NKX3.1 stabilizes p53, inhibits AKT activation, and blocks prostate cancer initiation caused by PTEN loss

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Summary

We demonstrate that PTEN loss causes reduced NKX3.1 expression in both murine and human prostate cancers. Restoration of *Nkx3.1* expression in vivo in *Pten* null epithelium leads to decreased cell proliferation, increased cell death, and prevention of tumor initiation. Whereas androgen receptor (AR) positively regulates NKX3.1 expression, NKX3.1 negatively modulates AR transcription and consequently the AR-associated signaling events. Consistent with its tumor suppressor functions, NKX3.1 engages cell cycle and cell death machinery via association with HDAC1, leading to increased p53 acetylation and half-life through MDM2-dependent mechanisms. Importantly, overexpression of *Nkx3.1* has little effect on *Pten* wild-type epithelium, suggesting that PTEN plays a predominant role in PTEN-NKX3.1 interplay. Manipulating NKX3.1 expression may serve as a therapeutic strategy for treating PTEN-deficient prostate cancers.

Introduction

Prostate cancer is the second leading cause of cancer-related death in males (Gregorakis et al., 1998; McDavid et al., 2004). Its development proceeds through a series of defined steps, including prostatic intraepithelial neoplasia (PIN), invasive cancer, and hormone-dependent or -independent metastasis. Although different stages of prostate cancer have been well defined histologically, relatively little is known about the molecular mechanisms contributing to the initiation and progression of prostate cancer.

The *PTEN* (phosphatase and tensin homolog deleted on chromosome 10) tumor suppressor gene is frequently mutated in human cancers (Dahia, 2000; Maehama et al., 2001; Parson et al., 1998). The major function of PTEN relies on its phosphatase activity toward PIP3 (phosphatidyl inositol 3,4,5-triphosphate) and, consequently, antagonism of the PI3K (phosphatidylinositol 3-kinase) signaling pathway (Di Cristofano et al., 2001; Maehama et al., 2001). Loss of *PTEN* function results in accumulation of PIP3 and activation of its downstream effectors, such as AKT/PKB (Maehama et al., 2001). AKT, a serine/threonine protein kinase, phosphorylates key intermediate signaling molecules, leading to increased cell metabolism, growth, survival, and invasiveness, all hallmarks of cancer (Di Cristofano et al., 2001; Hanahan and Weinberg, 2000; Vivanco and Sawyers, 2002).

PTEN alteration is strongly implicated in prostate cancer development, as mutations of the *PTEN* gene are found in 30% of primary prostate cancers (Dahia, 2000; Sellers and Sawyers, 2002) and 63% of metastatic prostate tissue samples (Suzuki et al., 1998). Thus, *PTEN* mutations are among the most frequent genetic alterations in human prostate cancer. As PTEN-controlled signaling pathways are frequently altered in human prostate cancers, inhibiting the resultant signaling aberrations will likely serve as promising targets for therapeutic strategies

SIGNIFICANCE

Gene expression profiling of mouse tumor models or human cancers has identified many dysregulated genes that may contribute to tumor development. These wealthy data sets, upon functional validation, may help in elucidating the molecular mechanisms underlying tumorigenesis and providing potential novel targets for cancer therapies. Using a powerful prostate epithelial tissue reconstitution assay, we demonstrated the importance of NKX3.1 in prostate cancer initiation caused by PTEN loss. Our finding emphasizes the cooperative effects between ubiquitously expressed *PTEN* tumor suppressor genes and prostate-specific expressed *NKX3.1* in prostate cancer development. Our study further indicates that validation of candidate genes using mouse models can yield valuable molecular insights that impact human cancer research.

(DeMarzo et al., 2003; Sellers and Sawyers, 2002; Vivanco and Sawyers, 2002).

We and others have developed murine models of prostate cancers by deleting the *Pten* tumor suppressor gene specifically in the prostatic epithelium (Chen et al., 2005; Ma et al., 2005; Trotman et al., 2003; Wang et al., 2003). The *Pten* prostate cancer model recapitulates many features of the disease progression seen in humans with defined kinetics: initiation of prostate cancer with PIN lesions, followed by progression to locally invasive adenocarcinoma, and subsequent metastasis (Wang et al., 2003). Similar to human cancer, *Pten* null murine prostate cancers regress in response to androgen ablation therapy but subsequently relapse and proliferate in the absence of androgens (Wang et al., 2003).

Global assessment of molecular changes caused by homozygous *Pten* deletion identified key genes known to be relevant to human prostate cancer, including those "signature" genes associated with human cancer metastasis (Wang et al., 2003). Among the genes that are downregulated in *Pten* null prostate cancer is *Nkx3.1*, a homeobox gene specifically expressed in the prostate epithelium. NKX3.1 is one of the earliest markers for prostate development and is continuously expressed at all stages during prostate development and in adulthood (Bhatia-Gaur et al., 1999). Human *NKX3.1* maps to chromosome 8p21, a region that frequently undergoes loss of heterozygosity (LOH) at early stages of prostate carcinogenesis (He et al., 1997; Voeller et al., 1997). *Nkx3.1* mutant mice develop prostatic hyperplasia and dysplasia. However, these early lesions failed to progress to metastatic cancers (Abdulkadir et al., 2002; Bhatia-Gaur et al., 1999), consistent with a role for *Nkx3.1* inactivation in prostate cancer initiation.

In this study, we employed a dissociated prostatic epithelial regeneration system to directly test the significance of *Nkx3.1* loss in *Pten* null prostate cancer formation. Our data show that NKX3.1 plays an important role in prostate cancer initiation caused by PTEN loss and that forced *Nkx3.1* expression prevents *Pten* null prostate cancer initiation and progression. Thus, decreased *Nkx3.1* expression contributes to prostate cancer development caused by PTEN loss.

Results

PTEN loss leads to reduced *NKX3.1* expression in both murine and human prostate cancers

Our previous gene expression profiling analysis revealed that *Nkx3.1* mRNA level is downregulated in the *Pten* null prostate cancers (Wang et al., 2003). In this study, consecutive sections of ventral prostate lobe from 4-week-old (4W) *Pten* conditional knockout animals were probed with antibodies to either NKX3.1 or phospho-AKT (P-AKT/Ser 473) (Figure 1A). In the acini where P-AKT levels are low, intense NKX3.1 staining can be observed (Figure 1A, arrows). In contrast, areas with high P-AKT are either low or negative for NKX3.1 staining (Figure 1A, arrowheads). Since increased AKT phosphorylation is a consequence of PTEN loss (Figure S1 in the Supplemental Data available with this article online), these staining patterns suggest that *Nkx3.1* downregulation is an early event linked to *Pten* deletion and prostate cancer initiation.

To test whether PTEN-regulated *Nkx3.1* expression can be observed in human prostate cancers, we conducted double immunofluorescent analysis of PTEN and NKX3.1, using human

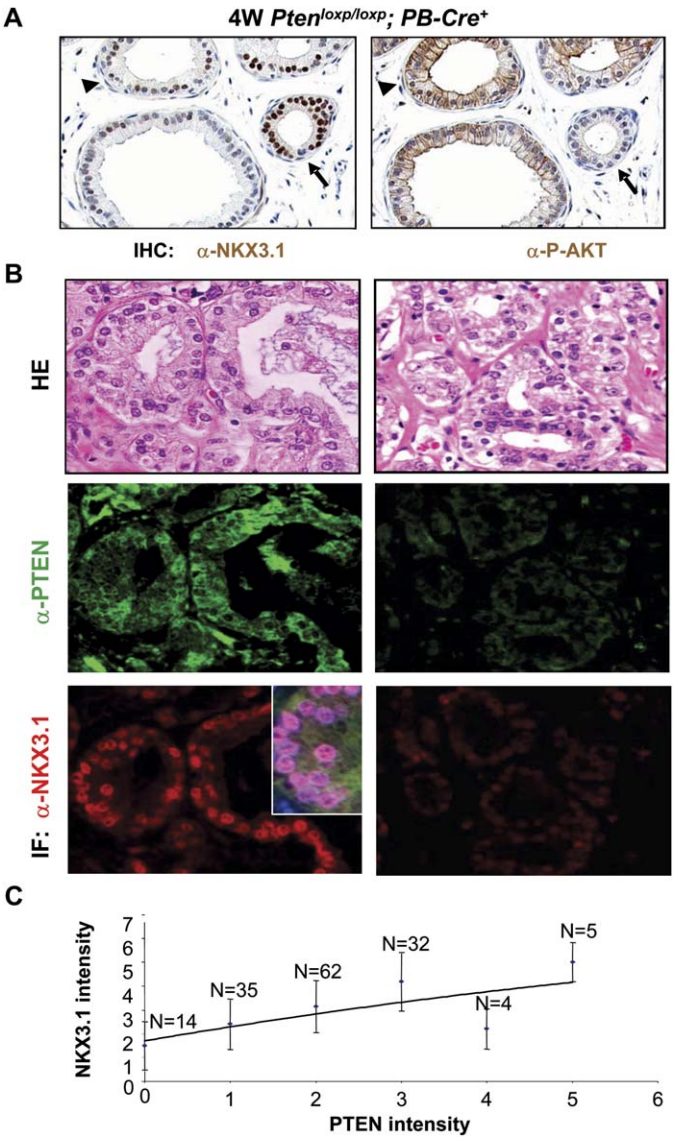

Figure 1. PTEN loss leads to decreased NKX3.1 protein levels in both murine and human prostate epithelium

A: Consecutive sections of 4-week-old *Pten* mutant prostates were probed for NKX3.1 (left) and phospho-AKT (Ser 473) (P-AKT, right) expression. Arrows and arrowheads point to the same duct. Note that NKX3.1 expression reversely correlates with P-AKT staining.

