Characterization of two functional NKX3.1 binding sites upstream of the PCAN1 gene that are involved in the positive regulation of PCAN1 gene transcription

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Abstract

Background: NKX3.1 and PCAN1 are both prostate-specific genes related to prostate development and prostate cancer. So far, little is known about the regulatory mechanisms of the expression of these two genes. In the present study, we found that NKX3.1 upregulated PCAN1 gene transcription in LNCaP prostate cancer cells. To understand the regulatory mechanisms, our work focused on identifying the functional NKX3.1 binding sites upstream of the PCAN1 gene, which might be involved in the positive regulation of PCAN1 expression by NKX3.1.

Results: We cloned and characterized a 2.6 kb fragment upstream of the PCAN1 gene. Analysis of the 2.6 kb sequence with MatInspector 2.2 revealed five potential binding sites of NKX3.1 transcription factor. Luciferase reporter assays, electrophoretic mobility shift assays, chromatin immunoprecipitation and RNA interference were performed to study the effects of NKX3.1 on PCAN1 gene expression in prostate cancer cells. Our results showed that PCAN1 promoter activity and mRNA expression were increased by transfection with the NKX3.1 containing plasmid (pcDNA3.1-NKX3.1) and that PCAN1 mRNA expression was decreased by RNA interference targeting human NKX3.1 in LNCaP prostate cancer cells. The results of electrophoretic mobility shift assays and chromatin immunoprecipitation showed that NKX3.1 bound to NBS1 (-1848 to -1836) and NBS3 (-803 to -791) upstream of the PCAN1 gene. The luciferase reporter assays showed that NBS1 and NBS3 enhanced the promoter activity in pGL3-promoter vector with cotransfection of the NKX3.1 containing plasmid. Furthermore, the deletion of NBS1 or both NBS1 and NBS3 reduced PCAN1 promoter activity and abolished the positive regulation of PCAN1 expression by NKX3.1.

Conclusion: Our results suggested that two functional NKX3.1 binding sites located at -1848 to -1836 and -803 to -791 upstream of the PCAN1 gene were involved in the positive regulation of PCAN1 gene transcription by NKX3.1.
Background
Prostate cancer is the most frequently diagnosed neoplasia in men and one of the leading causes of cancer-related deaths in men over 60 [1]. Although early prostate specific antigen (PSA) detection and surgery have decreased the death rate, most of the patients still die of metastasis and recurrence of prostate cancer [2,3]. However, the mechanisms involved in the onset and progression of prostate cancer are poorly understood at the molecular level. It is important to understand the molecular biology of this cancer for its prevention, early diagnosis, and effective treatment.

**PCAN1** (prostate cancer gene 1, also known as GDEP) is highly expressed in prostate epithelial tissue and frequently mutated in prostate tumors [4-6]. **PCAN1** expression is undetectable in the highly undifferentiated DU145 and PC-3 prostate cancer cell lines and weakly detected in the more differentiated LNCaP cell line [5]. This gene is localized to chromosome 4q21, a region of the genome that experiences frequent loss of heterozygosity (LOH) in prostate cancer. It is mutated in 35% of the tumor samples [5]. Therefore, **PCAN1** has been proposed to have tumor suppressing function in prostate cancer.

**NKX3.1** is a prostate-specific homeobox gene that is thought to play an important role in the normal development of prostate and carcinogenesis. In mice **Nkx3.1** is exclusively expressed in prostate epithelium [7,8]. Its targeted disruption leads to aberrations in prostate ductal morphogenesis and secretory protein production, and epithelial hyperplasia and dysplasia [9]. Notably **Nkx3.1** mutant mice display the pathologic changes of prostatic intraepithelial neoplasia (PIN) [10] that is the presumed precursor to prostate cancer in humans, implying that loss of **Nkx3.1** expression correlates with the initiation of prostate carcinogenesis. Human **NKX3.1** has been mapped to human chromosome 8p21, a region with frequent loss of heterozygosity in human prostate cancer [11]. This gene has been proposed to have tumor suppressing function [12]. It also inhibits the growth of cultured prostate cancer cells [13-15] in vitro. The strong association of **NKX3.1** with prostate development and prostate cancer makes this gene an attractive molecular target for further study.

