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Loss of PTEN Accelerates NKX3.1 Degradation to Promote Prostate Cancer Progression

Cai Bowen[1], **Michael C. Ostrowski**[2], **Gustavo Leone**[3], **Edward P. Gelmann**[1,4]

[1]Departments of Medicine and of Pathology and Cell Biology, Columbia University Medical Center, Herbert Irving Comprehensive Cancer Center, Columbia University, 177 Ft. Washington Ave., MHB 6N-435, New York, NY, 10032

[2]Department of Biochemistry and Molecular Biology, Hollings Cancer Center, Medical University of South Carolina, Charleston, SC 29425

[3]Medical University of South Carolina, Hollings Cancer Center, 86 Jonathan Lucas Street, MSC 955, Charleston, SC 29425

[4]Corresponding author present address: University of Arizona Medical Center, Division of Hematology/Oncology, 1515 N Campbell Avenue, Room 1969K, Tucson, AZ 85724-5024

Abstract

NKX3.1 is the most commonly deleted gene in prostate cancer and a gatekeeper suppressor. NKX3.1 is a growth suppressor, mediator of apoptosis, inducer of antioxidants, and enhancer of DNA repair. PTEN is a ubiquitous tumor suppressor that is often decreased in prostate cancer during tumor progression. Steady state turnover of NKX3.1 is mediated by DYRK1B phosphorylation at NKX3.1 serine 185 that leads to polyubiquitination and proteasomal degradation. In this study, we show PTEN is an NKX3.1 phosphatase that protects NKX3.1 from degradation. PTEN specifically opposed phosphorylation at NKX3.1(S185) and prolonged NKX3.1 half-life. PTEN and NKX3.1 interacted primarily in the nucleus as loss of PTEN nuclear localization abrogated its ability to bind to and protect NKX3.1 from degradation. The effect of PTEN on NKX3.1 was mediated via rapid enzyme-substrate interaction. An effect of PTEN on *Nkx3.1* gene transcription was seen *in vitro*, but not *in vivo*. In gene-targeted mice Nkx3.1 expression significantly diminished shortly after loss of Pten expression in the prostate. Nkx3.1 loss primarily increased prostate epithelial cell proliferation in vivo. In these mice *Nkx3.1* mRNA was not affected by Pten expression. Thus, the prostate cancer suppressors PTEN and NKX3.1 interact and loss of PTEN is responsible, at least in part, for progressive loss of NKX3.1 that occurs during tumor progression.

Keywords

NKX3.1; PTEN; prostate cancer; DYRK1B; tumor suppressor

Address correspondence to: Dr. Edward P. Gelmann, University of Arizona Medical Center, Division of Hematology/Oncology, 1515 N Campbell Avenue, Room 1969K, Tucson, AZ 85724-5024, (520) 626-8096, gelmanne@email.arizona.edu.

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Introduction

Two key suppressor proteins of prostate cancer are NKX3.1 and PTEN. Genetic studies have repeatedly and consistently implicated 8p21, the locus of *NKX3.1,* as the most commonly deleted chromosomal locus of prostate cancer (1–3). Furthermore, *NKX3.1*, is the most commonly deleted gene in human prostate cancer (4). *NKX3.1* is a prostate-specific homeobox gene that controls differentiation (5), epithelial cell proliferation (6), and stem cell maintenance (7). In early prostate cancer reduced levels of the haploinsufficient NKX3.1 protein are seen in the majority of prostate cancers (8). During prostate cancer progression there is continued selective pressure to decrease NKX3.1 expression further demonstrating the selection for loss of NKX3.1 growth suppression in advanced disease (9). NKX3.1 protein loss may result from genetic loss, decreased mRNA, or protein degradation, all effecting reduced NKX3.1 protein levels early in the evolution of the most prostate cancers (8,10). NKX3.1 also acts to enhance the DNA repair response in the prostate and, for example, accelerates activation of ATM in response to DNA damage (11,12). Early reductions of NKX3.1 in prostate carcinogenesis predispose to the pathognomonic translocation that fuses the *TMPRSS2* promoter and the *ERG* gene that characterizes more than half of all prostate cancers (10,13).

The tumor suppressor PTEN is a lipid and protein phosphatase (14,15). PTEN is down regulated or deleted in many human cancers and is often a target for inactivation in prostate cancer [for example see (16–18)]. It acts as a phosphatase for phosphatidylinositol-tris-phosphate to counteract PI3 kinase signaling and decrease AKT activation (19). PTEN is also a protein phosphatase with many substrates (20) that has an effect on cell phenotype even after relatively small degrees of reduction in protein levels (21). Some PTEN is found in the nucleus where it interacts with components of the DNA damage signaling pathway and plays a role in the maintenance of DNA integrity and promotes repair after DNA damage (22–24). Thus both PTEN and NKX3.1 enhance DNA repair.

Studies of gene-targeted mice have established that Nkx3.1 and Pten interact to suppress prostate cancer and reductions in the levels of both proteins act synergistically to augment prostate neoplasia. In fact, engineered loss of murine Pten has been known for some time to be accompanied by spontaneous loss of Nkx3.1 protein (25,26). The effect of Nkx3.1 on cell proliferation may to some degree mediate prostate epithelial growth suppression attributed to Pten (26). NKX3.1 is extensively regulated by phosphorylation that controls both protein turnover and intermolecular interactions (27). Steady state levels of NKX3.1 are regulated predominantly by phosphorylation at serine 185 as shown by replacement of serine with alanine that markedly prolonged NKX3.1 half-life (28). Thus, inhibition of the kinase(s) that trigger protein degradation can increase intracellular NKX3.1 levels and may decrease growth and increase the differentiation state of prostate cancer. We identified DYRK1B as a key kinase that phosphorylates NKX3.1 on serine 185 and triggers protein degradation (29). In this report we show that PTEN is an NKX3.1 phosphatase and that PTEN maintains NKX3.1 protein levels under conditions that induce NKX3.1 phosphorylation and degradation. Moreover, nuclear PTEN directly opposes the effect of DYRK1B on NKX3.1(S185). Lastly, we show that stochastic loss of Pten expression induced by Cre recombinase in mice engineered with *loxP* sites flanking *Pten* results in coordinated loss of

Nkx3.1 protein expression and that variation in Pten activity correlates with degrees of Nkx3.1 protein reduction in *in vivo*. Thus we show that expression of Pten is critical to the maintenance of Nkx3.1 expression in the prostate.

Material and Methods

Cell culture, transfection, gRNA and reagents.

LNCaP, LAPC4, VCAP, 22RV1 and 293T cells were cultured in IMEM medium (Invitrogen) supplemented with 5% serum. Transfection was performed with Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. gRNAs for human PTEN were provided by Horizon. siCtr, siPTEN smart pool were from Dharmacon. Antibodies for immunofluorescence and immunoblotting are enumerated in Supplementary Table 1. PTEN inhibitors [Bpv(HOpic) and SF1670] are from Santa Cruz and Biovision Inc., respectively. TNFα was purchased from Sigma. siRNA sequences are detailed in Supplementary Table 1. All cell lines used in the study were obtained from the American Type Culture Collection (Rockville, MD) and were used within 20 passages of thawing. Cells were tested for *Mycoplasma* contamination every six months. All cell lines were authenticated in the lab by verification of cell line-specific known polymorphisms or missense mutations.

Expression Plasmids.

The Flag-PTEN plasmid was obtained from Addgene. MYC-PTEN and SFB-PTEN plasmids were provided by Li Ma (The University of Texas MD Anderson Cancer Center, Houston, TX). MYC-PTEN mutants (C124S, G129E, Y138L, and K254R) were generated by point mutation with QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies). Primers used for site-directed mutagenesis are detailed in Supplementary Table 1. All mutations were confirmed by DNA sequencing of the resultant expression plasmids.

Immunoblotting was performed as previously described (11). Antibody reagents are detailed in Supplementary Table 2.

Immunoprecipitation.

