Suppression of PTEN Expression Is Essential for Antiapoptosis and Cellular Transformation by Oncogenic Ras

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Abstract

Ras is one of the most commonly mutated oncogenes in the array of human cancers. The mechanism by which Ras induces cellular transformation is, however, not fully elucidated. We present here evidence that oncogenic Ras suppresses the expression of the tumor suppressor phosphatase and tensin homologue deleted from chromosome 10 (PTEN), and this action of oncogenic Ras is mediated by the Raf-mitogen-activated protein kinase/extracellular signal–regulated kinase (ERK) kinase (MEK)-ERK pathway via up-regulation of c-Jun. $Jun^{+/+}$ cells undergo cellular transformation by oncogenic Ras, and restoration of wild-type PTEN, but not a phosphate-defective mutant of PTEN, induces apoptosis in these cells. Conversely, in $Jun^{-/-}$ cells, oncogenic Ras neither suppresses PTEN nor causes transformation, but rather it induces PTEN-dependent apoptosis. An apoptotic response to oncogenic Ras in $Jun^{-/-}$ cells can be prevented by suppressing PTEN expression. These findings imply that oncogenic Ras suppresses the apoptotic gene *PTEN* via the Raf-MEK-ERK-c-Jun pathway to induce antiapoptosis and cellular transformation. Together, our findings identify a novel molecular interface between the oncogenic and tumor suppressor pathways that regulates cellular transformation and survival. [Cancer Res 2007;67(21):10343–50]

Introduction

The tumor suppressor phosphatase and tensin homologue deleted from chromosome 10 (PTEN) is a dual specificity phosphatase that plays an important role in cell cycle arrest and apoptosis (1). As a consequence, PTEN is one of the most commonly altered genes in human cancer, with a mutation frequency approaching that of p53 (2). The *PTEN* gene is mutated in a wide variety of human cancers, most notably glioblastomas, endometrial carcinomas, melanomas, and advanced prostate adenocarcinomas. Homozygous deletion of the *PTEN* gene in mice causes embryonic lethality. PTEN heterozygous mice are highly susceptible to the development of multiple organ tumors and show defects in Fas-inducible apoptosis (3). These observations point to a role for PTEN as a critical regulator of tumorigenesis in both mice and humans.

PTEN functions primarily as a lipid phosphatase and dephosphorylates phosphatidylinositol-3,4,5-triphosphate, the second messenger produced by phosphoinositol-3-kinase (2). In doing so, PTEN negatively regulates the activity of the serine/threonine protein kinase Akt, which is involved in cell survival, apoptosis prevention, and growth stimulation of different tissues (4). Activated Akt protects cells from apoptotic death by phosphorylating and inactivating proapoptotic substrates such as Par-4 (5), BAD, procaspase-9, and the forkhead family of transcription factors (1). The antiapoptotic role of Akt accounts for its transforming potential (6) and for the resistance of cancer cells to the action of chemotherapeutic agents and ionizing radiation (7). Expression of exogenous PTEN in mutant cells restores the endogenous pattern of Akt phosphorylation as well as corresponding sensitivity to agonist-induced apoptosis. Moreover, PTEN-deficient mouse embryonic fibroblasts (MEF) exhibit decreased sensitivity to cell death in response to a number of apoptotic stimuli. This observation is accompanied by the constitutively elevated phosphorylation and activity of Akt (5, 8), thus providing *in vivo* evidence that PTEN negatively regulates cell survival. Besides the genetic alteration in the PTEN locus in certain tumors, many other cancers, such as those of the lung and thyroid, possess wild-type PTEN alleles, but expression of the *PTEN* gene is often diminished (9). Recently, we reported that the *PTEN* gene is targeted for inactivation in human tumor cells by the NF-κB cell survival pathway (10).

Ras activation is one of the most common genetic changes associated with human cancer. Activating Ras mutations are found in nearly all pancreatic cancers, one half of colon and thyroid cancers and one third of lung cancers (11). Ras is a small GTPase that acts as a molecular switch by regulating the passage of extracellular signals to intracellular pathways that control the expression of various effector genes (12). In this manner, Ras executes a regulatory effect on diverse cellular functions, such as proliferation, cytoskeletal organization, and survival (13). Oncogenic Ras, which is locked in a constitutively active (GTP-bound) state, contributes to the malignant transformation of various cell types (14). In addition to promoting cell proliferation, oncogenic Ras also shows antiapoptotic effects. In many tumors, activation of Ras correlates with very low rates of apoptosis (15). Moreover, tumor development and maintenance necessitates continued expression of activated Ras to prevent apoptosis: withdrawal of doxycycline-inducible oncogenic Ras expression in transgenic mice bearing melanomas causes apoptosis in both the melanoma and endothelial cells of the tumor (16).

Ras activates proliferation and survival/antiapoptotic signaling via the phosphatidylinositol 3-kinase (PI3K)-Akt pathway (17) and the RAF-mitogen-activated protein kinase (MAPK)/extracellular signal–regulated kinase (ERK) kinase (MEK)-ERK pathway (18). Oncogenic Ras also induces the necessary NF-κB transcriptional activity for suppressing p53-independent induction of apoptosis,

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which would typically occur on oncogenic Ras expression (19). Thus, oncogenic transformation is inseparably coupled with suppression of apoptosis. Because PTEN negatively regulates the cell survival pathway, we examined the possibility that oncogenic Ras may regulate the expression of the proapoptotic gene *PTEN*. We present evidence that oncogenic Ras effectively inhibits PTEN expression via the RAF-MEK-ERK-c-Jun pathway, and thereby prevents apoptosis to promote cellular transformation.

Materials and Methods

Cell culture. NIH 3T3 cells and MCF-7 cells were purchased from American Type Culture Collection. NIH 3T3/iRas cells (19) and NIH 3T3 cells stably expressing vector, oncogenic H-*ras* (V12), or activated-*raf* (20) were from Albert Baldwin, Jr. (University of North Carolina at Chapel Hill, NC). $p53^{+/+}$ and $p53^{-/-}$ immortalized MEFs were from Tyler Jacks (MIT, Cambridge, MA); $p65^{-/-}$ immortalized MEFs (RelA 3T3 cells) were from Michael Karin (University of California at San Diego, San Diego, CA); and c-$Jun^{+/+}$ and c-$Jun^{-/-}$ immortalized MEFs were from Keith Laderoute (SRI International, Menlo Park, CA). $PTEN^{+/+}$ and $PTEN^{-/-}$ immortalized MEFs were from Hong Wu (University of California at Los Angeles, Los Angeles, CA). Human lung cancer cells H157 and H838 were from John Yannelli (Department of Microbiology, Immunology and Molecular Genetics, University of Kentucky, Lexington, KY). Lung cancer cells were grown in RPMI 1640 with 10% fetal bovine serum. NIH 3T3 cells were grown in DMEM with 10% calf serum supplemented with penicillin (1,000 units/mL) and streptomycin (1,000 μg/mL). All other cells were grown in DMEM supplemented with 10% fetal bovine serum, L-glutamine (2 mmol/L), penicillin (1,000 units/mL), and streptomycin (1,000 μg/mL).

