Published in final edited form as:

Biol Psychiatry. 2018 August 15; 84(4): 265–277. doi:10.1016/j.biopsych.2017.11.025.

Nuclear-Excluded Autism-Associated PTEN Mutations Dysregulate Neuronal Growth

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Abstract

Background—Phosphatase and tensin homolog (PTEN) negatively regulates downstream AKT signaling resulting in decreased cellular growth and proliferation. PTEN is mutated in a subset of children with autism spectrum disorder (ASD); however, the mechanism by which specific point mutations alter PTEN function is largely unknown. Here, we assess how ASD-associated single-nucleotide variations in PTEN (ASD-PTEN) affect function.

Methods—We use viral-mediated molecular substitution of human PTEN into Pten knockout mouse neurons and assess neuronal morphology to determine the functional impact of ASD-PTEN. We employ molecular cloning to examine how PTEN's stability, subcellular localization, and catalytic activity impact neuronal growth.

Results—We identify a set of ASD-PTEN mutations displaying altered lipid phosphatase function and subcellular localization. We demonstrate that wild-type PTEN can rescue the neuronal hypertrophy, while PTEN H93R, F241S, D252G, W274L, N276S and D326N fail to rescue this hypertrophy. A subset of these mutations lacks nuclear localization prompting us to examine the role of nuclear PTEN in regulating neuronal growth. We find that nuclear PTEN alone

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Financial Disclosures

The authors report no biomedical financial interests or potential conflicts of interest.

Author Contributions

C.J.F. and B.W.L. contributed to manuscript preparation. C.J.F, B.W.L., and M.R.W. contributed to experimental design. C.J.F., S.A.G., A.A.Z., T.D., M.R.W. and P.W.F. conducted experiments and contributed to analysis. M.L. and M.R.W. cloned and packaged the viruses used in this research. A.J.O. and E.L.M. performed the statistical analysis of the data and created the scripts used in analysis.

is sufficient to regulate soma size. Further, forced localization of the D252G and W274L mutations into the nucleus partially restore regulation of soma size.

Conclusions—ASD-PTEN mutations display decreased stability, catalytic activity, and/or altered subcellular localization. Mutations lacking nuclear localization uncover a novel mechanism whereby lipid phosphatase activity in the nucleus can regulate mTOR signaling and neuronal growth.

Keywords

Autism; dentate gyrus; PTEN; neuron; hypertrophy; ASD

Introduction

Phosphatase and tensin homolog (PTEN) is a phosphatase that antagonizes PI3K and downstream AKT signaling by dephosphorylating phosphatidylinositol-3,4,5-triphosphate (PIP3) to phosphatidylinositol-4,5-bisphosphate (PIP2). Dysregulation of this pathway at multiple points has been highly implicated in autism spectrum disorder (ASD), including *TSC1/2* (1), *FMRP* (2), *AKT* (3) and *PI3K*. *PTEN* mutations are found in patients with autism and macrocephaly in many case reports (for review, (4)). *De novo* *PTEN* mutations linking *PTEN* function to ASD have also been identified in large-scale exome sequencing studies (5–8). While rare variants in *PTEN* show high penetrance for autism risk, variable expressivity is observed (9–11). Although many *PTEN* mutations have been identified in autism, the effect of these mutations on neurons *in vivo* is unknown.

The prevalence of *PTEN* mutations in ASD has led to the creation of diverse *PTEN* loss-of-function mouse models. Neuron-specific conditional knockout (KO) mice have social deficits associated with ASD, concurrent with macrocephaly due to neuronal hypertrophy (12). *PTEN* mutations are heterozygotic in patients with ASD, similarly, *Pten* haploinsufficient mice display widespread brain overgrowth, altered sociability, and anxiety (13). The *Pten*^{m3m4} mouse, a knock-in mouse model with *Pten* R233Q, R234Q, K266N, and K267N mutations, results in increased brain weight, cell number, and neuronal hypertrophy (14). While these mouse models recapitulate behavioral and morphological abnormalities associated with *PTEN*-ASD, it is still unknown whether specific *PTEN* point mutations found in ASD can support normal neuronal growth and function.

There are a constellation of cellular phenotypes associated with *Pten* knock-out (15), and we are just beginning to understand the contribution of *PTEN* subcellular localization and catalytic activity to these morphological changes (16). Cell membrane-bound *PTEN* has been shown to regulate dendritic and axonal growth cone guidance, synapse formation, growth, and proliferation via its lipid phosphatase activity (17–19). Nuclear *PTEN* has been shown to maintain chromosomal integrity, regulate cell cycle, and regulate DNA repair responses via its function as a protein phosphatase (20, 21). Interestingly, *PTEN*'s nuclear lipid phosphatase activity has not been extensively studied, and nuclear *PTEN* has not been linked to neuronal hypertrophy.