Either 293T cells transfected with Flag-PTEN and MYC-NKX3.1, or LNCaP cells transfected with MYC-PTEN were used for protein interaction analysis. Association of endogenous PTEN and NKX3.1 was assayed in extracts of LAPC4 cells. Immunoprecipitation was performed with Catch and Release Reversible Immunoprecipitation Kit (Millipore) with monoclonal PTEN antibody, and NKX3.1 antiserum (Santa Cruz). Immunoblotting was performed with monoclonal PTEN antibody (Santa Cruz) and NKX3.1 antiserum.

In vitro kinase and phosphatase.

His-tagged NKX3.1 (200 nanomoles) were incubated with 10 nanomoles DYRK1B (Invitrogen) in 45 μl 25 mM HEPES pH7.5, 2.5 mM $MgCl_2$, 0.5 mM DTT, 250 μM ATP for 30 min at 30°C. Four microliters of the kinase reaction were incubated with 0, 100, 150 or 200 nanomoles PTEN (Thermo Scientific) in the above mentioned buffer solution to a total

volume of 15 μl for 60 minutes at 30°C. Reactions were stopped by addition of 3x protein loading buffer and heated at 95°C for 5 min. Ten μl of sample were resolved on 8% Phos-tag SDS-PAGE. Phos-tag SDS-PAGE was prepared with 10 μM of Phos-tag™ acrylamide according to Phos-tag™ PAGE guidebook (Wako). Immunoblot transfer was performed for 3 hours at 25°C at 330 mA and dephosphorylation of NKX3.1 was detected by NKX3.1 antiserum.

Mass spectroscopy.

One μg His-tagged NKX3.1 was incubated with 150 ng DYRK1B in 60 μl of 25 mM Hepes, pH7.5, 2.5 mM $MgCl_2$, 0.5 mM DTT, 250 μM ATP for 30 minutes at 30°C. Fifteen μl of the kinase reaction were incubated with 1000 ng PTEN (Thermo Scientific) in 30 μl of the buffer cited above, but without ATP, for 60 minutes at 30°C. Reactions were stopped by the addition of protein loading buffer (4x) and heated at 95°C for 5 minutes. Forty μl of the reaction were resolved on SDS-PAGE and stained with Coomassie blue. Protein bands excised from the gel were subject to trypsin digestion and analyzed by LC-MS/MS. Peptides were run on LC/MS/MS (Thermo Orbitrap Fusion Tribrid) in targeted analysis mode to analyze the unphosphorylated and phosphorylated sites at NKX3.1(S185). Dephosphorylation was calculated by subtracting the fraction of phosphorylated peptides in the NKX3.1 sample with DYRK1B plus PTEN from the fraction of phosphorylation of the NKX3.1 sample with DYRK1B alone, and then normalized to the phosphorylation of the NKX3.1 sample with DYRK1B alone.

In vivo phosphorylation assay.

Cells were pretreated with 100 μM bortezomib for 30 minutes, followed by exposure to either TNFα or etoposide. Five hundred micrograms of cellular extract isolated with RIPA buffer containing protease inhibitors and phosphatase inhibitors were incubated with 2 μg of NKX3.1 antibody at 4°C overnight. The protein complex was isolated with Catch & Release Reversible Immunoprecipitation Kit (Millipore), and then resolved by SDS-PAGE. Phosphorylation of NKX3.1 was detected by anti-phosphoserine (Chemicon), anti-phosphotyrosine (Upstate), and anti-phosphothreonine antibodies (Cell Signaling).

Ubiquitination assay.

LNCaP cells cultured in 10 cm dishes at $2x10^6$ cells/well were transfected with 2 μg of His-tagged ubiquitin plasmid and either 3 μg MYC or MYC-PTEN expression plasmids. Cells were pretreated with 100 nM bortezomib for 30 min, then treated with either etoposide (50 μM) or TNFα. Cellular extract was incubated with 50 μl of Ni-NTA-His–binding resin (Novagen) overnight. The His-ubquitin-NKX3.1 complex was eluted using 4x SDS loading buffer, resolved by SDS-PAGE, and detected by immunoblotting with anti-NKX3.1, and anti-PTEN antibodies (Cell Signaling).

Mapping the binding sites of PTEN on NKX3.1.

To map binding region of PTEN and NKX3.1, Flag-PTEN and MYC-NKX3 expression constructs were transfected into 293T cells. The associated proteins were immunoprecipitated with anti-MYC antibodies with Catch and Release V2.0 kit, and

captured tagged NKX3.1 and PTEN peptides were detected by immunoblotting with monoclonal anti-PTEN and anti-MYC antibodies.

RNA analysis.

LNCaP cells were grown in 12-well plates at $3x10^5$ cells/well for 24 hours and then transfected with 1 μg of MYC-PTEN plasmid with Lipofectamin 3000. 22RV1 cells, cultured similarly, were transfected with either 30 nM of siLuc, or siPTEN, or with 1 μg of either gPTEN(47990) or gPTEN(47991) using Lipofectamin 3000. Cells were harvested and processed for immunoblotting and for quantitative PCR. Quantitative analysis of *NKX3.1* mRNA was done using RNeasy Mini Kit (QIAGEN), synthesizing cDNA with SuperScript First-Strand Kit (Invitrogen), and assaying RNA expression by real time PCR. Relative expression of NKX3.1 was obtained by normalizing Cq values of *NKX3.1* to Cq values of *GAPDH*. Prostate tissue mRNA was isolated from anterior prostates of 5 month-old $Pten^{+/+}$ and $Pten^{+/-}$ mice using the Direct-zol™ RNA MiniPrep Plus kit (ZYMO Research) according to the supplier's instructions. cDNA was synthesized with SuperScript First-Strand Kit (Invitrogen). Quantitative analysis of *Nkx3.1* mRNA compared to *Gapdh* was done as described above.

RT-PCR primers for murine *Nkx3.1* and *Gapdh* were:

Nkx3.1 forward: ACCAAGTATCCGGCATAG

Nkx3.1 reverse: CTACCAGAAAGATGGATG

Gapdh forward: AGGTCGGTGTGAACGGATTTG

Gapdh reverse: TGTAGACCATGTAGTTGAGGTCA

Tamoxifen administration.

Twenty mg/ml tamoxifen solution in olive oil was shaken rigorously at 55°C and divided in aliquots of 250 μl. Tamoxifen was warmed to 37°C for 5 min before administration. Mice were fed orally by introducing 250 μl of the tamoxifen solution with a syringe. Tamoxifen was given daily for 4 days.

Immunofluorescence.

For cultured cells, after transfection and treatment, cells were fixed with 4% formaldehyde for 10 min, followed by permeabilization with 0.5% Triton-X 100 in PBS for 15 min. Cells were incubated with primary antibodies at 4°C overnight, then incubated with Alexa Fluor 488 or 568 secondary antibodies for 30 minutes. For detection of PTEN HRP-conjugated antirabbit or antimouse IgG were used with TSA amplification. (tyramide from PerkinElmer). Tissue sections were deparaffinized and subject to antigen retrieval in 10 mM citrate buffer pH6.0 for 40 minutes in a vegetable steamer. The sections were permeabilized with 0.5% Triton-X 100 for 30 minutes and treated with 0.3% H_2O_2 for 60 minutes to quench endogenous peroxidase activity. The slides were blocked with 5% goat serum in PBS for 30 minutes and incubated with primary antibodies overnight. For Ki67, CK5, CK8, pAKT staining, slides were incubated with Alexa Fluor 633, 568 or 488 conjugated

antibodies (1:500) for 30 min. For Nkx3.1, slides were incubated with biotinylated goat antirabbit IgG (1:100) for 30 min and then Texas Red (1:500) for 30 mi. For PTEN and pS6 staining, slides were incubated with HRP-conjugated goat anti-rabbit or mouse IgG (1:200) for 30 minutes. TSA plus tyramide was applied on slides for 6–8 min and slides were mounted with mounting medium with DAPI (Vector). Antibody reagents are detailed in Supplementary Table 2.

Genotyping.