Plasmids and chemical reagents. The human *PTEN* gene promoter-luciferase reporter construct was a gift from Eileen Adamson (Burnham Institute, San Diego, CA). The PTEN-luc reporter construct was made by subcloning a 1,978-bp genomic DNA region upstream of human *PTEN* gene into empty pGL3-basic-luc vector (21). Wild-type c-Jun expression construct and c-Jun deletion mutant constructs TAM67 (which lacks c-Jun amino acids 3–122) and LZM1 (deletion mutant of c-Jun lacking the leucine zipper domain) were from Michael Birrer (National Cancer Institute, Frederick, MD). The GFP-PTEN wild-type and GFP-PTEN C124S mutant expression constructs were from K. M. Yamada (National Institute of Dental and Craniofacial Research, Bethesda, MD). Dominant-negative Akt1 (K179A, T308A, and S473A) was from Naoya Fujita (University of Tokyo, Tokyo, Japan). The expression construct for constitutively active Akt (myr-Akt), pSV-βgal reporter construct (Promega Corp.), and pGL-3-basic-luc reporter construct have previously been described (5, 22). Adenovirus expression vector for oncogenic Ras (61L) was from Joseph Nevins (Duke University, Durham, NC). Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Promega. Wortmannin, PD98059, LY294002, SB203580, and SP600125 were from Calbiochem-Novabiochem Corp.

Antibodies and other reagents. Mouse monoclonal antibodies for PTEN (A2B1) and H-Ras (F-235) and rabbit polyclonal antibodies for c-Fos (H125), Raf1 (C-20), ERK-2 (K-23), and Akt1/Akt2 (H-136) were from Santa Cruz Biotechnology, Inc. The monoclonal antibody for β-actin was from Sigma Chemical Corp. Rabbit polyclonal antibodies for phospho-Akt(Ser^{473}) (587F11) and phospho-ERK and rabbit monoclonal antibody for c-Jun (60A8) were from Cell Signaling Corp. The Annexin V apoptosis kit was from Clontech.

Northern blot and Western blot analyses. Cells treated with either vehicle or with IPTG were harvested after various time points for preparation of total RNA by the guanidinium isothiocyanate extraction method. Twenty micrograms of total RNA were resolved on formaldehyde-agarose gels, transferred onto nylon membranes, and subjected to Northern blot analysis by using the human PTEN cDNA or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA as probe, as previously described (23).

Whole-cell protein extracts were resolved by SDS-PAGE, transferred onto polyvinylidene difluoride membrane, and subjected to Western blot analysis for PTEN, H-Ras, Raf1, c-Jun, c-Fos, phospho-ERK, total ERK 1/2, phospho-Akt(Ser^{473}), total Akt, or β-actin with the indicated antibodies. Blots were developed by using enhanced chemiluminescence (Amersham Corp.).

Transfection, reporter assays, and adenovirus infection. Cells were transfected with the luciferase reporter and various driver plasmids at a reporter to driver ratio of 1:4, along with the β-galactosidase expression construct for an internal control. Whole-cell extracts from the transfectants were examined for luciferase activity by using the LucLite kit (from Packard Bioscience) or for β-galactosidase activity. The luciferase activity in each reaction was normalized with respect to the corresponding β-galactosidase activity and expressed as relative luciferase activity. For adenovirus infection, cells were transduced with the GFP control adenovirus and H-Ras adenovirus, as previously described (22).

Apoptosis assay. After transfection, the cells were fixed and apoptotic nuclei were revealed by 4,6-diamidino-2-phenylindole (DAPI) or terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling as previously described (5). A total of three independent experiments were done; in each experiment, ~200 cells expressing the transfected construct (GFP-positive cells only) were scored for apoptosis under a confocal microscope.

Statistical analysis. All experiments were done in triplicate to verify the reproducibility of the findings. Statistical analyses were carried out with Statistical Analysis System software (SAS Institute) and P values were calculated using the Student's t test.

Results

Oncogenic Ras down-regulates PTEN expression. To determine whether oncogenic Ras controls the expression of the proapoptotic tumor suppressor PTEN, we analyzed NIH 3T3 fibroblasts and MCF-7 breast cancer epithelial cells stably transfected with constitutively activated Ras (V12) for PTEN protein levels by Western blot analysis. Compared with vector-transfected cells, Ras-transformed cells showed dramatically reduced levels of PTEN expression (Fig. 1*A*). Similarly, PTEN levels were severely diminished in MCF-7 cells stably expressing oncogenic Ras compared with the MCF-7 cells expressing control vector (Fig. 1*A*). These initial findings indicate that Ras down-regulates PTEN in both fibroblast and epithelial cell backgrounds.

To ascertain that down-regulation of PTEN was not a consequence of random gene aberrations that occurred on transformation of the cells constitutively expressing oncogenic Ras, we tested nontransformed NIH 3T3 cells for PTEN expression following regulated induction of the *ras* oncogene. NIH 3T3/iRas cells (19), which contain a stably integrated oncogenic H-*ras* (VI2) gene under the control of an IPTG-inducible promoter, were treated with IPTG to induce oncogenic Ras expression, and PTEN expression was examined by Western blot analysis at various time points. IPTG treatment resulted in strong induction of Ras V12 expression and was accompanied by a time-dependent decrease in PTEN protein levels relative to vehicle-treated cells (Fig. 1*A*). These findings suggest that down-regulation of PTEN is an effect of oncogenic Ras activation, and not a secondary consequence associated with a transformed cell phenotype.