Here, we have identified a class of ASD-associated mutations, F241S, D252G, W274L, and N276S, that are all loss of function with altered subcellular localization. We examined the relationship of catalytic activity and subcellular localization to neuronal growth using various lipid and phosphatase dead PTEN mutants. Surprisingly, we found that lipid phosphatase function in the nucleus is sufficient to regulate soma size. We also found that trafficking ASD-associated point mutations to the nucleus improves their ability to rescue soma size, signifying a potentially important and novel role for nuclear PTEN in regulating the PI3K pathway.

Methods and Materials

See the supplemental material for the details of the techniques outlined below.

Molecular Cloning

All vectors, primers, and detailed.gb vector maps are available on request. We obtained the *PTEN* point mutations F241S, D252G, and W274L in pCDNA3 from the laboratory of Alonzo Ross (22). All other mutations and motif insertions were generated with site directed mutagenesis (QuikChange II XL Site-Directed Mutagenesis Kit, Agilent).

Viral Packaging and Injection

The viral packaging and injection procedure was as previously described (23). All viral injections were performed on P7 mice.

Histology

All Histology was performed at 21–24 days post virus-injection following transcardial perfusion with PBS and 4% PFA+Sucrose. All sections were 50µm thick free floating sections cut using a Leica VT1200s vibratome.

Biochemistry

For Western blotting standard buffers and SDS-polyacrylamide gels were used and transferred to nitrocellulose membranes. The PI(3,4,5)P₃ mass ELISA kits (Echelon Bioscience Inc., Catalog # K-2500s, Salt Lake City, UT) were used for the PIP3 assays according to the manufacturer's protocol.

Statistical Research Design

All values are reported in tables as the mean of all neurons in each group ± the standard error of the mean. All statistical comparisons were calculated using a mixed-effect regression model, unless otherwise indicated in the results (24).

Results

ASD-associated PTEN point mutations are loss-of-function

We knocked out endogenous *Pten* and concurrently expressed human *PTEN* *in vivo* by co-injecting *Pten*^{Flox/Flox} neonatal (P7) mice with two lentiviruses: one expressing mCherry-T2A-Cre and the other expressing GFP-PTEN or GFP-T2A-Pten (Figure 1A). The GFP-

PTEN construct translates into a GFP-PTEN fusion protein, the T2A sequence in the GFP-T2A-PTEN construct codes for a ribosomal skip which allows the translation of two separate proteins, GFP and PTEN (25, 26). Therefore, the T2A construct controls for steric effects that may be caused by a larger fusion protein or the tagging at the N-terminus. By injecting less than 10^6 particles/ μ l of each virus we ensured sparse infection allowing for fields of view in which uninfected, singly infected, and co-infected (PTEN + Cre) neurons were visible, providing in-tissue controls. We have previously characterized the specificity of targeting the dentate gyrus with this methodology (23). Immunostaining confirmed loss of the endogenous Pten protein. We also observed increased S6 protein expression and phosphorylation which is consistent with the upregulation of the PI3K signaling pathway due to Pten loss of function (27). (Figure 1B, C). Because staining for Pten and S6 require antigen retrieval for robust staining we used pS6 staining as an assay for downstream Pten signaling. At 21–24 days-post injection (DPI), *Pten* knockout (KO) neurons are hypertrophic and display increased pS6 signaling downstream of AKT. This hypertrophy is rescued by reconstitution of either GFP-PTEN or GFP-T2A-PTEN (Figure 1D–F, Table S1).

To test the function of *PTEN* mutations found in patients with ASD, we co-injected mCherry-T2A-Cre and each ASD-associated point mutations (H93R, F241S, D252G, W274L, N276S, and D326N) as a GFP-fusion into *Pten^{Flox/Flox}* mice (Figure 2A,B). We found that the H93R mutation, in the WPD loop of the phosphatase domain, maintained nuclear and cytoplasmic subcellular localization indistinguishable from wild-type PTEN (Figure 2 A,B,C). However, a set of PTEN variants with mutations in the CBR3 loop of the C2 domain—F241S, D252G, W274L, and N276S displayed decreased nuclear localization (Figure 2A,B,C; Table S2). F241S and D252G had a more severe loss of nuclear localization than did W274L and N276S indicating subtle variation in the functional significance of these mutations ($p < 0.01$ with post-hoc multiple comparisons). The D326N mutation in the C-alpha2 loop was undetectable (Figure 2A).

To assay *in vivo* function of these mutants we measured the cross-sectional area of the soma of neurons co-infected with the Cre and PTEN viruses. While GFP-PTEN was able to rescue the neuronal hypertrophy caused by the Pten KO, none of the point mutations were able to rescue this hypertrophy (Figure 2D, Table S3). The point mutations were also unable to rescue the increased pS6 signaling resulting from PTEN KO (Figure 2E, Table S4). We next injected the PTEN mutations H93R, F241S, D252G, and W274L into wild-type mice. We found a significant increase in soma size in neurons infected with the F241S and W274L mutations (Figure 2F, Table S4). It is important to note that the degree of hypertrophy observed due to the mutations alone with no loss of endogenous Pten is minimal compared to complete *Pten* KO (note scale in Figure 2D versus F). These data suggest that these ASD-associated *PTEN* point mutations result in a small degree of dominant negative function because they induce neuronal hypertrophy when expressed in neurons with normal, functional levels of endogenous PTEN.

