

Functional analysis of the protein phosphatase activity of PTEN

Xiaoqun Catherine ZHANG, Antonella PICCINI¹, Michael P. MYERS¹, Linda VAN AELST and Nicholas K. TONKS²

Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, NY 11724, U.S.A.

In vitro, the tumour suppressor PTEN (phosphatase and tensin homologue deleted on chromosome 10) displays intrinsic phosphatase activity towards both protein and lipid substrates. *In vivo*, the lipid phosphatase activity of PTEN, through which it dephosphorylates the 3 position in the inositol sugar of phosphatidylinositol derivatives, is important for its tumour suppressor function; however, the significance of its protein phosphatase activity remains unclear. Using two-photon laser-scanning microscopy and biolistic gene delivery of GFP (green fluorescent protein)-tagged constructs into organotypic hippocampal slice cultures, we have developed an assay of PTEN function in living tissue. Using this bioassay, we have demonstrated that overexpression of wild-type PTEN led to a decrease in spine density in neurons. Furthermore, it was the protein phosphatase

activity, but not the lipid phosphatase activity, of PTEN that was essential for this effect. The ability of PTEN to decrease neuronal spine density depended upon the phosphorylation status of serine and threonine residues in its C-terminal segment and the integrity of the C-terminal PDZ-binding motif. The present study reveals a new aspect of the function of this important tumour suppressor and suggest that, in addition to dephosphorylating the 3 position in phosphatidylinositol phospholipids, the critical protein substrate of PTEN may be PTEN itself.

Key words: organotypic hippocampal slice culture, PDZ domain, phosphatase and tensin homologue deleted on chromosome 10 (PTEN), protein phosphatase, spine density, two-photon laser-scanning microscopy.

INTRODUCTION

PTEN (phosphatase and tensin homologue deleted on chromosome 10; also known as MMAC1 and TEP1) is the tumour suppressor encoded by the chromosome 10q23 locus [1]. It is one of the most frequently lost or mutated tumour suppressors in human cancer and is generally associated with advanced and metastatic disease [1]. Germline mutations in *PTEN* are also associated with a series of autosomal-dominant cancer-predisposition syndromes, including Cowden, Bannayan–Riley–Ruvalcaba and Proteus syndromes and Lhermitte–Duclos disease, which have been categorized together as the PTEN hamartoma tumour syndromes [2]. These syndromes, which are associated with the formation of multiple benign tumours (hamartomas) and increased rates of malignant breast and thyroid tumours, reveal that even minor disruption of PTEN function can increase the risk of cancer [3]. More recently, changes in PTEN function have also been implicated in non-neoplastic disease, including Alzheimer's and Parkinson's diseases, and other neuronal disorders [4,5]. Some PTEN hamartoma tumour syndrome patients develop macrocephaly, an abnormal enlargement of the head, which led to recognition of PTEN as a gene marker for autistic behaviour [4,5]. Taken together, these observations emphasize the diverse roles of PTEN *in vivo* and highlight the importance of understanding its function and the mechanisms by which it exerts its effects.

The PTEN protein comprises 403 amino acid residues and features a PTP (protein tyrosine phosphatase) domain at the N-terminal segment of the protein (Figure 1). Structural studies revealed an unusual architecture of the active site of PTEN, which is sufficiently large to accommodate the sugar headgroup of inositol phospholipids as a substrate [6]. In fact, there are now extensive lines of evidence that illustrate the importance

of this lipid phosphatase activity of PTEN, in particular the ability to antagonize PI3K (phosphoinositide 3-kinase) function by dephosphorylating the 3 position in the inositol sugar ring, for its tumour suppressor function and its ability to control cell proliferation, survival and motility [1]. Considering the importance of PTEN in a broad array of functions *in vivo*, one would anticipate that its activity would be tightly regulated. In fact, there are additional prominent structural features of the PTEN protein that are important in this regard. The PTP domain forms an extensive interface with the C2 domain, which serves to bind phospholipid membranes [6]. The C-terminus of the protein, which is also important for its tumour suppressor function, is characterized by multiple phosphorylation sites and a binding motif (-Thr-Lys-Val-COOH) for PDZ domain-containing proteins [1]. Several protein–protein interactions involving binding partners of the C-terminus of PTEN have the potential to be of regulatory significance [7,8].

A significant development that facilitated the appreciation of the physiological importance of PTEN has been the generation of sophisticated animal models to define the consequences of the loss of function of this phosphatase [9]. In addition to providing a deeper understanding of its role as a tumour suppressor, animal models have revealed the importance of PTEN in neuronal systems, including insights into the structural and behavioural consequences of its loss of function. For example, conditional knockout of PTEN in specific neuronal populations of the cerebral cortex and hippocampus generated mice that display hyperactivity and increased anxiety-like behaviour, coupled with deficits in social interactions, consistent with a linkage to atypical autism spectrum disorders [10]. These behavioural changes coincided with structural changes within the brain, including macrocephaly. The mice also displayed disruption of axonal and

Abbreviations used: DsRed, *Discosoma* red fluorescent protein; DTT, dithiothreitol; GFP, green fluorescent protein; HEK, human embryonic kidney; NMDA, *N*-methyl-D-aspartate; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homologue deleted on chromosome 10; PTP, protein tyrosine phosphatase.

¹ Present address: The International Centre for Genetic Engineering and Biotechnology, AREA Science Park, Padriciano 99, 34012 Trieste, Italy

² To whom correspondence should be addressed (email tonks@cshl.edu).

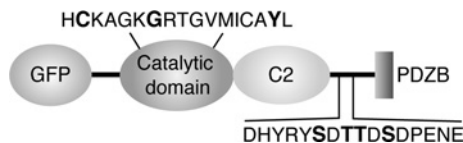


Figure 1 The domain structure of PTEN

Schematic representation to illustrate the major domains of PTEN, the catalytic and C2 domains and the C-terminal PDZ-binding motif (PDZB). The signature motif of the catalytic domain is shown, with the residues that were altered in the phosphatase activity mutants highlighted in bold. The acidic sequence motif in the C-terminal segment of the protein is also shown with the four phosphorylation sites highlighted in bold.

dendritic growth, including hypertrophy that was characterized by increased density of spines, the structures that underlie functional neural connections [10,11]. Importantly, these effects were cell autonomous, which suggested that it may be possible to use spine density as a biological read-out of PTEN function.

Following its identification as the 10q23 tumour suppressor, as predicted by its sequence, we demonstrated that PTEN possessed intrinsic phosphatase activity towards protein substrates *in vitro* [12]. In an important breakthrough for the field, it became apparent that PTEN also had the ability to dephosphorylate the lipid second messenger molecule PtdIns(3,4,5) P_3 , which suggested a potential mechanism for tumour suppression as an antagonist of PI3K-dependent signalling [13]. Initially, we had characterized the enzymatic activity of disease-derived mutations in PTEN, which revealed the properties of the G129E mutant that was identified originally in the germline of patients with Cowden syndrome [14]. This mutant maintains the ability to dephosphorylate protein substrates, but its ability to dephosphorylate inositol phospholipids is impaired, thus highlighting the importance of the lipid phosphatase activity for PTEN tumour suppressor function [14]. Nevertheless, the significance of its protein phosphatase activity remains unknown and represents a gap in our understanding of the function of this important regulator of cell signalling. Such PTEN mutants form the basis for our experimental strategy to define the significance of this activity.

