Ubiquitination of PTEN (Phosphatase and Tensin Homolog) Inhibits Phosphatase Activity and Is Enhanced by Membrane Targeting and Hyperosmotic Stress*§

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The PTEN (phosphatase and tensin homolog) tumor suppressor is a phosphatase that inhibits phosphoinositide 3-kinase-dependent signaling by metabolizing the phosphoinositide lipid phosphatidylinositol 3,4,5-trisphosphate (PtdInsP3) at the plasma membrane. PTEN can be mono- or polyubiquitinated, and this appears to control its nuclear localization and stability, respectively. Although PTEN phosphorylation at a cluster of C-terminal serine and threonine residues has been shown to stabilize the protein and inhibit polyubiquitination and plasma membrane localization, details of the regulation of ubiquitination are unclear. Here, we show that plasma membrane targeting of PTEN greatly enhances PTEN ubiquitination and that phosphorylation of PTEN in vitro does not affect subsequent ubiquitination. These data suggest that C-terminal phosphorylation indirectly regulates ubiquitination by controlling membrane localization. We also show that either mono- or polyubiquitination in vitro greatly reduces PTEN phosphatase activity. Finally, we show that hyperosmotic stress increases both PTEN ubiquitination and cellular PtdInsP3 levels well before a reduction in PTEN protein levels is observed. Both PTEN ubiquitination and elevated PtdInsP3 levels were reduced within 10 min after removal of the hyperosmotic stress. Our data indicate that ubiquitination may represent a regulated mechanism of direct reversible control over the PTEN enzyme.

PTEN§ is a lipid phosphatase that, through dephosphorylation of the second messenger phosphatidylinositol 3,4,5-trisphosphate (PtdInsP3), inhibits phosphoinositide 3-kinase-dependent signaling (1, 2). This appears to be the dominant mechanism by which PTEN can control cell proliferation, survival, and growth and the reason why PTEN is one of the most frequently lost tumor suppressors in human cancer (3). In contrast to the classical model of two-hit loss of tumor suppressor function, in vivo models of tumor formation, best analyzed in the prostate, suggest that PTEN acts in a haploinsufficient and dose-dependent manner (4–6). Several binding partners and regulators of the PTEN protein also appear to act as oncogenes and tumor suppressors principally through their ability to control PTEN (7–12). These findings highlight the importance of mechanisms by which PTEN expression and activity are regulated.

It has been known for some years that PTEN can become polyubiquitinated and that this appears to target PTEN for degradation by the proteasome (13–15). Also, C-terminal phosphorylation of PTEN causes the enzyme to be less ubiquitinated and more stable (13, 14, 16). More recently, NEDD4-1 has been identified as an efficient E3 ubiquitin ligase for PTEN in vitro that is capable of controlling PTEN ubiquitination and expression in cells (11). Stemming from this work have been further studies implying much greater complexity in the regulation of PTEN function by ubiquitination. These include the indication that the nuclear/cytoplasmic shuttling of PTEN can be controlled by monoubiquitination (17) and regulated deubiquitination (18) and that NEDD4-1 may not be a universal regulator of PTEN ubiquitination (19), implicating further unidentified E3 ligases such as XIAP (X-linked inhibitor of apoptosis protein) (20). Here, we investigate the link between PTEN phosphorylation and ubiquitination and analyze the effects of ubiquitination on PTEN enzyme activity.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—U87MG glioblastoma cells and HEK293T cells were obtained from the European Collection of Animal Cell Cultures and maintained in the recommended medium (10% fetal bovine serum). Standard cell culture medium, additives, and sera were from Invitrogen. Transfection of U87MG cells was performed using TransIT-LT1 reagent (Mirus) when U87MG cells were ~50% confluent. Plasmids encoding PTEN, the PTEN A3 mutant (S380A/T382A/T383A), or the myristoylated PTEN construct and/or FLAG-tagged ubiquitin were used in transfection as indicated. After transfection, cells were treated with or without inhibitor(s) for the indicated times. Cells were then washed with phosphate-buffered saline and lysed. The following agents were purchased from Calbiochem: proteasome inhibitor I (PSI), calpeptin, and the CK2 inhibitors DMAT and 4,5,6,7-tetramethoxy-
benzotriazole. The specificity of these inhibitors has been studied previously and described (21–23). 293T cells were transfected using a standard calcium phosphate protocol.

