Direct identification of PTEN phosphorylation sites

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Abstract **The PTEN tumor suppressor gene encodes a phosphatidylinositol 3′-phosphatase that is inactivated in a high percentage of human tumors, particularly glioblastoma, melanoma, and prostate and endometrial carcinoma. Previous studies showed that PTEN is a seryl phosphoprotein and a substrate of protein kinase CK2 (CK2). However, the sites in PTEN that are phosphorylated in vivo have not been identified directly, nor has the effect of phosphorylation on PTEN catalytic activity been reported. We used mass spectrometric methods to identify Ser^{370} and Ser^{385} as in vivo phosphorylation sites of PTEN. These sites also are phosphorylated by CK2 in vitro, and phosphorylation inhibits PTEN activity towards its substrate, PIP3. We also identify a novel in vivo phosphorylation site, Thr^{366}. Following transient over-expression, a fraction of CK2 and PTEN co-immunoprecipitate. Moreover, pharmacological inhibition of CK2 activity leads to decreased Akt activation in PTEN+/+ but not PTEN−/− fibroblasts. Our results contrast with previous assignments of PTEN phosphorylation sites based solely on mutagenesis approaches, suggest that CK2 is a physiologically relevant PTEN kinase, and raise the possibility that CK2-mediated inhibition of PTEN plays a role in oncogenesis.**

Key words: PTEN; Phosphatase; Protein phosphorylation; Protein kinase CK2

1. Introduction

The tumor suppressor gene PTEN plays a key role in mammalian growth control. PTEN is absent or mutated in a large number of human tumors, especially glioblastoma, melanoma, and prostate and endometrial carcinoma (reviewed in [1–4]). With the exception of endometrial carcinomas [5], somatic PTEN mutations typically are associated with more advanced tumors [6–12]. Germline mutation of PTEN causes Cowden disease [13,14], Lhermitte–Duclos disease [13], and Bannayan–Zonana syndrome [15,16], which are characterized by benign hamartomas of the thyroid, intestine, breast, and central nervous system and a markedly increased cancer incidence. Although homozygotic PTEN mutation in mice results in early embryonic lethality, PTEN+/− mice are tumor-prone, exhibiting hyperplasia of the prostate, skin, and colon and an increased incidence of breast, prostate and endometrial carcinomas, T cell lymphomas and leukemia [17–19]. Understanding how PTEN is regulated should provide new insight into cell signaling mechanisms and may suggest novel approaches to treatment of PTEN-deficient tumors.

PTEN belongs to the dual specificity phosphatase (DSP) family of protein phosphatases [6,7,20]. DSPs typically dephosphorylate phosphotyrosyl (pY), phosphothreonyl (pT) and phosphoseryl (pS) residues in vitro, although they usually are highly selective for particular pT-X-pY or pT-pY motifs in vivo [21]. Although PTEN has DSP activity in vitro [22], its physiological target is not a phosphoprotein, but instead, a phospholipid: specifically, the D3-phosphate group of phosphatidylinositol 3,4,5-triphosphate (PIP3) ([23–28], reviewed in [1,29]). By dephosphorylating PIP3, PTEN antagonizes growth-promoting and anti-apoptotic pathways that are stimulated by phosphatidylinositol 3-kinase (PI3K), itself an oncogene [30]. Cells lacking wild-type (WT) PTEN display constitutive activation of Akt and other downstream targets of the PI3K pathway, whereas ectopic over-expression of PTEN inhibits several PI3K actions [18,24–27,31,32].

Surprisingly little is known about PTEN regulation. Besides its catalytic domain, PTEN contains a C2 domain and a PDZ binding motif. The C2 domain directs PTEN binding to phospholipid vesicles in vitro, and mutations that abrogate vesicle binding activity without affecting catalysis disrupt PTEN function in vivo [33]. However, there is no evidence for modulation of C2 domain/membrane association in response to different stimuli. Several groups have reported association of PTEN with PDZ domain-containing proteins; in some cases, these potentiate the effects of limiting amounts of PTEN on PI3K-dependent functions in transient transfection assays [34–36]. Mutations of the PDZ domain binding motif in PTEN appear to impair some, but not all PTEN functions [37]. The role of these interactions in tumor suppression remains to be demonstrated.

Phosphorylation is a third possible mode of PTEN regulation. Using mutagenesis approaches, two groups have suggested that PTEN is phosphorylated at specific C-terminal seryl and threonyl residues. Vazquez and colleagues found that deletion of the PTEN tail (residues 354–403) abrogated most PTEN phosphorylation. They mutated every serine and threonine in the tail to alanine, either singly or in clusters. Because mutation of the cluster Ser^{380}, Thr^{382}, Thr^{383}, and

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Abbreviations: CK2, protein kinase CK2 or casein kinase II; DMEM, Dulbecco's modified Eagle's medium; MS, mass spectrometry; PIP3, phosphatidylinositol 3,4,5-triphosphate; PI3K, phosphatidylinositol 3-kinase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate

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Ser^{385} significantly reduced incorporation of orthophosphate into PTEN, they proposed that these are the predominant phosphorylated residues [38]. Torres and Pulido [39] reported that protein kinase CK2 (CK2) (formerly casein kinase II) catalyzes phosphorylation of PTEN to high stoichiometry. Mutation of residues Ser^{370} and Ser^{385} nearly abolished CK2-induced phosphorylation and PTEN phosphorylation in vivo, whereas mutations in residues Ser^{380}, Thr^{382} and Thr^{383} had a smaller effect. Vazquez et al. [38] also reported that mutation of Ser^{380}, Thr^{382} or Thr^{383} to alanine resulted in a PTEN protein that is less stable but more active in its ability to block downstream targets of PIP3 and promote cell cycle arrest, mimicking removal of the PTEN C-terminus [38,40]. In contrast, Torres and Pulido found that mutation of Ser^{370}, Ser^{385}, Ser^{380}, Thr^{382} or Thr^{383} resulted in decreased PTEN stability. Furthermore, PTEN instability was prevented by treatment with the proteasome inhibitor MG132 [39,41]. Very recently, it was reported that mutation of Thr^{382} or Thr^{383} [41] or substitution of alanine for residues Ser^{380}, Thr^{382}, Thr^{383}, and Ser^{385} increases PTEN association with PDZ domain-containing proteins [42]. Thus, the prevailing view is that phosphorylation keeps PTEN stable but less active, whereas dephosphorylation activates PTEN and may enhance its interaction with PDZ domain-containing binding partners, but also targets it for degradation. Notably, the sites on PTEN that are phosphorylated in vivo have not been identified directly, but only inferred from mutagenesis. Likewise how phosphorylation affects PTEN catalytic activity has not been analyzed.