**NKX3.1** and **PCAN1** are both prostate-specific genes strongly related to prostate development and prostate cancer. Studying the regulatory mechanisms of their expression is important for understanding their roles in prostate development and prostate cancer. In this study, we cloned and characterized a 2.6 kb fragment upstream of the **PCAN1** gene. Analysis of the 2.6 kb sequence with MatInspector 2.2 revealed potential binding sites of some important transcription factors, including **NKX3.1**, **P53**, **Sp1**, **cEBP** and **PPAR/RXR** heterodimers. In our study, the eukaryotic expression plasmids containing **NKX3.1**, **P53**, **Sp1**, **cEBP** and **PPARγ** were respectively used to study their effects on **PCAN1** expression. We found that **NKX3.1** upregulated **PCAN1** promoter activity and mRNA expression. To explore the regulatory mechanisms of **NKX3.1** on **PCAN1** transcription, we focused on identifying the functional **NKX3.1** binding sites (NBSs) upstream of the **PCAN1** gene. We demonstrated that **NKX3.1** upregulated **PCAN1** gene transcription through direct binding with NBSs upstream of the **PCAN1** gene. Our study provided a molecular mechanism for the regulation of **PCAN1** gene expression.

Results
**Effects of NKX3.1 on PCAN1 promoter activity and mRNA expression**
In our previous work, a 2.6 kb promoter fragment (+32 to -2598) of the **PCAN1** gene amplified by PCR was inserted into pGL3-basic vector to form the **PCAN1** promoter-luciferase reporter plasmid designated as pGL3-pPCAN1. Firefly luciferase expression driven by the 2.6 kb **PCAN1** promoter was used to evaluate the promoter activity [16]. To detect the effects of **NKX3.1** on **PCAN1** gene expression, LNCaP and PC-3 cells were harvested 48 h after cotransfection with pGL3-pPCAN1 and pcDNA3.1-NKX3.1. The control cells were cotransfected with pGL3-pPCAN1 and pcDNA3.1 (+) plasmid. The **PCAN1** promoter activity detected by dual-luciferase reporter assays was respectively used to study their effects on **PCAN1** expression. We found that **NKX3.1** upregulated **PCAN1** promoter activity and mRNA expression. To explore the regulatory mechanisms of **NKX3.1** on **PCAN1** transcription, we focused on identifying the functional **NKX3.1** binding sites (NBSs) upstream of the **PCAN1** gene. We demonstrated that **NKX3.1** upregulated **PCAN1** gene transcription through direct binding with NBSs upstream of the **PCAN1** gene. Our study provided a molecular mechanism for the regulation of **PCAN1** gene expression.

**Figure 1**
**Effects of NKX3.1 on the PCAN1 promoter in LNCaP and PC-3 cells.** LNCaP and PC-3 cells were cotransfected with pGL3-pPCAN1 and pcDNA3.1-NKX3.1 (grey bars). The control cells were cotransfected with pGL3-pPCAN1 and pcDNA3.1 (+) plasmid (blank bars). The cells were harvested 48 h after transfection and **PCAN1** promoter activity was detected by dual-luciferase reporter assays. Results were expressed as relative luciferase activities (M1/M2). The data were represented as the mean of four individual values ± SD. *p < 0.05, **p < 0.01, grey bar vs blank bar.
The PCAN1 mRNA expression level in LNCaP cells, as detected by RT-PCR, was increased significantly by NKX3.1 cotransfection, compared with that of the control cells transfected with pcDNA3.1 (+) plasmid (Fig. 2A and 2B). The PCAN1 mRNA expression level in PC-3 cells was undetectable by RT-PCR (Fig. 2A and 2B).

The expression of endogenous NKX3.1 in LNCaP cells was knocked down by RNAi, which made the PCAN1 mRNA expression decrease, as detected by RT-PCR (Fig. 2D).

**Identification of specific NKX3.1 binding sites (NBSs) with nuclear extracts**

Analysis of the 2.6 kb promoter sequence with MatInspector 2.2 revealed five potential NKX3.1 transcription factor binding sites (sequences are shown in Fig. 3A). They were located at -1848 to -1836 (NBS1), -1080 to 1068 (NBS2), -803 to -791 (NBS3), -179 to 166 (NBS4) and -131 to -119 (NBS5), upstream of the PCAN1 gene. To investigate the binding activities of these five NBSs with NKX3.1 transcription factor, we carried out electrophoretic mobility shift assays (EMSA). It was performed with NKX3.1-transfected nuclear extracts from LNCaP cells and synthesized oligonucleotide probes containing these five NBS sequences. The results showed that DNA-protein binding complexes were identified for the NBS1 probe and the NBS3 probe (Fig 3B). The bindings of NBS1 and NBS3 with nuclear extracts proved to be specific, as they were blocked by a 250-fold excess of unlabeled NBS1 probe (Fig. 3C) or NBS3 probe (Fig. 3D) and by anti-NKX3.1 antibody (Fig. 3C and 3D), but not by unlabeled mutant NBS1 probe or mutant NBS3 probe (Fig. 3C and 3D).