**DNA Expression Vectors**—Mammalian expression vectors for untagged PTEN were originally generated by cloning the PTEN cDNA from pCR2.1-TOPO-PTEN (24) into pcDNA3.1/H11001 (Invitrogen) as a BamHI-XbaI fragment. The generation of myristoylated hemagglutinin-PTEN vectors has been described previously (25), and the production of a second myristoylated vector was performed by non-directional EcoRI restriction enzyme cloning from pEGFP-PTEN (24) into this site of the myristoylated vector pCMF. The generation of mutants PTEN C124S and PTEN A3 has been described previously (24, 26). A mammalian expression vector for FLAG-ubiquitin was kindly provided by Philip Cohen (University of Dundee). A bacterial expression vector for the E3 ligase NEDD4-1 was produced as follows. The 5′-end of NEDD4-1 was amplified from I.M.A.G.E. expressed sequence tag 5575951 (Geneservice) using Phusion DNA polymerase (Finnzymes) and primers NEDD4 F1 (5′-AAAAGAATTCTGGCAACTTGCGCGTGGAGGTGTT-CGGG) and NEDD4 R1 (5′-TGTTCAGCTACTGATGAGATGTGTGTGGTGACTG-GAGGGAC) and NEDD4 R2 (5′-TTTCTCGAGCTAATTCAACTCATCAAAGCCCTGGGTGTTC-TCAATTGCCATCTGA). The full-length PCR fragment was assembled and cloned into pCR2.1 (Invitrogen) and sequenced to completion. Full-length NEDD4-1 was subsequently excised using EcoRI and XhoI and inserted into pGEX6P-1.

**Preparation of Whole Cell Extracts, SDS-PAGE, and Western Blotting**—Cells were washed twice with ice-cold phosphate-buffered saline and lysed in ice-cold lysis buffer (25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1 mM EGTA, 1 mM EDTA, 5 mM sodium pyrophosphate, 10 mM β-glycerophosphate, and 50 mM sodium fluoride) containing 0.1% 2-mercaptoethanol and protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Equal amounts of proteins were separated by SDS-PAGE using precast 4–12% gradient gels (Invitrogen) and blotted onto polyvinylidene difluoride membranes (Polyscreen, PerkinElmer Life Sciences). Most reagents for electrophoresis and blotting were purchased from Invitrogen, and standard manufacturers’ protocols were followed. Mouse anti-PTEN monoclonal antibody (clone A2B1) was purchased from Santa Cruz Biotechnology. Polyclonal antibodies to phospho-Ser380/Thr382/Thr383 PTEN and phospho-Ser380 PTEN were purchased from Cell Signaling Technologies. Anti-FLAG antibody M2 and β-actin antibody were purchased from Sigma.

**Ubiquitination of PTEN in U87MG Cell Culture**—Whole cell extracts were prepared using lysis buffer containing fresh 0.2 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 mM N-ethylmaleimide (Sigma). PTEN was immunoprecipitated using monoclonal antibody A2B1 precoated with agarose beads. The proteins bound to beads were released by boiling in SDS-PAGE sample buffer (Invitrogen) for 10 min. The samples were then resolved by 4–12% SDS-PAGE, followed by immunoblot analysis using anti-FLAG antibody M2 or anti-PTEN antibody.
PTEN Ubiquitination Regulates Phosphatase Activity

