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NKX3.1 binding to *GPX2, QSCN6, SOD1,* and *SOD2* promoters contributes to antioxidant response regulation via transactivation

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Abstract: NKX3.1 is a prostate-specific transcription factor that is regulated by the androgen receptor in the presence of androgens. It functions as a tumor suppressor against the development of prostatic intraepithelial neoplasia and primary prostate tumors. Here, a recognized approach combining in silico analysis and chromatin immunoprecipitation (ChIP) was used to identify the genes directly regulated by NKX3.1 promoter binding in LNCaP cells. Quantitative PCR using ChIP-captured DNAs as templates verified a subset of NKX3.1 binding motifs. Thus, in the presence of androgens, significant NKX3.1 binding occurs to promoters of *GPX2, QSCN6, SOD1,* and *SOD2* genes that contribute to oxidative stress regulation. Our data demonstrate that NKX3.1 is found in a DNA-bound state transiently at a basal level even in the absence of androgens; an increase in androgens promotes NKX3.1 binding, perhaps temporally rather than spatially, to the specific sites. The overall changes potentiate the transcriptional regulatory activity of NKX3.1, although they are dependent on the androgen receptor for the target promoters. The results suggest that NKX3.1 contributes to an antioxidant response by regulating the transcription of oxidative stress regulators by direct promoter binding.

Key words: Chromatin immunoprecipitation, promoter binding, androgen, NKX3.1, MatInspector

1. Introduction

Prostate cancer is the most common malignancy among males in developing countries and constitutes 29% of all cancer cases among males in the United States (Weir et al., 2003; Siegel et al., 2013). While surgical treatment of organ-confined disease may be curative, prostate cancer that has progressed to the hormone-independent stage is nearly always fatal. Although the outcome is nearly inevitable once conventional hormone ablation therapy has been initiated, it is important to understand the spectrum of genes regulated in hormone-sensitive disease and recognize whether any of these are active in hormone refractory tumors of the prostate. One of the androgen receptor (AR)-regulated genes (Nelson et al., 2002; Sato et al., 2003; Masuda, 2005; Beier et al., 2009) is *NKX3.1*, and it encodes a homeobox-containing protein with an important function in normal prostate development during embryogenesis under the regulation of androgens. It also acts as a tumor suppressor in prostate cancer (Korkmaz et al., 2000a; Abate-Shen et al., 2008). As described independently by 2 groups, it functions in the DNA damage repair response by activating ataxia telangiectasia mutated (ATM) kinase (Bowen et al., 2013; Erbaykent-Tepedelen et al., 2014), and compromises the oxidative damage-mediated response in prostate cancer cells. Loss of NKX3.1 expression is frequently observed in prostatic intraepithelial neoplasia and advanced prostate tumors. However, its expression is restored in castration-resistant prostate tumors. Additionally, the decreased proliferation in cell culture and alleviated tumorigenesis in xenografts supports the suppressor role of NKX3.1 (Lei et al., 2006; Abate-Shen et al., 2008).

Furthermore, the DNA-binding motif of NKX3.1 was determined as TAAGT(N) using an in vitro protein–DNA binding assay in a previous study (Steadman et al., 2000). As a transcription factor, NKX3.1 functions by activating or repressing the expression of target genes transcriptionally via its homeo- or TN-domains. Expression of smooth muscle gamma actin is activated by NKX3.1 along with a synergistic interaction to serum response factor (Carson et al., 2000), whereas human ether-a-go-go-related gene (*HERG*) promoter activity is repressed through one functional NKX3.1 binding site on the *HERG* promoter (Bowen et al., 2007). As NKX3.1 also represses vascular endothelial growth factor (VEGF) transcription via its specific binding site on the *VEGF* promoter (Zhang et

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al., 2008), NKX3.1 binding on the 2 specific sites on the *PCAN1* promoter was also reported (Abate-Shen et al., 2008).

In a study involving *NKX3.1*-deficient mice, NKX3.1 function was compared by microarray, and 29 positively and 28 negatively (57 potential) regulated targets in hemizygous (monoallelic) and nullizygous (0 alleles) mutants were determined in comparison to the wild type (2 alleles) (Magee et al., 2003). Another microarray study in *NKX3.1*-deficient mice showed that 299 genes and 339 genes were up- and downregulated in 638 target candidates, respectively. Furthermore, in that study, glutathione peroxidase 2 (*Gpx2*), glutathione peroxidase 3 (*Gpx3*), peroxiredoxin 6 (*Prdx6*), and sulfhydryl oxidase Q6 (*Qscn6*), which are oxidative stress regulators, were altered in expression by the loss of NKX3.1 (Ouyang et al., 2005).