Here, we use mass spectrometric methods to directly identify the major in vivo phosphorylation sites of PTEN as Ser^{370} and Ser^{385}; Thr^{366} also is phosphorylated to a lower extent. CK2 phosphorylates Ser^{370} and Ser^{385} to high stoichiometry in vitro, and CK2 associates with PTEN in transient co-transfection assays. Furthermore, CK2-catalyzed phosphorylation inhibits PTEN activity against its physiologically relevant target, PIP3, and pharmacological inhibition of PTEN activity leads to decreased Akt activity in a PTEN-dependent fashion.

2. Materials and methods

2.1. Cell culture, drug treatments and transfections

293-ϕnx human embryonic kidney cells, a generous gift of Dr. Gary Nolan, Stanford University Medical School (www.stanford.edu/group/nolan/retroviral_systems/phx.html), U87-MG human glioblastoma cells (U87), and PTEN+/+ and PTEN−/− fibroblast lines derived from teratomas derived from the corresponding embryonal stem cells (supplied by Dr. P. Pandolfi, Memoral Sloan-Kettering Cancer Center) [17] were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamine and 10% fetal bovine serum at 37°C in a humidified 10% CO_2 atmosphere. In some experiments, the selective CK2 inhibitors apigenin (a plant flavonoid) [43] or emodin (an anthraquinone) [44] were prepared in dimethyl sulfoxide (DMSO) and added to the culture media at the indicated concentrations. Cells were transfected using Fugene-6 (Boehringer-Mannheim) according to the manufacturer's instructions. U87 cells expressing wild-type PTEN were generated by retroviral infection using supernatant from 293-ϕnx cells previously transfected with a pBabepuro plasmid expressing human PTEN cDNA. Puromycin-resistant clones were isolated, and PTEN expression verified by immunoblotting. At least three independent clones were tested for each parameter and gave similar results.

2.2. Plasmids

pGEX-PTEN-351–403, pGEX-PTEN-366–403, and pGEX-PTEN-376–403 were generated by polymerase chain reaction (PCR) using a common downstream primer (5′-CGGAATTCAGACTTTTGTAATTTGTGTATG-3′) and the following 5′ primers: 5′-GCGCGGATCCGTAGAGGAGCCGTCAAATCC-3′ (351–403), 5′-GCGCGGATC CACACCAGATGTTAGTGAC-3′ (366–403), 5′-GCGCGGATCCCCATTATAGATATTCTGAC-3′ (376–403). The resultant PCR products were cloned into the pGEX4T1 vector (Amersham/Pharmacia). PTEN point mutants were generated by overlap extension PCR by using as flanking primers: 5′-CGGAATTCAGACTTTTGTAATTTGTGTATG-3′ (upstream) and 5′-GAGGATCCACCATGACAGCCATCATCAAAGA-3′ (downstream), and the following overlapping mutagenesis oligonucleotides: S370A: 5′-CACCAGATGTTGCTGACAATGAACC-3′ and 5′-GGTTCATTGTCAGCAACATCTGGTG-3′; S370D: 5′-CACCAGATGTTGATGACAATGAACC-3′ and 5′-GGTTCATTGTCATCAACATCTGGTG-3′; S385A: 5′-GACACCACTGACGCTGATCCAGAG-3′ and 5′-CTCTGGATCAGCGTCAGTGGTGTC-3′; S385D: 5′-GACACCACTGACGATGATCCAGAG-3′ and 5′-CTCTGGATCATCGTCAGTGGTGTC-3′; S385T: 5′-GACACCACTGACACTGATCCAGAG-3′ and 5′-CTCTGGATCAGTGTCAGTGGTGTC-3′.

2.3. Production of recombinant PTEN

Cultures of bacteria (DH5α) expressing pGEX2T-PTEN (400 ml) were induced at $A_{600} = 0.5–1.0$ with 0.1 mM isopropyl thiogalactose (IPTG), incubated overnight at 25°C, pelleted, and lysed in 25 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid (EDTA) containing 0.1 mg/ml lysozyme, 5 mM dithiothreitol (DTT), 20 μg/ml leupeptin, 2 μg/ml pepstatin A, 2 μg/ml antipain, 4 μg/ml aprotinin, and 40 μg/ml phenylmethylsulfonyl fluoride. Triton X-100 was added to a final concentration of 1% and the lysate was sonicated and clarified by centrifugation at 18 000 × *g*. Cleared lysates were incubated with glutathione–agarose beads with rocking at 4°C overnight. The fusion protein was eluted into 20 mM reduced glutathione (pH 8.0) for 10 min at 25°C and dialyzed extensively against storage buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM DTT, 50% glycerol).

2.4. In vitro kinase assays

Recombinant GST-PTEN or His-PTEN (a generous gift of Drs. S. Dhe-Paganon and S. Shoelson; Joslin Diabetes Center) was labeled in vitro with 500 U of CK2 (Calbiochem) in a 30 μl reaction containing 200 μM adenosine triphosphate (ATP), 20 mM Tris–HCl, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, and 1–10 μCi [γ-^{32}P]ATP for 10 min at 37°C. Reactions were stopped with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer, boiled, and subjected to electrophoresis and autoradiography.

