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# Negative Regulation of PKB/Akt-Dependent Cell Survival by the Tumor Suppressor PTEN

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## Summary

**PTEN is a tumor suppressor with sequence homology to protein tyrosine phosphatases and the cytoskeletal protein tensin. *mPTEN*-mutant mouse embryos display regions of increased proliferation. In contrast, *mPTEN*-deficient immortalized mouse embryonic fibroblasts exhibit decreased sensitivity to cell death in response to a number of apoptotic stimuli, accompanied by constitutively elevated activity and phosphorylation of protein kinase B/Akt, a crucial regulator of cell survival. Expression of exogenous PTEN in mutant cells restores both their sensitivity to agonist-induced apoptosis and normal pattern of PKB/Akt phosphorylation. Furthermore, PTEN negatively regulates intracellular levels of phosphatidylinositol (3,4,5) trisphosphate in cells and dephosphorylates it in vitro. Our results show that PTEN may exert its role as a tumor suppressor by negatively regulating the PI3′K/PKB/Akt signaling pathway.**

## Introduction

Loss of heterozygosity on human chromosome 10 is the most common genetic alteration in the development of malignant gliomas (Bigner et al., 1998). Recent identification of *PTEN* (also known as *MMAC1* and *TEP1*) (Li and Sun, 1997; Li et al., 1997; Steck et al., 1997) as the candidate tumor suppressor gene located on chromosome 10 initiated a number of studies that have demonstrated a significant rate of mutations of the *PTEN* gene not only in gliomas (Rasheed et al, 1997) but also in endometrial (Tashiro et al., 1997), prostate (Cairns et al., 1997), and breast cancers (Rhei et al., 1997). In addition to frequent mutations in sporadic tumors, germline mutations of *PTEN* are believed to cause Cowden disease (Liaw et al., 1997), an autosomal-dominant hamartoma syndrome, which results in increased risk for the development of breast, thyroid, and brain tumors (Eng, 1998).

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The *PTEN* gene encodes a 403-amino-acid polypeptide with a high degree of homology to protein tyrosine phosphatases and tensin (Li et al., 1997; Steck et al., 1997). PTEN is capable of dephosphorylating both phospho-tyrosine and phospho-serine/threonine-containing synthetic substrates in vitro (Myers et al., 1997). Recent evidence also demonstrates the ability of PTEN to dephosphorylate position D3 of phosphatidylinositol (3,4,5) trisphosphate, a direct product of phosphatidylinositol 3′ kinase (PI3′K) activity (Maehama and Dixon, 1998). The importance of an intact PTEN phosphatase domain for its tumor suppressor function is underscored by findings that a large proportion of tumor-associated PTEN mutations map to the region encoding the phosphatase domain (Rasheed et al., 1997; Marsh et al., 1998). Furthermore, unlike wild-type PTEN, catalytically inactive PTEN is unable to suppress growth and tumorigenicity of PTEN-deficient glioblastoma cells (Furnari et al., 1997).

The N-terminal domain of PTEN shows extensive homology to tensin, a protein associated with the actin cytoskeleton at focal adhesions (Lo et al., 1994). Recent evidence demonstrates that overexpressed PTEN interacts directly with focal adhesion kinase (FAK) and reduces its tyrosine phospholylation. In addition, PTEN was shown to inhibit cell migration, integrin-mediated cell spreading, and formation of focal adhesions (Tamura et al., 1998). Thus, both genetic and biochemical evidence has implicated PTEN in the regulation of several different cellular processes: cell growth, extracellular matrix interactions, and/or cell migration.

To determine the role of the PTEN in normal development and tumorigenesis, we have generated *mPTEN*-deficient mice by targeted disruption of exon 3 to 5. Homozygous *mPTEN*$^{3\text{-}5}$ mutant mice die by day 9.5 of development and show abnormal patterning and overgrowth of the cephalic and caudal regions (A. S. et al., submitted). Analysis of *mPTEN*$^{3\text{-}5}$ embryos reveals regions of increased proliferation. In contrast, immortalized mouse embryonic fibroblast cell lines from *mPTEN*$^{3\text{-}5}$ homozygous mutant embryos show decreased sensitivity to various apoptotic stimuli, including UV irradiation, heat shock, osmotic stress, and tumor necrosis factor $\alpha$ stimulation. *mPTEN* mutant cells display constitutively elevated activity of PKB/Akt, which can be restored to normal levels by reexpression of mPTEN. Consistent with these findings, an active mutant of PKB/Akt suppresses apoptosis induced by mPTEN overexpression. Our results demonstrate that PTEN negatively regulates intracellular levels of PI(3,4,5)$P_3$, most likely via direct dephosphorylation, providing a mechanism for PTEN action. We propose that PTEN exerts its role as a tumor suppressor by negatively regulating the PI′3K/PKB/Akt signaling pathway.

## Results

### Enhanced Proliferation in E8.5 *mPTEN*$^{3\text{-}5}$ Mutant Embryos

*mPTEN*-deficient mice were generated by targeted disruption of exon 3 to 5 of the murine *PTEN* gene. Homozy-

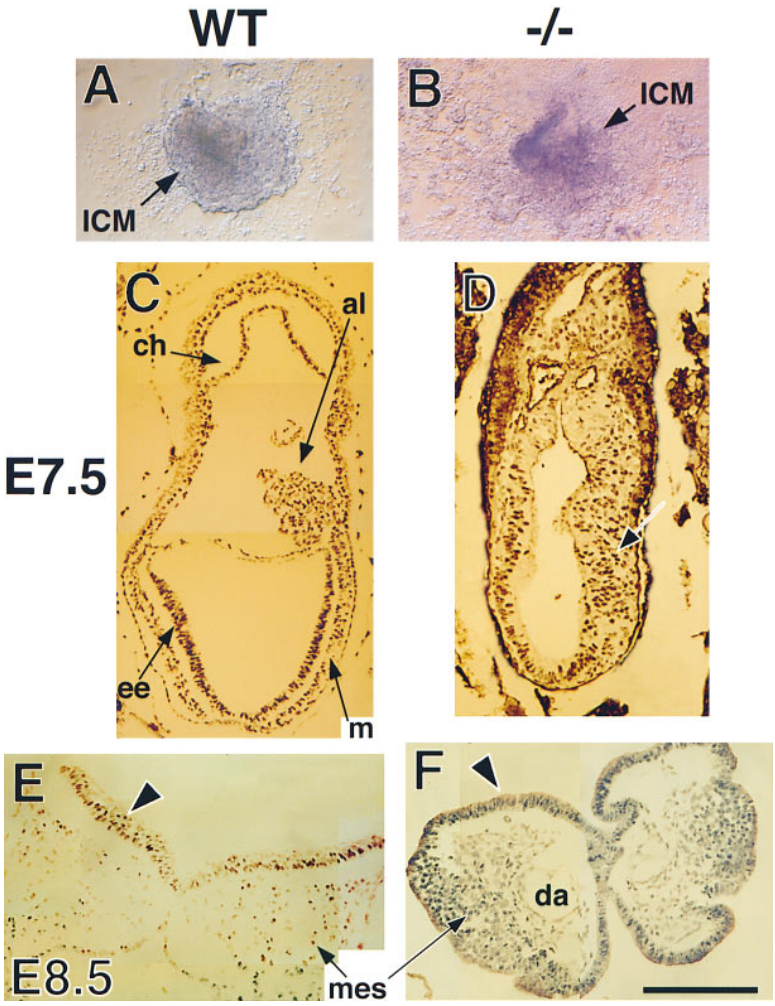


Figure 1. Extensive Proliferation of *mPTEN*$^{3-5}$ Mutant Embryos In Vitro and In Vivo

(A) Wild-type blastocyst outgrowth after 10 days of culture. The inner cell mass (ICM) has grown extensively and remains coherent.