To eliminate the possibility that the GFP-fusion was contributing to the loss-of-function we expressed GFP-T2A-PTEN mutations and confirmed that they are incapable of rescuing soma size on the Pten KO background (Figure 3A, B Table S5) or on an shRNA-mediated Pten knockdown background (Figure S1, Table S6). Further we found that the nuclear

excluded mutations, F241S, D252G and W274L resulted in a modest increase in soma size when expressed in wild-type granule neurons (Figure 3C). Thus, we conclude that H93R, F241S, D252G, W274L, N276S, and D326N are all loss-of-function because they are unable to rescue *Pten* KO induced neuronal hypertrophy and pS6 signaling.

We used *in vivo* immunohistochemistry to determine the level of expression that we achieved for each PTEN point mutation (Figure 4). We measured the fluorescence intensity for uninfected cells, *Pten* KO cells, and *Pten* KO cells coinfecting with GFP-PTEN in the same field of view and normalized the intensity to the uninfected cells (Figure 4A,B). We found that control cells infected with lentiviruses expressing GFP-alone expressed PTEN equivalent to uninfected cells (Figure 4A,B). *Pten* KO cells displayed a significant loss of *Pten* immunoreactivity, whereas co-expression of wild-type PTEN restored immunoreactivity to a level equivalent to endogenous *Pten*. For the H93R mutant, expression was equivalent to wild-type (Figure 4A,B). For the F241S, D252G, W274L, and D326N mutants, the expression was less than what was achieved for wild-type PTEN (Figure 4A,B). Further, D326N was indistinguishable from *Pten* KO (Figure 4A,B).

We next plotted PTEN fluorescence intensity as a function of soma size (Figure 4C–H). There was no correlation of soma size to PTEN fluorescence intensity for control GFP or wild-type PTEN infected cells indicating that *Pten* is normally present in catalytic excess (Figure 4C). For H93R we found a negative correlation of soma size to PTEN expression indicating that driving mutant expression can result in control of soma size (Figure 4D $r = -0.5734$; $p < 0.02$; Pearson's correlation with 1-tailed t-test). For F241S, D252G, W274L and D326N there was no correlation between the level of PTEN expression and soma size (Figure 4E–H). Further, comparison of soma size (x-axis) of the F241S, D252G and W274L to wild-type cells with equivalent *Pten* intensity (y-axis) demonstrates a significant increase in soma size. This indicates that loss of function of these mutations cannot be entirely attributable to decreased expression. The D326N mutation was undetectable.

Localization, Stability, and Activity of ASD-Associated PTEN Mutations

We infected HEK293 cells with the GFP-PTEN viruses and again observed normal subcellular localization for H93R, a lack of nuclear localization for GFP-F241S, GFP-D252G, and GFP-W274L mutations, and no fluorescence for GFP-D326N (Figure 5A). The shift in size of the GFP-PTEN fusion protein allowed us to examine the expression levels of exogenously expressed PTEN compared to endogenous *Pten* in Western blots of in HEK293 cells. All point mutations other than D326N were expressed at levels 4–8 fold greater than endogenous PTEN (Figure 5B). Next, we used qPCR to measure mRNA levels of *PTEN*, *GFP*, and *WRE* in HEK293 cells infected with each of the point mutations. qPCR revealed no change in the *PTEN* transcript levels for mutant *PTEN* versus wild-type (Figure 5C). Further, the D326N transcript was expressed equivalently to all other mutations. Thus, the presence of transcript but lack of protein expression indicates that D326N results in instability of the PTEN protein.

To test the stability of the other variant proteins, we treated GFP-PTEN infected HEK293 cells with cycloheximide and quantified the relative expression of PTEN over time. The H93R mutation displayed no change in stability compared to control. However, the nuclear

excluded point mutations (F241S, D252G, and W274L) displayed decreased expression compared to GFP-PTEN at 8 and 10 hours post-treatment (Figure 5D).

Finally, to measure the catalytic activity of the ASD-associated PTEN mutants we transfected HEK293 cells with CRISPR constructs targeting intron/exon junctions to knockout endogenous Pten and then expressed free PTEN (GFP-T2A-PTEN) in these cells using lentiviral infection (Figure 5E). We titrated the concentration of lentiviral particles to achieve physiological levels of PTEN expression and confirmed expression levels with Western blots (Figure 5E, F). For D326N, even at high multiplicity of infection (50MOI, data not shown), we were unable to achieve significant expression levels. We next measured the level of PIP3 using ELISA and found that knockout of endogenous Pten resulted in an increase in the PIP3 levels (Figure 5G). Reconstitution of wild-type PTEN decreased PIP3 levels to that of controls, however, expression of the ASD-associated PTEN mutations was unable to restore PIP3 levels (Figure 5G). Thus, when expressed at physiological levels, the H93R, F241S, D252G and W274L mutations have a decreased ability to dephosphorylate PIP3 which is the mechanism by which PTEN inhibits PI3K/AKT signaling.

