ISSN 1672-9145 **Acta Biochimica et Biophysica Sinica** 2005, **37**(11): 773–778 **CN** 31-1940/Q

Identification of a Positive *Cis*-Element Upstream of Human *NKX3.1* Gene

An-Li JIANG, Peng-Ju ZHANG, Xiao-Yan HU, Wei-Wen CHEN, Feng KONG, Zhi-Fang LIU, Hui-Qing YUAN, and Jian-Ye ZHANG*

Department of Biochemistry, Medical School of Shandong University, Jinan 250012, China

Abstract *NKX3.1* is a prostate-specific homeobox gene related to prostate development and prostate cancer. In this work, we aimed to identify precisely the functional *cis*-element in the 197 bp region (from –1032 to –836 bp) of the *NKX3.1* promoter (from –1032 to +8 bp), which was previously identified to present positive regulatory activity on *NKX3.1* expression, by deletion mutagenesis analysis and electrophoretic mobility shift assay (EMSA). A 16 bp positive *cis*-element located between –920 and –905 bp upstream of the *NKX3.1* gene was identified by deletion mutation analysis and proved to be a functional positive *cis*-element by EMSA. It will be important to further study the functions and regulatory mechanisms of this positive *cis*-element in *NKX3.1* gene expression.

Key words human *NKX3.1*; *cis*-acting element; positive regulation; mutagenesis

NKX3.1 is an androgen regulated prostate-specific homeobox gene [1,2] that is thought to play important roles in prostate development and carcinogenesis [3–5]. The strong association between *NKX3.1* and prostate development and prostate cancer makes this gene an attractive molecular target for further study. It provides an excellent model to explore the relationship between embryogenesis and oncogenesis. So far, little is known about the regulatory mechanisms of *NKX3.1* gene expression or the relevant regulatory elements and factors. To study its transcriptional regulation, a 1040 bp promoter (from –1032 to +8 bp) of the human *NKX*3.1 gene was cloned upstream of the luciferase reporter gene in pGL_3-basic plasmid and a 197 bp region extending from –1032 to –836 bp upstream of the *NKX3.1* gene was identified presenting positive regulatory activity for luciferase reporter expression in our previous experiments [6].

In our present study, we have identified a functional positive *cis*-element between –1032 and –836 bp upstream of the *NKX3.1* gene; it plays an important role in upregulating *NKX3.1* gene transcription. It will be important to further study the functions and regulatory mechanisms of this positive element in *NKX3.1* gene expression.

Materials and Methods

Construction of luciferase reporter plasmids

pGL_3-1040 containing the 1040 bp *NKX3.1* promoter was constructed as previously described [6] and its 5′ deletion mutants were generated by the polymerase chain reaction (PCR) method using pGL_3-1040 as the template. The primers used in PCR were one lower primer PF+8 and 10 upper primers, PR-1032, PD-999, PD-966, PD-945, PD-936, PD-920, PD-904, PD-883, PD-869 and PD-835 (**Table 1**). The PCR was conducted at 94 ºC for 2 min followed by 35 cycles at 94 ºC for 30 s, 60 ºC for 30 s, and 72 ºC for 1 min. The PCR products were separated by 1% agarose gel electrophoresis and purified with QIAquick gel extraction kit (QIAgene, Ontario, Canada) cut with *Xho*I and *Sac*I, and inserted into the pGL_3-basic vector digested with *Xho*I and *Sac*I to generate 10 constructs that were designated pGL_3-1040, pGL_3-1007, pGL_3-974, pGL_3-953, pGL_3-944, pGL_3-928, pGL_3-912, pGL_3-891, pGL_3-877 and pGL_3-843. All were confirmed by restriction enzyme digestion and DNA sequencing and tested by reporter assay.