(B) *mPTEN*$^{3-5}$ mutant blastocyst showing a large ICM that spreads out in the dish.

(C) E7.5 wild-type embryo. Strongly BrdU-positive cells can be seen throughout all embryonic regions. al, allantois; ch, chorion; ee, embryonic ectoderm; m, mesoderm.

(D) E7.5 *mPTEN*$^{3-5}$ mutant embryo showing strong BrdU incorporation and a very thick ectoderm region (arrow).

(E) Cross section through the headfold region of a E8.5 wild-type embryo, showing BrdU staining in the neuroepithelium (arrowhead) and underlying mesenchyme (mes).

(F) Cross section of the headfold of a *mPTEN*$^{3-5}$ mutant embryo. Very strong staining can be seen in the neuroepithelium (arrowhead) and underlying mesenchyme. Bar, 40 μm in (A) and (B); 140 μm in (C)–(F).

gous *mPTEN*$^{3-5}$ mutant mice die by day 9.5 of development (E9.5), and, at E8.5, a typical phenotypic feature of the mutants is dramatic overgrowth in the cephalic and caudal regions (A. S. et al., submitted), suggesting a possible defect in programmed cell death or cellular proliferation. Apoptosis, as measured by TUNEL assay (Gavrieli et al., 1992) or DAPI staining, showed no obvious differences between wild-type and mutant embryos (data not shown).

Next, we analyzed the proliferative capacity of *mPTEN*$^{3-5}$ mutant embryos in vitro and in vivo. E3.5 blastocysts cultured in vitro organize into a compact inner cell mass and a surrounding trophoblast (Figure 1A). *mPTEN*$^{3-5}$ mutant blastocysts grow similarly to wild-type littermates for a period of up to 10 days, although they tend to appear more expanded and disorganized relative to normal littermates (Figure 1B). To measure directly the effects of the *mPTEN*$^{3-5}$ mutation on cellular proliferation, we examined the incorporation of 5-bromo-2′-deoxyuridine (BrdU) into DNA during the S phase of the cell cycle. Analysis was performed at E7.5 and E8.5, at times when the mitotic activity in the mouse embryo undergoing organogenesis is high (Snow, 1977). A proliferative index based on the ratio of proliferating cells (BrdU-positive nuclei) to total cell number was calculated. Wild-type embryos showed extensive BrdU labeling, particularly in the ectoderm region (Figure 1C). A total of three wild-type embryos were analyzed, and they showed a proliferative index of 0.7 ± 0.1. *mPTEN*$^{3-5}$ mutant embryos appear more primitive and show very strong BrdU staining in all germ layers (Figure 1D). Three mutant embryos analyzed gave an index of 0.8 ± 0.1. At E8.5, the differences in proliferation between wild-type and mutant embryos were apparent. Figure 1E shows BrdU staining in the cephalic region of a E8.5 wild-type embryo. BrdU-positive cells appear scattered throughout the neuroepithelium of the head folds and underlying cephalic mesenchyme. Three wild-type embryos were analyzed at E8.5, and they gave a proliferative index of 0.7 ± 0.05. In contrast, *mPTEN*$^{3-5}$ mutant embryos show BrdU staining throughout the deformed head folds and in the cephalic mesenchyme that is significantly stronger than in wild-type embryos (Figure 1F). Three mutant embryos analyzed gave an index of 0.9 ± 0.03. These values were statistically significantly different ($p < 0.05$, Student's t test). Thus, BrdU incorporation is quantitatively increased in mutant embryos, suggesting that enhanced proliferation is involved in the abnormal development of the cephalic region of *mPTEN*$^{3-5}$ mutant embryos. Similarly, increased BrdU incorporation was

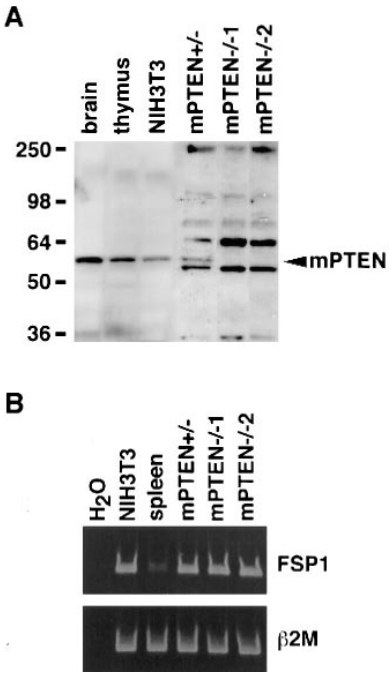


Figure 2. Characterization of Immortalized Mouse Embryonic Fibroblasts from mPTEN Mutant Mice

(A) Western blot analysis of whole-cell lysates from brain, thymus, and mPTEN$^{+/-}$ and mPTEN$^{-/-}$ cell lines. The positions of molecular weight markers (kDa) are shown on the left. The position of PTEN is indicated by an arrow.

(B) RT-PCR analysis of mPTEN$^{+/-}$ and mPTEN$^{-/-}$ cell lines. FSP1 indicates the reactions performed using fibroblast-specific protein 1–specific primers. β2M indicates the reactions performed using β2-microglobulin-specific primers. $H_2O$, water control (no RNA); spleen, total RNA from normal mouse splenocytes; mPTEN$^{+/-}$, total RNA from mPTEN$^{+/-}$ cells; mPTEN$^{-/-}$1, total RNA from mPTEN$^{-/-}$1 cells; mPTEN$^{-/-}$2, total RNA from mPTEN$^{-/-}$2 cells.

observed in the allantois at the posterior end of the embryo (data not shown).

