

ABSTRACT

SIMMONS, STEVEN O'NEAL. Biochemical and Functional Analysis of Homeoprotein Nkx3.1. (Under the direction of Jonathan M. Horowitz.)

Nkx3.1 is a homeodomain-containing transcription factor that plays an important role in the development and differentiation of prostatic epithelia. Loss of Nkx3.1 protein expression is often an early event in prostate tumorigenesis, and the abundance of Nkx3.1-negative epithelial cells increases with disease progression. Herein I report that Nkx3.1 collaborates with Sp-family members in the regulation of *prostate specific antigen (PSA)* in prostate-derived cells. Nkx3.1 forms protein complexes with Sp proteins dependent on their respective DNA-binding domains and an amino-terminal segment of Nkx3.1, and negatively regulates Sp-mediated transcription via Trichostatin A-sensitive and -insensitive mechanisms. Nkx3.1 DNA-binding activity is not required for *trans*-repression of Sp proteins, suggesting that Nkx3.1 regulates Sp-mediated transcription via direct protein/protein interactions.

I report that Nkx3.1 homeodomain encodes at least one nuclear localization signal (NLS) as well as sequences that facilitate the association of Nkx3.1 with the nuclear matrix. I show further that a functionally intact homeodomain is not required for nuclear localization but is required for the association of Nkx3.1 with the nuclear matrix. In contrast to many transcription factors, I show that Nkx3.1 is associated with mitotic chromatin throughout most, if not all, of mitosis and that a functionally intact Nkx3.1 homeodomain is sufficient to facilitate inclusion within mitotic chromosomes.

Finally, I report my efforts to identify Nkx3.1 target genes using a genome-wide approach. This genome-wide screen for putative Nkx3.1 target genes yielded 42 clones

containing novel, human genomic DNA. Ten of these clones harbored unique genomic fragments, while the remaining 32 clones carried sequences that were isolated repeatedly and could be subdivided into three sequence classes. Many of the recovered sequences mapped to locations that are within or near known genes and most carried one or more consensus Nkx3.1 DNA-binding sites. Further work must be performed to corroborate that the results from this genome-wide screen represent *in vivo* Nkx3.1 targets.

Biochemical and Functional Analysis of Homeoprotein Nkx3.1

by

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LIST OF ABBREVIATIONS

AR.....	androgen receptor
ARE.....	androgen response element
BPH.....	benign prostate hyperplasia
BSA.....	bovine serum albumin
CAT.....	chloramphenicol acetyltransferase
CDK.....	cyclin dependent kinase
cDNA.....	complementary DNA
CGH.....	comparative genomic hybridization
ChIP.....	chromatin immunoprecipitation
CIS.....	carcinoma <i>in situ</i>
CK.....	casein kinase
CPM.....	counts per minute
CSP.....	common salivary protein
DNA.....	deoxyribonucleic acid
d.p.c.....	days post-coitum
DTT.....	dithiothreitol
EDTA.....	ethylenediaminetetraacetic acid
EGTA.....	ethylene glycol bis (2-aminoethyl ether)-N,N,N'N'-tetraacetic acid
ERV.....	endogenous retroviral
EST.....	expression sequence tag
EYFP.....	enhanced yellow fluorescent protein
FBS.....	fetal bovine serum

GST	glutathione-S-transferase
HA	influenza hemagglutinin
HDAC	histone deacetylase
HGPIN	high-grade PIN
HIPK	homeodomain-interacting protein kinase
HPC	hereditary prostate cancer
IHC	immunohistochemical
IPTG	isopropyl-1-thio- β -D-galactopyranoside
K_d	dissociation constant
LGPIN	low-grade PIN
LOH	loss of heterozygosity
Lux	luciferase
MBP	maltose-binding protein
mRNA	messenger RNA
NLS	nuclear localization signal
NMTS	nuclear matrix targeting sequence
PAGE	polyacrylamide gel electrophoresis
PAS	protein A sepharose
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDEF	prostate-derived Ets factor
PIN	prostatic intraepithelial neoplasia
PKC	protein kinase C