Received: June 27, 2005 Accepted: August 11, 2005

This work was supported by the grants from the National Natural Science Foundation of China (No. 30470952) and the Natural Science Foundation of Shandong Province (No. Y2004C26)

*Corresponding author: Tel, 86-531-88382092; E-mail, zhjy@sdu.edu.cn

DOI: 10.1111/j.1745-7270.2005.00104.x

Table 1 Primers used in the polymerase chain reaction method for mutagenesis analysis

Primer	Sequence (5′→3′)
PF+8	GGCCTCGAGCGCACCGCTTTCACTTTC
PR-1032	CGCGAGCTCAAGGCAGGAGGATCACTTG
PD-999	CGCGAGCTCAGGCTGCAGTGAGTTATG
PD-966	CGCGAGCTCTTCCAGCCTGGGCGGCAGA
PD-945	CGCGAGCTCAGACCCTGTCCGTAAAAA
PD-936	CGCGAGCTCCCGTAAAAAAAAGAAGAG
PD-920	CGCGAGCTCAGAAAAGACAAGGAAAGA
PD-904	CGCGAGCTCGAAAATACTTCCATAATC
PD-883	CGCGAGCTCGTTCCACTTTCGTCTGTT
PD-869	CGCGAGCTCCTGTTGTCACGGTACCGT
PD-835	CGCGAGCTCACCGGTTGGACCAATCTG

Restriction enzyme sites are underlined.

A 16 bp positive *cis*-element from –920 to –905 bp was identified by 5′ deletion mutation analysis, as described above. To confirm its positive regulatory activity, the 16 bp *cis*-element was inserted upstream of the heterogeneous promoters to generate the plasmid of the 16 bp *cis*-element heterogeneous promoter-luciferase reporter gene. The sequence of the synthetic 16 bp *cis*-element was 5′-TCGAGTTTCCTTGTCTTTTCTGAGCT-3′ (sense strand), and 3′-CAAAGGAACAGAAAAGAC-5′(antisense strand). In the sense strand, a 5′ overhang *Xho*I and a 3′ overhang *Sac*I sites (underlined) at the 5′ and 3′ ends, respectively, were produced when the two strands were annealed.

The double-stranded 16 bp fragment was generated by annealing the equimolar complementary oligonucleotides in sterilized water at 95 ºC for 10 min, then the reaction mixture was slowly cooled to room temperature. The product was inserted upstream of the maspin gene promoter in pGL_3-maspin (a gift from Dr. Charles Y. F. YOUNG, Mayo Clinic, Rochester, USA), upstream of the SV40 gene promoter in pGL_3-promoter plasmid (Promega, Madison, USA), or upstream of the luciferase gene reporter in pGL_3-basic plasmid, which was used as a promoter-less control. The constructs were designated pGL_3-M-A, pGL_3-P-A and pGL_3-B-A, respectively. All constructs were confirmed by DNA sequencing analysis, then were tested by transient transfection and dual-luciferase reporter assay described following.

Cell culture and transient transfection

The human prostate cancer cell line LNCaP was obtained from the American Type Culture Collection (ATCC, Manassas, USA). The cell line was established from a lymph node metastasis of a prostate cancer patient. It expresses the androgen receptor gene and the *NKX3.1* gene. The cells were routinely grown at 37 ºC in a 5% CO_2 incubator with RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml ampicillin and 100 U/ml streptomycin.

LNCaP cells were seeded in 24-well plates to approximately 90% confluence and transiently transfected by pGL_3-construct using Lipofectamine 2000 (Invitrogen, Carlsbad, USA). Each well included 2 μl Lipofectamine 2000, 1.0 μg pGL_3-construct (10 constructs of pGL_3-*NKX3.1* promoter, pGL_3-M-A, pGL_3-P-A, or pGL_3-B-A), 0.04 μg internal control vector pRL-TK and 500 μl RPMI 1640 medium without serum or antibiotics. Cells were harvested for dual-luciferase activity assay after 48 h of transfection.

