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Stabilization of the Prostate-Specific Tumor Suppressor NKX3.1 by the Oncogenic Protein Kinase Pim-1 in Prostate Cancer Cells

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ABSTRACT

Loss of NKX3.1 is an early and consistent event in prostate cancer and is associated with increased proliferation of prostate epithelial cells and poor prognosis. NKX3.1 stability is regulated post-translationally through phosphorylation at multiple sites by several protein kinases. Here, we report the paradoxical stabilization of the prostate-specific tumor suppressor NKX3.1 by the oncogenic protein kinase Pim-1 in prostate cancer cells. Pharmacologic Pim-1 inhibition using the small molecule inhibitor CX-6258 decreased steady state levels and half-life of NKX3.1 protein but mRNA was not affected. This effect was reversed by inhibition of the 26S-proteasome, demonstrating that Pim-1 protects NKX3.1 from proteasome-mediated degradation. Mass spectrometric analyses revealed Thr89, Ser185, Ser186, Ser195, and Ser196 as Pim-1 phospho-acceptor sites on NKX3.1. Through mutational analysis, we determined that NKX3.1 phosphorylation at Ser185, Ser186, and within the N-terminal PEST domain is essential for Pim-1-mediated stabilization. Further, we also identified Lys182 as a critical residue for NKX3.1 stabilization by Pim-1. Pim-1-mediated NKX3.1 stabilization may be important in maintaining normal cellular homeostasis in normal prostate epithelial cells, and may maintain basal NKX3.1 protein levels in prostate cancer cells. J. Cell. Biochem. 114: 1050–1057, 2013.

KEY WORDS: PROSTATE CANCER; NKX3.1; PIM-1 KINASE; PHOSPHORYLATION

NKX3.1 is a homeodomain protein and a member of the NK-family of transcription factors whose expression in adult animals is restricted primarily to the prostate [Bieberich et al., 1996; He et al., 1997; Bhatia-Gaur et al., 1999]. In addition to its role in prostate development, NKX3.1 functions as a prostate-specific tumor suppressor [He et al., 1997; Bhatia-Gaur et al., 1999]. Diminished NKX3.1 expression is a consistent feature in both human and mouse prostate cancers and is observed in prostatic intraepithelial neoplasia (PIN) lesions [Abdulkadir et al., 2002; Kim et al., 2002; Bethel and Bieberich, 2007]. The genomic region harboring *NKX3.1* (8p21) undergoes frequent loss of heterozygosity in human prostate cancer [He et al., 1997; Magee et al., 2003]. Targeted *Nkx3.1* disruption in mice leads to mouse-PIN, prostate epithelial dysplasia, and hyperplasia, whereas ectopic NKX3.1 expression in prostate cancer cells suppresses their growth and tumorigenic potential [Bhatia-Gaur et al., 1999; Abdulkadir et al., 2002; Kim et al., 2002; Bethel et al., 2006; Bethel and Bieberich, 2007].

Although these data establish the role of NKX3.1 as a prostate-specific tumor suppressor, the regulation of NKX3.1 in prostate epithelial cells is complex. Discordance between NKX3.1 mRNA and protein levels was reported in prostate cancer cases, suggesting that NKX3.1 is post-translationally regulated in prostate cancer cells [Bethel et al., 2006]. Reports have since emerged linking protein phosphorylation to NKX3.1 stability. Phosphorylation at Thr89 and Thr93 by CK2 was shown to stabilize NKX3.1 [Li et al., 2006]. In contrast, the pro-inflammatory cytokines TNF-α and IL1-ß induced phosphorylation in an NKX3.1 C-terminal domain, causing rapid NKX3.1 ubiquitination and degradation [Markowski et al., 2008]. TNF-α-mediated phosphorylation at Ser196 was shown to significantly decrease NKX3.1 half-life, and phosphorylation at Ser185 and Ser195 was also shown to be critical in maintaining NKX3.1 steady-state levels in prostate epithelial cells [Markowski et al., 2008].

Here, we report the stabilization of the tumor suppressor NKX3.1 by the protein kinase Pim-1, a serine/threonine protein kinase

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suspected to function as an oncoprotein. We demonstrate that Pim-1 directly phosphorylates NKX3.1 in N-terminal and C-terminal domains and protects it from proteasome-mediated degradation. Taken together, these data reveal a new molecular mechanism that operates to control the steady-state level of NKX3.1, and further underscores the complex regulatory signaling network that acts to regulate this key tumor suppressor post-translationally.

MATERIALS AND METHODS

ANTIBODIES, REAGENTS, AND VECTOR CONSTRUCTS

Antibodies were as follows: NKX3.1 (AthenaES, #0314), c-MYC (N-term; Epitomics, #1472-1), HA antibody (Roche, #11867423001), donkey anti-rabbit, donkey anti-mouse, and goat anti-rat HRP-linked (Roche). Reagents were as follows: CX-6258 (Cylene Pharmaceuticals), MG132 (Sigma).

CELL CULTURE AND TRANSFECTION

PC3, LNCaP, and 22RV1 cells (Invitrogen) were cultured in RPMI 1640 medium containing L-glutamine and supplemented with 10% heat-inactivated fetal bovine serum in 5% CO_2 at 37°C. NKX3.1 (wild-type and mutants) were cloned into pCDNA3 vector for expression in mammalian cells. Transfections were performed using LipoD293 (Signagen) as per the manufacturer's instructions.

EXPRESSION AND PURIFICATION OF RECOMBINANT PROTEINS

NKX3.1 was cloned into pQE80L vector with an N-terminal 6xHis tag and expressed in *E. coli* (BL21 strain). Recombinant protein was purified using Ni-affinity chromatography. The final buffer condition for protein used in in vitro kinase assay is 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM Imidazole, and 0.1% Tween.