For *Nkx3.1*$^{CreERT2/+}$;*Pten*$^{flox/flox}$ FVB mouse genotyping PCR was performed with primers for *Pten*$^{flox/flox}$, *Nkx3.1,* and *Cre* at 94°C 3 min, 38 cycles of 94°C 30 sec, 55°C or 58°C 30 sec (55°C for *Pten*, 58°C for *Nkx3.1* and *Cre*), 72°C 45 sec, 72°C 3 min. PCR products were resolved on a 2% agarose gel. For *Pten* deletion and Pten mutant C124R and G129E genotyping PCR was performed with primers for Pten KO and primers for C124R/G129E at 94°C 3 min, 38 cycles of 94°C 30 sec, 55°C 30 sec, 72°C 45 sec, 72°C 3 min. PCR products was resolved on a 2% agarose gel. All experiments with animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Columbia University.

Statistical analysis.

Scoring of immunofluorescence and immunoblotting was quantified using Image J software and values were analyzed by t-test. A $p<0.05$ was considered to be statistically significant.

Results

LNCaP prostate cancer cells that express NKX3.1 but lack PTEN were transfected to express each of three different epitope-tagged PTEN constructs. In each instance the half-life of endogenous NKX3.1 was markedly prolonged (Figure 1A). NKX3.1 has a half-life *in vitro* of less than 30 minutes (28). The effect of PTEN on NKX3.1 expression could be seen in individual cells where PTEN expression permitted the persistence of high levels of NKX3.1 expression one hour after exposure to cycloheximide (Supplementary Figure 1A). Further confirmation of this result was seen with two other cell lines, VCaP and 22RV1, that express both NKX3.1 and PTEN in which knockdown of PTEN reduced detectable NKX3.1 (Supplementary Figure 1B). Our observation was not likely due to off-target effects of the gRNAs since two different guide RNAs targeting PTEN had similar effects on NKX3.1 expression and PTEN activity (Supplementary Figure 1C). The effects of PTEN knockdown by the two guide RNAs was well demonstrated by immunohistochemistry in individual cells (Supplementary Figure 1D).

NKX3.1 half-life is reduced by a variety of cell stresses including TNFα, peroxide, both UV and γ-irradiation, and etoposide (11,12,28,30). The effect of PTEN on degradation of NKX3.1 in response to these stresses was studied using MYC-tagged PTEN expression in LNCaP cells. PTEN prolonged NKX3.1 half-life after cells were exposed to TNFα and etoposide, but not after exposure to peroxide, UV, or γ-irradiation (Figure 1B). Transient expression of MYC-tagged PTEN prolonged NKX3.1 half-life after TNFα exposure only in those cells that acquired PTEN expression as shown by sorting of transfected cells with a coexpressed GFP marker (Supplementary Figure 2A). Consistent with the observation that

PTEN affected NKX3.1 half-life after cell exposure either to TNFα or etoposide, PTEN expression also diminished NKX3.1 phosphorylation under those two conditions (Supplementary Figure 2B). Visualization of phosphorylated NKX3.1 was facilitated by exposure of cells to bortezomib to block proteasomal degradation of phospho-NKX3.1 that is well demonstrated four hours after TNFα exposure (Supplementary Figure 2C). Serine phosphorylation is a known trigger for ubiquitination of NKX3.1 and NKX3.1 ubiquitination was also inhibited by PTEN (Supplementary Figure 2D) but not by PTEN(K245R) that is deficient in nuclear localization (Supplementary Figure 2E). An explanation for the difference in PTEN effects on NKX3.1 became apparent when subcellular localization of PTEN was examined after exposures to the different cell stresses. Substantially more PTEN was seen in the nucleus after TNFα and etoposide exposure than after peroxide exposure, UV, or γ-irradiation (Figure 2A). Therefore there was a correlation between colocalization of PTEN and NKX3.1 and the decrease in NKX3.1 degradation. It is also noteworthy that TNFα increased the levels of exogenous PTEN expression in LNCaP cells. The subcellular localization of endogenous PTEN was affected by the different cell stresses similarly to exogenously expressed PTEN (Figure 2B). The correlation of PTEN nuclear localization and increased NKX3.1 levels was further demonstrated in LAPC4 cells where endogenous nuclear PTEN correlated with decreased NKX3.1 phosphorylation after etoposide exposure, but not after exposure to ionizing irradiation (Figure 2C). Etoposide induced phosphorylation of NKX3.1 had been shown in Figure S2C. Lastly PTEN(K254R) deficient in nuclear localization failed to prolong NKX3.1 half-life or to decrease phosphorylation of NKX3.1 (Figure 2D) (31). Nuclear colocalization of endogenous NKX3.1 and PTEN was seen by immunomicroscopy after LAPC4 cells were exposed to etoposide or TNFα, but not to γ-irradiation (Figure 2E).

We next asked whether PTEN could oppose the effect of the major antagonistic kinase of NKX3.1, DYRK1B (29). In an *in vitro* kinase assay adding increasing amounts of PTEN protein reversed DYRK1B phosphorylation of recombinant NKX3.1 (Figure 3A). The primary phosphorylation site and DYRK1B target that triggers NKX3.1 ubiquitination is serine 185 (28,29). *In vitro*, DYRK1B phosphorylated serine 185 as we previously showed and, in excess, also phosphorylated serine 186. PTEN dephosphorylated S185 by 40% and S186 by only 5%, suggesting a preferential interaction with the key target site for NKX3.1 degradation (Figure 3B). Serine 186 was phosphorylated in vitro, but only in the presence of excess DYRK1B (29). In fact, in cultured cells PTEN had no effect on the half-life of NKX3.1(S185A), demonstrating that the most significant target of PTEN phosphatase activity on NKX3.1 is serine 185 (Figure 3C). PTEN was also able to dephosphorylate NKX3.1 at threonine 179, a target of protein kinase C (32), but not threonine residues 89 and 93, targeted by casein kinase 2 (33) (Supplementary Figure 3). Thus PTEN manifests some degree of specificity for sites of NKX3.1 dephosphorylation.

NKX3.1 and PTEN were shown to interact physically in a manner dependent to some degree on a functional interaction. Both exogenously expressed and endogenous PTEN and NKX3.1 could be seen to bind in cells by coimmunoprecipitation (Figure 4A). Mutation of NKX3.1 serine 185, a target amino acid residue for PTEN phosphatase, to alanine diminished NKX3.1 binding to PTEN (Figure 4B). Moreover, pretreatment of cell extracts

with calf intestinal alkaline phosphatase abrogated binding of PTEN to NKX3.1 underscoring the requirement that PTEN bind to phosphorylated NKX3.1 (Figure 4C).

Because of the apparent control of NKX3.1 levels by PTEN and the antagonistic role of PTEN in DYRK1B-medidated phosphorylation of NKX3.1, we wanted to reexamine the effect of PTEN expression on the transcription of *NKX3.1* mRNA. Previously, the expression of exogenous PTEN had been shown in cultured cells and tissues to increase both human and murine *NKX3.1* mRNA (26). We confirmed that expression or knock-down of PTEN in cultured cells after three days markedly affected *NKX3.1* mRNA as previously reported (Figures 5A and B). Importantly, the effect on *NKX3.1* transcription was not seen at 24 hours and required up to 72 hours to manifest fully (Figure 5C). In contrast, there was no chronic effect of Pten expression on *Nkx3.1* mRNA in 5 month-old $Pten^{+/-}$ mice compared to wild-type mice (Figure 5D). Moreover, missense mutants of Pten deficient in either protein, lipid, or both protein and lipid phosphatase activity all increased NKX3.1 mRNA after 24 hours in culture whereas only PTEN deficient in nuclear localization had no effect on NKX3.1 mRNA (Figure 5E). This will be shown to contrast with the effect of these mutant constructs on Nkx3.1 protein *in vivo* (see below).