In the present paper, we describe the development of a biological assay of PTEN function. The approach used two-photon laser-scanning microscopy to monitor the effects of biologically delivered GFP (green fluorescent protein)-tagged PTEN constructs into organotypic slices of the hippocampus. Through this approach we observed that expression of wild-type PTEN led to a decrease in spine density, which could be used as a read-out of PTEN function. Comparison of the effects of expressing various PTEN mutants revealed that the decrease in density of neuronal spines in cultured hippocampal slices was triggered by its protein phosphatase activity, rather than its ability to dephosphorylate inositol phospholipids. The results of the present study illustrate that dephosphorylation of the PTEN C-tail (C-terminal tail), which is a substrate of PTEN itself, is of regulatory significance in the control of spine density in this system, perhaps through controlling interactions between PTEN and PDZ domain-containing proteins. Overall, our results suggest that PTEN autodephosphorylation may be a critical event in this process; thus a major protein substrate for PTEN may be PTEN itself.

EXPERIMENTAL

Plasmids

All GFP-PTEN constructs were generated using the GFP-C2 vector (Clontech) to produce N-terminally GFP-tagged

proteins. GFP-PTEN(C124S)-4A and GFP-PTEN(C124S)-4D were generated from GFP-PTEN-4A or GFP-PTEN-4D by site-directed mutagenesis using the QuikChange[®] II site-directed mutagenesis kit (Stratagene).

Hippocampal slice culture and transfection

Hippocampal slices were prepared from P7 rats as described previously [15]. Animal experimentation was carried out according to the Animal Care and Use Committee of Cold Spring Harbor Laboratory guidelines (protocol number 08-04-02). Genes were delivered at 6 DIV (days *in vitro*) using biolistic gene transfer [180 psi (1 psi = 6.9 kPa), Helios Gene Gun, Bio-Rad Laboratories] [16]. Plasmid DNAs were coated on to 1.6 μ m gold beads; co-expression was achieved by coating the beads with DsRed (*Discosoma* red fluorescent protein) and GFP-PTEN or one of the GFP-PTEN mutant constructs. For a standard preparation, we used 14 μ g of DsRed DNA and 36 μ g of GFP-PTEN wild-type or mutant DNA with 12.5 mg of gold beads.

Two-photon laser-scanning microscopy and image analysis

The procedures were based on those described in [17–19]. At 48 h after transfection, slices were perfused with ACSF [artificial cerebrospinal fluid; 127 mM NaCl, 25 mM NaHCO₃, 1.25 mM NaH₂PO₄, 25 mM D-glucose, 2.5 mM KCl, 4 mM MgCl₂, 2 mM CaCl₂ and 1 mM MgSO₄ (pH 7.28 at 23°C) aerated with 95% O₂/5% CO₂] at room temperature (23°C). Transfected CA1 pyramidal neurons were identified by epifluorescence illumination. For each cell, a standard region encompassing the initial bifurcation of the CA1 neuron was chosen for data acquisition and two to three segments of apical dendrites were sampled. High-resolution three-dimensional image stacks, consisting of sections of 512 pixels \times 512 pixels were collected in 0.5 μ m steps on a custom-built instrument based on a Fluoview laser-scanning microscope (Olympus America) with an excitation wavelength of 910 nm. All clear protrusions emanating laterally from the dendritic shaft, irrespective of shape, were counted in the DsRed images and the spine density was measured manually using custom MATLAB software [18] by an observer who was blinded to genotype. All images in the Figures are projections of three-dimensional stacks. Values are means \pm S.E.M., and statistical significance was determined using Student's *t* test. Expression levels of GFP-tagged proteins were compared by measuring the fluorescence intensity of single spines at a fixed region of interest, standardized according to the co-transfected cytoplasmic marker DsRed. No significant differences in expression were detected.

PTEN purification, PTEN C-tail preparation and PTEN phosphatase assays

GST-PTEN, GST-PTEN G129E, GST-PTEN C124S and GST-PTEN Y138L were expressed and purified as described previously [12]. PTEN C-tail peptide (DHYRYSDDTTDSDPEN, 0.5 mM) was phosphorylated with recombinant casein kinase II (a gift from Dr D. Litchfield, Department of Biochemistry, Schulich School of Medicine and Dentistry, University of Western Ontario, London, Ontario, Canada) in a reaction mixture consisting of 50 mM Tris/HCl (pH 7.6), 2 mM DTT (dithiothreitol), 150 mM NaCl, 10 mM MgCl₂, 0.2 mg/ml BSA and 0.1 mM [γ -³²P]ATP (specific activity 100 c.p.m./pmol) and incubated at 30°C for 10 min. Phosphorylated peptide then was purified with minitrapp G-10 columns (GE Healthcare, catalogue number 28-9180-10). Purified peptide substrates were freeze-dried to dryness and resuspended

in phosphatase assay buffer, consisting of 50 mM Hepes (pH 7.0), 10 mM MgCl₂ and 10 mM DTT. The standard phosphatase assay contained 5 μM substrate, 50 mM Hepes (pH 7.0), 10 mM MgCl₂ and 10 mM DTT. The reaction was initiated by the addition of 0.2 μg of enzyme to pre-warmed (30°C) substrate mixture, resulting in a final volume of 30 μl. The reactions were allowed to proceed at 30°C for various times up to 15 min and stopped by the addition of a suspension of activated charcoal in 900 mM HCl, 90 mM sodium pyrophosphate and 2 mM sodium phosphate.

Protein extraction, immunoprecipitation and immunoblotting

GFP or GFP-tagged PTEN constructs were transfected into HEK (human embryonic kidney)-293 cells. At 2 days after transfection, cells were harvested in ice-cold lysis buffer [20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM 2-glycerophosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin and 1 mM PMSF]. Protein extracts (1 mg) were incubated with a mouse anti-GFP antibody (Invitrogen) bound to magnet beads (Invitrogen) overnight at 4°C. Antibodies used for immunoblotting [anti-phospho-PTEN antibody and anti-(total PTEN) antibody] were from Cell Signaling Technology.

RESULTS

Phosphatase activity of PTEN and regulation of dendritic spine density

We developed a tissue assay of PTEN function to test the significance of its protein phosphatase activity and extended this assay to discover non-catalytic functions of PTEN. We used a biolistic particle delivery system to express plasmids encoding GFP-tagged PTEN (to visualize PTEN expression) and DsRed (used as a cell marker) in individual neurons in organotypic slices of the hippocampus. At 2 days after transfection, two-photon laser-scanning microscopy was performed to generate whole-cell images, which revealed that GFP-PTEN was distributed throughout CA1 pyramidal neurons, including in the dendrites and spines (Figure 2A). The levels of expression of the various GFP-tagged forms of the PTEN protein were consistent (Figure 2B).