B: High-magnification views of two representative samples from human prostate cancer tissue microarray are shown here. Upper panels: H&E staining; middle and lower panels: double immunofluorescent staining using anti-PTEN (middle) and anti-NKX3.1 (lower) antibodies. Insert: high-power overlay of NKX3.1 and DAPI staining showing NKX3.1 nuclear localization.

C: Correlation of PTEN and NKX3.1 protein levels in 153 human prostate samples. SPSS liner regression was used to analyze data, and the standardized coefficient value was 0.52 ($p < 0.01$ level; $n = 153$).

prostate tissue microarrays (Rocchi et al., 2004). Among 153 samples surveyed (see Experimental Procedures), positive PTEN expression was significantly correlated with NKX3.1 staining, whereas PTEN loss was associated with decreased NKX3.1 staining (Figure 1C; $p < 0.01$). Photos from two representative samples are shown in Figure 1B. Therefore, PTEN loss leads to decreased NKX3.1 expression in both human and murine prostate cancers, implying that NKX3.1 may serve

as an important regulator downstream of PTEN-controlled signaling pathway in prostate cancer development.

Forced *Nkx3.1* expression in *Pten* null epithelium using an exogenous promoter

Several mechanisms have been proposed for loss of NKX3.1 expression in human prostate cancers, including both transcriptional and posttranscriptional regulations (Asatiani et al., 2005; Bowen et al., 2000; He et al., 1997; Korkmaz et al., 2004; Ornstein et al., 2001; Voeller et al., 1997). To understand how PTEN controls NKX3.1 expression and to evaluate the functional relevance of *Nkx3.1* downregulation in *Pten* null prostate cancer initiation, we employed a dissociated prostate cell regeneration method (Xin et al., 2003, 2005) to test (1) whether we can restore NKX3.1 expression in *Pten* null epithelium to a level comparable to the wild-type (wild-type) by using an exogenous promoter, and (2) the consequence of forced *Nkx3.1* expression in *Pten* null prostate epithelium. In order to circumvent the low transfection efficiency of mouse prostate epithelium, we cloned Flag-tagged *Nkx3.1* into a lentiviral vector (Xin et al., 2003, 2005) in which *Nkx3.1* expression is driven by the ubiquitin promoter followed by an IRES-eGFP expression cassette (Figure 2A) (Lois et al., 2002). NKX3.1 expression can be detected indirectly via eGFP expression (Figure 2B, left panels) and by Western blot analysis of transfected 293T cells using total protein lysate (Figure 2B, upper row in the right panel) or eGFP-sorted cells (Figure 2B, middle row in the right panel) with an anti-Flag antibody.

We then infected epithelium from 4W *Pten* null mice (Mut), corresponding to the hyperplastic stage, and the epithelium from their wild-type littermates with *Nkx3.1* expressing (NKX3.1 group) or control lentivirus (GFP group). Infected epithelium was then mixed with mesenchyme isolated from embryonic day 16 wild-type urogenital sinus mesenchyme (UGSM) and grafted under the renal capsule of $CB17^{SCID/SCID}$ mice and propagated for 6 weeks. Low but detectable levels of PTEN expression can be found in the mutant grafts by Western blot analysis (Figure 2C, insert), most likely attributable to the wild-type UGSM cells used for reconstitution. Importantly, lentivirus-mediated gene expression in *Pten* null grafts restored NKX3.1 protein levels so that they were comparable to those of the WT graft (Figure 2C, insert). The fact that NKX3.1 protein expression can be restored and maintained near the wild-type levels via an exogenous promoter suggests that PTEN modulates NKX3.1 function largely through regulation of its transcription, e.g., by controlling its promoter activity.

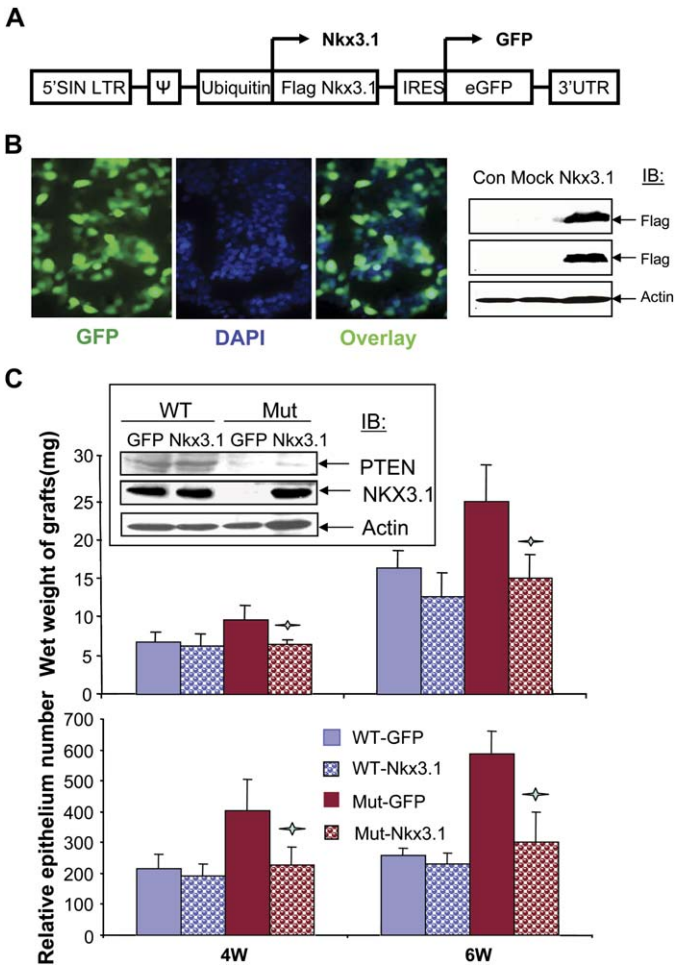

Figure 2. Overexpression of *Nkx3.1* reduces the growth of reconstituted *Pten* null epithelium graft

A: The lentivirus FUGW-IRES-eGFP vector used for expressing *Nkx3.1*.

B: NKX3.1 expression can be indirectly detected by the expression of GFP from the same vector (left panels). Right panels show the expression of Flag-tagged NKX3.1 protein in 293T cells (upper row) and infected, eGFP-sorted cells (middle row) by Western blot analysis.

C: A comparison of the wet weight (upper panel) and relative epithelial cell number (lower panel) of regenerated tissue without or with forced *Nkx3.1* expression (mean ± SD). Similar results were obtained in three independent experiments ($p \leq 0.05$). Insert: Western blot shows the levels of PTEN and NKX3.1 in mock-infected (GFP group) and NKX3.1 viral-infected (Nkx3.1 group) grafts.

Introducing *Nkx3.1* into *Pten* null prostatic epithelium leads to reduced graft growth

To evaluate the effects of PTEN loss and forced NKX3.1 expression, we quantified the graft weight (Figure 2C, upper panel) and DNA contents (Figure S2). To increase the confidence of our analysis, we considered only the epithelial compartment, instead of both the mesenchymal and epithelial ones, as a function of PTEN loss or forced *Nkx3.1* expression (Figure 2C, lower panel). Compared to wild-type grafts (blue), *Pten* null grafts (in red) are significantly larger and include greater number of epithelium (Figure 2C, left, compare wild-type-GFP and Mut-GFP groups) while forced *Nkx3.1* expression consistently reduced *Pten* null graft weight and epithelial cell numbers (Figure 2C, compare Mut-GFP and Mut-Nkx3.1 grafts; $p < 0.05$). To evaluate whether the effect of forced *Nkx3.1* expression depends on the stage of cancer, epithelium from PIN lesions (6W) were harvested and yielded similar observations (Figure 2C, right). No significant difference was observed when mock-infected wild-type grafts were compared to *Nkx3.1*-infected wild-type grafts (Figure 2C, compare wild-type-GFP and wild-type-Nkx3.1 groups). These data show that forced *Nkx.3.1* expression can reverse the growth advantage of *Pten* null epithelium but has little effects on its wild-type counterpart.