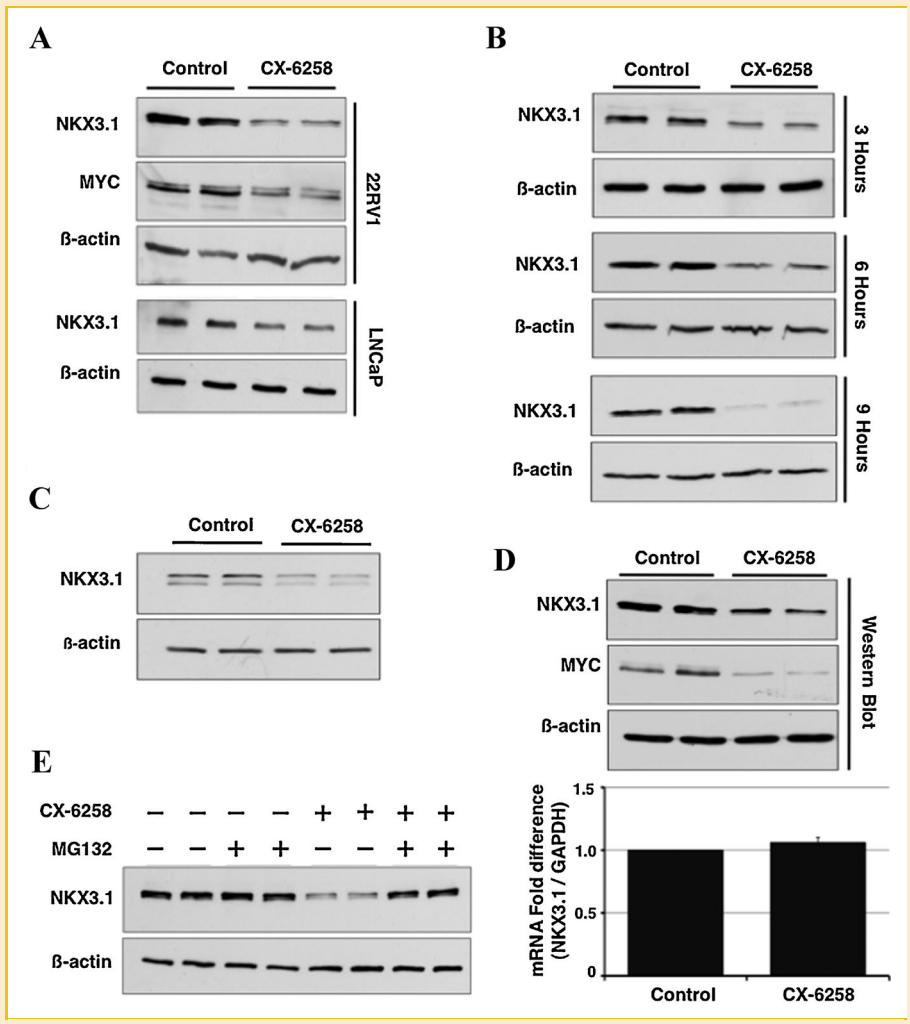

Fig. 1. Pim-1 stabilizes NKX3.1 post-translationally. A: Pim-1 inhibition using the small molecule inhibitor CX-6258 (12 μM, 3 h) diminishes endogenous NKX3.1 steady state levels in 22RV1 and LNCaP cells. Reduction in MYC levels served as a positive control for Pim-1 inhibition. β-actin was used as the loading control. B: Short- and long-term Pim-1 inhibition destabilizes endogenous NKX3.1. C: CX-6258 treatment (12 μM, 3 h) treatment diminished steady-state levels of ectopic NKX3.1 in PC3 cells. D: Effect of Pim-1 inhibition on NKX3.1 is post-transcriptional. CX-6258 treatment resulted in decreased NKX3.1 protein (top panel) but did not affect NKX3.1 mRNA (bottom panel). Normalized RT-PCR data (25 cycles) from 22RV1 cells (n = 3) treated with CX-6258 or vehicle control (DMSO) is represented. E: Pim-1 activity protects NKX3.1 from 26S proteasome-mediated degradation. Treatment of 22Rv1 cells with proteasome inhibitor MG132 rescued the effect of Pim-1 inhibition on NKX3.1.

WESTERN BLOTTING AND IMMUNOPRECIPITATION

For Western blot analysis, mammalian cells were harvested 24–48 h post-transfection and lysed using 150 mM NaCl, 50 mM Tris (pH 7.5), 0.5% NP-40 and complete protease inhibitor (Roche). Equal amounts of total protein was resolved on a SDS–PAGE gel and transferred to a PVDF membrane. Western blot was performed as described previously [Guan et al., 2008]. All samples were loaded in duplicate to minimize technical error.

IN VITRO KINASE ASSAY

In vitro kinase assay was performed as previously described [Li et al., 2007]. Kinase assays was carried out for 2 h at room temperature and stopped using 2× SDS buffer.

RT-PCR ANALYSIS

Total RNA was extracted using the RNeasy Kit (Qiagen) and reverse-transcribed using the Superscript cDNA synthesis kit (Biorad). Semi-quantitative PCR was performed to detect changes in levels of mRNA. RT-PCR was performed on 1 μg of total isolated RNA (total reaction volume was maintained at 20 μl). One microliter of RT-PCR product was used as template for PCR reaction using the following primers: NKX3.1 (134 bp product): Fwd 5′CTTCCCCAAACCCC TAAGC3′; Rev 5′TCCTCTCCAACTCGATCAC3′; GAPDH (226 bp): Fwd 5′GAAGGTGAAGGTCGGAGT3′; Rev 5′GAAGATGGTGATGGG ATTTC3′. The GAPDH levels were used as internal loading controls.

