Transcriptional regulation of Nkx3.1 in prostate luminal stem cell specification and cancer initiation via its 3’ genomic region

Qing Xie (谢青), and Zhu A. Wang (王竹)

From the Department of Molecular, Cell, and Developmental Biology, University of California, Santa Cruz, CA 95064

Running title: Nkx3.1 in prostate stem cells and cancer

To whom correspondence should be addressed: Zhu A. Wang, Department of Molecular, Cell, and Developmental Biology, University of California Santa Cruz, 1156 High Street, Mailstop: MCDB, Santa Cruz, CA 95064, Telephone: (831) 459-5137; FAX: (831) 502-7301; E-mail: zwang36@ucsc.edu

Keywords: Nkx3.1, transcription, androgen, Pten, stem cells, prostate cancer

ABSTRACT

Nkx3.1, a transcription factor expressed in the prostate epithelium, is crucial for maintaining prostate cell fate and suppressing tumor initiation. Nkx3.1 is ubiquitously expressed in luminal cells of hormonally-intact prostate, but upon androgen deprivation, exclusively labels a type of luminal stem cells named castration-resistant Nkx3.1-expressing cells (CARNs). During prostate cancer initiation, Nkx3.1 expression is frequently lost in both humans and mouse models. Therefore, investigating how Nkx3.1 expression is regulated in vivo is important for understanding the mechanisms of prostate stem cell specification and cancer initiation. Here, using a transgenic mouse line with destabilized GFP, we identified an 11-kb genomic region 3’ of the Nkx3.1 transcription start site to be responsible for alterations in Nkx3.1 expression patterns under various physiological conditions. We found that androgen cell-autonomously activates Nkx3.1 expression through androgen receptor (AR) binding to the 11-kb region in both normal luminal cells and CARNs, and discovered new androgen response elements in the Nkx3.1 3’ UTR. In contrast, we found that in Pten-/- prostate tumors, loss of Nkx3.1 expression is mediated at the transcriptional level through the 11-kb region, despite functional AR in the nucleus. Importantly, the GFP reporter specifically labeled CARNs in the regressed prostate only in the presence of cell-autonomous AR, supporting a facultative model for CARN cell specification.

INTRODUCTION

The homeodomain-containing protein Nkx3.1 is the earliest known transcription factor expressed in prostate development (1,2), and orchestrates a transcriptional regulatory network important for prostate cell fate specification (3). The mature prostate gland contains stromal layers and an epithelium that is composed of secretory luminal cells, basal cells, and rare neuroendocrine cells. Nkx3.1 is primarily expressed in luminal epithelial cells of the adult prostate, with low levels present in a small subset of basal cells (4,5). It plays a tumor-suppressing role, as both homozygous and heterozygous Nkx3.1 mutant mice display increases of epithelial hyperplasia and defects in prostate branching and protein secretion as they age (6-9). In humans, Nkx3.1 down-regulation is considered one of the earliest events in prostate cancer initiation (10). Mechanistically, Nkx3.1 has been shown to play a critical role in protecting prostate cells from DNA damage (11-14).

Despite its functional significance, how Nkx3.1 expression is regulated in normal and tumorigenic prostate in vivo remains elusive. Nkx3.1 mRNA is detected in prostatic buds in E17.5 mouse embryos (2), and studies using urogenital sinus explant culture have demonstrated the involvement of Fgf10 and Wnt signaling pathways in activating Nkx3.1 expression during prostate organogenesis (15-18). In postnatal and adult prostate, androgen receptor (AR) signaling
has been shown to maintain Nkx3.1 expression. In particular, androgen deprivation via castration in mice induced prostate regression accompanied by apoptosis in the majority of luminal cells and loss of Nkx3.1 expression in the ones that survived (1,2,5), although the relative contribution of cell-autonomous luminal AR versus non-cell-autonomous stromal AR in this process has yet to be determined. Notably, a small subset of the survived luminal cells retained Nkx3.1 expression in the regressed prostate. Those cells, named castration-resistant Nkx3.1-expressing cells (CARNs), were shown to behave as luminal stem cells that contribute to prostate regeneration upon androgen re-administration and could also serve as a cell of origin for prostate cancer (5). How CARNs are specified is unclear, for the retention of their Nkx3.1 expression could be due to an intrinsically different cellular program from other luminal cells or alternatively, stochastically determined by local microenvironment. Another important question concerning Nkx3.1 expression arises from studies of prostate cancer. Under prostate tumor-initiating conditions such as the loss of Pten, luminal Nkx3.1 expression is abolished in both human samples and mouse models (19-22). How this is accomplished remains unclear. The decrease of Nkx3.1 mRNA in Pten-/--tumors suggests that the regulation may occur at the transcriptional level (20,23), while the loss of Nkx3.1 protein but not mRNA in Pten+/+-tumors suggests the existence of translational or post-translational regulations (19).