Nkx3.1 blocks *Pten* null prostate cancer initiation and progression

Histological analysis indicated that wild-type and *Pten* null epithelium reconstituted prostate grafts recapitulate the

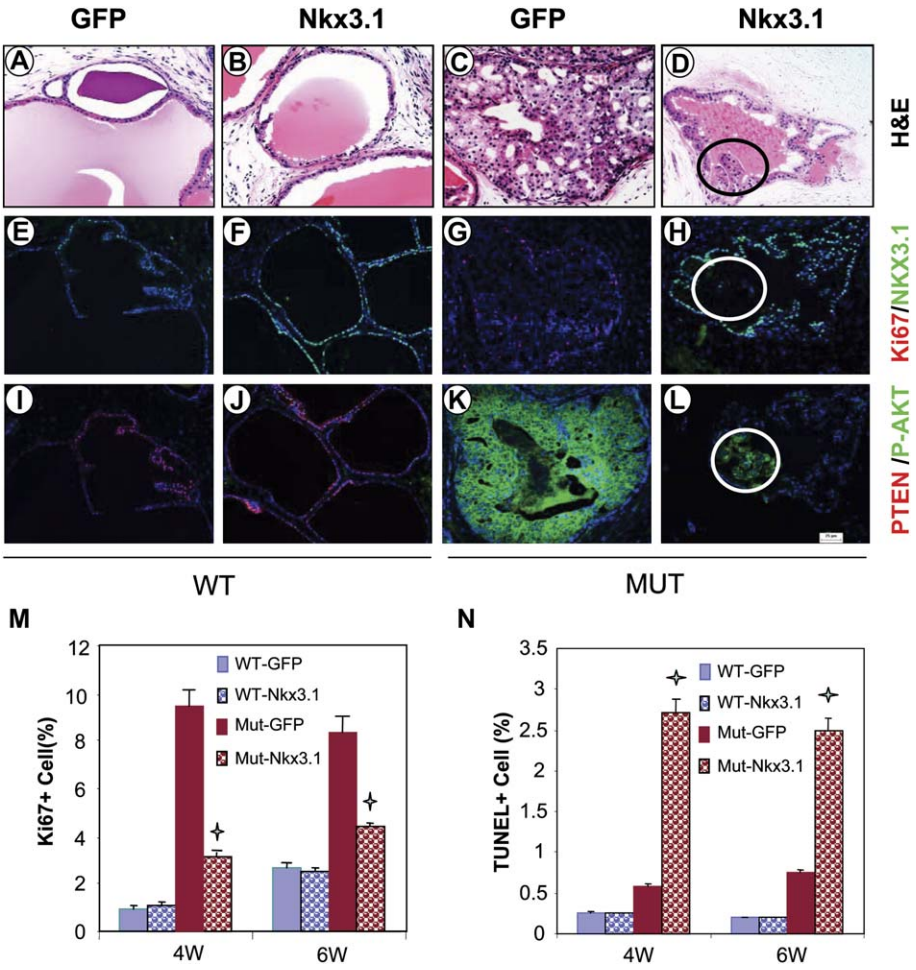

Figure 3. *Nkx3.1* reexpression in *Pten* null epithelium leads to decreased cell proliferation, increased cell death, and reverse of hyperplasia phenotype

A–D: Histopathology of regenerated grafts. H&E-stained paraffin sections of grafts generated from 4-week-old wild-type (**A** and **B**) and *Pten* null (MUT) epithelium (**C** and **D**). Circled area in **D** appears hyperplastic in otherwise normal duct.

E–L: Consecutive sections from upper panels were double immunofluorescent stained with anti-Ki67 and -NKX3.1 antibodies (**E**–**H**), as well as anti-PTEN and -P-AKT antibodies (**I**–**L**) and then counterstained with DAPI. Circled area shows negative correlation of P-AKT staining (**L**) with NKX3.1 expression (**H**).

M and N: *Nkx3.1* reexpression in *Pten* null epithelium leads to quantitatively decreased cell proliferation (M) and increased cell death (N). Data are represented as mean ± SD from three independent experiments ($p \leq 0.05$).

histological characterization of the donor epithelium (Figures 3A and 3C)(Wang et al., 2003). No significant difference was observed when comparing wild-type-GFP and wild-type-Nkx3.1 grafts (compare Figures 3A and 3B). In contrast, tissue recombinants from *Pten* null epithelium resulted in very differential phenotypes: those from the GFP group demonstrated hyperplasia and early mPIN lesions, whereas grafts from the *Nkx3.1* expression group showed relative preservation of normal prostatic duct structure with protein secretion in the lumen (compare Figures 3C and 3D). Occasionally, isolated areas with hyperplastic phenotype were observed (Figure 3D, circled area). Further studies using consecutive sections suggest that these hyperplastic lesions are most likely due to incomplete viral infection, as evidenced by the low to undetectable NKX3.1 protein expression and high P-AKT staining in the same region (circled areas in Figures 3H and 3L, respectively). These results suggest that forced *Nkx3.1* expression can rescue the hyperplastic phenotype caused by PTEN loss.

Forced *Nkx3.1* expression leads to decreased cell proliferation and increased apoptosis of *Pten* null grafts

Previous studies showed that overexpression of *NKX3.1* in human PC3 prostate cancer cells and rodent AT 6 cells leads to inhibition of cell growth (Kim et al., 2002a), whereas increased epithelial cell proliferation is observed in *Nkx3.1* knockout mice (Abdulkadir et al., 2002; Bhatia-Gaur et al., 1999; Magee et al., 2003; Tanaka et al., 2000). We examined whether forced *Nkx3.1* expression could inhibit cell proliferation in *Pten* null epithelium grafts. Detection of the Ki-67 epitope indicated that *Nkx3.1* expression significantly decreased the proliferative index of 4W and 6W mutant grafts (compare Figures 3G and 3H; quantification shown in Figure 3M; $p \leq 0.05$) consistent with the growth-suppressive function of NKX3.1 in previous studies. Interestingly, *Pten* null epithelium appears to be hypersensitive to NKX3.1's suppression effect, since NKX3.1 at similar protein levels (Figure 2C, insert) has no significant effect on cell proliferation in the wild-type grafts (Figures 3E, 3F, and 3M).

Given that *Pten* null grafts infected with NKX3.1 were consistently smaller than those of the control GFP group (Figure 2C), the apoptotic index was evaluated by way of the TUNEL assay. In both 4W and 6W *Pten* null grafts, a significant increase in TUNEL-positive cells was observed in the presence of NKX3.1 (Figure 3N; $p \leq 0.05$). When consecutive tissue sections were probed for the presence of NKX3.1, TUNEL-positive cells were consistently associated with areas of strong NKX3.1 expression (Figure S3). No increase in TUNEL-positive cells was observed in 4W or 6W wild-type grafts (Figure 3N). Taken together, our data demonstrate that the effects of NKX3.1 on cell proliferation and cell death depend on the PTEN status.