### *mPTEN*-Deficient Mouse Embryonic Fibroblasts Exhibit Decreased Sensitivity to Apoptosis

To further investigate the role of PTEN in regulation of cellular processes, immortalized mouse embryonic fibroblast cell lines from two *mPTEN*[3-5] homozygous mutant embryos (mPTEN$^{-/-}$1 and mPTEN$^{-/-}$2, respectively) and one heterozygous mutant embryo (mPTEN$^{+/-}$) were generated. Reverse transcription–polymerase chain reaction (RT-PCR) using RNA from generated cells shows the expression of fibroblast-specific protein 1 (FSP1) (Strutz et al., 1995) in all three cell lines, demonstrating that the generated cells are fibroblasts (Figure 2). Immunoblotting of cell lysates from the established cell lines confirmed the loss of mPTEN expression in both mPTEN$^{-/-}$1 and mPTEN$^{-/-}$2 cell lines, whereas mPTEN$^{+/-}$ expressed a protein of the molecular mass of approximately 55 kDa (Figure 2). The apparent molecular mass of mPTEN is consistent with a major immunoreactive species identified in brain, thymus, and NIH3T3 cells (Figure 2) and of recombinant mPTEN (data not shown). Both mPTEN$^{-/-}$ cell lines showed no morphological differences in comparison to their heterozygous counterpart (data not shown).

Surprisingly, we observed no significant difference in the rate of proliferation between the *mPTEN* homo- and heterozygous cells (data not shown). We next examined the rate of agonist-induced cell death in heterozygous and mPTEN-deficient cells. As measured by viability dye staining, more than 80% of mPTEN$^{-/-}$1 and mPTEN$^{-/-}$2 cells were viable 24 hr following treatment with UV irradiation (780 J/m$^2$), sorbitol (0.4 mM), TNFα (10 ng/ml)/cycloheximide (5 μg/ml), anisomycin (10 μg/ml), or 30 min of heat shock at 42.5°C (Figure 3A). In contrast, these same treatments resulted in extensive cell death in mPTEN$^{+/-}$ cells. Strikingly, even 1 day after a 2 hr heat shock, almost 40% of mPTEN$^{-/-}$ cells were still viable (Figure 3A). Similar results were obtained using early and late markers of programmed cell death, annexin V, and propidium iodide, respectively, suggesting that the mPTEN$^{+/-}$ cells were dying from apoptosis (data not shown). To further test this possibility, 8 hr after treatment, nuclear morphology of mPTEN$^{+/-}$ and mPTEN$^{-/-}$ cells was examined. Nuclei of mPTEN$^{+/-}$ cells display chromatin condensation indicative of apoptosis, whereas nuclei of mPTEN$^{-/-}$ appear normal (Figure 3B). Prolonged monitoring of mPTEN$^{-/-}$ cells indicates that they eventually die in response to UV irradiation and sorbitol treatment (Figure 3C), as well as other apoptotic stimuli (data not shown), although at a significantly reduced rate in comparison to the mPTEN$^{+/-}$ cells. A similar decrease in sensitivity to apoptosis was observed in mPTEN-deficient ES cells (data not shown). Thus, reduced sensitivity of mPTEN-negative cells to apoptosis suggests that PTEN might exert its function as a tumor suppressor by negatively regulating cell survival.

### Expression of Exogenous mPTEN in *mPTEN*-Deficient Cells Restores Sensitivity to Apoptosis

To further investigate whether resistance to apoptosis in mPTEN$^{-/-}$ cells was a result of the loss of PTEN function, mPTEN was expressed in all three cell lines using recombinant retrovirus carrying an HA-tagged copy of wild-type mPTEN, or green fluorescence protein (GFP) as a control. As measured by fluorescence of control cells, the efficency of retroviral infection was higher than 70% in all three cell lines (data not shown). In contrast to GFP, expression of mPTEN in mPTEN-deficient cell lines restored normal apoptosis in response to all stimuli tested (Figure 4). Retrovirus-mediated expression of mPTEN in mouse NIH 3T3 cells also increased their sensitivity to cell death (data not shown), confirming the role of PTEN as a negative regulator of cell survival.

### Increased Activity of Cell Survival Kinase PKB/Akt in PTEN Mutant Cells

One of the key regulatory molecules involved in the regulation of cell survival is protein kinase B/Akt (PKB/Akt). PKB/Akt is implicated in protection from apoptosis in response to several growth factors and cytokines, and it protects cells from apoptosis induced by withdrawal of survival signals, c-Myc overexpression (Kauffmann-Zeh et al., 1997), or matrix detachment (Khwaja et al., 1997;

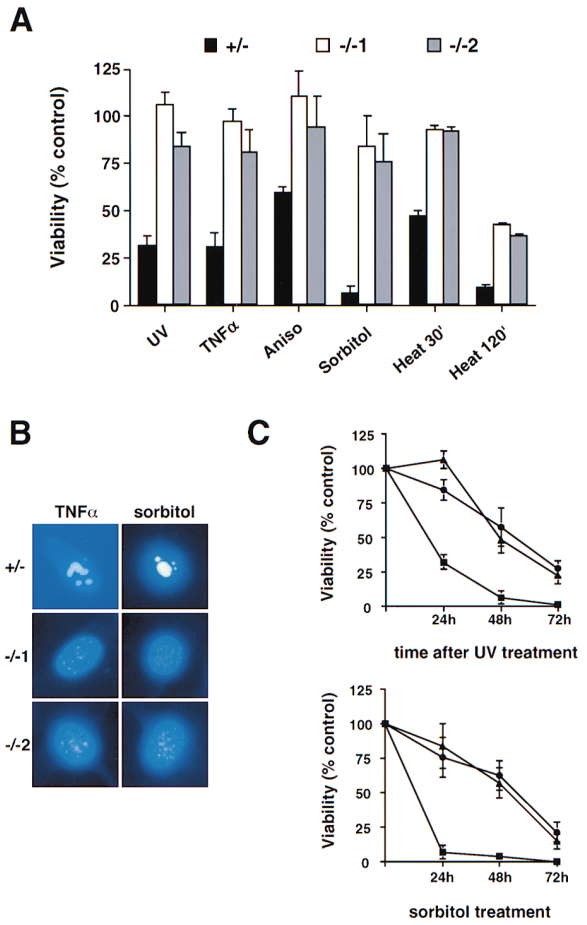


Figure 3. PTEN-Deficient Cells Show Decreased Sensitivity to Apoptosis

(A) PTEN-deficient cells are resistant to apoptosis. Twenty-four hours after the indicated treatment. UV, UV irradiation (780 J/m$^2$); TNFα, TNFα (10 ng/ml)/cycloheximide (5 μg/ml); Aniso, anisomycin (10 μg/ml); sorbitol (400 μM); heat 30', 30 min at 42.5°C; heat 120', 120 min at 42.5°C; viable cells were determined by negative staining with 7-amino-actinomycin D (7-A-AD). Viability is expressed normalized to the untreated control (5% FCS). Solid bars correspond to mPTEN$^{+/-}$; open bars correspond to mPTEN$^{-/-}$1; gray bars correspond to mPTEN$^{-/-}$2. The presented data are from at least four independent experiments. Error bars represent standard error of the mean expressed in percentage.

(B) Fluorescence microscopy visualization of nuclear DAPI staining of treated mPTEN$^{+/-}$ and mPTEN$^{-/-}$ cells. Cell line annotations are on the left. Treatments (same as in [A]) are indicated on the top. Chromatin condensation is apparent solely in the mPTEN$^{+/-}$ cell line.