2.5. Antibodies, immunoprecipitations and immunoblotting

Anti-PTEN polyclonal antisera were generated by immunizing rabbits with GST-full length PTEN, and affinity-purified antibodies were prepared by passing antisera over GST-PTEN bound to Affi-Gel 15 (Bio-Rad), according to standard procedures [45]. Anti-pAkt (Ser^{473}) antibodies were obtained from Santa Cruz Biotechnology and used as recommended by the manufacturer. For whole-cell extracts, cells were washed twice in phosphate-buffered saline and lysed in Nonidet P-40 (NP-40) buffer (1% NP-40, 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA), containing 20 μg/ml leupeptin, 2 μg/ml pepstatin A, 2 μg/ml antipain, 4 μg/ml aprotinin, 40 μg/ml phenylmethylsulfonyl fluoride (PMSF), 2 mM sodium fluoride, 2 mM sodium vanadate, and 25 mM β-glycerol phosphate. Lysates were cleared by centrifugation at 4°C for 10 min. Immunoprecipitations and immunoblotting were performed as described previously [46], except that following immunoprecipitation, beads were washed once in 20 mM Tris–HCl (pH 8.0), 250 mM NaCl, 1 mM EDTA, 0.5% NP-40, and four times in 20 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% NP-40.

2.6. Lipid phosphatase assays

PIP3 radiolabeled at the D-3 position was generated by incubating phosphatidylinositol 4,5-bisphosphate (Sigma) in 50 μM ATP, 20 μCi [γ-^{32}P]ATP, 10 mM $MgCl_2$, and baculovirus-purified PI3K (a generous gift of Dr. L. Cantley, Beth Israel-Deaconess Medical Center) for 60–120 min at 25°C in a total volume of 50 μl. Reactions were terminated by the addition of 1 N HCl (80 μl), and lipids were extracted into 160 μl chloroform:methanol (1:1). Aliquots of radiolabeled PIP3 were dried under N_2, sonicated in 10 μl of 10 mM Tris–HCl, pH 7.5,

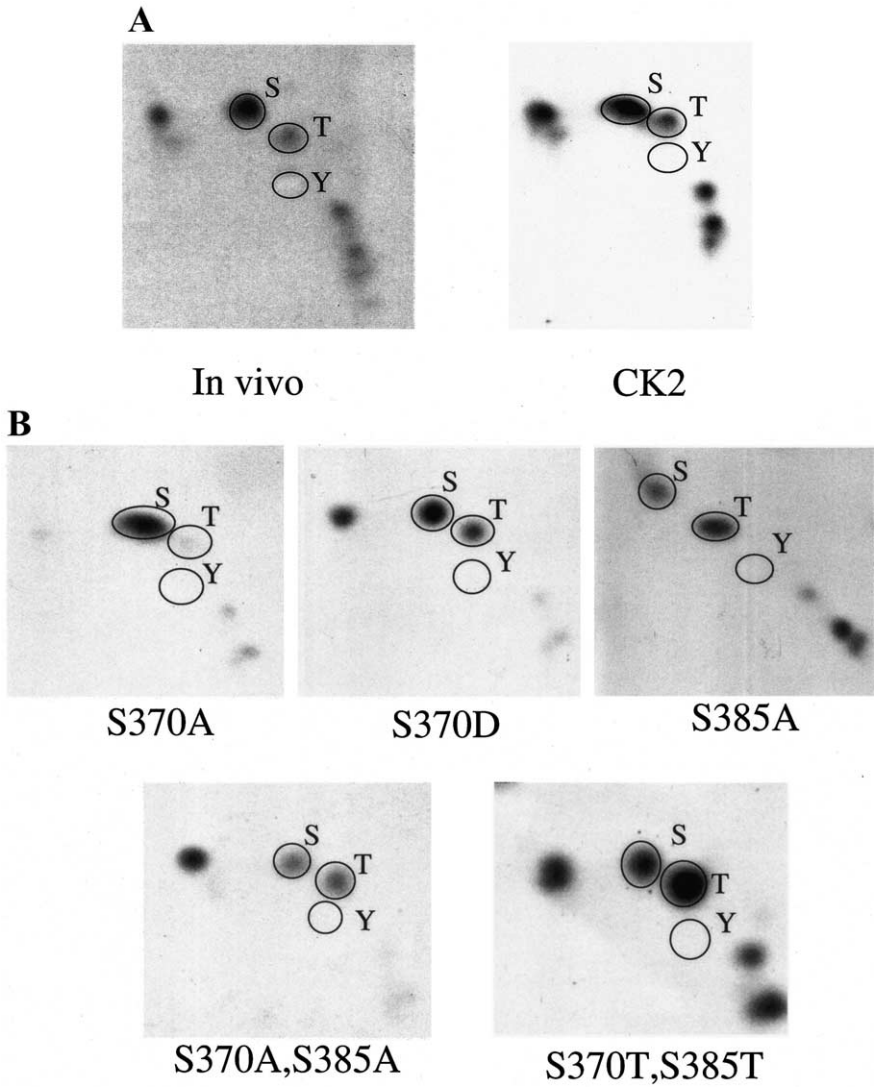

Fig. 1. Phosphoamino acid analyses. A: Two-dimensional phosphoamino acid analysis of PTEN isolated in vivo (left) or phosphorylated in vitro by CK2 (right). B: Phosphoamino acid analyses of WT and PTEN mutants phosphorylated by CK2. Note shift in ratio of phosphoserine and phosphothreonine, particularly in the S370T, S385T mutant. Cold phosphoamino acid standards were used to determine the locations of phosphotyrosine (Y), phosphothreonine (T), and phosphoserine (S), as indicated.

and used in 25 μl reactions containing 50 mM Tris–HCl, pH 7.5, 50 mM NaCl, 2 mM DTT and recombinant PTEN. Reactions were incubated at 37°C for 5–10 min and stopped with acid-washed charcoal (800 μl). A fraction of the supernatant (600–700 μl) was used to quantify phosphate release by scintillation counting.

For assaying the effect of CK2-catalyzed phosphorylation on PTEN activity, kinase reactions were terminated by the addition of 5 mM EDTA and chilled on ice. Reactions were adjusted to the appropriate Tris–HCl, NaCl, and DTT concentrations for lipid phosphatase assays, radiolabeled PIP3 was added, and incubation and analysis were carried out as described above.

2.7. GST fusion protein binding assays

Lysates from asynchronous U87 cells were prepared as above. Lysate (2 mg) was incubated with 20 μg of fusion protein at 4°C for 3.5 h. The beads were washed as above, and bound proteins were resolved by SDS–PAGE and analyzed by immunoblotting.