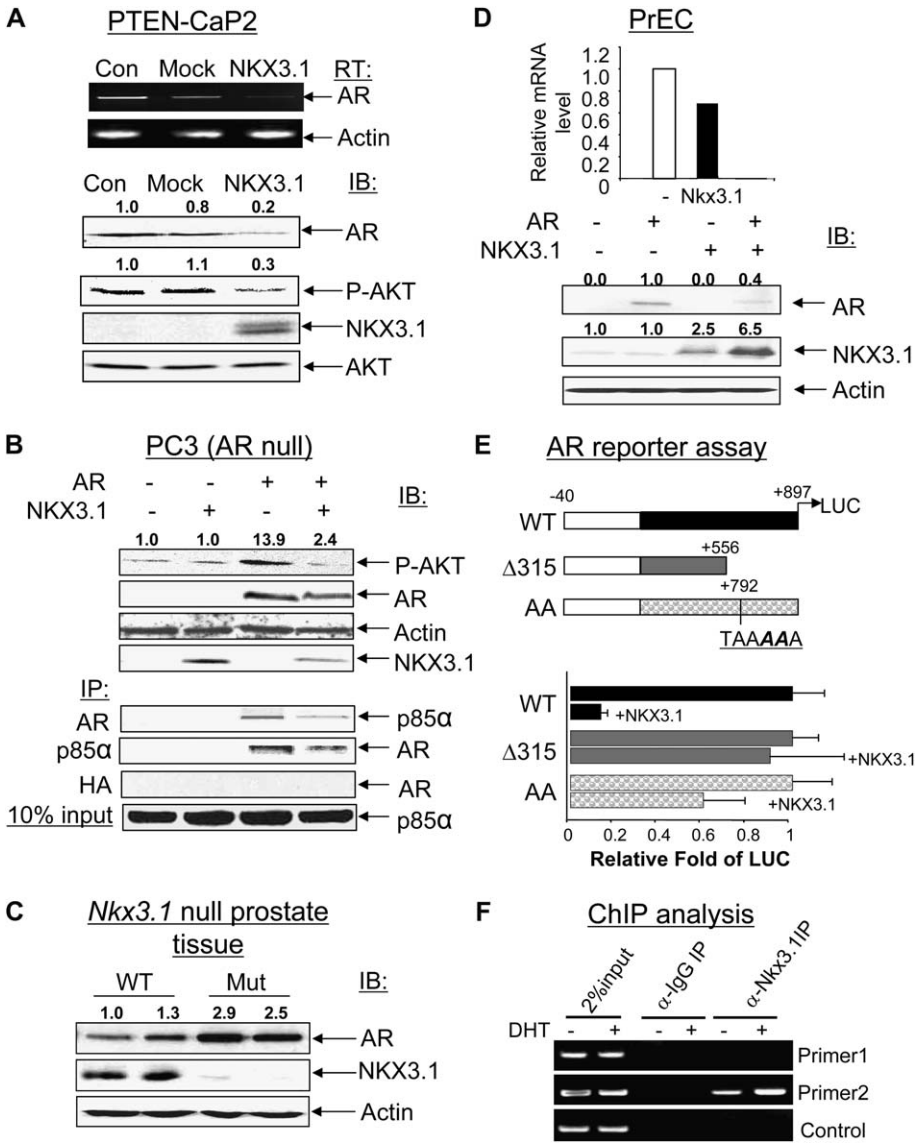

Figure 4. NKX3.1 negatively regulates AR promoter and modulates AKT phosphorylation through an AR-dependent mechanism

A: NKX3.1 regulates AR transcription and reduces AKT phosphorylation in vitro. Total RNA and protein were harvested from PTEN-CaP2 cells transfected with *Nkx3.1*. RT-PCR (upper panel), and Western blot analysis (lower panel) was performed as indicated.

B: NKX3.1-regulated AKT phosphorylation depends on AR expression. PC3 cells were transfected with *Nkx3.1* without *AR* (left two lanes) and with *AR* (right two lanes). Cell lysates were immunoprecipitated with indicated antibodies and analyzed by Western blotting. P-AKT levels were normalized to actin levels.

C: Loss of NKX3.1 leads to increased AR expression in vivo. Western blot analysis of wild-type and *Nkx3.1* null prostate tissues. AR expression levels were normalized to actin loading control.

D: NKX3.1 regulates AR expression in normal human primary prostatic cells. PrEC cells were transfected with indicated plasmid. Thirty-six hours later, RNA and protein were harvested for real-time PCR (upper panel) and Western blot analysis (lower panel), respectively. AR expression and NKX3.1 expression levels were normalized to actin levels.

E: NKX3.1 suppresses AR transcription. PTEN-CaP2 cells were cotransfected with AR luciferase reporter constructs and NKX3.1 expression vector. Relative AR transcriptional activity was measured 24 hr posttransfection. Three independent tests were performed for each experiment and presented as mean ± SD.

F: Association of endogenous NKX3.1 with the AR promoter. LNCaP cells were treated with or without DHT (10 nM) for 16 hr, and ChIP analysis was performed according to Experimental Procedures.

NKX3.1 negatively regulates AKT activity in an AR-dependent manner

To investigate possible changes in the known PTEN-controlled signaling pathways in the presence and absence of NKX3.1, we first examined AKT status on consecutive sections of the grafts. No difference in either PTEN or P-AKT levels can be detected in the wild-type grafts with or without *Nkx3.1* overexpression (Figures 3I and 3J). Robust P-AKT staining can be detected in the mock-infected *Pten* null grafts (Figure 3K), whereas forced *Nkx3.1* expression, at a level comparable to that of wild-type prostatic epithelium (compare Figures 3F and 3H, and Figure 2C), leads to a dramatic decrease in P-AKT levels (Figure 3L). In the uninfected area where NKX3.1 staining is weak or undetectable (Figure 3H, circled area), P-AKT level remains high, thus serving as an important internal control (Figure 3L, circled area).

To develop a system more amenable to in vitro biochemical analysis, we generated several prostate epithelial cell lines from the prostates of *Pten* conditional knockout mice. One such line, PTEN-CaP2, is characterized by positive AR expression but undetectable PTEN and NKX3.1 protein expression (our unpublished data). *Nkx3.1* overexpression in PTEN-CaP2 cells suppressed AKT phosphorylation by 3-fold (Figure 4A, lower panel).

Given previous studies that androgen receptor (AR) can modulate AKT activation via a PI3K-dependent mechanism (Baron et al., 2004; Sun et al., 2003), we investigated whether AR serves as a mediator for NKX3.1-regulated AKT activity. For this, we used PC3 cells, a human prostate cancer cell line known to be null for AR, PTEN, and NKX3.1. When we compared P-AKT levels in the presence or absence of NKX3.1 without cotransfection of AR, we did not detect any significant difference (Figure 4B, first two lanes in the upper panel). While introduction of AR significantly increased the level of P-AKT (Figure 4B, compare first and third lanes in the upper panel), this effect was diminished by cotransfection of the *Nkx3.1*-expressing vector (Figure 4B, compare the third and fourth lanes). Previous studies also showed that AR modulates AKT phosphorylation via binding of the p85α subunit of PI3-kinase (Baron et al., 2004; Sun et al., 2003). We performed reciprocal coimmunoprecipitation experiments and showed that *Nkx3.1* overexpression decreases the

amount of AR able to interact with p85α (Figure 4B, lower panels), further supporting the notion that NKX3.1 controls AKT phosphorylation via an AR/PI3K-dependent mechanism.

NKX3.1 negatively regulates the AR promoter

Since AR serves as a mediator for NKX3.1-controlled AKT activation, we investigated the possible role of NKX3.1 on AR expression in the PTEN-CaP2 cells and found that NKX3.1 also inhibits AR expression at both mRNA (Figure 4A, upper panel) and protein levels (Figure 4A, lower panel). To determine if NKX3.1 negatively regulates AR in vivo, we examined AR protein levels in the prostate glands of *Nkx3.1* knockout mice (Kim et al., 2002b) and found that AR levels are indeed increased (Figure 4C). The role of NKX3.1 in negatively regulating AR level was also confirmed in human primary prostatic PrEC cells (Figure 4D, upper panel; compare lanes 2 and 4 in the lower panel).

Similar to previous reports (Bieberich et al., 1996; He et al., 1997; Magee et al., 2003; Prescott et al., 1998), our study indicates that NKX3.1 expression can be positively modulated by androgen (Figure 4D, compare lanes 3 and 4; Figure 6B). That overexpression of NKX3.1 leads to AR downregulation (upper panels in Figures 4A and 4D) while NKX3.1 loss results in an increased level of AR (Figure 4C) suggests that AR and NKX3.1 may form an important feedback loop. Within this feedback loop, NKX3.1 may serve as an important negative regulator for AR expression as well as AR-controlled signaling pathway. To test this hypothesis, we searched sequences surrounding the murine AR promoter region and found a potential NKX3.1 consensus binding site, TAAGTA, within the 5′ UTR. The functional significant of this consensus site was further investigated using a series of luciferase reporter constructs with truncated promoter regions (Figure 4E, upper panel). NKX3.1 significantly suppressed the wild-type reporter construct, while it had little effect on a Δ315 construct in which sequences surrounding the putative binding motif have been deleted (Figure 4E, lower panel). Changing ''TAAGTA'' to ''TAAAAA'' also decreased NKX3.1's effect (Figure 4E, lower panel).

To assess whether NKX3.1 can associate with the endogenous AR promoter, we conducted chromosome immunoprecipitate (ChIP) analysis on the human prostate cancer cell line LNCaP, which expresses endogenous NKX3.1 and AR. Using a primer set that contains the human NKX3.1 ''CAAG'' motif (nt 1223–1386), we showed that the endogenous NKX3.1 can physically associate with the AR promoter and that this association can be further enhanced in the presence of androgen (Figure 4F, primer 2). We further demonstrate that this association is site specific: primer 1 set derived from nt 1113–1311 of the human AR promoter containing the ''CAAG'' motif, as well as control primers (nt 17–170) without the CAAG motif, can not detect NKX3.1 binding activity. Therefore, NKX3.1 inhibits AR transcriptional activity, at least in part, through its consensus binding site.

