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THE p85 β REGULATORY SUBUNIT OF PI3K SERVES AS A SUBSTRATE FOR PTEN PROTEIN PHOSPHATASE ACTIVITY DURING INSULIN MEDIATED SIGNALING

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Abstract

Phosphatase and tensin homolog *deleted on chromosome 10* (PTEN) is a tumor suppressor gene that is frequently mutated in brain, uterine, and prostate cancers. The protein phosphatase activity is poorly defined. We demonstrate that insulin stimulates phosphorylation of tyrosine and threonine/proline residues on the p85 regulatory subunit of PI3K in Huh-7, and HEK 293 cells. The specificity of PTEN binding and dephosphorylation of PI3K appears to reside on the p85 β subunit. Therefore, the PTEN phosphatase is active against the PI3K p85 β subunit and dephosphorylates a protein involved in insulin signaling where known downstream consequences are increased cell migration, motility, and invasion.

Keywords

p85 binding; insulin signals; PTEN protein phosphatase activity

Introduction

PTEN is an important tumor suppressor gene frequently deleted or mutated in a wide variety of human cancers [1;2;3;4;5;6]. This gene encodes a phosphatase that has both protein and lipid activity in vitro [7]. The main lipid substrate identified for PTEN is PIP₃ which serves as an important second messenger for many signaling pathways [5;8]. However, intracellular substrates and functional consequences of protein phosphatase activity are poorly understood in vivo [9]. The N-terminal domain contains the phosphatase catalytic site. Like other phosphatases, PTEN contains a substrate-binding pocket in its catalytic domain; however, this structure is deeper and broader than that found in other protein phosphatases. The expanded width of the pocket is considered necessary to encompass the larger size of the PIP₃ substrate whereas the width and depth are consistent with its ability to dephosphorylate protein substrate containing serine/threonine and tyrosine residues [10;11].

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Constitutive activation of the PI3K/Akt pathway has been reported to occur in breast, ovarian, pancreatic, esophageal, as well as other tumor types. The PI3K is a heterodimer comprised of a p85 regulatory and p110 catalytic subunit. The p85 regulatory subunit is a phosphoprotein substrate for a variety of cytoplasmic and receptor kinases. In some instances, the p85 protein-kinase interactions occur indirectly through intermediates as exemplified by insulin receptor substrate-1 (IRS-1) or insulin receptor substrate-2 (IRS-2) which are key docking proteins that transmit insulin signaling from the cell surface to the nucleus via the interaction of tyrosyl phosphorylated IRS-1 and IRS-2 with PI3K [12]. A pre-formed, inactive p85–p110 complex is present in the cytoplasm of resting cells poised for activation in response to appropriate growth factor stimulation [13]. The phosphorylated state of p85 may functionally promote, or inhibit, the action of PTEN [13;14;15;16;17;18;19;20]. In COS 7 cells, p85 is tyrosyl phosphorylated in response to EGF stimulation which releases its inhibitory activity on the 110 subunit and thus activates PI3K [14]. Furthermore in NIH3T3 cells, p85 is tyrosyl phosphorylated which results in PI3K activation following stimulation with platelet derived growth factor [19]. In addition, the p85 subunit of PI3K is phosphorylated on serine/ threonine residues via a kinase co-purified with PI3K from rat liver [21;22].

We have demonstrated that PTEN is closely associated with PI3K during kinase activation [23]; similar observations have been made by others [24] and appears to regulate growth factor mediated signal transduction associated with enhanced cell survival, migration, motility and proliferation. The PI3K catalyzes conversion of phosphatidylinositol 4,5-bisphosphate (PIP₂) to PIP₃, a major substrate for PTEN. PTEN converts PIP₃ back to PIP₂ via its phosphatase activity, blunts insulin, and other growth factor mediated signals. Conditional knock-out mice lacking the p85 regulatory subunit of PI3K have diminished PTEN activity in the liver due to reduced protein expression [25]. Furthermore, PTEN activity loss has been associated with a PI3K mutation [26]. Recently we demonstrated that PTEN inhibits PI3K/Akt signaling by direct interaction between PTEN and the p85 α regulatory subunit of PI3K contributing to alcohol-induced insulin resistance [23].