FIGURE 2. Mutation of the PTEN C-terminal phosphorylation sites reduces PTEN stability and enhances ubiquitination. A, the cellular stability of the wild-type PTEN (PTEN WT) and PTEN A3 (S380A/T382A/T383A) proteins was analyzed by metabolic 35S-amino acid labeling of PTEN-null U87MG cells transduced with viruses encoding either wild-type PTEN or PTEN A3 and chased with unlabeled amino acid-containing growth medium for the indicated times. PTEN proteins were visualized by autoradiography after immunoprecipitation. B, U87MG cells transiently expressing PTEN A3 were treated as indicated for 24 h with either 10 μM PSI or 10 μM calpeptin before Western blotting for PTEN expression along with a β-actin control. C, the ubiquitination of wild-type PTEN and PTEN A3 was compared in U87MG cells. U87MG cells were transfected with vectors encoding FLAG-tagged ubiquitin and either wild-type PTEN or PTEN A3 24 h before cells were treated with PSI at 10 μM for 2 h. Cells were lysed, and PTEN was immunoprecipitated (IP) before Western blotting (W blot) these immunoprecipitates and the lysates with antibodies raised against the FLAG epitope, PTEN, and β-actin.

Analysis of Cellular PTEN Stability—U87MG cells were infected with baculoviruses encoding PTEN and then, 24 h after transduction, washed and incubated for 1 h in methionine/cysteine-free medium containing 10% (v/v) dialyzed fetal bovine serum. Cells were labeled with [35S]methionine/cysteine (70 μCi/ml; Amersham Biosciences) for 2 h. After three washes with complete culture medium without label, cells were incubated in more culture medium and chased for different times before lysis. PTEN proteins were isolated by immunoprecipitation and resolved by SDS-PAGE. The labeled PTEN present at each time point was quantified using a Fuji FLA-2000 phosphorimager and AIDA software.
RESULTS

Phosphorylation of PTEN Increases Stability and Inhibits Ubiquitination in Cells—To better understand the role of ubiquitination in regulating PTEN function, we established assays for PTEN ubiquitination in cells and in vitro. These cell-based experiments were performed using the PTEN-null glioblastoma cell line U87MG, coexpressing recombinant PTEN with FLAG epitope-tagged ubiquitin. In these cells, only when both PTEN and FLAG-ubiquitin were coexpressed was a smear of high molecular weight polyubiquitinated PTEN observed when PTEN immunoprecipitates were blotted with anti-FLAG antibody (Figs. 1A and 2B). Similar results were obtained when anti-FLAG immunoprecipitation was followed by anti-PTEN Western blotting (data not shown). This apparent polyubiquitinated protein accumulated when cells were treated with a proteasome inhibitor for 2 or 24 h, correlating with the increased abundance of PTEN protein caused by similar treatment for 24 h (Fig. 1, A and B, and supplemental Fig. S1) and in accordance with previous data (13–15). PTEN levels were not affected by cellular treatment with calpeptin, an inhibitor of calpain proteases. Initially, we chose to use this system to investigate the details of the connection between C-terminal phosphorylation and ubiquitination. PTEN can be stabilized by phosphorylation of a cluster of serine and threonine residues by the kinase CK2, correlating with reduced polyubiquitination (13, 14, 29, 30). In agreement with this model, treatment of U87MG cells expressing wild-type PTEN with well validated selective inhibitors of CK2, 4,5,6,7-tetrabromobenzotriazole and DMAT (22), led to a significant reduction in PTEN expression level, which could be reversed by a proteasome inhibitor (Fig. 1B and supplemental Fig. S1). Similarly, blocking C-terminal phosphorylation by mutation of three residues in this cluster of serine and threonine residues to alanine (PTEN A3) led to a marked destabilization of PTEN in amino acid pulse-chase labeling experiments (Fig. 2A). This mutant protein was also stabilized by treatment of cells with a proteasome inhibitor (Fig. 2B), and when the ubiquitination of PTEN A3 was analyzed, enhanced ubiquitination of this mutant relative to the wild-type protein was observed (Fig. 2C).