PMSF	phenylmethylsulphonylfluoride
PSA	prostate-specific antigen
PVDF	polyvinylidifluoride
qRT-PCR.....	quantitative real-time PCR
ROS.....	reactive oxygen species
RPM	revolutions per minute
RT-PCR.....	reverse transcriptase PCR
S/MAR	scaffold/matrix attachment region
SAAB.....	selective amplification and binding
SBP	spermine-binding protein
SDS	sodium dodecyl sulphate
SMGA.....	smooth muscle gamma actin
SRF	serum response factor
TGCT	testicular germ cell tumor
TR	testosterone replacement
TSA.....	trichostatin A
UGM	urogenital mesenchyme
UTR.....	untranslated region
X-GAL	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

CHAPTER I
Nkx3.1 and Prostate Cancer

1.1 Prostate cancer

Prostate cancer is the most commonly diagnosed cancer in American men, excluding basal and squamous skin cancers (Jemal et al., 2004). In the United States, approximately 230,000 men were diagnosed with prostate cancer in 2004. These cases comprised one third of all non-skin cancers diagnosed in American males in 2004. Roughly, one in six American men will develop prostate cancer in his lifetime. Prostate cancer is also the second leading cause of male cancer deaths in the U.S. Approximately 30,000 American men died from prostate cancer in 2004. These deaths represent 10% of all cancer related deaths in American men in 2004, and worldwide approximately 200,000 men succumb to prostate cancer annually (Parkin et al., 2001).

Three epidemiological observations regarding prostate cancer are worth noting. First, there is a strong relationship between incidence of prostate cancer and age (Ross et al., 1998). This explains in part why developed countries with higher life expectancies, such as the U.S., incur a disproportionately large number of prostate cancer cases and deaths. Over 70% of prostate cancers are detected in men over the age of 65. Prostate cancer is extremely rare before age 50, but it is still the most commonly diagnosed cancer in American men due largely to the increased rate of incidence with age. Secondly, there are profound variations of incidence between international and racial-ethnic groups. African-Americans are up to 80 times more likely to develop prostate adenocarcinoma than native Japanese and Chinese populations (Ross et al., 1996). Thirdly, there is an occurrence of occult, subclinical prostate cancer at a relatively comparable prevalence across populations (Yatani et al., 1982).

1.2 Prostate anatomy and tumor classification

The prostate gland is located posterior to the bladder and surrounds the urethra (Figure 1). The necessity of the prostate gland is debatable, but it is believed that the prostate is not an absolute requirement for fertility. The prostate gland produces seminal fluid proteins that enhance sperm motility. The prostate is composed of branching glands, with ducts lined with secretory epithelial cells and basal cells (McNeal, 1988). A stroma comprised of fibroblasts, smooth muscle, nerves, and lymphatics surround the prostate gland, and interactions between stroma and epithelium are important for normal prostate development, maintenance as well as prostate cancer (Chung, 1995). The secretory epithelial cells constitute the major cell type in the gland and are androgen dependent for growth (Issacs, 1983). Basal cells are not androgen dependent and provide the stem cell population for the epithelium. Over 95% of prostate cancers are adenocarcinomas that arise from prostatic epithelial cells.

The human prostate divides into four anatomical zones (McNeal, 1981) (Figure 2). The relationship of each to the urethra provides a central anatomic reference point. The peripheral zone constitutes over 70% of the glandular prostate. It forms a disc of tissue whose ducts radiate laterally from the urethra lateral and distal to the verumontanum. Almost all prostatic carcinomas arise here. The central zone constitutes 25% of the glandular prostate. Its ducts arise close to the ejaculatory duct orifices and follow these ducts proximally, branching laterally near the prostate base. Its lateral border fuses with the proximal peripheral zone border, completing in continuity with the peripheral zone, a full disc of secretory tissue oriented in a coronal plane. The transition zone is comprised of two small lobules that surround the urethra and is the region where