**NKX3.1 binds to NBS1 and NBS3 upstream of the PCAN1 gene in living cells**

To determine whether NKX3.1 also binds to the NBSs in vivo, we performed chromatin immunoprecipitation (ChIP) assays, which are used to define interactions of proteins with specific DNA elements in living cells. ChIP was carried out in LNCaP cells transfected with pcDNA3.1-NKX3.1. After cross-linking with formaldehyde, cell lysates were immunoprecipitated with anti-NKX3.1 antibody or rabbit IgG (negative control). The DNA purified from this coprecipitation was analyzed by PCR with primers (sequences are shown in Table 1) spanning the NBSs in the PCAN1 promoter. As shown in Fig. 4, we observed a clear PCR product using NBS1 primer or NBS3 primer but no band was observed using NBS2 primer or NBS4,5 primer, and no PCR product was identified for all primers with rabbit IgG precipitation complexes, indicating that NKX3.1 bound to NBS1 and NBS3 upstream of the PCAN1 gene in living cells.

**Figure 2**

**Effects of NKX3.1 on PCAN1 mRNA expression in LNCaP and PC-3 cells.** The effects of NKX3.1 on PCAN1 mRNA expression as detected by RT-PCR. A. PCAN1 mRNA expression following transfection of LNCaP cells with 1. pcDNA3.1-NKX3.1; 2. pcDNA3.1 (+); or following transfection of PC-3 cells with 3. pcDNA3.1-NKX3.1; or 4. pcDNA3.1 (+). B. Relative expression levels were presented as the ratio of densities of NKX3.1 or PCAN1 to β-actin bands. The results were expressed as mean ± SD (n = 3). C. The expression of NKX3.1 protein in LNCaP and PC-3 cells after transfection of pcDNA3.1-NKX3.1 (1, 3, 5, 7) or pcDNA3.1 (+) (2, 4, 6, 8). D. NKX3.1 and PCAN1 mRNA expression following stable transfection of LNCaP cells with 1. pRNAT-RNAi1 targeting human NKX3.1; 2. pRNAT-RIN as a control vector.
Interaction of NKX3.1 with NBS1 or NBS3 stimulates luciferase reporter expression driven by SV40 promoter

To investigate the interactions between NKX3.1 and these five potential NBSs in vivo, five pGL3-NBS-promoter luciferase plasmids were constructed (Fig. 5) and cotransfected with pcDNA3.1-NKX3.1 plasmid respectively into LNCaP cells, while the control cells were cotransfected with pcDNA3.1 (+) vector. The luciferase reporter assays showed that when cotransfected with NKX3.1 expression plasmid, NBS1 and NBS3 enhanced SV40 promoter activity by 1.7-fold and 2.1-fold, respectively, compared with that of the control cells that were cotransfected with pcDNA3.1 (+) vector. NBS2, NBS4, NBS5 showed no significant effects on SV40 promoter activity. These results suggested that NBS1 and NBS3 were the functional cis-elements in vivo for the upregulation by NKX3.1.

Deletion of NBS1 and NBS3 in the PCAN1 promoter abolishes the positive regulation by NKX3.1

The binding assays of five NBSs in vivo and in vitro suggested that NBS1 and NBS3 in the PCAN1 promoter were involved in the positive regulation of PCAN1 expression by NKX3.1. To further confirm this observation, the sequences of NBS1, or both NBS1 and NBS3 were deleted from pGL3-pPCAN1 to examine the effects of the deletions on PCAN1 promoter activity. The results in Fig. 6 showed that deletion of NBS1 (pGL3-NBS1id-pPCAN1) or both NBS1 and NBS3 (pGL3-NBS1,3idd-pPCAN1) reduced the promoter activity to 75% or 50% of the wild-type promoter (pGL3-pPCAN1). With cotransfection of the NKX3.1 expression plasmid, deletion of NBS1 partially abolished, and deletion of both NBS1 and NBS3 completely abolished NKX3.1 stimulation of the PCAN1 promoter. These findings suggested that NBS1 and NBS3 upstream of the PCAN1 gene were functional cis-elements mediating the positive regulation by NKX3.1 of PCAN1 gene transcription.

