

# Occurrence of *NKX3.1* C154T Polymorphism in Men with and without Prostate Cancer and Studies of Its Effect on Protein Function<sup>1</sup>

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## ABSTRACT

**NKX3.1, a member of the NK class of homeodomain proteins, is expressed primarily in the adult prostate and has growth suppression and differentiating effects in prostate epithelial cells. A C→T polymorphism at nucleotide 154 (*NKX3.1* C154T) is present in ~11% of healthy men with equal distribution among whites and blacks. In a cohort of 1253 prostate cancer patients and age-matched controls, the presence of the polymorphism was associated with a 1.8-fold risk of having stage C or D prostate cancer or Gleason score  $\geq 7$  (confidence interval, 1.01–3.22). The *NKX3.1* C154T polymorphism codes for a variant protein that contains an arginine-to-cysteine substitution at amino acid 52 (R52C) adjacent to a protein kinase C phosphorylation site at serine 48. Substitution of cysteine for arginine 52 or of alanine for serine 48 (S48A) reduced phosphorylation at serine 48 *in vitro* and *in vivo*. Phosphorylation of wild-type *NKX3.1*, but not of *NKX3.1* R52C or *NKX3.1* S48A, diminished binding *in vitro* to a high-affinity DNA binding sequence. *NKX3.1* also serves as a transcriptional coactivator of serum response factor. Treatment of cells with 12-*O*-tetradecanoylphorbol-13-acetate to phosphorylate *NKX3.1* had no effect on *NKX3.1* coactivation of serum response factor. Neither the R52C nor the S48A substitution affected serum response factor coactivation by *NKX3.1*. We conclude that the polymorphic *NKX3.1* allele codes for a variant protein with altered DNA binding activity that may affect prostate cancer risk.**

## INTRODUCTION

Prostate cancer is a neoplasm with a variable natural history that ranges from indolent to aggressive. Low-grade or early-stage disease may have little impact on survival. However, patients with advanced stages or higher histological grades suffer substantial disease-related mortality (1). The occurrence of prostate cancer is influenced to a substantial degree by genetic factors (2, 3). Genetic determinants may affect individual risk for aggressive prostate cancer and, therefore, mortality from prostate cancer. For example, a polymorphic region in the androgen receptor gene affects the incidence of aggressive prostate cancer (4, 5).

*NKX3.1* is an androgen-regulated NK-class homeobox gene with expression in adult mice and humans localized primarily in the prostate (6–9). The *NKX3.1* gene has been conserved during evolution; the murine and human proteins share 63% amino acid identity. The human *NKX3.1* has been mapped to chromosome 8p21 (10), a locus frequently deleted in prostate cancer (11–13). However, no tumor-specific mutations of the *NKX3.1* protein-coding region have been identified by genetic analysis of human prostate cancer samples (10).

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Nevertheless, loss of *NKX3.1* expression was found in 6–15% of early-stage prostate cancer, 22% of locally advanced disease, 34% of hormone-refractory localized prostate cancer, and 78% of metastases (14). Decreased expression of *NKX3.1* may have a role in prostate cancer pathogenesis because heterozygous *Nkx3.1* gene-targeted mice displayed a phenotype of prostatic hyperplasia, suggesting that *NKX3.1* haploinsufficiency may be dominant.

In the course of analyzing tumor samples for *NKX3.1* mutations, we found a C→T polymorphism at nucleotide 154 (C154T) that coded for a variant protein with a substitution of cysteine for arginine at amino acid 52 (R52C) of *NKX3.1* (10). The polymorphism lay NH<sub>2</sub>-terminal to the homeodomain in a region of the protein that was not conserved between mouse and human. We have determined the frequency of the polymorphism in a population of healthy men and examined its role as a possible risk factor for prostate cancer. We also show that the amino acid change coded by the polymorphism alters *in vitro* and *in vivo* properties of the protein.

## MATERIALS AND METHODS

**Plasmid Construction.** Plasmids expressing full-length wild-type or polymorphic *NKX3.1* fused to maltose-binding protein were generated as described previously (15). A plasmid encoding amino acids 1–184 (nucleotides 1–581) of wild-type *NKX3.1* with an NH<sub>2</sub>-terminal FLAG epitope was constructed. *NKX3.1* point mutants were generated using a Quikchange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Mutant *NKX3.1* cDNAs were fully sequenced to confirm the presence of mutations and to ensure that no additional mutations were introduced.

**Bacterial Expression and Purification of *NKX3.1* Fusion Proteins.** Plasmids expressing wild-type or *NKX3.1* R52C were used to transform competent *Escherichia coli* strain BL21. The proteins were expressed and purified as described previously, using an amylose column that was eluted with 10 mM maltose (New England Biolabs, Beverly, MA; Ref. 15).

***In Vivo* Phosphorylation and Immunoprecipitation.** For labeling of exogenous *NKX3.1*, TSU-Pr1 or LNCaP cells were plated on a 6-cm dish in DMEM containing 5% fetal bovine serum (Life Technologies, Inc., Rockville, MD). At ~90% confluence, cells were transfected with 10  $\mu$ g of wild-type or polymorphic *NKX3.1* expression vector or empty vector, using Lipofectamine 2000 according to the manufacturer's protocol (Life Technologies, Inc.). The *NKX3.1* constructs contained cDNA for expression of amino acids 1–184, including the NH<sub>2</sub> terminus and homeodomain of *NKX3.1*, because our data and those of others had shown that under the control of a cytomegalovirus promoter, the COOH-terminal truncated protein is expressed at higher levels than the wild-type protein (16).