We reason that studying Nkx3.1 transcriptional regulation by analysis of the Nkx3.1 gene locus should shed light on the above questions. A pioneer study using transgenic LacZ reporter mice discovered that a 32-kb fragment containing 20-kb upstream and 12-kb downstream of the Nkx3.1 transcription start site (TSS) could drive endogenous Nkx3.1 expression pattern in most organs during embryogenesis (4). Within this fragment, the downstream-most 5-kb region acted as a urogenital enhancer that partially restored prostatic Nkx3.1 expression (4). Based on this finding, we hypothesize that change of Nkx3.1 expression in adult prostate in vivo is regulated transcriptionally through its 3’ local genomic region. Our data reported below support this hypothesis by testing it in the contexts of both prostate regression-regeneration and Pten-loss-induced cancer initiation. They also support a facultative model for CARN cell specification.

RESULTS
An 11-kb Nkx3.1 region drives normal gene expression in adult prostate
To test the hypothesis that change of prostatic Nkx3.1 expression is mediated transcriptionally through its 3’ genomic sequence in vivo, we took a transgenic reporter mouse approach by constructing an ~11-kb DNA fragment containing the 240-bp Nkx3.1 proximal promoter, the 4.0-kb Nkx3.1 gene sequence, and its adjacent 3’ 6.5-kb intergenic region (Fig. 1A). In addition, for visualizing loss of Nkx3.1 expression under androgen deprivation and tumor initiation conditions, we inserted the d2EGFP sequence encoding a destabilized GFP (24) with SV40 PolyA sequence right after the start codon (Fig. 1A). With a half-life of two hours, the d2EGFP should dynamically report the Nkx3.1 transcriptional activities in vivo. The final construct (named Nkx3.1(11)-d2EGFP) was used for pronuclear injection, and 5 independent transgenic mouse lines were obtained. Robust GFP signal was observed specifically in the adult prostate, as 4 out of the 5 lines showed strong to moderate GFP expression in various prostate lobes while 1 line showed weak expression (Figs. 1B and S1; Table S1). For subsequent analyses, we focused on transgenic line 18 (Ng18), since it displayed strongest expression in all the lobes of the prostate (Fig. 1B).

We next examined reporter gene expression during embryogenesis and prostate development. No GFP was observed in somite of E10.5 embryos in any of the lines, consistent with previous report showing that key elements for somite Nkx3.1 expression lie in the 5’ region of the gene locus (4). We also did not detect GFP in E18.5 prostate buds, where cells mostly expressed the basal marker CK5 (Fig. 1C). GFP began to be expressed in the prostate around neonatal stage P4. By neonatal stage P8, GFP was expressed in the prostate around neonatal stage P4. By neonatal stage P8, GFP was expressed throughout the prostate epithelium, which contained basal (CK5+), luminal (CK18+) and intermediate cells (CK5+CK18+), and co-localized with Nkx3.1 expression (Figs. 1D and S2A). Throughout adult stage, GFP expression was persistently present in the luminal layer and co-localized with Nkx3.1 expression (Figs. 1E and
The proportions of luminal cells that were GFP⁺ in the anterior, ventral, and dorsal-lateral prostate lobes (AP, VP DLP) were 98.2% (n=2019/2055), 67.2% (n=839/1248), and 87.1% (n=1283/1473), respectively (Figs. 1G and S2B-D). Notably, approximately 5.8% (n=95/1638) of basal cells were also GFP⁺ (Figs. 1G and 1H), in agreement with the percentage of Nkx3.1⁺ basal cells reported previously (4,5). Overall, Nkx3.1(11)-d2EGFP recapitulates endogenous Nkx3.1 expression pattern in adult prostate homeostasis.

Androgen activates Nkx3.1 transcription through cell-autonomous AR in both luminal cells and CARNs

To test whether androgen regulates Nkx3.1 expression through the 11-kb region, we castrated Nkx3.1(11)-d2EGFP mice at 8-week of age and performed three rounds of androgen-mediated serial prostate regression-regeneration (Fig. 2A). We found that GFP signal is dramatically decreased in the regressed prostate (Figs. 2B and 2C), consistent with loss of Nkx3.1 expression in most luminal cells upon androgen deprivation. Importantly, cells that retained GFP expression were strictly luminal and expressed Nkx3.1 (Fig. 2C), and they comprised of 1.4% (n=29/2053) of all luminal cells (Fig. 2K), suggesting they correspond to the previously identified CARN cells (5). Upon androgen re-administration, GFP signal was again detected in most luminal cells (95.6%, n=1883/1970) of the regenerated prostate, and the same results were observed for both 1-round and 3-round regeneration experiments (Figs. 2D, 2E, and 2K). These data demonstrate that the Nkx3.1 11-kb region is fully responsive to androgen regulation.