Membrane Localization Enhances PTEN Ubiquitination—It has been shown that mutation of the C-terminal phosphorylation sites of PTEN, including work with the PTEN A3 mutant, leads to enhanced plasma membrane localization (31, 32). This, along with other lines of evidence, indicates that the C-terminal phosphorylation of PTEN leads to a conformational switch, whereby the phosphorylated tail binds to the C2 and phosphatase domains and inhibits membrane binding (29, 31–35). This leaves a question as to whether phosphorylation inhibits ubiquitination directly through this conformational change or perhaps indirectly through, for example, inhibiting the co-localization of PTEN with a plasma membrane-localized ubiquitin ligase. To test this latter hypothesis, we analyzed the ubiquitination of PTEN proteins artificially targeted to the plasma membrane by the addition of the N-terminal myristoylation/palmitoylation signal from Lck (25, 36). Both wild-type and phosphatase-dead PTEN proteins were ubiquitinated to a far greater level when myristoylated compared with their corresponding non-myristoylated proteins (Fig. 3A) despite being C-terminally phosphorylated to a similar degree (Fig. 3B). The N-terminal myristoylation signal contains no lysine residues, and similar results were obtained using two different myristoylated PTEN expression constructs with different linker regions, one of which has no additional lysine residues other than those in PTEN.

PTEN Ubiquitination Inhibits Catalytic Activity in Vitro—We then chose to directly address the effect of phosphorylation on ubiquitination in vitro (Fig. 4). Recombinant purified PTEN was first phosphorylated with purified CK2 and immunoprecipitated before being ubiquitinated in vitro (Fig. 4A). In vitro phosphorylation of PTEN with CK2 consistently achieved a stoichiometry of >3 and often approaching 4. Ubiquitination used recombinant purified Ubc5Hb and NEDD4-1 as the ubiquitin E2 and E3 components, respectively. In these experiments, we used both purified untagged ubiquitin and a mutant in which each lysine residue within ubiquitin is mutated to arginine (referred to as “ubiquitin K all R”) to block the formation of polyubiquitin chains. Phosphorylation had little or no inhibitory effect on subsequent ubiquitination, with some experiments with wild-type ubiquitin showing an increase in high
molecular weight polyubiquitination relative to unphosphorylated PTEN (Fig. 4, B and C).

Next, we were able to use the in vitro ubiquitination assay to analyze the effects of ubiquitination on PTEN catalytic activity. PTEN has been described to be both polyubiquitinated and monoubiquitinated (17). Therefore, we addressed the effects of monoubiquitination by performing ubiquitination in vitro using the ubiquitin K all R mutant, with each lysine residue replaced by arginine, blocking the formation of polyubiquitin chains (represented in Fig. 5A). Experimentally, ubiquitin K all R led to an apparent block in the polyubiquitination of PTEN, with a range of molecular weights being evident that presumably correspond to monoubiquitination and multisite mono-ubiquitination (Fig. 5B). Purified PTEN was put through this in vitro ubiquitination reaction in parallel with control samples, in which ubiquitin was omitted from the incubation. PTEN was then immunoprecipitated, washed, and assayed against both PtdInsP_3 lipid vesicles and the peptide polymer substrate poly-(Glu-Tyr(P)), with, in each case, greatly reduced activity being observed with ubiquitinated PTEN relative to a sham- ubiquitinated control (Fig. 5, C and D). Similarly, when the PTEN product of the monoubiquitination assay was immunoprecipitated and assayed, it was also found to show a similar loss of activity to polyubiquitination, suggesting that either mono- or polyubiquitination may greatly inhibit PTEN activity. In this case, although the mean activity against both substrates was reduced, this difference achieved statistical significance only using the lipid substrate (Fig. 5D). Given the incomplete reaction in the ubiquitination assay and that, in this series of experiments, the degree of ubiquitination appeared to correlate closely with the degree of PTEN activity loss, this suggests that the ubiquitination of PTEN in these experiments must lead to almost complete inactivation. Western blotting of both the completed crude ubiquitination assay and the subsequent PTEN immunoprecipitates demonstrated that the ubiquitination procedure did not affect the efficiency of PTEN immunoprecipitation (supplemental Fig. S2).