Dual-luciferase reporter assay

The activities of firefly luciferase in pGL_3 and Renilla luciferase in pRL-TK were determined by the dual-luciferase reporter assay following the protocol supplied by Promega. The cells were rinsed with phosphate-buffered saline, then harvested using 1×passive lysis buffer. Twenty microliters of cell lysate was transferred into the luminometer tube containing 100 μl luciferase assay reagent II. The firefly luciferase activity (M1) was measured first, then the Renilla luciferase activity (M2) was determined after the addition of 100 μl Stop & Glo reagent (Promega). M1/M2 was taken as the relative activity of the pGL_3-constructs.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from LNCaP cells using a nuclear extraction kit (Active Motif, Carlsbad, USA) according to the manufacturer's instructions. The sense strand of the 16 bp positive *cis*-element and its mutants with four-base substitution are shown in **Table 2**. Equal amounts of sense and antisense oligonucleotides were mixed and annealed in a buffer (10 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA) by heating to 95 ºC for 5 min and cooling slowly to room temperature.

The double-stranded 16 bp *cis*-element was labeled with digoxigenin (DIG). Binding reactions were performed for 20 min on ice in a 20 μl mixture containing 0.2% (*W*/*V*) Tween-20, 1 mM EDTA, 1 mM dithiothreitol, 30 mM KCl, 20 mM HEPES (pH 7.6), 1 μg of poly(dI-dC), 0.1 μg of poly(*L*-Lys), 10 μg of nuclear extract and 0.8 ng of DIG-labeled 16 bp *cis*-element. For the competition experiment, unlabeled 16 bp *cis*-element or its mutants in 150-fold ex-

Table 2 Sequences of the probes used in electrophoretic mobility shift assay

Probe	Sequence of the sense strand
16bp-A	TTTCCTTGTCTTTTCT
16bp-m1	GGGACTTGTCTTTTCT
16bp-m2	TTTCAGGTTCTTTTCT
16bp-m3	TTTCCTTGGAGGTTCT
16bp-m4	TTTCCTTGTCTTGGAG
ARE	TGCAGAACAGCAAGTGCTAGC

Underlined sequences are the mutated bases of 16bp-A. ARE, unlabeled random sequence.

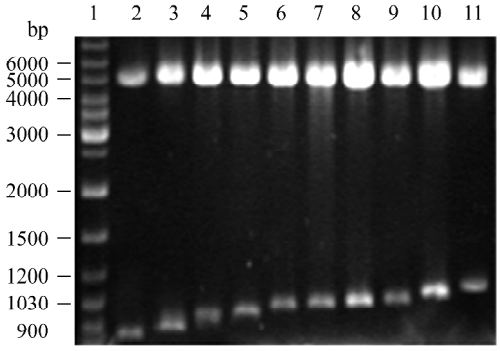

Fig. 1 Identification of deletion mutants by restriction enzyme digestion

1, DNA marker; 2, pGL_3-843 cut with *Sac*I and *Xho*I to produce an 843 bp fragment; 3, pGL_3-877 cut with *Sac*I and *Xho*I to produce an 877 bp fragment; 4, pGL_3-891 cut with *Sac*I and *Xho*I to produce an 891 bp fragment; 5, pGL_3-912 cut with *Sac*I and *Xho*I to produce a 912 bp fragment; 6, pGL_3-928 cut with *Sac*I and *Xho*I to produce a 928 bp fragment; 7, pGL_3-944 cut with *Sac*I and *Xho*I to produce a 944 bp fragment; 8, pGL_3-953 cut with *Sac*I and *Xho*I to produce a 953 bp fragment; 9, pGL_3-974 cut with *Sac*I and *Xho*I to produce a 974 bp fragment; 10, pGL_3-1007 cut with *Sac*I and *Xho*I to produce a 1007 bp fragment; 11, pGL_3-1040 cut with *Sac*I and *Xho*I to produce a 1040 bp fragment.

cess were added to the binding reaction mixture and co-incubated. DNA-protein binding complexes were separated by 5% nondenaturing polyacrylamide gel electrophoresis in 0.25×Tris-Boric acid (TBE) buffer. Electroblotting and chemiluminescence detection were performed based on the instructions of the manufacturer of the DIG gel shift kit (Roche, Penzberg, Germany).