Androgen-regulated luminal Nkx3.1 expression could be mediated cell-autonomously through luminal AR and/or through paracrine signals induced by stromal AR. To distinguish these two possibilities, we analyzed prostate in the midst of regression. We found that, two weeks after castration of Nkx3.1(11)-d2EGFP mice (Fig. 2A), the shrinking prostate showed a mosaic GFP expression pattern, with the GFP⁺ regions overlapping with luminal nuclear AR signals (Fig. 2F). To further confirm the cell-autonomous role of androgen, we deleted AR specifically in luminal cells using the CK18-CreERT² driver (25) by tamoxifen induction of 8-week old CK18-CreERT²; ARfloxt/Y mice. Two weeks after induction, AR-null luminal cells could be identified as clusters with condensed nuclei and enhanced membrane CK18 expression as we reported previously (26), and we found that Nkx3.1 expression was diminished in those cells (Fig. 2G). Direct staining of AR and Nkx3.1 in adjacent sections also confirmed this result (Fig. S3A). Importantly, we found that GFP signal was significantly reduced in AR-null luminal cells of tamoxifen-induced CK18-CreERT²; ARfloxt/Y; Nkx3.1(11)-d2EGFP mice (Figs. 2H and S3B), indicating that luminal cell-autonomous AR directly activates Nkx3.1 transcription through the 11-kb region. To determine whether such mechanism also applies to CARN cells, we ablated AR specifically in CARNs using the Nkx3.1CreERT²/+ driver (5) in the fully regressed prostate and analyzed GFP reporter activity. In Nkx3.1CreERT²/+; ARfloxt/Y; Nkx3.1(11)-d2EGFP mice that first underwent castration and then tamoxifen induction (Fig. 2I), the percentage of GFP⁺ luminal cells in the regressed prostate was dramatically reduced by almost ten-fold to 0.16% (n=4/2450) (Figs. 2J and 2K), and no Nkx3.1⁺ cells were found by immunofluorescence staining. Therefore, Nkx3.1 expression in both luminal cells and CARNs are transcriptionally regulated by cell-autonomous AR through the 11-kb region.

AR preferentially binds to the Nkx3.1 3’ gene locus in vivo and in vitro

To confirm that AR interacts with the Nkx3.1 3’ gene locus, we next performed quantitative chromatin immunoprecipitation (qChIP) analyses across a 17-kb Nkx3.1 region that encompasses both 7-kb upstream and 10-kb downstream sequences to its TSS (Fig. 3A). We chose to analyze this region since it was previously shown to be capable of driving prostatic Nkx3.1 expression (4). Consistent with our reporter mouse data, we found that AR had low affinity for the region 5’ to the Nkx3.1 TSS, but instead occupied multiple loci in the 3’ 11-kb region (Fig. 3A). In particular, our statistical analysis (see Methods) highlighted two binding sites with the highest affinity in the 3’ UTR (“Peak+3kb”) and the intron (“Peak+1kb”), respectively (Fig. 3A). To test whether these two peaks could serve as transcriptional enhancers, we
performed the luciferase reporter assay by transfection of synthetic constructs containing an E4TATA minimal promoter in combination with the peak regions into prostate LnCaP cells (Fig. 3B), which express AR and are androgen responsive (27). Upon dihydrotestosterone (DHT) treatment, luciferase activity for the construct containing the ~290-bp “Peak+3kb” sequence was significantly enhanced, while no difference was observed for the construct containing the “Peak+1kb” sequence (Fig. 3C). To test any potential spatial effect of the binding site arrangement, we also generated constructs in which the “Peak+1kb” and “Peak+3kb” sequences were positioned downstream of the luciferase sequence, and obtained similar results (Fig. S4). We next used the FIMO software (28) to search for potential AR responsive elements (AREs) within the “Peak+3kb” fragment based on the AR DNA-binding consensus motif obtained from TRANSFAC (29). Three new candidate AREs were identified, with ARE1 being a composite site containing two 6-bp half sites with one overlapping nucleotide and ARE2 and ARE3 both being optimal palindromic sequences with 15 bp in length (Fig. 3D). In a series of luciferase assays using synthetic reporters containing six copies of the individual predicted AREs (Fig. 3B), we found that both ARE2 and ARE3, but not ARE1, significantly enhanced luciferase activities upon DHT treatment (Fig. 3E). Cumulatively, these molecular analyses support that AR directly binds to the 11-kb region, particularly the Nkx3.1 3’ UTR, to activate Nkx3.1 transcription.