Pten protein expression however did affect Nkx3.1 levels *in vivo*. We compared $Nkx3.1^{+/-}$ mice in which one *Nkx3.1* allele had been replaced by a tamoxifen-inducible gene for Cre recombinase with tamoxifen-treated $Pten^{flox/flox};Nkx3.1^{+/CreERT2}$ mice (34). Genotyping of the mice demonstrated the presence of the desired alleles (Supplementary Figures 4A and B). Mice were administered tamoxifen and analyzed at 2-week intervals for 8 weeks. Loss of *Pten* was accompanied by prostate epithelial dysplasia seen on H&E staining (Figure 6). There were visible changes in histology of the *Nkx3.1* heterozygous mice consistent with published reports that hyperplastic and dysplastic changes are seen in these mice at 6 months (5,6). *Pten* loss caused a marked reduction in Nkx3.1. As Pten was lost, resulting in Nkx3.1 loss, Ki-67 staining was seen to increase, reflecting increased cell proliferation. Consistent with the known functions of Pten, both Akt and S6 phosphorylation increased as Pten was lost.

To get a better understanding of the relative contributions of Pten loss and Nkx3.1 loss on the phenotypic changes seen in Figure 6, we studied the effects of phosphatase-deficient *Pten* mutant proteins on Nkx3.1 and on other markers in gene-targeted mice (31,35). In LNCaP cells, whereas Pten prolonged NKX3.1 half-life, PTEN(C124S) that has lost both lipid and protein phosphatase activity had no detectable effect on NKX3.1 half-life (Supplementary Figure 5A). Pten(G129E) that lacks lipid phosphatase activity had a diminished, but visible, effect on prolonging NKX3.1 half-life, but much less than the wild type protein, indicating that Nkx3.1 phosphatase activity was also affected by the mutation thought to confer preferential loss of lipid phosphatase. Pten(Y138L) that lacks protein phosphatase activity, like Pten(C124S), had no detectable effect on NKX3.1 half-life. Importantly, all the missense mutants were able to bind to NKX3.1 (Supplementary Figure 5B). As a negative control for protein interaction, Pten(K245R) that lacks a nuclear localization signal, did not bind NKX3.1, suggesting that nuclear colocalization of the two proteins mediated protein binding (Supplementary Figure 5B).

We also examined the effects of *Pten* phosphatase mutant proteins in mice heterozygous for production of either Pten(C124R) or Pten(G129E). Partial loss of PTEN is seen in many cases of prostate cancer and was shown to cause phenotypic changes in mouse models, so even mice heterozygous for *Pten* inactivation were expected to have phenotypic consequences (Figure 7). Consistent with this observation, we saw that, compared to intact mice, $Pten^{+/-}$ mice demonstrated hyperplasia, substantial loss of Nkx3.1 expression, increased Ki-67 expression, and elevated levels of phosphorylated Akt and S6. Mice with the $Pten^{+/C124R}$ resembled the *Pten* heterozygous mice, but showed less Ki-67 expression and S6 phosphorylation and more Nkx3.1 expression. The $Pten^{+/G129E}$ mice retained greater cytologic differentiation, more Nkx3.1 expression, and showed similar levels of Ki-67 expression to mice with $Pten^{+/C124R}$, but lower than the *Pten* heterozygous mice (Figure 7A and B).

$Nkx3.1^{-/-}$ mice were examined to determine the effects of Nkx3.1 loss in an intact *Pten* background. Prostate sections from $Nkx3.1^{-/-}$ mice are shown that have a high degree of dysplasia on H&E, increased Ki-67 staining compared to $Pten^{+/-}$ mice, and an unexpected increase in expression of S6 kinase (Figure 7A). Importantly we demonstrated that Akt activation resulted from loss of Pten activity and was not seen with loss of Nkx3.1 alone. Remarkably, increased proliferative index as assayed by Ki-67 immunostaining was largely driven by Nkx3.1 loss. This latter finding reflects the known antiproliferative effect of NKX3.1 (6,36).

Discussion

In early prostate cancer reduced levels of the haploinsufficient NKX3.1 protein are seen in the majority of prostate cancers (8). The degree of NKX3.1 protein loss in primary prostate cancer is related to Gleason grade, suggesting that prostate cancer phenotype is, to some degree, affected by NKX3.1 protein levels (8,37). During prostate cancer progression there is continuing selective pressure to decrease NKX3.1 expression further demonstrating the growth suppressive role NKX3.1 plays in advanced disease (9). However, some amount of NKX3.1 expression is sustained in both high grade and metastatic prostate cancer and can serve as a tissue-specific marker to aid histologic diagnosis (38). *PTEN* undergoes both genetic and epigenetic alteration in prostate cancer (39,40). These changes ae infrequent in early stage prostate cancer (13,41), but increase in frequency with tumor progression (42). We suggest that temporally *PTEN* loss or reduced PTEN expression occurs after NKX3.1 loss and may therefore be a contributing factor, and perhaps the major factor, for continued NKX3.1 loss during tumor progression.

It was previously suggested that Pten controls Nkx3.1 expression via transcription. In tumor explants expression of Pten driven by viral vectors increased Nkx3.1 expression (26). We too found that in cultured cells exogenous expression of PTEN increased NKX3.1 expression after 48–72 hours. However, NKX3.1 has a half-life of less than 30 minutes and therefore physiologic control of protein expression is likely to affect expression in that short time frame. In cultured cells PTEN expression does affect NKX3.1 protein expression within just one hour. Moreover, PTEN directly opposes DYRK1B phosphorylation at NKX31(S185) suggesting further a physiologic role for PTEN in the post-translational control of NXK3.1.

Lastly, when we examined levels of *Nkx3.1* mRNA in mice with *Pten* loss, we found that *Pten* genotype had no effect on Nkx3.1 mRNA in contrast to the marked effect of *Pten* copy number on Nkx3.1 protein levels.

We present *in vitro* and *in vivo* data demonstrating the interaction between PTEN and NKX3.1. The cell culture data demonstrate the direct enzyme-substrate interaction of the two proteins and show that alteration of PTEN expression has a nearly immediate effect on NKX3.1 protein levels in cultured cells. Moreover, in response to certain cellular stresses, PTEN can undergo nuclear translocation resulting in decreased NKX3.1 phosphorylation and preservation of NKX3.1 levels perhaps to enhance the DNA damage response (11). The interaction of NKX3.1 and PTEN described by findings with cultured cells are complemented by observations of prostates sampled from gene-targeted mice. In the latter studies loss of Pten expression had an enduring effect on Nkx3.1 protein levels and on markers of cell proliferation up to 8 weeks after Pten loss.

NKX3.1 plays physiologic roles in the cell's response to certain stresses such as irradiation and oxidation. In the case of exposure to γ-irradiation NKX3.1 is activated by tyrosine phosphorylation and then phosphorylated on threonines by ATM to trigger degradation within an hour of radiation exposure (12). We showed that PTEN did not affect NKX3.1 degradation after radiation response, however, PTEN did participate in modulating NKX3.1 levels after exposure to etoposide and to TNFα. In these two instances PTEN was able to affect NKX3.1 loss by translocating to the nucleus. PTEN clearly plays a role in the cell response to TNFα as others have also observed that PTEN displays nuclear localization after cells are exposed to TNFα (43). In fact, TNFα induces monoubiquitination of PTEN that licenses nuclear translocation of the phosphatase (24).

The direct effect of PTEN on NKX3.1 suggests that in prostate cancer, loss of PTEN has effects that extend beyond the activation of PI3 kinase (PI3K). Thus the application of PI3K inhibitors to prostate cancer treatment may have limited efficacy in cancers that have attenuation of PTEN activity. NKX3.1 expression is never completely lost in advanced prostate cancer, except perhaps in the event of transformation to neuroendocrine cancer (44). However, there is a progressive decrease in NKX3.1 expression during tumor progression (9). NKX3.1 loss is clearly an activator of cell proliferation and its expression is highly inhibitory of cell proliferation. Thus the very common finding of PTEN loss in prostate cancer is accompanied by increased turnover and loss of NKX3.1, compounding the effect on tumor progression and potentially on susceptibility to therapeutic intervention. Lastly, therapeutic efficacy may be compromised further by the well-documented loss of p53 during the later stages of prostate cancer progression that may be accelerated by loss of NKX3.1 (26).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Significance

PTEN functions as a phosphatase of NKX3.1, a gatekeeper suppressor of prostate cancer