Nuclear PTEN's lipid phosphatase activity is sufficient to regulate neuronal hypertrophy

We next examined the effect of PTEN subcellular localization and catalytic activity on the regulation of neuronal soma size. We fused an SV40-NLS (nuclear localization sequence) onto the N-terminus of the GFP-*PTEN* constructs to traffic them into the nucleus (Figure S2 and Figure 6A). Pten KO neurons infected with either 1X-NLS-GFP-*PTEN* or 2X-NLS-GFP-*PTEN* displayed a normalization of soma size similar to that seen in KO neurons infected with GFP-*PTEN* (Figure 6B, Table S7). This demonstrates that nuclear PTEN is sufficient to decrease the soma size of Pten KO neurons. p-S6 levels were also rescued in KO neurons infected with NLS-GFP-*PTEN*, showing that nuclear PTEN can regulate downstream activation of the mTOR pathway (Figure 6C). Thus, PTEN action in the nucleus is sufficient to influence soma size.

To determine if PTEN's nuclear lipid or protein phosphatase function is responsible for rescuing soma size, we created NLS-*PTEN* constructs with mutations specific for each phosphatase function. The C124S mutation creates a completely catalytically dead PTEN protein, while the G129E mutation renders a lipid phosphatase dead protein and the Y138L mutation is protein phosphatase dead. We confirmed the lipid phosphatase activity of these mutants by isolating the proteins via immunoprecipitation and assaying for PIP3 dephosphorylation *ex vivo* (Figure S3). When lentiviruses expressing these mutant phosphatase forms of *PTEN* were injected *in vivo* there was no rescue in soma size of KO neurons infected with NLS-C124S or NLS-G129E. Conversely, injection of NLS-Y138L showed a reduction of soma size (Figure 6D, E, Table S8). These experiments indicate that nuclear lipid phosphatase activity is necessary for regulating soma size *in vivo*.

To confirm that PTEN could regulate nuclear PIP3 levels, the lipid phosphatase activity of 2XNLS-GFP-PTEN was assessed via a PIP3 ELISA for endogenous PIP3 in HEK293 cells. HEK293 cells were transfected with Pten shRNA (shPten), shPten and GFP-PTEN, or shPten and GFP-2XNLS-PTEN (Figure S4). There was an increase in endogenous PIP3 levels in cells infected with shPten, demonstrating that knocking down Pten increases the

amount of its available substrate. Co-transfecting these cells with WT PTEN or 2XNLS-PTEN decreased PIP3 levels. Therefore, nuclear localized PTEN was able to dephosphorylate PIP3 *in vitro*. We performed immunohistochemistry to determine if PTEN could act on the nuclear pool of PIP3 *in vivo* (Figure S5). We found that knockout of endogenous Pten resulted in an increase in PIP3 signal in both the somatic cytoplasm and in the nucleus. Reconstitution of 2XNLS-PTEN into PTEN KO neurons decreased levels of PIP3 in the nucleus of KO cells, but had no effect on levels of PIP3 in the soma. These results indicate that endogenous Pten effects nuclear PIP3 levels and that nuclear localized PTEN can restore PIP3 levels in this cellular compartment.

Restoring subcellular localization of ASD-PTEN mutations partially restores growth regulation

To determine whether altered subcellular localization contributes to the loss of function phenotype exhibited by the D252G and W274L mutants, we cloned NLS-GFP-D252G and NLS-GFP-W274L constructs to force these mutations into the nucleus. In HEK293 cells fusion of the NLS sequence to D252G and W274L, altered the subcellular distribution so that they now exist in the cytoplasm and nucleus (Figure 7A,B). We were also able to increase the nuclear localization of D252G and W274L *in vivo* where they localize evenly to the cytoplasm and nucleus (Figure 7C). KO neurons infected with NLS-GFP-D252G are slightly smaller than neurons infected with GFP-D252G, but this decrease was not significant ($p=0.053$). However, there is a significant decrease in KO neurons infected with NLS-GFP-W274L compared to GFP-W274L (Figure 7D, Table S9). To determine if fusing the NLS was restoring activity simply by increasing the stability of PTEN we examined expression of the NLS-PTEN using immunohistochemistry for neurons *in vivo* and western blots for HEK 293 cells (Figure S6). We find that these constructs were expressed at levels equivalent to wild-type Pten. These data indicate that restoring the subcellular localization of the nuclear excluded PTEN results in a partial functional restoration.