Forty-eight h post-transfection, cells were labeled with 1 mCi/ml [<sup>32</sup>P]P<sub>i</sub> in carrier-free HCl (Amersham Pharmacia Biotech, Piscataway, NJ) for 3–4 h in phosphate-free DMEM containing 5% dialyzed fetal bovine serum (Life Technologies, Inc.). Labeling of endogenous *NKX3.1* was done in LNCaP cells treated with 10 nM methyltrienolone (R1881; DuPont, Boston, MA). Cells were then treated with 100 nM TPA<sup>3</sup> (Sigma, St. Louis, MO) for 30 min before cell lysis. Labeled *NKX3.1* was immunoprecipitated with either 1.5  $\mu$ g of

<sup>3</sup> The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; SRF, serum response factor; SMGA, smooth muscle  $\gamma$ -actin; FAM, 6-carboxyfluorescein; TET, 6-carboxy-4,7,2',7'-tetrachlorofluorescein; TAMRA, 6-carboxy-*N,N,N',N'*-tetramethylrhodamine.

anti-NKX3.1 polyclonal antiserum or 20  $\mu\text{g}$  of anti-FLAG M2 antibody (Stratagene). Immunoprecipitates were electrophoresed on denaturing 10–20% gradient polyacrylamide gels followed by gel drying and autoradiography for visualization of radiolabeled proteins. Western blot analysis to determine protein levels was performed as described previously (14).

**Phosphoamino Acid Analysis of NKX3.1.** Labeled proteins were excised and eluted from polyacrylamide gels. The eluted protein was digested with 0.15 mg/ml trypsin overnight at 37°C, followed by hydrolysis with 1 ml of 6 N HCl at 105°C for 1 h. The HCl was removed by lyophilization, and the pellet was washed with 1 ml of H<sub>2</sub>O and dried. Phosphoamino acids were separated by one-dimensional thin-layer electrophoresis as described previously (17). The identity of *in vivo* phosphorylated amino acids was determined by autoradiography followed by comparison of the autoradiogram with phosphoamino acid standards.

**In Vitro Phosphorylation.** Synthetic peptides (30  $\mu\text{g}$ ) obtained from Research Genetics, Inc. (Huntsville, AL) or purified fusion proteins (200 ng) were incubated at 30°C for 30 min with 10 ng of a purified protein kinase C  $\alpha$ ,  $\beta$ , and  $\gamma$  isoform mixture (Upstate Biotechnology, Lake Placid, NY) in a buffer containing 20 mM HEPES (pH 7.4), 10 mM MgCl<sub>2</sub>, 0.2 mM EGTA, 5 mM DTT, 0.5 mM CaCl<sub>2</sub>, 100  $\mu\text{g}/\text{ml}$  phosphatidylserine, 50  $\mu\text{M}$  ATP, and 0.11  $\mu\text{Ci}$  of [ $\gamma$ -<sup>32</sup>P]ATP.

**Electrophoretic Mobility Shift Assay.** Gel shift assays were performed as described previously with modifications (15). Double-stranded DNA representing a consensus NKX3.1 binding site had the sequence 5'-GTATATAAGTAGTTG-3' (15).

**Transcription Assay.** CV-1 fibroblasts were maintained in Modified Improved MEM (Life Technologies, Inc.) supplemented with 5% fetal bovine serum. Cells were plated at  $\sim 1\text{--}2 \times 10^5$  cells/well in 12-well plates. Cells were transfected 24 h after plating, using Lipofectamine Plus according to the manufacturer's protocol (Life Technologies, Inc.). Each transfection reaction contained either 0.25  $\mu\text{g}$  of SMGA reporter plasmid (a gift from Warren Zimmer, University of South Alabama (Mobile, AL); Ref. 16) or 0.2  $\mu\text{g}$  of various NKX3.1 expression plasmids. SRF expression plasmid (0.5  $\mu\text{g}$ ; a gift from Ron Prywes, Columbia University, New York, NY; Ref. 18) was used as indicated. Total transfected DNA was always kept the same and balanced to 0.5  $\mu\text{g}$  with empty vector. Cells were lysed 48 h after transfection, and the lysate was assayed for firefly luciferase activities with Dual Luciferase Reporter Assay Reagents (Promega, Madison, WI).

**TaqMan Assay.** The TaqMan allelic discrimination assay (19) was used to determine the frequency of the polymorphism at nucleotide 154 in prostate DNA samples. Genomic DNA was isolated using the Easy DNA Genomic DNA Isolation Kit (Invitrogen, Carlsbad, CA). The probe used to detect the wild-type codon was 5'-CAGAGACAGCGACCCGG-3', and the probe used to detect the polymorphic codon was 5'-CAGAGACAGTGCACCCGGAGC-3'. The wild-type probe contained a 5'-FAM reporter dye, whereas the polymorphic probe had a 5'-TET reporter dye. Both probes had a 3'-TAMRA quencher dye. Probes used for allelic discrimination were synthesized by Biosearch Technologies, Inc. (Novato, CA). The forward primer used for PCR was 5'-CGCAGCGCAAGGC-3', and the reverse primer was 5'-GGTGCTCAGCTGGTCTCT-3' (Life Technologies, Inc., Rockville, MD). TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) was used in the PCR reaction according to the manufacturer's protocol. DNA (100 ng), primers (900 nM each), and probe (100 nM FAM-tagged or 200 nM TET-tagged) were added to the TaqMan Universal PCR Master Mix in a total volume of 50  $\mu\text{L}$ . PCR was carried out on an ABI Prism 7700 Sequence Detection System (Applied Biosystems), using the following program: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 62°C for 1 min. Allelic discrimination analysis was performed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). When necessary, samples that contained the C154T polymorphism were confirmed by *HhaI* restriction digestion of PCR-amplified DNA. C154T abrogates a *HhaI* restriction endonuclease recognition site in the PCR transcript. The wild-type transcript was digested to 50-, 48-, and 21-nucleotide fragments, and the polymorphic allele to 98- and 21-nucleotide fragments.

**Population Prevalence of NKX3.1 (C154T).** For the purpose of determining the frequency of *NKX3.1* (C154T) in different racial groups, *NKX3.1* genotype was assessed in a cohort of healthy male controls ( $n = 246$ ; age range, 40–79 years) residing in Detroit, Michigan, or in 10 counties in New Jersey who had participated in a population-based case-control study (20).

DNA was extracted from peripheral blood and assessed for *NKX3.1* genotype. Blood samples were obtained after informed consent. Cases from this case-control study were not included in the analysis.