Results

Construction and identification of luciferase reporter constructs

PCR methods were used in the construction of *NKX3.1* promoter-luciferase reporter plasmids and its 5′ deletion mutants. The lengths of PCR products were 1040 bp, 1007 bp, 974 bp, 953 bp, 944 bp, 928 bp, 912 bp, 891 bp, 877 bp and 843 bp. The related primers used in PCR are listed in **Table 1**. All the constructs were confirmed to be correct by restriction enzyme digestion (**Fig. 1**) and sequence analysis.

Deletion mutagenesis analysis of *NKX3.1* promoter

To identify precisely the functional *cis*-element within the 197 bp region between −1032 and −836 bp, we dissected the 1040 bp promoter by deletion mutagenesis and tested their activities by using transient transfection and dual-luciferase reporter assay. The schematic depiction of the construction is shown in **Fig. 2**. The results in **Fig. 2** show that the deletion from −1032 to −921 bp presented no significant effects on promoter activity while the deletion from −920 to −905 bp showed a 3-fold reduction in the promoter activity, which suggested that the 16 bp sequence from −920 to −905 bp was important in the positive regulation of *NKX3.1*.

Effects of the 16 bp *cis*-element on heterogeneous promoters

To confirm the positive regulatory activity of the 16 bp *cis*-element and to assess whether it alone possesses positive regulatory activity, the 16 bp *cis*-element sequence was synthesized *in vitro* and inserted upstream of the SV40 gene promoter in the pGL_3-promoter, and the maspin gene promoter in pGL_3-maspin. By transient transfection assay, the effects of the 16 bp *cis*-element on heterologous promoter activities were tested. The results in **Fig. 3** show that the 16 bp *cis*-element presented significant positive regulatory effects on heterogeneous promoters and it enhanced the promoter activity to 4.3-fold for the SV40 gene promoter, and 2-fold for the maspin gene promoter.

Binding ability of the 16 bp *cis*-element to nuclear extracts

To confirm whether the 16 bp *cis*-element we identified is functional, its binding ability to nuclear extracts was determined by EMSA. The 16 bp *cis*-element sequence (16bp-A) was synthesized, DIG-labeled, and reacted with nuclear extracts from LNCaP cells. A specific DNA-protein binding complex was identified from LNCaP nuclear extracts (**Fig. 4**, lane 2). The competitors used in EMSA were 16bp-m1, 16bp-m2, 16bp-m3, 16bp-m4 and unlabeled random sequence (ARE) as shown in **Table 2**. The

