). This value was not affected by the presence of calcium ions at intracellular (0.1–0.4 μM) or extracellular (1.2 mM) concentrations (data not shown).

Thermostability of PTEN-L

We assessed the conformational stability of PTEN-L, the “long” isoform of PTEN, with DSF. Strikingly, we found that PTEN-L, which has 173 additional residues on its N-terminus, has a T_m of $(46.7 \pm 0.2)^\circ\text{C}$ (Figure 2). This value is 6.4°C greater than that of wild-type PTEN.

Effect of Inorganic Phosphate and Heating Rate on the Value of T_m

Ligands have long been known to increase the conformational stability of proteins²⁷⁻²⁹ along with their resistance to proteolysis.³⁰ As PTEN is a phosphatase, we assessed its thermostability in PBS, a buffer that is isotonic with human cells and contains 11.8 mM inorganic phosphate. We found that the T_m value of wild-type PTEN is $(42.9 \pm 0.1)^\circ\text{C}$ in PBS (Figure 2), an increase of 2.6°C compared to that in HEPES buffer. In contrast, the T_m value of PTEN-L is $(46.2 \pm 0.1)^\circ\text{C}$ in PBS, which is actually a slight decrease compared to that in HEPES buffer. These data suggest that wild-type PTEN has greater affinity for inorganic phosphate than does PTEN-L.

Like the binding of ligands, the rate of sample heating is also known to affect the measured value of T_m .³¹ Slower rates enable the folded and unfolded states of a protein to equilibrate more extensively and can give rise to lower T_m values. Instead of the typical heating rate of $1^\circ\text{C}/\text{min}$ for a DSF experiment,²⁶ we used a rate of $0.2^\circ\text{C}/\text{min}$. We found that the measured T_m values for wild-type PTEN and PTEN-L in HEPES buffer decrease to $(35.6 \pm 0.1)^\circ\text{C}$ and $(41.6 \pm 0.2)^\circ\text{C}$, respectively, with the slower heating rate (Figure 2). Likewise, the T_m values in PBS decrease upon slow heating to $(39.0 \pm 0.1)^\circ\text{C}$ and $(42.4 \pm 0.2)^\circ\text{C}$. Again, PTEN-L is much less affected by inorganic phosphate than is wild-type PTEN. Indeed, the $T_m = 6^\circ\text{C}$ in HEPES buffer and $T_m = 3^\circ\text{C}$ in PBS are independent of heating rate. We performed all subsequent measurements of thermostability in the absence of inorganic phosphate to avoid confounding ligand-binding with conformational stability.

Effect of Molecular Crowding on the Value of T_m

The concentration of molecules in the cytosol approaches 300 mg/mL ,³² and is even greater in the nucleus.³³ The conformational stability of proteins can be increased by the ensuing molecular crowding.^{32,34,35} To determine whether molecular crowding affects the conformational stability of PTEN, we determined the value of T_m for wild-type PTEN in the presence of a molecular crowding agent, Ficoll[®] PM 70 ($0\text{--}300\text{ mg/mL}$), which is a high molecular-weight, highly branched polymer of sucrose. We found that the T_m value increases with the concentration of Ficoll[®] PM 70 (Figure 3). At 300 mg/mL Ficoll[®] PM 70, which mimics the concentration of macromolecules in the cytosol,³² the T_m value is increased by 2°C . To control for specific interactions with the sucrose units, we also determined the value of T_m for wild-type PTEN in the presence of sucrose itself ($0\text{--}300\text{ mg/mL}$). We found that the T_m value has an even greater dependence on the concentration of sucrose than on that of Ficoll[®] PM 70. At 300 mg/mL sucrose, the T_m value of PTEN is increased by 4°C .

Cancer-linked Variants of PTEN

We produced and purified three variants of PTEN that have been found in somatic tumors as well as in the germline of patients with PHTSs (Figure 1). We began with an active-site variant. Catalysis by PTEN entails nucleophilic attack by the sulfur of Cys124 on the C-3 phosphoryl group of PIP₃. The substitution of Cys124 with a serine residue is found in some somatic tumors.³⁶ The C124S substitution leads to a variant with undetectable catalytic activity (Table 1). We did, however, find that this variant has a T_m of $(40.8 \pm 0.1)^\circ\text{C}$, indicative of thermostability comparable to that of wild-type PTEN. Thus, the C124S substitution devastates catalysis by PTEN, but has little effect on conformational stability.