2.8. Metabolic labeling and phosphoamino acid analysis

U87 cells expressing wild-type PTEN were radiolabeled at 70% confluence with 1 mCi of [^{32}P]orthophosphate/10 cM plate in phosphate-free DMEM for 4 h. PTEN immunoprecipitates (prepared as described above) were resolved by SDS–PAGE. Following electroblotting onto Immobilon-P (Millipore), radiolabeled PTEN was hydrolyzed in 6 N HCl, and phosphoamino acid analysis was performed as described [47].

2.9. Mass spectrometric analysis of phosphopeptides

Following SDS–PAGE, gels were stained with Coomassie blue, destained, and the PTEN band was excised and subjected to reduction, carboxyamidomethylation, and digestion with trypsin. Tryptic peptides were analyzed by microcapillary reverse-phase high-performance liquid chromatography (HPLC) nano-electrospray tandem mass spectrometry (μLC-MS/MS) on a ThermoFinnigan LCQ DECA quadrupole ion trap mass spectrometer.

Two strategies were used to enhance the detection of low stoichiometry-phosphorylated peptides. In one, phosphorylated peptides were selectively enriched by performing bimodal chromatography on a Fe^{3+} metal affinity/reverse-phase microcolumn directly coupled to the ion trap MS. The ion trap repetitively surveyed MS (395–1295 *m/z*), executing data-dependent scans on the three most abundant ions in the survey scan, allowing high-resolution (zoom) scans to determine charge state and exact mass and MS/MS spectra for peptide sequence information. MS/MS spectra were acquired with a relative collision energy of 30% and an isolation width of 2.5 Da. Recurring ions were dynamically excluded. After database correlation with SEQUEST, phosphorylated peptides were confirmed by manual, de novo interpretation of the MS/MS spectra using FuzzyIons [48]. Second sets

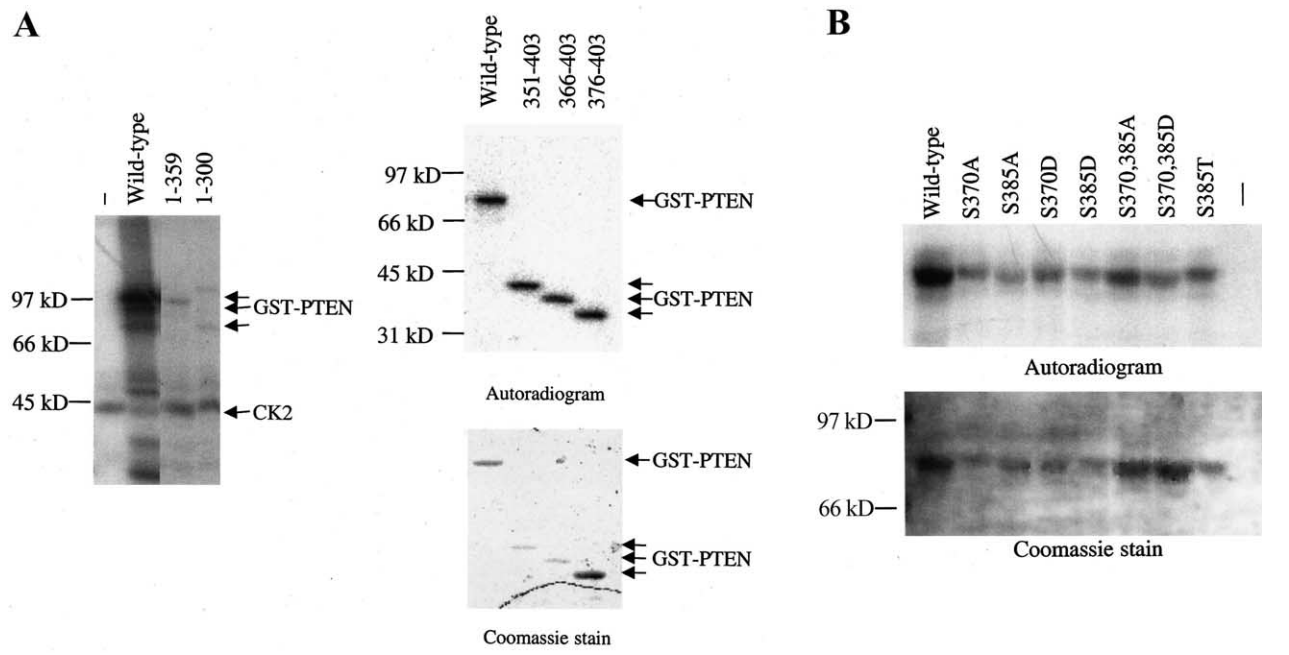

Fig. 2. In vitro phosphorylation of PTEN by CK2. A: Phosphorylation of PTEN truncation mutants. GST fusion proteins containing the indicated PTEN amino acids or GST-full length PTEN ('wild-type') were used as substrates for in vitro kinase assays with CK2. The arrows indicate the expected positions of the various GST fusion proteins (indicated at the top of each lane). Molecular weight markers also are shown. The autoradiogram in the left panel was exposed approximately five times longer than the right panel (compare intensity of GST-WT bands). (−) no exogenous substrate. Note CK2 autophosphorylation in the left panel, which is diminished by the presence of the good substrate, GST-PTEN ('wild-type'). CK2 phosphorylation is not evident in the autoradiogram in the right panel, because of the shorter exposure time and because all of the GST fusion proteins in this experiment were good CK2 substrates. B: Phosphorylation of PTEN point mutants. GST fusion proteins to WT PTEN and the indicated PTEN mutants were phosphorylated by CK2. Autoradiograms and Coomassie stains for protein loading are shown.

of μLC-MS/MS experiments were conducted for each tryptic peptide containing the putative phosphorylation. In these targeted ion MS/MS (TIMM) experiments, the predicted *m/z* of the peptide is targeted for the entire chromatographic run. This significantly increases MS/MS sensitivity by capturing only the peptide of interest while removing interfering chemical and peptidic background.