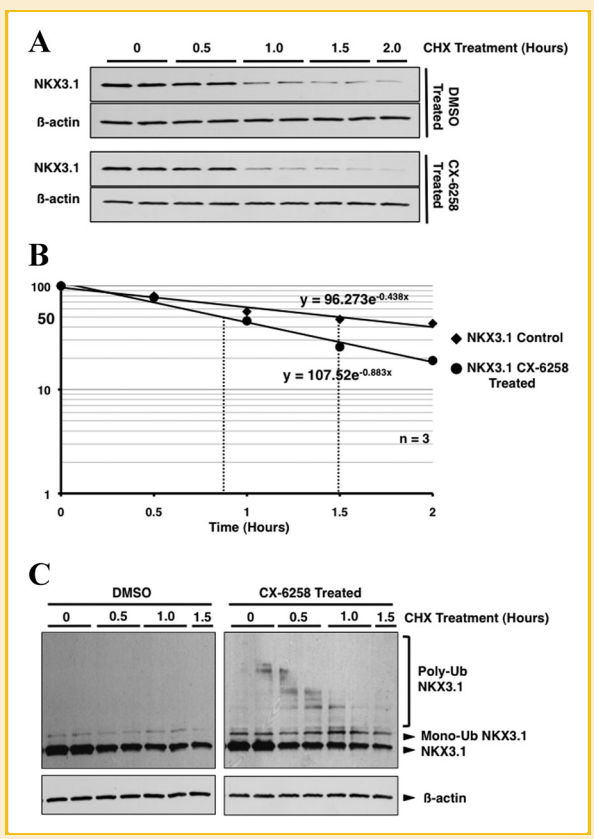

Fig. 2. Reduced half-life of NKX3.1 upon Pim-1 inhibition. A: Western blot analysis of whole-cell extracts of PC3 cells expressing HA-tagged NKX3.1. The cells were either treated with either DMSO (vehicle-control) or CX-6258 for 1 h prior to cycloheximide treatment. The cells were sampled at the indicated times after cycloheximide (CHX) treatment. B: Quantification of Western blot data in panel A. The NKX3.1 levels were normalized to that of β-actin and graphed on a semi-log plot. The calculated half-life values are indicated. C: CX-6258 treatment caused increased accumulation of mono- and poly-ubiquitinated forms of NKX3.1 in cells.

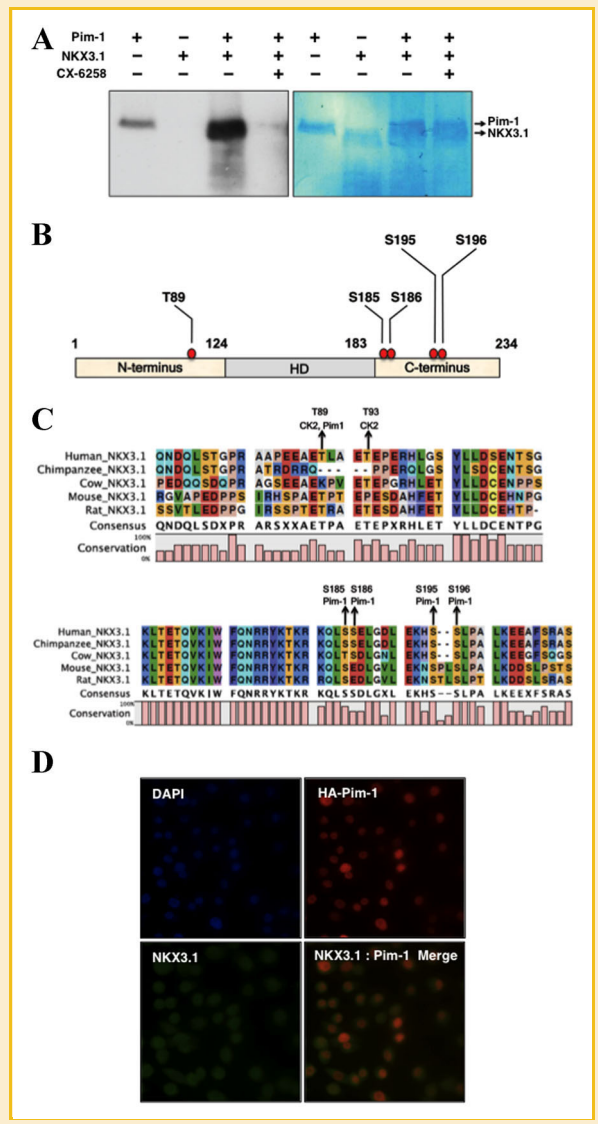

Fig. 3. NKX3.1 is a Pim-1 substrate. A: Pim-1 phosphorylates NKX3.1 in vitro as seen in the autoradiogram (left). In vitro phosphorylation was inhibited by the addition of Pim-1 inhibitor (CX-6258). The right panel shows Coomassie-stained PVDF membrane. B: Pim-1 phosphorylation sites on NKX3.1 mapped by mass spectrometry. NKX3.1 N-terminal, homeodomain (HD) and C-terminal domains are also shown in the cartoon. C: Pim-1 phosphorylation sites on NKX3.1 are evolutionarily conserved. Alignment for regions containing Pim-1 phosphorylation sites is shown above. Residues phosphorylated by Pim-1 and CK2 are also indicated. D: Endogenous Pim-1 (anti-Pim-1: red) co-localizes with ectopically expressed HA-NKX3.1 (anti-HA: green) in the nucleus of prostate epithelial cells. Immunofluorescence analysis was carried out in PC3 cells. DAPI (blue) staining shows nucleus.

The PCR products were run on a 1.5% agarose gel and the bands were visualized by ethidium bromide staining. The intensity of the bands was quantified using the Image J software. The values obtained from three independent experiments were normalized to the corresponding GAPDH levels and the data were presented as fold difference ±SEM.