This information is important not only for proper understanding of the role of androgen-regulated NKX3.1 expression in prostate cancer but also for describing its role in the development and maintenance of the prostate gland under stress conditions, such as oxidative damage or inflammation (Mora et al., 1996; Coffey et al., 2002). Loss or alteration of NKX3.1-dependent regulation of these interactions might be crucial for understanding the progression of prostate cancer. In physiological samples, NKX3.1 binding sites may deviate considerably from the putative consensus sequences (Nelson et al., 2002; Beier et al., 2009). It is unclear how specificity of transcription factors such as AR and NKX3.1 to the response element is achieved. As NKX3.1 binding to these promoter and enhancer sequences facilitates interactions with the repressor transcriptional machinery (Groucho), leading to gene transcription or repression, finding the sole targets of the NKX3.1-regulated promoters is essential for understanding the biology of prostate cancer (Schule et al., 1988; Truss and Beato, 1993; Suzuki et al., 2003).

In order to define the genes driven by the NKX3.1 transcriptional complex in vivo, we used the combination of in silico analysis of the NKX3.1 binding motif on promoter sequences with chromatin immunoprecipitation (ChIP) as a means to identify genes captured by ChIP for recognition of endogenous transcription factor assemblies on gene promoters in vivo (Hecht et al., 1999; Adamson et al., 2003; Hayakawa et al., 2004; Li et al., 2005; Masuda et al., 2005; Sun et al., 2006). Finally, binding rates of NKX3.1 on the native promoters of *ACTG2*, *PCAN1*, *HERG*, *GPX2*, *GSCN6*, *SOD1*, and *SOD2* were validated using quantitative PCR (Q-PCR), and the androgen effect was also investigated in LNCaP cells.

2. Materials and methods

2.1. Cell culture, serum starvation, and R1881 induction

An LNCaP cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured at 37 °C in RPMI 1640 with 10% FBS in a humidified atmosphere of 5% CO_2. The day after cells split, the medium was changed to 2% CT-FBS and incubated for 48 h. The medium was then changed to 0.5% CT-FBS and incubated for an additional 24 h, and synthetic androgen R1881 (10^{-8} M) was added for up to 24 h.

2.2. Analysis of NKX3.1 binding motif on the promoters

Promoter sequences (from –9999 to +1; +1 indicates transcription start site) of the target gene candidates were extracted in FASTA format from the databases (UCSC Genome Browser and Eukaryotic Promoter Database). Promoter sequences were then analyzed to extract the specific NKX3.1 binding motif [TAAGT(N)] by using the MatInspector tool (www.genomatix.de). Analysis parameters were adjusted to: library, "transcription factor binding sites", and core similarity, "1". Finally, specific binding sites were indicated on the schematic promoter maps of each gene.

2.3. Antibodies

The anti-NKX3.1 antibody was a gift from Dr Saatçioğlu, Oslo Biotechnology Center, Norway, and nonspecific rabbit IgG (Santa Cruz) and HRP-antirabbit secondary antibody (Amersham) were purchased and used according to the manufacturer's recommendations.

2.4. Chromatin immunoprecipitation

LNCaP cells (10^8) were cultured in 15-cm cell culture dishes and treated with 10^{-8} M R1881 for 24 h. Cells were then fixed by adding 1% formaldehyde to cells for 10 min at room temperature, and then 2.5 M glycine at 1/20 volume was added for 5 min with gentle shaking. Media were discarded, and cells were washed with PBS and pelleted. Cell pellets were washed with lysis buffer [10 mM Tris-Cl (pH 7.5), 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Igepal, 1 mM PMSF, 1X Roche protease inhibitor cocktail] 3 times and centrifuged at 500 × *g* for 5 min. The pellet was resuspended in 1 mL of pre-IP dilution buffer [10 mM Tris-Cl (pH 7.5), 10 mM NaCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 4% Igepal, 1 mM PMSF, 1X Roche protease inhibitor cocktail], and 1% SDS and 200 mM NaCl were added. Cells were sonicated 3 times for 10 s with 45% power and 50% pulse. Centrifugation was performed at 13,000 × *g* for 10 min at 4 °C. Supernatant was transferred into a new tube, and 1 mg of protein from each sample was subjected to preclearance with 20 µL of protein A/G agarose beads. Samples were then incubated with 2 µg of anti-NKX3.1 antibody in a rotator at 4 °C overnight. Nonspecific rabbit IgG was used as a negative control, and 40 µL of protein A/G agarose beads were added and incubated for an

additional 3 h. After centrifugation, the supernatant was removed, and beads were washed with washing buffers I, II, and III subsequently [wash buffer I: 20 mM Tris-Cl (pH 8.0), 2 mM EDTA, 1% Triton X-100, 150 mM NaCl, 1 mM PMSF; wash buffer II: 20 mM Tris-Cl (pH 8.0), 2 mM EDTA, 1% Triton X-100, 0.1% SDS, 500 mM NaCl, 1 mM PMSF; wash buffer III: 10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 0.25 M LiCl, 0.5% Igepal, 0.5% sodium deoxycholate]. Elution of the protein–DNA complexes was done by incubating the beads with elution buffer [25 mM Tris-Cl (pH 7.5), 10 mM EDTA, 0.5% SDS] at 65 °C for 30 min. Reverse cross-linking was then performed by incubation with proteinase-K at 65 °C overnight.