Discussion

We have found that the H93R, F241S, D252G, W274L, N276S and D326N autism-associated PTEN mutations are loss-of-function in dentate granule neurons *in vivo*. We focused on dentate granule neurons as a model neuron of the developing CNS but hypertrophy after Pten loss occurs broadly across CNS neuron types (28–31). Thus, our findings are likely applicable across the CNS. Every mutated residue we have examined in this study has not only been found in ASD, but has also been observed in cancer (<http://cancer.sanger.ac.uk/cosmic>). Given the widespread occurrence of these mutations in patients with autism or cancer and clear heterogeneity in patient phenotypes with germ-line mutations, it must be considered that the ultimate phenotypic consequence of a specific mutation may depend on gene-environment and gene-gene interactions rather than variations in functionality associated with specific mutations.

The mechanisms through which ASD-associated PTEN mutations result in loss-of-function vary. The H93R mutation is in the catalytic WPD-loop of the PTEN phosphatase domain and, in support of our results, has been demonstrated to decrease lipid phosphatase activity

(32, 33). We find that this protein is as stable as wild-type PTEN and increasing its expression increases catalytic activity. But, reconstitution in Pten KO HEK293 cells did not restore PIP3 levels to that of control. Thus, while H93R displays decreased catalytic activity its stability and proper subcellular localization likely support partial function. In contrast, D326N cannot be expressed at detectable levels despite abundant transcript. In neurons infected with the GFP-T2A-D326N lentivirus we could measure soma size due to the expression of GFP independently of the PTEN protein. In these neurons the soma size was indistinguishable from neurons with Cre-mediated deletion of endogenous Pten. We, therefore, conclude that D326N loss-of-function is due to protein instability and that this mutation acts essentially as a null allele.

The F241S, D252G, W274L and N276S mutations are in the C2 domain of PTEN and these mutations displayed decreased nuclear localization in both neurons *in vivo* and in HEK293 cells. We find that the stability of F241S, D252G, and W274L is decreased; thus, the decreased expression level of these proteins likely contributes to their loss of function in patients. However, when expressed *in vivo* at near wild-type levels these point mutations remain incapable of rescuing soma size. Further, *in vitro*, these mutants are catalytically dead when expressed at levels equivalent to wild-type PTEN. We also find that these specific mutations have the strongest hypertrophic phenotype in cells also expressing endogenous Pten indicating the ability of homodimeric intramolecular interaction to regulate Pten activity (34). In contrast to Papa et al, we saw a modest dominant negative effect, this could be due to differences in the specific PTEN mutations we analyzed or due to the fact that we were examining an effect of human PTEN mutations in the context of endogenous mouse Pten.

We find that PTEN is capable of dephosphorylating PIP3 in the nucleus and that nuclear PTEN phosphatase activity is sufficient to regulate neuronal size. Further, restoration of subcellular localization can partially restore function of nuclear excluded mutations. Thus, loss of activity for these mutations is likely due to a combination of decreased stability, activity, and altered subcellular localization. This discovery implies that pharmacological manipulation of PTEN subcellular localization could restore cellular function in autism or cancer patients.

Previous research using a heterologous yeast reconstitution system to assess the lipid phosphatase activity of ASD/DD associated *PTEN* mutations also demonstrated low lipid phosphatase activity in F241S and D252G with increased activity in W274L (32). The increased lipid phosphatase function of W274L in this study compared to ours could be due to higher levels of expression from transfection. F241S, D252G, and N276S have also been shown to reduce protein stability when expressed at physiological levels in U87MG cells (35). Overall, our *in vivo* and *in vitro* results show that the nuclear excluded mutations F241S, D252G, W274L and N276S are all loss-of-function mutations. Further, we have demonstrated that we are able to express these proteins at near physiological levels both *in vitro* and *in vivo* demonstrating that this loss-of-function is not solely a consequence of decreased protein stability.

The altered stability, nuclear localization, and phosphatase activity of these mutants may be mechanistically related. A nearby mutation, K289E, found in a patient with Cowden's syndrome displayed a similar lack of nuclear localization and demonstrated that monoubiquitination regulates nuclear transport of PTEN (36). PTEN has been hypothesized to exist in two conformations: an open, unphosphorylated form that can interact with the cell membrane and dephosphorylate PIP3, and a closed, phosphorylated form where the C-tail folds onto the C2 domain and inhibits molecular interactions (37). Mutations promoting the dissociation of the C-tail from the C2 domain have been shown to increase nuclear localization (38). The subset of nuclear excluded ASD-associated *PTEN* mutations we have identified are near the CBR3 loop (AA 260–269), in a region that may participate in the intramolecular interaction with the C-tail (39). The C2 interaction domain is generally basic and positively charged while the C-tail is acidic and negatively charged. Serine and tyrosine phosphorylation in the C-tail would increase the acidity and decrease the charge thus promoting C2 interaction with the C-tail. D252 and N276 are negatively charged hydrophilic residues that would normally repel the C-tail. Mutation to glycine and serine would decrease the ability of this region to repel the C-tail potentially promoting the closed state. While it is difficult to predict intramolecular mechanism based on an incomplete structure, we speculate that the additional mutations we have identified may regulate the access of ubiquitinases and deubiquitinases to this region of the PTEN C2 domain. The decreased stability of the nuclear excluded *PTEN*-ASD mutations also supports the hypothesis that they are more likely to exist in the closed state (40).