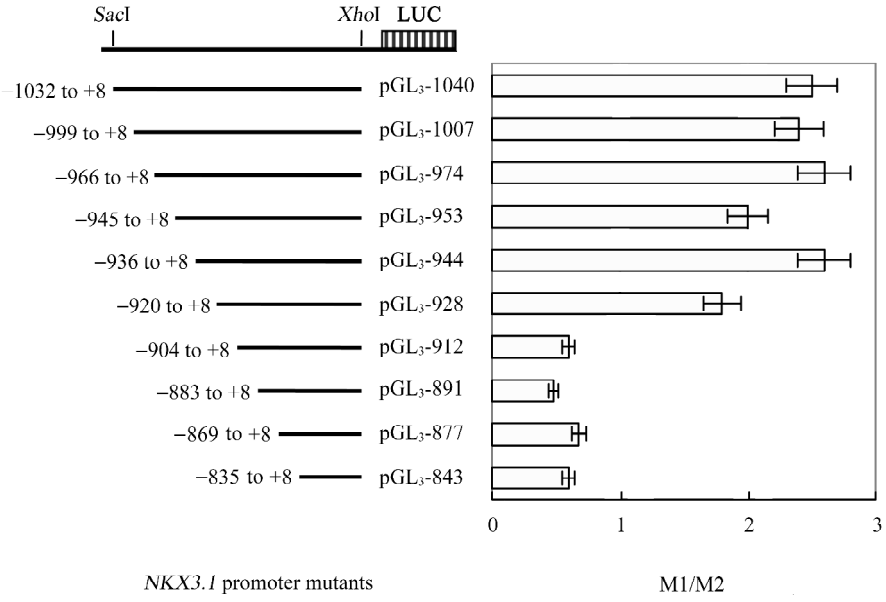

Fig. 2 Deletion mutagenesis analysis from −1032 to −836 bp of the *NKX3.1* gene

Progressive 5′ deletion mutants of the *NKX3.1* promoter from −1032 to −836 bp were linked to the luciferase reporter plasmid pGL_3-basic between the *Sac*I and *Xho*I sites. The promoter activities of various deletion mutants were determined by transient transfection into LNCaP cells and dual luciferase assay. Results are expressed as relative luciferase activity (M1/M2) that are the ratio of firefly luciferase activity (M1) in pGL_3 plasmid to Renilla luciferase activity (M2) in pRL-TK plasmid. The data are represented as mean±SD (n=6).

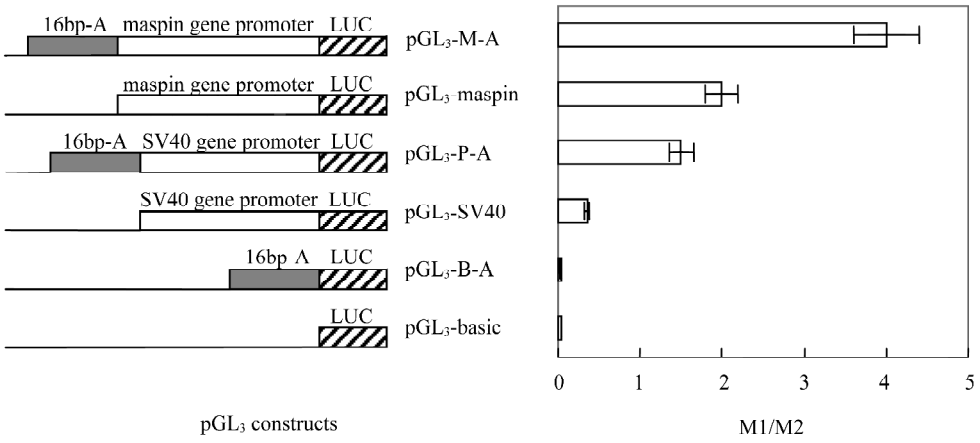

Fig. 3 Effects of the 16 bp *cis*-element on heterogeneous promoter activities

The double-stranded sequence of the 16 bp *cis*-element was synthesized *in vitro* and inserted upstream of the *maspin* and SV40 promoters in the pGL_3 plasmids at the *Xho*I and *Sac*I sites. pGL_3-basic construct was used as the control. In **Fig. 3**, 16bp-A donated the 16 bp *cis*-element sequence and the luciferase reporter gene (LUC). The promoter activities of the constructs were determined by transient transfection into LNCaP cells and dual luciferase assay. Results are expressed as relative luciferase activity (M1/M2), the ratio of firefly luciferase activity (M1) in pGL_3 plasmid to Renilla luciferase activity (M2) in pRL-TK plasmid. The data are represented as mean±SD (n=4).

results are shown in **Fig. 4**. The binding of labeled 16bp-A to nuclear extract can be blocked by a 150-fold excess amount of unlabeled 16bp-A (lane 3), 16bp-m1 (lane 4) and 16bp-m4 (lane 7), but not unlabeled 16bp-m2 (lane 5), 16bp-m3 (lane 6) or ARE (lane 8). The results indicated that the 16 bp *cis*-element presented a binding ability to a specific protein in nuclear extracts from LNCaP cells. The sequence CTTGTCTT is very important for the bind-