NKX3.1 negatively regulates AR expression in both murine and human prostatic cancer samples

To test whether the inverse relationship between NKX3.1 and AR is present in human prostate cancer samples, we conducted double immunofluorescent analysis of NKX3.1 and AR and found two basic scenarios: areas where NKX3.1 levels negatively correlate with AR levels (Figure 5A) and areas where NKX3.1 and AR are coexpressed (Figure 5B). Interestingly, even in the areas where both NKX3.1 and AR are expressed, mosaic patterns of NKX3.1 and AR expression can be easily detected (Figure 5B, high-powered image on the right), suggesting that the NKX3.1-AR feedback loop may function at a single cell level.

Importantly, despite upregulated AR levels (Figure 5C), NKX3.1 mRNA levels are downregulated in both PIN (Figure 5C) and *Pten* null prostate cancer (Wang et al., 2003), suggesting that PTEN either plays a predominant role over AR, the known positive regulator of *Nkx3.1*, or is essential for AR-mediated *Nkx3.1* regulation. We then compared the relative expression levels of those NKX3.1 target genes identified by Magee et al. (2003) that are also present in our microarray data sets (Wang et al., 2003 and our unpublished data). Intriguingly, the trends of gene regulation in the *Pten* null prostate are very similar to what has been observed in the *Nkx3.1* null prostate: *Probasin*, which is known to be positively regulated by NKX3.1, is downregulated in the *Pten* null PIN lesions, whereas *Elafin-like II*, which belongs to the repression group, is upregulated upon *Pten* deletion (Figure 5C). Furthermore, the mosaic expression patterns of NKX3.1 and AR shown in Figure 5B may provide an explanation for the stochastic expression patterns of NKX3.1-targeted genes within the same prostatic acini (Magee et al., 2003).

NKX3.1 stabilizes p53 through MDM2-dependent and AKT-independent mechanisms

PTEN regulates p53 protein levels and transcription activity via AKT-MDM2-dependent and -independent mechanisms (Freeman et al., 2003; Mayo and Donner, 2001; Zhou et al., 2001). The fact that overexpression of NKX3.1 inhibits AKT phosphorylation in *Pten* null grafts prompts us to analyze whether NKX3.1 also alters p53 levels. Western blot analysis demonstrated that forced *Nkx3.1* expression significantly increases p53 protein levels in *Pten* null grafts in vivo (Figure 6A, upper panels), whereas knockout of *Nkx3.1* leads to reduced p53 levels (Figure 6A, lower panels).

To understand the molecular mechanisms involved in NKX3.1-regulated p53 activity, we first measured endogenous p53 levels in the LNCaP cells. The androgen analog R1881 can stimulate the endogenous NKX3.1 expression with a peak around 4 hr (Figure 6B, upper panel). Following this trend, p53 protein levels are also increased by 1.5-fold 4 hr after R1881 addition (Figure 6B, upper panel). Consistently, overexpression of *Nkx3.1* leads to increased p53 protein levels without changing of its mRNA level (Figure S4, upper panel), suggesting that NKX3.1 modulates p53 at the posttranscriptional level. Furthermore, *Nkx3.1* expression leads to an increase in p53 half-life from 21 min to 30 min in LNCaP cells (Figure 6B, lower panel). Similar results were also obtained when using the *Pten* null murine PTEN-CaP2 prostate cancer cell line (Figure S4, lower panel).

To determine whether NKX3.1 stabilizes p53 in a MDM2-dependent manner, we introduced *Nkx3.1* into *p53/Mdm2* double null ($p53^{-/-};Mdm2^{-/-}$) mouse embryonic fibroblasts (Jones et al., 1995). Without MDM2, NKX3.1 has no significant influence on p53 expression levels (Figure 6C, compare lanes 3 and 4). When cotransfected with a vector containing *Mdm2*, NKX3.1 expression partially reverses the increased p53 degradation brought about by *Mdm2* overexpression (Figure 6C, compare lanes 5 with 6), suggesting that NKX3.1 controls p53 half-life via a MDM2-dependent mechanism.

MDM2 nuclear translocation and stability are known to be controlled by AKT phosphorylation (Mayo and Donner, 2001;

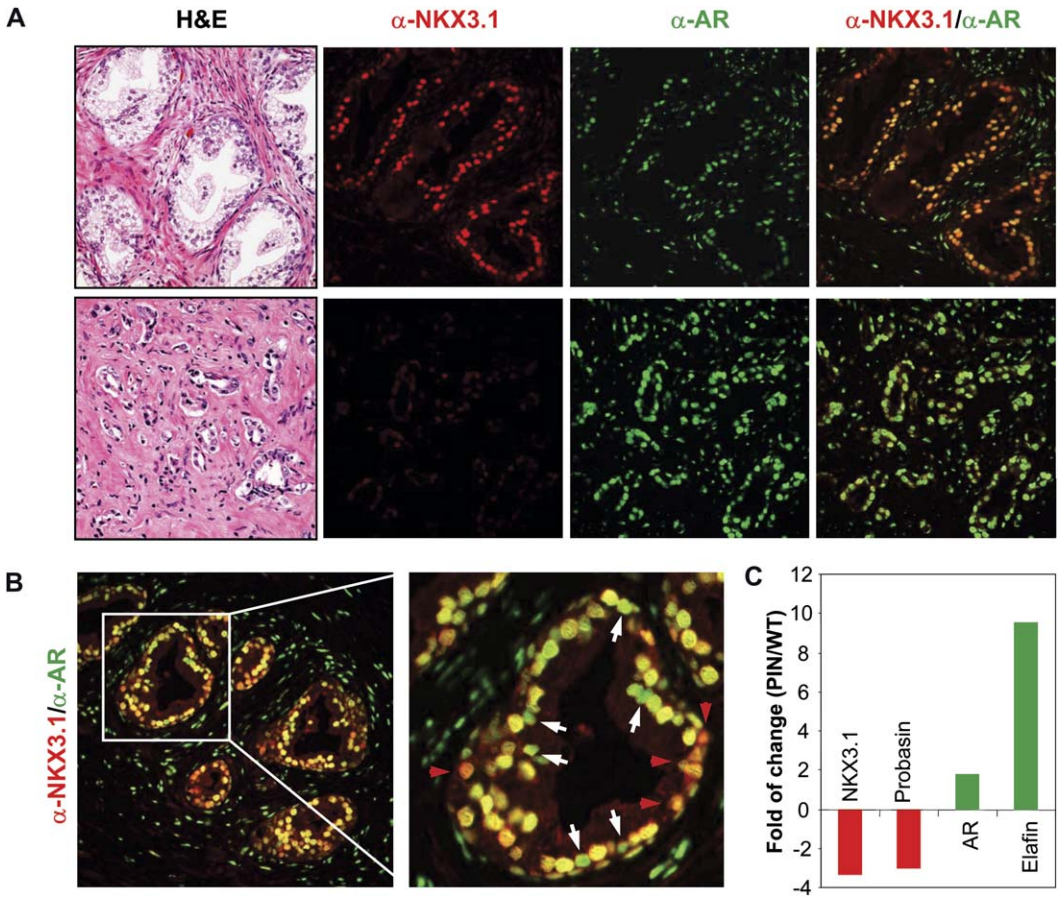

Figure 5. NKX3.1 controls AR level in human prostate cancers and modulates its target gene expression in *Pten* null prostate cancer model

A: Inverse correlation of NKX3.1 and AR levels in human prostate cancer samples. Representative images of double immunohistochemistry analysis using anti-NKX3.1 and AR antibodies.

B: Mosaic expression patterns of NKX3.1 and AR in human prostate samples. Double immunohistochemical analysis shows overlapping NKX3.1 and AR expression in most of the epithelial cells (in yellow), with some cells that have higher levels of either NKX3.1 (in red; red arrowheads) or AR (in green; white arrowheads) expression.

C: The relative expression levels of Nkx3.1, AR, and their target genes in *Pten*-deleted prostate glands. Microarray analysis was performed using mRNA prepared from the prostates of 6-week-old *Pten* null mice (PIN lesion stage) and their wild-type littermates (n = 5). Fold of changes in indicated genes was calculated as the relative expression levels of PIN versus wild-type ($p \leq 0.01$).