2.5. Q-PCR

To study the amplification of specific NKX3.1 binding sites, quantitative RT-PCR was performed using a SYBR Green PCR Kit and the LC480 PCR system (Roche, Germany). The primers specific for each NKX3.1 binding site on promoters of the genes are given in the Table. The relative abundance of each amplicon was calculated using a comparative cycle of threshold (CT) method with rIgG-precipitated DNA as an invariant control for promoter binding analysis. The formula is: (target unknown / reference unknown) / (target calibrator / reference calibrator), where 'target unknown' is precipitated with NKX3.1 ab from R1881-treated cells, 'target calibrator' is precipitated with rabbit IgG from R1881-treated cells, 'reference unknown' is precipitated with NKX3.1 ab from untreated cells, and 'reference calibrator' is precipitated with rabbit IgG from untreated cells. ChIP DNA samples were used as templates. For Q-PCR analysis on the NKX3.1 mRNA level, mRNA isolation (RNeasy mRNA Isolation Kit, QIAGEN, the Netherlands) and cDNA synthesis (Omniscript cDNA Synthesis Kit, QIAGEN) were performed. The relative mRNA level was calculated using a comparative CT method, using GAPDH as an invariant control. Primers were NKX3.1_F: TCTATCAGCATCTGACAGGTGAA, NKX3.1_R: AGCAGGGTTTGTTATGCATGTAG, GAPDH_F: CATTGCCCTCAACGACCACTTT, GAPDH_R: GGTGGTCCAGGGGTCTTACTCC.

Table. List of primers used to amplify the target regions covering NKX3.1 binding motif.

GPX2_1_F ProLC	GCCTCACCAAGTATATTCCATTAG
GPX2_1_R ProLC	CCTCCAGAGACACCTCTTAT
GPX2_2_F ProLC	CAAGTCCTTGTGACTCAGTG
GPX2_2_R ProLC	TTTCCATGGCTGTTCAAGTAT
GPX2_3_F ProLC	TCCACTAAGTTTATCCACCTGT
GPX2_3_R ProLC	TCAGTGCCTCAGCTCTT
QSCN6_F ProLC	GTCCCTGATCTTCGTGTG
QSCN6_R ProLC	CTCTAGGGACGTGGAACT
SOD1_1_F ProLC	ACTACCAAATACAACAGGCA
SOD1_1_R ProLC	AGTGTGAAACAACAATAGAAGC
SOD1_2_F ProLC	ATATTAAGTACTAGGCTGGACG
SOD1_2_R ProLC	CGCCATCATAGCTCACTG
SOD2_F ProLC	GTTGCAAGAAGCAACGGA
SOD2_R ProLC	GTAGCCTAGTAAGCTGTTAAGTAT
HERG_F ProLC	GTGCAGCTCCTATGCAGA
HERG_R ProLC	CAAGACAATCTGAAGGCCC
ACTG2_F ProLC	CACTCTGGGCAGCTTAT
ACTG2_R ProLC	CCAGCTGACGGTGTGTTA
PCAN1_1_F ProLC	CACTGCCTCTATGCACT
PCAN1_1_R ProLC	GGTATAACTGAACATGCTACCA
PCAN1_2_F ProLC	GCTGCCTGATAACTCATAAGTAAT
PCAN1_2_R ProLC	CTTAGCATTGCCAAGCTG
PCAN1_3_F ProLC	TTTAAGCTCACAGCATTCACA
PCAN1_3_R ProLC	AGCAGTTCACTTTTCTGTATCT

2.6. Cell lysis, protein extraction, and blotting

For protein extraction, cells were grown in 6-cm culture dishes (Sarstedt, Germany) and washed once with PBS prior to cell lysis. Cells were collected from culture plates using a cell scraper and transferred to Eppendorf tubes. Cells were resuspended in 250 µL of modified RIPA buffer [10 mM Tris-Cl (pH 8.0), 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 1 mM EDTA, 1 mM EGTA, 140 mM NaCl] containing protease and phosphatase inhibitors. Lysates were sonicated for 20 s (25% power, 0.5 cycles) and centrifuged at 12,000 × *g* for 10 min, and cleared supernatants were collected into new tubes. Protein concentrations were determined using BCA assay (Sigma, UK). SDS-PAGE and western blots were performed under standard conditions using 50 µg of protein lysate per lane; proteins were separated on 10%–12% gel and transferred to a PVDF membrane (Amersham, UK) using a wet transfer blotter. The PVDF membrane was blocked with 5% dry milk in TBS-T (Tris-borate-saline solution containing 0.1% Tween 20). Primary and secondary antibody incubations were performed using TBS-T containing 0.5% dry milk or 5% BSA at room temperature for 1 h or at 4 °C overnight. Membranes were developed using ECL prime reagent (Amersham) for 5 min and were photographed using Kodak X-ray film in a dark room.

2.7. Statistics and criteria of significance

The results were obtained from 3 independent experiments. Statistical analyses were performed using Student's t-test, and the P-values were calculated by comparing each treatment to the control.