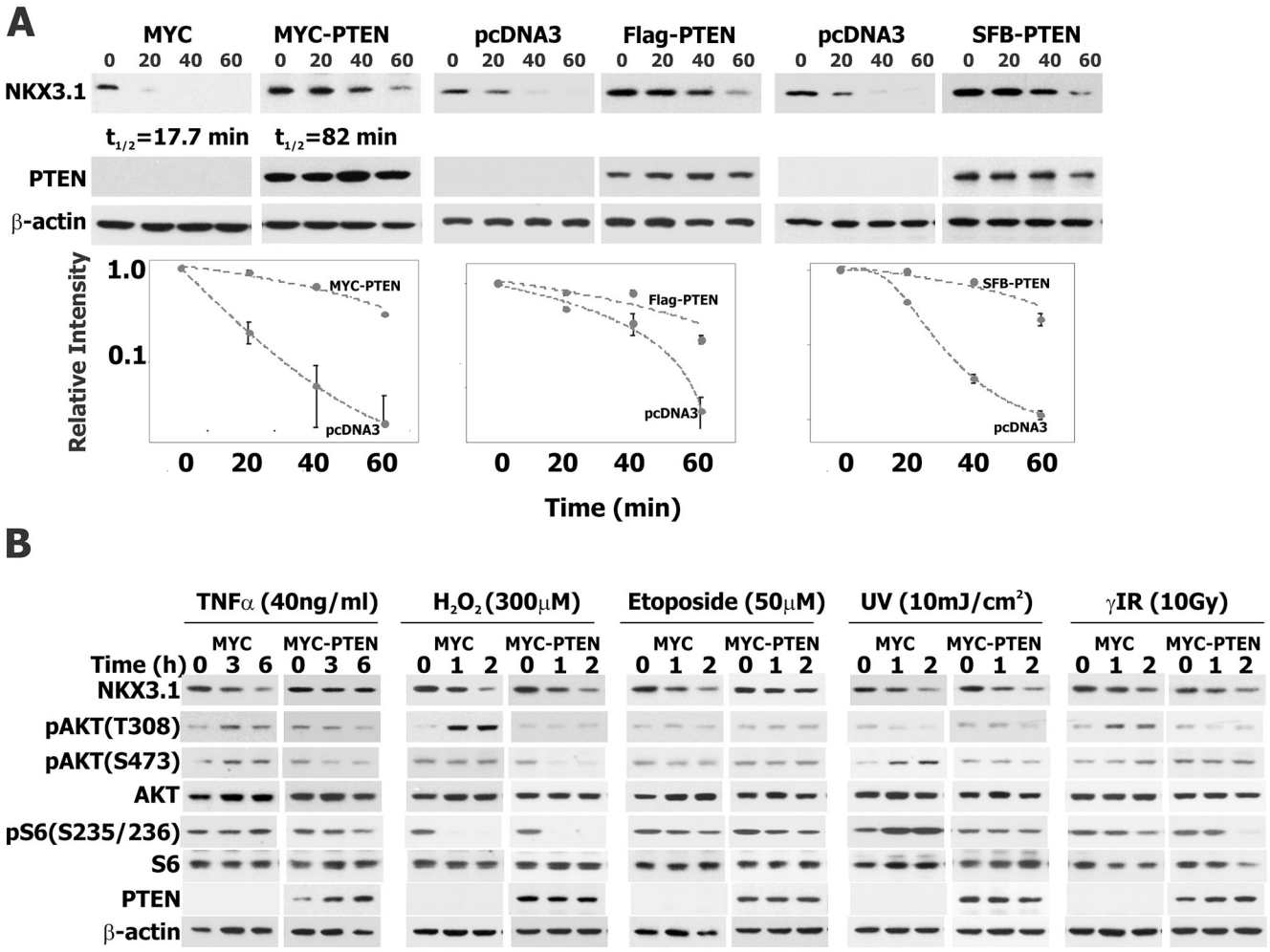

Figure 1.

PTEN prolongs NKX3.1 half-life. A. LNCaP cells transfected with expression plasmids as indicated and were exposed to 100 μM cycloheximide. Cells were harvested at the indicated times and processed for immunoblotting. The plots show relative intensity of NKX3.1 bands analyzed by Image J. B. PTEN prevents stress-induced NKX3.1 degradation. LNCaP cells transfected with MYC or MYC-PTEN plasmids were exposed to TNFα, H_2O_2, etoposide, UV or γIR as shown. Cells were processed for immunoblotting.

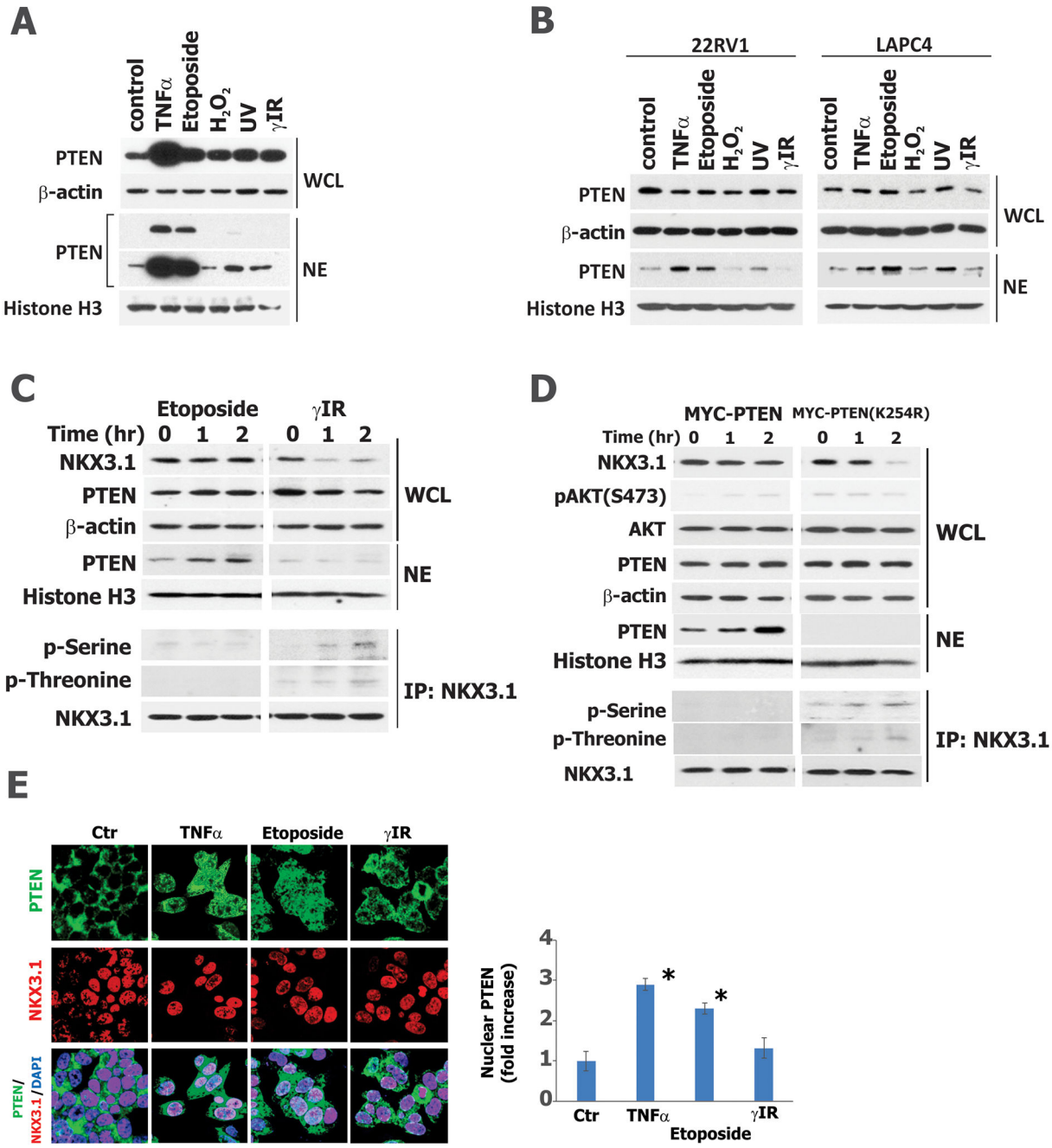

Figure 2.