Most studies of oncogenic Ras function have used H-*ras* cDNA constructs that direct supraphysiologic Ras expression levels. However, K-ras is the ras family member that is most commonly mutated in human cancer (7). Because oncogenic K-*ras* mutations are frequently noticed in human non–small cell lung cancers, we determined the relative levels of PTEN protein in lung cancer cells that carry naturally occurring K-*ras* mutations compared with cancer cells with wild-type K-*ras* alleles. Diminished levels of PTEN protein were noted in H157 cells with K-*ras* mutations relative to

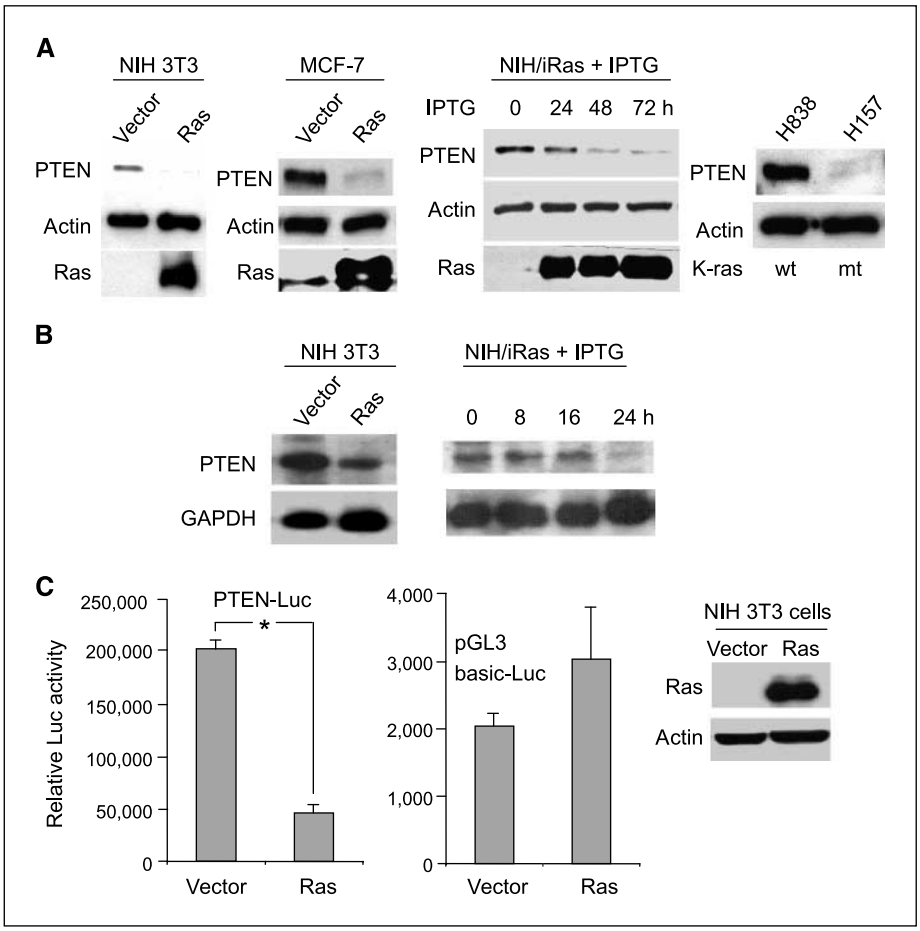

Figure 1. Oncogenic Ras down-regulates PTEN. *A,* Ras down-regulates PTEN protein. Whole-cell protein extracts from mouse fibroblast cells NIH 3T3 or human breast cancer cells MCF-7 that stably expressed either oncogenic Ras (V12) or vector for control; from NIH 3T3/iRas cells that were untreated or treated with IPTG for 24, 48 or 72 h; or from human lung cancer cells H838 (wild-type K-ras status) and H157 (mutant K-ras status), as indicated, were subjected to Western blot analysis as indicated for PTEN protein, H-Ras, or β-actin as a loading control. *B,* Ras down-regulates PTEN mRNA. Total RNA prepared from NIH 3T3 cells expressing vector or oncogenic Ras (*left*) or NIH 3T3/iRas cells that were untreated or treated with IPTG for 8, 16, or 24 h (*right*) was subjected to Northern blot analysis for PTEN and GAPDH as control. *C,* Ras represses PTEN promoter. NIH 3T3 cells were cotransfected with PTEN promoter-luc (*left*) or pGL3-basic-luc (*middle*) reporter construct, as indicated, and H-Ras (V12) expression construct or vector for control, along with β-galactosidase expression plasmid. Luciferase activity was measured after 48 h of transfection and relative luc activity, normalized to the corresponding β-galactosidase activity, is shown (*left* and *middle*). Whole-cell protein extracts prepared from the transfected cells in luciferase assay were analyzed for Ras or β-actin protein expression by Western blot analysis (*right*). *, $P < 0.0001$, significant difference in promoter activity.

H838 cells, which contain wild-type K-*ras* alleles (Fig. 1*A*), suggesting a clinical relevance to these findings.

We then assessed whether Ras activation resulted in diminished mRNA levels of PTEN by Northern blot analysis. Similar to the findings with PTEN protein, PTEN mRNA levels were diminished in Ras-transformed NIH 3T3 cells relative to control vector–transfected NIH 3T3 cells (Fig. 1*B*). Moreover, NIH 3T3/iRas cells treated with IPTG showed a marked down-regulation of PTEN mRNA at ~24 h of Ras induction (Fig. 1*B*). These findings indicate that oncogenic Ras down-regulates PTEN mRNA levels.

To determine whether Ras regulates PTEN expression at the transcription level, we cotransfected NIH 3T3 cells with a luciferase reporter construct containing an ~2-kb promoter region of the human *PTEN* gene along with a Ras V12 expression construct or an empty vector for control, and determined the effect of oncogenic Ras on PTEN promoter activity. Consistent with the changes noted in the PTEN protein and mRNA levels, Ras significantly suppressed ($P < 0.0001$) PTEN promoter activity relative to the vector control (Fig. 1*C*). In contrast, Ras did not alter the promoter activity of the control pGL3-basic-luc construct lacking the PTEN promoter region (Fig. 1*C*). Together, these results suggest that Ras down-regulated *PTEN* gene expression at the promoter level.