3. Results

3.1. MatInspector (in silico) analysis supports the regulation data

In order to identify the NKX3.1 binding sites on *GPX2*, *QSCN6*, *SOD1*, and *SOD2* promoters, the sequences from –9999 to +1 (transcription start site) were extracted from the experimentally verified EPD (Cavin Perier et al., 1998) and UCSC, and the specific binding motif for NKX3.1 was analyzed using the MatInspector tool. NKX3.1 binding sites were then schematically mapped (Figure 1). We found 36 NKX3.1 consensus sites [TAAGT(N)] in 4 promoter regions, and 7 out of 36 proximal promoters were chosen for validation as potential binding regions. Since we described the list of genes with promoters containing NKX3.1 sequences and presumed that altered expression of the promoters previously reported by Ouyang et al. (2005) might be controlled directly by NKX3.1 recruitment into transcriptional complexes, further validations in specific binding regions were carried out. Primers covering particular promoter sequences were designed using LightCycler primer-probe design software. The PCR validations were then performed using genomic DNA as a template. Finally, the binding sequences were examined in silico as regions of interest for ChIP studies.

3.2. Validation of site specificity of NKX3.1 binding

Since LNCaP is a well-known androgen-responsive prostate cancer cell line, an increase in the expression of NKX3.1 was shown as an indicator of androgen induction. RNA was prepared from LNCaP cells, either untreated or treated with synthetic androgen R1881 (10^{-8} M), and transcriptional alteration of androgen-responsive gene *NKX3.1* was induced for up to 24 h; a time-dependent increase in NKX3.1 mRNA level by Q-PCR, in comparison to GAPDH, was detected, which was the quality control of expression (Figure 2A). Consistent with transcriptional increase, after administration of R1881 for 24 h, a clear increase was also observed in protein expression of NKX3.1 (Figure 2B).

In order to analyze the promoters regulated by direct recruitment of NKX3.1 in vivo, ChIP was performed using a specific antibody against NKX3.1 in LNCaP cells. Q-PCR studies were then performed using primers covering the [TAAGT(N)] consensus sites to validate the precipitates from ACTG2, HERG, and PCAN1 that are known targets of NKX3.1, as an experimental control. Finally, binding ratios of NKX3.1 were measured using Q-PCR and calculated for each promoter region of interest by comparing the amplified precipitation of an R1881-treated sample to an untreated sample. NKX3.1 antibody-precipitated samples were normalized to nonspecific IgG. When known transcriptional targets from previous studies were verified in LNCaPs, the binding ratios were given as fold-change of 6.1 for *ACTG2*, 8.7 for *HERG*, and 24.6-, 57.3-, and 5.3-fold for PCAN1_1, PCAN1_2, and PCAN1_3 on the *PCAN1* promoter.

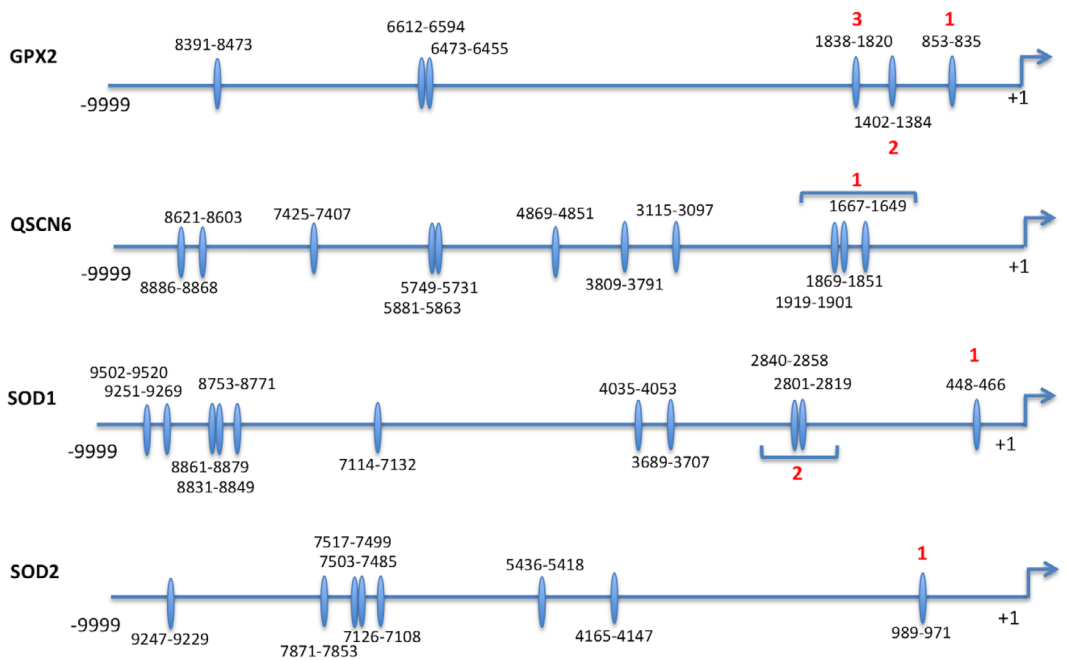

Figure 1. MatInspector analysis yielded 36 NKX3.1 putative binding sites in 4 promoter regions. DNA was analyzed using the criteria of high occurrence of TAAGT(N) sequences on proximal promoters. The regions of interest in proximal regions are numbered in red as amplicon numbers.