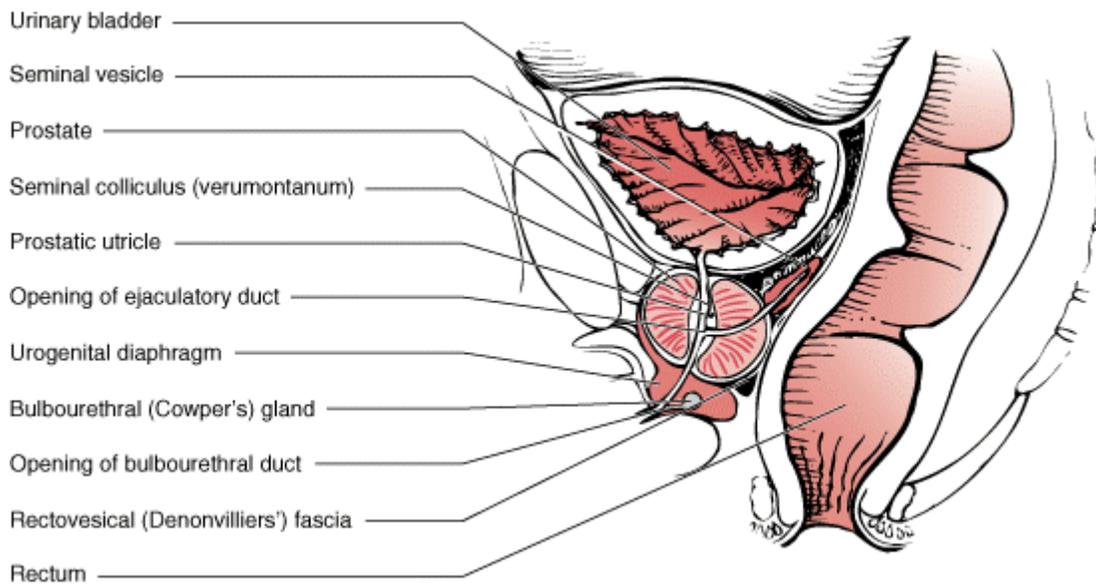


Figure 1. Normal human prostate anatomy. (adapted from Holland-Frei, Cancer Medicine 6th edition, 2003; Figure 111-3).