The Raf-MEK-ERK pathway, but not the PI3K-Akt pathway, mediates PTEN down-regulation by Ras. Ras induces cellular transformation via the MEK-ERK signaling cascade regulated by Raf1 and/or via the PI3K/Akt pathway. To determine whether these signaling pathways were responsible for down-regulation of *PTEN* gene expression, we used pharmacologic inhibitors of these downstream effector pathways. Interestingly, treatment of NIH 3T3 cells overexpressing oncogenic Ras with the MEK inhibitor PD98059 resulted in restoration of PTEN protein levels (Fig. 2*A*). As expected, the MEK inhibitor caused severe reduction of the ERK phosphorylation induced by oncogenic Ras (Fig. 2*A*). On the other hand, treatment of cells with the PI3K inhibitor wortmannin (Fig. 2*A*), LY294002 (data not shown), or the p38 MAPK inhibitor SB203580 (Fig. 2*A*) did not affect PTEN protein levels in Ras-transformed cells. Similarly, blocking c-jun NH_2-terminal kinase (JNK) activation by treating the cells with the JNK-specific inhibitor SP600125 did not result in restoration of PTEN levels in Ras-transformed cells (data not shown), suggesting the JNK pathway is not involved in PTEN down-regulation by Ras. These results reveal that MEK activation is necessary for the inhibitory effect of Ras on PTEN.

We also verified the involvement of Raf-MEK-ERK pathway by using specific downstream Ras pathway effectors, such as constitutively active forms of Raf1. Consistent with the importance of MEK in the down-regulation of PTEN by oncogenic Ras, overexpression of constitutively active Raf1 in NIH 3T3 cells showed down-regulation of PTEN protein (Fig. 2*B*), showing that activation of the Raf-ERK pathway is sufficient to cause down-regulation of PTEN protein. Consistent with the changes in the PTEN protein levels, a constitutively active MEK construct down-regulated PTEN promoter activity (data not shown), whereas constitutively active Akt (myr-Akt) caused induction of PTEN

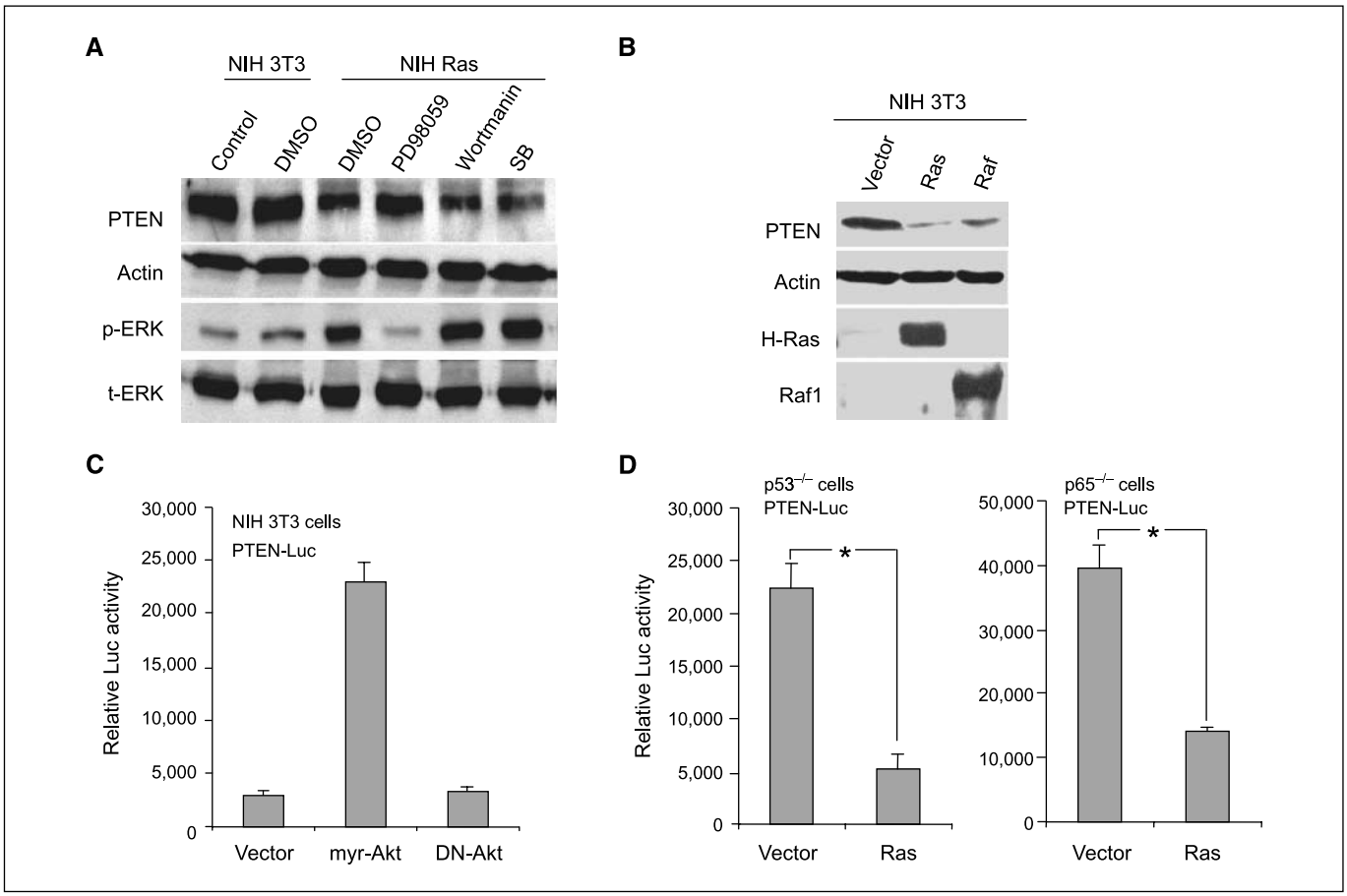

Figure 2. Raf-MEK-ERK pathway mediates down-regulation of PTEN by Ras, independent of Akt, p53, or p65/NF-κB. *A,* NIH 3T3/Ras cells were exposed to DMSO (vehicle control), PD98059 (50 μmol/L), wortmannin (2 μmol/L added at 0 and 12 h), or SB203580 (10 μmol/L) for 48 h, and whole-cell protein extracts were subjected to Western blot analysis for PTEN, Ras, phospho-ERK, total ERK, or β-actin (*A*). Whole-cell protein extracts from NIH 3T3 parent cells were loaded as controls (*A, first* and *second columns*). *B,* whole-cell protein extracts prepared from NIH 3T3 fibroblast cells expressing vector, oncogenic Ras (V12), or oncogenic Raf1 were subjected to Western blot analysis for PTEN protein, H-Ras, Raf1, or β-actin as a loading control. *C* and *D,* NIH 3T3 cells were cotransfected with PTEN promoter-luc reporter construct and constitutive Akt (myristylated Akt), dominant negative Akt (179A/308A/473A) expression construct, or vector for control, along with β-galactosidase expression plasmid (*C*). $p53^{-/-}$ (*D, left*) or $p65^{-/-}$ (*D, right*) 3T3 cells were cotransfected with PTEN promoter-luc reporter construct and oncogenic Ras expression construct or vector for control, along with β-galactosidase expression plasmid. Luciferase activity was measured after 48 h of transfection, and relative luc activity, normalized to the corresponding β-galactosidase activity, is shown. *, $P < 0.0001$, significant difference in promoter activity.

promoter activity relative to vector-transfected cells (Fig. 2*C*). Collectively, these data reveal that Ras-inducible down-regulation of PTEN is mediated through the Raf-MEK-ERK pathway.