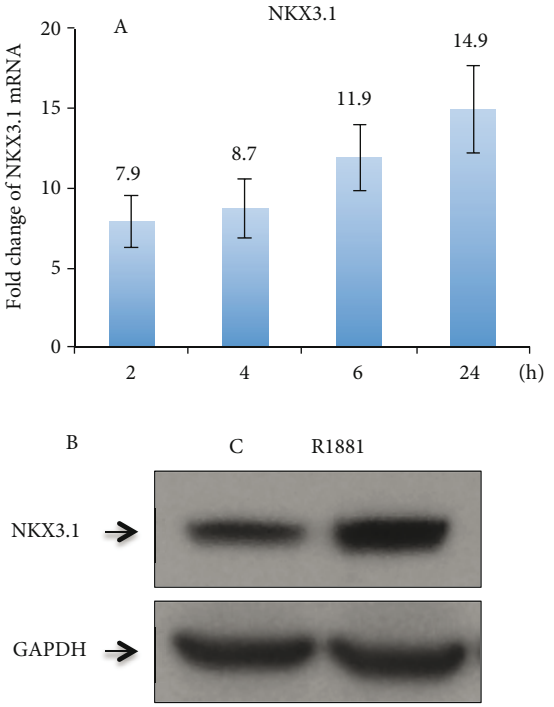

Figure 2. NKX3.1 expression is inducible with androgens. **A)** *NKX3.1* gene expression was induced by R1881 at the mRNA level at about 15-fold of control at 2, 4, 6, and 24 h; $P < 0.001$. **B)** NKX3.1 expression also increased at the protein level at 24 h. GAPDH western blot was presented as the loading control. Representative figure was obtained from 3 independent experiments.

Because the expression of genes was altered by NKX3.1 expression, as reported in a previous study (Ouyang et al., 2005), NKX3.1 binding ratios on the 3 specific motifs of the *GPX2* promoter were also examined, and the regions GPX2_1, GPX2_2, and GPX2_3 increased 10.3-, 24.2-, and 9.6-fold, respectively. In addition, the 3 adjacent NKX3.1 motifs on the *QSCN6* promoter resulted in a 22-fold increase in binding. The associations on the 2 binding sites of the *SOD1* promoter, SOD1_1 and SOD1_2, also increased 9.3- and 36.4-fold, respectively; the SOD2 promoter was 24-fold higher (Figure 3) in comparison to androgen-negative controls. Thus, we observed that NKX3.1 precipitations resulted in similar representations of the promoters analyzed where the overall changes were significantly altered with R1881 treatment in comparison to untreated control samples.

Taken together, we found that 7 promoter regions significantly bound 4 nonredundant promoters with NKX3.1, either with or without R1881 (Figure 3). NKX3.1 binding on the known transcriptional target (*ACTG2*, *HERG*, and *PCAN1*) promoters was validated by enhanced NKX3.1 expression upon R1881 treatment in LNCaP cells; additionally, NKX3.1 binding was first demonstrated at the *GPX2*, *QSCN6*, *SOD1*, and *SOD2* promoters. Therefore, we suggest that NKX3.1 regulates these genes transcriptionally.

4. Discussion

Antiandrogen therapy usually results in involution of both normal prostate glands and prostatic tumors in the early stages of the disease if the disease is still androgen-dependent. However, in the case of the latter, recurrence of the tumor in almost all cases in a few months or years is described as an androgen-independent state of the prostate disease. Such tumors appear autonomous in that a variety of AR-target genes are expressed (e.g., *NKX3.1*) without the ligand (Korkmaz et al., 2000b; Skotheim et al., 2003). Since the originated tumor cells are usually error-prone, this may render the development of a clone that is selected during hormone ablation therapy (Dryhurst et al., 2012). There is strong evidence that selected clones represent a loss of oxidative stress regulation as well as androgen response. Consistent with a recent expression profiling study showing alterations in several prooxidant and antioxidant enzyme genes involving *Gpx2* and *Qscn6* in NKX3.1-mutant mice (Ouyang et al., 2005), our study demonstrated that NKX3.1 bound to *GPX2* and *QSCN6* promoters and that the binding increased with androgen administration. In addition, we used R1881 instead of 5α-dihydrotestosterone (DHT) in our experiments, since R1881 is a synthetic androgen and is not metabolized as rapidly as DHT in cell culture (Asselin et al., 1979). Further, it is 10 times more potent than DHT and corresponds to physiological levels (1–100 nM) covering all ages (Ripple et al., 1997). Our data imply that transcriptional changes could also be regulated via NKX3.1 binding directly. Although the SOD1 and SOD2 were not changed in NKX3.1-mutant mice in a previous report (Ouyang et al., 2005), we observed significant NKX3.1 binding to *SOD1* and *SOD2* proximal promoters. This discrepancy suggests that enhanced NKX3.1 binding on *SOD1* and *SOD2* promoters did not lead to altered expressions of these enzymes due to posttranscriptional regulation of mRNA or a requirement for an additional coactivator after NKX3.1 binding.