The use of two-photon microscopy allowed us to image apical dendrites and to analyse spine density. Importantly, these experiments were conducted blind with respect to the identity of the GFP-PTEN constructs that were being expressed. Consistent with the increase in spine density that has been shown to accompany the loss of PTEN in knockout mouse models [10,11], we observed that expression of wild-type GFP-PTEN resulted in a dramatic decrease in spine density compared with the control neurons transfected with GFP alone. Furthermore, GFP-PTEN(C124S), the catalytically dead mutant form of the phosphatase [12], which differs from the wild-type enzyme by a single atom, was without apparent effect. These data demonstrate that spine density can be used as a measure of PTEN activity *in vivo*. Furthermore, this decrease in spine density was also observed in neurons expressing the GFP-PTEN(G129E) mutant, in which the protein phosphatase activity is maintained but lipid phosphatase activity is attenuated (Table 1) [14]. Interestingly, expression of GFP-PTEN(Y138L), the converse mutant in which lipid phosphatase activity is preserved, but protein phosphatase activity is attenuated [20], did not alter spine density (Figure 3). These results reveal striking specificity in the effects of the PTEN mutants when expressed at similar levels in the context of an organotypic brain slice culture system. Furthermore, they indicate

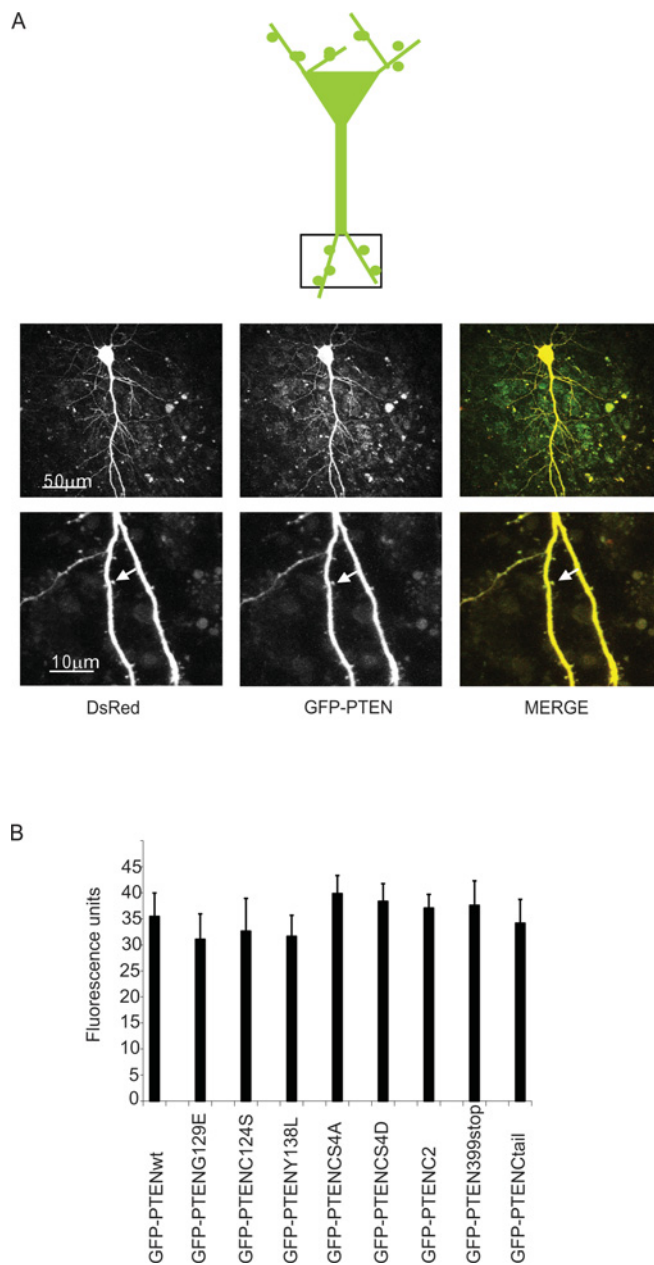


Figure 2 Expression of GFP-PTEN in hippocampal CA1 pyramidal neurons

(A) Top panel: a schematic representation of the position of dendrites imaged in the neurons. Examples of GFP-PTEN/DsRed fluorescence of a transfected hippocampal CA1 neuron (middle panels) and of apical dendrites in a transfected hippocampal CA1 neuron (bottom panels) are presented. White arrows highlight the spines. (B) Comparison of the levels of expression of the various GFP-tagged forms of PTEN, made by measurement of fluorescence intensity of single spines at a fixed region of interest, standardized according to the co-transfected cytoplasmic marker DsRed.

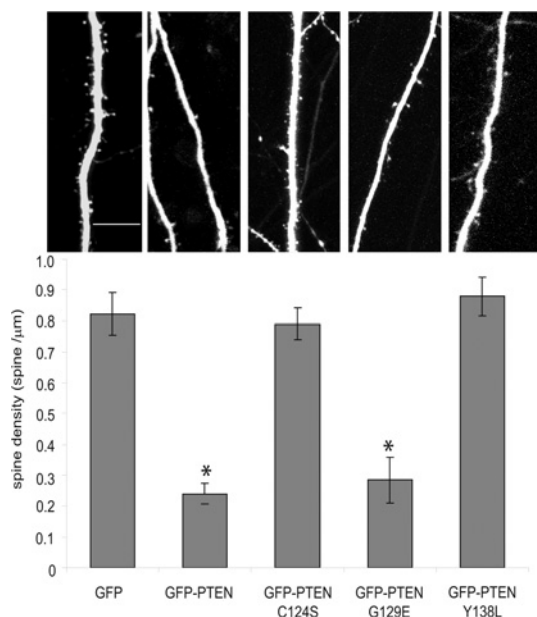
that in CA1 hippocampal pyramidal neurons, expression of PTEN regulated spine density in a manner that was dependent on its protein phosphatase, but not its lipid phosphatase, activity.

Dephosphorylation of PTEN, by PTEN

A striking feature of the protein phosphatase activity of PTEN is its preference for acidic substrates [12]. Inspection of the sequence surrounding the cluster of phosphorylation sites

Table 1 Summary of the enzymatic properties of PTEN mutants

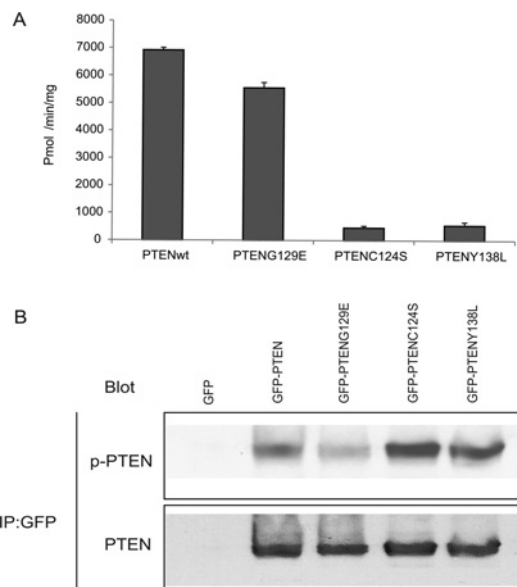
Mutant	Lipid phosphatase activity	Protein phosphatase activity
GFP-PTEN	Yes	Yes
GFP-PTEN(C124S)	No	No
GFP-PTEN(G129E)	No	Yes
GFP-PTEN(Y138L)	Yes	No