**Pten promotes Nkx3.1 expression in vivo through transcriptional regulation on the 11-kb region**

Finally, we sought to address the question how loss of the tumor suppressor Pten leads to down-regulation of Nkx3.1 expression in both human and murine prostate tumors (19-21). We generated lineage-marked Pten-null prostate cell clones, which were identifiable as YFP+ or phospho-Akt+, by tamoxifen-induction of CK18-CreER 

To determine whether Pten regulation of Nkx3.1 expression occurs at the transcriptional level, we analyzed GFP reporter expression in tamoxifen-induced CK18-CreER 

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**DISCUSSION**

In the present study, we show that the 11-kb region 3’ next to the Nkx3.1 TSS is responsible for Nkx3.1 expression alterations in adult prostate under various physiological conditions (Fig. 4F). The 11-kb transgene was able to recapitulate normal Nkx3.1 expression pattern in adult prostate and induce the same levels of expression as the previously characterized 32-kb and 17-kb transgenes (4), suggesting that the 5’ sequence of the Nkx3.1 locus is dispensable for this purpose. Notably, all three fragments induced Nkx3.1 expression in most but not all luminal cells of the DLP and VP, suggesting that distant cis-regulatory elements exist beyond the 32-kb region to strengthen Nkx3.1 expression in those lobes. In contrast, the 11-kb fragment induced much stronger expression in all lobes than the previously
identified 5-kb urogenital enhancer at its distal 3' location (4), suggesting that the 3' region adjacent to the Nkx3.1 TSS is also important. Indeed, our qChIP experiments identified multiple AR binding sites in vivo in the 11-kb region, but not in the 5' region. In particular, the “Peak+3kb” sequence in the 3' UTR was discovered as a new important androgen response region. Using prostate cancer LnCaP cells, previous studies have identified potential AREs in various locations of the Nkx3.1 locus, including the intron (31), the 3' UTR (32,33), and a site 5' to the Nkx3.1 TSS (34). However, whether those sites are functional in vivo are unknown. Using the reporter mice, our prostate regression-regeneration experiments and AR conditional knockout experiments showed that androgen regulation of Nkx3.1 expression in the adult prostate is primarily mediated by cell-autonomous AR through the 11-kb region. We note that reporter GFP signal was not completely abolished in some AR-null luminal cell clones, indicating that other transcription factors can also bind to the 11-kb region to enhance Nkx3.1 expression in the absence of AR (Fig. 4F).

An interesting finding from our study is that the reporter mice labeled rare GFP+ luminal cells in the regressed prostate. The frequency of those cells and their positivity for Nkx3.1 suggest that they are the previously identified luminal stem cell CARNs. CARNs are AR+ (5), and we recently showed that they require AR for proper daughter cell differentiation (26). However, how CARNs are specified remains unclear. Is their exclusive Nkx3.1 expression in the regressed prostate due to AR expression or some other intrinsic properties that are different from the rest of the luminal cells? Our conditional knockout and reporter mouse experiments indicate that Nkx3.1 expression in CARNs is also mediated by cell-autonomous AR through the 11-kb region. We hypothesize that, after castration, residual levels of androgen secreted from the adrenal gland may induce AR nuclear translocation in a few localized niches, thereby specifying CARNs in the regressed prostate and sensitizing them to AR levels. Such a model would argue that CARNs are facultative stem cells dependent on microenvironmental cues (local androgen) rather than a pre-existing fixed population. This model can also explain the previously reported phenomena that CARNs tend to be clustered and that some initially YFP+ lineage-marked CARNs lost Nkx3.1 expression over time (5). Our Nkx3.1(11)-d2EGFP mice should provide a useful and convenient tool for isolation of bona fide CARNs from the regressed prostate at any particular time and studying their molecular and functional properties. Notably, CARNs are not the only source for prostate luminal cell regeneration since many regressed luminal cells contribute to this process (22,35), and Bmi1+ cells were recently shown to be a distinct type of luminal progenitor cells competent for regeneration (36). Future work should shed light on how this new type of progenitor cells is specified and its relationship with CARNs.

Nkx3.1 down-regulation has been reported in a variety of mouse prostate cancer models (20,21,37,38), and is well documented in human prostate cancer initiation (10). In human prostate cancer samples, decreases in Nkx3.1 and Pten expression levels were reported to be significantly correlated (21,39). Our data showing that loss of Pten abolished the GFP reporter signal provide definitive evidence that Pten promotes Nkx3.1 expression at the transcriptional level. The presence of nuclear AR in Pten-null clones as well as the down-regulation of a subset but not all of the AR target genes indicate that AR protein is still functional but unknown transcriptional repressors activated by the PI3K pathway are involved and exert their negative effects through the 11-kb region (Fig. 4F). It was proposed that Egr1, c-Jun, and Ezh2 are up-regulated upon Pten deletion and interact with AR to suppress its transcriptional output (23). Further analyses of the 11-kb sequence and molecular analyses of Pten-null luminal cells should provide potential candidates to test. It will also be interesting to determine whether similar transcriptional regulatory mechanisms exist for Nkx3.1 down-regulation under other tumor-initiating conditions.