In a patient with early onset breast cancer, the codon for Asn82 in PTEN is replaced with one for a threonine residue.³⁷ Residue 82 is on the surface of the phosphatase domain of the protein (Figure 1). We found that N82T PTEN has a k_{cat}/K_M value of $(27 \pm 6) \mu\text{M}^{-1}\text{min}^{-1}$ (Table 1), which is 15% that of the wild-type enzyme. This decrease in activity arises from both a reduction in the value of k_{cat} and an increase in the value of K_M . Thermostability was compromised as well to give a T_m of $(39.2 \pm 0.3)^\circ\text{C}$. We conclude that the N82T substitution is detrimental to both catalysis and conformational stability.

In some patients with Cowden syndrome, which is a PHTS, Phe337 is replaced with a serine residue.³⁸ Residue 337 is located in the interior of the C2 domain of PTEN. We found that the F337S variant has a k_{cat}/K_M value of $(16 \pm 5) \mu\text{M}^{-1}\text{min}^{-1}$ (Table 1), which is 9% that of wild-type PTEN. The F337S variant has a T_m of $(32.2 \pm 0.2)^\circ\text{C}$ (Figure 1A), which is the lowest observed herein and nearly 10°C less than that of wild-type PTEN (though still well above 25°C , which was the temperature in assays of catalytic activity). Thus, the F337S substitution is highly detrimental to catalysis by PTEN and to its conformational stability.

Autism-linked Variants of PTEN

We also produced and purified three variants of PTEN that have been found in patients on the autism spectrum. Among these variants was one in which His93 is replaced with an arginine residue. As had been shown previously,³⁹ the catalytic activity of H93R PTEN is affected severely by this substitution, which is located close to the interface between the phosphatase and C2 globular domains of PTEN (Figure 1A). We found that H93R PTEN has a k_{cat}/K_M value of $(4.5 \pm 0.4) \mu\text{M}^{-1}\text{min}^{-1}$ (Table 1), which is 3% that of the wild-type enzyme. Previous work indicated that the H93R substitution causes the protein to bind less strongly to liposomes containing the PIP₂ product.³⁹ The binding of PIP₂ causes PTEN to shift into a relaxed state, resulting in higher activity.^{17,24} Accordingly, an inability to bind to PIP₂ should lead to low levels of activity, as observed herein. H93R PTEN had a low T_m of $(38.1 \pm 0.3)^\circ\text{C}$, which is close to physiological temperature in humans.

The tyrosine residue at position 176 of PTEN is replaced with cysteine in an autistic boy.⁴⁰ At 9 years old, this boy suffered from delayed speech and inhibited social interactions. Like His93, Tyr176 is located in the interface between the two globular domains (Figure 1A). Y176C PTEN has the highest catalytic activity of all variants tested herein, with a k_{cat}/K_M value of $(55 \pm 10) \mu\text{M}^{-1}\text{min}^{-1}$, which is 32% that of wild-type PTEN. The Y176C variant

has a slightly diminished value of T_m of $(39.0 \pm 0.4)^\circ\text{C}$. Thus, Y176C PTEN suffers from only a modest decrease in catalytic efficacy and conformational stability.

In a pair of twins, one exhibiting a partial developmental delay and another on the autism spectrum,⁹ Glu157 was replaced with a glycine residue.¹¹ This residue is located on the surface of the phosphatase domain. Like the Y176C variant, E157G PTEN has relatively high activity, with a k_{cat}/K_M value of $(49 \pm 11) \mu\text{M}^{-1}\text{min}^{-1}$ (Table 1). Moreover, this variant also has a T_m of $(38.4 \pm 0.5)^\circ\text{C}$ (Figure 1A), which is only 2°C less than that of the wild-type enzyme. Thus, E157G PTEN is similar to Y176C PTEN in its catalytic activity and conformational stability. These two variants, which are active but only marginally stable, might be able to act in some cellular environments but be impaired in others.

Discussion

PTEN has a Fragile Conformation

Human PTEN has an elongated structure composed of two distinct domains (Figure 1). The conformational stability of this structure had not been assessed previously. The raw data from thermal denaturation experiments were indicative of a transition between two states, not three (Figure S1). Apparently, the two domains of PTEN unfold cooperatively rather than independently. This single unfolding transition gave rise to a T_m value that is close to the physiological temperature of humans (Figure 2).