While the ability of the ASD-*PTEN* mutations to catalyze the conversion of PIP3 to PIP2 is impaired, its ability to access membrane bound PIP3 may also be hindered. Transition of PTEN between the closed and open state regulates its ability to associate and dissociate with the cell membrane and access membrane bound PIP3 (39, 41–44). We speculate that forcing D252G and W274L into the nucleus improves their ability to regulate soma size by allowing them to access PIP3 that is not membrane associated. Ultrastructural analysis of the subcellular localization of PIP3 has demonstrated that 20–30% of the total PIP3 population exists in the nuclear matrix while less than 5% exists at the nuclear membrane (45). Our work contrasts with this study in showing that PTEN can catalyze the conversion of nuclear PIP3. Further, the ability to access the pool of nuclear PIP3 may be better retained by mutant forms of PTEN that have a decreased ability to interact with PIP3 in the membrane. Thus, restoring nuclear localization of mutant PTEN allows greater catalytic access to substrate and thus exerts greater control over the activation of downstream signaling. In summary, we have identified a set of related PTEN loss-of-function mutations found in autism and implicate a common aberrant intramolecular interaction in their dysfunction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by National Institutes of Health Grant R01 MH097949 to B.W.L. and Autism Speaks Pilot Grant 7359 to B.W.L., and the Optical Cellular Imaging Shared Resource and the Norris Cotton Cancer Center at the Geisel School of Medicine at Dartmouth, P30 CA023108.

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Figure 1.

Reconstitution of human *PTEN* restores function in mouse *Pten* KO neurons. (A) A combination of lentiviruses was injected into the dentate gyrus of P7 *Pten* flx/flx mice to KO (mCherry-T2A-Cre) or reconstitute (GFP-*PTEN* or GFP-T2A-*PTEN*) *PTEN* expression. (B) Granule neurons in the dentate gyrus of *Pten* flx/flx mice were immunostained for *Pten* (gray). Neurons infected with mCherry-T2A-Cre (red) demonstrated a lack of *Pten* expression compared to neighboring, uninfected neurons. (C) Immunostaining for total S6 (gray; S6 IHC) and pS6 (gray; pS6 IHC) demonstrate an upregulation in the expression and

phosphorylation of the S6 protein in the mCherry positive PTEN KO cells (red). (D) Pten KO neurons (red, arrowheads) show an increase in soma size and pS6 staining (gray) 21 days post injection. Neurons that are co-infected with mCherry-T2A-Cre and GFP-*PTEN* or GFP-T2A-*PTEN* (red and green, arrows) had a decrease in neuronal hypertrophy and pS6 (gray). (E) Quantification of soma cross sectional area indicates that there was a significant increase in soma size of Pten KO neurons when compared to control neurons ($p < 0.001$). KO neurons co-infected with GFP-*PTEN* or GFP-T2A-*PTEN* had a significant decrease in soma size when compared to KO neurons (Pten KO vs. Pten KO + GFP-Pten, $p < 0.001$; Pten KO vs. KO + GFP-T2A-Pten, $p < 0.001$). The GFP-T2A-*PTEN* neurons were smaller than the GFP-*PTEN* neurons ($p < 0.001$) demonstrating that the GFP fusion is less active than the free PTEN. (F) Quantitation revealed increased pS6 in Pten KO neurons compared to control neurons ($p < 0.001$). Neurons co-infected with mCherry-T2A-Cre and GFP-*PTEN* or GFP-T2A-*PTEN* demonstrated a decrease in pS6 signaling compared to Pten KO cells (Pten KO vs. Pten KO + GFP-Pten = $p < 0.001$; Pten KO vs. Pten KO + T2A-Pten = $p < 0.001$). There was no statistical difference between the GFP-*PTEN* and T2A-*PTEN* constructs in decreasing pS6 levels. The numbers in parentheses in (E) and (F) indicate the number of mice in each group.

Figure 2.

Expression of ASD-associated GFP-PTEN point mutations result in neuronal hypertrophy. (A) A schematic of PTEN indicating functional domains and positions of mutations that have been identified in patients with ASD. Below the schematic is the crystal structure of PTEN indicating the position of the ASD-associated mutations analyzed. (B) Lentiviruses expressing mCherry-T2A-Cre (red) were co-injected with lentiviruses that express GFP fused to *PTEN* mutations (green) into *Pten*^{Flx/Flx} mice and were stained for phosphorylated ribosomal subunit S6 (pS6, gray). The arrowheads indicate co-labeled cells. Subcellular