Since transcriptional control has been an attractive area for drug development research in recent years, identification of the molecular targets of NKX3.1 transcription factor can lead to new therapeutic strategies for prostate cancer. Providing insight into a more complete spectrum of the development of inhibitors for certain targets may circumvent the function of NKX3.1 and the development of ligand-independent AR action. The development of inhibitors of key targets in late-stage hormone-independent disease will greatly increase our basic understanding of the

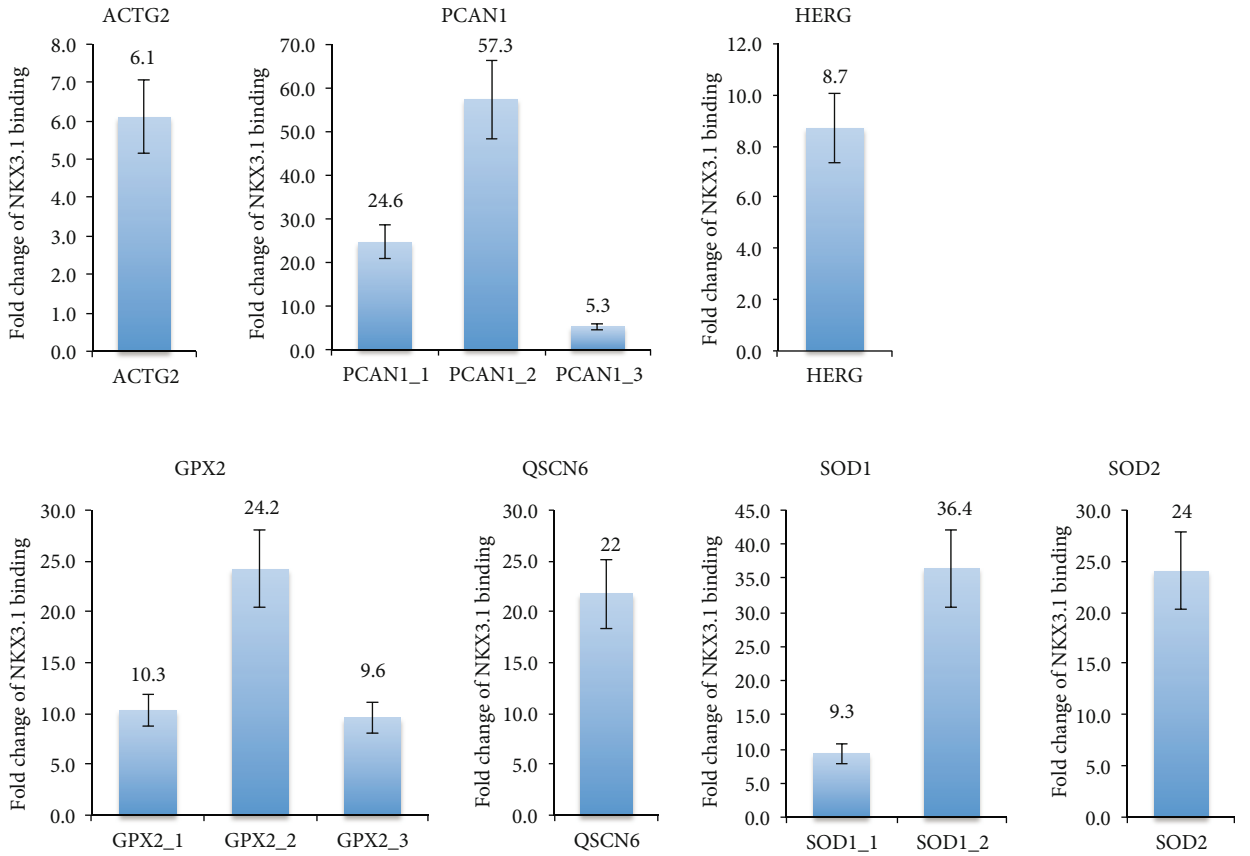

Figure 3. Q-PCR verified that 4 genes are regulated by NKX3.1. Binding ratios (antibody-specific amount of precipitated DNA compared to nonspecific IgG) of NKX3.1 were measured using Q-PCR and calculated for each promoter region of interest by comparing binding ratios. Graphics showed that the relative NKX3.1 binding to proximal promoters increases when androgen is administered to the cells. The ratio was calculated in comparison to untreated binding; $P < 0.01$.

role of AR and NKX3.1 and their effects on prostate cells, as well as providing new therapeutic approaches.