We found that the T_m value of PTEN correlates with the concentration of Ficoll[®] PM 70 (Figure 3), a sucrose polymer that can mimic molecular crowding *in cellulo*.³² We also found, however, that much of this added stability arises from specific interactions between the sucrose units and PTEN, as a sucrose molecule confers approximately twice the increase in T_m value as does a sucrose unit within the Ficoll[®] PM 70 polymer. We conclude that molecular crowding does not lead to a substantial increase in the thermostability of PTEN. Hence, PTEN is poised on the edge of instability and a point mutation that leads to a small decrease in PTEN thermostability can have detrimental consequences *in cellulo*.

The fragility of PTEN is in marked contrast to that of another important tumor suppressor, p53. Recently, Veprintsev, Fersht, and coworkers used DSF to show that human p53 has a T_m value of 46°C ,²⁰ which is well above that of human PTEN under any condition used herein (Figures 2–4). These data suggest that PTEN is more vulnerable than is p53 to inactivation *in cellulo* by an amino-acid substitution.

Unlike human PTEN, human PTEN-L is not a fragile protein. Its T_m value is 10°C above physiological temperature. Previously, the 173 appended residues were shown to enable secretion of PTEN-L and uptake by other cells.¹⁴ Our data indicate that these residues also serve to endow PTEN with greater conformational stability. That attribute befits a protein that must survive in the extracellular matrix, and provides humans with a reservoir of PTEN activity that is less vulnerable to inactivation.

The three-dimensional structure of PTEN-L is unknown. Like wild-type PTEN, PTEN-L unfolds with a single transition (Figure S1). This cooperative unfolding, along with the

conformational stability conferred by the appended residues, is consistent with the 173 N-terminal residues of PTEN-L adopting a defined three-dimensional structure that interacts intimately with one or both of its globular domains. Kinetic data suggest that the N-terminal residues of PTEN-L interacts with the PIP₂-binding motif (residues 6–14 of wild-type PTEN).¹⁷ These experimental data conflict with a bioinformatics analysis predictive of a disordered N-terminus in PTEN-L.⁴¹

Cancer-linked Variants of PTEN

The importance of PTEN to cancer progression and many other cellular functions and malfunctions cannot be understated.³ We characterized three variants of PTEN that are linked to cancers. We found that two of these three variants are deficient in either conformational stability or catalytic activity. C124S PTEN lacked detectable catalytic activity; F337S PTEN has a T_m that is nearly 10 °C lower than that of wild-type PTEN, and would be unfolded *in cellulo*. In contrast, N82T PTEN is less compromised than the other two variants. This finding is consistent with the *in vivo* data of Newschaffer and coworkers, who reconstituted a humanized PI3K/PTEN system in *Saccharomyces cerevisiae* cells.⁴² The N82T substitution is linked with early-onset breast cancer. We note that Asn82 is located on the surface of the protein and could play a role in PTEN–protein interactions.⁴³ Moreover, the installation of a threonine residue at position 82 converts this segment of the protein into a putative substrate for the cellular kinases STE, STE7, and MAP2K2.⁴⁴ Phosphorylation there could be detrimental to PTEN activity *in cellulo*.

Autism-linked Variants of PTEN

All three of the variants of PTEN tested herein exhibited T_m values between 38 and 39 °C. Two of these variants (E157G and Y176C) were still efficacious catalysts with k_{cat}/K_M values near 50 $\mu\text{M}^{-1}\text{s}^{-1}$, which is 1/3 that of the wild-type enzyme. These data are again consistent with observations in *S. cerevisiae* cells.⁴² Another variant (H93R) is found in patients with PHTS as well as in somatic cancer.^{36,45} This variant had been analyzed previously³⁹ and found to be defective in binding to the product PIP₂, an event that is important for the manifestation of the catalytic activity of PTEN.¹⁷ We too found that the H93R PTEN has low catalytic activity.