**Physicians Health Study Population.** Blood samples were obtained in 1982 from 14,916 men enrolled in the Physicians Health Study. Follow-up questionnaires were completed by 99% of the men through 1995, and follow-up for vital status was 100%. Whenever prostate cancer was diagnosed in the cohort, we sought permission to obtain the medical records to determine stage at diagnosis, tumor grade, and Gleason score. If pathological staging was not available, the case was considered of indeterminate stage unless metastases were clinically evident. We categorized cases as high stage/grade if they were diagnosed at stages C or D, had a Gleason score  $\geq 7$ , or had poor histological differentiation (4). We selected one or two controls at random for each case among the men who returned a blood specimen. Controls were men who had not undergone a radical prostatectomy, had not been diagnosed with prostate cancer at the time of the case diagnosis, and were matched by age and smoking status. DNA was extracted from peripheral blood and sent to E. G. for assay; all assays were performed blinded to case-control status. Samples from 558 cases and 695 controls were assayed. We calculated odds ratios as estimates for the relative risks and 95% confidence intervals from logistic regression models (21), controlling for the matching factors.

## RESULTS

**Occurrence of NKX3.1 R52C Variant in Prostate Cancer Patients and Healthy Men.** To determine the frequency of the *NKX3.1* C154T polymorphism in the population, we tested DNA from healthy American white and black men. Prostate cancer incidence is substantially higher in black Americans than in whites, and prostate cancer deaths among blacks also exceed the rate in whites (22–24). We therefore wanted to determine whether there were racial differences in the occurrence of *NKX3.1* R52C. We analyzed *NKX3.1* genotype in a cohort of 246 healthy men. Overall, 11% of men in the study population were found to carry the *NKX3.1* R52C polymorphism. There was a no statistically significant difference in *NKX3.1* genotype distribution between the groups of white and black men (Table 1).

To ask whether *NKX3.1* genotype influenced prostate cancer risk, we analyzed a nested case-control study of 558 men with prostate cancer from the Physicians Health Study and 695 age-matched controls (4). The results for the total study population (Table 2) showed no statistically significant difference between the two groups. When we analyzed only men who presented with aggressive prostate cancer defined as stage C or D or Gleason score  $\geq 7$ , we found a statistically significant increase in the frequency of *NKX3.1* R52C among the cases (relative risk, 1.8; confidence interval, 1.01–3.22). No significant differences between cases and controls were found for nonaggressive cancers or for those men with unknown stage and grade. Because there appeared to be an effect of the polymorphic allele on prostate cancer risk, we sought to investigate whether the variant protein differed in activity from its wild-type counterpart.

**Wild-Type and Variant NKX3.1 Are Differentially Phosphorylated by Protein Kinase C.** The software program Phosphobase v2.0 was used to analyze the *NKX3.1* amino acid sequence for possible phosphorylation sites (25). Three consensus sites were identified at serine 48 (44-GRTSSQRQR-52), threonine 117 (115-RLPQTPKQP-123), and threonine 179 (175-RRYKTKRKRQ-184). Serine 48 was a candidate phosphorylation site for calmodulin kinase II, protein kinase

Table 1 *NKX3.1* genotype in white and black American men

|       | <i>NKX3.1</i> (genotype nt 154), n (%) |     |     | Total |
|-------|--|-----|-----|-------|
|       | C/C                                    | C/T | T/T |       |
| White | 116 (88%)                              | 15  | 1   | 132   |
| Black | 103 (90%)                              | 9   | 2   | 114   |
| Total | 219 (89%)                              | 24  | 3   | 246   |

A, and protein kinase C. This site was of interest because it is located in close proximity to the NKX3.1 polymorphism at amino acid 52. Previously, Zannini *et al.* (26) showed that Nkx2.1 could be phosphorylated by protein kinase C. Wild-type NKX3.1 fused with maltose-binding protein was phosphorylated *in vitro* by protein kinase C (Fig. 1A). In addition to NKX3.1 phosphorylation, a minor level of protein kinase C autophosphorylation was present, represented by the 80-kDa band. Phosphobase v2.0 also identified four consensus protein kinase C phosphorylation sites in the amino acid sequence of the maltose-binding protein affinity tag. However, protein kinase C did not phosphorylate maltose-binding protein alone, suggesting that phosphorylation of the fusion protein was specific for the NKX3.1

Table 2 Relative risk of prostate cancer according to the CGC→TGC polymorphism in NKX3.1

| NKX3.1 genotype                         | No. cases (%) | No. controls (%) | RR <sup>a</sup> | 95% CI     |
|---|---------------|------------------|-----------------|------------|
| <b>Overall cancer</b>                   |               |                  |                 |            |
| CC                                      | 499 (89.4)    | 637 (91.7)       | 1.00            | Reference  |
| CT                                      | 57 (10.2)     | 55 (7.9)         | 1.32            | 0.90–1.95  |
| TT                                      | 2 (0.4)       | 3 (0.4)          | 0.85            | 0.14–5.11  |
| CT + TT                                 |               |                  | 1.30            | 0.89–1.90  |
| <b>Nonaggressive cancer<sup>b</sup></b> |               |                  |                 |            |
| CC                                      | 234 (90.4)    | 285 (89.6)       | 1.00            | Reference  |
| CT                                      | 25 (9.7)      | 31 (9.8)         | 0.98            | 0.56–1.71  |
| TT                                      | 0             | 2 (0.6)          | 0               |            |
| CT + TT                                 |               |                  | 0.92            | 0.53–1.60  |
| <b>Aggressive cancer<sup>b</sup></b>    |               |                  |                 |            |
| CC                                      | 229 (88.8)    | 313 (93.4)       | 1.00            | Reference  |
| CT                                      | 27 (10.5)     | 21 (6.3)         | 1.76            | 0.97–3.19  |
| TT                                      | 2 (0.8)       | 1 (0.3)          | 2.73            | 0.25–30.33 |
| CT + TT                                 |               |                  | 1.80            | 1.01–3.22  |
| <b>Unknown aggressiveness</b>           |               |                  |                 |            |
| CC                                      | 36 (87.8)     | 39 (92.9)        | 1.00            | Reference  |
| CT                                      | 5 (12.2)      | 3 (7.1)          | 1.81            | 0.40–8.10  |
| TT                                      | 0             | 0                | 0               |            |
| CT + TT                                 |               |                  | 1.81            | 0.40–8.10  |

<sup>a</sup> RR, relative risk; CI, confidence interval.

<sup>b</sup> Aggressive cancers were those cases identified at presentation as stages C or D or Gleason score  $\geq 7$ .