Moreover, in previous studies, researchers developed and utilized arrays of all intergenic DNA, as in the yeast genome, to identify promoters bound and activated during the cell cycle. They also correlated gene expression changes to a network of coexpressed genes of the steps of the cell cycle (Hartemink et al., 2001; Bar-Joseph et al., 2003). These studies were immediately extended to mammalian cells by analysis of the transcription factor E2F (Ren et al., 2000) and further extended to an analysis of c-myc binding (Li et al., 2003). Thus, it is important to appreciate that the initial approach was one of sampling by use of portions of promoters and a fraction of the promoters of the genome. Nevertheless, by careful validation and functional analysis, numerous E2F and myc-regulated genes were identified and correlated with tumor phenotype. Moreover, these studies suggested that more AR-target genes would be determined, and these genes could be observed in a physiological context of coordinate regulation by endogenous levels of AR in living cells. Thus, while this study is focused on prostate cancer and oxidative stress regulation via NKX3.1, it can be extrapolated to other transcriptional factors and/or types of cancers. The methodology proposed here is not limited to prostate cell lines and may describe other tissue-specific genes whose expression might be modulated by androgens via NKX3.1. Whether they exert any transcriptional changes in prostate cell lines, this type of data has to be evaluated in a different setting where expression correlation can be studied. NKX3.1 binding sites regulating transcription might vary in the absence and presence of R1881; perhaps the AR-binding is influenced by recruiting to complexes at different magnitudes, although a different context of promoters needs to be studied.

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References

Abate-Shen C, Shen MM, Gelmann E (2008). Integrating differentiation and cancer: the *Nkx3.1* homeobox gene in prostate organogenesis and carcinogenesis. Differentiation 76: 717–727.

Adamson E, de Belle I, Mittal S, Wang Y, Hayakawa J, Korkmaz K, O'Hagan D, McClelland M, Mercola D (2003). Egr1 signaling in prostate cancer. Cancer Biol Ther 2: 617–622.

Asselin J, Melancon R, Gourdea Y, Labrie F, Bonne C, Raynaud JP (1979). Specific binding of [3H]-methyltrienolone to both progestin and androgen binding components in human benign prostatic hypertrophy (BPH). J Steroid Biochem 10: 483–486.

Bar-Joseph Z, Gerber GK, Lee TI, Rinaldi NJ, Yoo JY, Robert F, Gordon DB, Fraenkel E, Jaakkola TS, Young RA et al. (2003). Computational discovery of gene modules and regulatory networks. Nat Biotechnol 21: 1337–1342.

Beier CP, Schmid C, Gorlia T, Kleinletzenberger C, Beier D, Grauer O, Steinbrecher A, Hirschmann B, Brawanski A, Dietmaier C et al. (2009). RNOP-09: pegylated liposomal doxorubicine and prolonged temozolomide in addition to radiotherapy in newly diagnosed glioblastoma—a phase II study. BMC Cancer 9: 308.

Bowen C, Ju JH, Lee JH, Paull TT, Gelmann EP (2013). Functional activation of ATM by the prostate cancer suppressor NKX3.1. Cell Rep 4: 516–529.

Bowen C, Stuart A, Ju JH, Tuan J, Blonder J, Conrads TP, Veenstra TD, Gelmann EP (2007). NKX3.1 homeodomain protein binds to topoisomerase I and enhances its activity. Cancer Res 67: 455–464.

Carson JA, Fillmore RA, Schwartz RJ, Zimmer WE (2000). The smooth muscle gamma-actin gene promoter is a molecular target for the mouse bagpipe homologue, mNkx3-1, and serum response factor. J Biol Chem 275: 39061–39072.

Cavin Perier R, Junier T, Bucher P (1998). The Eukaryotic Promoter Database EPD. Nucleic Acids Res 26: 353–357.

Coffey RN, Watson RW, O'Neill AJ, McEleny K, Fitzpatrick JM (2002). Androgen-mediated resistance to apoptosis. Prostate 53: 300–309.

Dryhurst D, McMullen B, Fazli L, Rennie PS, Ausio J (2012). Histone H2A.Z prepares the prostate specific antigen (PSA) gene for androgen receptor-mediated transcription and is upregulated in a model of prostate cancer progression. Cancer Lett 315: 38–47.

Erbaykent-Tepedelen B, Karamil S, Gonen-Korkmaz C, Korkmaz KS (2014). DNA damage response (DDR) via NKX3.1 expression in prostate cells. J Steroid Biochem Mol Biol 141: 26–36.

Hartemink AJ, Gifford DK, Jaakkola TS, Young RA (2001). Using graphical models and genomic expression data to statistically validate models of genetic regulatory networks. Pac Symp Biocomput: 422–33.

Hayakawa J, Mittal S, Wang Y, Korkmaz KS, Adamson E, English C, Ohmichi M, McClelland M, Mercola D (2004). Identification of promoters bound by c-Jun/ATF2 during rapid large-scale gene activation following genotoxic stress. Mol Cell 16: 521–535.

Hecht A, Litterst CM, Huber O, Kemler R (1999). Functional characterization of multiple transactivating elements in beta-catenin, some of which interact with the TATA-binding protein in vitro. J Biol Chem 274: 18017–18025.