Conclusions

We have reported the first measurements of the thermostability of human PTEN. We find that PTEN is a quasi-stable protein at physiological temperature, even in the presence of a ligand (inorganic phosphate) or a molecular crowding agent. PTEN dysfunction leads to the overgrowth, excessive proliferation, and accelerated differentiation of human cells.^{3,12} Accordingly, we have also reported a biochemical analysis of six PTEN variants that are implicated in somatic cancers and diseases on the autism spectrum. We find that those variants linked to cancers are more damaged than those linked to autism. This conclusion, which is based on biochemical data (Table 1), is consistent with observed effects of allelic mutations *in cellulo*.⁴⁶ Notably, the PTEN-L isoform is more stable than wild-type PTEN. As both PTEN and PTEN-L are encoded by the same gene, the uptake of PTEN-L could provide a cell with some relief from a deleterious mutation. In addition to underpinning an

understanding of the biology and pharmacology of PTEN, these findings encourage the development of small-molecule ligands that stabilize disease-related variants of PTEN and thus enhance their enzymatic activity *in cellulo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Dr. D. J. Aceti (University of Wisconsin–Madison) for help with DSF experiments.

Funding: This work was supported by Grant R01 GM044783 (NIH). S.B.J. was supported by a Dennis Weatherstone Predoctoral Fellowship from Autism Speaks and by Molecular Biosciences Training Grant T32 GM007215 (NIH). The ViiA™ 7 Real-Time PCR System for DSF was obtained with support from Grant U01 GM094622 (NIH).

References

1. Maehama T, Dixon JE. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem*. 1998; 273:13375–13378. [PubMed: 9593664]
2. Eng C. PTEN: One gene, many syndromes. *Human Mut*. 2003; 22:183–198.
3. Song MS, Salmena L, Pandolfi PP. The functions and regulation of the PTEN tumour suppressor. *Nat Rev Mol Cell Biol*. 2012; 13:283–296. [PubMed: 22473468]
4. Worby CA, Dixon JE. PTEN. *Annu Rev Biochem*. 2014; 83:641–669. [PubMed: 24905788]
5. Bassi C, Ho J, Srikumar T, Dowling RJO, Gorrini C, Miller SJ, Mak TW, Neel BG, Raught B, Stambolic V. Nuclear PTEN controls DNA repair and sensitivity to genotoxic stress. *Science*. 2013; 341:395–399. [PubMed: 23888040]
6. Song MS, Carracedo A, Salmena L, Song SJ, Egia A, Malumbres M, Pandolfi PP. Nuclear PTEN regulates the APC-CDH1 tumor-suppressive complex in a phosphatase-independent manner. *Cell*. 2011; 144:187–199. [PubMed: 21241890]
7. Salmena L, Carracedo A, Pandolfi PP. Tenets of PTEN tumor suppression. *Cell*. 2008; 133:403–414. [PubMed: 18455982]
8. Mester JL, Tilot AK, Rybicki LA, Frazier TW II, Eng C. Analysis of prevalence and degree of macrocephaly in patients with germline *PTEN* mutations and of brain weight in *Pten* knock-in murine model. *Eur J Hum Genet*. 2011; 19:763–768. [PubMed: 21343951]
9. Varga EA, Pastore M, Prior T, Herman GE, McBride KL. The prevalence of *PTEN* mutations in a clinical pediatric cohort with autism spectrum disorders, developmental delay, and macrocephaly. *Genet Med*. 2009; 11:111–117. [PubMed: 19265751]
10. Caronna EB, Milunsky JM, Tager-Flusberg H. Autism spectrum disorders: Clinical and research frontiers. *Arch Dis Child*. 2008; 93:518–523. [PubMed: 18305076]
11. Newschaffer CJ, Croen LA, Daniels J, Giarelli E, Grether JK, Levy SE, Mandell DS, Miller LA, Pinto-Martin J, Reaven J, Reynolds AM, Rice CE, Schendel D, Windham GC. The epidemiology of autism spectrum disorders. *Annu Rev Public Health*. 2007; 28:235–258. [PubMed: 17367287]
12. Lv JW, Cheng TL, Qiu ZL, Zhou WH. Role of the PTEN signaling pathway in autism spectrum disorder. *Neurosci Bull*. 2013; 29:773–778. [PubMed: 24136242]
13. Kreis P, Leondaritis G, Lieberam I, Eickholt BJ. Subcellular targeting and dynamic regulation of PTEN: Implications for neuronal cells and neurological disorders. *Front Mol Neurosci*. 2014; 7:23. [PubMed: 24744697]
14. Hopkins BD, Fine B, Steinbach N, Dendy M, Rapp Z, Shaw J, Pappas K, Yu JS, Hodakoski C, Mense S, Klein J, Pegno S, Sulis ML, Goldstein H, Amendolara B, Lei L, Maurer M, Bruce J, Canoll P, Hibshoosh H, Parsons R. A secreted PTEN phosphatase that enters cells to alter signaling and survival. *Science*. 2013; 341:399–402. [PubMed: 23744781]