PTEN nuclear localization is required for interaction with NKX3.1. A. LNCaP cells transfected with a MYC-PTEN expression plasmid were exposed to 40ng/ml TNFα, 50 μM etoposide, 300 μM H_2O_2, 10 mJ/cm^2 UV, or 10 Gy γ-irradiation. Cells were processed for immunoblotting to analyze either whole cell lysates (WCL) or nuclear extracts (NE). B. 22RV1 and LAPC4 cells were treated as in Figure 2A without MYC-PTEN transfection to analyze nuclear localization of endogenous PTEN in response to stimuli. C. LAPC4 cells were exposed to 50 μM etoposide or 10 Gy γ-irradiation. Cells were harvested at the

indicated times and processed as total lysates or nuclear extracts for immunoblotting (upper panel). In the lower panel cells were pretreated with 100 nM bortezomib prior to etoposide exposure or irradiation and then lysates were subjected to immunoprecipitation with NKX3.1 antiserum and then immunoblotting with the indicated antisera. D. LNCaP cells were transfected with the indicated expression plasmids, exposed to 10 Gy γ-irradiation, and harvested at the indicated times. Cells were processed similar to the experiment shown in panel C. E. LAPC4 cells cultured on polylysine-coated slides were fixed in 10% formalin 1 hour after. Staining was performed with monoclonal anti-PTEN antibody (Santa Cruz) and polyvalent NKX3.1 antiserum. The signals were amplified with HRP-conjugated antimouse IgG plus TSA, or biotin-conjugated antirabbit IgG and Texas Red. Nuclear PTEN was quantitated in three fields by Image J and then normalized to the average intensity in control samples. * Indicates a statistically different result from control by t-test, $p<0.05$.

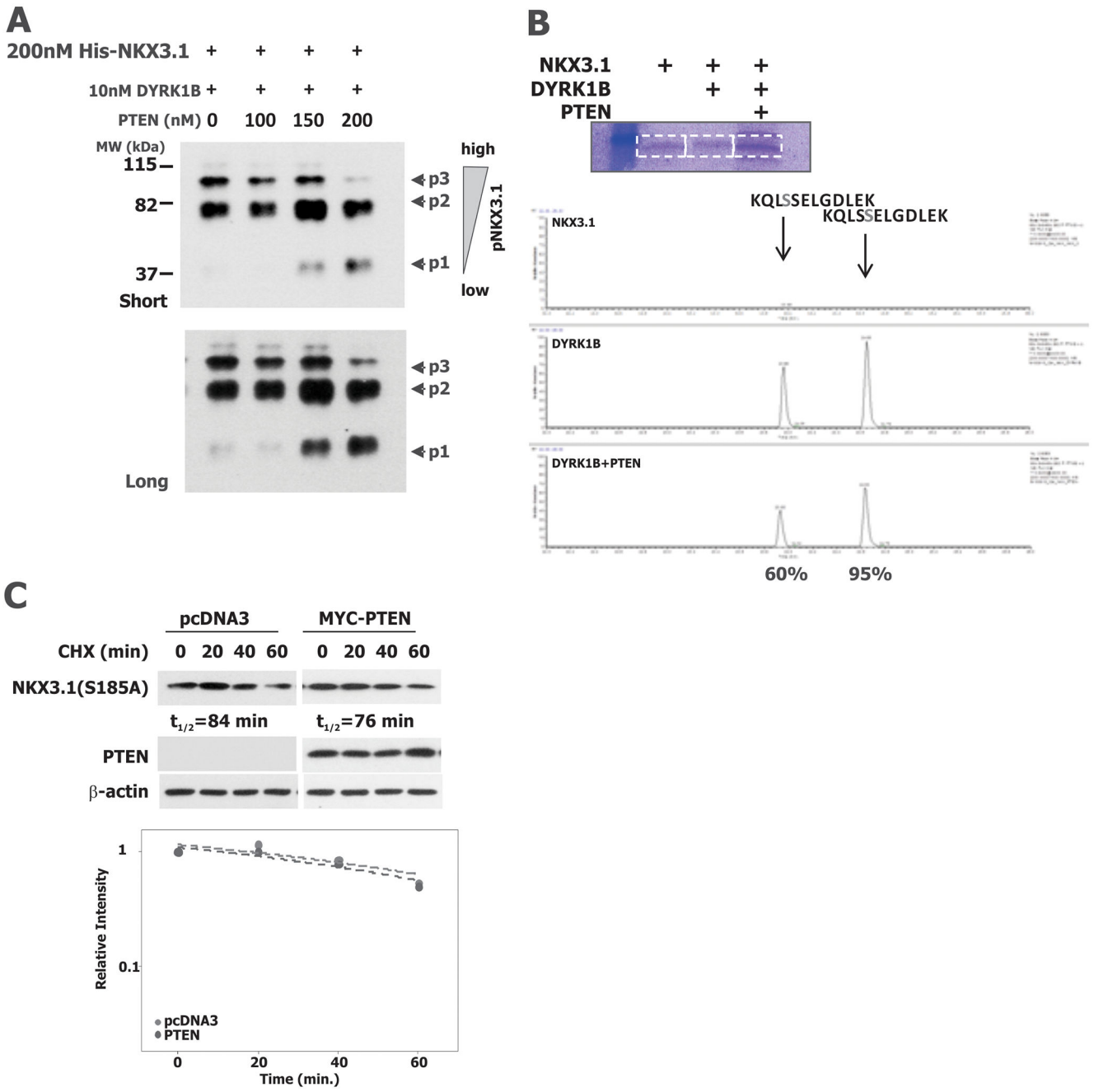

Figure 3.

In vitro dephosphorylation of NKX31 by PTEN. His-tagged recombinant NKX3.1 was incubated with DYRK1B for 30 minutes and then incubated with PTEN for 60 minutes. A. Phosphorylated NKX3.1 were resolved on 8% of Phos-tag SDS-PAGE and revealed by NKX3.1 antiserum. B. Phosphorylated NKX3.1 was resolved on SDS-PAGE and stained with Coomassie blue. Bands were extracted for mass spectrometer analysis to identify dephosphorylation sites. Dephosphorylation was determined by subtracting the phosphorylated fraction of peptides in the reaction with both DYRK1B and PTEN from phosphorylation fraction of peptides in the sample with DYRK1B alone. The resulting

differences were normalized to the phosphorylation fraction of NKX3.1 peptides extracted from the exposure to DYRK1B alone. C. LNCaP cells were transfected with expression plasmids for MYC-PTEN, pcDNA3 or MYC-NKX3.1(S185A). After 24 hours cells were treated with 100μM cycloheximide for the indicated times. Immunoblotting was performed with polyvalent NKX3.1 antiserum and PTEN antibody.