Discussion

NKX3.1 is a prostate-specific homeobox gene that is thought to play important roles in normal prostate development. NKX3.1 protein has been proposed to act as a specific tumor suppressor in prostate. Loss of NKX3.1 expression correlates with prostate carcinogenesis [15] and prostate tumor progression [17]. It was reported that NKX3.1 could collaborate with other transcription factors,
such as serum response factor (SRF) [18], Sp-family protein [19] and prostate derived Ets factor (PDEF) [20], to regulate the expression of target genes. The potential for NKX3.1 to exert a differentiating and growth suppressing effect on prostatic epithelium was confirmed by targeted gene disruption of \( \textit{Nkx3.1} \) in mice [21]. Deletion of either one or both copies of \( \textit{Nkx3.1} \) resulted in prostatic epithelial hyperplasia and dysplasia that increased in severity with age. Magee et al. [21] has analyzed the expression profiles of prostate tissues from wild-type, \( \textit{Nkx3.1}^{+/+} \), and \( \textit{Nkx3.1}^{-/-} \) mice and identified \( \textit{Nkx3.1} \) target genes. However, the genes directly regulated by human NKX3.1 have yet to be identified. In the present study, we found that NKX3.1 upregulated the expression of the \( \textit{PCAN1} \) gene in LNCaP prostate cancer cells and identified two functional NKX3.1 binding sites upstream of the \( \textit{PCAN1} \) gene.

\( \textit{PCAN1} \) is highly expressed in prostate epithelial tissue and was initially identified in a screen for prostate-specific genes. As an important gene in prostate cancer initiation or progression [22], it has been shown that \( \textit{PCAN1} \) is frequently mutated or deleted in prostate tumor samples [5] and differentially expressed in tumor versus normal prostate tissue, demonstrating a prostate tumor suppressor role [6]. So far, little is known about the regulatory mechanisms of \( \textit{PCAN1} \) gene expression as well as the relevant regulatory elements and factors. Cross et al. [4] has identified \( \textit{PCAN1} \) gene in prostate epithelial tissue and demonstrated its expression pattern in different cells. They did an initial characterization of 2.5 kb sequence upstream of the initiation sites with the MatInspector program, in which they found several important transcription factor binding sites, including NKX3.1 binding sites. In our study, we cloned a 2.6 kb \( \textit{PCAN1} \) promoter sequence and five NKX3.1 binding sites were found in this region with the MatInspector (core/matrix sim: 0.75/optimized). We further performed reporter assays, RNAi, EMSA and ChIP to demonstrate that NKX3.1 could directly bind to NKX3.1 binding sites in the \( \textit{PCAN1} \) promoter to enhance \( \textit{PCAN1} \) gene expression in prostate cancer cells. This finding provides a foundation for future studies of the regulatory mechanisms and roles of NKX3.1 on \( \textit{PCAN1} \) gene expression in prostate development and prostate cancer.

Table 1: PCR primers used in chromatin immunoprecipitation

| Names          | Sequences                      | Product sizes     |
|----------------|--------------------------------|-------------------|
| NBS1 primers   | F: GATTCTTTGACTGGTCTGGCACAC    | 170 bp (spanning NBS1) |
|                | R: TTATCCATTGTGCCGGACTGGAG    |                   |
| BNS2 primers   | F: TCCTACTAAAGGGGACTGAAGAGG   | 170 bp (spanning NBS2) |
|                | R: ATGGGCAATTTGGAAGGATTG      |                   |
| NBS3 primers   | F: AAGAATGAGCTGATCCTTACCGA    | 150 bp (spanning NBS3) |
|                | R: GGTATTAGAAATAGCATGGGCAACA  |                   |
| NBS4,5 primers | F: GTGTAAGCAGGTAACATGGTGGAG   | 180 bp (spanning NBS4 and 5) |
|                | R: TCAGCTGACAGCAACTCTCAATTCC  |                   |

NBS1 F and R span NBS1;
NBS2 F and R span NBS2; NBS3 F and R span NBS3; NBS4,5 F and R span NBS4 and NBS5
Cotransfection of pGL3-NBS-promoter with pcDNA3.1 (+) on the activity of the SV40 heterogeneous promoter. pcDNA3.1-vector was used as the control. The promoter activities expressed as relative luciferase activity (M1/M2). The data were determined by dual luciferase assays. Results were represented as the mean of four individual values ± SD. *p < 0.01, **p < 0.01, strip bar vs blank bar.

 NKX3.1 upregulates which in turn prevents cancer initiation. Our finding that tumor modulator, serving as a regulator of differentiation, are not consistent with activities of “classic” tumor suppressor genes. Instead, NKX3.1 is a prostate-specific gene required for maintaining the normal differentiated state of the prostate epithelium. It is proposed to have tumor suppressing function. However, it is not a classic tumor suppressor gene. Despite that loss of function of NKX3.1 predisposes to prostate cancer, it is not sufficient for tumorigenesis. Moreover, while one allele of NKX3.1 is lost by means of chromosomal deletion in prostate cancer, the other allele does not undergo mutational inactivation. These features are not consistent with activities of “classic” tumor suppressor genes. Instead, NKX3.1 appears to act more like a tumor modulator, serving as a regulator of differentiation, which in turn prevents cancer initiation. Our finding that NKX3.1 upregulates PCAN1 expression will give a clue for further exploring the relationships between NKX3.1 and PCAN1 and understanding the functional importance of NKX3.1 in regulating PCAN1 in prostate differentiation and carcinogenesis. Identification of the specific biological functions of NKX3.1 in prostate cancer may provide targets for the early diagnosis and prevention of prostate cancer.