3. Results and discussion

3.1. Direct identification of PTEN phosphorylation sites

Consistent with previous publications [38,39], anti-PTEN immunoprecipitations from PTEN-expressing cells metabolically labeled with [$^{32}P_i$]orthophosphate contained a ^{32}P-labeled protein of size predicted for PTEN (data not shown). Phosphoamino acid analysis revealed that PTEN is phosphorylated predominantly on seryl residues, although there was a small amount of threonyl phosphorylation (Fig. 1A). To directly identify PTEN phosphorylation sites, GST-PTEN was expressed in 293-ϕnx cells, purified on glutathione agarose beads, and subjected to microcapillary HPLC tandem mass spectrometry (μLC-MS/MS) with bimodal chromatography and targeted ion MS/MS (TIMM) analyses. In such analyses, each of the phosphorylated peptides is subjected to collision-induced dissociation in the mass spectrometer, and the resulting products of that fragmentation are recorded as a mass spectrum that can be assigned to a specific linear sequence of amino acids. For a given peptide, each possible phosphorylation site generates different and unique product ions in the MS/MS spectrum. For each peptide in Table 1, we observed product ions that were characteristic of addition of phosphate only at the sites indicated. This analysis unambiguously identified Ser^{370}, Ser^{385} and Thr^{366} as in vivo phosphorylation sites.

Because the PTEN C-terminus contains CK2 consensus phosphorylation sites, and because CK2 over-expression [49] or loss of PTEN (see Section 1) can cause T cell lymphomas, we asked whether CK2 could phosphorylate and/or regulate PTEN. Indeed, CK2 phosphorylated a glutathione-*S*-transferase-PTEN fusion protein (GST-PTEN) to a stoichiometry of

Table 1
Mass spectrometry analysis of PTEN phosphorylation

Peptide no.	Peptide	Phosphorylated residue
1	TVEEPSNPEASSSTSVp**T**PDVSDNEpDHYR[a]	T366
2	TVEEPSNPEASSSTSVTPDVp**S**DNEPDHYR[b]	S370
3	YSDTTDp**S**DPENEPFDEDQHTQITK[c]	S385
4	YSDTTDp**S**DPENEPFDEDQHTQITKV[b]	S385

[a]Observed in vivo.
[b]Observed both in vitro and in vivo.
[c]Observed in vitro.

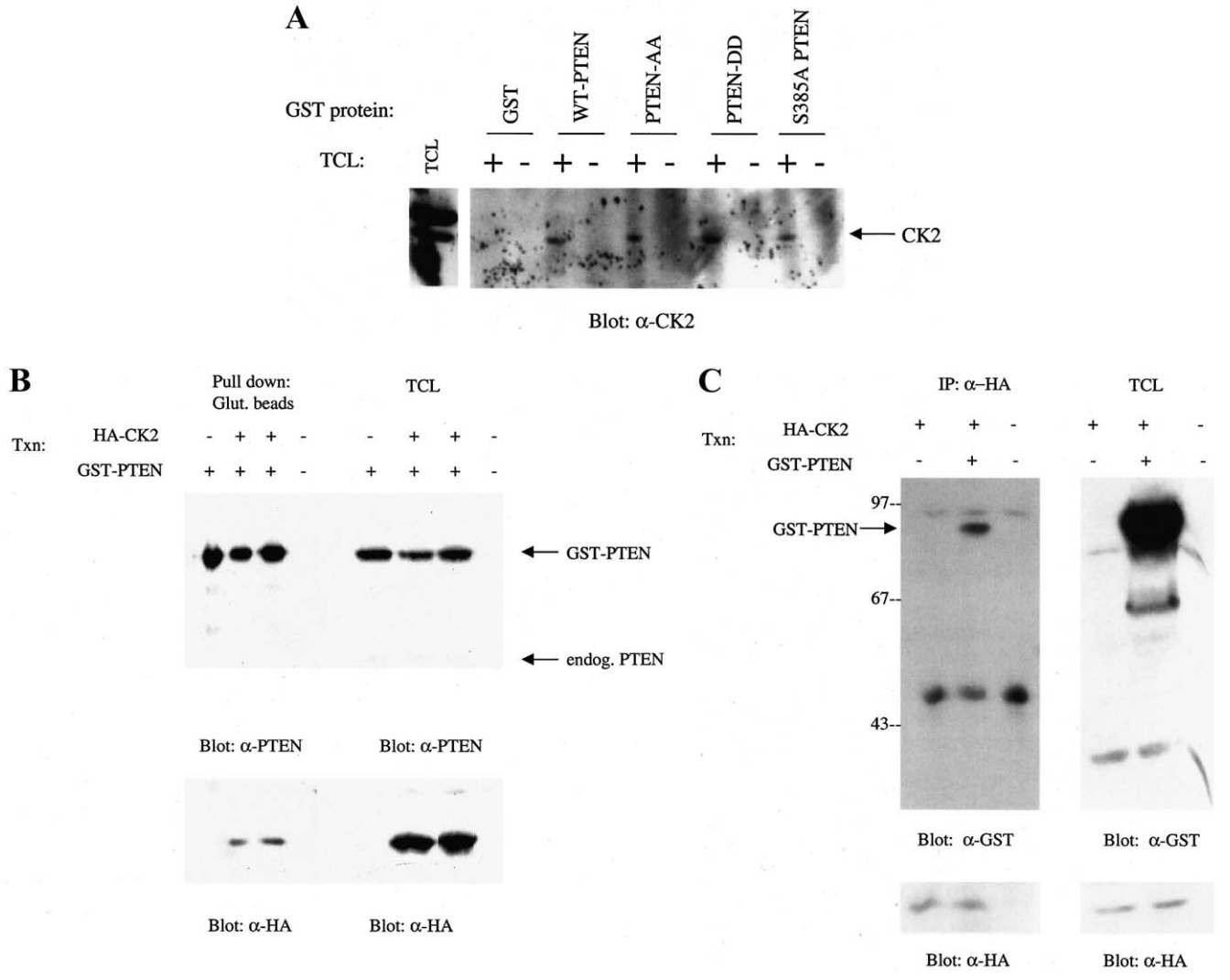

Fig. 3. Association of PTEN and CK2. A: PTEN interacts with CK2 in vitro. The indicated GST-PTEN fusion proteins or GST were incubated with 2 mg U87 cell lysate (+) or lysis buffer alone (−) to exclude non-specific cross-reactivity of the GST fusion protein with anti-CK2 antibodies. Bound proteins were collected, resolved by SDS–PAGE and immunoblotted with anti-CK2 antibodies. Also shown is anti-CK2 immunoblot of 50 μg of total U87 cell lysate (TCL). B, C: PTEN interacts with CK2 in vivo. 293-ϕnx cells were transfected with expression plasmids for HA-CK2 and GST-PTEN separately or together. TCL and proteins bound to glutathione–agarose were resolved by SDS–PAGE and immunoblotted with anti-HA antibodies, as indicated (B). Alternatively, anti-HA immunoprecipitates were prepared, and bound proteins and TCL were immunoblotted with anti-GST antibodies (to detect GST-PTEN) or anti-HA antibodies (to detect CK2), as indicated (C).