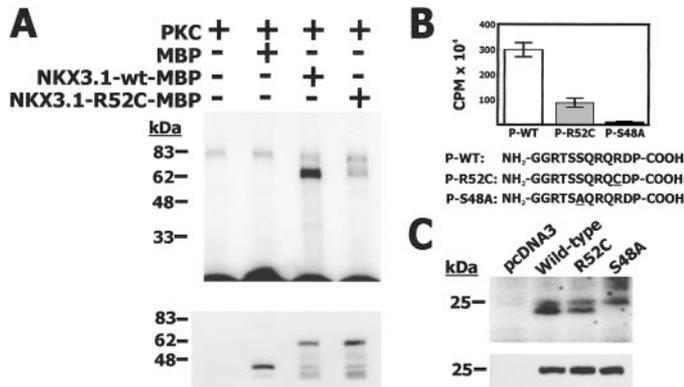


Fig. 1. Protein kinase C preferentially phosphorylates wild-type NKX3.1. A, purified maltose-binding protein (MBP) or NKX3.1 fusion proteins (200 ng) were used as substrates in kinase reactions with protein kinase C (10 ng) and 0.11  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP. After the kinase reactions, samples were electrophoresed on a denaturing 10–20% gradient polyacrylamide gel. Phosphorylated proteins were identified by autoradiography (top). Western blotting with rabbit antiserum to maltose-binding protein (5  $\mu$ g) was used to control for protein loading (bottom). B, peptides (30  $\mu$ g) representing amino acids 43–54 of wild-type (P-WT), R52C (P-R52C), or S48A (P-S48A) NKX3.1 were used in an *in vitro* kinase assay with 10 ng of protein kinase C and 0.11  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP. After the kinase reaction, samples were transferred to phosphocellulose discs and washed, and the incorporated radioactivity was measured as cpm by liquid scintillation counting. Amino acid sequences of the peptides are shown below the graph. C, LNCaP cells were transfected with vectors expressing wild-type, R52C, or S48A NKX3.1 with an NH<sub>2</sub>-terminal FLAG tag. The cells were treated with R1881, and 48 h later, the cells were exposed to 1 mCi/ml [<sup>32</sup>P]P<sub>i</sub>. Cells were lysed, and NKX3.1 was immunoprecipitated with an anti-FLAG antibody. Immunoprecipitates were electrophoresed, and radiolabeled proteins were visualized by autoradiography (top). Western blotting with an anti-FLAG antibody (20  $\mu$ g) was used to control for protein loading (bottom).

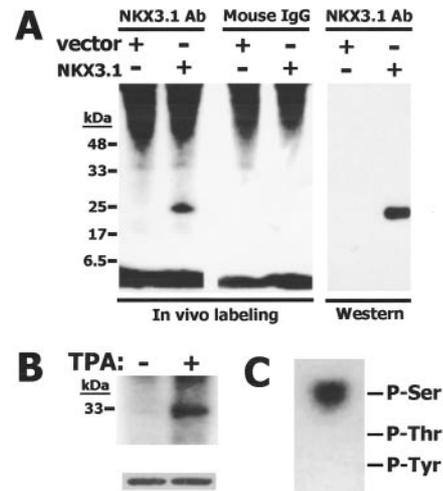


Fig. 2. NKX3.1 is phosphorylated *in vivo*. A, TSU-Pr1 cells were transfected with a wild-type NKX3.1 expression vector or empty vector. Forty-eight h post-transfection, cells were treated with 1 mCi/ml [<sup>32</sup>P]P<sub>i</sub>. Cells were then lysed, and NKX3.1 was immunoprecipitated with an anti-NKX3.1 antiserum or mouse IgG. Immunoprecipitates were electrophoresed by SDS-PAGE, and phosphorylated protein was visualized by autoradiography. The same antibody used for immunoprecipitation of NKX3.1 was used in a Western blot of lysates from cells transfected with either empty vector or NKX3.1 expression vector. B, LNCaP cells were treated with R1881, and 48 h later the cells were exposed to 1 mCi/ml [<sup>32</sup>P]P<sub>i</sub>. Cells were then treated with or without TPA (100 nM) for an additional 30 min. Cells were lysed, NKX3.1 was immunoprecipitated with an anti-NKX3.1 antibody and electrophoresed, and radiolabeled proteins were visualized by autoradiography (top). Western blotting with an anti-NKX3.1 antibody (1.5  $\mu$ g) was used to control for protein loading (bottom). C, endogenous radiolabeled NKX3.1 was excised from a polyacrylamide gel, eluted, and treated with 0.15 mg/ml trypsin. The digested protein was hydrolyzed with 6 N HCl for 1 h at 105°C. Phosphoamino acids were separated by one-dimensional thin-layer electrophoresis. The identity of the phosphorylated amino acids was determined by autoradiography and comparison with phosphoamino acid standards.

moiety. In contrast, protein kinase C phosphorylation of NKX3.1 R52C was noticeably decreased relative to phosphorylation of wild-type NKX3.1 (Fig. 1A). The results of the Western blotting with anti-maltose-binding protein shown in the bottom panel of Fig. 1A indicate that equal amounts of fusion protein with either wild-type or NKX3.1 R52C were present in the reaction.

To confirm that the arginine-to-cysteine variation specifically affected phosphorylation by protein kinase C, synthetic peptide substrates representing amino acids 43–54 of NKX3.1 were used as protein kinase C substrates (Fig. 1B). Relative phosphorylation of peptide from wild-type NKX3.1 was 3-fold higher than for the NKX3.1 R52C peptide. Phosphorylation of peptide with an alanine replacing serine 48 was decreased 33-fold relative to the wild-type sequence. To determine the effects of amino acid alterations on the serine 48 phosphorylation *in vivo*, NKX3.1, NKX3.1 R52C, or NKX3.1 S48A expression vectors with a FLAG tag were used to transfect LNCaP prostate cancer cells that expressed endogenous NKX3.1 as well (7). The level of phosphorylation in the cells transfected with the NKX3.1 R52C variant was half that in the cells transfected with the wild-type protein. The mutation of serine 48 to alanine essentially eliminated *in vivo* phosphorylation of Flag-tagged NKX3.1 (Fig. 1C), providing evidence that serine 48 is a major *in vivo* phosphoacceptor.