PROTEIN IDENTIFICATION AND PHOSPHOSITE MAPPING MASS SPECTROMETRY

In-gel tryptic digests were performed as previously described [Li et al., 2007]. Desalted tryptic peptides were analyzed by nano LC–MS/MS on a linear ion-trap mass spectrometer (LTQ, Thermo Fischer). Acquired data were searched against a *Homo sapiens* protein database or phospho-proteome database using the Turbo-SEQUEST algorithm (Thermo Fischer).

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Cells were plated on poly-L-lysine-treated glass cover slips in six well plates. PC3 cells were grown on the coverslips and transfected using lipoD293T (Signagen) according to the manufacturer's instructions. Forty-eight hours post-transfection, cells were fixed using 2% paraformaldehyde in PBS. Immunofluorescence analysis was performed as described previously [Guan et al., 2008].

RESULTS

PIM-1 STABILIZES NKX3.1 IN PROSTATE CANCER CELLS

Since increased Pim-1 activity has been suggested to play an important role in the progression of prostate cancer, we sought to identify the mechanisms through which Pim-1 contributes to this outcome. Pim-1 inhibition in prostate cancer cells using the small-molecule Pim-1 inhibitor CX-6258, resulted in decreased NKX3.1 steady state level (Fig. 1A). Given the established role of Pim-1 as an oncogene and NKX3.1 as a tumor suppressor, this was unexpected. To determine whether the effect of prolonged Pim-1 inhibition on NKX3.1 differs from the short-term effect, we treated prostate cancer cells with CX-6258 for longer time points. Similar to 3-h Pim-1 inhibition, both 6- and 9-h Pim-1 inhibition resulted in decreased NKX3.1 levels (Fig. 1B). To further confirm the paradoxical stabilization of NKX3.1 by Pim-1 kinase, we transfected HA-tagged NKX3.1 into PC3 cells, which were subsequently treated with CX-6258. Similar to endogenous NKX3.1, ectopically expressed NKX3.1 was also destabilized upon Pim-1 inhibition (Fig. 1C). The

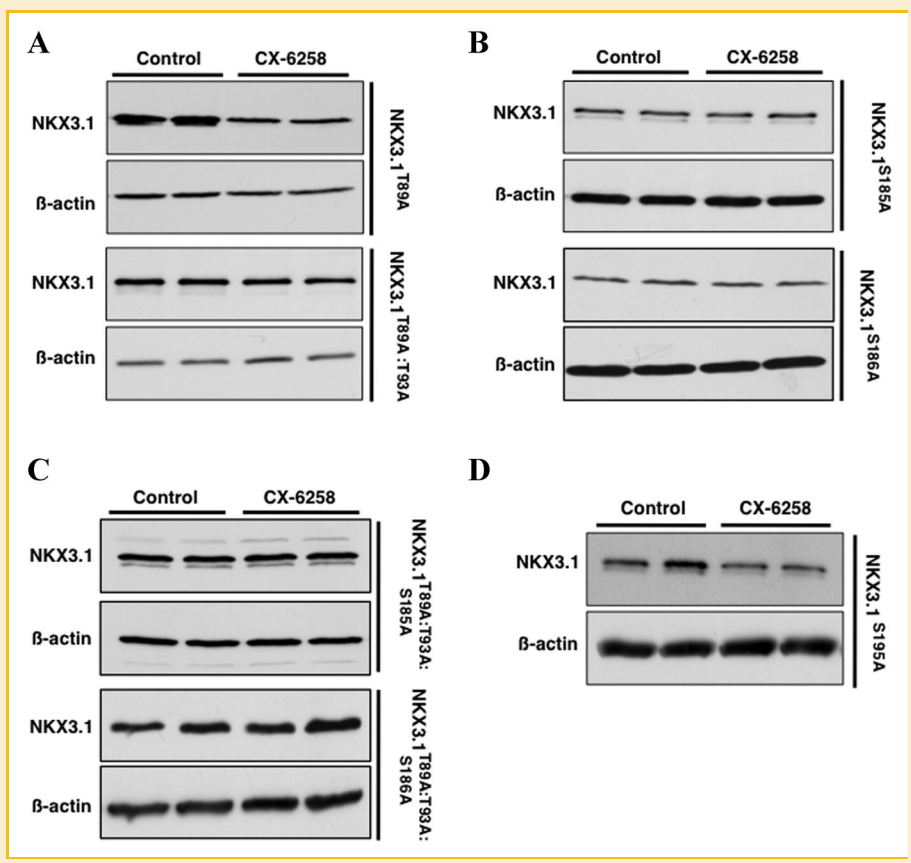

Fig. 4. Mutational analysis of Pim-1 phosphorylation sites on NKX3.1. A: NKX3.1^{T89A} responds to CX-6258 treatment (top panel) whereas the double mutant NKX3.1$^{T89A:T93A}$ reverses the effect of Pim-1 inhibition (bottom panel). B: Mutation of both Ser185 (top panel) and Ser186 (bottom panel) to alanine abolished the effect of CX-6258 on NKX3.1 in PC3 cells. C: NKX3.1$^{T89A:T93A:S185A}$ (top panel) and NKX3.1$^{T89A:T93A:S186A}$ (bottom panel) triple mutants are non-responsive to Pim-1 inhibition. D: NKX3.1 S195A mutant is destabilized upon CX-6258 treatment. Ectopic NKX3.1 was detected using anti-HA antibody. β-actin was used as the loading control.

ectopically expressed HA-NKX3.1 lacks endogenous *NKX3.1* promoter elements. Hence, these data also suggested that the effect of Pim-1 on NKX3.1 is primarily post-transcriptional. In addition, the endogenous NKX3.1 mRNA did not change upon Pim-1 inhibition, further demonstrating that the effect of Pim-1 on NKX3.1 is post-transcriptional (Fig. 1D). To evaluate whether Pim-1 inhibition targets NKX3.1 for degradation through the 26S-proteasome, we determined if the blocking the 26S-proteasome could reverse the effect of Pim-1 inhibition on NKX3.1. Treatment of 22RV1 cells with the proteasome inhibitor MG132 completely reversed the effect of Pim-1 inhibition on NKX3.1, suggesting that Pim-1 phosphorylation protects NKX3.1 from proteasome-mediated degradation (Fig. 1E).