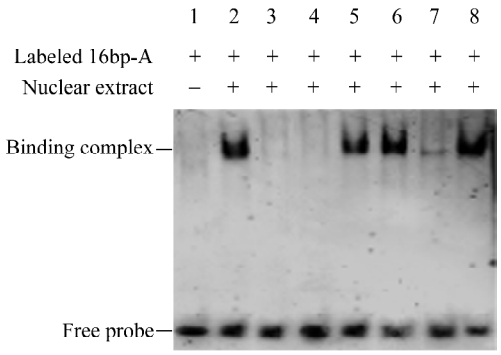

Fig. 4 Binding activity of the 16 bp *cis*-element to LNCaP cell nuclear extract

Electrophoretic mobility shift assay (EMSA) experiments were performed to assay the binding activity of the 16 bp *cis*-element (16bp-A) to the nuclear extract from LNCaP cells. The probes used in EMSA are shown in **Table 2**. 1, labeled 16bp-A without nuclear extract; 2, labeled 16bp-A with nuclear extract; 3–8, labeled 16bp-A with nuclear extract plus a 150-fold excess amount of unlabeled 16bp-A (lane 3), 16bp-m1 (lane 4), 16bp-m2 (lane 5), 16bp-m3 (lane 6), 16bp-m4 (lane 7) or unlabeled random sequence (lane 8).

ing activity of the 16 bp *cis*-element.

Discussion

Recent studies of human cancers [7] and a mutant mouse model [8] have implicated that the *NKX3.1* homeobox gene plays a key role in prostate carcinogenesis. In mice, *NKX3.1* is a key regulator of prostatic epithelial differentiation. *NKX3.1* null mutant mice display abnormal prostatic differentiation as well as epithelial hyperplasia and dysplasia [9]. Notably, *NKX3.1* mutant mice display the pathologic changes of prostatic intraepithelial neoplasia [10] that is the presumed precursor to prostate cancer in humans. The *NKX3.1* gene maps to the chromosomal region 8p21 [11], a region with high loss of heterozygosity in about 80% of human prostate cancers [12–14]. Loss of *NKX3.1* protein expression is closely related with the initiation of prostate carcinogenesis and with prostate tumor progression [7]. No mutations in the *NKX3.1* gene have been found in prostate tumor specimens [15] and its second allele is inactivated by mechanisms other than mutations in the coding region.

In this report, a 16 bp positive *cis*-element was identified in the 197 bp region of *NKX3.1* gene promoter. This 16 bp positive *cis*-element proved to be functional by the assay of its binding ability to nuclear extracts in EMSA, and its positive regulatory effects on heterogeneous promoters. In EMSA experiments, the DNA-protein complex is specific because the binding complex can be blocked by competition from the excess amount of unlabeled 16 bp *cis*-element and cannot be blocked by competition from the excess amount of ARE. The results indicated that this *cis*-element presented a binding ability to a specific protein in nuclear extracts from LNCaP cells. We also synthesized four mutants of 16 bp *cis*-element with four-base substitution that were used as competitors in the EMSA. The results showed that the binding can be blocked by competition from a 150-fold excess amount of unlabeled 16bp-m1 and 16bp-m4 and can not be blocked by competition from a 150-fold excess amount of unlabeled 16bp-m2 and 16bp-m3, which suggested that the CTTGTCTT is the key sequence for the binding activity of the 16 bp *cis*-element.

In summary, we have identified a 16 bp potent positive *cis*-element between –920 and –905 bp upstream of the *NKX3.1* gene. Its activity is promoter type-independent and it is likely to play an important role in regulating *NKX3.1* gene transcription. It will provide an insight into the regulatory mechanisms of *NKX*3.1 gene expression in further study.