distribution of H93R was similar to PTEN while F241S, D252G, W274L and N276S had decreased nuclear localization. Note that we were unable to detect GFP-positive cells after injection of the D326N virus. (C) Quantitative analysis of GFP-PTEN fluorescence intensity as a ratio of the nucleus/cytoplasm indicates a decrease in nuclear localization of all mutations in the C2 domain. (D) Analysis of soma cross-sectional area shows that ASD-associated point mutations H93R, F241S, D252G, W274L, and N276S were not able to rescue the hypertrophy in Pten KO cells. KO cells have an increase in soma size compared to wild-type control cells ($p < 0.001$). Expressing GFP-PTEN restores soma size ($p < 0.001$), while none of the point mutations rescue soma size (KO + GFP-PTEN vs KO + F241S, $p < 0.05$; KO + GFP-PTEN vs KO + D252G, $p < 0.001$; KO + GFP-PTEN vs KO + W274L, $p < 0.001$; KO + GFP-PTEN vs KO + N274S, $p < 0.001$). (E) There is an increase in pS6 with Pten KO that remains elevated in cells co-infected with any of the ASD associated point mutations (KO + GFP-PTEN vs KO + F241S, $p < 0.001$; KO + GFP-PTEN vs KO + D252G, $p < 0.001$; KO + GFP-PTEN vs KO + W274L, $p < 0.05$; KO + GFP-PTEN vs KO + N274S, $p < 0.001$). KO cells co-infected with GFP-PTEN have a decrease in pS6 signaling ($p < 0.001$). (F) Lentiviral expression of the point mutations in wildtype neurons demonstrates an increase in soma size for F241S and W274L compared with control neurons ($p < 0.001$). The numbers in parentheses in (C), (D), (E), and (F) indicate the number of mice in each group.

Figure 3.

Expression of free ASD-associated PTEN mutations via T2A peptide results in neuronal hypertrophy. (A) Confocal images show Pten KO neurons (red) co-infected with GFP-T2A-PTEN constructs (green) in Pten^{Flx/Flx} animals. All GFP-T2A-PTEN constructs appear nuclear and cytoplasmic because GFP freely diffuses throughout the cell when not fused to PTEN. Cells co-infected mCherry-T2A-Cre and GFP-T2A-PTEN displayed restoration of soma size and pS6 levels (arrows) while co-infection with the ASD-associated point mutations did not rescue soma size or the increase in pS6 staining (arrowheads). (B) Quantification of soma size indicates that Pten KO increases soma size, GFP-T2A-PTEN restores size to wild-type levels and GFP-T2A-H93R, GFP-T2A-F241S, GFP-T2A-D252G, GFP-T2A-W274L, and GFP-T2A-D326N are all significantly larger than KO neurons co-infected with GFP-T2A-PTEN ($p < 0.001$). (C) Expression of GFP-T2A-PTEN, GFP-T2A-H93R, GFP-T2A-F241S, GFP-T2A-D252G, GFP-T2A-W274L, and GFP-T2A-D326N in wild-type neurons with normal endogenous Pten expression reveals a significant increase in soma size for the F241S, D252G, and W274L mutations.

Figure 4.

Expression levels resulting from PTEN lentivirus infection *in vivo*. (A) mCherry-T2A-Cre lentivirus was co-injected with GFP-T2A-PTEN lentivirus into the dentate gyrus of *Pten^{Flox/Flox}* mice at P7 and immunostained at P18. Analysis of normalized PTEN intensity revealed identical levels of PTEN in uninfected cells (Control), GFP-only (FUGW) cells, and KO and reconstitution of human PTEN. KO and reconstitution with H93R resulted in expression equivalent to wild-type PTEN ($p=1.000$), while F241S ($p=0.002$), D252G ($p=0.0001$), W274L ($p=0.0001$), and D326N ($p=0.0001$) all have lower levels of PTEN

compared to KO and reconstitution with PTEN. Further, D326N is the only point mutation that is not different from Pten KO ($p=0.137$; PTEN $p=0.0001$, H93R $p=0.0001$, F241S $p=0.0001$, D252G $p=0.0008$, W274L $p=0.0003$; A). (B-H) Soma size was measured and compared to the PTEN fluorescence intensity. (C) KO and reconstitution of PTEN revealed similar soma sizes and normalized PTEN intensity compared to FUGW, rather than KO. (D-G) H93R, F241S, D252G, and W274L resulted in decreased PTEN expression and increased soma sizes compared to wild-type Pten. (H) KO and reconstitution with D326N revealed somal hypertrophy and normalized PTEN levels equivalent KO. In B, the scale bar represents 10 μm , and the arrows denote Pten KO. Arrowheads denote either GFP only (FUGW) or KO with reconstitution of point mutations, while asterisks denote uninfected within tissue controls. In A, the brackets with stars denote statistical comparisons to KO with reconstitution of PTEN, while stars directly above each group represents statistical comparisons to KO. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Figure 5.