**Figure 3 Effect of PTEN expression on spine density in CA1 pyramidal neurons requires its protein, but not lipid, phosphatase activity**

Representative images of apical dendrites from biolistically transfected CA1 pyramidal neurons expressing the indicated forms of PTEN in organotypic slice cultures of rat hippocampus. Scale bar = 10 μ m. The histogram shows quantification of the effects of each plasmid on spine density and each bar is presented below the appropriate image (means \pm S.E.M., * $P < 0.0001$). $n = 8$ cells for each group.

in the C-terminal segment of PTEN revealed that this portion of the molecule is rich in acidic residues (Figure 1). Therefore we tested the possibility that a peptide modelled on this sequence, and phosphorylated *in vitro* by casein kinase 2 α , may be recognized as a substrate by PTEN. As shown in Figure 4(A), both wild-type and G129E mutant forms of PTEN dephosphorylated the PTEN C-tail peptide, whereas the inactive PTEN(C124S) and protein phosphatase-deficient PTEN(Y138L) mutants did not. These results are consistent with the model that PTEN dephosphorylates itself.

Furthermore, we have investigated the ability of PTEN to autodephosphorylate by determining the phosphorylation status of the various forms of PTEN in lysates of HEK-293 cells. At 2 days post-transfection, we used anti-GFP antibodies to immunoprecipitate PTEN and then immunoblotted with phospho-specific antibodies directed against the C-terminal cluster of phosphorylation sites. In contrast with inactive GFP-PTEN(C124S) or protein phosphatase-deficient GFP-PTEN(Y138L), which were recognized by the phospho-specific antibodies, the signal from wild-type PTEN and the protein phosphatase-active lipid phosphatase-deficient mutant, GFP-PTEN(G129E) was decreased (Figure 4B). Importantly, only those PTEN alleles that could dephosphorylate the synthetic

**Figure 4 PTEN displays potential for autodephosphorylation**

(A) PTEN dephosphorylated the C-tail peptide *in vitro*. Purified wild-type and mutant PTEN was tested for protein phosphatase activity using [32 P] P_i -labelled phosphorylated PTEN C-tail peptide as substrate. Activity is expressed as pmol of [32 P] P_i released/min per mg of enzyme. The assay was performed in triplicate. Values are means \pm S.D. (B) The phosphorylation status of the C-tail segment of PTEN in cells coincided with protein phosphatase activity. GFP or GFP-tagged PTEN constructs were transfected into HEK-293 cells and the expressed proteins were immunoprecipitated (IP) with an antibody against GFP, followed by immunoblotting first with an antibody against phospho-PTEN (Ser³⁸⁰/Thr^{382/383}) (top panel) and secondly with an antibody against total PTEN (bottom panel).

C-tail peptide *in vitro* also displayed reduced anti-phospho-PTEN reactivity when isolated from cells. Therefore these results are consistent with the ability of PTEN to utilize its protein phosphatase activity to dephosphorylate its C-terminal segment.

Effects of phosphorylation site-mutant forms of PTEN on dendritic spine density

As phosphorylation of the C-terminal portion of PTEN has been proposed to exert a regulatory influence, we sought to exploit our bioassay to examine the importance of the C-terminal phosphorylation sites for the effects of PTEN on spine density. We constructed inactive (C124S) PTEN mutants in which the four phosphorylation sites (Ser³⁸⁰, Thr³⁸², Thr³⁸³ and Ser³⁸⁵) were converted into non-phosphorylatable alanine residues (4A mutation), to mimic the dephosphorylated form of the protein, or to negatively charged aspartate residues (4D mutation), designed to mimic phosphorylated PTEN. Surprisingly, even in the absence of any intrinsic phosphatase activity in the protein, expression of the PTEN(C124S)-4A mutant led to a significant decrease in spine density (Figure 5A). In contrast, neither the C124S mutant, which would be incapable of catalysing autodephosphorylation, nor the (C124S)-4D mutant, which potentially mimics the phosphorylated state of PTEN, led to a decrease in spine density. Similarly, the 4D mutation blocked the effects of wild-type PTEN (Figure 5B). These results indicate that rather than phosphatase activity, it was the phosphorylation status of these C-terminal sites, specifically the absence of phosphate, that was important for the ability of PTEN to reduce dendritic spine density, indicating the presence of a heretofore

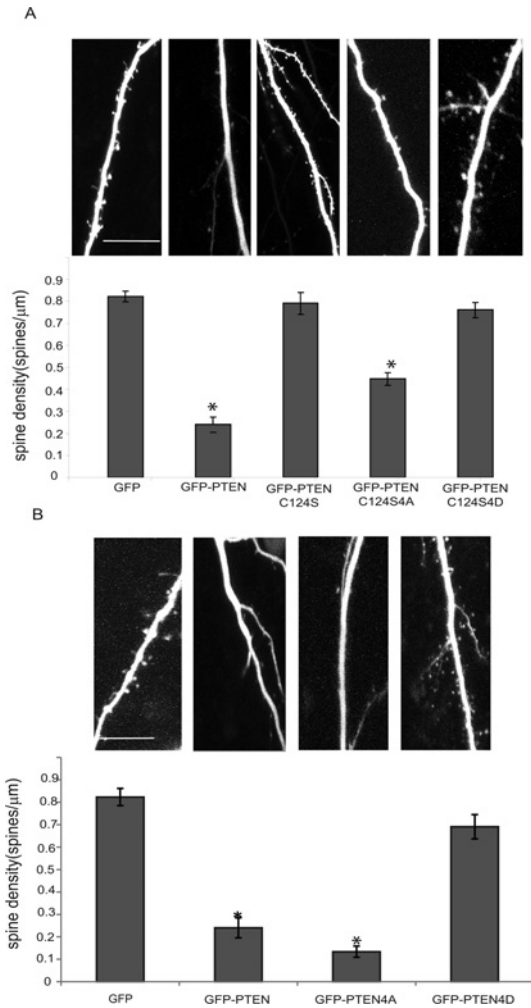


Figure 5 Effect of mutating phosphorylation sites in the C-terminal segment of wild-type and C124S inactive mutant forms of PTEN on its ability to reduce spine density

Representative images of apical dendrites from biologically transfected CA1 pyramidal neurons expressing the indicated forms of PTEN in organotypic slice cultures of rat hippocampus. Scale bar = 10 μm . The histograms show quantification of the effects of each plasmid on spine density (means \pm S.E.M., * $P < 0.0001$). $n = 8$ cells for each group.

uncharacterized function of PTEN that is regulated by its phosphorylation status.

Identification of other structural features of PTEN that are important for the decrease in dendritic spine density

In addition to the catalytic domain, PTEN contains a C2 domain and a C-terminal tail. The C2 domain of PTEN lacks residues that are important for the normal Ca^{2+} -binding function of such domains, but it has been shown to bind phospholipid membranes *in vitro* [6]. In addition, it has been shown that PTEN can inhibit cell migration via its C2 domain, independently of its function as a lipid phosphatase [21]. In light of the importance of the PTEN C2 domain, we examined its effects on spine density in our system. In contrast with these previous studies, we observed a minimal decrease in spine density following expression of the GFP-PTEN C2 domain (Figure 6).