Two important point mutations at amino acid 124 and 129 have been found within the PTEN phosphatase catalytic site in a variety of primary tumors [8;27;28;29;30;31]. The G129E at position 129 reportedly abolishes the ability of PTEN to recognize phospholipids as substrates; however, it retains protein phosphatase activity at least against peptides in vitro [32;33]. In the present study, we demonstrated that the C124S but not G129E mutation abolishes PTEN binding resulting in loss of PTEN protein phosphatase activity against p85 of PI3K.

Materials and Methods

Cell Culture

Huh-7 hepatocellular carcinoma cells (HCC) and human embryonic kidney (HEK) 293 T-cells were cultured with Dulbecco's modified Eagle's medium (DMEM) in 10% fetal serum with the addition of L-glutamine. Cells were serum starved overnight before the addition of human recombinant insulin for stimulation at a concentration of 0.075U/ml at indicated times for each experiment.

Plasmids and Transfection Protocol

The plasmids pSG5L-HA, pSG5L-HA-PTEN; pSG5L-HA-PTEN-C124S; pSG5L-HAG129E; pSG5L-HA-G129R, pSG5L-HA-PTEN1-274; pSG5L-HA-PTEN1-336 were kindly provided by Dr. William R. Sellers [34]. Cells were transfected in 60 mm dishes using 12 μ g of DNA diluted in 1 ml of OPTI-MEM (Invitrogen Laboratory, Carlsbad, CA)

combined with 20 μ l of Lipofectamine LTX (Invitrogen). The mixture was incubated for 25 min at 20°C and then added onto the cells. After 24 hours of incubation, the medium was replaced with serum free medium for 16 hours prior to assay.

Protein Studies

Protein expression was examined by Western blot analysis or immunoprecipitation followed by immunoblotting as described [23]. Immunoreactivity was detected with the horseradish peroxidase-conjugated secondary antibody (1:100 dilution) and enhanced chemiluminescence reagents, and then quantified with a Kodak Digital Science imaging station (NEN Life Sciences, Boston, MA). The levels of immunoreactivity are reported in arbitrary densitometry units.

The immunoprecipitation studies were performed as indicated previously [35]. The immunoprecipitants were resuspended in an SDS-PAGE sample buffer with a loading dye for Western blot analysis [35].

Source of the Reagents

Recombinant human insulin was purchased from Novo Nordisk (Copenhagen, Denmark). Antibodies to Akt and p-Akt (Ser⁴⁷³) (AF887) were obtained from R & D System, Inc (Minneapolis, MN). Antibodies to PI3K 85 α (sc-71892), 85 β (sc-56934), PTEN (N-19), PTEN (sc-9145) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody to GAPDH (MAB374) was obtained from Chemicon (Millipore, Billerica, MA). Antibody to phosphotyrosine (06-427) was purchased from Upstate (Lake Placid, NY); antibody to PI3K 85 906-497 phosphotyrosine (AB1607), and phosphothreonine-proline (p-thr-pro) (AB3192), and to HA tag (05-904) were obtained from Millipore (Billerica, MA). Lipofectamine TMLTX reagent (15338-100 used for plasmid transfection) were purchased from Invitrogen (Carlsbad, CA). Western lighting plus-ECL oxidizing reagent plus was obtained from Perkin Elmer (Freehold, NJ). RIPA buffer was provided by Thermo-Fisher Scientific (Waltham, MA). Protein A/G beads were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Antibody to PTEN for immunoprecipitation studies was provided by Dr. Nicholas Leslie.

Results

PTEN deactivates insulin stimulated phosphorylation of p85, and downregulates transmission of the insulin signal to Akt

Insulin binding to cells activate the insulin receptor, IRS-1, PI3K and Akt to promote survival signals by inhibiting programmed cell death cascades. Previous studies report that tyrosyl phosphorylation of p85 activates PI3K [14]. We determined if PTEN inhibited p85 phosphorylation. Under insulin stimulation, the p85 subunit of PI3K was phosphorylated on tyrosine and threonine-proline residues in the human Huh-7 HCC cell lines (Fig. 1A; left panel) as well as HEK 293 T-cells (Fig 1C; right-hand panel). In this context the insulin signal was transmitted downstream as shown by p-Akt formation. Thus, insulin not only stimulates tyrosyl but also threonine/proline phosphorylation on p85 in both cell lines as well. Next, we evaluated the effect of PTEN on insulin mediated tyrosyl and threonine/proline phosphorylation of p85. Following transfection of WT PTEN, there was striking inhibition of tyrosyl and threonine-proline phosphorylation in both Huh-7 and HEK 293 cells as shown in Figure 1B and D. Furthermore, PTEN inhibited downstream Akt phosphorylation in both cell lines. These results suggest that insulin stimulated phosphorylation of p85 is an important substrate for PTEN protein phosphatase activity in vivo.