Ras-induced down-regulation of PTEN expression is independent of p53 and NF-κB/p65. Disproportionate signaling by oncogenic Ras may provoke a p53 response designed to cause apoptosis, and thereby mitigate the threat of the oncogenic stimulus. Similarly, oncogenic Ras may induce premature senescence in primary MEFs by activation of the Arf-p53 pathway via Raf-MEK-ERK signaling (24). Oncogenic Ras has also been shown to activate the NF-κB cell survival pathway to prevent p53-independent apoptosis associated with cellular transformation (19). We have previously shown that the p65 component of NF-κB by itself can negatively regulate PTEN expression (10). We therefore examined whether p53 or p65 was involved in PTEN suppression by Ras. Thus, $p53^{-/-}$ and $p65^{-/-}$ 3T3 cells were transiently transfected with either Ras V12 expression construct or vector as control and with PTEN-Luc reporter construct, and luciferase reporter activity was measured 48 h after transfection. Both in $p53^{-/-}$ cells and in $p65^{-/-}$ cells, oncogenic Ras overexpression resulted in significant suppression ($P < 0.0001$) of PTEN promoter activity (Fig. 2*D*). These findings indicate that neither p53 nor p65 is involved in the Ras-induced down-regulation of PTEN.

c-Jun is a negative regulator of PTEN expression. To identify downstream transcription factors that effect oncogenic Ras-mediated down-regulation of PTEN, we surveyed the literature for genes that (*a*) encode transcription factors with putative consensus DNA binding sites in the PTEN promoter and (*b*) are induced by Ras via the MEK-ERK pathway. We then tested those genes for *PTEN* gene regulation. During the course of these studies, we noted that c-Jun was up-regulated in NIH 3T3/Ras cells relative to control NIH 3T3/vector cells. c-Jun is one of the key transcription factors activated by mitogenic signaling cascades, most importantly by the Ras/MAPK pathway (25), and c-Jun function is required for Ras-induced cell survival and transformation (26, 27).

Because PTEN is a proapoptotic protein, we considered the possibility that c-Jun may cause down-regulation of PTEN expression to promote cell survival. We first determined the basal expression level of PTEN in wild-type and $c\text{-}Jun^{-/-}$ 3T3 fibroblasts. When compared with the wild-type cells, c-Jun–deficient cells contained elevated levels of PTEN (Fig. 3*A*), suggesting that

endogenous c-Jun represses the basal level of *PTEN* gene expression. Consistently, ectopic overexpression of c-Jun in NIH 3T3 cells resulted in down-regulation of PTEN protein levels compared with vector-transfected cells (Fig. 3*A*). In contrast, overexpression of c-Fos did not alter the PTEN levels compared with vector-transfected cells (Fig. 3*A*). We also examined PTEN mRNA levels in c-Jun wild-type and c-Jun–deficient cells. Consistent with the data on PTEN protein, c-Jun–deficient cells showed elevated levels of PTEN mRNA compared with wild-type cells (Fig. 3*A*).

As an extension of these studies, we carried out transient transfection assays to test whether c-Jun suppresses PTEN expression at the *PTEN* promoter level. Luciferase expression directed by a 1,978-bp DNA fragment of the human *PTEN* promoter was significantly higher ($P < 0.0001$) in c-Jun$^{-/-}$ fibroblasts relative to wild-type cells (Fig. 3*B*), and ectopic reintroduction of c-Jun into c-Jun$^{-/-}$ cells resulted in suppression ($P < 0.0001$) of PTEN promoter activity (Fig. 3*B*). Similarly, blockade of endogenous c-Jun activity by TAM67, a dominant negative (transactivation domain deletion) mutant of c-Jun, resulted in significant induction ($P < 0.0001$) of *PTEN* promoter activity relative to the vector-transfected NIH 3T3 cells, whereas the c-Jun deletion mutant LZM, in which the leucine zipper domain is deleted, did not induce the PTEN promoter activity (Fig. 3*C*). These findings support the role of c-Jun as a negative regulator of PTEN expression.

c-Jun is overexpressed in Ras- or Raf-transformed cells. We next ascertained c-Jun expression in NIH 3T3 cells transformed with either oncogenic Ras or oncogenic Raf. Compared with nontransformed NIH 3T3 cells, Ras- and Raf-transformed cells were found to contain elevated levels of c-Jun protein (Fig. 3*D*). Similarly, Ras- and Raf-transformed cells showed reduced expression of PTEN (Fig. 3*D*), suggesting that c-Jun induced by the Ras-Raf pathway may mediate the suppression of PTEN.

Ras is unable to down-regulate PTEN in the absence of c-Jun. To further assess the requirement for c-Jun in PTEN suppression by Ras, wild-type or c-Jun$^{-/-}$ 3T3 cells were infected with adenoviral constructs expressing oncogenic Ras or GFP for control and, 48 h later, whole-cell extracts were subjected to