(C) PTEN-deficient cells show decreased sensitivity to apoptosis. The viability of cells treated as indicated was monitored for 3 days as in (A). Squares correspond to mPTEN$^{+/-}$; triangles correspond to mPTEN$^{-/-}$1; circles correspond to mPTEN$^{-/-}$2. The presented data are from at least three independent experiments. Error bars represent standard error of the mean expressed in percentage.

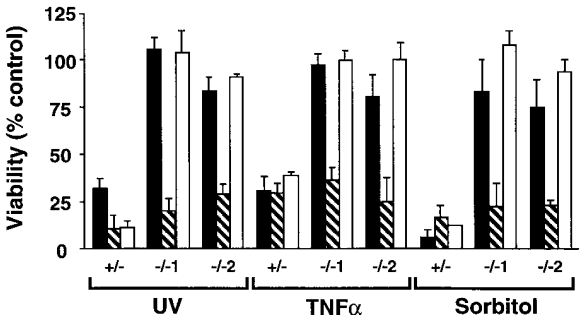


Figure 4. Expression of PTEN Restores Susceptibility of PTEN-Deficient Cells to Agonist-Induced Apoptosis

Two days following infection with retroviruses carrying PTEN (hatched bars) or GFP (open bars), cells were treated as indicated and analyzed for viability as in Figure 3. Viability is expressed normalized to the untreated control (5% FCS). Solid bars correspond to noninfected cells. The individual cell lines and cell treatments, respectively, are indicated at the bottom. The presented data are from at least three independent experiments. Error bars represent standard error of the mean expressed in percentage.

reviewed in Downward, 1998). Furthermore, loss of PKB/Akt function in *Drosophila* results in ectopic cell death (Staveley et al., 1998). Phosphorylation of PKB/Akt on Thr-308 and Ser-473 is required for its full activation (Alessi et al., 1997; Stokoe et al., 1997). Using an antibody specific for PKB/Akt phosphorylated at Ser-473 and kinase assays of immunoprecipitated PKB/Akt proteins, we tested the phosphorylation status and activity of PKB/Akt in our cells (Figure 5A). Under resting (5% FCS) or serum-starvation (0.5% FCS) conditions, the levels of PKB phosphorylation were elevated in both mPTEN$^{-/-}$ cell lines in comparison to mPTEN$^{+/-}$ cells. Hyperphosphorylation of PKB/Akt was accompanied by increased activity of PKB/Akt toward a previously characterized PKB/Akt-specific peptide substrate (Cross et al., 1995) (Figure 5A). Stimulation of serum-starved cells with platelet-derived growth factor (PDGF) (50 ng/ml) resulted in similar increase in phosphorylation and activity of PKB/Akt in all three cell lines, demonstrating that the pathway leading to PKB/Akt activation was intact in mPTEN$^{-/-}$ cells. In addition, basal activity and PDGF-induced activation of mitogen-activated protein kinases Erk1 and Erk2 appeared normal in all three cell lines (data not shown). Pretreatment of serum-starved cells with the specific inhibitor of PI3′K, wortmannin (Figure 5A), or LY294002 (data not shown) completely eliminated basal as well as PDGF-dependent increase in PKB/Akt phosphorylation/activity in all cell lines. This result indicates that PI3′K is responsible for both increased PKB phosphorylation/activity in nontreated mPTEN$^{-/-}$ cells and activation following PDGF stimulation in all three cell lines. The reduced basal phosphorylation of PKB/Akt (Figure 5B) and activity (data not shown) in mPTEN$^{-/-}$ cells could be restored by retroviral expression of mPTEN in these cells, demonstrating a functional interaction between mPTEN and PKB/Akt and an active role of mPTEN in negative regulation of PKB/Akt phosphorylation and activity. In support of such a role is the observation of PKB/Akt hyperphosphorylation in T cell lymphomas that frequently develop in *mPTEN* heterozygous mice and are associated with the loss of heterozygocity at the *mPTEN* locus (A. S. et al., submitted).

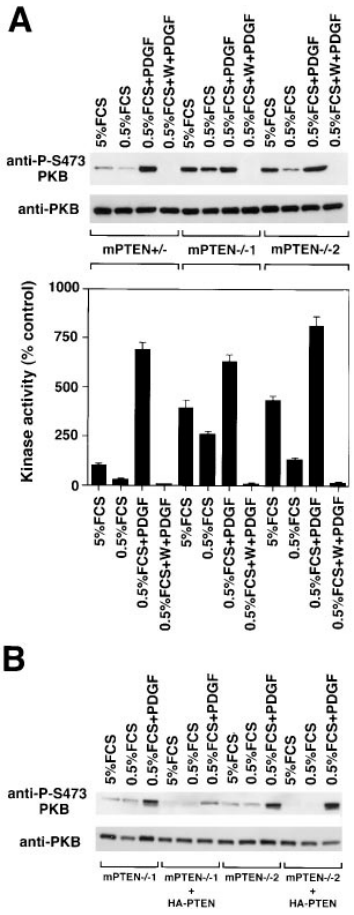


Figure 5. Negative Regulation of PI3′K/PKB/Akt Signaling Pathway by PTEN

(A) Increased phosphorylation and activity of PKB/Akt in PTEN-deficient cells. (Top) Western blot analysis of cell lysates from cells treated as indicated. 0.5% FCS indicates incubation of cells in 0.5% FCS for 24 hr prior to treatment. PDGF indicates treatment with PDGF (50 ng/ml) for 15 min. W indicates pretreatment of cells with wortmannin (100 nM) for 20 min prior to addition of PDGF. Antibodies used in Western blotting are indicated on the left. Comparable results were obtained from at least four independent experiments. (Bottom) PKB kinase activity in mPTEN$^{+/-}$ and mPTEN$^{-/-}$ cell lines. PKB proteins were immunoprecipitated from cells treated as indicated (see above) and analyzed for incorporation of $^{32}PO_4$ into a previously characterized peptide substrate (see Experimental Procedures). The data are presented normalized to the activity of PKB/Akt from untreated (5% FCS) mPTEN$^{+/-}$ cells. The presented data are from at least three independent experiments. Error bars represent standard error of the mean expressed in percentage.

(B) Expression of PTEN reduces basal phosphorylation of PKB/Akt in mPTEN$^{-/-}$ cells. Forty-eight hours after mock infection or infection with the retrovirus expressing HA-PTEN, cells were treated as indicated on top (see [A]). Antibodies used in Western blotting are indicated on the left. Comparable results were obtained from at least three independent experiments.