TSU-Pr1 cells, which do not express NKX3.1, were used for [<sup>32</sup>P]P<sub>i</sub> labeling of COOH-terminal truncated NKX3.1 (Fig. 2A). Western blotting confirmed that only exogenous NKX3.1 protein was detected in the cells (Fig. 2A). Endogenous full-length NKX3.1 in LNCaP cells was also phosphorylated *in vivo*, and the level of phosphorylation was increased by the presence of 100 nM TPA (Fig. 2B), suggesting that NKX3.1 was phosphorylated *in vivo* by a TPA-induced kinase, such as protein kinase C. Protein kinase C did not affect levels of endog-

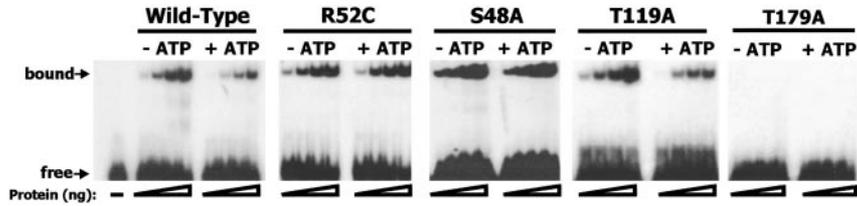


Fig. 3. R52C polymorphism affects phosphorylation-regulated DNA binding. Purified fusion proteins (200 ng) were treated with protein kinase C (10 ng) in the presence or absence of cold ATP. After protein kinase C treatment, the proteins (2, 5, 10, or 25 ng) were used in gel shift assays with a radiolabeled *NKX3.1* consensus DNA binding sequence. Protein-bound DNA was separated from free probe by 8% native PAGE, and the results were visualized by autoradiography.

enous *NKX3.1* as determined by Western blotting (Fig. 2B). Phosphoamino acid analysis of the radiolabeled endogenous protein in LNCaP cells indicated that *NKX3.1* was phosphorylated only at serine (Fig. 2C). Similar phosphoamino acid analysis results were obtained when we labeled exogenous *NKX3.1* in transfected TSU-Pr1 cells (data not shown). Moreover, no phosphorylation was seen in several attempts to label *NKX3.1* S48A *in vivo*.

**Phosphorylation at Serine 48 Regulates *in Vitro* *NKX3.1* DNA Binding.** Recombinant purified *NKX3.1* fusion proteins were treated with protein kinase C in the presence or absence of ATP, and the proteins were included in gel shift assays with a radiolabeled *NKX3.1* high-affinity DNA binding sequence (15). Phosphorylation of wild-type *NKX3.1* decreased the apparent binding affinity of the protein for the consensus sequence by 3-fold relative to the nonphosphorylated protein (Fig. 3). However, the DNA binding of *NKX3.1* R52C was not noticeably altered after treatment with protein kinase C in the presence of ATP. Similarly, *NKX3.1* S48A lost regulation of DNA binding by phosphorylation (Fig. 3). To compare the effects of phosphorylation at two other consensus protein kinase C phosphorylation sites on DNA binding, either threonine 119 or threonine 179 was mutated to alanine. Protein kinase C treatment of *NKX3.1* T119A yielded DNA binding data similar to those for wild-type *NKX3.1* (Fig. 3). Interestingly, the T179A mutation, located in the homeodomain, abrogated *NKX3.1* DNA binding (Fig. 3).

**Effect of Polymorphism on *NKX3.1* Transcriptional Activity.** *NKX3.1* and the heart-specific NK family protein *NKX2.5* have very similar *in vitro* properties. The activities of these proteins are mediated largely by the homeodomains, which are nearly identical in their three major homeodomain helices and coincide at 39 of 60 amino acids. Both proteins bind SRF (16, 27), and *NKX2.5* was shown to bind SRF *via* the homeodomain (27). Because of similarities in their homeodomain primary structure, *NKX3.1* is likely also to bind SRF *via* the homeodomain. To assess *NKX3.1* coactivation of SRF, we used a transcription assay with a reporter construct under control of the SMGA promoter, similar to the transactivation experiment reported by Carson *et al.* (16). We found that human *NKX3.1* can act as a coactivator for SRF activation of transcription from the SMGA promoter, similar to previously published results for murine *Nkx3.1* (16). In general, the presence of *NKX3.1* resulted in a 3–5-fold increase in SMGA promoter activity. We compared full-length wild-type *NKX3.1* expression vector with mutant constructs that coded for *NKX3.1* R52C and *NKX3.1* S48A protein variants. As a control we used an expression construct with *NKX3.1* in reverse orientation that coded for no protein. The three coding constructs had similar levels of *NKX3.1* protein expression and similar levels of SRF coactivation. Treatment of the cultures with 100 nM TPA, which had been shown to cause phosphorylation of *NKX3.1* in culture, had a minimal inhibitory effect on the activity of each construct, but did not differentially affect the coactivation by mutant and wild-type constructs (Table 3).

## DISCUSSION

A common polymorphism in the prostate-specific homeoprotein may have an effect on prostate cancer pathogenesis as risk factor for aggressive disease. A tumor suppressor function of *NKX3.1* has been suggested by studies of gene-targeted mice. Targeted disruption of murine *Nkx3.1* suggested that the gene exerts growth suppression and differentiating effects on prostatic epithelium (9, 28). Importantly, animals heterozygous for loss of *Nkx3.1* demonstrated histological disarray of the prostate and bulbourethral gland, suggesting that haploinsufficiency was dominant. Because the murine gene lacks an amino acid similarity at arginine 52 and lacks the protein kinase C phosphorylation site at serine 48, it is hard to predict the role of this putative regulatory region in the mouse.

We found that the R52C polymorphism occurs with similar frequency among whites and blacks in the United States. Prostate cancer is more common among blacks than whites in the United States, with a higher mortality among blacks than whites (22–24). Therefore, it does not appear that disparities in the frequency of *NKX3.1* C154T contribute to the difference in prostate cancer between the races. Approximately 5–10% of prostate cancer is inherited in a Mendelian fashion that has been traced to at least three susceptibility loci on chromosomes 1, X, and 17 (29–32).