> 1 mol phosphate/mol protein, consistent with the observations of Torres and Pulido [39]. CK2-phosphorylated PTEN was predominantly seryl phosphorylated with lesser amounts of threonyl phosphorylation (Fig. 1A).

We confirmed that CK2 phosphorylates PTEN within its C-terminus by using nested GST-PTEN fusion proteins. CK2 did not phosphorylate PTEN lacking residues 360–403 (Fig. 2A; left panels). However, CK2 phosphorylated GST-PTEN-351–403 and GST-PTEN-366–403 equivalently, whereas GST-PTEN-376–403 was phosphorylated to a lower extent (Fig. 2A, right panels; note the higher amount of GST-PTEN-376–403 fusion protein in the Coomassie stain). These data indicate that at least one phosphorylated residue resides in the region between amino acids 366 and 376, with an additional phosphorylated residue(s) downstream of position 376 (Fig. 2A).

To directly identify the residues phosphorylated by CK2, in vitro phosphorylated PTEN was subjected to μLC-MS/MS analysis. Both Ser^{370} and Ser^{385} were identified as CK2 phosphorylation sites (Table 1). Thus, the two seryl residues of PTEN phosphorylated in vivo are identical to those phosphorylated to high stoichiometry by CK2 in vitro. Conceivably, the third in vivo phosphorylation site, Thr^{366}, also is phosphorylated by CK2, because phosphoamino acid analysis showed that CK2 phosphorylation of PTEN is partially on threonyl residues (Fig. 1A). Mass spectrometric analysis did not detect this residue in PTEN phosphorylated in vitro. Although this technology is extremely sensitive, low stoichiometric events can be missed. A mixture of singly phosphorylated Thr^{366} and Ser^{370} peptides might be expected to co-chromatograph and co-fragment in the μLC-MS/MS analysis. The more abundant peptides would dominate, masking the sequence interpretation of the minor component. However, we were able to identify the Thr^{366}-phosphorylated peptide in PTEN in vivo (Table 1). Since it usually is easier to detect phosphorylation events in vitro compared to in vivo, we sus-

pect that another kinase is responsible for the low stoichiometry phosphorylation of Thr^{366} in vivo (and that CK2 phosphorylates another threonyl residue in vitro). Notably, Thr^{366} is followed by a prolyl residue, and does not conform to the CK2 consensus, making it far more likely that this site is the target of a prolyl-directed kinase.

Surprisingly, following mutagenesis of PTEN residues Ser^{370} and/or Ser^{385} to alanine or aspartic acid, CK2 remained capable of phosphorylating the mutant PTEN protein (Fig. 2B). Thus, when its preferred sites are mutated, CK2 can phosphorylate other sites in PTEN. However, only Thr^{366}, Ser^{370} and Ser^{385} were detected in PTEN in vivo by MS/MS, suggesting that these 'default' sites are not significantly phosphorylated in the wild-type protein. Phosphoamino acid analysis of doubly-point-mutated PTEN (either PTEN (Ala^{370} and Ala^{385}) or PTEN (Asp^{370} and Asp^{385})) suggests that at least some of the other residues phosphorylated by CK2 after mutagenesis of Ser^{370} and Ser^{385} are threonines, because the ratio of seryl to threonyl phosphorylation changed from primarily seryl phosphorylation (roughly 80%) to nearly equivalent seryl and threonyl phosphorylation (compare Fig. 1A, right panel with Fig. 1B, lower left panel). Mutation of Ser^{385} alone resulted in primarily threonyl phosphorylation of PTEN by CK2 (Fig. 1B, upper right panel), whereas comparable mutations at Ser^{370} resemble the phosphoamino acid ratio of wild-type PTEN (compare Fig. 1A with Fig. 1B). CK2 phosphorylation of PTEN containing both Ser^{370} and Ser^{385} converted to threonine revealed a switch from primarily seryl to primarily threonyl phosphorylation, confirming that these residues are the preferred substrates for CK2 (compare Fig. 1A with Fig. 1B, lower right panel).

Previous studies showed that mutation of Ser^{380}, Thr^{382} or Thr^{383} decreases PTEN stability [38,39] and increase its ability to inhibit downstream targets of PIP3 in transient transfections [38], leading to the conclusion that phosphorylation of these sites regulates PTEN. However, our direct sequence analysis of PTEN phosphopeptides reveals no significant phosphorylation of these residues either in vitro (with CK2) or in vivo. Moreover, phosphoamino acid analysis reveals only a minor amount of threonyl phosphorylation of PTEN (Fig. 1A), which presumably is due, at least in part, to phosphorylation at Thr^{366} (Table 1). Failure to detect phosphorylation of Thr^{382} or Thr^{383} (or, for that matter, Ser^{380}) by mass spectrometry does not exclude the possibility that one or both of these residues is phosphorylated, particularly if either is phosphorylated at low stoichiometry (see above). However, it is difficult to see how sub-stoichiometric threonyl phosphorylation (Fig. 1A, B) could regulate the stability of the entire pool of PTEN. Moreover, since Ser^{380} lies within the same tryptic peptide as Ser^{385}, it should have been detectable by MS/MS were it phosphorylated to comparable levels. For these reasons, we suspect that mutation of Ser^{380}, Thr^{382} and/or Thr^{383} probably affects the ability of Ser^{370} and/or Ser^{385}, the real in vivo phosphorylation sites, to be phosphorylated and/or improves recognition of PTEN by an F-box protein required to target it to the proteasome. Notably, Vazquez et al. [38] found that mutation of Ser^{385} to alanine had no effect on PTEN stability, whereas Torres and Pulido [39] reported decreased stability of either this mutant or a mutant of the other major PTEN phosphorylation site identified here, Ser^{370}. It is not obvious how to explain this discrepancy. However, our data indicate that CK2 can phosphorylate secondary sites on PTEN when the primary sites are mutated (Figs. 1B and 2B); conceivably, this could occur to different extents in different cell systems (perhaps dependent on the levels of expression of PTEN and the activity of CK2). Regardless, our results emphasize the danger of relying on mutagenesis approaches alone for the identification of phosphorylation sites and/or for inferring the consequences of phosphorylation.