**EXPERIMENTAL PROCEDURES**

**Generation of Nkx3.1(11)-d2EGFP mice**

The Nkx3.1(11)-d2EGFP construct is comprised of the 10.5 kb mouse genomic sequence (UCSC genome browser, mm9 Chr 14: 69808515-69819104) at the Nkx3.1 locus (-234/+10356) containing a 240-bp proximal promoter, exon 1, intron, exon 2 and ~6.5 kb 3’ region, as well as the d2EGFP (Clontech) and SV40 polyA sequences inserted right after the Nkx3.1 start codon. The
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construct (11671-bp total length) was synthesized by GenScript and cloned into a pUC57 vector. MluI and XhoI restriction enzyme sites were engineered at both the end of the synthesized sequence for plasmid linearization. The transgenic mouse lines were generated by pronuclear injection of fertilized eggs by Cyagen Biosciences.

**Mouse genotyping and operations**

The CK18-CreERT2 transgenic line (25), Nkx3.1CreERT2/+ targeted allele (5), AR<sup>lox</sup> allele (40), Pten<sup>lox</sup> allele (41), and R26R-YFP line (42) were described previously. Animals were maintained in C57BL/6N background. Genotyping was performed by PCR using tail genomic DNA, with primer sequences listed in Table S2. The Nkx3.1(11)-d2EGFP transgene was genotyped by a set of primers amplifying the junction between the proximal promoter and the d2EGFP sequence. The following primers generated a 319-bp PCR product: 5'-AAGGGCTCTGGAGCCTAATC -3' (forward), 5'-GAACTTCAGGGTCAGCTTGC -3' (reverse).

For tamoxifen induction, mice were administered 9 mg per 40 g body weight tamoxifen (Sigma) suspended in corn oil by oral gavage once daily for 4 consecutive days. Castration of adult male mice was performed using standard techniques. For prostate regeneration, testosterone (Sigma) was dissolved at 25 mg/ml in 100% ethanol and diluted in PEG-400 to a final concentration of 7.5 mg/ml. Testosterone was administered for 4 weeks at a rate of 1.875 µg/h delivered by subcutaneous implantation of mini-osmotic pumps (Alzet). All animal experiments received approval from the Institutional Animal Care and Use Committee at UCSC.

**GFP visualization, immunofluorescence staining and cell quantitation**

Prostate tissue dissection and direct GFP visualization were performed under a Nikon SMZ-1000 stereomicroscope with fluorescence and CCD digital camera. Tissues were fixed in 4% paraformaldehyde for subsequent cryo-embedding in OCT compound (Sakura). Immunofluorescence staining was performed as described previously (26). Slides were mounted with VectaShield mounting medium with DAPI (Vector Labs). Images were obtained using a Leica TCS SP5 spectral confocal microscope in the Microscopy Shared Facility of UCSC. All primary antibodies and dilutions used are listed in Table S3. Nkx3.1, AR, and pAkt primary antibodies were all raised in rabbit, but their expression can be distinguished in co-staining since pAkt signal is membrane while AR and Nkx3.1 signals are nuclear. For AR and Nkx3.1 co-staining, adjacent cryosections of 3 µm thickness were used.

Cell numbers were counted manually using confocal ×40 and ×63 photomicrographs across tissue sections. Three animals were counted for each group. Basal cells were identified based on lack of CK18 staining, positivity for CK5 staining, and/or shape of the cells (oval or triangular) and their positions at the basement of the epithelium. Luminal cells were determined based on positive CK18 staining and/or shape of the cells (columnar) and their positions at the apical side of the epithelium.

**Flow cytometry**

To sort luminal cells, lineage-marked prostate tissues were dissected and minced to small clumps, followed by enzymatic dissociation with 0.2% collagenase I (Invitrogen) in DMEM media with 5% FBS for 3 h at 37°C. Tissues were digested with 0.25% Trypsin-EDTA (StemCell Technologies) for 1 h at 4°C, passed through 21-to 26-gauge syringes and filtered through a 40-µm cell strainer to obtain single-cell suspensions. Dissociated prostate cells were suspended in Hanks’ Balanced Salt Solution Modified/2% FBS. ROCK inhibitor Y-27632 (StemCell Technologies) was added at 10µM throughout the whole process to inhibit luminal cell death and dead cells were excluded by propidium iodide staining. Luminal cells were sorted based on YFP positivity on a BD FACS Aria II instrument in the Flow Cytometry Shared Facility of UCSC.