The occurrence of sporadic prostate cancer, however, is likely to be influenced subtly by many genes that affect susceptibility. Much attention has been directed to variations in the polyglutamine tract in the NH<sub>2</sub> terminus of the androgen receptor. Shorter polyglutamine repeat lengths are associated with increased androgen receptor activity and more aggressive prostate cancer (4, 5, 33). Other genetic factors that may have a subtle effect on prostate cancer risk in the general population include the vitamin D receptor (34–36), *CYP17* (37), 5 $\alpha$ -reductase A49T (38), and glutathione *S*-transferase  $\theta$  (39). The *NKX3.1* C154T genotype may be one of those subtle genetic influences on prostate cancer risk, in particular for aggressive disease.

The *NKX3.1* C154T polymorphism appears to affect a region of the protein that can affect DNA binding. The exact role of the region containing amino acids 48–52 has not been determined, but it is clear that the region is important for phosphorylation. Homeoproteins are known to undergo posttranslational modification by phosphorylation. Homeoprotein phosphorylation has been shown to affect protein-protein interactions (40), subcellular localization (41), DNA binding affinity (42), and transcriptional activity (43). Generally, these effects

Table 3 SRF coactivation assay of *NKX3.1* and mutant constructs

| Construct          | Fold SRF coactivation |                 |
|--------------------|-----------------------|-----------------|
|                    | –TPA                  | +TPA            |
| Control            | 1.06                  | NT <sup>a</sup> |
| <i>NKX3.1</i>      | 4.4                   | 3.7             |
| <i>NKX3.1</i> R52C | 5.8                   | 4.6             |
| <i>NKX3.1</i> S48A | 6.8                   | 5.6             |

<sup>a</sup> NT, not tested.

have been attributed to electrostatic repulsion or a conformational change in the protein (44). Members of the NK family of homeoproteins have been shown to undergo phosphorylation. The kinases responsible for phosphorylating NK-class homeoproteins include casein kinase II (43), MST2 kinase (45), extracellular signal-regulated kinase (46), homeodomain-interacting protein kinase (47), protein kinase A (48), and protein kinase C (26).

Although we believe that the cellular activity of NKX3.1 R52C is different from that of the wild type, the precise impact of the polymorphism on NKX3.1 function is unclear. Although relatively little is known about the protein interactions of NKX3.1, the NK family member NKX2.5 has been characterized more extensively and has been shown to interact with DNA and with at least two other transcription factors, GATA-4 and SRF (27, 49, 50). Moreover, the protein-protein interactions of NKX3.1 are mediated by the homeodomain as well (27, 49, 50). It is entirely possible that NKX3.1, like NKX2.5, undergoes multiple interactions that are involved in manifestation of its biological effects. However, the analogy between the two homeoproteins has yet to be proved. It should be remembered that although the two proteins share nearly identical homeodomain sequences, they have very little amino acid identity in the NH<sub>2</sub>- and COOH-terminal regions.