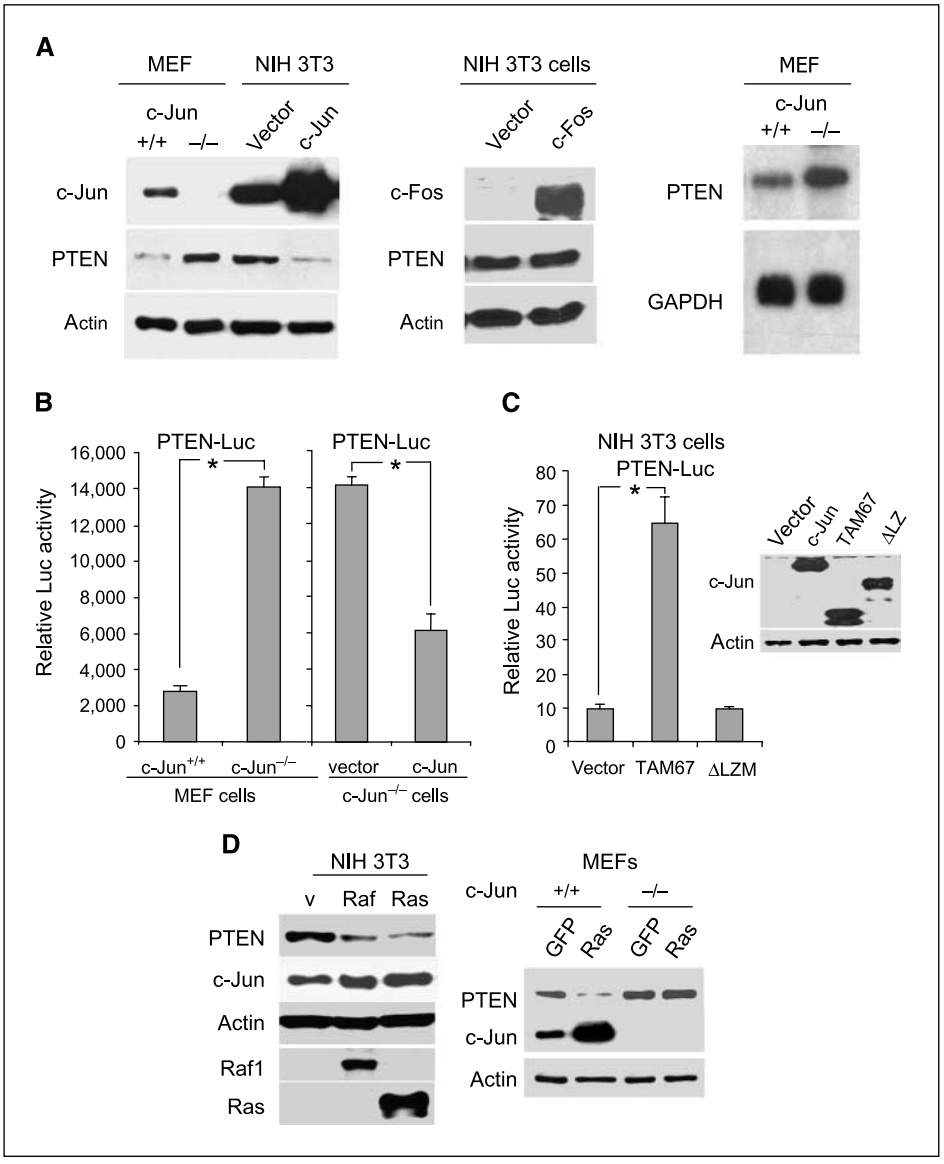

Figure 3. c-Jun down-regulates PTEN expression. *A,* c-Jun negatively regulates PTEN. Whole-cell protein extracts from c-Jun$^{+/+}$ and c-Jun$^{-/-}$ immortalized MEFs or NIH 3T3 cells transiently transfected with c-Jun, c-Fos expression constructs, or vector as control for 48 h were subjected to Western blot analysis for expression of PTEN, c-Fos, c-Jun, and actin. Moreover, total RNA prepared from c-Jun$^{+/+}$ and c-Jun$^{-/-}$ immortalized MEFs was subjected to Northern blot analysis for PTEN or GAPDH as control. *B,* c-Jun suppresses the PTEN promoter. c-Jun$^{+/+}$ and c-Jun$^{-/-}$ cells were cotransfected with the PTEN promoter-luc reporter construct along with empty vector or c-Jun expression vector. β-gal expression construct was also cotransfected to normalize the transfection efficiency. Cell lysates, prepared 48 h after transfection, were subjected to luciferase activity and β-galactosidase activity assays. Relative luc activity, normalized to the corresponding β-galactosidase activity, is shown. *C,* dominant negative c-Jun mutant induced PTEN promoter activity. NIH 3T3 cells were cotransfected with the PTEN promoter-luc reporter construct along with dominant negative c-Jun mutant (TAM67), a leucine zipper mutant of c-Jun (LZM), or vector as control. The β-gal expression construct was also cotransfected to normalize the transfection efficiency, and 48 h after transfection, cell lysates were subjected to luciferase activity and β-galactosidase activity assays. Relative luc activity, normalized to the corresponding β-galactosidase activity, is shown in arbitrary units. *, $P < 0.0001$, significant difference in promoter activity. The cell lysates were subjected to Western blot analysis for c-Jun and actin (*inset*). *D,* Ras-transformed cells show elevated levels of c-Jun protein, and Ras requires c-Jun to down-regulate PTEN. Whole-cell protein extracts prepared from NIH 3T3 fibroblast cells expressing vector, oncogenic Ras (V12), or oncogenic Raf were subjected to Western blot analysis for PTEN, H-Ras, Raf1, c-Jun, or β-actin as a loading control (*left*). c-Jun$^{+/+}$ and c-Jun$^{-/-}$ cells were infected with adenovirus constructs expressing oncogenic Ras (61L) or GFP virus, and after 48 h of infection, whole-cell protein extracts were subjected to Western blot analysis for PTEN, c-Jun, Ras, or actin (*right*).

Western blot analysis for PTEN. As seen in Fig. 3*D*, Ras strongly up-regulated c-Jun protein levels in c-Jun$^{+/+}$ cells. Importantly, Ras caused down-regulation of PTEN protein in wild-type (i.e., c-Jun$^{+/+}$ cells), but not c-Jun$^{-/-}$, cells (Fig. 3*D*). Together, these findings reveal that c-Jun up-regulation by Ras is required for PTEN down-regulation.

Restoration of PTEN inhibits Ras-induced cell survival. We analyzed the functional relevance of Ras-mediated PTEN down-regulation by determining the effect of PTEN on Ras-induced cell survival. Ras-transformed NIH 3T3 cells were transfected with constructs expressing wild-type GFP-PTEN, the C124S mutant of PTEN that lacks phosphatase activity, or GFP vector (as control), and apoptotic cell death was assessed. Overexpression of wild-type PTEN resulted in significant apoptotic cell death ($P < 0.0001$) relative to GFP-transfected cells (Fig. 4*A*). On the other hand, overexpression of GFP-PTEN C124S caused only a marginal increase in apoptotic cell death compared with GFP vector–transfected cells (Fig. 4*A*). These findings confirm that PTEN reconstitution is necessary to induce apoptosis in Ras-transformed cells.