In parallel, we examined the importance of other features of the non-catalytic portion of PTEN, specifically the C-terminal

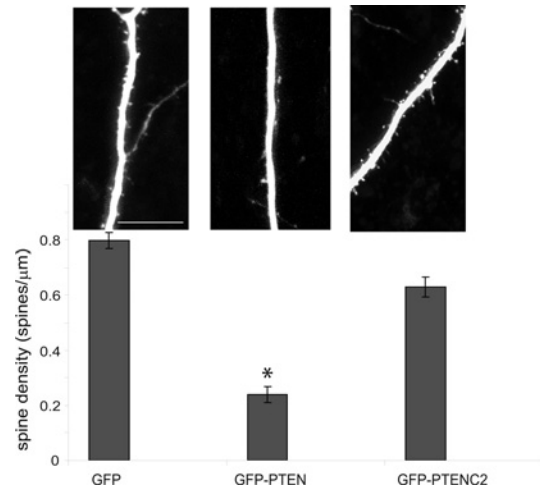


Figure 6 Expression of the PTEN C2 domain alone did not reduce the spine density in CA1 pyramidal neurons

Effects on spine density of wild-type and isolated PTEN C2 domain were compared. The top panels are representative images of apical dendrites of biologically transfected CA1 pyramidal neurons expressing the indicated forms of PTEN in organotypic slice cultures of rat hippocampus. Scale bar = 10 μm . The histogram shows quantification of the effects of each plasmid on spine density (means \pm S.E.M., * $P < 0.0001$). $n = 8$ cells for each group.

PDZ-binding motif. First, we expressed GFP-PTEN(1–399stop), a mutant that lacks the C-terminal five residues. Interestingly, we observed that, unlike wild-type PTEN, expression of this mutant did not reduce dendritic spine density (Figure 7A), thus illustrating that the PDZ-binding motif of PTEN was necessary for this effect. To test whether the C-tail segment of PTEN, containing the PDZ-binding motif, was sufficient to reduce dendritic spine density, we expressed a GFP-PTEN-(354–403) truncation mutant. Strikingly, we observed that expression of this C-tail segment of PTEN alone reduced dendritic spine density and that this effect was dependent upon the presence of the PDZ-binding motif (Figure 7B). Furthermore, we observed no effect of the 4A mutation, whereas incorporation of the 4D mutation into the C-tail segment of PTEN blocked its ability to reduce dendritic spine density (Figure 7B). This indicates that the observed effects are not artefacts of ectopic expression of PTEN, for example, loss of substrate specificity due to overexpression. In contrast, the results suggest that the phosphorylation of PTEN exerts a direct influence on its interaction with PDZ domain-containing proteins [38,39] and plays an important role in regulating spine density in this biological assay.

DISCUSSION

The importance of PTEN as a phosphatidylinositol phospholipid-directed phosphatase, and antagonist of PI3K signalling, is well established; however, there are additional features of the PTEN molecule for which the functional significance remains to be fully established, but which may exert regulatory influence. Evidence is accumulating to support the classification of PTEN as haploinsufficient, which means that inactivation of one allele, leaving only one functional copy, is not sufficient to support the wild-type state [1,2]. Consequently, cells may also be particularly susceptible to indirect means of functional inactivation of PTEN, via disruption of regulatory mechanisms [3]. There have been reports of lipid phosphatase-independent functions of PTEN. For example, expression of the catalytically dead C124S mutant was sufficient to impair glioma cell invasion [22] and PTEN can inhibit

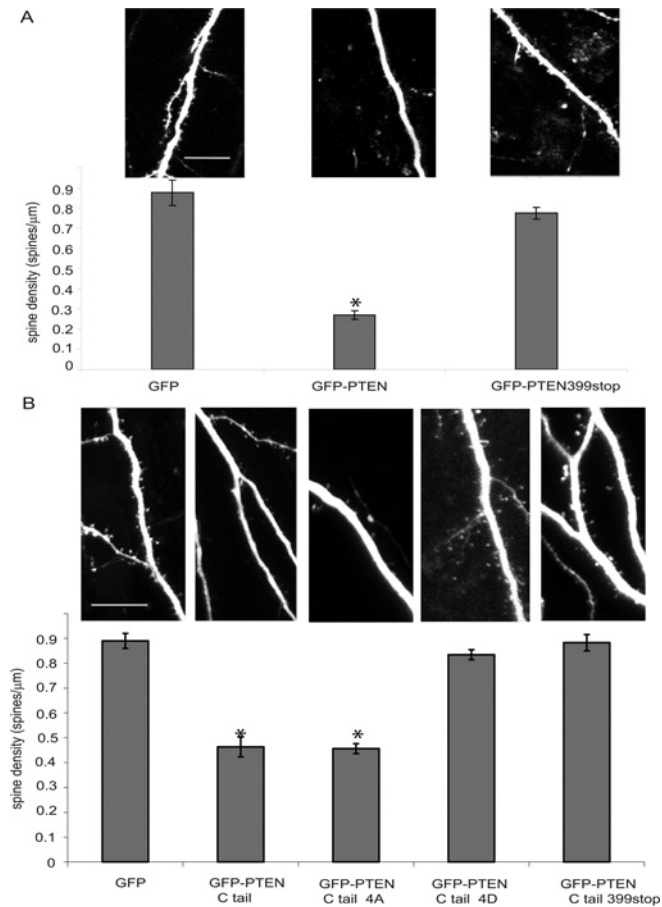


Figure 7 Effect of the PTEN PDZ domain-binding motif on spine density in neurons

(A) The PTEN PDZ-binding domain is required for the effects of PTEN on spine density in CA1 pyramidal neurons. The effects on spine density of wild-type and mutant PTEN-(1–398), which lacked the extreme C-terminal five residues of the protein, were compared. (B) Effects on spine density of a PTEN-(354–403) truncation mutant, which comprised only the C-terminal segment of the protein, were tested to determine the importance of the phosphorylation sites and the PDZ domain-binding motif in this construct. The top panels are representative images of apical dendrites of biolistically transfected CA1 pyramidal neurons expressing the indicated forms of PTEN in organotypic slice cultures of rat hippocampus. Scale bar = 10 μm. The histograms show quantification of the effects of each plasmid on spine density (means ± S.E.M., ** $P < 0.0001$). $n = 8$ cells for each group.

glioma cell migration through its C2 domain alone, in the absence of the catalytic domain [21]. In addition, the isolated C2 domain and catalytically dead forms of PTEN can affect p53 levels [23]. Such observations reinforce the importance of understanding how these activities of PTEN may be controlled *in vivo*. In the present study, we have developed a biological assay of PTEN function on the basis of its ability to influence the dendritic spine density of hippocampal CA1 neurons. We have used it to focus attention on the protein phosphatase activity of PTEN and its importance in the regulation of the phosphatase itself by reversible phosphorylation.