The PTEN C124S and G129E phosphatase catalytic site mutants fail to inhibit Akt activation

In these experiments, HEK 293 T-cells were transfected with various expression plasmids and Akt phosphorylation was evaluated one day after overnight serum starvation. Cells were stimulated with insulin or vehicle (DMEM) for 10 minutes. Cell lysates were analyzed by immunoblotting using antibodies to phospho-Akt, PTEN, and actin. As expected WT PTEN inhibited Akt phosphorylation in the presence or absence of insulin stimulation, whereas the C124S and G129E mutant constructs did not. It has been previously established that the G129E PTEN mutant lacks lipid phosphatase activity whereas the C124S construct is considered to be both lipid and protein phosphatase inactive [33]. Thus, HEK 293 T-cells transfection of the two mutant PTEN constructs resulted in increased Akt phosphorylation in both insulin-treated and non-treated cells (Fig. 2).

The C124S PTEN mutant lacks protein phosphatase activity for the p85 substrate

Since previous experiments have established that both the C124S and G129E PTEN mutants restore the PI3K/Akt signal following insulin stimulation, we then evaluated their effect on insulin stimulated p85 phosphorylation. The results presented in Figure 2C demonstrate that WT PTEN and the G129E PTEN mutant were active as shown by dephosphorylation of tyrosine and threonine-proline sites on the p85 subunit of PI3K. However, The C124S mutant construct was inactive with respect to protein phosphatase activity. These results are consistent with previous studies demonstrating that the G129E PTEN mutant retains protein phosphatase activity. In contrast, the C124S mutation appears to abolish protein phosphatase activity towards insulin stimulated tyrosyl and threonine/proline phosphorylation on p85 of PI3K.

PTEN phosphatase activity is active against the β but not α -subunit of p85

The PI3K has two α and β p85 regulatory isoforms. We explored the effect of WT PTEN and the C124S and G129E PTEN mutants on insulin stimulated tyrosyl and threonine/proline phosphorylation of these two subunits. It was found that all three PTEN constructs were inactive with respect to their protein phosphatase activity against the p85 α subunit. In contrast, both WT and the G129E PTEN mutant dephosphorylated tyrosine and threonine/proline residues on the p85 β subunit of PI3K, whereas the C124S PTEN mutant was phosphatase inactive. These results suggest that the major activity of WT PTEN with respect to regulation of the insulin signal is through dephosphorylation of these residues on the p85 β isoform (Fig. 3).

The PTEN C124S mutant does not bind to the p85 subunit of PI3K

In these experiments, we tested the capability of WT, C124S, G129E, I-336 and I-273 PTEN deletion mutants to bind to the p85 subunit of PI3K. In these experiments, HEK 293 T-cells were transfected with the various PTEN constructs plus a p85 expression plasmid followed by immunoprecipitation with PTEN and blotting with antisera to p85 or PTEN. As shown in Fig. 4A both the WT and G129E mutant protein associated with the p85 subunit of PI3K whereas the C124S species did not. Similarly, the PTEN I-273 and I-336 deletion mutants also bound to the p85 subunit of PI3K as shown in Fig. 4B. The p-Akt formation was inhibited by WT PTEN whereas the PTEN I-273 and I-336 deletion mutants, and the C124S and G129E mutants have no effect on insulin-stimulated phosphorylation of Akt (Fig. 4C). Thus, the binding site for p85 on PTEN appears to be at the N-terminal region of the molecule, and the C124S mutation appears critical for this protein-protein interaction to occur.