To determine the relationship between PTEN expression and suppression of Ras-inducible transformation, wild-type MEFs (c-Jun$^{+/+}$ cells) were infected with adenoviral GFP-vector, PTEN, and oncogenic Ras (61L), separately or in combination, and transformed colonies (foci) were scored after 5 days. As seen in Fig. 4*B*, oncogenic Ras caused cellular transformation, which is denoted by the presence of foci formation. However, foci formation by oncogenic Ras was severely inhibited ($P < 0.0001$) in the presence of the PTEN adenovirus but not with the control GFP adenovirus (Fig. 4*B*), implying that PTEN restoration suppresses transformation by oncogenic Ras.

Finally, to determine whether c-Jun function, which suppresses PTEN, was necessary for cell survival in Ras-transformed cells, c-Jun$^{+/+}$ or c-Jun$^{-/-}$ MEFs were transduced with either the oncogenic Ras adenovirus or control GFP adenovirus, and apoptotic cells were scored. As seen in Fig. 4*C*, oncogenic Ras induced apoptosis in c-Jun$^{-/-}$ cells but failed to induce apoptosis in c-Jun$^{+/+}$ cells ($P < 0.0001$). Because oncogenic Ras fails to suppress PTEN in c-Jun$^{-/-}$ cells, we further explored whether apoptosis by oncogenic Ras in c-Jun$^{-/-}$ cells was dependent on basal PTEN expression. Accordingly, c-Jun$^{+/+}$ and c-Jun$^{-/-}$ cells were treated with control small interfering RNA (siRNA) or PTEN siRNA and knockdown of endogenous PTEN was confirmed by Western blot analysis (Fig. 4*C*). The cells were then transduced with the GFP control or oncogenic Ras adenovirus and apoptosis was quantified. As seen in Fig. 4*C*, c-Jun$^{-/-}$ cells treated with control siRNA readily underwent apoptosis with oncogenic Ras, but those treated with PTEN siRNA failed to undergo apoptosis with

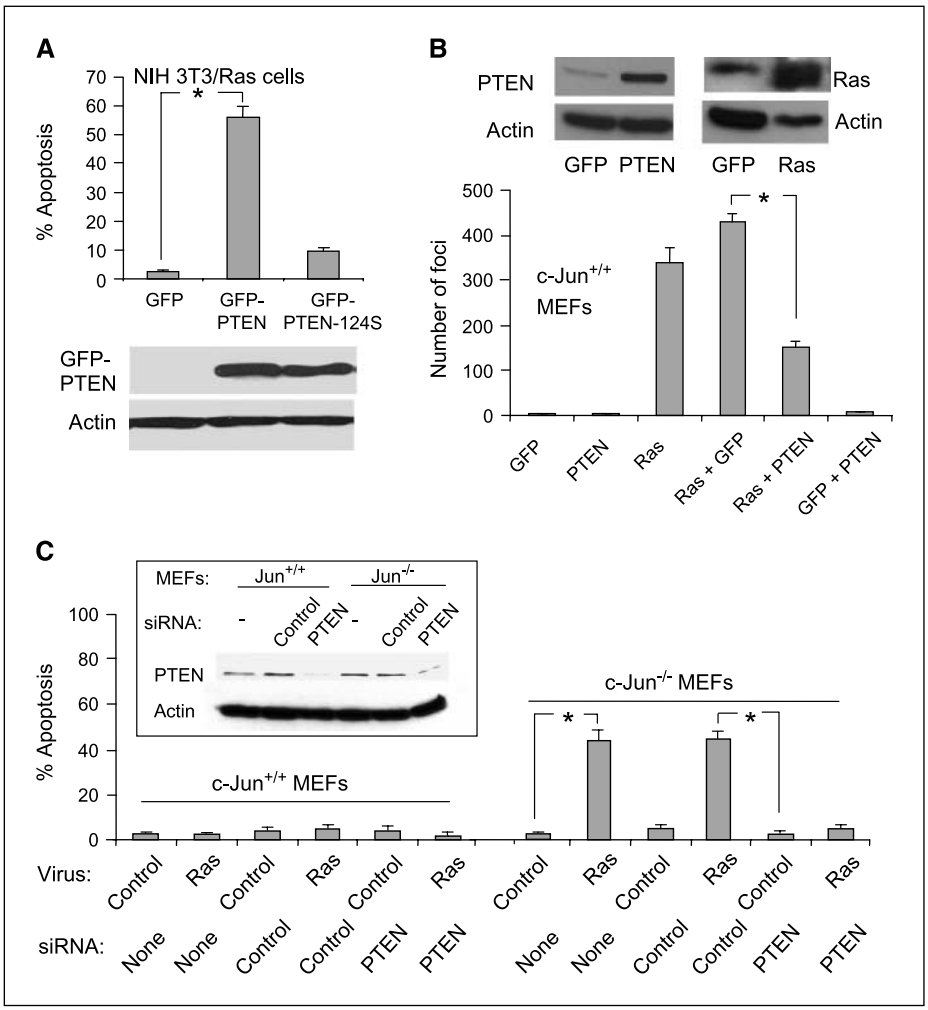

Figure 4. PTEN induces apoptosis and prevents transformation by Ras. *A,* PTEN induces apoptosis in Ras-expressing cells. NIH 3T3/Ras cells were transfected with GFP-vector, GFP-wild-type PTEN, or GFP-PTEN C124S mutant in chamber slides; 60 h after transfection, cells were stained with DAPI and apoptotic cells were scored as described in Materials and Methods. *, $P < 0.0001$, significant difference in apoptosis. *B,* PTEN prevents Ras-inducible transformation. c-Jun$^{+/+}$ cells were infected with adenoviral GFP-vector, PTEN, and oncogenic Ras (61L), separately or in the combinations indicated, and transformed colonies (foci) were scored after 5 d. Expression of the PTEN or Ras adenovirus was confirmed by Western blot analysis. *, $P < 0.0001$, significant difference in focus formation. *C,* PTEN inhibition by RNA interference prevents Ras-inducible apoptosis. c-Jun$^{+/+}$ and c-Jun$^{-/-}$ MEFs were transfected for 24 h with control or PTEN siRNA duplexes and suppression of PTEN expression was verified by Western blot analysis (*inset*). The cells were then infected with adenoviral GFP-vector or oncogenic Ras (61L) as indicated, and apoptotic cells were scored after 48 h. *, $P < 0.0001$, significant difference in apoptosis.

oncogenic Ras ($P < 0.0001$). c-Jun$^{+/+}$ did not show apoptosis in the presence of oncogenic Ras or PTEN siRNA (Fig. 4*C*). As a whole, these results indicate that oncogenic Ras confers protection from cell death by down-regulating PTEN expression via c-Jun. Thus, down-regulation of PTEN expression is critical for Ras-induced antiapoptosis action and cellular transformation.