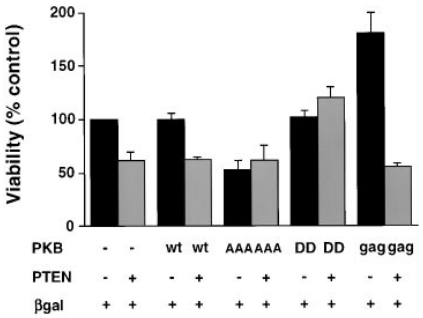


Figure 6. PTEN and PKB/Akt Act in the Same Signaling Pathway

NIH 3T3 cells were transfected with constructs expressing the indicated proteins (bottom) in the presence of tracer amounts of a β-gal construct. PKB/Akt alleles are as follows: wt, wild-type; AAA, alanine-substituted dominant-negative mutant; DD, aspartate-substituted constitutively active mutant; gag, gag-PKB/Akt fusion protein (see text for details). Forty-eight hours posttransfection cells were fixed and stained with X-gal. The number of surviving blue cells was counted in ten randomly selected high-power fields under a microscope and expressed as a percentage relative to the control transfection. The presented data are from at least three independent experiments. Error bars represent standard error of the mean expressed in percentage.

### A Constitutively Active Mutant of PKB/Akt Rescues Cells from PTEN-Induced Apoptosis

Expression of mPTEN using transient transfection of NIH 3T3 cells resulted in a decrease in the number of surviving transfected cells in comparison to the mock-transfected controls, indicating that high levels of mPTEN expression can result in cell death (Figure 6). Using a cotransfection approach, we examined the ability of wild-type or mutant PKB/Akt to alter mPTEN-induced cell death in NIH 3T3 cells 48 hr after transfection. Expression of wild-type PKB/Akt had no influence on mPTEN-mediated cell death (Figure 6). However, coexpression of PKB-DD, a constitutively active PKB/Akt mutant in which Thr-308 and Ser-473 have been changed to aspartate to mimic phosphorylation (Welch et al., 1998; Jing Jin and James R. Woodgett, personal communication), with mPTEN increases the number of surviving transfected cells to the levels comparable to mock-transfected cells (Figure 6). Thus, expression of constitutively active PKB/Akt rescues transfected cells from mPTEN-induced apoptosis and indicates a potential function of mPTEN upstream of PKB/Akt.

PKB-AAA is a dominant-negative PKB/Akt mutant in which both phosphorylation sites and the lysine in the ATP-binding pocket have been mutated to alanine residues, respectively, resulting in a complete loss of kinase activity (Meier et al., 1997; Stephens et al., 1998; Jing Jin and James R. Woodgett, personal communication). Expression of PKB-AAA induced apoptosis in NIH 3T3 cells comparable to the levels achieved by mPTEN transfection alone (Figure 6). Significantly, coexpression of PKB-AAA and mPTEN did not result in an additive effect on cell death (Figure 6), suggesting that PKB and mPTEN act in the same signaling pathway.

The role of mPTEN in PKB/Akt regulation is further supported by the effect of mPTEN on gag-PKB-mediated cell survival. *gag-PKB* is the oncogenic form of *PKB/Akt* that was identified as an oncogene carried by AKT8, the acute transforming virus from a rodent T cell lymphoma (AKR) (Bellacosa et al., 1991). The myristyl group of viral gag protein serves to bring gag-PKB fusion protein to the cell membrane, where it becomes activated by upstream kinases (Ahmed et al., 1993; Kennedy

et al., 1997; Alessi and Cohen 1998). In our studies, transfection of *gag-PKB* into NIH 3T3 cells resulted in an increase in the number of surviving cells, possibly due to the ability of gag-PKB to protect from transfection-induced death (Figure 6). However, cotransfection of mPTEN with gag-PKB resulted in a decrease in the number of transfected cells comparable to that induced by mPTEN alone (Figure 6). Considering that gag-PKB requires phosphorylation of Thr-308 and Ser-473 for activity (Kennedy et al., 1997), mPTEN is most likely negatively regulating the phosphorylation of both PKB and gag-PKB, consistent with our previous findings (Figure 6).

### Phosphatidylinositol (3,4,5)$P_3$ Is a Direct Substrate of PTEN

The activity of PI3′K under normal or PDGF-stimulation conditions was identical in mPTEN$^{+/-}$ and mPTEN$^{-/-}$ cells (data not shown), suggesting that mPTEN was affecting the PKB/Akt phosphorylation and activity by acting downstream of PI3′K. Treatment with wortmannin, a specific inhibitor of PI3′K, sensitized mPTEN$^{-/-}$ cells to UV irradiation– and sorbitol treatment–induced cell death (Figure 7A), demonstrating that an immediate product of PI3′K activity was responsible for the altered sensitivity of mPTEN$^{-/-}$ cells to apoptosis. Furthermore, constitutively active PKB/Akt rescues transfected cells from mPTEN-induced apoptosis, implying a function of mPTEN upstream of PKB/Akt.

PKB/Akt phosphorylation on both Thr-308 and Ser-473 is directly dependent on the levels of intracellular phosphatidylinositol(3,4,5)$P_3$, the key product of PI3′K activity (Alessi et al., 1996, 1997; M. Delcommene et al., submitted). In mPTEN$^{-/-}$ cells, PKB/Akt is hyperphosphorylated under resting conditions (5% FCS) (Figure 5A). Under those conditions, mPTEN$^{-/-}$ cells exhibit elevated levels of intracellular PI(3,4,5)$P_3$ in comparison to their heterozygous counterpart (Figure 7B), implying a direct role of mPTEN in dephosphorylation of PI(3,4,5)$P_3$. Furthermore, bacterially expressed mPTEN dephosphorylates the D3 position of PI(3,4,5)$P_3$ in vitro (Figure 7C). Thus, PI(3,4,5)$P_3$ is a physiological substrate of PTEN.

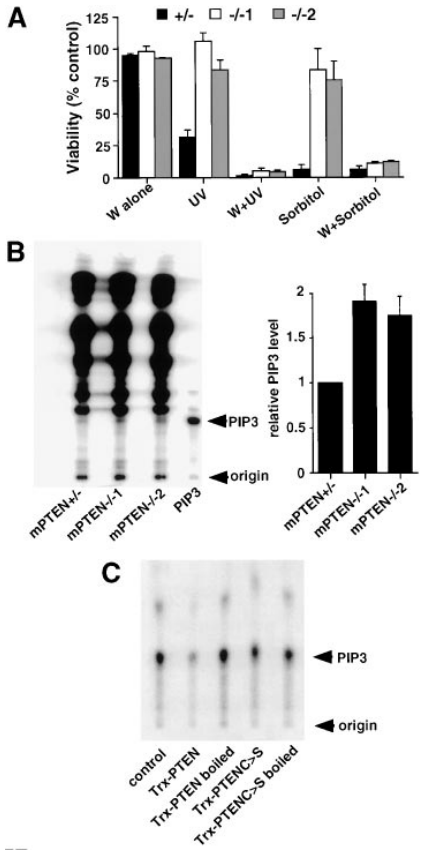


Figure 7. PTEN Dephosphorylates PI(3,4,5)$P_3$

(A) Wortmannin restores the sensitivity of mPTEN$^{-/-}$ cells to apoptosis. Twenty-four hours after the indicated treatment. W, wortmannin (50 nM); UV, UV irradiation (780 J/m$^2$); sorbitol (400 μM); viable cells were determined by negative staining with 7-amino-actinomycin D (7-A-AD). Viability is expressed normalized to the untreated control (5% FCS). Solid bars correspond to mPTEN$^{+/-}$; open bars correspond to mPTEN$^{-/-}$1; hatched bars correspond to mPTEN$^{-/-}$2. The presented data are from at least three independent experiments. Error bars represent standard error of the mean expressed in percentage.