Discussion

This study evaluates the protein phosphatase activity of PTEN with respect to its interaction with the p85 regulatory subunit of PI3K. Previous investigations have established that the p85 regulatory subunit of PI3K serves to both stabilize and inactivate the p110 catalytic subunit [14]. Phosphorylation of Tyr⁶⁸⁸ on p85 leads to upregulation of PI3K activity as measured by phosphorylation of the downstream target protein Akt. Indeed, dephosphorylation of Tyr⁶⁸⁸ by Shp1 was associated with reduced p-Akt formation. Likewise as shown in Fig. 1, insulin stimulation of Huh-7 and HEK 293 cells reveals not only enhanced tyrosyl phosphorylation of p85 but phosphorylation of threonine-proline residues as well. The functional consequences were enhanced p-Akt formation. Cells transfected with WT PTEN demonstrated the reverse phenomenon in that both p-Tyr and p-Thr-Pro formation were lowered due to its protein phosphatase activity. These results are consistent with previous observations indicating that tyrosyl phosphorylation of the p85 regulatory subunit of PI3K is an important regulatory mechanism for PI3K activation.

The intracellular targets for PTEN protein phosphatase activity have not been extensively explored. One physiologically relevant target has been defined by Tamura et al [36]. PTEN was found to interact with focal adhesion kinase (FAK) reducing tyrosyl phosphorylation of the protein. Since FAK antagonizes the effect of PTEN, this phosphatase may negatively regulate cell interactions with the extracellular matrix. There are functional studies that demonstrate reduced cell motility and migration following transfection and expression of PTEN G129E mutant indicating a potential role for the protein phosphatase in these cellular activities [9]. Our investigations suggest that the p85 regulatory subunit of PI3K is a target for PTEN protein phosphatase activity. To experimentally address this issue, we employed the PTEN G129E mutant which has no lipid phosphatase activity against PIP₃. This naturally occurring mutation has been found in Cowden disease as well as in endometrial cancer [33]. However, the PTEN G129 mutant retains its protein phosphatase activity. In contrast, another naturally occurring mutation found in tumors is the C124S mutation which has been shown to lose both lipid and protein phosphatase function [32;33]. Both of these mutations emphasize the role of PTEN as a tumor suppressor gene since there is unregulated growth factor mediated signaling through PI3K and Akt. Loss of PTEN function affects many cellular functions including proliferation, survival, motility and migration for example. With respect to insulin stimulation, Fig. 1 demonstrates that both the C124S and G129E PTEN mutants allow unopposed signaling through PI3K to promote pAkt formation, whereas WT PTEN exerts its regulatory influence on transmission of the insulin signal.

To better understand the role of C124S and G129E mutation(s) on PTEN function with respect to its protein phosphatase activity, we performed experiments as illustrated in Fig. 2. HEK 293 cells were transfected with WT, C124S and G129E PTEN constructs followed by insulin stimulation, and the phosphatase activity against p-Tyr and p-Thr-Pro residues of the p85 subunit of PI3K were evaluated. We found that the G129E mutant behaved like WT PTEN since it still retains protein phosphatase activity even though the lipid phosphatase function had been lost. In contrast the C124S PTEN mutant, which is both lipid and phosphatase inactive, had no effect on dephosphorylation of such residues on p85. This observation indicates that PTEN may function during insulin mediated signal transduction through PI3K is to dephosphorylate p-Tyr and p-Thr-Pro residues on p85.

The hypothesis was tested that the C124S and G129E mutations in PTEN might affect their binding to the p85 subunit of PI3K. We also employed C-terminal PTEN deletion mutants to estimate if the contact site of PTEN to p85 was likely to reside in the N- or C-terminal region of the molecule. It has been demonstrated that the phosphatase and the C2 domains formed a minimal catalytic unit [10;37;38] in which the C2 domain may play a dual role of

promoting membrane recruitment and assisting in the orientation of the phosphatase domain at the cell surface membrane [37]. Deletion mutants retaining the N-terminal catalytic domain but with different deletions in the C2 domain were employed. The results shown in Fig. 4B reveal that the C2 domain deletion mutant still retained binding activity to p85 but lost inhibitory activity for p-Akt formation compared to WT PTEN. Likewise, the C124S and G129E mutants had no effect on insulin-stimulated phosphorylation of Akt.

It is important to note that PTEN G129E retains its protein phosphatase activity as shown in Fig. 3. In this regard, we demonstrated that PTEN protein phosphatase activity was active for the p85 β rather than α -regulatory subunit of PI3K. However, the PTEN C124S mutant cannot bind to the p85 subunit of PI3K, whereas the PTEN G129E mutant, although lacking lipid phosphatase activity, still maintains its capability of dephosphorylating p-Tyr and p-Tyr-Pro residues on p85 following insulin stimulation. Therefore, the C124S mutation does not permit a protein-protein interaction between PTEN and the p85 subunit of PI3K. These investigations emphasize the potential importance of PTEN protein phosphatase activity and further studies are required to explore the cellular consequences on insulin mediated signaling events.