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## REFERENCES

- Albertsen, P. C., Hanley, J. A., Gleason, D. F., and Barry, M. J. Competing risk analysis of men aged 55 to 74 years at diagnosis managed conservatively for clinically localized prostate cancer. *JAMA*, 280: 975–980, 1998.
- Lichtenstein, P., Holm, N. V., Verkasalo, P. K., Iliadou, A., Kaprio, J., Koskenvuo, M., Pukkala, E., Skytthe, A., and Hemminki, K. Environmental and heritable factors in the causation of cancer—analyses of cohorts of twins from Sweden, Denmark, and Finland. *N. Engl. J. Med.*, 343: 78–85, 2000.
- Steinberg, G. D., Carter, B. S., Beaty, T. H., Childs, B., and Walsh, P. C. Family history and the risk of prostate cancer. *Prostate*, 17: 337–347, 1990.
- Giovannucci, E., Stampfer, M. J., Krithivas, K., Brown, M., Brufsky, A., Talcott, J., Hennekens, C. H., and Kantoff, P. W. The CAG repeat within the androgen receptor gene and its relationship to prostate cancer. *Proc. Natl. Acad. Sci., USA*, 94: 3320–3323, 1997.
- Hsing, A. W., Gao, Y. T., Wu, G., Wang, X., Deng, J., Chen, Y. L., Sesterhenn, I. A., Mostofi, F. K., Benichou, J., and Chang, C. Polymorphic CAG and GGN repeat lengths in the androgen receptor gene and prostate cancer risk: a population-based case-control study in China. *Cancer Res.*, 60: 5111–5116, 2000.
- Kim, Y., and Nirenberg, M. *Drosophila* NK-homeobox genes. *Proc. Natl. Acad. Sci. USA*, 86: 7716–7720, 1989.
- He, W. W., Sciovolino, P. J., Wing, J., Augustus, M., Hudson, P., Meissner, P. S., Curtis, R. T., Shell, B. K., Bostwick, D. G., Tindall, D. J., Gelmann, E. P., Abate-Shen, C., and Carter, K. C. A novel human prostate-specific, androgen-regulated homeobox gene (*NKX3.1*) that maps to 8p21, a region frequently deleted in prostate cancer. *Genomics*, 43: 69–77, 1997.
- Prescott, J. L., Blok, L., and Tindall, D. J. Isolation and androgen regulation of the human homeobox cDNA *NKX3.1*. *Prostate*, 35: 71–80, 1998.
- Bhatia-Gaur, R., Donjacour, A. A., Sciovolino, P. J., Kim, M., Desai, N., Norton, C. R., Gridley, T., Cardiff, R. D., Cunha, G. R., Abate-Shen, C., and Shen, M. M. Roles for *NKx3.1* in prostate development and cancer. *Genes Dev.*, 13: 966–977, 1999.
- Voeller, H. J., Augustus, M., Madlike, V., Bova, G. S., Carter, K. C., and Gelmann, E. P. Coding region of *NKX3.1*, prostate-specific homeobox gene on 8p21, is not mutated in human prostate cancers. *Cancer Res.*, 57: 4455–4459, 1997.
- Lundgren, R., Kristofferson, U., Heim, S., Mandahl, N., and Mitelman, F. Multiple structural chromosome rearrangements, including del(7q) and del(10q), in an adenocarcinoma of the prostate. *Cancer Genet. Cytogenet.*, 35: 103–108, 1988.
- Carter, B. S., Ewing, C. M., Ward, W. S., Treiger, B. F., Aalders, T. W., Schalken, J. A., Epstein, J. I., and Isaacs, W. B. Allelic loss of chromosomes 16q and 10q in human prostate cancer. *Proc. Natl. Acad. Sci. USA*, 87: 8751–8755, 1990.
- Phillips, S. M., Morton, D. G., Lee, S. J., Wallace, D. M., and Neoptolemos, J. P. Loss of heterozygosity of the retinoblastoma and adenomatous polyposis susceptibility gene loci and in chromosomes 10p, 10q and 16q in human prostate cancer. *Br. J. Urol.*, 73: 390–395, 1994.
- Bowen, C., Bubendorf, L., Voeller, H. J., Slack, R., Willi, N., Sauter, G., Gasser, T. C., Koivisto, P., Lack, E. E., Kononen, J., Kallioniemi, O. P., and Gelmann, E. P. Loss of *NKX3.1* expression in human prostate cancers correlates with tumor progression. *Cancer Res.*, 60: 6111–6115, 2000.
- Steadman, D. J., Giuffrida, D., and Gelmann, E. P. DNA-binding sequence of the human prostate-specific homeodomain protein *NKX3.1*. *Nucleic Acids Res.*, 28: 2389–2395, 2000.
- Carson, J. A., Fillmore, R. A., Schwartz, R. J., and Zimmer, W. E. 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, 2000.
- Hirano, A. A., Greengard, P., and Hagan, R. L. Protein tyrosine kinase activity and its endogenous substrates in rat brain: a subcellular and regional survey. *J. Neurochem.*, 50: 1447–1455, 1988.
- Prywes, R., Dutta, A., Cromlish, J. A., and Roeder, R. G. Phosphorylation of serum response factor, a factor that binds to the serum response element of the c-FOS enhancer. *Proc. Natl. Acad. Sci. USA*, 85: 7206–7210, 1988.
- Heid, C. A., Stevens, J., Livak, K. J., and Williams, P. M. Real time quantitative PCR. *Genome Res.*, 6: 986–994, 1996.
- Hayes, R. B., Pottern, L. M., Strickler, H., Rabkin, C., Pope, V., Swanson, G. M., Greenberg, R. S., Schoenberg, J. B., Liff, J., Schwartz, A. G., Hoover, R. N., and Fraumeni, J. F., Jr. Sexual behaviour, STDs and risks for prostate cancer. *Br. J. Cancer*, 82: 718–725, 2000.
- Kleinbaum, D. G., Kupper, L. L., and Morgenstern, H. *Epidemiologic Research: Principles and Quantitative Methods*. New York: Van Nostrand Reinhold, 1982.
- Stanford, J. L., Stephenson, R. A., Coyle, L. M., Cerhan, J., Correa, R., Eley, J. W., Gilliland, F., Hankey, B., Kolonel, L. N., Kosary, C. L., Ross, R., Severson, R., and West, D. *Prostate Cancer Trends, 1973–1995*, SARY Program. Bethesda, MD: National Cancer Institute, 1998.
- Feuer, E. J., Merrill, R. M., and Hankey, B. F. Cancer surveillance series: interpreting trends in prostate cancer—part II: cause of death misclassification and the recent rise and fall in prostate cancer mortality. *J. Natl. Cancer Inst. (Bethesda)*, 91: 1025–1032, 1999.
- Hankey, B. F., Feuer, E. J., Clegg, L. X., Hayes, R. B., Legler, J. M., Prorok, P. C., Ries, L. A., Merrill, R. M., and Kaplan, R. S. Cancer surveillance series: interpreting trends in prostate cancer—part I: evidence of the effects of screening in recent prostate cancer incidence, mortality, and survival rates. *J. Natl. Cancer Inst. (Bethesda)*, 91: 1017–1024, 1999.
- Kreepipuu, A., Blom, N., and Brunak, S. PhosphoBase, a database of phosphorylation sites: release 2.0. *Nucleic Acids Res.*, 27: 237–239, 1999.
- Zannini, M., Acebron, A., De Felice, M., Arnone, M. I., Martin-Perez, J., Santisteban, P., and Di Lauro, R. Mapping and functional role of phosphorylation sites in the thyroid transcription factor-1 (TTF-1). *J. Biol. Chem.*, 271: 2249–2254, 1996.
- Chen, C. Y., and Schwartz, R. J. Recruitment of the tinman homolog *Nkx-2.5* by serum response factor activates cardiac  $\alpha$ -actin gene transcription. *Mol. Cell Biol.*, 16: 6372–6384, 1996.
- Schneider, A., Brand, T., Zweigerdt, R., and Arnold, H. Targeted disruption of the *nkx3.1* gene in mice results in morphogenetic defects of minor salivary glands: parallels to glandular duct morphogenesis in prostate. *Mech. Dev.*, 95: 163–174, 2000.
- Carter, B. S., Bova, G. S., Beaty, T. H., Steinberg, G. D., Childs, B., Isaacs, W. B., and Walsh, P. C. Hereditary prostate cancer. Epidemiology and clinical features. *J. Urol.*, 150: 797–802, 1993.
- Smith, J. R., Freije, D., Carpten, J. D., Gronberg, H., Xu, J., Isaacs, S. D., Brownstein, M. J., Bova, G. S., Guo, H., Bujnovszky, P., Nusskern, D. R., Damber, J. E., Bergh, A., Emanuelsson, M., Kallioniemi, O. P., Walker-Daniels, J., Bailey-Wilson, J. E., Beaty, T. H., Meyers, D. A., Walsh, P. C., Collins, F. S., Trent, J. M., and Isaacs, W. B. Major susceptibility locus for prostate cancer on chromosome 1 suggested by a genome-wide search. *Science (Wash. DC)*, 274: 1371–1374, 1996.
- Xu, J., Meyers, D., Freije, D., Isaacs, S., Wiley, K., Nusskern, D., Ewing, C., Wilkens, E., Bujnovszky, P., Bova, G. S., Walsh, P., Isaacs, W., Schleutker, J., Matikainen, M., Tammela, T., Visakorpi, T., Kallioniemi, O. P., Berry, R., Schaid, D., French, A., McDonnell, S., Schroeder, J., Blute, M., Thibodeau, S., and Trent, J. Evidence for a prostate cancer susceptibility locus on the X chromosome. *Nat. Genet.*, 20: 175–179, 1998.
- Tavtigian, S. V., Simard, J., Teng, D. H., Abtin, V., Baumgard, M., Beck, A., Camp, N. J., Carrillo, A. R., Chen, Y., Dayananth, P., Desrochers, M., Dumont, M., Farnham, J. M., Frank, D., Frye, C., Ghaffari, S., Gupte, J. S., Hu, R., Iliev, D., Janecki, T., Kort, E. N., Laitly, K. E., Leavitt, A., Leblanc, G., McArthur-Morrison, J., Pederson, A., Penn, B., Peterson, K. T., Reid, J. E., Richards, S., Schroeder, M., Smith, R., Snyder, S. C., Swedlund, B., Swensen, J., Thomas, A., Tranchant, M., Woodland, A. M., Labrie, F., Skolnick, M. H., Neuhausen, S., Rommens, J., and Cannon-Albright, L. A. A candidate prostate cancer susceptibility gene at chromosome 17p. *Nat. Genet.*, 27: 172–180, 2001.
- Beilin, J., Ball, E. M., Favaloro, J. M., and Zajac, J. D. Effect of the androgen receptor CAG repeat polymorphism on transcriptional activity: specificity in prostate and non-prostate cell lines. *J. Mol. Endocrinol.*, 25: 85–96, 2000.
- Correa-Cerro, L., Berthon, P., Haussler, J., Bochum, S., Drelon, E., Mangin, P., Fournier, G., Paiss, T., Cussenot, O., and Vogel, W. Vitamin D receptor polymorphisms as markers in prostate cancer. *Hum. Genet.*, 105: 281–287, 1999.
- Ingles, S. A., Ross, R. K., Yu, M. C., Irvine, R. A., La Pera, G., Haile, R. W., and Coetzee, G. A. Association of prostate cancer risk with genetic polymorphisms in vitamin D receptor and androgen receptor. *J. Natl. Cancer Inst. (Bethesda)*, 89: 166–170, 1997.
- Taylor, J. A., Hirvonen, A., Watson, M., Pittman, G., Mohler, J. L., and Bell, D. A. Association of prostate cancer with vitamin D receptor gene polymorphism. *Cancer Res.*, 56: 4108–4110, 1996.