(B) Lack of PTEN increases intracellular levels of PI(3,4,5)$P_3$. Total cellular lipids were extracted from cells metabolically labeled with $^{32}$P-orthophosphate as described in Experimental Procedures and resolved by ascending thin-layer chromatography together with PI(3,4,5)$P_3$ prepared as described in Experimental Procedures. Source of lipid preparations is indicated at the bottom. Quantification of relative levels of PI(3,4,5)$P_3$ is shown on the right; PI(3,4,5)$P_3$ content relative to total cellular lipid was normalized to the relative amount of PI(3,4,5)$P_3$ in mPTEN$^{+/-}$ cells. Positions of the origin and PI(3,4,5)$P_3$ (PIP3) are indicated. The presented data are from at least three independent experiments. Error bars represent standard error of the mean.

(C) PTEN dephosphorylates PI(3,4,5)$P_3$ in vitro. PI(3,4,5)$P_3$ was incubated with 0.5 mg of purified wild-type PTEN protein (Trx-PTEN) or catalytically inactive mutant of PTEN (Trx-PTENC>S) as indicated, extracted, and resolved by ascending thin-layer chromatography. Positions of the origin and PI(3,4,5)$P_3$ (PIP3) are indicated.

## Discussion

### Role of PTEN in Proliferation

*mPTEN$^{3-5}$* mutant embryos die by E9.5 with abnormal patterning and extensive overgrowth of the cephalic and caudal regions. At E7.5 and more dramatically at E8.5, BrdU incorporation is increased in *mPTEN$^{3-5}$* mutants. In the head, proliferation is enhanced in the neuroepithelium of the cephalic neural folds and in the underlying mesenchyme. Increased proliferation in *mPTEN$^{3-5}$* embryos is consistent with the ability of ectopic PTEN to suppress cell growth (Furnari et al., 1997, Cheney et al., 1998). Nuclear integrity dye staining of *mPTEN$^{3-5}$* embryos does not reveal any alterations in apoptosis associated with the *mPTEN* loss. In contrast, *mPTEN*-deficient fibroblasts display no increase in proliferation but decreased sensitivity to apoptosis. This apparent discrepancy between the phenotypes of *mPTEN$^{3-5}$* embryos and cells could be explained by the fact that programed cell death during early embryogenesis is a rare event (Coucovanis and Martin, 1995), preventing, using

the available methods, the detection of a potential decrease in apoptosis in *mPTEN*$^{3-5}$ embryos. Hence, hyperproliferation in *mPTEN*$^{3-5}$ embryos could be a consequence of a differentiation defect associated with decreased apoptosis due to *mPTEN* deficiency. In support of such a view, immunostaining of wild-type mouse embryos demonstrated that tissue distribution of mPTEN coincides with regions where differentiation occurs (data not shown). Alternatively, immortalized embryonic fibroblasts derived from *mPTEN*$^{3-5}$ embryos might not be analogous to the affected cells involved in the overgrowth in the *mPTEN*$^{3-5}$ embryo and therefore might not display an equivalent proliferation defect.

### Role of PTEN in Apoptosis

Loss of *mPTEN* in immortalized mouse embryonic fibroblasts results in decreased sensitivity to cell death in response to diverse apoptotic stimuli. The effect of *mPTEN* loss on apoptosis appears to be pleiotropic considering that mPTEN$^{-/-}$ cells no longer die in response to a number of treatments known to affect a variety of different signaling pathways. Such a broad role of mPTEN in regulation of cell survival suggests that mPTEN functions in a general pathway that regulates cell death and survival. The evidence presented here identifies a crucial regulator of cell survival, PKB/Akt, as a major target of mPTEN activity. mPTEN regulates phosphorylation and activity of PKB/Akt in cells. Furthermore, expression of a constitutively active mutant of PKB/Akt rescues cells from PTEN-induced apoptosis. Activation of PKB/Akt has been implicated in protection from apoptosis in response to several growth factors and cytokines, including IGF-1 (Kulik et al., 1997), IL-2 (Ahmed et al., 1997), and NGF (Ulrich et al., 1998). Moreover, elevated PKB/Akt activity protects cells from apoptosis induced by withdrawal of survival signals (reviewed in Downward, 1998), c-*myc* overexpression (Kauffmann-Zeh et al., 1997), UV irradiation (Kulik et al., 1997), CD95/Fas-induced cell killing (Hausler et al., 1998), and matrix detachment (Khwaja et al., 1997). Decreased sensitivity of *mPTEN* mutant cells to apoptosis in response to TNF$\alpha$, sorbitol, and anisomycin suggests that active PKB/Akt also protects cells from death induced by these agents.

Activation of SAPK/JNK, whose function in induction and protection from apoptosis remains controversial (Kyriakis and Avruch, 1996; Nishina et al., 1997), was normal in our cells in response to UV irradiation, anisomycin, and sorbitol (data not shown), indicating that this pathway is not affected by the loss of *mPTEN*. Furthermore, TNF$\alpha$ stimulation of all three cell lines resulted in a comparable level of phosphorylation of I$\kappa$B (data not shown), suggesting that NF-$\kappa$B activation in our mutant cells was intact and is therefore an unlikely cause of their altered cell death. A recent report (Tamura et al., 1998) has identified direct interaction between focal adhesion kinase (FAK) and PTEN and demonstrated a role of PTEN in regulation of cell migration and spreading. Even though our preliminary evidence does not reveal any differences in matrix attachment between mPTEN$^{+/-}$ and $^{-/-}$ cells (V. S., unpublished observations), it will be interesting to utilize our mPTEN-deficient cells to further characterize the interaction between FAK and PTEN in vivo.

### PI(3,4,5)$P_3$ Is a Physiological Substrate of PTEN

The intact PI3′K activity in mPTEN$^{-/-}$ cells and the ability of wortmannin to sensitize mPTEN$^{-/-}$ cells to agonist-induced apoptosis suggested that the mechanism of mPTEN action might be at the level of the products of PI3′K activity. The evidence presented here shows that mPTEN negatively regulates the intracellular levels of PIP(3,4,5)$P_3$, most likely via direct dephosphorylation, resulting in decreased phosphorylation and activity of PKB/Akt. Consistent with our findings, a recent report (Maehama and Dixon, 1998) has shown that PTEN is capable of dephosphorylating PI(3,4,5)$P_3$ in vitro and down-regulating its levels in insulin-stimulated 293 cells. Taken together, our results establish PI(3,4,5)$P_3$ dephosphorylation as a major mechanism of PTEN action. Thus, in the absence of mPTEN, elevated levels of PI(3,4,5)$P_3$ result in increased PKB/Akt phosphorylation. Conversely, expression of mPTEN negatively regulates PKB/Akt phosphorylation/activity via dephosphorylation of PIP(3,4,5)$P_3$.