The identification of mutant forms of PTEN in which lipid and protein phosphatase activities are attenuated selectively [14,20] has furnished us with a powerful tool with which to explore the regulation of PTEN function. In particular, the G129E mutant, in which we first demonstrated that lipid phosphatase activity was selectively ablated leaving protein phosphatase activity intact [14], has been used extensively in the field, not only to highlight the importance of the lipid phosphatase function for the effects of PTEN on apoptosis [24], cell cycle [25] and resistance of breast

cancer cells to therapeutics [26], but also to reveal the importance of the protein phosphatase activity in invasion [27], regulation of the function of Src family kinases [28] and control of the epithelial–mesenchymal transition [29]. The G129E mutant was also used to illustrate that PTEN protein phosphatase activity is important for its effects on NMDA (*N*-methyl-D-aspartate) receptor function [30]. We have used the G129E mutant, and its more recently discovered counterpart, the Y138L mutant [15], in which protein phosphatase activity is selectively ablated, to determine which activity, protein or lipid phosphatase, is required for PTEN-dependent regulation of spine density in organotypic brain slices. Interestingly, we observed that it was the protein phosphatase activity that was important; therefore we believe that spine density can be used as a read-out to assess the role of this poorly characterized activity of PTEN.

Such studies prompt questions regarding the identity of the critical protein substrates of PTEN. Initially, there were suggestions that PTEN may influence cell migration through dephosphorylation of the cytoskeletal-associated proteins FAK (focal adhesion kinase) and p130^{CAS} [31]. It has also been implicated in dephosphorylation of the PDGF (platelet-derived growth factor) receptor [32]. However, in our initial characterization of PTEN, we noted that it demonstrated an unusual specificity, with a marked preference for acidic substrates [12]. Various studies have demonstrated that PTEN is itself a phosphoprotein, and that the major sites of phosphorylation are found in an acidic stretch (DHYRYSDDTTDSDPEN_E) near the C-terminus [1]. This prompted us to consider whether PTEN may autodephosphorylate these sites. Indeed, PTEN alleles that maintain protein phosphatase activity were able to dephosphorylate this peptide *in vitro*. Significantly, these alleles also exhibited decreased phosphorylation *in vivo*, consistent with autodephosphorylation of PTEN. We found that regulation of spine density by ectopically expressed PTEN depended on the phosphorylation status of the phosphatase, in that expression of a form of PTEN that cannot be phosphorylated resulted in a dramatic decrease in spine density, whereas a form of PTEN that mimics the phosphorylated state did not. Somewhat surprisingly, the regulation of spine density by dephospho-PTEN did not require catalytic activity, indicating that this is a non-catalytic function of PTEN that only required the catalytic domain for autodephosphorylation.

As might be expected for a motif that is rich in acidic residues, the C-terminal segment of PTEN is phosphorylated by protein kinase CK2 [33]. The potential regulatory importance of this modification is suggested by acute changes in phosphorylation in response to signals, such as leptin [34,35], phosphorylation-dependent feedback regulation of PTEN following activation of PI3K-PKB (protein kinase B)/Akt signalling [36] and the association of PTEN phosphorylation with disease, such as acute myeloid leukaemia [37]. Various functions have been suggested for the phosphorylation of PTEN, including control of the stability of the protein [38] and its susceptibility to cleavage by caspases [39]. Phosphorylation has also been implicated in regulating the subcellular distribution and activity state of PTEN. Initially, it was shown that the segment of PTEN comprising the PTP and C2 domains has the capacity to direct the protein to interact with membranes, where it can dephosphorylate its phospholipid substrate; however, this is regulated by the C-terminal tail segment, phosphorylation of which was proposed to serve as an ‘electrostatic switch’ to antagonize membrane binding [40]. Subsequently, an intramolecular association was reported between the N-terminal PTP and C2 domains and the C-terminal portion of the molecule, which results in a ‘closed’ conformation of PTEN that limits its association with the membrane [41]. Dephosphorylation of the C-terminal

tail serves to open up the conformation, promoting both membrane recruitment and activation [42], which, in turn, may play an important role in determining the distribution of its phosphatidylinositol phospholipid substrates in the membrane [43]. Our results suggest that reversible phosphorylation may also regulate a non-catalytic function of PTEN, its ability to interact with regulatory proteins via its C-terminal PDZ-binding domain, which may control its localization, stability and activity [7]. Several binding proteins have been identified that have the potential to regulate PTEN function [7,8]. It has been shown that phosphorylation of the C-tail segment of PTEN influences its ability to interact with the scaffold protein MAGI-2 (membrane-associated guanylate kinase inverted 2) [44,45]. Interaction with PICT-1 [protein interacting with C-terminus (of PTEN)-1], which itself displays properties of a tumour suppressor, is important for PTEN stability and phosphorylation [46]. There are also binding proteins with intrinsic kinase activity [47]. Overall, the results of the present study reveal that the C-terminal PDZ-binding segment of PTEN is both necessary and sufficient for the effects of the phosphatase on spine density in this bioassay. This is consistent with a model in which the C-terminal segment of PTEN is not accessible to binding proteins in the phosphorylated state, whereas autodephosphorylation promotes association with PDZ domain-containing proteins. At present the identity of the critical binding protein(s) remains to be established. The recent identification of an interaction between PTEN and PSD-95 (postsynaptic density protein 95), which has been implicated in anchoring the phosphatase at the postsynaptic membrane during NMDA receptor-dependent long-term depression, suggests one candidate [48].

In the present study, we have introduced an organotypic brain slice culture system in which to examine PTEN function. Although the system is not amenable to biochemical analysis, it has the advantage of maintaining the tissue architecture of the hippocampus, producing a 'mosaic' that permits analysis of the transfected neuron in the context of a tissue. By combining this with two-photon imaging, we have produced a system in which images with subcellular resolution can be taken, in real-time, from living tissues, permitting us to use the density of dendritic spines as a biological read-out of PTEN function. Our results illustrate that spine density was decreased following ectopic expression of both wild-type PTEN and the protein phosphatase-competent G129E mutant; in contrast, catalytically dead (C124S) and protein phosphatase-deficient but lipid phosphatase-active (Y138L) mutants were without effect. Furthermore, our results are consistent with a model in which the PTEN C-tail segment is itself a substrate of PTEN protein phosphatase activity, with dephosphorylation of the C-tail being a major requirement for the reduction in spine density, perhaps through controlling interactions between PTEN and PDZ domain-containing proteins. These results reveal a striking parallel with PI3K, which phosphorylates exogenous lipid substrates and displays protein kinase activity through autophosphorylation [49], illustrating symmetry in the regulation of signalling via phosphorylation and dephosphorylation of inositol phospholipids. It is well known that PDZ domain proteins play significant roles in forming and maintaining spines in neurons [50]. Our results highlight the potential significance of PTEN itself as a scaffolding protein, rather than simply being the subject of scaffolding molecules that target its catalytic function to particular subcellular locales. Although at present we are only using the effects of PTEN on spine density as a read-out of phosphatase function in a bioassay, the identification of those proteins that interact with PTEN to control spine density in this system may also provide new insights into the regulation of neuronal function in normal and disease states.

AUTHOR CONTRIBUTION

Xiaoqun Catherine Zhang was the primary person responsible for conducting the experiments described in the paper. Antonella Piccini introduced the organotypic brain culture system to the laboratory and undertook the initial characterization of PTEN and its mutant forms in this system, which formed the basis for the project. Michael Myers and Linda Van Aelst assisted in the scientific direction of the project. Nicholas Tonks directed the study, obtained grant support and wrote the paper.