Zhou et al., 2001). To determine whether NKX3.1 antagonizes MDM2 function via an AKT-dependent mechanism, we treated PC3 cells with or without LY294002, a specific inhibitor for PI3K. As shown in Figure 6D, although P-AKT levels were significantly diminished, LY294002 treatment had no significant effect on either p53 (compare lanes 2 and 5) or NKX3.1-regulated p53 level (compare lanes 3 and 6). This result suggests that NKX3.1 regulates p53 half-life by modulating nuclear MDM2 activity, independent of AKT activation.

NKX3.1 can physically associate with HDAC1 and promotes p53 acetylation by recruiting HDAC1 from p53-MDM2-HDAC1 complex

Besides its E3 ubiquitin ligase activity, MDM2 can negatively regulate p53 half-life by recruiting a nuclear protein HDAC1, thereby promoting p53 deacetylation and degradation (Kobet et al., 2000; Ito et al., 2002; Brooks and Gu, 2003). To determine if enhanced p53 acetylation is one of the mechanisms involved in NKX3.1-mediated p53 half-life control, we examined acetylated p53 levels without or with *Nkx3.1* overexpression and found that NKX3.1 indeed enhances p53 acetylation (Figure 6E, upper panel). In vivo, NKX3.1 can physically associate with either endogenous or exogenous HDAC1 (Figure 6E, middle and lower panels), suggesting that NKX3.1 can form a complex with HDAC1 and change p53 acetylation status. Furthermore, NKX3.1 cannot further increase p53 levels in the presence of TSA, an inhibitor for HDAC activity, suggesting that NKX3.1 indeed depends on HDAC activity to regulate p53 level (Figure S5).

We then tested whether NKX3.1 can recruit HDAC1 from p53-MDM2-HDAC1 complex. To do this, *Mdm2* was introduced into $p53^{-/-};Mdm2^{-/-}$ cells, without or with *Nkx3.1*, followed by measurement of the relative amount of p53 associating with HDAC1. It was observed that *Mdm2* expression leads to increased p53-HDAC1 association by 4-fold, resulting in decreased total p53 protein level (compare lanes 1 and 2 of Figure 6F). In contrast, coexpressing *Nkx3.1* reduces p53-HDAC1 association, partially rescuing MDM2-mediated p53 degradation (Figure 6F, compare lanes 2 and 3). Thus, NKX3.1 regulates p53 half-life, as least in part, by recruiting HDAC1

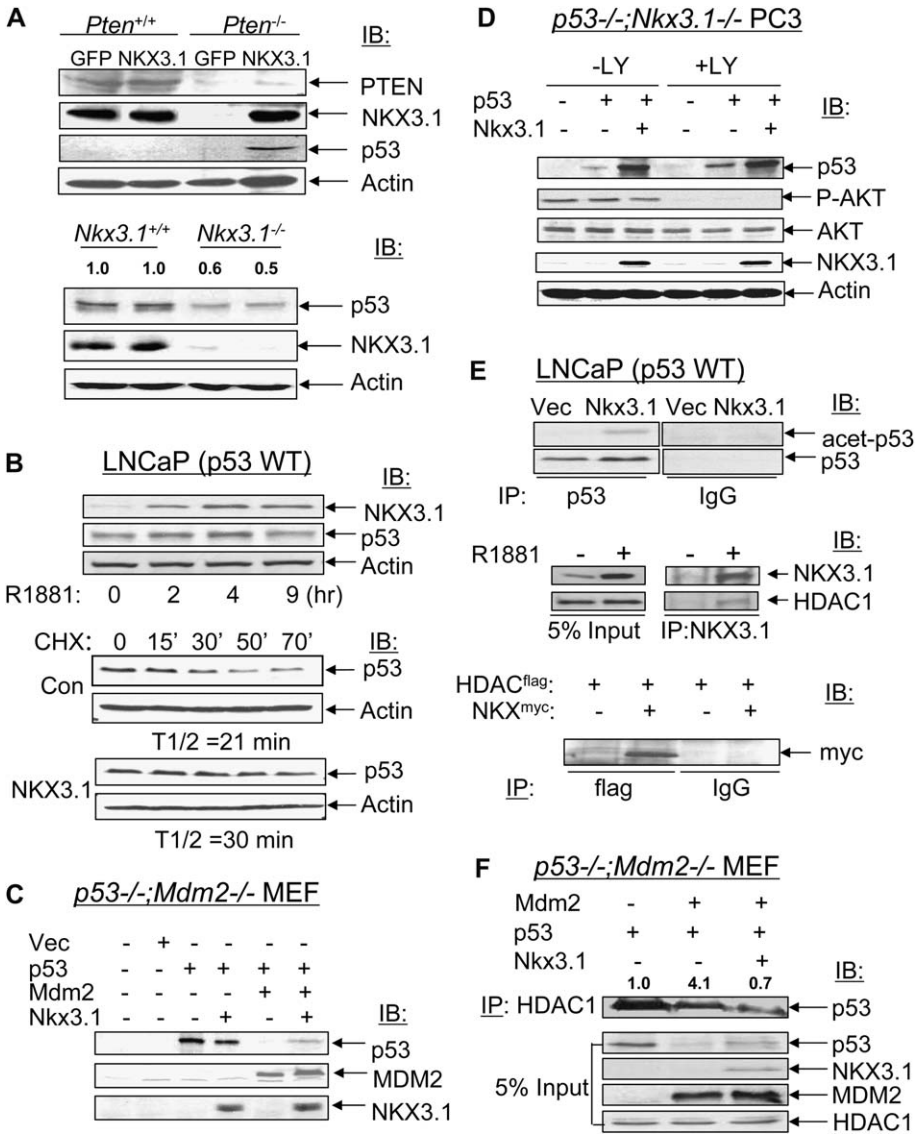

Figure 6. NKX3.1 regulates the level of p53 in a MDM2-dependent and AKT-independent manner

A: NKX3.1 regulates the level of p53 in vivo. Upper panel: reintroducing *Nkx3.1* into *Pten* null epithelium increases p53 protein level in regenerated grafts. Lower panel: knocking out *Nkx3.1* leads to decreased p53 protein levels. Cell lysates from *Nkx3.1* wild-type and null prostate tissues were analyzed using Western blot and normalized to actin loading control.

B: NKX3.1 regulated p53 level and half-life. Upper panel shows androgen-induced NKX3.1 expression and p53 increase in LNCaP cells. Lower panel: *Nkx3.1*-transfected LNCaP cells were incubated with cycloheximide for the indicated time, and total proteins were analyzed by Western blot. p53 half-life was calculated using Quantity One (Bio-Rad).

C: NKX3.1 stabilizes p53 via MDM2-dependent mechanism. Western blot analysis of cell lysates from $p53^{-/-};Mdm2^{-/-}$ MEFs after transfection of indicated plasmids.

D: NKX3.1 stabilizes p53 in an AKT-independent manner. PC3 cells were transfected with indicated plasmids with or without LY treatment before harvesting. Cell lysates were analyzed by Western blot.

E: NKX3.1 increases p53 acetylation and can physically associate with HDAC1. Upper panel: LNCaP cells were transfected without or with *Nkx3.1*, and the cell lysates were first immunoprecipitated with anti-p53 antibody and then blotted with antibodies recognizing either acetylated p53 or total p53, respectively. Middle panel: LNCaP cells were treated with androgen analog R1881 for 4–6 hr to induce *Nkx3.1* expression, and cell lysates were immunoprecipitated with anti-NKX3.1 antibody and then blotted with indicated antibodies. Lower panel: LNCaP cells were cotransfected with Flag-tagged HDAC1 and myc-tagged NKX3.1. Cell lysates were immunoprecipitated with either anti-Flag or control IgG and blotted with anti-myc antibody.

F: NKX3.1 modulates the level of p53 by recruiting HDAC1 from the MDM2-p53 complex. PC3 cells were cotransfected with indicated plasmid. Cell lysates were immunoprecipitated with anti-HDAC1 antibody and blotted with anti-p53 antibody. The relative amounts of p53 within the p53-HDAC1-MDM2 complex, as indicated above the Western blot, were calculated as HDAC1-associated p53:total p53 from the input, then normalized against lane 1.

from the HDAC1-MDM2-p53 complex, thereby promoting p53 acetylation.