 NKX3.1 is an androgen regulated gene and its expression is upregulated by androgens. It is presumed that androgens upregulate PCAN1 expression through increasing NKX3.1 expression. In the experiments we have analyzed the effect of androgen (R1881) on endogenous PCAN1 and NKX3.1 expression in LNCaP cells. Our results showed that R1881 (10^-8~10^-10 M) increased NKX3.1 expression but have no significant effects on PCAN1 expression in RT-PCR (results are not shown in this paper) and PCAN1 promoter activity in luciferase reporter assays (results are shown in our paper in Cell Mol Biol Lett). So far, it is not very clear whether or not PCAN1 gene expression is regulated directly by androgens. It may be possible that PCAN1 is negatively regulated by androgens and NKX3.1 compensates this effect through positively regulating PCAN1. In addition, AR, NKX3.1 and PCAN1 play different roles in prostate development and cancer, and their interactions in prostate are very complicated and need further to be investigated.

**Conclusion**

In conclusion, we cloned and characterized the human PCAN1 promoter, and identified two functional NKX3.1 binding sites upstream of the PCAN1 gene, which were involved in the positive regulation by NKX3.1 of PCAN1 expression. This suggests that NKX3.1 is involved in the positive regulation of PCAN1 expression, which may contribute to the development and progression of prostate cancer. Further studies are required to explore the regulatory mechanisms and cofactors involved in the positive regulation by NKX3.1 of PCAN1 expression.
gene expression. Both NKX3.1 and PCAN1 are related to prostate development and prostate cancer. Our findings will contribute to the understanding of molecular regulatory mechanisms of PCAN1 gene expression in prostate development and cancer.

Methods

Construction of the NKX3.1 eukaryotic expression plasmid (pcDNA3.1-NKX3.1)

PCR2.1-NKX3.1, a T-clone of NKX3.1 cDNA containing the complete sequence of NKX3.1 (a gift from Dr. Charles Young, Mayo Clinics, USA), was digested with EcoR I (Takara, Shiga, Japan) to release the 971 bp fragment of NKX3.1 cDNA. The NKX3.1 cDNA sequence (971 bp) contains the CDS of NKX3.1 including a start codon, a stop codon and a partial 3' UTR. It was then inserted into pcDNA3.1 (+) vector (Invitrogen Life Technologies, San Diego, CA, USA), which had been digested with EcoR I and dephosphorylated with calf intestine alkaline phosphatase (Takara), to generate the NKX3.1-cDNA eukaryotic expression plasmid pcDNA3.1-NKX3.1. The recombinant plasmid was digested with EcoR I to identify the size of the insert and digested with Not I (Takara) to identify the correct insert orientation. The NKX3.1 cDNA was also confirmed by DNA sequencing.

Construction of the PCAN1 promoter-luciferase reporter plasmid (pGL3-pPCAN1)

The 2.6 kb promoter fragment (+32 to -2598) of the PCAN1 gene was amplified by PCR using human genomic DNA as the template. The primer pairs were PCANF 5' - GCCATACCCAGTTGGTGCGAGTGAATCC - 3' and PCARN 5' - CCGCTCTGCCCTGCTTTGCAC - 3' with a Nhe I site at the 5'-end and PCANR 5' - CCGCTCTGCCCTGCTTTGCAC - 3' with Hind III at the 5'-end. The PCR was conducted at 96°C for 2 min followed by 35 cycles at 98°C for 20 s, 68°C for 10 min. The amplified fragment was isolated and purified following agarose electrophoresis using a Gel Extract Kit (Omega Bil-Tek, Inc. Doraville, GA, USA), digested with Nhe I and Hind III (Takara), and ligated into the equivalent sites of the pGL3-basic vector (Promega, Madison, WI, USA) to generate the pGL3-pPCAN1 construct. The resulting construct was confirmed by restriction enzyme digestion and DNA sequence analysis.