15. Pulido R, Baker SJ, Barata JT, Carracedo A, Cid VJ, Chin-Sang ID, Davé V, den Hertog J, Devreotes P, Eickholt BJ, Eng C, Furnari FB, Georgescu MM, Gericke A, Hopkins B, Jiang X, Lee SR, Lösche M, Malaney P, Matias-Guiu X, Molina M, Pandolfi PP, Parsons R, Pinton P, Rivas C, Rocha RM, Rodríguez MS, Ross AH, Serrano M, Stambolic V, Stiles B, Suzuki A, Tan SS, Tonks NK, Trotman LC, Wolff N, Woscholski R, Wu H, Leslie NR. A unified nomenclature and amino acid numbering for human PTEN. *Sci Signal*. 2014; 7:pe15. [PubMed: 24985344]
16. Liang H, He S, Yang J, Jia X, Wang P, Chen X, Zhang Z, Zou X, McNutt MA, Shen WH, Yin Y. PTEN α , a PTEN isoform translated through alternative initiation, regulates mitochondrial function and energy metabolism. *Cell Metab*. 2014; 19:836–848. [PubMed: 24768297]
17. Johnston SB, Raines RT. Catalysis by the tumor-suppressor enzymes PTEN and PTEN-L. *PLoS ONE*. 2015; 10:e0116898. [PubMed: 25607987]
18. Knowles JR. Tinkering with enzymes: What are we learning? *Science*. 1987; 236:1252–1258. [PubMed: 3296192]
19. Bullock AN, Henckel J, DeDecker BS, Johnson CM, Nikolova PV, Proctor MR, Lane DP, Fersht AR. Thermodynamic stability of wild-type and mutant p53 core domain. *Proc Natl Acad Sci USA*. 1997; 94:14338–14342. [PubMed: 9405613]
20. Brandt T, Kaar JL, Fersht AR, Veprintsev DB. Stability of p53 homologs. *PLoS ONE*. 2012; 7:e47889. [PubMed: 23112865]
21. Crabtree B, Thiagarajan N, Prior SH, Wilson P, Iyer S, Ferns T, Shapiro R, Brew K, Subramanian V, Acharya KR. Characterization of human angiogenin variants implicated in amyotrophic lateral sclerosis. *Biochemistry*. 2007; 46:11810–11818. [PubMed: 17900154]
22. Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, Smith HO. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods*. 2009; 6:343–345. [PubMed: 19363495]
23. Lukesh JC III, Palte MJ, Raines RT. A potent, versatile disulfide-reducing agent from aspartic acid. *J Am Chem Soc*. 2012; 134:4057–4059. [PubMed: 22353145]
24. Campbell R, Liu F, Ross A. Allosteric activation of PTEN phosphatase by phosphatidylinositol 4,5-bisphosphate. *J Biol Chem*. 2003; 278:33617–33620. [PubMed: 12857747]
25. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976; 72:248–254. [PubMed: 942051]
26. Niesen FH, Berglund H, Vedadi M. The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability. *Nat Protoc*. 2007; 2:2212–2221. [PubMed: 17853878]
27. O'Sullivan C, Tompson FW. LX—Invertase: A contribution to the history of an enzyme or unorganised ferment. *J Chem Soc*. 1890; 57:834–931.
28. Schellman JA. Macromolecular binding. *Biopolymers*. 1975; 14:999–1018.
29. Molina Martinez D, Jafari R, Ignatushchenko M, Seki T, Larsson EA, Dan C, Sreekumar L, Cao Y, Nordlund P. Monitoring drug target engagement in cells and tissues using the cellular thermal shift assay. *Science*. 2013; 341:84–87. [PubMed: 23828940]
30. Park C, Marqusee S. Pulse proteolysis: A simple method for quantitative determination of protein stability and ligand binding. *Nat Methods*. 2005; 2:307–312.
31. Rüegg M, Moor U, Blanc B. A calorimetric study of the thermal denaturation of whey proteins in simulated milk ultrafiltrate. *J Dairy Res*. 1977; 44:509–520.
32. Ellis RJ. Macromolecular crowding: Obvious but underappreciated. *Trends Biochem Sci*. 2001; 26:597–604. [PubMed: 11590012]
33. Dhar A, Girdhar K, Singh D, Gelman H, Ebbinghaus S, Gruebele M. Protein stability and folding kinetics in the nucleus and endoplasmic reticulum of eucaryotic cells. *Biophys J*. 2011; 101:421–430. [PubMed: 21767495]
34. Zhou HX, Rivas G, Minton AP. Macromolecular crowding and confinement: Biochemical, biophysical, and potential physiological consequences. *Annu Rev Biophys*. 2008; 37:375–397. [PubMed: 18573087]
35. Elcock AH. Models of macromolecular crowding effects and the need for quantitative comparisons with experiment. *Curr Opin Struct Biol*. 2010; 20:196–206. [PubMed: 20167475]