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References

1. Maxwell GL, Risinger JI, Gumbs C, Shaw H, Bentley RC, Barrett JC, Berchuck A, Futreal PA. Mutation of the PTEN tumor suppressor gene in endometrial hyperplasias. *Cancer Res.* 1998; 58:2500–2503. [PubMed: 9635567]
2. Mutter GL, Lin MC, Fitzgerald JT, Kum JB, Baak JP, Lees JA, Weng LP, Eng C. Altered PTEN expression as a diagnostic marker for the earliest endometrial precancers. *J Natl Cancer Inst.* 2000; 92:924–930. [PubMed: 10841828]
3. Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH, Langford LA, Baumgard ML, Hattier T, Davis T, Frye C, Hu R, Swedlund B, Teng DH, Tavtigian SV. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat Genet.* 1997; 15:356–362. [PubMed: 9090379]
4. Teng DH, Hu R, Lin H, Davis T, Iliev D, Frye C, Swedlund B, Hansen KL, Vinson VL, Gumpfer KL, Ellis L, El-Naggar A, Frazier M, Jasser S, Langford LA, Lee J, Mills GB, Pershouse MA, Pollack RE, Tornos C, Troncso P, Yung WK, Fujii G, Berson A, Steck PA, et al. MMAC1/PTEN mutations in primary tumor specimens and tumor cell lines. *Cancer Res.* 1997; 57:5221–5225. [PubMed: 9393738]
5. Li DM, Sun H. TEPI, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor beta. *Cancer Res.* 1997; 57:2124–2129. [PubMed: 9187108]
6. Stokoe D. Pten. *Curr Biol.* 2001; 11:R502.
7. 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]
8. Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliareis C, Rodgers L, McCombie R, Bigner SH, Giovanella BC, Ittmann M, Tycko B, Hibshoosh H, Wigler MH, Parsons R. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science.* 1997; 275:1943–1947. [PubMed: 9072974]
9. Leslie NR, Maccario H, Spinelli L, Davidson L. The significance of PTEN's protein phosphatase activity. *Adv Enzyme Regul.* 2008
10. 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]