37. Gsur, A., Bernhofer, G., Hinteregger, S., Haidinger, G., Schatzl, G., Madersbacher, S., Marberger, M., Vutuc, C., and Micksche, M. A polymorphism in the *CYP17* gene is associated with prostate cancer risk. *Int. J. Cancer*, *87*: 434–437, 2000.
38. Makridakis, N. M., Ross, R. K., Pike, M. C., Crocitto, L. E., Kolonel, L. N., Pearce, C. L., Henderson, B. E., and Reichardt, J. K. Association of mis-sense substitution in *SRD5A2* gene with prostate cancer in African-American and Hispanic men in Los Angeles, USA. *Lancet*, *354*: 975–978, 1999.
39. Rebbeck, T. R., Walker, A. H., Jaffe, J. M., White, D. L., Wein, A. J., and Malkowicz, S. B. Glutathione *S*-transferase- $\mu$  (GSTM1) and - $\theta$  (GSTT1) genotypes in the etiology of prostate cancer. *Cancer Epidemiol. Biomark. Prev.*, *8*: 283–287, 1999.
40. Li, C., and Manley, J. L. Allosteric regulation of even-skipped repression activity by phosphorylation. *Mol. Cell*, *3*: 77–86, 1999.
41. Moede, T., Leibiger, B., Pour, H. G., Berggren, P., and Leibiger, I. B. Identification of a nuclear localization signal, RRMKWKK, in the homeodomain transcription factor PDX-1. *FEBS Lett.*, *461*: 229–234, 1999.
42. Bourbon, H. M., Martin-Blanco, E., Rosen, D., and Kornberg, T. B. Phosphorylation of the *Drosophila* engrailed protein at a site outside its homeodomain enhances DNA binding. *J. Biol. Chem.*, *270*: 11130–11139, 1995.
43. Kasahara, H., and Izumo, S. Identification of the *in vivo* casein kinase II phosphorylation site within the homeodomain of the cardiac tissue-specifying homeobox gene product Csx/Nkx2.5. *Mol. Cell. Biol.*, *19*: 526–536, 1999.
44. Karin, M. Signal transduction from the cell surface to the nucleus through the phosphorylation of transcription factors. *Curr. Opin. Cell Biol.*, *6*: 415–424, 1994.
45. Aurisicchio, L., Di Lauro, R., and Zannini, M. Identification of the thyroid transcription factor-1 as a target for rat MST2 kinase. *J. Biol. Chem.*, *273*: 1477–1482, 1998.
46. Missero, C., Pirro, M. T., and Di Lauro, R. Multiple ras downstream pathways mediate functional repression of the homeobox gene product TTF-1. *Mol. Cell. Biol.*, *20*: 2783–2793, 2000.
47. Kim, Y. H., Choi, C. Y., Lee, S. J., Conti, M. A., and Kim, Y. Homeodomain-interacting protein kinases, a novel family of co-repressors for homeodomain transcription factors. *J. Biol. Chem.*, *273*: 25875–25879, 1998.
48. Yan, C., and Whitsett, J. A. Protein kinase A activation of the surfactant protein B gene is mediated by phosphorylation of thyroid transcription factor 1. *J. Biol. Chem.*, *272*: 17327–17332, 1997.
49. Sepulveda, J. L., Belaguli, N., Nigam, V., Chen, C. Y., Nemer, M., and Schwartz, R. J. GATA-4 and Nkx-2.5 coactivate Nkx-2 DNA binding targets: role for regulating early cardiac gene expression. *Mol. Cell. Biol.*, *18*: 3405–3415, 1998.
50. Chen, C. Y., Croissant, J., Majesky, M., Topouzis, S., McQuinn, T., Frankovsky, M. J., and Schwartz, R. J. Activation of the cardiac  $\alpha$ -actin promoter depends upon serum response factor, Tinman homologue, Nkx-2.5, and intact serum response elements. *Dev. Genet.*, *19*: 119–130, 1996.