To determine the effect of Pim-1 inhibition on NKX3.1 turnover and half-life, HA-tagged NKX3.1 was transfected into PC3 cells. These cells were either treated with the Pim-1 inhibitor CX-6258 or DMSO (vehicle) for 1 h and subsequently treated with cycloheximide for varying time points to determine the effect of Pim-1 inhibition on NKX3.1 half-life (Fig. 2A). CX-6258 treatment resulted in a significant reduction in NKX3.1 half-life (Fig. 2B). While ectopically expressed NKX3.1 in control cells had a half-life of ~90 min, Pim-1 inhibition reduced the half-life to ~52 min (Fig. 2B). Furthermore, we consistently observed increased mono- and poly-ubiquitinated forms of NKX3.1 in the CX-6258-treated samples in these experiments using anti-HA antibody upon longer exposure, suggesting that Pim-1 protects NKX3.1 from ubiquitination and subsequent degradation (Fig. 2C).

PIM-1 PHOSPHORYLATES NKX3.1 AT MULTIPLE SITES

Since the effect of Pim-1 inhibition on NKX3.1 was post-transcriptional, we determined whether Pim-1 could phosphorylate NKX3.1. An in vitro kinase reaction was performed using purified recombinant 6xhis-tagged NKX3.1 and 6x-his tagged Pim-1 in the presence of γ^{32}P-ATP. Pim-1 phosphorylated NKX3.1 efficiently in vitro and the phosphorylation was extinguished in the presence of CX-6258 (Fig. 3A). Mass spectrometric analysis of Pim-1-phosphorylated NKX3.1 identified five potential Pim-1 phospho-acceptor sites: Thr89, Ser185, Ser186, Ser195, and Ser196 (Fig. 3B and Tables S1–S5). These identified Pim-1 phosphorylation sites on NKX3.1 were well conserved across species (Fig. 3C). In cases were conservation was not perfect, the residues were replaced by a phosphomimetic residue (either Glu or Asp). Further, to determine whether NKX3.1 and Pim-1 have overlapping spatial localization in prostate cancer cells, we performed immunofluorescence analysis. The ectopically expressed NKX3.1 co-localized with endogenous Pim-1 in PC3 cells (Fig. 3D).

IN VIVO ANALYSIS OF PIM-1 PHOSPHORYLATION SITES ON NKX3.1

To determine the Pim-1 phosphorylation site(s) on NKX3.1 critical for Pim-1 mediated stabilization, we generated alanine substitution mutants at these sites and evaluated the effect of Pim-1 inhibition on their stability. NKX3.1^{T89A} responded to Pim-1 inhibition in a manner similar to wildtype NKX3.1 (NKX3.1^{WT}; Fig. 4A). Since Thr89 and Thr93 were identified previously as CK2 phospho-acceptor sites important in NKX3.1 turnover, we sought to determine if the double mutant had any effect on Pim-1 inhibition. Interestingly unlike NKX3.1^{T89A}, NKX3.1$^{T89A:T93A}$ was found to be non-responsive to CX-6258 treatment, suggesting that Thr93 is critical for Pim-1-mediated stabilization of NKX3.1 (Fig. 4A). Although Thr93 was not identified by mass spectrometry as a Pim-1 phospho-acceptor site on NKX3.1, it is likely that identification of this site could have been missed during the analysis due to technical difficulty in precisely distinguishing adjacent phospho-acceptor sites from the same ion. Similar to NKX3.1$^{T89A:T93A}$, the NKX3.1^{S185A}, and NKX^{S186A} mutants did not respond to CX-6258 treatment, demonstrating that these residues are critical for Pim-1-mediated NKX3.1 stabilization (Fig. 4B). As expected, the steady-state levels of NKX3.1$^{T89A:T93A:S185A}$ and NKX3.1$^{T89A:T93A:S186A}$ triple mutant also did not change upon CX-6258 treatment (Fig. 4C). However, in contrast, the NKX3.1^{S195A} mutant responded to Pim-1 inhibition demonstrating this residue to be non-critical for Pim-1-mediated NKX3.1 stabilization (Fig. 4D). To ensure that the lack of response of the mutants was not the result of mis-localization, we performed immunofluorescence analysis on ectopically transfected NKX3.1 mutants in PC3 cells. The NKX3.1 mutants localized primarily to the nucleus in these cells. No significant change in localization was observed in any of the NKX3.1 mutants relative to the wild type (Fig. 5).