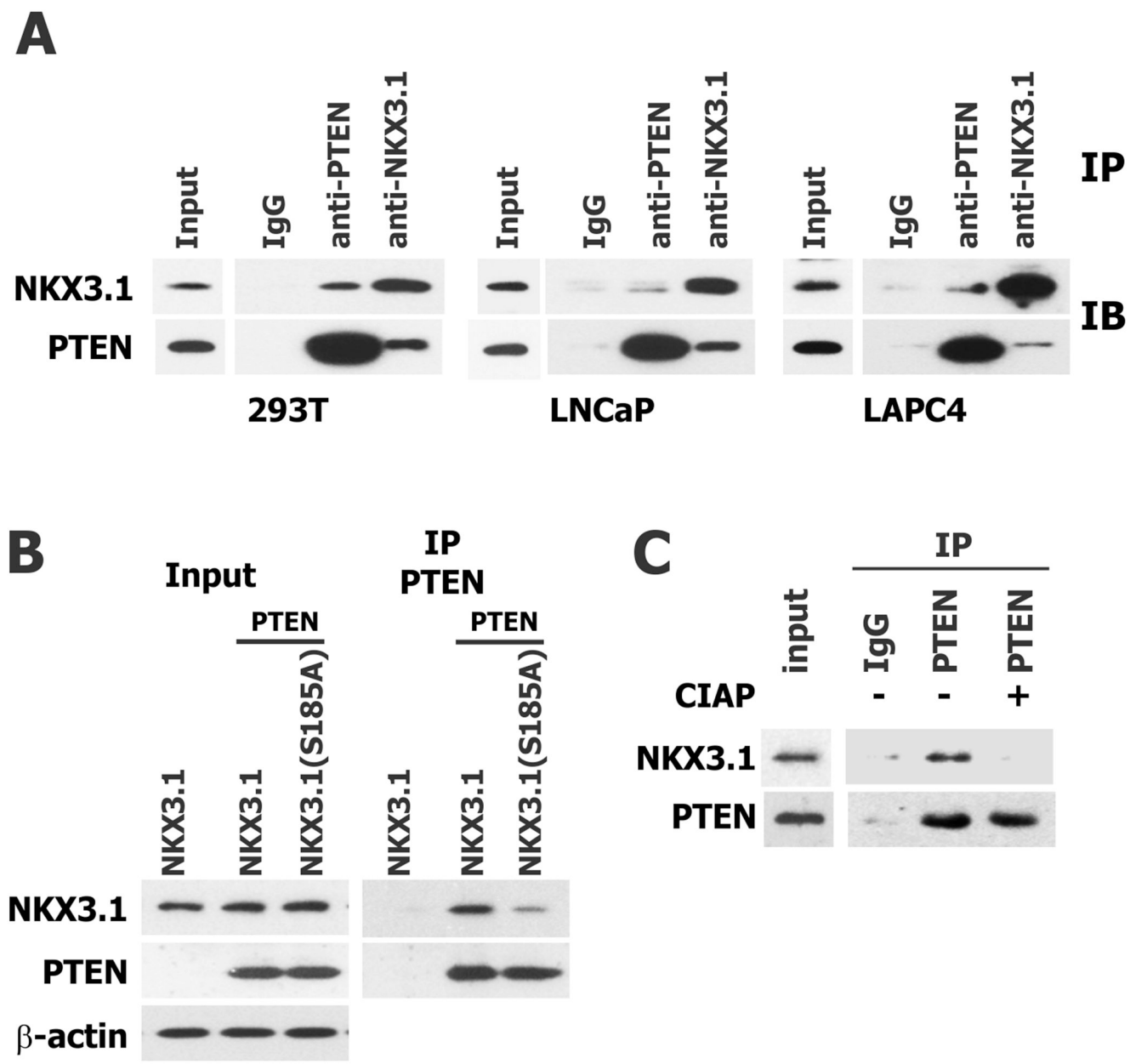

Figure 4.

Intracellular association of NKX3.1 and PTEN. A. Either 293T cells transfected with both Flag-PTEN and MYC-NKX3.1 plasmids or LNCaP cells transfected with a MYC-PTEN expression plasmid were processed for immunoprecipitation and immunoblotting for interaction of exogenously expressed proteins. LAPC4 cells were analyzed similarly for interaction of endogenous PTEN and NKX3.1. B. 293T cells were transfected with MYC-NKX3.1 or MYC-NKX3.1(S185A) plasmids with or without MYC-PTEN and were used for interaction analysis. Immunoprecipitation (right panel) was done with PTEN antibody. C. Cell lysates were treated with 100 units calf intestinal alkaline phosphatase for 60 minutes. The interaction of NKX3.1 and PTEN was abrogated.

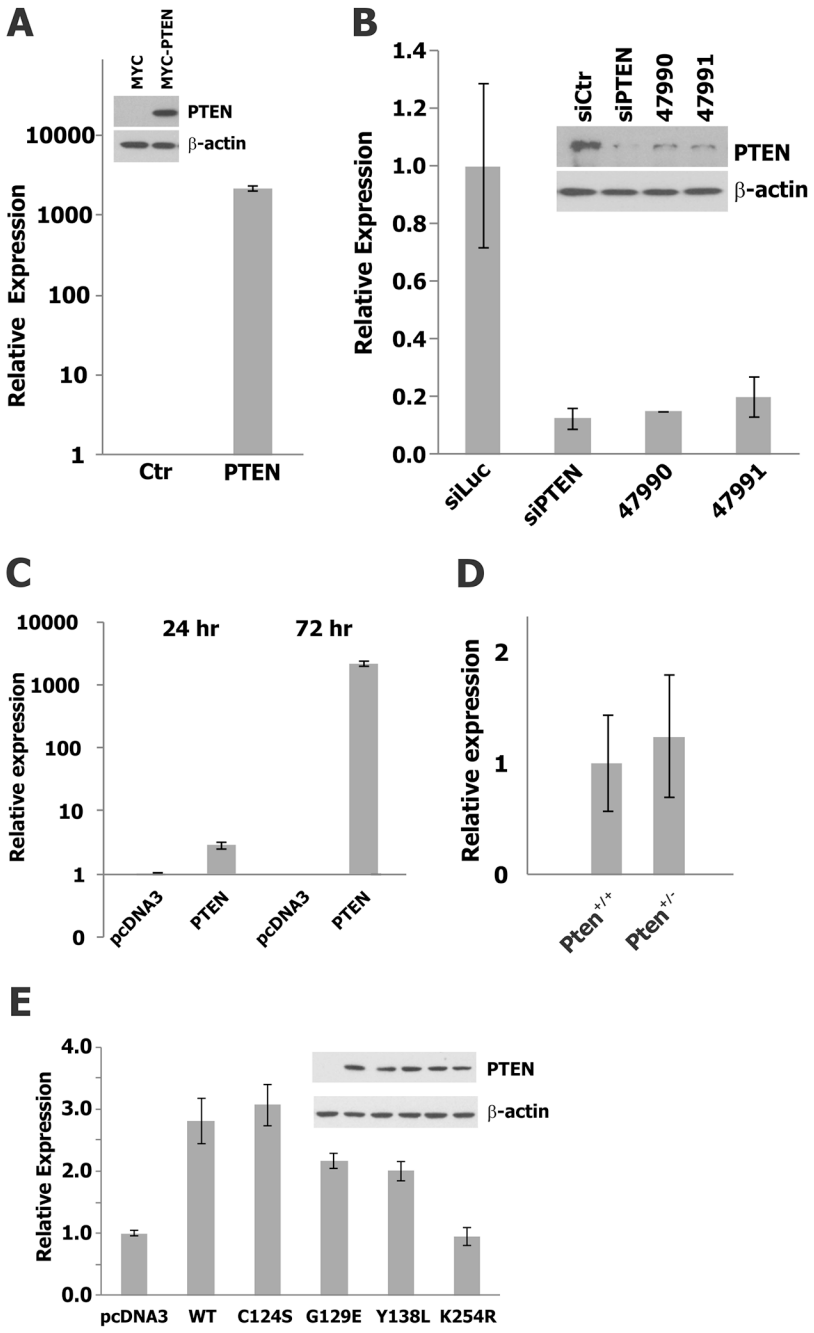

Figure 5.

Effect of PTEN on *NKX3.1* transcription. A. LNCaP cells were cultured and transfected with the indicated expression plasmids. After three days cells were processed for immunoblotting (inset in histogram) and quantitative PCR. B. 22Rv1 cells were cultured and transfected with either siRNA or guide RNA vectors to decreased expression of PTEN. After three days cells were processed as in A. C. LNCaP cells were cultured and transfected as in A except cells were processed at both 24 and 72 hours. D. Prostates from 5-month old mice were processed for RNA and analyzed by quantitative PCR. Expression of *Nkx3.1* relative to

Gapdh is shown. E. Effect of Pten phosphatase-deficient mutants on NKX3.1 mRNA 24 hours after transfection of the indicated constructs into LNCaP cells.