**Quantitative real-time PCR analysis**

Total RNA from FACS-purified luminal cells was isolated using the RNeasy Micro Kit (Qiagen) and reverse transcribed into cDNA using the SuperScript IV VILO Master Mix (Invitrogen). Quantitative real-time PCR was carried out using Power SYBR Green PCR Master Mix (Life Technology) in the ViiA 7 Real-Time PCR instrument. cDNA samples were diluted 1:100 for all analyses, which were performed in quadruplicate. Expression values were obtained
using the ΔΔCT method and normalized to β-actin (Actb) expression; average values are shown as the mean ± standard deviation (SD). Welch two sample t-test was performed. Primer sequences for qRT-PCR are provided in Table S4.

**Quantitative ChIP and statistics**

Magna ChIP™ HiSens Chromatin Immunoprecipitation Kit (Millipore) was used according to the manufacturer's instructions. Formaldehyde cross-linked chromatin was obtained from adult mouse prostate and sheared by sonication (average size, 600 bp). Aliquots of chromatin from 2 mg prostate tissue were incubated with 2 µg of anti-AR antibody (Sigma Cat# A9853) bound to 40 µl protein A/G-coated magnetic beads from the kit. The immunoprecipitates were washed three times by SCW buffer of the kit with protease inhibitor cocktail and resuspended in ChIP Elution buffer. After one hour incubation at 55 °C, the cross-links were reversed by 3 hour incubation at 65 °C. Genomic DNA was resuspended into 150 µl of water.

The amounts of each specific DNA fragment in immunoprecipitates were determined by quantitative PCR reactions using a standard curve generated for each primer set with 0.04, 0.2, and 1% input DNA samples. Primer sequences are provided in Table S5. The PCR products were between 80 and 120 bp. Reactions were carried out using the Viia™ 7 Real-Time PCR System and a 2xSYBR mix (ABI). Cycle threshold (Ct) values were transformed into DNA copy numbers using a standard curve. The copy number of each specific DNA fragment was compared with the value before immunoprecipitation (input DNA). The control antibody (rabbit normal IgG, Millipore Cat# CS200581) was included for each set of the quantitative PCR experiments. The enrichment obtained with IgG was subtracted from the corresponding value obtained with anti-AR antibody. To determine a threshold value that distinguishes real binding signals from background noise, statistical analysis was conducted using the R Software (Version 2.13.1). Analysis of variance (ANOVA) was performed for the AR signals obtained from all 16 qChIP amplicon sites (7 amplicons 5’ to and 9 amplicons 3’ to the TSS), and Fisher's least significant difference (LSD) test was performed to separate the 16 sites into the following 6 groups with a descending order in the signal intensity: +3 kb (3’UTR) > +1 kb (intron), +2 kb (3’UTR) > +5 kb, +4 kb > +6 kb, +10 kb > -0.2 kb > the other eight sites (background noise). Student's t-test for the last group showed the 99% confidence interval of the background signal value to be 0.107–0.242, indicating that all regions having signals higher than 0.242 should be considered as specific AR-binding regions.

**Plasmid construction, transfection, and luciferase reporter assays**

The sequences of “Peak+1kb” and “Peak+3kb” regions and six copies of predicted AREs with a minimal E4TATA promoter were synthesized by GenScript, and then subcloned into the firefly luciferase reporter vector in pGL3-Basic plasmid (Promega). Transient transfections were conducted by Lipofectamine 2000 (Invitrogen) in LnCaP cells (ATCC CRL-1740). The cells were cultured in RPMI-1640 Medium with 10% FBS in 24-well plates for 36 hours before transfection to ensure complete cell attachment and growth to ~70% confluence. For transfection, 1.6 µg of firefly luciferase reporter plasmid and 40 ng of Renilla-TK (Promega) internal control plasmid were mixed with 2.5 µl Lipofectamine 2000 in 100 µl Opti-MEM medium (Life Technologies). The mixture was then incubated with the cells for 5 hours before being replaced with cell culture medium with or without 10⁻⁶M DHT. The dual luciferase reporter assays (Promega) were performed after 48 hours DHT stimulation according to the manufacturer's instructions, and were repeated twice in triplicates. Statistical evaluation of luciferase activities was performed using two-sided student’s t-test.

**Prediction of AREs in the “Peak+3kb” region**

To search for candidate AREs in the “Peak+3kb” region, FIMO (Find Individual Motif Occurrence), a motif-based sequence analysis software of MEME Suite 4.11.3 (http://meme-suite.org/index.html) was run with a 15-bp AR consensus motif (V$AR_01 – AR) from TRANSFAC, a manually curated database of eukaryotic transcription factors’ binding sites (http://gene-regulation.com/pub/databases.html). The 15-bp optimal AR binding motif is a palindromic sequence containing two 6-bp AR
binding half sites separated by 3 bp random nucleotides with a head-to-head arrangement. Prediction was also performed by FIMO using the AR binding half site sequence. Filter criteria with the match p-value $< 10^{-4}$ was used to identify the three potential AREs.