36. Forbes SA, Beare D, Gunasekaran P, Leung K, Bindal N, Boutselakis H, Ding M, Bamford S, Cole C, Ward S, Kok CY, Jia M, De T, Teague JW, Stratton MR, McDermott U, Campbell PJ. COSMIC: Exploring the world's knowledge of somatic mutations in human cancer. *Nucleic Acids Res.* 2015; 43:D805–D811. [PubMed: 25355519]
37. Figer A, Kaplan A, Frydman M, Lev D, Paswell J, Papa MZ, Goldman B, Friedman E. Germline mutations in the *PTEN* gene in Israeli patients with Bannayan–Riley–Ruvalcaba syndrome and women with familial breast cancer. *Clin Genet.* 2002; 62:298–302. [PubMed: 12372056]
38. Lachlan KL, Lucassen AM, Bunyan D, Temple IK. Cowden syndrome and Bannayan–Riley–Ruvalcaba syndrome represent one condition with variable expression and age-related penetrance: results of a clinical study of *PTEN* mutation carriers. *J Med Genet.* 2007; 44:579–585. [PubMed: 17526800]
39. Redfern RE, Daou MC, Li L, Munson M, Gericke A, Ross AH. A mutant form of PTEN linked to autism. *Protein Sci.* 2010; 19:1948–1956. [PubMed: 20718038]
40. Orrico A, Galli L, Buoni S, Orsi A, Vonella G, Sorrentino V. Novel *PTEN* mutations in neurodevelopmental disorders and macrocephaly. *Clin Genet.* 2009; 75:195–198. [PubMed: 18759867]
41. Malaney P, Uversky VN, Davé V. The PTEN Long N-tail is intrinsically disordered: Increased viability for PTEN therapy. *Mol BioSyst.* 2013; 9:2877–2888. [PubMed: 24056727]
42. Rodriguez-Escudero I, Oliver MD, Andres-Pons A, Molina M, Cid VJ, Pulido R. A comprehensive functional analysis of *PTEN* mutations: Implications in tumor- and autism-related syndromes. *Hum Mol Genet.* 2011; 20:4132–4142. [PubMed: 21828076]
43. Hopkins BD, Hodakoski C, Barrows D, Mense SM, Parsons RE. PTEN function: The long and the short of it. *Trends Biochem Sci.* 2014; 39:183–190. [PubMed: 24656806]
44. Xue Y, Liu Z, Cao J, Ma Q, Gao X, Wang Q, Jin C, Zhou Y, Wen L, Ren J. GPS 2.1: Enhanced prediction of kinase-specific phosphorylation sites with an algorithm of motif length selection. *Protein Eng Des Sel.* 2011; 24:255–260. [PubMed: 21062758]
45. Hobert JA, Embacher R, Mester JL, Frazier TW, Eng C. Biochemical screening and *PTEN* mutation analysis in individuals with autism spectrum disorders and macrocephaly. *Eur J Hum Genet.* 2014; 22:273–276. [PubMed: 23695273]
46. Spinelli L, Black FM, Berg JN, Eickholt BJ, Leslie NR. Functionally distinct groups of inherited *PTEN* mutations in autism and tumour syndromes. *J Med Genet.* 2015; 52:128–134. [PubMed: 25527629]
47. Lee JO, Yang H, Georgescu MM, Di Cristofano A, Maehama T, Shi Y, Dixon JE, Pandolfi P, Pavletich NP. Crystal structure of the PTEN tumor suppressor: Implications for its phosphoinositide phosphatase activity and membrane association. *Cell.* 1999; 99:323–334. [PubMed: 10555148]