PTEN Ubiquitination Can Be Stimulated by Hyperosmotic Stress and Correlates with PtdInsP_3 Levels—Finally, we wanted to test the hypothesis that ubiquitination directly inactivates PTEN catalytic activity in cell-based experiments. Previous experiments in our laboratory had shown that treatment of HEK293T cells with hyperosmotic stress led to a reduction in PTEN expression levels over a period of several hours (Fig. 6 and data not shown). Therefore, we analyzed the ubiquitination of PTEN in HEK293T cells treated with 0.5 M sorbitol. We found that hyperosmotic stress rapidly increased PTEN polyubiquitination (Fig. 6A). This treatment also led to a modest decrease in PTEN protein levels, ~50% reduction in 4 h (Fig. 6, A–C), which could be blocked by proteasome inhibition (Fig. 6B).

The phosphorylation of the downstream PtdInsP_3-regulated kinase Akt could not be used as an indicator of the cellular PtdInsP_3 level in this case, as hyperosmotic stress appeared to block the activation of Akt (Fig. 6C), as described in a previous study despite a reported increase in the cellular PtdInsP_3 level (37). Therefore, we used direct measurements of PtdInsP_3 levels in these cells as a readout of PTEN activity. Sorbitol treatment of HEK293T cells caused cellular PtdInsP_3 levels to increase rapidly (Fig. 6C), correlating with the stimulated ubiquitination, long before effects on PTEN expression level became evident. In these experiments, sorbitol stimulation led to an increase in the activating phosphorylation of the recognized hyperosmotically responsive kinase p38 MAPK at Thr_180/Tyr_182 but did not affect the phosphorylation of PTEN at Ser_380/Thr_382/Thr_383 (Fig. 6C). Furthermore, both PTEN ubiquitination and cellular PtdInsP_3 levels were reduced within 10 min if cells were returned to normal medium after 1 h of sorbitol treatment (Fig. 6, D and E). These data support a direct connection between PTEN ubiquitination, cellular PTEN activity, and cellular PtdInsP_3 levels.

DISCUSSION

Our data support two novel conclusions. First, PTEN ubiquitination directly inhibits its phosphatase activity. Second,
PTEN ubiquitination is greatly enhanced by hyperosmotic stress and membrane localization, and this latter indirect mechanism is how C-terminal phosphorylation controls PTEN ubiquitination.

Several previous studies have established that C-terminal phosphorylation of PTEN increases the stability of the enzyme and reduces polyubiquitination (13–16). Our data here indicate that a dominant mechanism by which this effect is mediated is indirectly via the recognized effect of phosphorylation in suppressing PTEN localization at the plasma membrane (31, 32, 35). It has recently been reported that the phosphorylated PTEN C terminus inhibits ubiquitination in cells and in vitro by...

![Image of a scheme and a Western blot experiment showing PTEN ubiquitination and its effects on phosphatase activity.](image-url)