Construction of luciferase reporter plasmids of NKX3.1 binding sites (pGL3-NBS-promoter)

Analysis of the 2.6 kb promoter sequence using MatInspector 2.2 revealed five potential binding sites for the NKX3.1 transcription factor. To confirm the functional binding sites, we synthesized oligonucleotides corresponding to these five sequences (NBS1~NBS5) shown in Table 2. Each NBS sequence was synthesized with an overhanging Mlu I site (CCGGT) at the 5'-end of the sense strand and an overhanging Xho I site (TGGAG) at the 5'-end of the antisense strand. The double-stranded NBS was generated by annealing equal amounts of sense and antisense oligonucleotides at 95°C for 10 min, then cooling to room temperature. The double-stranded NBS was inserted upstream of the SV40 promoter in the pGL3-promoter (Promega) vector to generate the recombinant plasmid of NBS-SV40 promoter-luciferase reporter gene. All constructs were confirmed by DNA sequencing.

Construction of the NBS deletion plasmids of pGL3-pPCAN1

The construction of NBS1 or both NBS1 and NBS3 deletion plasmids was made by a two-step PCR procedure. In the first step, pGL3-pPCAN1 was used as the template for the construction of pGL3-NBS1id-pPCAN1, and two PCR fragments were generated with two primer pairs, NBS1idd-up 5' - CCGCTCTGCCCTGCTTTGCAC - 3', NBS1idd-down 5'- TGGAGCCCGCCCAAGTCAGGGGGTTAAC - 3' and PCANF 5' - CGCTCTGCCCTGCTTTGCAC - 3' and PCARN 5' - CGCTCTGCCCTGCTTTGCAC - 3'. PCR conditions were 94°C for 2 min, 98°C for 10 s, 68°C for 5 min, 32 cycles. The two PCR fragments were purified by agarose electrophoresis and used together as templates in the second step of PCR with primers PCANF and PCARN. The PCR conditions were 94°C for 2 min, 98°C for 30 s, 72°C for 5 min, followed by addition of PCANF and PCARN primers and PCR at 98°C for 10 s, 68°C for 6 min, 32 cycles. The PCR fragments and pGL3-basic were both digested with Hind III and Nhe I and ligated together to construct pGL3-NBS1idd-pPCAN1. A similar approach was used to generate pGL3-NBS1, 3idd-pPCAN1, in which both NBS1 and NBS3 sequences were deleted. pGL3-NBS1idd-pPCAN1 was used as the template and other two primer pairs, NBS3idd-up 5' - GAGATGGCTCGTATTTTCCTATCCAGCAAGGAC - 3' and PCANF, NBS3idd-down 5' - ATAAAGAAATACGGCGATTTCCCCTCACAAGGAC - 3' and PCARN, were used. The constructed deletion plasmids were validated by DNA sequencing.

Cell culture

The human prostate cancer cell lines LNCaP and PC-3 were obtained from the American Type Culture Collection (ATCC). They were grown at 37°C in 5% CO2 with RPMI...
1640 media (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Gibco, BRL Grand Island, NK, USA), ampicillin 100 U/ml and streptomycin 100 U/ml.

**Transient transfection**

For the promoter activity assay of pGL3-pPCAN1, LNCaP and PC-3 cells were transfected with lipofectamin™ 2000 (Invitrogen) in 24-well plates. Each well included 1.5 x 10^5 cells, 1.0 µg pGL3-pPCAN1, 0.04 µg internal control vector pRL-TK (Promega), 2 µl lipofectamin™ 2000 and 500 µl RPMI 1640 media without serum and antibiotics. The cells were analyzed using a dual-luciferase reporter assay system (Promega) 48 h after completion of the transfection procedure.

For cotransfection experiments of the NPKX3.1 expression plasmid (pcDNA3.1-NPKX3.1) with pGL3-construct (pGL3-pPCAN1, pGL3-NBS-promoter, pGL3-NBS1id-pPCAN1 or pGL3-NBS1idd-pPCAN1), LNCaP cells were transfected with lipofectamin™ 2000 in 24-well plates, and each well included 1.5 x 10^5 cells, 0.8 µg pGL3-construct, 0.4 µg pcDNA3.1-NPKX3.1, 0.04 µg pRL-TK, 2 µl lipofectamin™ 2000 and 500 µl RPMI 1640 media without serum and antibiotics.