ACKNOWLEDGMENTS

We thank Robert Malinow for providing the image analysis software, Z. Josh Huang and Bo Li for sharing their two-photon microscope, and Peter Downes and Nick Leslie (The Inositol Lipid Signalling Laboratory, College of Life Sciences, Dundee University, Dundee, Scotland, U.K.) for sharing PTEN constructs, particularly the Y138L mutant.

FUNDING

This work was supported by the National Institutes of Health [grant numbers CA53840 (to N.K.T.) and MH082808 (to L.V.A.)], and the National Institutes of Health Cold Spring Harbor Laboratory Cancer Centre Support Grant [grant number CA45508]. M.P.M. and A.P. are supported by funds from the AIRC (Associazione Italiana per la Ricerca sul Cancro). We are also grateful for support from the Irving Hansen Foundation and the Robertson Research Fund of Cold Spring Harbor Laboratory.

REFERENCES

- 1 Salmena, L., Carracedo, A. and Pandolfi, P. P. (2008) Tenets of PTEN tumor suppression. *Cell* **133**, 403–414
- 2 Hollander, M. C., Blumenthal, G. M. and Dennis, P. A. (2011) PTEN loss in the continuum of common cancers, rare syndromes and mouse models. *Nat. Rev. Cancer* **11**, 289–301
- 3 Leslie, N. R. and Foti, M. (2011) Non-genomic loss of PTEN function in cancer: not in my genes. *Trends Pharmacol. Sci.* **32**, 131–140
- 4 Chang, N., El-Hayek, Y. H., Gomez, E. and Wan, Q. (2007) Phosphatase PTEN in neuronal injury and brain disorders. *Trends Neurosci.* **30**, 581–586
- 5 Endersby, R. and Baker, S. J. (2008) PTEN signaling in brain: neuropathology and tumorigenesis. *Oncogene* **27**, 5416–5430
- 6 Lee, J. O., Yang, H., Georgescu, M. M., Di Cristofano, A., Maehama, T., Shi, Y., Dixon, J. E., Pandolfi, P. and Pavletich, N. P. (1999) Crystal structure of the PTEN tumor suppressor: implications for its phosphoinositide phosphatase activity and membrane association. *Cell* **99**, 323–334
- 7 Gericke, A., Munson, M. and Ross, A. H. (2006) Regulation of the PTEN phosphatase. *Gene* **374**, 1–9
- 8 Bonifant, C. L., Kim, J. S. and Waldman, T. (2007) NHERFs, NEP, MAGUKs, and more: interactions that regulate PTEN. *J. Cell. Biochem.* **102**, 878–885
- 9 Nardella, C., Carracedo, A., Salmena, L. and Pandolfi, P. P. (2010) Faithful modeling of PTEN loss driven diseases in the mouse. *Curr. Top. Microbiol. Immunol.* **347**, 135–168
- 10 Kwon, C. H., Luikart, B. W., Powell, C. M., Zhou, J., Matheny, S. A., Zhang, W., Li, Y., Baker, S. J. and Parada, L. F. (2006) Pten regulates neuronal arborization and social interaction in mice. *Neuron* **50**, 377–388
- 11 Fraser, M. M., Bayazitov, I. T., Zakharenko, S. S. and Baker, S. J. (2008) Phosphatase and tensin homolog, deleted on chromosome 10 deficiency in brain causes defects in synaptic structure, transmission and plasticity, and myelination abnormalities. *Neuroscience* **151**, 476–488
- 12 Myers, M. P., Stolarov, J. P., Eng, C., Li, J., Wang, S. I., Wigler, M. H., Parsons, R. and Tonks, N. K. (1997) P-TEN, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 9052–9057
- 13 Maehama, T. and Dixon, J. E. (1998) The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.* **273**, 13375–13378
- 14 Myers, M. P., Pass, I., Batty, I. H., Van der Kaay, J., Stolarov, J. P., Hemmings, B. A., Wigler, M. H., Downes, C. P. and Tonks, N. K. (1998) The lipid phosphatase activity of PTEN is critical for its tumor suppressor function. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 13513–13518
- 15 Shi, S., Hayashi, Y., Esteban, J. A. and Malinow, R. (2001) Subunit-specific rules governing AMPA receptor trafficking to synapses in hippocampal pyramidal neurons. *Cell* **105**, 331–343
- 16 Arnold, D., Feng, L., Kim, J. and Heintz, N. (1994) A strategy for the analysis of gene expression during neural development. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 9970–9974