**FIGURE 5.** PTEN ubiquitination *in vitro* inhibits phosphatase activity. A, a scheme is shown that distinguishes among polyubiquitination, multiubiquitination (multisite monoubiquitination), and monoubiquitination of different lysine (K) residues on a ubiquitinated substrate protein. Using a ubiquitin mutant with each lysine residue mutated to arginine (ubiquitin K all R mutant), the formation of polyubiquitin chains is blocked. B, an anti-PTEN Western blot (W.blot) is shown comparing an *in vitro* ubiquitinated purified PTEN protein sample with a similar reaction using the ubiquitin K all R mutant and with a sham-ubiquitinated sample. Other immunoreactive bands within this negative control sample represent residual GST-tagged PTEN after an incomplete cleavage of this affinity purification tag. C and D, PTEN was immunoprecipitated from these ubiquitination and sham ubiquitination reactions along with a no-PTEN control, and each immunoprecipitate was assayed *in vitro* against PtdInsP₃ (PIP₃) vesicles (C) or the peptide substrate poly(Glu-Tyr(P)) (D). The mean phosphatase activity ± S.D. from triplicate samples is shown. Asterisks represent statistically significant reductions in activity compared with the relevant sham-ubiquitinated control (p < 0.05). In control blots, ubiquitination did not interfere with the efficiency of the immunoprecipitation (supplemental Fig. S2).
FIGURE 6. Osmotic stress induces PTEN ubiquitination and increases PtdInsP3 levels. HEK293T cells were transfected as indicated with vectors encoding wild-type PTEN (PTEN WT) and/or FLAG-tagged ubiquitin (Flag-Ub). A, cells were treated with 0.5 M sorbitol for 1, 2, or 4 h. Cells were then lysed, and PTEN was immunoprecipitated (IP) before Western blotting (W.blot) these immunoprecipitates and lysates with antibodies raised against the FLAG epitope, PTEN, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). B, cells were treated with sorbitol (0.5 M, 4 h) alone or in the presence of PSI (10 µM), and PTEN expression was analyzed by Western blotting along with a glyceraldehyde-3-phosphate dehydrogenase loading control. C, cells were treated with sorbitol as indicated (0.5 M, 10 min to 4 h). Cellular PtdInsP3 (PIP3) content was measured using a time-resolved fluorescence resonance energy transfer sensor complex by comparison with a standard curve of known PtdInsP3 concentrations as described previously (41). Parallel lysates were analyzed for the phosphorylation of Akt, PTEN, and the hyperosmotically responsive protein kinase p38 MAPK by Western blotting with phosphospecific and total protein antibodies. In these experiments, ~60% of cells were successfully transfected. D, after transfection as indicated, some cells were treated with 0.5 M sorbitol for 1 h as indicated, and two sets of samples were then washed with normal medium lacking sorbitol for 10 or 30 min. Cells were lysed, and PTEN was immunoprecipitated before Western blotting of these immunoprecipitates and lysates with antibodies raised against the FLAG epitope, PTEN, and glyceraldehyde-3-phosphate dehydrogenase. E, cells transfected as indicated with wild-type PTEN were treated with sorbitol for 1 h before some samples were washed with normal medium for 10 or 30 min. Cellular PtdInsP3 content was then assessed as described previously (41).
PTEN Ubiquitination Regulates Phosphatase Activity

NEDD4-1 (38). Because the phosphorylated C terminus inhibits its membrane binding of PTEN (31), these cellular data seem consistent with our conclusions. The reported effects of PTEN C-terminal deletion on ubiquitination in vitro appear to us far less strong than those in cells (38). From this we conclude that although both mechanisms may apply, the direct enhancement of ubiquitination in vitro caused by deletion of the C-terminal tail is weaker than the effect of membrane localization in cells.

Recent data indicate that the regulation of PTEN via ubiquitination is complex. Both mono- and polyubiquitination appear to have separable effects on PTEN function, with mono- and polyubiquitination targeting PTEN for nuclear localization and proteasomal degradation, respectively (11, 17, 18). Although NEDD4-1 has been identified as an E3 ligase capable of controlling PTEN abundance in several cultured cells (11), subsequent data suggest that this may not be a universal effect and that other E3 ligases may exist, such as XIAP (19, 20). Additionally, other proteins implicated in ubiquitin-dependent signaling have been identified as PTEN-interacting proteins, including the NEDD4-related proteins WWP2 and UbcH7 (39, 40). Our demonstration that ubiquitination inhibits PTEN activity implies that, if PTEN is polyubiquitinated and targeted for degradation, it is far less active once ubiquitinated even before it is degraded by the proteasome. Because our data suggest that this ubiquitination may occur at the plasma membrane, where the lipid substrate for PTEN is found, they also favor the speculation that ubiquitination may act as a mechanism to control PTEN activity. Ubiquitination is well recognized as a reversible modification, and the identification of HAUSP/USP7 as a PTEN-deubiquitinating enzyme supports this model for PTEN (18). The regulated inactivation of PTEN would appear significant whether PTEN is polyubiquitinated and targeted for degradation or monoubiquitinated either for targeting to the nucleus or simply as a mechanism of reversible inactivation at other cellular locations.