### The Role of *PTEN* in Tumorigenesis

*PTEN* mutations have been identified in a large number of different tumors, indicating that PTEN regulates fundamental cellular processes. Our results provide evidence for such a critical role of PTEN in control of normal cell growth. In certain tumors, loss of *PTEN* may result in decreased sensitivity of mutant cells to apoptotic stimuli. In other tumors, mutations of PTEN may lead to an increase in proliferative potential. Both survival and proliferative advantages of *PTEN*-deficient cells would promote cellular overgrowth and tumorigenesis.

*PTEN* mutations in humans have initially been identified in glioblastomas (Li et al., 1997). Frequent amplification of platelet-derived growth factor receptor (*PDGFR*) and epidermal growth factor receptor (*EGFR*) genes have also been demonstrated in glioblastoma (Chaffanet et al., 1992; Smits and Funa, 1998). Furthermore, the production and cellular response to fibroblast growth factor (FGF) are often elevated in astrocytomas and gliomas (Takahashi et al., 1992). PI3′K is regulated by the activity of receptor-tyrosine kinases and is potentially up-regulated in cells overexpressing PDGFR and EGFR, as well as in cells exposed to higher than normal levels of FGF. Hypothetically, this would result in increased levels of intracellular PI(3,4,5)$P_3$ and elevated activity of PKB/Akt, giving the cells a survival advantage. The ability of PKB/Akt to protect cerebral neurons from apoptosis supports such a scenario (Dudek et al., 1997). Moreover, increased expression of PDGFR and EGFR, or FGF overproduction in glioblastoma, could result in increased proliferation. Thus, other genetic alterations in glioblastoma leading to PI3′K activation might have similar phenotypic consequences on tumor development as the loss of PTEN function.

v-*akt*, an oncogenic form of PKB/Akt resulting in increased kinase activity, causes mouse T cell lymphoma

(AKR) (Staal and Hartley, 1988; Bellacosa et al., 1991). Interestingly, mice heterozygous for *mPTEN* frequently develop T cell lymphomas that are associated with the loss of heterozygosity at the *mPTEN* locus (A. S. et al., submitted). These tumors exhibit elevated phosphorylation of PKB/Akt in comparison to normal tissue, consistent with our findings that PTEN negatively regulates PKB/Akt signaling. In humans, overexpression of PKB/Akt has been demonstrated in 15% ovarian (Cheng et al., 1992), 12% of pancreatic (Cheng et al., 1996), and 3% of breast cancers (Bellacosa et al., 1995). Significantly, in addition to mutations shown in other tumors, LOH in the human *PTEN* locus has been observed in ovarian, pancreatic, and breast tumor specimens (Teng et al., 1997). Such correlation in the rate of LOH of *PTEN* and up-regulation of PKB/Akt in human cancer, together with the evidence presented here, emphasizes the importance of PTEN/PKB/Akt interaction in tumor progression.

Our results suggest that *PTEN* belongs to a class of "gatekeeper" tumor suppressor genes together with *p53*, *Rb*, and *APC*. These genes participate in regulation of normal cell growth, and their mutations directly contribute to the neoplastic growth of the cell (Kinzler and Vogelstein, 1998). We propose that negative regulation of PI3′K/PKB/Akt cell survival signaling pathway is a likely mechanism by which PTEN exerts its tumor suppressor function. Thus, loss of *PTEN* results in general alteration of the normal cell homeostasis due to increased survival potential, which could subsequently lead to tumorigenesis. Further investigations, including those aimed at elucidating modes of PTEN regulation, will provide valuable insight into the processes of cell transformation and tumorigenesis.

## Experimental Procedures

### BrdU Labeling of Mouse Embryos

Generation of *mPTEN*$^{3\text{-}5}$ mutant embryonic stem cells and mice will be described elsewhere. BrdU labeling of cells in the S phase of the cell cycle was performed according to the protocol described by Hayashi et al. (1988). BrdU (100 mg/g of body weight) was injected intraperitoneally into pregnant females at E7.5 and E8.5 of gestation. Females were sacrificed 1 hr after injection, the uteri removed, and complete deciduas or embryos fixed in 4% paraformaldehyde at 4°C overnight and processed for immunohistochemistry. The sections were incubated with an anti-BrdU monoclonal antibody (Boehringer Mannheim) at a 1:10 dilution. Staining was performed according to the protocol described by Mishina et al. (1995).

### Establishment of Immortalized Embryonic Fibroblast Cell Lines

Day 8.5 heterozygous and homozygous *mPTEN*$^{3\text{-}5}$ embryos (A. S. et al., submitted) were dissected, minced well by scissors, and cultured individually in gelatinized 24-well plates containing Dulbecco modified Eagle's medium (DMEM), 10% fetal calf serum (FCS), L-glutamine, and β-mercaptoethanol. After 7 days of culture, cells were trypsinized, replated, incubated for 1 hr, and washed three times with PBS to remove nonadherent cells. Adherent cells were continuously cultured for 3 months using the 3T3 protocol (Goldman, 1998). Embryo genotyping was performed by PCR with DNA from the yolk sac, using specific primer sets to detect wild-type and the mutant mPTEN allele, respectively.

### RT-PCR

Total RNA was prepared from $5 \times 10^6$ cells using TRIZOL reagent as per manufacturer's protocols (Life Technologies). Intron-spanning oligonucleotide primer pairs specific for mouse β2-microglobulin (sense primer 5′-ATGGCTCGCTCGGTGACCCTAG-3′; antisense primer 5′-TCATGATGCTTGATCACATGTCTCG-3′) or mouse fibroblast-specific protein-1 (sense primer 5′-CAGCGAAAGAGGGTGAC AAGTTCA-3′; antisense primer 5′-ATGTGCGAAGAAGCCAGAGTA AGG-3′) were used to amplify 1 μg of total RNA employing the Titan one-tube RT-PCR system as per manufacturer's instructions (Boehringer Mannheim/Roche). Reaction products were resolved by 6% polyacrylamide gel electrophoresis and detected by ethidium bromide staining.

### Cloning, Expression, and Purification of mPTEN

A cDNA encoding the entire mouse PTEN open reading frame was amplified from mouse brain mRNA by RT-PCR as above using sense primer 5′-GGATCCGACATGACAGCCATCATCAAAG −3′ and antisense primer 5′-CTCGAGTCAGACTTTTGTAATTTGTGAATG CTG-3′, sequenced, and subcloned in-frame with two N-terminal influenza virus hemagglutinin (HA) epitopes into the eukaryotic expression vector pcDNA3.1 (Invitrogen). An EcoRI-XhoI fragment from the resultant pcDNA3.1HA2mPTEN vector, encoding N-terminally HA-tagged mPTEN open reading frame, was ligated into the EcoRI-SalI sites of the retroviral vector pBabeMN-z (a kind gift of Dr. Garry Nolan). Full-length mPTEN cDNA and a fragment encoding a C-terminal portion of mPTEN (amino acids 151–403 of SwissProt O08586) were separately cloned into the thioredoxin-hexahistidine tag (Trx) fusion vector pET32a (Novagen) and transfected into *E. coli* strain BL21(DE3)pLysS or BL21(DE3), respectively (Stratagene, La Jolla, CA).