References

1 Bieberich CJ, Fujita K, He WW, Jay G. Prostate-specific and androgen-dependent expression of a novel homeobox gene. J Biol Chem 1996, 271: 31779–31782
2 Sciavolino PJ, Abrams EW, Yang L, Austenberg LP, Shen MM, Abate-Shen C. Tissue-specific expression of murine *Nkx*3.1 in the male urogenital system. Dev Dyn 1997, 209: 127–138
3 Shen MM, Abate-Shen C. Roles of the *Nkx3.1* homeobox gene in prostate organogenesis and carcinogenesis. Dev Dyn 2003, 228: 767–778
4 Tanaka M, Lyons GE, Izumo S. Expression of the *Nkx3.1* homeobox gene during pre and postnatal development. Mech Dev 1999, 85: 179–182
5 Xu LL, Srikantan V, Sesterhenn IA, Augustus M, Dean R, Moul JW, Carter KC *et al.* Expression profile of an androgen regulated prostate specific homeobox gene *NKX3.1* in primary prostate cancer. J Urol 2000, 163: 972–979
6 Jiang AL, Zhang JY, Young C, Hu XY, Wang YM, Liu ZF, Hao ML. Molecular cloning and characterization of human homeobox gene *Nkx*3.1 promoter. Acta Biochim Biophys Sin 2004, 36: 64–67
7 Bowen C, Bubendorf L, Voeller HJ, Slack R, Willi N, Sauter G, Gasser TC *et al.* Loss of NKX3.1 expression in human prostate cancers correlates with tumor progression. Cancer Res 2000, 60: 6111–6115
8 Kim MJ, Bhatia-Gaur R, Banach-Petrosky WA, Desai N, Wang Y, Hayward SW, Cunha GR *et al.* *Nkx3.1* mutant mice recapitulate early stages of prostate carcinogenesis. Cancer Res 2002, 62: 2999–3004
9 Bhatia-Gaur R, Donjacour AA, Sciavolino PJ, Kim M, Desai N, Young P, Norton CR *et al.* Roles for *Nkx*3.1 in prostate development and cancer. Genes Dev 1999, 13: 966–977
10 Abdulkadir SA, Magee JA, Peters TJ, Kaleem Z, Naughton CK, Humphrey PA, Milbrandt J. Conditional loss of *Nkx3.1* in adult mice induces prostatic

intraepithelial neoplasia. Mol Cell Biol 2002, 22: 1495–1503

11 Voeller HJ, Augustus M, Madike V, Bova GS, Carter KC, Gelmann EP. Coding region of *NKX3.1*, a prostate-specific homeobox gene on 8p21, is not mutated in human prostate cancers. Cancer Res 1997, 57: 4455–4459

12 Cher ML, Bova GS, Moore DH, Small EJ, Carroll PR, Pin SS, Epstein JI *et al*. Genetic alterations in untreated metastases and androgen-independent prostate cancer detected by comparative genomic hybridization and allelotyping. Cancer Res 1996, 56: 3091–3102

13 Dong JT. Chromosomal deletions and tumor suppressor genes in prostate cancer. Cancer Metastasis Rev 2001, 20: 173–193

14 Vocke CD, Pozzatti RO, Bostwick DG, Florence CD, Jennings SB, Strup SE, Duray PH *et al*. Analysis of 99 microdissected prostate carcinomas reveals a high frequency of allelic loss on chromosome 8p12-21. Cancer Res 1996, 56: 2411–2416

15 Ornstein DK, Cinquanta M, Weiler S, Duray PH, Emmert-Buck MR, Vocke CD, Linehan WM *et al*. Expression studies and mutational analysis of the androgen regulated homeobox gene *NKX3.1* in benign and malignant prostate epithelium. J Urol 2001, 165: 1329–1334

Edited by
Ding-Gan LIU