In summary, our data imply that PTEN ubiquitination occurs at the plasma membrane or at least is indirectly enhanced by PTEN membrane localization and that either mono- or polyubiquitination inhibits PTEN catalytic activity. These findings raise the possibility that PTEN ubiquitination may be driven by extracellular stimuli and may be a mechanism of reversible control over the enzyme during hyperosmotic stress and other conditions. Given the emerging complexity of mechanisms controlling PTEN ubiquitination, this seems likely to have significant implications for future functional studies.

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REFERENCES
1. Carracedo, A., and Pandolfi, P. (2008) Oncogene 27, 5527–5541
2. Maehama, T., and Dixon, J. E. (1998) J. Biol. Chem. 273, 13375–13378
3. Keniry, M., and Parsons, R. (2008) Oncogene 27, 5477–5485
4. Kwabi-Addo, B., Giri, D., Schmidt, K., Podsyspanina, K., Parsons, R., Greenberg, N., and Ittmann, M. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 11563–11568
5. Salmena, L., Carracedo, A., and Pandolfi, P. (2008) Cell 133, 403–414
6. Trotman, L. C., Niki, M., Dotan, Z. A., Koutcher, J. A., Di Cristofano, A., Xiao, A., Khoo, A. S., Roy-Burman, P., Greenberg, N. M., Van Dyke, T., Cordon-Cardo, C., and Pandolfi, P. (2003) PLoS Biol. 1, e59
7. Cao, J., Schulte, J., Knight, A., Leslie, N. R., Zagozdzon, A., Bronson, R., Manevich, Y., Beeson, C., and Neumann, C. A. (2009) EMBO J. 28, 1505–1517
8. Fine, B., Hodakoski, C., Koyuk, S., Su, T., Saal, L. H., Maurer, M., Hopkins, B., Keniry, M., Sulis, M. L., Mense, S., Hibshoosh, H., and Parsons, R. (2009) Science 325, 1261–1265
9. Leslie, N. R., Spinelli, L., Tabarew, P., Zilidis, G., Weerasinghe, N., Lim, J. C., Maccario, H., and Downes, C. P. (2010) Adv. Enzyme Regul., in press
10. Okahara, F., Itoh, K., Nakagawara, A., Murakami, M., Kanaho, Y., and Maehama, T. (2006) Mol. Biol. Cell 17, 4888–4895
11. Wang, X., Trotman, L. C., Koppie, T., Alimonti, A., Chen, Z., Gao, Z., Wang, J., Erdjument-Bromage, H., Tempst, P., Cordon-Cardo, C., Pandolfi, P. P., and Jang, X. (2007) Cell 128, 129–139
12. Yim, E. K., Peng, G., Dai, H., Hu, R., Li, K., Lu, Y., Mills, G. B., Meric-Bernstam, F., Hennessy, B. T., Craven, R. J., and Lin, S. Y. (2009) Cancer Cell 15, 304–314
13. Tolkacheva, T., Boddapati, M., Sanfiz, A., Tsuchida, K., Kimmelman, A. C., and Chan, A. M. (2001) Cancer Res. 61, 4985–4989
14. Torres, J., and Pulido, R. (2001) J. Biol. Chem. 276, 993–998
15. Wu, W., Wang, X., Zhang, W., Reed, W., Samet, J. M., Whang, Y. E., and Ghio, A. J. (2003) J. Biol. Chem. 278, 28258–28263
16. Vazquez, F., Ramaswamy, S., Nakamura, N., and Sellers, W. R. (2000) Mol. Biol. Cell. 10, 5010–5018
17. Trotman, L. C., Wang, X., Alimonti, A., Chen, Z., Teruya-Feldstein, J., Yang, H., Pavletich, N. P., Carver, B. S., Cordon-Cardo, C., Erdjument-Bromage, H., Tempst, P., Chi, S. G., Kim, H. J., Misteli, T., Jang, X., and Pandolfi, P. P. (2007) Cell 128, 141–156