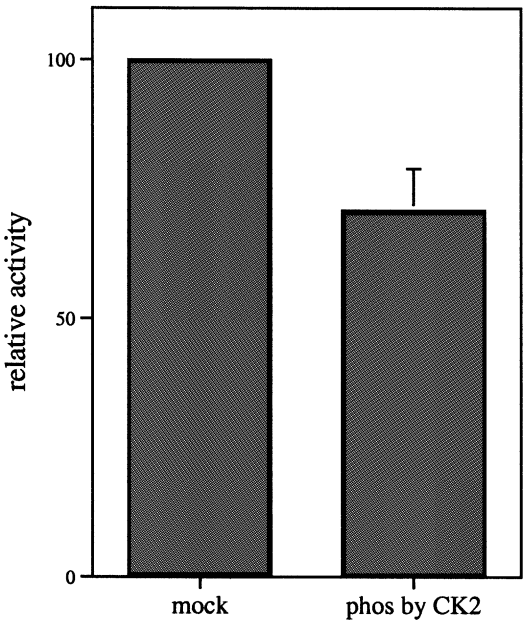

Fig. 4. CK2 phosphorylation suppresses PTEN activity. Following phosphorylation by CK2, PTEN activity against radiolabeled PIP3 substrate was assessed. Shown is the mean and standard error of seven independent experiments; each experiment was normalized such that the activity of unphosphorylated PTEN is set at 100%. The stoichiometry of phosphorylation of PTEN was approximately 2 mol P/mol PTEN. The difference in activity of phosphorylated PTEN compared to unphosphorylated PTEN is highly significant ($P < 0.0005$; Student's t-test).

3.2. CK2 and PTEN physically associate

To test whether CK2 and PTEN can physically associate, we incubated GST-PTEN with U87 cell lysates, collected bound proteins on glutathione agarose beads and performed CK2 immunoblots. A small amount of CK2 bound to GST-PTEN, but not to GST in this assay (Fig. 3A). CK2 interacted similarly with catalytically inactive PTEN (C124S) and PTEN mutants that had one or more of the preferred CK2 phosphorylation sites altered (Fig. 3A and data not shown). We asked whether CK2 and PTEN associate in vivo by co-transfecting expression plasmids for each into 293-ϕnx cells and performing reciprocal co-immunoprecipitations. Indeed, CK2 (HA-tagged) could be detected in PTEN immunoprecipitates and PTEN (GST-tagged) could be detected in CK2 immunoprecipitates (Fig. 3B, C). Thus, PTEN and CK2 can form a complex in vitro and in vivo. However, despite multiple attempts, we did not detect significant co-immunoprecipitation of endogenous PTEN and CK2; whether this reflects inadequacy of our reagents or a low-affinity interaction between PTEN and CK2 as observed with most enzyme/substrate complexes, remains unclear.

3.3. Phosphorylation by CK2 inhibits PTEN activity

Following phosphorylation by CK2, the ability of PTEN to dephosphorylate PIP3 was reduced by approximately 30% (Fig. 4) compared with PTEN subjected to a mock kinase reaction without CK2. These results argue that phosphorylation of PTEN by CK2 directly suppresses PTEN catalytic activity. Because, as indicated above, mutation of the preferred CK2 sites results in new (probably non-physiological) sites of CK2 phosphorylation, we could not assess whether phosphorylation of Ser^{370}, or Ser^{385}, or both is responsible for inhibition of PTEN activity.

How phosphorylation inhibits PTEN remains unclear. An intriguing possibility is raised by the acidic nature of CK2 phosphorylation sites and PTEN's physiological substrate, PIP3. The PTEN catalytic domain is designed specifically to accommodate highly negatively charged residues [33], and the only reasonable in vitro peptide substrate of PTEN (polyGlu-Tyr) is highly negatively charged [22]. This suggests that the phosphorylated tail of PTEN (368-DVpSDNEPDHYRYS-DTTDpSDPENE-390) may serve as a pseudosubstrate.

Although phosphorylation by CK2 affects PTEN's activity directly, phosphorylation also could affect PTEN's cellular localization and/or its ability to associate with other proteins. PTEN has been shown to interact via its extreme C-terminus with PDZ domain-containing proteins, including members of the MAGUK family. The sites that are phosphorylated by CK2 are in fairly close proximity (15–30 amino acids) to the PDZ domain interacting residues and indeed, recent data suggest that mutations within this region can affect PTEN interactions with PDZ-containing proteins [41,42].

3.4. CK2 regulates the Akt pathway in a PTEN-dependent fashion

Our results, together with previous studies [38,39], strongly suggest that CK2 is a physiologically relevant PTEN kinase and that CK2-mediated phosphorylation inhibits PTEN function. To assess the functional relevance of phosphorylation of PTEN by CK2, PTEN+/+ and PTEN−/− fibroblast lines were treated with the structurally unrelated, CK2-selective inhibitors apigenin or emodin. Since Akt phosphorylation is negatively regulated by PTEN, phospho-Akt (pAkt) levels were used as an indirect measure of PTEN bioactivity. As expected, cells lacking PTEN (Fig. 5A, B; left lanes) had constitutively high pAkt. Importantly, there was no change in pAkt following inhibition of CK2 with either apigenin (Fig. 5A) or emodin (Fig. 5B). In contrast, PTEN+/+ cells