Discussion

The involvement of NKX3.1 in human prostate cancer development has been intensively studied since its cloning (for review see Abdulkadir, 2005). Despite strong correlation of loss of NKX3.1 expression in human prostate cancer initiation and progression, several outstanding questions remain to be answered; these include but are not limited to the following. (1) How and at what level is NKX3.1 expression controlled in the prostate epithelium? (2) What is the normal function of NKX3.1 in vivo? And (3) what are the targets of NKX3.1, and how do those targets, upon NKX3.1 loss, contribute to prostate cancer initiation and progression? Our current study suggests that PTEN and its controlled signal pathway regulate NKX3.1 expression in the prostatic epithelium. Within the prostate epithelium, AR and NKX3.1 form a signaling feedback loop in which NKX3.1 is the negative modulator that keeps AR level and AR-controlled pathways in check. NKX3.1 regulates prostate cell proliferation and survival not only via its transcription factor activity, i.e., by negatively modulating the AR promoter, but also through protein-protein interaction with HDAC1 and consequently regulating p53 half-life and activity (Figure 7). Our finding emphasizes the cooperative effects between ubiquitously expressed *PTEN* tumor suppressor genes and prostatic-specific expressed *NKX3.1* in prostate cancer development.

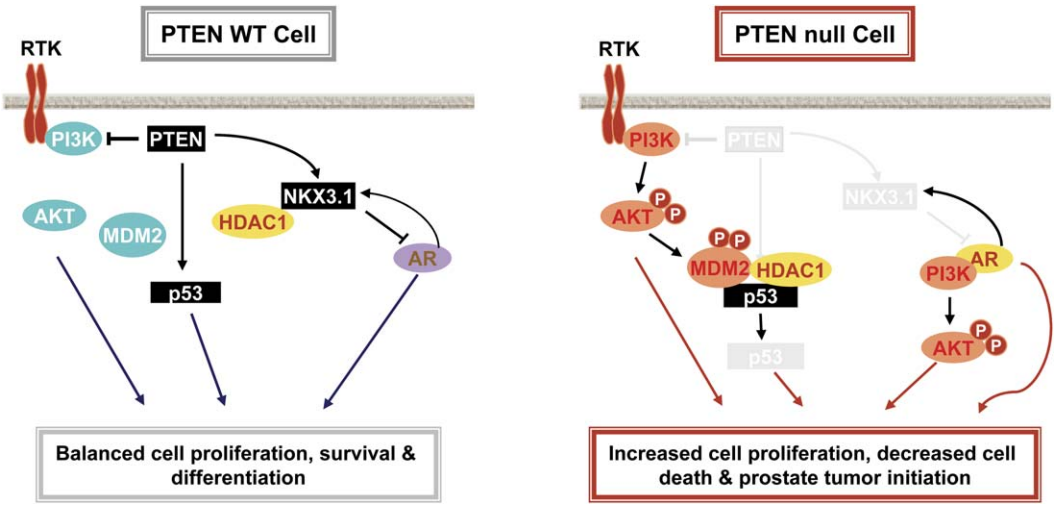

Figure 7. A schematic diagram illustrating the involvement of NKX3.1 in PTEN-controlled prostate tumorigenesis

In *Pten* wild-type prostatic epithelium (left panel), PTEN negatively regulates PI3K/AKT pathway but positively modulates p53 level and activity. The transcription level of *Nkx3.1* is controlled predominantly by PTEN as well as by AR. NKX3.1 in turn negatively regulates AR promoter activity and keeps AR and AR-controlled pathway in check. NKX3.1 also binds HDAC1 and releases p53 from p53-MDM2-HDAC1 complex, promoting p53 acetylation and activity. The net result of these NKX3.1-mediated PTEN functions is the balanced cell proliferation, differentiation, and cell death, which prevents prostate cancer initiation. Upon PTEN loss (right panel), the balance is broken, and NKX3.1 and its controlled signaling pathways are severely downregulated. AR, no longer under the control of NKX3.1, becomes overexpressed and activates its targets and downstream pathways, including PI3K/AKT pathway. In the absence of NKX3.1, most of the p53 is in the MDM2-HDAC1 complex, leading to p53 degradation. Activation of PI3K/AKT pathway, together with downregulated p53 activity and increased AR level and activity, leads to increased cell proliferation, decreased cell death, and prostate cancer initiation.

Several mechanisms have been proposed for loss of NKX3.1 expression in human prostate cancers, including both posttranscriptional modification, such as protein degradation, as well as transcriptional and epigenetic regulation (Asatiani et al., 2005; Bowen et al., 2000; He et al., 1997; Korkmaz et al., 2004; Ornstein et al., 2001; Voeller et al., 1997). The facts that NKX3.1 mRNA (Figure 5C and Wang et al., 2003) and protein levels (Figure 1A and Wang et al., 2003) are concomitantly downregulated in the *Pten* null prostate, and we can successfully restore *Nkx3.1* expression using an exogenous promoter and maintain near wild-type levels of NKX3.1 in different cell lines and in the renal capsule grafts (Figure 2C), suggest that PTEN modulates NKX3.1 function largely through regulation of its promoter activity. This conclusion is consistent with the strong correlation of NKX3.1 mRNA and protein levels in human prostate cancer specimens reported by a recent study (Korkmaz et al., 2004).

Nkx3.1 is positively regulated by androgen and its receptor AR at the transcriptional level (Bieberich et al., 1996; He et al., 1997; Magee et al., 2003; Prescott et al., 1998). Our results suggest that PTEN either predominantly controls NKX3.1 transcription or contributes significantly to AR-controlled mechanism. In the presence of PTEN, *Nkx3.1* expression can be modulated by AR, while in its absence, *Nkx3.1* expression is almost completely silenced despite increases in AR levels in *Pten* null prostate cancers (Wang et al., 2003; Figures 1A, 5C, and 7). Mechanisms involving enhancer/promoter and transcription activator/repressor interactions, epigenetic changes such as DNA methylation (Asatiani and Gelmann, 2005), and histone modification (Plass, 2002) are all potential possibilities by which NKX3.1 and AR interact and are regulated by PTEN and warrant a separate and more elaborate study.

Recently, Magee et al. identified NKX3.1 target genes that are sensitive to NKX3.1 dosage in a stochastic manner and, at the same time, are influenced by the androgen status in vivo (Magee et al., 2003). Among those target genes is a set regulated by androgens only in the absence of NKX3.1, suggesting that NKX3.1 functions as a selector for modulation of the expression of potential androgen target genes. We demonstrate that NKX3.1 loss, in either human prostate cancer samples (Figure 5A) or murine prostates of *Pten* and *Nkx3.1* knockout mice (Figures 5C and 4C), leads to increased AR levels. Conversely, overexpression of *Nkx3.1* decreases AR mRNA and protein expression (Figures 4A and 4D). These results imply that NKX3.1 is a negative regulator for AR expression, and AR and NKX3.1 form a feedback loop important for both prostate development and cancer formation. With *Nkx3.1* at steady-state levels, androgen/AR induces NKX3.1 expression, which in turn inhibits AR expression. Interestingly, the mosaic expression pattern of NKX3.1/AR target genes showed by Magee and colleagues (Magee et al., 2003) are similar to NKX3.1 and AR expression patterns observed in human prostate samples (Figure 5B), suggesting that the balance of NKX3.1-AR expression may ultimately determine the levels of target gene expression. The natural "set point" of this feedback loop and its range of action are currently unknown and require further investigation. Loss of NKX3.1 in the prostatic epithelium will impair this feedback system (Figure 7), which in turn leads to AR overexpression and may contribute to PIN lesion in *Nkx3.1* knockout mice (Abdulkadir et al., 2002; Kim et al., 2002a) and prostate cancer development in *Pten* null model (Wang et al., 2003), similar to what has been concluded from AR transgenic animals (Stanbrough et al., 2001) and human prostate cancer studies (Chen et al., 2004).

The growth-suppressive activities of NKX3.1 have been demonstrated in vitro in cell culture system and in vivo in knockout mice (Bhatia-Gaur et al., 1999; Kim et al., 2002a; Schneider et al., 2000; Tanaka et al., 2000). NKX3.1 haploinsufficiency or

loss leads to significant delay in exiting from the cell cycle (Magee et al., 2003), but how NKX3.1 engages the cell cycle machinery to regulate prostatic epithelium growth is currently unknown. Here, we showed that restoration of *Nkx3.1* expression to the wild-type level in *Pten* null prostatic epithelium leads to increased p53 protein levels in vivo and in vitro. NKX3.1 can physically associate with HDAC1 and recruit HDAC1 from the HDAC1-MDM2-p53 complex, protecting p53 from deacetylation and degradation. Furthermore, NKX3.1 stabilizes p53 through modulation of MDM2 activity in the nucleus, independent of AKT-mediated MDM2 phosphorylation and nuclear translocation. Whether the above-mentioned pathways involve or collaborate with NKX3.1-regulated oxidative damage or p53-dependent senescence requires further investigation (Chen et al., 2005; Ouyang et al., 2005).