Figure 1. Ribbon diagram of the three-dimensional structure of human PTEN. The enzyme has a phosphatase domain (green) and C2 domain (gray). The side chains of amino-acid residues that are substituted in cancers (red) and autism spectrum disorders (blue) are shown explicitly. The enzymic active site contains an L(+)-tartrate ion, which is rendered in ball-and-stick format. The structure lacks 7 and 49 residues at the N- and C-termini, respectively, and a 24-residue internal loop. The image was created with the program PyMOL from Schrödinger (New York, NY) and PDB entry 1d5r.⁴⁷

Figure 2.

Thermostability of human PTEN and PTEN-L. Values of T_m were determined with DSF (T = 1 °C/min or 0.2 °C/min) in 100 mM HEPES–NaOH buffer, pH 7.4, containing NaCl (100 mM), DTBA (10 mM), and SYPRO® Orange Protein Gel Stain (15×), or in PBS. Each data point is for an individual experiment.

Figure 3.

Effect of molecular crowding on the thermostability of human PTEN. Values of T_m were determined with DSF ($T = 1$ °C/min) in 100 mM HEPES–NaOH buffer, pH 7.4, containing NaCl (100 mM), DTBA (10 mM), SYPRO® Orange Protein Gel Stain (15×), and Ficoll® PM 70 or sucrose. Each data point is for an individual experiment. Data in the absence of additive are from Figure 2.

Figure 4.

Thermostability of human PTEN and its variants. Values of T_m were determined with DSF ($T = 1$ °C/min) in 100 mM HEPES–NaOH buffer, pH 7.4, containing NaCl (100 mM), DTBA (10 mM), and SYPRO® Orange Protein Gel Stain (15×). Each data point is for an individual experiment. Data for wild-type PTEN and PTEN-L are from Figure 2. Raw data are shown in Figure S1. Values of T_m are listed in Table 1.

Table 1
Parameters of wild-type PTEN and dysfunctional variants

Enzyme	T_m (°C) ^a	k_{cat}/K_M (μM ⁻¹ min ⁻¹) ^b	k_{cat} (min ⁻¹) ^b	K_M (μM) ^b
Wild-type PTEN	40.3 ± 0.1	174 ± 40 ^d	4000 ± 300 ^d	23 ± 5 ^d
C124S	40.8 ± 0.1	<0.03	ND ^c	ND ^c
N82T	39.2 ± 0.3	27 ± 6	1300 ± 200	48 ± 18
F337S	32.2 ± 0.2	16 ± 5	700 ± 140	45 ± 21
H93R	38.7 ± 0.3	4.5 ± 0.4	171 ± 9	37 ± 5
Y176C	39.0 ± 0.4	55 ± 10	1800 ± 180	32 ± 9
E157G	38.4 ± 0.5	49 ± 11	1400 ± 150	28 ± 9
PTEN-L	46.7 ± 0.2	100 ± 25 ^d	477 ± 28 ^d	4.8 ± 1.1 ^d

^a Values (±SE) were determined by DSF in 100 mM HEPES–NaOH buffer, pH 7.4, containing NaCl (100 mM), DTBA (10 mM), and SYPRO® Orange Protein Gel Stain (15×) with heating at 1 °C/min. Data are shown in Figures 2 and S1.

^b Values (±SE) are for the turnover of diC8-phosphatidylinositol-3,4,5-trisphosphate in 50 mM Tris–HCl buffer, pH 7.6, containing NaCl (0, 100, or 200 mM), EDTA (2.0 mM), MESG (0.20 mM), and DTBA (40 mM), as initiated by the addition of PTEN to 20 nM. Values (± SE) were derived by fitting initial velocity data to eq 1.

^c ND, not determined.

^d Values are from ref 17.