Discussion

Tumor development favors adoption of both specific and restricted pathways of apoptosis suppression. Although several possible mechanisms for prevention of apoptosis by oncogenic Ras have been proposed, the actual mechanism of Ras-mediated antiapoptosis remains debatable. In the present study, we noted PTEN suppression by oncogenic Ras in both fibroblast and epithelial cell types and in human cancer cells. Down-regulation of PTEN by oncogenic Ras occurs at the transcriptional level and is executed through the Raf-MEK-ERK pathway. This study further identified the transcription factor c-Jun as a novel mediator of Ras-dependent PTEN suppression. Consistent with the physiologic implications of these findings, ectopic expression of PTEN in Ras-transformed cells severely impairs Ras-induced cell survival. Our findings expose a novel nonmutational mechanism by which PTEN expression may be negatively regulated in human tumors.

PTEN down-regulation by oncogenic Ras occurs in lieu of *PTEN* gene mutations in cancer. Recent mutational analyses of the *ras* proto-oncogenes and *PTEN* in several human tumors reveal an intriguing pattern. The absence of concurrent *PTEN* alteration and *N-ras* or K-*ras* mutation has been reported for melanoma cell lines (28) and endometrial cancer (29), respectively. Similarly, *PTEN* inactivation and H-*ras* activation are mutually exclusive in experimentally induced murine malignant skin carcinomas, implying that these events are functionally redundant and that coexistent alterations in both genes do not confer a greater advantage to the process of tumorigenesis (30). However, the molecular basis for these distinct and consistently exclusive mutational events was not clear. The present study reveals a likely mechanism for this mutually exclusive mutational pattern between the *ras* and *PTEN* loci in the development of these tumor types. Based on our observations, it is reasonable to speculate that abrogation of the proapoptotic function of PTEN via negative regulation of its expression by oncogenic Ras eliminates the requirement to mutate the *PTEN* gene locus, and, in settings such as those occurring in the absence of *ras* mutations wherein *PTEN* expression is not down-regulated, the *PTEN* gene locus is targeted for mutational inactivation to suppress apoptosis. This hypothesis, however, needs direct experimental validation.

Raf-MEK pathway in cell survival. The use of constitutively active mutants corresponding to downstream prosurvival effectors of the Ras pathway, such as Raf or Akt, establish the Raf-MEK pathway as being critical for PTEN suppression by oncogenic Ras. Interestingly, constitutively active Akt induced the PTEN promoter (Fig. 2), as previously reported (31). Such induction of PTEN by Akt is likely mediated through the transcription factor Egr-1 (31). Whereas the significance of PTEN induction by Akt is not clear, it may constitute a feedback response to counterbalance Akt signaling. The finding that Ras promotes cell survival by down-regulating PTEN expression through the Raf-MEK-ERK pathway underscores a critical role for this pathway in the prevention of apoptosis by oncogenic Ras. The MAPK pathway is critical for tumorigenesis in murine cells, and it is also commonly activated in human tumors (32). The Raf-MEK-ERK pathway also contributes to the ability of oncogenic Ras to provide a prosurvival function by down-regulating expression of Par-4, a proapoptotic transcriptional corepressor (33). These findings illustrate the significance of the Raf-MEK-ERK pathway in inhibition of apoptosis. In this regard, our finding that PTEN is a novel target of the Ras-Raf-MEK pathway defines yet another potential mechanism by which the ras oncogene decreases the cellular apoptotic capacity by inactivating a critical component of the cell death machinery. Moreover, our data support the view that targeting the Raf-MEK-ERK pathway may have a therapeutic advantage in certain tumor types.

Integration of survival signaling by targeting PTEN. The two most prominent downstream effector pathways induced by activated Ras, the Raf-MEK-ERK pathway and the PI3K-Akt pathway, are generally believed to operate in a co-parallel, yet independent, manner. However, down-regulation of PTEN, a PI3K-Akt antagonist, by Ras through the Raf-MEK-ERK pathway suggests that there is efficient integration of these two cell survival pathways by Ras to prevent apoptosis. This implies that Raf-MEK-ERK–inducible antiapoptosis is mediated, at least in part, by activation of the PI3K-Akt cell survival pathway via down-regulation of PTEN. Indeed, this hypothesis is supported by studies wherein dominant-negative Akt and the PI3K inhibitor wortmannin suppress the ability of Raf to promote cell survival on growth factor loss (34). Thus, our studies have identified a critical integration point for the two major pathways induced by Ras. A logical advantage of integrating these two pathways would be a bolstered antiapoptotic program essential for tumor development.

During the search for downstream transcription factors that may mediate the suppressor effect of oncogenic Ras on the PTEN promoter, we noted that c-Jun negatively regulates PTEN expression (Fig. 3) and mediates Ras-inducible suppression of PTEN transcription (Fig. 4). c-Jun is an oncogenic transcription factor that functions as a major component of the activator protein 1 (AP-1) transcription factor complexes. The expression of c-Jun is constitutively increased in many transformed cell lines and human cancers (35), and our findings imply that Ras requires c-Jun to suppress PTEN expression, which would otherwise counteract the transformation process by inducing apoptosis. Thus, our findings reveal that *PTEN* is a novel target of the Ras-Raf-MEK-MAPK-Jun survival pathway. During the preparation of this article, Hettinger et al. (36) reported that inducible expression of c-Jun promotes cellular survival via the Akt pathway by negatively regulating the expression of the tumor-suppressor PTEN. Consistent with our findings on the c-Jun/PTEN segment, they noted an inverse correlation between c-Jun and PTEN levels in a panel of human tumor cell lines and noted that c-Jun transcriptionally suppresses PTEN expression by binding to a variant AP-1 site found in the 5′ upstream sequences of PTEN promoter (36). The identification of PTEN as a target gene positioned specifically in the Ras survival pathway provides a valuable tool to better understand the role of Ras in normal development and tumorigenesis.

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