11. Myers MP, Stolarov JP, Eng C, Li J, Wang SI, Wigler MH, Parsons R, Tonks NK. P-TEN, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase. *Proc Natl Acad Sci U S A*. 1997; 94:9052–9057. [PubMed: 9256433]
12. White MF, Yenush L. The IRS-signaling system: a network of docking proteins that mediate insulin and cytokine action. *Curr Top Microbiol Immunol*. 1998; 228:179–208. [PubMed: 9401207]
13. Yu J, Zhang Y, McIlroy J, Rordorf-Nikolic T, Orr GA, Backer JM. Regulation of the p85/p110 phosphatidylinositol 3'-kinase: stabilization and inhibition of the p110alpha catalytic subunit by the p85 regulatory subunit. *Mol Cell Biol*. 1998; 18:1379–1387. [PubMed: 9488453]
14. Cuevas BD, Lu Y, Mao M, Zhang J, LaPushin R, Siminovitch K, Mills GB. Tyrosine phosphorylation of p85 relieves its inhibitory activity on phosphatidylinositol 3-kinase. *J Biol Chem*. 2001; 276:27455–27461. [PubMed: 11337495]
15. Hiles ID, Otsu M, Volinia S, Fry MJ, Gout I, Dhand R, Panayotou G, Ruiz-Larrea F, Thompson A, Totty NF, et al. Phosphatidylinositol 3-kinase: structure and expression of the 110 kd catalytic subunit. *Cell*. 1992; 70:419–429. [PubMed: 1322797]
16. Klippel A, Escobedo JA, Hirano M, Williams LT. The interaction of small domains between the subunits of phosphatidylinositol 3-kinase determines enzyme activity. *Mol Cell Biol*. 1994; 14:2675–2685. [PubMed: 8139567]
17. Hu Q, Klippel A, Muslin AJ, Fantl WJ, Williams LT. Ras-dependent induction of cellular responses by constitutively active phosphatidylinositol-3 kinase. *Science*. 1995; 268:100–102. [PubMed: 7701328]
18. Ueki K, Fruman DA, Yballe CM, Fasshauer M, Klein J, Asano T, Cantley LC, Kahn CR. Positive and negative roles of p85 alpha and p85 beta regulatory subunits of phosphoinositide 3-kinase in insulin signaling. *J Biol Chem*. 2003; 278:48453–48466. [PubMed: 14504291]
19. Jimenez C, Hernandez C, Pimentel B, Carrera AC. The p85 regulatory subunit controls sequential activation of phosphoinositide 3-kinase by Tyr kinases and Ras. *J Biol Chem*. 2002; 277:41556–41562. [PubMed: 12196526]
20. Luo J, Field SJ, Lee JY, Engelman JA, Cantley LC. The p85 regulatory subunit of phosphoinositide 3-kinase down-regulates IRS-1 signaling via the formation of a sequestration complex. *J Cell Biol*. 2005; 170:455–464. [PubMed: 16043515]
21. Carpenter CL, Auger KR, Chanudhuri M, Yoakim M, Schaffhausen B, Shoelson S, Cantley LC. Phosphoinositide 3-kinase is activated by phosphopeptides that bind to the SH2 domains of the 85-kDa subunit. *J Biol Chem*. 1993; 268:9478–9483. [PubMed: 7683653]
22. Dhand R, Hiles I, Panayotou G, Roche S, Fry MJ, Gout I, Totty NF, Truong O, Vicendo P, Yonezawa K, et al. PI 3-kinase is a dual specificity enzyme: autoregulation by an intrinsic protein-serine kinase activity. *EMBO J*. 1994; 13:522–533. [PubMed: 8313897]
23. He J, de la Monte S, Wands JR. Acute ethanol exposure inhibits insulin signaling in the liver. *Hepatology*. 2007; 46:1791–1800. [PubMed: 18027876]
24. Rabinovsky R, Pochanard P, McNear C, Brachmann SM, Duke-Cohan JS, Garraway LA, Sellers WR. Associates with unphosphorylated PTEN and the PTEN-associated complex. *Mol Cell Biol*. 2009; 29:5377–5388. [PubMed: 19635806]
25. Barber DF, Alvarado-Kristensson M, Gonzalez-Garcia A, Pulido R, Carrera AC. PTEN regulation, a novel function for the p85 subunit of phosphoinositide 3-kinase. *Sci STKE*. 2006; 2006:pe49. [PubMed: 17119157]
26. Perez-Tenorio G, Alkhori L, Olsson B, Waltersson MA, Nordenskjold B, Rutqvist LE, Skoog L, Stal O. PIK3CA mutations and PTEN loss correlate with similar prognostic factors and are not mutually exclusive in breast cancer. *Clin Cancer Res*. 2007; 13:3577–3584. [PubMed: 17575221]
27. Marsh DJ, Dahia PL, Zheng Z, Liaw D, Parsons R, Gorlin RJ, Eng C. Germline mutations in PTEN are present in Bannayan-Zonana syndrome. *Nat Genet*. 1997; 16:333–334. [PubMed: 9241266]