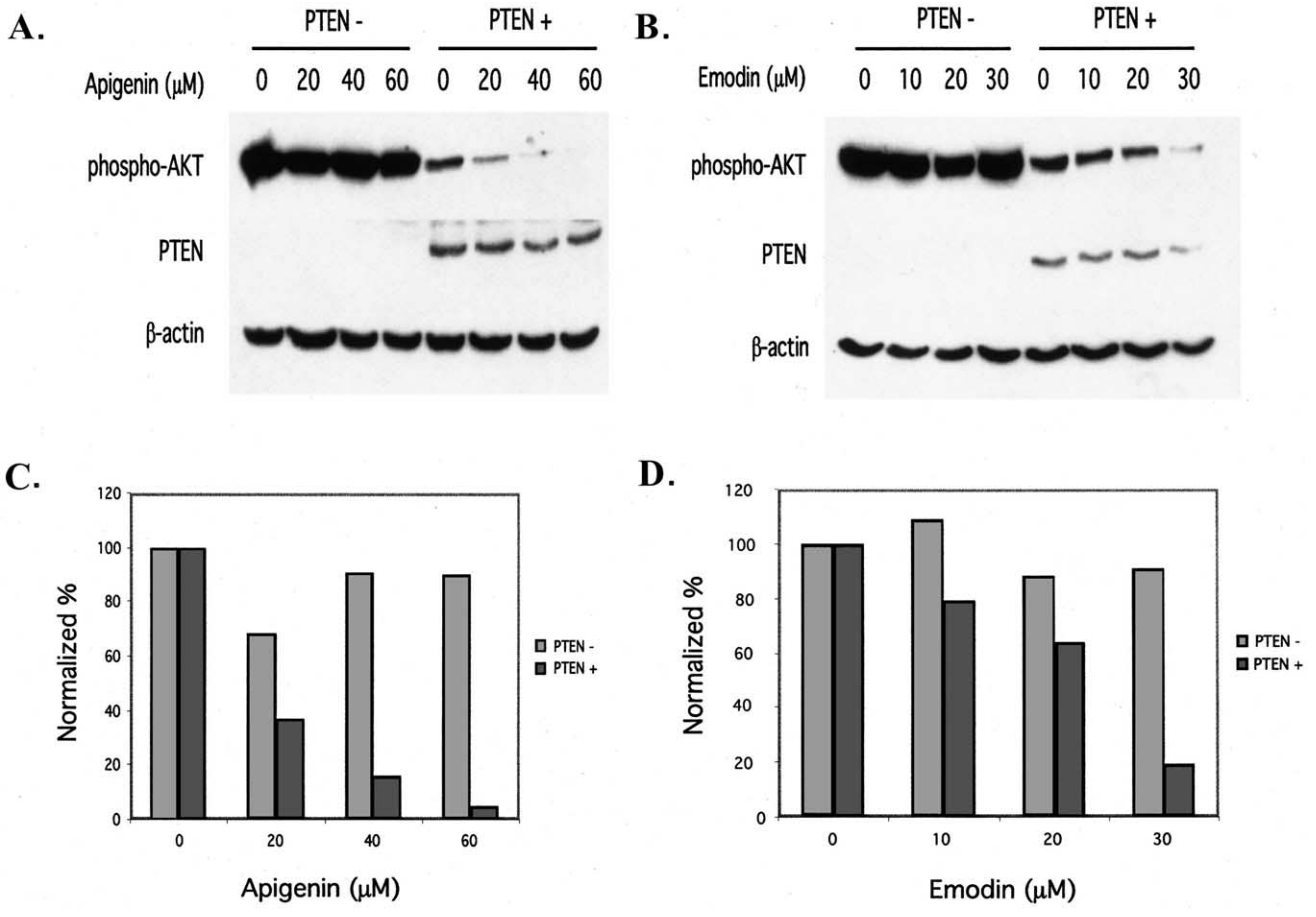

Fig. 5. Inhibition of CK2 reduces pAkt levels in a PTEN-dependent manner. Randomly growing PTEN−/− or PTEN+/+ fibroblasts were incubated with increasing doses of the selective CK2 inhibitors apigenin (A) or emodin (B). After 3 h, cells were lysed and immunoblotted for the indicated antigens. Densitometry was performed and pAkt levels were normalized to β-actin and compared to cells with no drug treatment set at 100% (C and D). Note that PTEN−/− cells have no immunoreactive PTEN and although their basal pAkt levels are much higher than in PTEN+/+ cells, they are not altered in response to the CK2 inhibitors. In contrast, PTEN+/+ cells have lower basal levels of pAkt, and pAkt is further reduced in a dose-dependent manner in response to CK2 inhibition. These data are consistent with a model in which CK2 inhibits PTEN in vivo, and relief of CK2-mediated inhibition leads to increased PTEN activity and consequently decreased Akt activity.

(Fig. 5A, B; right lanes) had lower basal levels of pAkt, and pAkt levels decreased further with apigenin (Fig. 5A) or emodin treatment (Fig. 5B). At the highest drug doses, a reduction in PTEN protein level was observed. Thus, CK2 can regulate Akt activity, but only in the presence of wild-type PTEN. These data strongly support the hypothesis that CK2 regulates PTEN activity and, possibly, stability in vivo (which in turn regulates PIP3 levels and thereby Akt phosphorylation). The effect of CK2 on PTEN stability is reminiscent of the stabilizing effect of CK2-mediated phosphorylation of β-catenin [50] and c-myc (Channavajhala and Seldin, submitted), and raises the possibility that a general role for CK2-mediated phosphorylation may be to regulate target protein stability.

CK2 is upregulated in many human cancers, as well as in cattle infected with the parasite *Theileria parva*. Transgenic expression of CK2 in lymphocytes [49,51] or the mammary gland [52] promotes neoplastic transformation, supporting a causal role for increased CK2 activity in oncogenesis. CK2 is purported to have multiple cellular targets and to affect many fundamental cell processes (reviewed in [53]) but the key proteins that mediate oncogenesis by CK2 remain unknown. Interestingly, lymphomagenesis in CK2 transgenic mice is accelerated by co-expression of the CK2 substrates myc [51] or by loss of p53 [54]. Our results, together with those of earlier workers, strongly suggest that PTEN is another key target of CK2-mediated oncogenesis. In this regard, CK2 over-expression might be expected to potentiate tumorigenesis in a heterozygotic PTEN+/− background. Moreover, by inactivating endogenous PTEN, CK2 over-expression might bypass the need for PTEN inactivation in tumors that typically express wild-type PTEN, such as breast carcinomas [55]. Determination of whether CK2 over-expression or PTEN absence, but not both, occur in the same tumor samples should clarify this issue and determine the importance of this newly identified regulation of PTEN.

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