Although this study suggests that NKX3.1 plays an essential role in PTEN-controlled prostate cancer development, NKX3.1 loss alone only mimics a part of *Pten* deletion phenotype, suggesting that other PTEN-controlled pathways may synergistically interact with those NKX3.1-dependent events (Figure 7). AKT hyperphosphorylation has been observed in isolated clusters of cells within regions of high grade PIN lesions in *Nkx3.1*$^{+/-}$ and *Nkx3.1*$^{-/-}$ mice, and deletion of one allele of *Pten* in *Nkx3.1* null mice leads to accelerated tumor initiation and increased incidence of high-grade PIN (Kim et al., 2002b). However, differing from the *Pten* null prostate cancer model (Wang et al., 2003), *Pten*$^{+/-}$*;Nkx3.1*$^{-/-}$ mice do not progress to metastatic prostate cancer (Kim et al., 2002b). These results suggest that the dosage of PTEN plays a dominant role in the cooperative effect of NKX3.1 and PTEN.

We showed that forced *Nkx3.1* expression in *Pten* null epithelium significantly induces cell apoptosis in the mutant grafts. This mechanism, together with NKX3.1's growth suppression function, leads to reduced graft size. Interestingly, the effects of NKX3.1 appear to depend on the PTEN status: *Nkx3.1* overexpression leads to increased cell death and decreased cell proliferation in the *Pten* null grafts but has no significant impact on the wild-type grafts. One possible explanation is that *Pten* null cells have become "addicted" to high levels of PI3K/AKT activity and, consequently, are hypersensitive to inhibition of this pathway. We have previously shown that PTEN-deficient human cancer cell lines and murine *Pten* null tumors are sensitive to inhibitors specific for mTOR, a downstream effector of the PI3K/AKT pathway (Neshat et al., 2001). In the present study, we demonstrate that NKX3.1 inhibits AKT phosphorylation/activation via an AR-dependent mechanism and show that NKX3.1 expression in vivo can block the hyperproliferative and antiapoptotic effects brought on by PTEN loss. This mechanism elucidates a pathway that can potentially be targeted with specific therapies for human prostate cancer, given that 30% of primary prostate cancers and as many as 60% of metastatic cases exhibit *PTEN* LOH (Sellers and Sawyers, 2002).

Experimental procedures

Immunohistochemical analysis

Tissues were fixed in 10% buffered Formalin for 6 hr, followed by transfer to 70% alcohol. These paraffin-embedded tissues were sectioned (4 μm) and stained with hematoxylin and eosin. Antigen retrieval was performed by incubating the slides in 0.01 M citric acid buffer (pH 6.0) at 95°C for 30 min. The endogenous peroxidase activity was inactivated in a solution containing 3% hydrogen peroxide (H_2O_2) in methanol. The following detection and visualization procedures were performed according to the manufacturer's protocol. Negative control slides were performed without primary antibody. Control slides known to be positive for each antibody were incorporated. For fluorescence double staining, pretreated sections were first blocked with mouse Ig blocking reagent in the VECTOR M.O.M. Immunodetection Kit (Vector Laboratories) and then incubated with mouse antibody PTEN (26H9, Cell Signaling Technology), Ki67 (NCL-Ki67-MM1), or NKX3.1 (a kind gift from Dr. Abate-Shen) at room temperature for 30 min, followed by signal amplification with TSA Plus Fluorescence Systems (PerkinElmer). After biotin blocking, the section was then stained with rabbit antibody P-AKT (9277, Cell Signaling Technology) or AR (PG21, Upstate), and signal was amplified with TSA system with different fluorescence. For human prostate tissue array, PTEN, NKX3.1 (SC-816 and SC-15022, Santa Cruz), and AR (PG21, Upstate) were used.

Tissue array analysis

Double immunofluorescent staining was performed as above. PTEN and NKX3.1 (Santa Cruz SC-816 and SC-15022, respectively) intensity was analyzed by Image Plus software, and only cores with epithelial structure were chosen for further analysis. Briefly, we chose four views for each core; measured the signal intensity using Image Plus software; and signed the signal intensity as 0–6, in which 0[<50]; 1[50-75]; 2[75–100]; 3[100–125]; 4[125–150]; 5[150–175]; 6[>175]. According to the average intensity value, samples were categorized into grades. By using the intensity grade, a stacked line was generated with value of each sample displayed. Linear regression analysis was performed for the correlation between PTEN and NKX3.1 expression by SPSS software and presented as Skattergram.

Preparation of prostate epithelial cells, virus infection, and prostate regeneration

Four- and six-week-old *Pten* wild-type and mutant mice were killed by carbon dioxide inhalation. Prostates were dissected, cut into small pieces with a steel blade, and digested with 0.8 mg/ml collagenase (GIBCO, 226 units/mg) in 10 ml of DMEM 10% FBS (GIBCO) at 37°C for 90 min. Cells were filtered through 100 μm nylon mesh (Becton Dickinson), washed twice with 10 ml of DMEM 10% FBS, resuspended in 1 ml of DMEM 10% FBS, and counted.

The lentivirus was prepared as described (Lois et al., 2002; Pfeifer et al., 2002). Infection was carried out according to Xin et al. (2003). Briefly, 1 × 10^5 prostate cells were mixed with GFP or NKX3.1 Lentivirus stock (titer 2 × 10^7) in the presence of 8 μg/ml polybrene (Sigma), then centrifuged at 1500 rpm with a Beckman GS-6R centrifuge (Beckman Coulter) for 2 hr at room temperature and washed twice with 1 ml of DMEM 10% FBS. All procedures were performed under University of California Los Angeles safety regulations for lentivirus usage.

Mouse prostate regeneration was performed according to previous reports (Cunha and Donjacour, 1987; Thompson et al., 1989; Xin et al., 2003). Lentivirus-infected cells (1 × 10^5) were combined with UGSM cells (1 × 10^5) and 25 μl of type I collagen (Roche) and then grafted under the renal capsule. Each experiment contained grafts of UGSM alone to ensure that tissue growth did not result from contaminating urogenital sinus epithelial cells. Grafts were harvested and weighed after 6–9 weeks. All surgical procedures were performed under Division of Laboratory Animal Medicine regulations of the University of California, Los Angeles.

Cell proliferation and apoptosis index

Cell proliferation index was examined by Ki67 staining. Five different fields were chosen, and then 200 cells were counted in each field. Ki67+ cells were presented as the percentage of nucleated cells. Cell apoptosis were determined by TUNEL assay using the In Situ Cell Death Detection Kit from Roche according to the manufacturer's instruction. Sections were dewaxed with xylene and rehydrated through graded alcohol. DNA fragmentation was labeled with fluorescein-conjugated dUTP and visualized with fluorescence. TUNEL-positive cells were counted and presented the percentage of nucleated cells.

Western blot analysis

Protein lysate was prepared by sonicating graft tissues and prostate tissue from *Nkx3.1* mice, R1881 treated or transfected LNCaP, PC3 and PTEN-CaP2 cells in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl,

0.1% SDS, 0.5% SD, 1% NP-40, 1 mM EDTA, 1 mM PMSF, 25 mM NaF, and cocktail protease inhibitors (Roche). Tissue lysate (40 μg) was resolved to SDS-PAGE followed by Western blot analysis using anti-p53 (Ab-1, Oncogene; DO-1, Santa Cruz), P-AKT (9271, Cell Signaling), total AKT (Cell Signaling), NKX3.1 (SC-15022, Santa Cruz), MDM2 (Ab-2, Oncogene), Flag (Stratagene), and Actin (#5060, Sigma) antibodies, respectively.

For immunoprecipitation experiments, 500 μg of cell lysate was incubated 16 hr at 4°C with 2 μg AR or p85α antibody (PG-21 and #06-496, Upstate), p53 antibody (DO-1, Santa Cruz), NKX3.1 or HDAC1 (SC-15022, SC-07872, Santa Cruz), or Flag antibody (Sigma, F1804) plus 50 μl Protein A agarose beads (#16-125, Upstate). Beads were washed three times with lysis buffer and centrifuged for 5 min at 5000 g between each wash. Protein was eluted from beads with 50 μl Laemmli sample buffer (Bio-Rad). Lysates were resolved on a 10% SDS-PAGE gel and transferred onto nitrocellulose (Bio-Rad).

For endogenous NKX3.1 induction, LNCaP cells were treated with 2 nM R1881 for different time periods after being plated in 10% charcoal serum for 2 days. For half-life experiments, *NKX3.1* was transfected into LNCaP cells 36 hr prior to the addition of 50 μg/ml cycloheximide (Cycloheximide, Calbiochem) in serum-free medium. Cells were then lysed at indicated time points and further analyzed by Western blot.

Supplemental data

The Supplemental Data include Supplemental Experimental Procedures and five supplemental figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/9/5/367/DC1/.

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