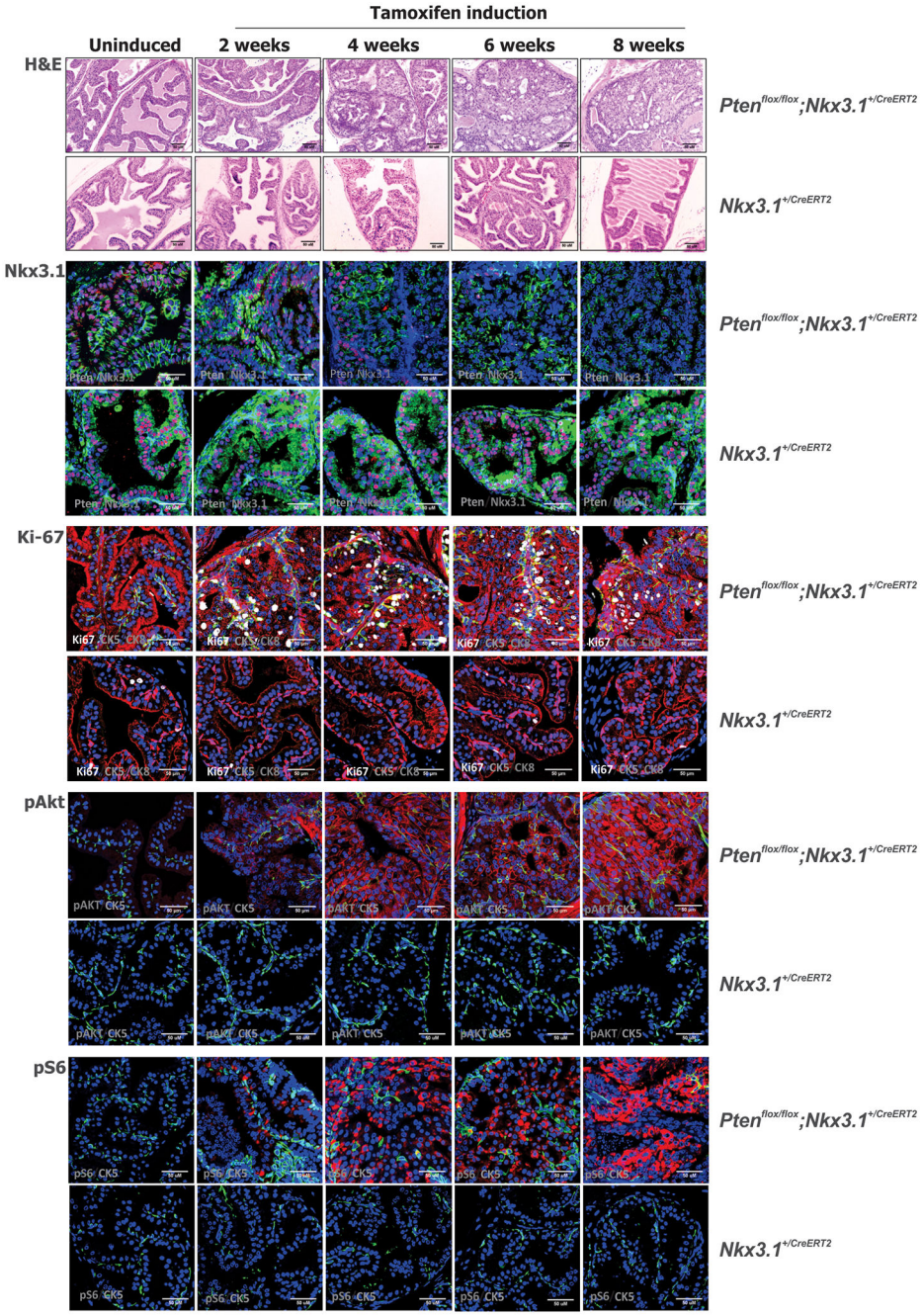

Figure 6.

Conditional knockout of *Pten* decreases both Pten and Nkx3.1 expression. *Nkx3.1*$^{CreERT2/+}$;*Pten*$^{flox/flox}$ mice and *Nkx3.1*$^{CreERT2/+}$;*Pten*$^{+/+}$ mice were administered tamoxifen and analyzed by immunohistochemical staining as shown. Microscopic images of the comparator mice are juxtaposed for ease of comparison. The Pten signal was amplified by incubation with HRP-conjugated IgG and revealed with tyramide. Nkx3.1 was amplified with biotin conjugated anti-rabbit IgG and Texas red. Ki67, CK5 and CK8 were detected by

Alexa fluor 488-, Alexa fluor 633- and Alexa fluor 568-conjugated IgG, respectively. The signals are shown as pseudocolors.

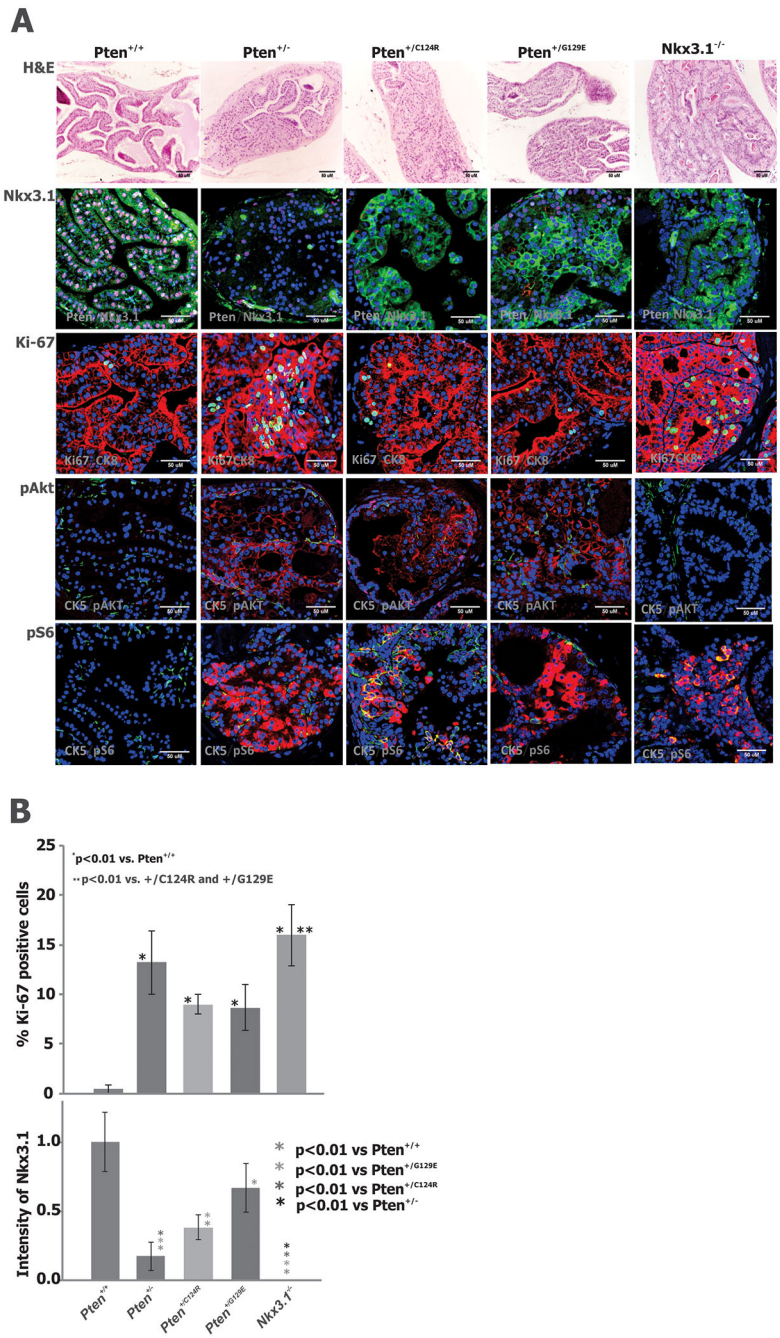

Figure 7.

Attenuation of Pten phosphatase reduces Nkx3.1 expression. Mice with the indicated *Pten* genotype were analyzed at 9 months of age as shown. The far right set of micrograms show prostates from *Nkx3.1*$^{-/-}$ mice at 9 months of age. Immunofluorescent staining was performed as in Figure 6. B. The upper panel shows image J quantitation of Ki-67 staining of the different prostates and the lower panel staining for Nkx3.1. Results of t-test for

significant differences are shown in the graphs. The data show general inverse relationship between Nkx3.1 and Ki-67 expression.