**Dual-luciferase reporter assays**

Forty-eight hours after the transfection, the activities of Firefly luciferase in pGL3-constructs and Renilla luciferase in pRL-TK were determined by RT-PCR with M-MuLV reverse transcriptase (Promega) in the presence of random hexamer and annealed in a buffer (10 mM Tris-HCl, pH 8.0, 200 mM KCl, 20 mM HEPES (pH 7.6), 1 µg of poly (dI-dC), 0.1 µg of poly (L-lys), 20 µg of nuclear extract and 0.8 ng of DIG labelled double-stranded NBS. For the competition experiment, unlabelled double-stranded NBS or mutant NBS (sequences are shown in Table 2) in 250-fold excess were added to the binding reaction mixture and incubated. For supershift assays, anti-NPKX3.1 antibody was pre-incubated with the nuclear extracts at room temperature for 30 min in the binding buffer, followed by an additional incubation for 20 min at room temperature with the reaction mixtures. Bound and free oligonucleotide probes were resolved by electrophoresis on an 8% nondenaturing polyacrylamide gel in 0.25 x Tris-Boric acid (TBE) buffer. Western blot analysis of NPKX3.1 protein expression

Expression of NPKX3.1 in prostate cancer cells was analyzed by Western blot analysis. Briefly, total protein was extracted from PC-3 cells or LNCaP cells using lysis buffer (containing 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1%SDS, 1%NP-40, 100 µg/ml PMSF) after transfection with pcDNA3.1-NPKX3.1 for 48 h to 72 h. The protein content of the samples was measured using the BCA protein assay kit (Shenergy Biocolor Bioscience & Technology Company, Shanghai, China). Thirty µg of each protein sample was used to detect the NPKX3.1 protein expression by Western blot analysis. The primary antibody was rabbit anti-human NPKX3.1 (RDI, Concord MA, USA) diluted 1: 2000; the second antibody was goat anti-rabbit IgG (Sigma) diluted 1: 2000. Relative protein levels were calculated in comparison to p-actin as standard. Immunoblots were detected using an ECL kit (Santa Cruz, CA, USA) and visualized after exposure to X-ray film.

**Reverse transcription-PCR**

Total RNA was isolated from LNCaP and PC-3 cells using Trizol reagent (Invitrogen) 48 h after transfection with pcDNA3.1-NPKX3.1, and expression of PCAN1 mRNA was determined by RT-PCR with M-Mul. V reverse transcriptase (Promega) in the presence of random hexamer primers. PCR primers for PCAN1 were PCAN-F 5’-GCGATGTCCTGGAAAATCTA-3’, PCAN-R 5’-CTTCACATTCCCGTGTTGCT-3’; for NPKX3.1 were NPKX-F 5’-GTACCTGGCCAGCCTTAACTC-3’, NPKX-R 5’-GCGATGGCTTATACGAGGAGCAG-3’. A β-actin mRNA was amplified and used to normalize the quantity of the PCAN1 mRNA in RT-PCR. The primers were β-actinF 5’-GCGATGTCAGAGATGTATATGCT-3’, β-actinR 5’-ACATTGACGTACA-3’. The PCR conditions were 94°C 3 min, 94°C 30 s, 56°C 30 s for PCAN1, 63°C 30 s for NPKX3.1, 72°C 50s, 32 cycles for PCAN1, 26 cycles for NPKX3.1, 72°C 6 min. The products were identified by 1.5% agarose gel electrophoresis.

**Electrophoretic mobility shift assays (EMSA)**

Nuclear extracts were prepared from LNCaP cells using a nuclear extraction kit (Active Motif, Carlsbad, CA, USA) following the manufacturer’s instructions. Oligonucleotides corresponding to the five binding sites shown in Table 2 were synthesized as probes. Equal amounts of sense and antisense oligonucleotides of NBSs were mixed and annealed in a buffer (10 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA) by heating to 95°C for 5 min and cooling slowly to room temperature. The five double-stranded NBSs were labelled with digoxigenin (DIG) (Roche). Binding reactions were performed for 20 min at room temperature 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), 20 µg of nuclear extract and 0.8 ng of DIG labelled double-stranded NBS. For the competition experiment, unlabelled double-stranded NBS or mutant NBS (sequences are shown in Table 2) in 250-fold excess were added to the binding reaction mixture and incubated. For supershift assays, anti-NPKX3.1 antibody was pre-incubated with the nuclear extracts at room temperature for 30 min in the binding buffer, followed by an additional incubation for 20 min at room temperature with the reaction mixtures. Bound and free oligonucleotide probes were resolved by electrophoresis on an 8% nondenaturing polyacrylamide gel in 0.25 x Tris-Boric acid (TBE) buffer. Western blot analysis of NPKX3.1 protein expression