Expression of Trx-mPTEN and Trx-mPTEN(151–403) fusion proteins was induced in bacterial cultures at an $OD_{600nm}$ of 0.8 by the incubation in the presence of 0.8 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 hr at 37°C or 6 hr at 28°C, respectively. Cells were lysed by sonication in lysis buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 20 mM imidazole, 10% glycerol, 5 mM β-mercaptoethanol, 0.5% Nonidet P-40, and protease inhibitors), cell lysate was clarified by centrifugation, and supernatents passed through nickel-nitrilotriacetic acid resin (Qiagen, Valencia, CA). Resin was washed with lysis buffer, and hexahistidine-tagged fusion protein was eluted using an imidazole concentration gradient. Eluted Trx-mPTEN(151–403) protein was dialysed against storage buffer (50 mM Tris-HCl [pH 8.0], 2 mM EDTA, 2 mM DTT, 150 mM NaCl) and concentrated by ultrafiltration.

Eluted Trx-mPTEN fusion protein was dialysed against ion-exchange column equilibration buffer (25 mM Tris-HCl [pH 8.0], 2 mM EDTA, and 2 mM DTT) and passed through a Q-Sepharose FF ion exchange column (Pharmacia Biotech, Inc.) equilibrated in the same buffer. The column was washed with 10 column bed volume of equilibration buffer, and Trx-mPTEN protein was eluted using 20 column bed volume of a 0–500 mM NaCl concentration gradient. The Trx-mPTEN containing fractions were pooled, dialysed against storage buffer (25 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 mM DTT), and concentrated by ultrafiltration.

### Generation of Anti-PTEN Antibody

Rabbit antisera was raised against purified Trx-mPTEN(151–403) protein (Antibodies, Inc., Davis, CA) and purified by preadsorption on Trx-coupled CNBr-Sepharose 4B followed by affinity purification on GST-PTEN fusion protein coupled to CNBr-Sepharose 4B. Low-affinity antibody was eluted in a pH 5.0 buffer, and high-affinity, PTEN-specific antibody (used in this paper) was eluted off the affinity column with 0.1 M glycine (pH 2.5).

### Cell Lysis

For the generation of cytoplasmic cell lysates, cells were washed in ice-cold PBS and lysed by incubation on ice in a buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10% glycerol, 1% Triton X-100, 50 mM NaF, 100 μM sodium vanadate, 1 mM benzamidine, and 5 μg/ml leupeptin. The lysates were centrifuged and supernatants analyzed.

For the generation of whole-cell lysates, the tissues were homogenized in a buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM $MgCl_2$, 1 mM EGTA, 0.5 mM CHAPS, 10% glycerol, 50 mM NaF, 100 μM sodium vanadate, 1 mM benzamidine, and 5 μg/ml leupeptin and

incubated on ice for 30 min. The lysates were centrifuged and supernatants analyzed.

**Western Blot Analysis**

Proteins were resolved by 12.5% SDS-PAGE, electroblotted to PVDF membrane (Boehringer Manheim), blocked in 4% skim milk, 1× PBS, 0.05% Tween-20, and probed with primary antibodies. Anti-PTEN is described above; anti-phosphoS473 PKB/Akt and anti-PKB/Akt are from New England Biolabs. Following incubation with horseradish peroxidase-conjugated goat anti-rabbit antibody (Amersham), bound immunoglobulins were detected using enhanced chemiluminescence (Amersham).

**Analysis of Cell Death**

For analysis of cell death $3 \times 10^5$ cells were plated and treated as indicated on the following day. Twenty-four hours after treatment, detached cells were collected, the remaining cells trypsinized, combined, and cell viability determined by negative 7-amino-actinomycin D staining (Schmid et al., 1994).

For analysis of chromatin condensation, cells were grown in chamber slides (Nunc), treated as indicated, fixed in freshly prepared 4% paraformaldehyde, and stained with DAPI (1 μg/ml). Mounted cells were imaged using a fluorescent microscope.

**Transient Transfection and Retroviral Infection**

Transient transfections of NIH 3T3 cells was performed using Lipofectamine (GIBCO-BRL) as per manufacturer's instructions. Briefly, $10^6$ cells were plated on 10 cm dishes and transfected the following day with a total of 10 μg of DNA mixed with the liposomes with the constructs indicated in figure legends. Retroviruses expressing HA-tagged PTEN were packaged within the ϕNX-Eco cell line and mPTEN$^{+/-}$ and mPTEN$^{-/-}$ cells infected as previously described (Hitoshi et al., 1998).

**Kinase and Phosphatase Assays**

PKB/Akt proteins were immunoprecipitated using an anti-PKB/Akt antibody (New England Biolabs) and analyzed for activity as described previously (Cross et al., 1995), except that the peptides were separated by tricine-SDS gel electrophoresis. Phosphorylated peptides were visualized by the PhosphorImager (Molecular Dynamics, Inc.) and incorporation of $^{32}P$ quantified using ImageQuant (MDI) software.

PI(3,4,5)$P_3$ labeled with $^{32}P$ at position D3 was generated as described previously (Sasaki et al., 1996). Phosphatase assays were performed by incubation of $^{32}P$-labeled PI(3,4,5)$P_3$ with 0.5 μg of purified Trx-PTEN or Trx-PTENC>S in a buffer containing 50 mM Tris (pH 7.5), 50 mM NaCl, 2 mM DTT for 30 min at 30°C. The reaction was terminated by the addition of $CH_3OH/CHCl_3$/6% $HClO_4$ (30/15/2, v/v/v). The lipids were extracted and separated as described previously (Sasaki et al., 1996).

**Metabolic Cell Labeling and Lipid Extraction**

Cells ($10^6$) were plated on 10 cm glass plates. The next day the cells were incubated for 2 hr in phosphate-free RPMI 1640 with 5% dialysed FCS, followed by a 2 hr incubation with phosphate-free RPMI 1640 with 5% dialysed FCS, 0.25 mCi/ml [$^{32}P$] orthophosphate (NEN/Dupont), 10 mM HEPES (pH 7.5). Cells were washed two times with phosphate-buffered saline and quenched by addition of chloroform/methanol/8% $HClO_4$ (5:10:4). Following vigorous vortexing, chloroform/$HClO_4$ (5:5) was added to separate the organic phase, which was then washed four times in chloroform-saturated 1% $HClO_4$ before being dried. Dried lipids were resolved in chloroform/methanol (95:5) and resolved as described previously (Sasaki et al., 1996).

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