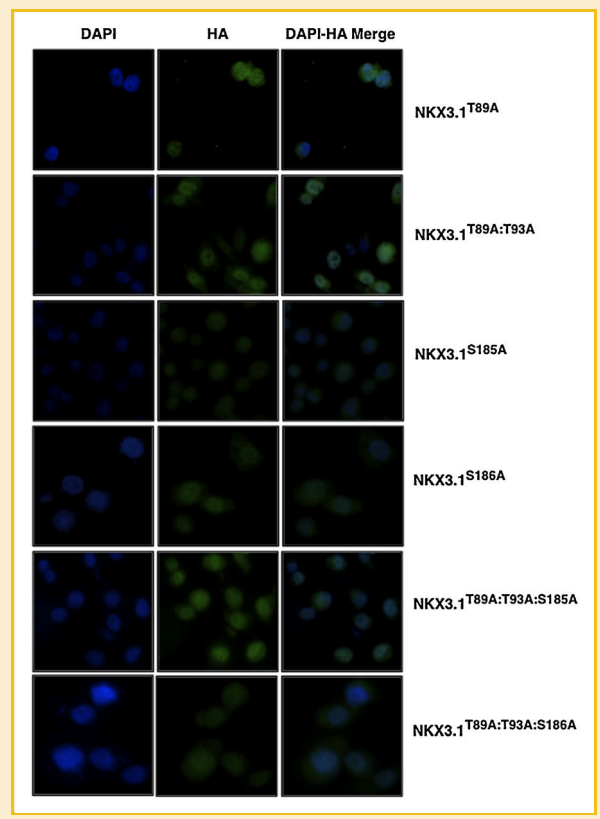

Fig. 5. Cellular localization of NKX3.1 alanine substitution mutants. NKX3.1 alanine substitution mutants localized primarily to the nucleus. Ectopically expressed HA-NKX3.1 mutants in PC3 cells were detected using anti-HA antibody (green). DAPI (blue) was used to stain the nucleus.

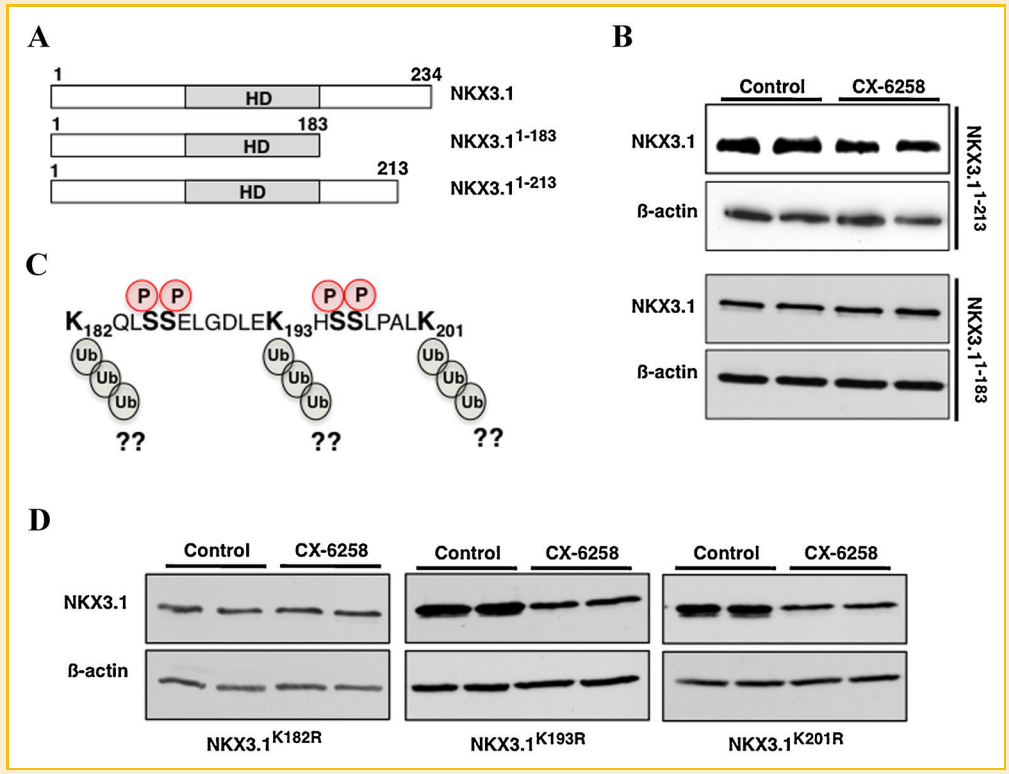

Fig. 6. Pim-1-mediated stabilization of NKX3.1 is dependent on K182. A: Cartoon illustrating the various truncated forms of NKX3.1 used in (B). NKX3.1 n-terminal, homeodomain (HD), and C-terminal domains are indicated. The amino acid numbering is also denoted. B: NKX3.1$^{1-213}$ is destabilized upon CX-6258 treatment (top panel). CX-6258 has no effect on NKX3.1$^{1-183}$ stability (bottom panel). C: Cartoon illustrating the region spanning amino acids 182–201 in NKX3.1. This region has four serines, which were identified as in vitro Pim-1 phosphorylation site, and three lysines, which can potentially be ubiquitinated. D: CX-6258 has no effect on the stability of NKX3.1^{K182R}. However, Pim-1 inhibition causes decreased steady-state levels of NKX3.1^{K193R} and NKX3.1^{K201R} mutants.