28. Liaw D, Marsh DJ, Li J, Dahia PL, Wang SI, Zheng Z, Bose S, Call KM, Tsou HC, Peacocke M, Eng C, Parsons R. Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. *Nat Genet.* 1997; 16:64–67. [PubMed: 9140396]
29. Tunca B, Bekar A, Cecener G, Egeli U, Vatan O, Tolunay S, Kocaeli H, Aksoy K. Impact of novel PTEN mutations in Turkish patients with glioblastoma multiforme. *J Neurooncol.* 2007; 82:263–269. [PubMed: 17151929]
30. Nelen MR, van Staveren WC, Peeters EA, Hassel MB, Gorlin RJ, Hamm H, Lindboe CF, Fryns JP, Sijmons RH, Woods DG, Mariman EC, Padberg GW, Kremer H. Germline mutations in the PTEN/MMAC1 gene in patients with Cowden disease. *Hum Mol Genet.* 1997; 6:1383–1387. [PubMed: 9259288]
31. Eng C. PTEN: one gene, many syndromes. *Hum Mutat.* 2003; 22:183–198. [PubMed: 12938083]
32. Furnari FB, Huang HJ, Cavenee WK. The phosphoinositol phosphatase activity of PTEN mediates a serum-sensitive G1 growth arrest in glioma cells. *Cancer Res.* 1998; 58:5002–5008. [PubMed: 9823298]
33. Myers MP, Pass I, Batty IH, Van der Kaay J, Stolarov JP, Hemmings BA, Wigler MH, Downes CP, Tonks NK. The lipid phosphatase activity of PTEN is critical for its tumor suppressor function. *Proc Natl Acad Sci U S A.* 1998; 95:13513–13518. [PubMed: 9811831]
34. Ramaswamy S, Nakamura N, Vazquez F, Batt DB, Perera S, Roberts TM, Sellers WR. 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.* 1999; 96:2110–2115. [PubMed: 10051603]
35. Yeon JE, Califano S, Xu J, Wands JR, De La Monte SM. Potential role of PTEN phosphatase in ethanol-impaired survival signaling in the liver. *Hepatology.* 2003; 38:703–714. [PubMed: 12939597]
36. Tsuruma M, Gu J, Matsumoto K, Aota S, Parsons R, Yamada KM. Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. *Science.* 1998; 280:1614–1617. [PubMed: 9616126]
37. Georgescu MM, Kirsch KH, Kaloudis P, Yang H, Pavletich NP, Hanafusa H. Stabilization and productive positioning roles of the C2 domain of PTEN tumor suppressor. *Cancer Res.* 2000; 60:7033–7038. [PubMed: 11156408]
38. Vazquez F, Ramaswamy S, Nakamura N, Sellers WR. Phosphorylation of the PTEN tail regulates protein stability and function. *Mol Cell Biol.* 2000; 20:5010–5018. [PubMed: 10866658]

Fig. 1.

Insulin stimulation induces phosphorylation of the p85 subunit of PI3K on tyrosine and threonine-proline residues and activates Akt (pAkt). (A) Huh7 cells or (C) HEK 293 T-cells were stimulated with insulin or vehicle (control) for 10 minutes after an overnight serum starvation. Note the phosphorylation of p85 and the activation of Akt in both cell lines. (B) Huh7 cells or (D) HEK 293 T-cells were transfected with a WT PTEN expression plasmid, or an empty vector control and then stimulated with insulin or vehicle. Note that WT PTEN dephosphorylates Tyr and Thr-Pro residues on the p85 subunit of PI3K following insulin stimulation.

Fig. 2.

The C124S and G129E mutations in the catalytic site for phosphatase activity preserve Akt activation following insulin stimulation. Cell lysates were analyzed by immunoblotting using antibodies to phospho-Akt, PTEN, and actin. Note that WT PTEN inhibits phosphorylation of Akt both with and without insulin stimulation, whereas the C124S and G129E mutant constructs do not. (A). The right hand panel represents densitometric scanning of the Western blots presented in Panel A. The experiment was repeated three times with similar results. (C). The PTEN C124S mutant has no protein phosphatase activity for the p85 subunit of PI3K following insulin stimulation. HEK 293 T-cells were transfected with expression plasmids and stimulated with insulin. Note that insulin stimulated p-tyrosine and p-threonine-proline on p85. However, the C124S mutant exhibited no protein phosphatase activity whereas the G129 mutant behaved like WT PTEN and reduced insulin stimulated p-tyrosine and p-threonine proline phosphorylation of p85.

Fig. 3. PTEN dephosphorylates p-tyrosine and p-threonine-proline on the p85 β subunit of PI3K. (A). Densitometric scanning of the Western blots are shown in Panel B. (B). Note that PTEN phosphatase is active for the p85 β but not the p85 α subunit of PI3K.

Fig. 4.

The PTEN C124S mutant does not bind to p85 subunit of PI3K. HEK 293 T-cells were transfected with expression plasmids, control, or PTEN 1–274 and 1–336 N-terminal deletion mutants, along with p85 expression plasmid and stimulated with insulin (0.075U/ml). Densitometric scanning and Western blot are presented in Panel A (left and right, respectively). **B**. Cell lysates were analyzed by immunoblotting for PTEN deletion mutant binding as well as determining the expression levels of different PTEN mutant proteins. Note that the PTEN C124S mutant does not bind to the p85 subunit of PI3K whereas WT PTEN, G129E and 1–336, and 1–273 deletion mutants physically interact with the p85

subunit of PI3K. (C). The PTEN I-273, (I-336, C124S and G129E mutants do not inhibit insulin stimulated pAkt formation compared to WT PTEN.