Acknowledgments: We thank the UCSC Special Research Grant for enabling the generation of the Nkx3.1(11)-d2EGFP mice. Q.X. was a postdoctoral fellow of the CIRM training program. This work was supported by NIH grant GM116872 to Z.A.W.

Conflict of interest: The authors declare no competing interests.

Author Contributions: Q.X. conducted experiments. Q.X. and Z.A.W. conceived the project, analyzed data, and wrote the manuscript.

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Figure 1. Nkx3.1(11)-d2EGFP recapitulates Nkx3.1 expression in adult prostate. 

(A) The DNA construct used for generating Nkx3.1(11)-d2EGFP transgenic mice showing the relative position of its sequences in the mouse Nkx3.1 gene locus. Conservation of DNA in multiple placental mammal species is shown as in the UCSC genome browser. (B) White field and GFP overlay images of dissected urogenital tissues showing strong prostate-specific GFP expression in the Ntg18 line. (C) Immunofluorescence (IF) images showing no GFP reporter expression in E18.5 prostate bud. (D) IF showing GFP expression in all prostate epithelial cells at P8. (E) IF showing GFP expression primarily in luminal cells at 8w. (F) IF showing co-localization of reporter GFP and Nkx3.1 signals in luminal cells at 5 months. (G) Quantitation of the percentages of GFP+ cells in luminal cells of different lobes and in basal cells. (H) Direct visualization of GFP signal showing most basal cells were GFP-negative (arrows) while a small proportion was GFP+ (arrowhead) in adult prostate. AP, anterior prostate, VP, ventral prostate, DLP, dorsal-lateral prostate, SV, seminal vesicle. Scale bars in B correspond to 1 mm and in C-F,H to 20 microns. Error bar corresponds to one s.d.

Figure 2. AR-dependent Nkx3.1 expression in luminal cells and CARNs revealed by Nkx3.1(11)-d2EGFP during prostate regression-regeneration. 

(A) Timeline of serial prostate regression-regeneration experiment for Nkx3.1(11)-d2EGFP mice. (B) Direct visualization showing relatively few GFP+ cells (arrow) in the regressed prostate. (C) IF images showing the rare GFP+ cells in the regressed prostate were luminal (CK5-, left) and Nkx3.1+ (right). (D) Direct visualization showing most luminal cells recovered GFP expression after 1-round prostate regeneration. (E) IF image showing most luminal cells were GFP+ after 3-round regression-regeneration. (F) IF image showing co-localization of reporter GFP and nuclear AR in prostate that is undergoing regression. (G) IF image showing loss of Nkx3.1 expression in AR-null luminal cells (revealed by enhanced CK18 expression, arrows). (H) GFP reporter signal was strongly reduced in AR-null luminal cells in the AP of tamoxifen-induced CK18-CreER2; ARflox/Y; Nkx3.1(11)-d2EGFP mice. (I) Timeline of experiment for AR ablation in CARNs in Nkx3.1CreERT2/+; ARflox/Y; Nkx3.1(11)-d2EGFP mice. (J) Almost no GFP signal was observed by direct visualization of the prostate in panel I. (K) Quantitation of the proportions of GFP+ cells among total luminal cells for experiments in panels B, D, E and J. ** p<0.01 by student’s t-test. Scale bars correspond to 20 microns. Error bars correspond to one s.d.

Figure 3. AR binds to the Nkx3.1 11-kb 3’ region to enhance its gene expression. 

(A) Distribution of AR detected by qChIP analysis over the 17-kb mouse Nkx3.1 locus in prostate chromatin. The relative enrichments are shown as the percentage of the DNA input. Locations of primer pairs for qChIP amplicons are marked by blue triangles. Conservations of DNA in vertebrate species and in human are shown as in the UCSC genome browser. Specific AR enrichments above the background noise (0.242, dashed grey line, see Methods for description) were concentrated in the 11-kb region 3’ next to the TSS, with the highest two peaks at locations +3kb and +1kb, respectively (arrows). (B) Constructs for transient transfection and luciferase reporter assays. (C) Luciferase reporter assay comparing the construct with the minimal promoter to those containing “Peak+1kb” and “Peak+3kb” sequences showing that the “Peak+3kb” region is androgen responsive. (D) Sequences of three candidate AREs in the “Peak+3kb” region. (E) Luciferase assay comparing construct containing six copies of each predicted ARE showing that ARE2 and ARE3 are androgen responsive. Error bars correspond to one s.d. * p < 0.05 by student’s t test.

Figure 4. Transcriptional regulation of Nkx3.1 by Pten and the overall model. 