LYSINE 182 IS IMPORTANT IN PIM-1 MEDIATED NKX3.1 STABILIZATION

To gain insight regarding how the signal initiated through NKX3.1 phosphorylation by Pim-1 results in increased NKX3.1 stability, we compared the effect of Pim-1 inhibition on truncated NKX3.1 mutants. As with NKX3,1^{WT}, the NKX3.1$^{1-213}$ mutant responded to CX-6258 treatment. However, the steady state-levels of the shorter version lacking the C-terminal domain, NKX3.1$^{1-183}$, did not change upon Pim-1 inhibition. This suggested that although phosphorylation in the N-terminus regulates NKX3.1 stability, the residues between amino acids 183 and 213 are critical in mediating the effect of NKX3.1 phosphorylation by Pim-1 (Fig. 6A). This region contains several residues important in NKX3.1 stability including potential Pim-1 phosphorylation sites. However, whereas NKX3.1^{WT} and NKX3.1$^{1-213}$ responded to Pim-1 inhibition, both NKX3.1$^{T89A:T93A}$ and NKX3.1$^{1-183}$ were non-responsive, leading us to hypothesize that NKX3.1 stabilization by phosphorylation of the N-terminus requires amino acids located between 183 and 213. Apart from the identified Pim-1 phosphorylation sites, this region also harbors two lysine residues, which could potentially be ubiquitinated downstream of Pim-1 phosphorylation at the N-terminus (Fig. 6B). We investigated whether any of these lysines contributed to Pim1-mediated NKX3.1 stability by generating substitution mutants wherein the Lys residues were replaced by the non-ubiquitinatable amino acid Arg. We also included Lys182 for our analysis since it was located just before the start of the C-terminal domain raising the possibility that this site might have been rendered inaccessible in the NKX3.1$^{1-183}$ mutant (Fig. 6B). While the K182R mutation rescued the CX-6258-mediated destabilization of NKX3.1, the K193R and K201R mutants did not, suggesting that Lys182 is critical for Pim-1-mediated NKX3.1 stabilization (Fig. 6C).

DISCUSSION

Diminished NKX3.1 level is a hallmark of human prostate cancers but the molecular mechanisms regulating NKX3.1 steady-state levels in these malignancies are not completely understood. Previous reports demonstrated a lack of correlation between NKX3.1 mRNA and protein levels in prostate cancer cases, suggesting an important role for post-translational regulation in maintenance of NKX3.1 steady state levels in prostate epithelial cells [Bethel et al., 2006]. It has become apparent through recent studies that NKX3.1 is phosphorylated at various sites across the protein by multiple protein kinases, thereby regulating its biology in response to diverse signals [Li et al., 2006; Markowski et al., 2008]. Mapping these phosphorylation events and the associated pathways is critical in gaining mechanistic insights into the molecular events determining the turnover and biology of this important prostate-specific tumor suppressor.

In this report, we demonstrate regulation of the tumor suppressor NKX3.1 by the oncogenic protein kinase Pim-1 in prostate cancer cells. We show that Pim-1 phosphorylates NKX3.1 within both N- and C-terminal domains, and protects from proteasome-mediated degradation. We further identify Lys182 as a residue downstream of Pim-1 phosphorylation critical in mediating the effect of Pim-1 on NKX3.1. We hypothesize that under Pim-1 inhibition conditions, ubiquitination at Lys182 targets NKX3.1 for proteasome-mediated degradation. In addition to providing novel insights into NKX3.1 regulation, our work further confirms previous observations that the N-terminal PEST domain (containing Thr89 and Thr93) and a C-terminal domain (amino acids 183–213) are critical in determining NKX3.1 stability. The region between amino acids 182 and 201 contains four serine and three lysine residues, which can be post-translationally modified to impact the biology of NKX3.1 (Fig. 6B). In addition, the four serines in this region are located in pairs (Fig. 6B). The increased local presence of post-translationally modifiable residues within this region raises the possibility of a complex crosstalk involving multiple signals converging in this region, leading to a confluence of signaling events that enables context-dependent regulation. This, in part, may have contributed to the difficulty in deducing the exact residues in this region important in eliciting a particular response.

In contrast to the results obtained from Pim-1 inhibition experiments reported here, Ser185 phosphorylation was previously demonstrated to promote NKX3.1 turnover. However, we report that NKX^{S185A} mutant is resistant to Pim-1 inhibition, suggesting that Ser185 increases its stability. We also identified Ser186 as a Pim-1 phosphorylation site. Taking these two independent observations into account, we speculate that Ser186 phosphorylation by Pim-1 precludes the adjacent Ser185 from being phosphorylated by another protein kinase, thereby increasing its stability (Fig. 7A). However, mutation of Ser185 to alanine would alter the local charge environment, which could indirectly affect phosphorylation at the adjacent Ser186 by Pim-1, which is critical for NKX3.1 stabilization (Fig. 7A). Clearly, a complex regulatory network operates in this region, and discerning the precise role of each residue will facilitate understanding how the NKX3.1 C-terminal domain functions to control steady-state protein level of this potent growth suppressor.

The seemingly paradoxical stabilization of a tumor suppressor by an oncogenic kinase could potentially serve as a feedback regulatory loop in normal prostate epithelial cells. Activation of the pro-survival kinase Pim-1 stabilizes NKX3.1, thus preventing normal cells from losing growth control upon sustained Pim-1 activation (Fig. 7B). However, in prostate cancers, NKX3.1 is diminished potentially through other mechanisms, which circumvents their stabilization by Pim-1 (Fig. 7B). As a result, Pim-1 activation, together with activation of another oncogene such as *MYC*, which causes decreased NKX3.1 level, may act synergistically to result in more aggressive cancers. Consistent with this hypothesis, it has been shown that Pim-1 overexpression in mouse prostate does not result in an overt phenotype, however, combined overexpression of Pim-1 and MYC leads to aggressive adenocarcinoma [Wang et al., 2012].

Taken together, the data reported here highlight the complexity of the signaling networks regulating key regulatory proteins such as Pim-1 and NKX3.1 in human prostate cancer cells. Although Pim-1 is known to function as a pro-survival oncogenic kinase, we demonstrate that, contrary to its expected role, Pim-1 stabilizes the tumor suppressor NKX3.1. Pim-1 is considered to be a promising therapeutic target but due to our lack of knowledge regarding Pim-1 substrates, its precise role has remained largely elusive. Further, it is evident that different protein kinases, in response to the pertinent upstream signal, phosphorylate NKX3.1 at their respective phospho-acceptor sites and regulate NKX3.1 differentially. Such context-dependent regulation, we believe, dictates and enables precise control over the turnover and function of NKX3.1 in prostate epithelial cells.