18. Song, M. S., Salmena, L., Carracedo, A., Egia, A., Lo-Cocco, F., Teruya-Feldstein, J., and Pandolfi, P. P. (2008) Nature 455, 813–817
19. Fouladkou, F., Landry, T., Kawabe, H., Neeb, A., Lu, C., Brose, N., Stambovlic, V., and Rotin, D. (2008) Proc. Natl. Acad. Sci. U.S.A. 105, 8585–8590
20. Van Themensche, C., Leblanc, V., Parent, S., and Asselin, E. (2009) J. Biol. Chem. 284, 20462–20466
21. Ostrowska, H., Wójcik, C., Wilk, S., Omura, S., Kozłowska, L., Stoklosa, T., Worowski, K., and Radziwon, P. (2000) Int. J. Biochem. Cell Biol. 32, 747–757
22. Pagano, M. A., Andrzejewska, M., Ruzzene, M., Sarno, S., Cesaro, L., Bain, J., Elliott, M., Meggio, F., Kazimierczuk, Z., and Pinna, L. A. (2004) J. Med. Chem. 47, 6239–6247
23. Traenckaer, E. B., Wilk, S., and Baeuerle, P. A. (1994) EMBO J. 13, 5433–5441
24. Leslie, N. R., Gray, A., Pass, I., Orchiston, E. A., and Downes, C. P. (2000) Biochem. J. 346, 827–833
25. Leslie, N. R., Bennett, D., Gray, A., Pass, I., Hoang-Xuan, K., and Downes, C. P. (2001) Biochem. J. 357, 427–435
26. Leslie, N. R., Yang, X., Downes, C. P., and Weijer, C. J. (2007) Curr. Biol. 17, 115–125
27. Davies, S. P., Reddy, H., Caivano, M., and Cohen, P. (2000) Biochem. J. 351, 95–105
28. Maccario, H., Perera, N. M., Caivano, M., and Cohen, P. (2004) Biochem. J. 379, 761–767
29. Filangi, S., Theodore, S. T., and D’Amore, M. (2005) J. Biol. Chem. 280, 28453–28462
30. Caro, J., Schulte, J., Knight, A., Leslie, N. R., Zagozdzon, A., Bronson, R., Manevich, Y., Beeson, C., and Neumann, C. A. (2009) EMBO J. 28, 1505–1517
31. Das, S., Dixon, J. E., and Cho, W. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 7491–7496
32. Vazquez, F., Matsuoka, S., Sellers, W. R., Yanagida, T., Ueda, M., and Devreotes, P. N. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 3633–3638
33. Ondrůzková, L., Singh, G., Hoang, T., and Chan, A. M. (2007) J. Biol. Chem. 282, 23306–23315
34. Rahdar, M., Inoue, T., Meyer, T., Zhang, J., Vazquez, F., and Devreotes, P. N. (2009) Proc. Natl. Acad. Sci. U.S.A. 106, 480–485
35. Vazquez, F., Grossman, S. R., Takahashi, Y., Rokas, M. V., Nakamura, N., and Sellers, W. R. (2001) J. Biol. Chem. 276, 48627–48630
36. McCabe, J. B., and Berthiaume, L. G. (1999) Mol. Biol. Cell 10, 3771–3786
37. Meier, R., Thelen, M., and Hemmings, B. A. (1998) EMBO J. 17, 7294–7303
38. Wang, X., Shi, Y., Wang, J., Huang, G., and Jiang, X. (2008) Biochem. J. 414, 221–229
39. Crockett, D. K., Fillmore, G. C., Elenitoba-Johnson, K. S., and Lim, M. S. (2005) Proteomics 5, 1250–1262
40. Waite, K. A., and Eng, C. (2003) Hum. Mol. Genet. 12, 679–684
41. Gray, A., Olsson, H., Batty, I. H., Priganica, L., and Peter Downes, C. (2003) Anal. Biochem. 313, 234–245
42. McConnachie, G., Pass, I., Walker, S. M., and Downes, C. P. (2003) Biochem. J. 371, 947–955