Expression of NPKX3.1 in prostate cancer cells was analyzed by Western blot analysis. Briefly, total protein was extracted from PC-3 cells or LNCaP cells using lysis buffer (containing 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1%SDS, 1%NP-40, 100 µg/ml PMSF) after transfection with pcDNA3.1-NPKX3.1 for 48 h to 72 h. The protein content of the samples was measured using the BCA protein assay kit (Shenergy Biocolor Bioscience & Technology Company, Shanghai, China). Thirty µg of each protein sample was used to detect the NPKX3.1 protein expression by Western blot analysis. The primary antibody was rabbit anti-human NPKX3.1 (RDI, Concord MA, USA) diluted 1: 2000; the second antibody was goat anti-rabbit IgG (Sigma) diluted 1: 2000. Relative protein levels were calculated in comparison to β-actin as standard. Immunoblots were detected using an ECL kit (Santa Cruz, CA, USA) and visualized after exposure to X-ray film.
Chromatin immunoprecipitation (ChIP)

In vivo binding of NKK3.1 to the NBs in the upstream region of the PCAN1 gene was investigated using the ChIP assay kit (Upstate Biotechnology, Inc., Lake Placid, NY, USA). Confluent human LNCaP prostate cancer cells were transfected with pcDNA3.1-NKK3.1. Forty-eight hours after the transfection, cells were treated with formaldehyde (1% final concentration) to cross-link NKK3.1 to the DNA. Cells were washed with cold phosphate-buffered saline and lysed in SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl pH 8.1). The lysate was sonicated to shear DNA to a length between 200 and 1000 bp. The sonicated supernatant was diluted 10-fold with ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 2 mM Tris-HCl pH 8.1), and 150 mM NaCl) and incubated with anti-NKK3.1 antibody (Santa Cruz) or rabbit IgG overnight at 4°C with rotation. After extensive washing of the pellet with a series of wash buffers, the pellet was dissolved with 250 µl of elution buffer and centrifuged to remove the agarose. The supernatant was treated with 20 µl of 5 M NaCl and heated to 65°C for 4 h to reverse the NKX3.1-DNA cross-link. After treatment with EDTA and proteinase K, the mixture was dissolved with 250 µl of elution buffer and centrifuged to remove the agarose. The supernatant was treated with 20 µl of 5 M NaCl and heated to 65°C for 4 h to reverse the NKK3.1-DNA cross-link. After treatment with EDTA and proteinase K, the mixture was dissolved with 250 µl of elution buffer and centrifuged to remove the agarose. The supernatant was treated with 20 µl of 5 M NaCl and heated to 65°C for 4 h to reverse the NKK3.1-DNA cross-link. After treatment with EDTA and proteinase K, the mixture was dissolved with 250 µl of elution buffer and centrifuged to remove the agarose.

RNA interference (RNAi)

pRNAT-U6.1/Neo, containing human U6 promoter, was used to generate a series of RNAi expression vectors by inserting annealed oligonucleotides between BamHI I and Hind III sites. The oligonucleotides RNAi1 (5’ GATCC CGGCCATgcaatcgactacgac CT 3’), RNAi2 (5’ GATCC CGGCCATgcaatcgactacgac CT 3’), RNAi3 (5’ GATCC CGGCCATgcaatcgactacgac CT 3’), RNAi4 (5’ GATCC CGGCCATgcaatcgactacgac CT 3’) were used for the construction of pRNAT-RNAi (1~3) expressing human NKK3.1. The oligonucleotides RIN (5’ GATCC CGGCCATgcaatcgactacgac CT 3’), RNAi5 (5’ GATCC CGGCCATgcaatcgactacgac CT 3’) were used for the construction of pRNAT-RNAi (1~3) expressing human NKK3.1. The oligonucleotides RIN (5’ GATCC CGGCCATgcaatcgactacgac CT 3’), RNAi5 (5’ GATCC CGGCCATgcaatcgactacgac CT 3’) were used for the construction of pRNAT-RNAi (1~3) expressing human NKK3.1.

Authors’ contributions

WL assisted in the design of the study, carried out the cloning of expression vectors and reporter plasmid constructs, transfection studies, RT-PCR, EMSA and ChIP; helped draft the manuscript. PZ assisted in the design of the study, participated in the sequence alignment and use of computer database; assisted in the construction of recombinant vectors, EMSA, ChIP and RNAi; helped draft the manuscript. WC participated in maintenance of cell lines, performed luciferase reporter assays and assisted with the transfection experiments. CY carried out the isolation of RNA and purification of plasmids, assisted with the RT-PCR and ChIP. FC carried out Western blot and assisted with the purification of plasmids. FK assisted with the maintenance of cell lines and the transfection experiments. JZ participated in the design of the study, assisted with the revising of the manuscript. AJ participated in the design of the study and revision of the manuscript, helped draft the manuscript, assisted with the construction of recombinant vectors, EMSA and ChIP.

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