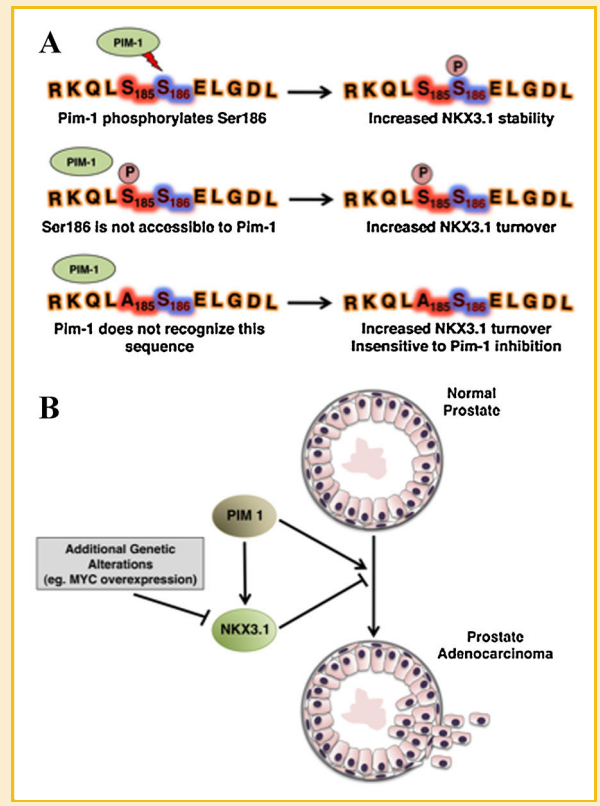

Fig. 7. A: Schematic illustration showing how NKX3.1 phosphorylation at Ser185 can interfere with subsequent phosphorylation at Ser186 and influence NKX3.1 stability. B: Model depicting possible role of the paradoxical stabilization of NKX3.1 by Pim-1 in prostate epithelial cells. In normal cells, sustained Pim-1 activation results in NKX3.1 stabilization. Increased NKX3.1 levels opposes Pim-1 mediated pro-survival signals and prevents normal cells from losing growth control and maintains homeostasis. In prostate cancer, additional mechanisms (such as MYC overexpression) causes diminished NKX3.1 expression, thereby eliminating the feedback regulatory loop. In such scenario, Pim-1 overexpression provides growth advantage to the predisposed $NKX3.1^{low}$ cells.

REFERENCES

Abdulkadir SA, Magee JA, Peters TJ, Kaleem Z, Naughton CK, Humphrey PA, Milbrandt J. 2002. Conditional loss of Nkx3.1 in adult mice induces prostatic intraepithelial neoplasia. Mol Cell Biol 22(5):1495–1503.

Bethel CR, Bieberich CJ. 2007. Loss of NKX3.1 expression in the transgenic adenocarcinoma of mouse prostate model. Prostate 67(16):1740–1750.

Bethel CR, Faith D, Li X, Guan B, Hicks JL, Lan F, Jenkins RB, Bieberich CJ, De Marzo AM. 2006. Decreased NKX3.1 protein expression in focal prostatic atrophy, prostatic intraepithelial neoplasia, and adenocarcinoma: Association with gleason score and chromosome 8p deletion. Cancer Res 66(22): 10683–10690.

Bhatia-Gaur R, Donjacour AA, Sciavolino PJ, Kim M, Desai N, Young P, Norton CR, Gridley T, Cardiff RD, Cunha GR, Abate-Shen C, Shen MM. 1999. Roles for NKX3.1 in prostate development and cancer. Genes Dev 13(8):966–977.

Bieberich CJ, Fujita K, He WW, Jay G. 1996. Prostate-specific and androgen-dependent expression of a novel homeobox gene. J Biol Chem 271(50): 31779–31782.

Guan B, Pungaliya P, Li X, Uquillas C, Mutton LN, Rubin EH, Bieberich CJ. 2008. Ubiquitination by TOPORS regulates the prostate tumor suppressor NKX3.1. J Biol Chem 283(8):4834–4840.

He WW, Sciavolino PJ, Wing J, Augustus M, Hudson P, Meissner PS, Curtis RT, Shell BK, Bostwick DG, Tindall DJ, Gelmann EP, Abate-Shen C, Carter KC. 1997. A novel human prostate-specific, androgen-regulated homeobox gene (NKX3.1) that maps to 8p21, a region frequently deleted in prostate cancer. Genomics 43(1):69–77.

Kim MJ, Bhatia-Gaur R, Banach-Petrosky WA, Desai N, Wang Y, Hayward SW, Cunha GR, Cardiff RD, Shen MM, Abate-Shen C. 2002. Nkx3.1 mutant mice recapitulate early stages of prostate carcinogenesis. Cancer Res 62(11): 2999–3004.

Li X, Guan B, Maghami S, Bieberich CJ. 2006. NKX3.1 is regulated by protein kinase CK2 in prostate tumor cells. Mol Cell Biol 26(8):3008–3017.

Li X, Guan B, Srivastava MK, Padmanabhan A, Hampton BS, Bieberich CJ. 2007. The reverse in-gel kinase assay to profile physiological kinase substrates. Nat Methods 4(11):957–962.

Magee JA, Abdulkadir SA, Milbrandt J. 2003. Haploinsufficiency at the NKX3.1 locus. A paradigm for stochastic, dosage-sensitive gene regulation during tumor initiation. Cancer Cell 3(3):273–283.

Markowski MC, Bowen C, Gelmann EP. 2008. Inflammatory cytokines induce phosphorylation and ubiquitination of prostate suppressor protein NKX3.1. Cancer Res 68(17):6896–6901.

Wang J, Anderson PD, Luo W, Gius D, Roh M, Abdulkadir SA. 2012. Pim1 kinase is required to maintain tumorigenicity in MYC-expressing prostate cancer cells. Oncogene 31(14):1794–1803.