(A) IF images showing loss of Nkx3.1 expression in Pten-null luminal cells marked by either YFP positivity (left) or phospho-Akt expression (right) of tamoxifen-induced CK18-CreER2; Ptenflox/flox; R26R-YFP/+ mice. Phospho-Akt staining (red) can be distinguished from Nkx3.1 or AR staining (red) by

Nkx3.1 in prostate stem cells and cancer
its membrane localization. (B) IF images showing nuclear AR expression in Pten-null luminal cells. (C, D) Adjacent sections of phospho-Akt staining and GFP direct visualization in AP (C) and DLP (D) of tamoxifen-induced CK18-CreER\textsuperscript{T2}, Pten\textsuperscript{floox}; Nkx3.1(11)-d2EGFP mice showing that GFP signal is abolished in Pten-null cells. Scale bars correspond to 20 microns. (E) qRT-PCR analyses comparing the expression levels of selected AR target genes in wild-type and Pten-null luminal cells by Welch t-test. *, p<0.01. Error bar corresponds to one s.d. (F) Model of Nkx3.1 transcriptional regulation in adult prostate epithelial cells. Androgen cell-autonomously maintains Nkx3.1 expression in adult prostate epithelial cells. Upon DHT binding, AR proteins in the cytoplasm dimerize and translocate into the nucleus. They preferentially bind to the 11-kb Nkx3.1 3’ region to the transcription start site (TSS), particularly the 3’ UTR, to activate Nkx3.1 transcription. Other transcription activators also bind to this region (shaded red triangle) and play a minor role in activating Nkx3.1 transcription (dashed arrow). Transcriptional repressors that are normally inhibited by Pten can also bind to this region (shaded yellow triangle) and strongly inhibit Nkx3.1 transcription even in the presence of nuclear AR.
Figure 1

A) Nkx3.1

B) SV, bladdor, AP, VP, DLP

C) E18.5

D) CK5, CK18, GFP, DAPI

E) CK5, CK18, GFP, DAPI

F) Nkx3.1, GFP, DAPI

G) Percent GFP+ cells

H) GFP, DAPI
Figure 2

(A) Nkx3.1(11)-d2EGFP

(panels F) (panels B,C,K) (panels D,K) (panels E,K)

Birth → Castration → Regress

Analysis → Analysis → Analysis

 Months 2 → 3 → 4 → 5 → 6 → 7 → 9 → Regress

Androgens present → Regenerate → Regenerate → Regenerate

Round 1 → Round 2 → Round 3

(B) GFP DAPI

(C) regressed

(panels CK5, GFP, DAPI)

(D) 1-round

(GFP, DAPI)

(E) 3-round

(CK5, GFP, DAPI)

(F) in regression (AR GFP, DAPI)

(G) CK18-CreERT2; AR<sup>LoxP/LoxP</sup>

(H) CK18-CreERT2; AR<sup>Acta2<i>c</i></sup>; Nkx3.1(11)-d2EGFP

(I) Nkx3.1<sup>C<sub>CreERT2</sub>/mm</sup>; AR<sup>LoxP/LoxP</sup>; Nkx3.1(11)-d2EGFP

(Birth → Castration → Regression → Tamoxifen)

(panels J,K) Analysis

Weeks 8 → 9 → 10 → 11 → 12

Androgens present → GFP DAPI

(J) Regressed

(K) Percent GFP+ luminal

(panel B) panel J) panel D) panel E)

**
Figure 3

A

Chrs: 14: conservation
vertebrate

qCHIP amplicons

E4 TATA

Firefly luciferase

Peak +1 kb

E4 TATA

Firefly luciferase

Peak +3 kb

E4 TATA

Firefly luciferase

6 copies of predicted ARE

B

Minimal promoter

C

Relative luciferase activities

D

AR DNA-binding consensus motif (15bp palindromic)

Peak +3 kb

E

Relative luciferase activities

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Figure 4

**CK18-CreER^{T2}; Pten^{floxed}; R26R-YFP/+**

A

Nkx3.1 YFP DAPI
Nkx3.1 pAkt

B

AR YFP DAPI
AR pAkt

**CK18-CreER^{T2}; Pten^{floxed}; Nkx3.1^{(11)}-d2EGFP**

C

AP
pAkt DAPI

D

GFP DAPI

E

<table>
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<tr>
<th>Gene</th>
<th>相对表达</th>
<th>Pten</th>
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<tr>
<td>Pbsn</td>
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F

Cytoplasm

DHT: AR

Nucleus

Activators

Repressors

AR

TSS

5’

3’

Nkx3.1

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Transcriptional regulation of Nkx3.1 in prostate luminal stem cell specification and cancer initiation via its 3' genomic region
Qing Xie and Zhu A. Wang

J. Biol. Chem. published online July 5, 2017

Access the most updated version of this article at doi: 10.1074/jbc.M117.788315

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