Korkmaz KS, Korkmaz CG, Ragnhildstveit E, Kizildag S, Pretlow TG, Saatcioglu F (2000a). Full-length cDNA sequence and genomic organization of human NKX3A—alternative forms and regulation by both androgens and estrogens. Gene 260: 25–36.

Korkmaz KS, Korkmaz CG, Ragnhildstveit E, Pretlow TG, Saatcioglu F (2000b). An efficient procedure for cloning hormone-responsive genes from a specific tissue. DNA Cell Biol 19: 499–506.

Lei Q, Jiao J, Xin L, Chang CJ, Wang S, Gao J, Gleave ME, Witte ON, Liu X, Wu H (2006). NKX3.1 stabilizes p53, inhibits AKT activation, and blocks prostate cancer initiation caused by PTEN loss. Cancer Cell 9: 367–378.

Li W, Meyer CA, Liu XS (2005). A hidden Markov model for analyzing ChIP-chip experiments on genome tiling arrays and its application to p53 binding sequences. Bioinformatics 21: i274–282.

Li Z, Van Calcar S, Qu C, Cavenee WK, Zhang MQ, Ren B (2003). A global transcriptional regulatory role for c-Myc in Burkitt's lymphoma cells. Proc Natl Acad Sci U S A 100: 8164–8169.

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: 273–283.

Masuda K, Werner T, Maheshwari S, Frisch M, Oh S, Petrovics G, May K, Srikantan V, Srivastava S, Dobi A (2005). Androgen receptor binding sites identified by a GREF_GATA model. J Mol Biol 353: 763–771.

Mora GR, Prins GS, Mahesh VB (1996). Autoregulation of androgen receptor protein and messenger RNA in rat ventral prostate is protein synthesis dependent. J Steroid Biochem Mol Biol 58: 539–549.

Nelson PS, Clegg N, Arnold H, Ferguson C, Bonham M, White J, Hood L, Lin B (2002). The program of androgen-responsive genes in neoplastic prostate epithelium. Proc Natl Acad Sci U S A 99: 11890–11895.

Ouyang X, DeWeese TL, Nelson WG, Abate-Shen C (2005). Loss-of-function of Nkx3.1 promotes increased oxidative damage in prostate carcinogenesis. Cancer Res 65: 6773–6779.

Ren B, Robert F, Wyrick JJ, Aparicio O, Jennings EG, Simon I, Zeitlinger J, Schreiber J, Hannett N, Kanin E et al. (2000). Genome-wide location and function of DNA binding proteins. Science 290: 2306–2309.

Ripple MO, Henry WF, Rago RP, Wilding G (1997). Prooxidant-antioxidant shift induced by androgen treatment of human prostate carcinoma cells. J Natl Cancer Inst 89: 40–48.

Sato K, Tsuchiya N, Habuchi T, Satoh S, Shimoda N, Kato T (2003). Total cystoprostatectomy in the treatment of locally advanced prostate carcinoma. Aktuelle Urol 34: 259–261.

Schule R, Muller M, Kaltschmidt C, Renkawitz R (1988). Many transcription factors interact synergistically with steroid receptors. Science 242: 1418–1420.

Siegel R, Naishadham D, Jemal A (2013). Cancer statistics, 2013. CA Cancer J Clin 63: 11–30.

Skotheim RI, Korkmaz KS, Klokk TI, Abeler VM, Korkmaz CG, Nesland JM, Fossa SD, Lothe RA, Saatcioglu F (2003). NKX3.1 expression is lost in testicular germ cell tumors. Am J Pathol 163: 2149–2154.

Steadman DJ, Giuffrida D, Gelmann EP (2000). DNA-binding sequence of the human prostate-specific homeodomain protein NKX3.1. Nucleic Acids Res 28: 2389–2395.

Sun H, Palaniswamy SK, Pohar TT, Jin VX, Huang TH, Davuluri RV (2006). MPromDb: an integrated resource for annotation and visualization of mammalian gene promoters and ChIP-chip experimental data. Nucleic Acids Res 34: D98–103.

Suzuki H, Ueda T, Ichikawa T, Ito H (2003). Androgen receptor involvement in the progression of prostate cancer. Endocr Relat Cancer 10: 209–216.

Truss M, Beato M (1993). Steroid hormone receptors: interaction with deoxyribonucleic acid and transcription factors. Endocr Rev 14: 459–479.

Weir HK, Thun MJ, Hankey BF, Ries LA, Howe HL, Wingo PA, Jemal A, Ward E, Anderson RN, Edwards BK (2003). Annual report to the nation on the status of cancer, 1975-2000, featuring the uses of surveillance data for cancer prevention and control. J Natl Cancer Inst 95: 1276–1299.

Zhang H, Muders MH, Li J, Rinaldo F, Tindall DJ, Datta K (2008). Loss of NKX3.1 favors vascular endothelial growth factor-C expression in prostate cancer. Cancer Res 68: 8770–8778.