- 17 Zito, K., Knott, G., Shepherd, G. M., Shenolikar, S. and Svoboda, K. (2004) Induction of spine growth and synapse formation by regulation of the spine actin cytoskeleton. *Neuron* **44**, 321–334
- 18 Kopec, C. D., Li, B., Wei, W., Boehm, J. and Malinow, R. (2006) Glutamate receptor exocytosis and spine enlargement during chemically induced long-term potentiation. *J. Neurosci.* **26**, 2000–2009
- 19 Li, B., Woo, R. S., Mei, L. and Malinow, R. (2007) The neuregulin-1 receptor erbB4 controls glutamatergic synapse maturation and plasticity. *Neuron* **54**, 583–597
- 20 Davidson, L., Maccario, H., Perera, N. M., Yang, X., Spinelli, L., Tibarewal, P., Glancy, B., Gray, A., Weijer, C. J., Downes, C. P. and Leslie, N. R. (2010) Suppression of cellular proliferation and invasion by the concerted lipid and protein phosphatase activities of PTEN. *Oncogene* **29**, 687–697
- 21 Raftopoulos, M., Etienne-Manneville, S., Self, A., Nicholls, S. and Hall, A. (2004) Regulation of cell migration by the C2 domain of the tumor suppressor PTEN. *Science* **303**, 1179–1181
- 22 Maier, D., Jones, G., Li, X., Schonthal, A. H., Gratzl, O., Van Meir, E. G. and Merlo, A. (1999) The PTEN lipid phosphatase domain is not required to inhibit invasion of glioma cells. *Cancer Res.* **59**, 5479–5482
- 23 Freeman, D. J., Li, A. G., Wei, G., Li, H. H., Kertesz, N., Lesche, R., Whale, A. D., Martinez-Diaz, H., Rozengurt, N., Cardiff, R. D., Liu, X. and Wu, H. (2003) PTEN tumor suppressor regulates p53 protein levels and activity through phosphatase-dependent and -independent mechanisms. *Cancer Cell* **3**, 117–130
- 24 Yuan, X. J. and Whang, Y. E. (2002) PTEN sensitizes prostate cancer cells to death receptor-mediated and drug-induced apoptosis through a FADD-dependent pathway. *Oncogene* **21**, 319–327
- 25 Ramaswamy, S., Nakamura, N., Vazquez, F., Batt, D. B., Perera, S., Roberts, T. M. and Sellers, W. R. (1999) Regulation of G1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 2110–2115
- 26 Steelman, L. S., Navolanic, P. M., Sokolosky, M. L., Taylor, J. R., Lehmann, B. D., Chappell, W. H., Abrams, S. L., Wong, E. W., Stadelman, K. M., Terrian, D. M. et al. (2008) Suppression of PTEN function increases breast cancer chemotherapeutic drug resistance while conferring sensitivity to mTOR inhibitors. *Oncogene* **27**, 4086–4095
- 27 Cai, X. M., Tao, B. B., Wang, L. Y., Liang, Y. L., Jin, J. W., Yang, Y., Hu, Y. L. and Zha, X. L. (2005) Protein phosphatase activity of PTEN inhibited the invasion of glioma cells with epidermal growth factor receptor mutation type III expression. *Int. J. Cancer* **117**, 905–912
- 28 Dey, N., Crosswell, H. E., De, P., Parsons, R., Peng, Q., Su, J. D. and Durden, D. L. (2008) The protein phosphatase activity of PTEN regulates SRC family kinases and controls glioma migration. *Cancer Res.* **68**, 1862–1871
- 29 Leslie, N. R., Yang, X., Downes, C. P. and Weijer, C. J. (2007) PtdIns(3,4,5)P3-dependent and -independent roles for PTEN in the control of cell migration. *Curr. Biol.* **17**, 115–125
- 30 Ning, K., Pei, L., Liao, M., Liu, B., Zhang, Y., Jiang, W., Mielke, J. G., Li, L., Chen, Y., El-Hayek, Y. H. et al. (2004) Dual neuroprotective signaling mediated by downregulating two distinct phosphatase activities of PTEN. *J. Neurosci.* **24**, 4052–4060
- 31 Tamura, M., Gu, J., Takino, T. and Yamada, K. M. (1999) Tumor suppressor PTEN inhibition of cell invasion, migration, and growth: differential involvement of focal adhesion kinase and p130Cas. *Cancer Res.* **59**, 442–449
- 32 Mahimainathan, L. and Choudhury, G. G. (2004) Inactivation of platelet-derived growth factor receptor by the tumor suppressor PTEN provides a novel mechanism of action of the phosphatase. *J. Biol. Chem.* **279**, 15258–15268
- 33 Torres, J. and Pulido, R. (2001) The tumor suppressor PTEN is phosphorylated by the protein kinase CK2 at its C terminus. Implications for PTEN stability to proteasome-mediated degradation. *J. Biol. Chem.* **276**, 993–998
- 34 Ning, K., Miller, L. C., Laidlaw, H. A., Burgess, L. A., Perera, N. M., Downes, C. P., Leslie, N. R. and Ashford, M. L. (2006) A novel leptin signalling pathway via PTEN inhibition in hypothalamic cell lines and pancreatic β -cells. *EMBO J.* **25**, 2377–2387
- 35 Ning, K., Miller, L. C., Laidlaw, H. A., Watterson, K. R., Gallagher, J., Sutherland, C. and Ashford, M. L. (2009) Leptin-dependent phosphorylation of PTEN mediates actin restructuring and activation of ATP-sensitive K⁺ channels. *J. Biol. Chem.* **284**, 9331–9340
- 36 Birle, D., Bottini, N., Williams, S., Huynh, H., deBelle, I., Adamson, E. and Mustelin, T. (2002) Negative feedback regulation of the tumor suppressor PTEN by phosphoinositide-induced serine phosphorylation. *J. Immunol.* **169**, 286–291
- 37 Cheong, J. W., Eom, J. I., Maeng, H. Y., Lee, S. T., Hahn, J. S., Ko, Y. W. and Min, Y. H. (2003) Phosphatase and tensin homologue phosphorylation in the C-terminal regulatory domain is frequently observed in acute myeloid leukaemia and associated with poor clinical outcome. *Br. J. Haematol.* **122**, 454–456
- 38 Vazquez, F., Ramaswamy, S., Nakamura, N. and Sellers, W. R. (2000) Phosphorylation of the PTEN tail regulates protein stability and function. *Mol. Cell. Biol.* **20**, 5010–5018
- 39 Torres, J., Rodriguez, J., Myers, M. P., Valiente, M., Graves, J. D., Tonks, N. K. and Pulido, R. (2003) Phosphorylation-regulated cleavage of the tumor suppressor PTEN by caspase-3: implications for the control of protein stability and PTEN-protein interactions. *J. Biol. Chem.* **278**, 30652–30660
- 40 Das, S., Dixon, J. E. and Cho, W. (2003) Membrane-binding and activation mechanism of PTEN. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 7491–7496
- 41 Odriozola, L., Singh, G., Hoang, T. and Chan, A. M. (2007) Regulation of PTEN activity by its carboxyl-terminal autoinhibitory domain. *J. Biol. Chem.* **282**, 23306–23315
- 42 Rahdar, M., Inoue, T., Meyer, T., Zhang, J., Vazquez, F. and Devreotes, P. N. (2009) A phosphorylation-dependent intramolecular interaction regulates the membrane association and activity of the tumor suppressor PTEN. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 480–485
- 43 Merlot, S. and Firtel, R. A. (2003) Leading the way: directional sensing through phosphatidylinositol 3-kinase and other signaling pathways. *J. Cell Sci.* **116**, 3471–3478
- 44 Tolkacheva, T., Boddapati, M., Sanfiz, A., Tsuchida, K., Kimmelman, A. C. and Chan, A. M. (2001) Regulation of PTEN binding to MAGI-2 by two putative phosphorylation sites at threonine 382 and 383. *Cancer Res.* **61**, 4985–4989
- 45 Vazquez, F., Grossman, S. R., Takahashi, Y., Rokas, M. V., Nakamura, N. and Sellers, W. R. (2001) Phosphorylation of the PTEN tail acts as an inhibitory switch by preventing its recruitment into a protein complex. *J. Biol. Chem.* **276**, 48627–48630
- 46 Okahara, F., Ikawa, H., Kanaho, Y. and Maehama, T. (2004) Regulation of PTEN phosphorylation and stability by a tumor suppressor candidate protein. *J. Biol. Chem.* **279**, 45300–45303
- 47 Valiente, M., Andres-Pons, A., Gomar, B., Torres, J., Gil, A., Tapparel, C., Antonarakis, S. E. and Pulido, R. (2005) Binding of PTEN to specific PDZ domains contributes to PTEN protein stability and phosphorylation by microtubule-associated serine/threonine kinases. *J. Biol. Chem.* **280**, 28936–28943
- 48 Jurado, S., Benoist, M., Lario, A., Knafo, S., Petrok, C. N. and Esteban, J. A. (2010) PTEN is recruited to the postsynaptic terminal for NMDA receptor-dependent long-term depression. *EMBO J.* **29**, 2827–2840
- 49 Vanhaesebroeck, B., Higashi, K., Raven, C., Welham, M., Anderson, S., Brennan, P., Ward, S. G. and Waterfield, M. D. (1999) Autophosphorylation of p110 δ phosphoinositide 3-kinase: a new paradigm for the regulation of lipid kinases *in vitro* and *in vivo*. *EMBO J.* **18**, 1292–1302
- 50 Kim, E. and Sheng, M. (2004) PDZ domain proteins of synapses. *Nat. Rev. Neurosci.* **5**, 771–781

Received 13 January 2012/22 February 2012; accepted 13 March 2012

Published as BJ Immediate Publication 13 March 2012, doi:10.1042/BJ20120098