ASD-associated PTEN point mutations have altered subcellular localization, stability and lipid phosphatase activity. (A) HEK293 cells infected with either GFP-F241S, GFP-D252G, or GFP-W274L show a lack of mutant-PTEN localization in the nucleus while wild-type GFP-PTEN and GFP-H93R show nuclear and cytoplasmic localization. (B) Western blots of HEK293 cells infected with GFP-fused *PTEN* constructs shows similar levels of exogenous PTEN protein expression. GFP-D326N protein was not detectable via western blot analysis. (C) qPCR was performed on infected HEK293 cells showing similar transcript levels for *PTEN*, *GFP*, and *WRE* for the mutations. (D) PTEN stability was assessed using cycloheximide (CHX) to inhibit protein synthesis. There was a decrease in protein levels for F241S, D252G, and W274L at hours 8 and 10 during treatment with CHX (two-way ANOVA with Bonferroni multiple comparisons post-hoc analysis; PTEN vs. F241S, $p < 0.01$).

at 8 and 10 hours; PTEN vs. D252G, $p < 0.0001$ at 8 hours and $p < 0.001$ at 10 hours; PTEN vs. W274L, $p < 0.01$ at 8 and 10 hours). (E) To measure lipid phosphatase activity of the PTEN mutations we transfected HEK293 cells with CRISPR plasmids to knockout endogenous Pten (KO) and then infected these cells with the GFP-T2A-PTEN lentiviruses to substitute the human ASD point mutations. Western blots of HEK293 cells infected with *PTEN* point mutant constructs were probed for PTEN, GFP and alpha-tubulin. (F) Quantification of PTEN expression from western blots showing average of 3 samples $\pm$ SEM indicate physiological levels of PTEN expression. (G) PIP₃ Mass ELISA values measure pmol of PIP₃ in 3 samples $\pm$ SEM. Quantified PIP₃ amounts are expressed relative to that of the PTEN KO sample for each independent experiment and are increased by the Pten KO and remain high after expression of the ASD-associated PTEN point mutants in Pten KO cells.

Figure 6.

Nuclear PTEN is sufficient to rescue soma size via its lipid phosphatase function. (A) Granule neurons show distinct nuclear PTEN when infected with either 1X-NLS-GFP-*PTEN* or 2X-NLS-GFP-*PTEN* (green). KO neurons (red) co-infected with NLS-GFP-*PTEN* (arrow heads) show a decrease in soma size compared to neighboring KO neurons. (B) Co-infection of KO neurons co-infected with NLS-GFP-*PTEN* reduced hypertrophy, demonstrating that nuclear PTEN is sufficient for regulating soma size (Pten KO vs. KO + 1X NLS-GFP- *PTEN*, $p < 0.001$; Pten KO vs. KO + 2X-NLS-GFP-*PTEN*, $p < 0.001$, KO + *PTEN* vs. KO + 1X-NLS-GFP- *PTEN* vs KO + 2X-NLS-GFP-*PTEN*, $p > 0.05$). (C) Nuclear PTEN is also capable of rescuing the increased p-S6 signaling in Pten KO neurons (Pten KO vs. KO + 1X-NLS-GFP- *PTEN*, $p < 0.001$). (D) NLS-GFP fusion viruses (green) were injected *in vivo* along with mCherry-T2A-Cre (Pten KO, red). Arrowheads indicate co-labeled cells. (E) The somas of Pten KO neurons co-infected with NLS-GFP-C124S or NLS-GFP-G129E were not significantly different than Pten KO neurons. KO neurons co-infected with NLS-GFP-Y138L were significantly smaller than Pten KO neurons ($p < 0.001$), demonstrating that Pten's nuclear lipid phosphatase activity regulates neuronal growth. The numbers in parentheses in (B) (C) and (E) indicate the number of mice in each group.

Figure 7.

Forced nuclear localization of ASD-associated PTEN mutations partially restores soma size. (A) GFP-fusion constructs (green) show that D252G and W274L are nuclear excluded in infected HEK293 cells. By adding an NLS to constructs, NLS-GFP-D252G and NLS-GFP-W274L are now present in the nucleus, stained with a Hoechst dye. (B) The percentage of PTEN in the nucleus and the cytoplasm was calculated for each construct, showing that the NLS constructs increased the amount of nuclear PTEN for each point mutation (D252G nucleus = 22.4%, n = 18; NLS-D252G nucleus = 52.7%, n = 19; W274L nucleus = 23.3%, n = 25; NLS-W274L nucleus = 75.3%, n = 23). (C) NLS-GFP-D252G and NLS-GFP-W274L appear nuclear and cytoplasmic when injected into dentate granule neurons. (D) Pten KO neurons co-infected with GFP-D252G have no difference in soma size compared to KO neurons, but there is a significant decrease in soma size in KO neurons co-infected with NLS-GFP-D252G compared to Pten KO neurons ($p < 0.001$). There is a trend towards a decrease in soma size when comparing GFP-D252G and NLS-GFP-D252G ($p = 0.053$). There is a significant difference in the soma size of KO neurons infected with GFP-W274L

versus NLS-GFP-W274L ($p < 0.01$), demonstrating improved regulation of neuronal growth when nuclear localization of these mutations is restored. The numbers in parentheses in (D) indicate the number of mice in each group.

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