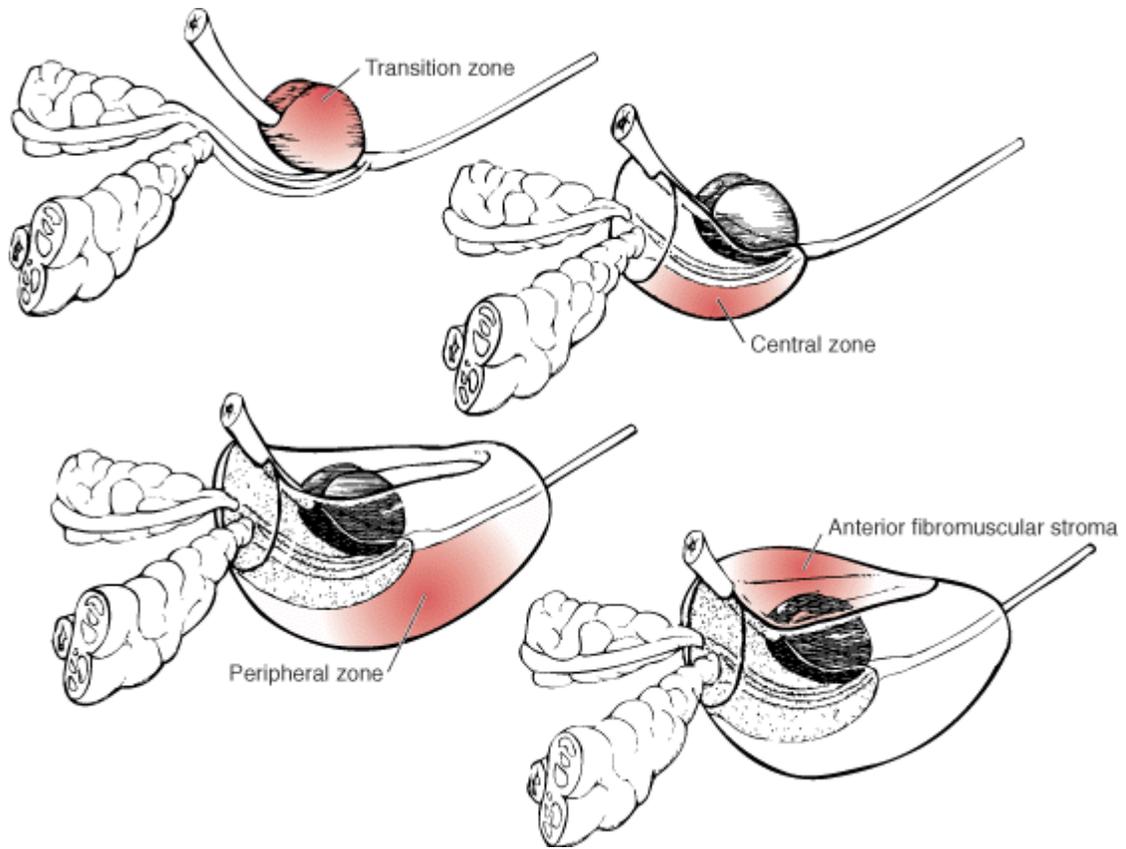


Figure 2. Zonal anatomy of the human prostate. (adapted from Holland-Frei, Cancer Medicine 6th edition, 2003; Figure 111-4).

benign prostatic hyperplasia (BPH) primarily originates. The anterior fibromuscular stroma forms the entire anterior surface of the prostate as a thick, nonglandular apron, shielding from view the anterior surface of the three glandular regions.

The most widely accepted grading scheme for adenocarcinoma of the prostate is that developed by Gleason (Gleason, 1966). The Gleason system recognizes the prognostic importance of histological differentiation of prostate adenocarcinoma. The Gleason system classifies prostate tumors on two levels of scoring. Primary differentiation pattern is assigned a score one to five based on specimen morphology and its departure from normal appearance. Secondary differentiation patterns are likewise scored one to five, and the two scores are summed for a final score. There is a clear association between a higher Gleason score and higher mortality (Gleason, 1974). While other indicators are used to assess prostate tumors, including blood prostate-specific antigen levels (PSA), clinical stage, tumor volume, and DNA content (ploidy), currently the Gleason score remains the most applied and useful histological grading system.

Prostatic intraepithelial neoplasias (PIN) are thought to be precursors of prostate cancer because they are commonly observed adjacent to prostate carcinomas and share many of the same cytogenetic abnormalities as their adjacent carcinomas. PIN lesions are defined cytologically as atypical or dysplastic epithelial cells arranged focally within prostate glands or ducts. There are three grades of PIN lesions: one (mild or low-grade), two (moderate), and three (severe). Grades two and three are collectively referred to as high-grade PIN (HGPIN) and grade one is commonly called low-grade PIN (LGPIN).

1.3 Prostate cancer initiation and progression

Prostate cancer, like other solid tumors, is thought to arise by a multi-step process involving successive genetic events that render a normal cell increasingly neoplastic. The genetic events associated with the etiology of prostate cancer are poorly understood compared to other epithelial cancers, *e.g.*, breast and colorectal cancers, where major susceptibility genes have been discovered. However, a number of genetic events have been implicated in prostate cancer initiation and progression to adenocarcinoma, and these events provide insights into genes that play a crucial role in prostate tumor formation.

Prostate tumors are derived from secretory luminal epithelial cells, which constitute the largest epithelial cell population in the prostate (Figure 3). In a normal prostate gland, these luminal epithelial cells are androgen-dependent, terminally differentiated and produce secretory proteins that are believed to aid fertility. With time however, genetic changes have been noted to exert a less differentiated phenotype on these cells. Among these early genetic events are hypermethylation of the *GSTP1* promoter and loss of human chromosomal region 8p (reviewed in Abate-Shen and Shen, 2000 and Porkka and Visakorpi, 2004). These changes convert normal prostatic epithelium to lesions, termed prostatic intraepithelial neoplasia or PIN, thought to represent the primary precursor of human prostate cancer. PIN lesions can be found in men in their twenties and is commonly found in men over the age of fifty.

Several lines of evidence implicate PIN lesions as precursors of human prostate cancer (reviewed in Abate-Shen and Shen, 2000). First, PIN lesions are found in immediate proximity to invasive prostate carcinomas. Second, the appearance of PIN

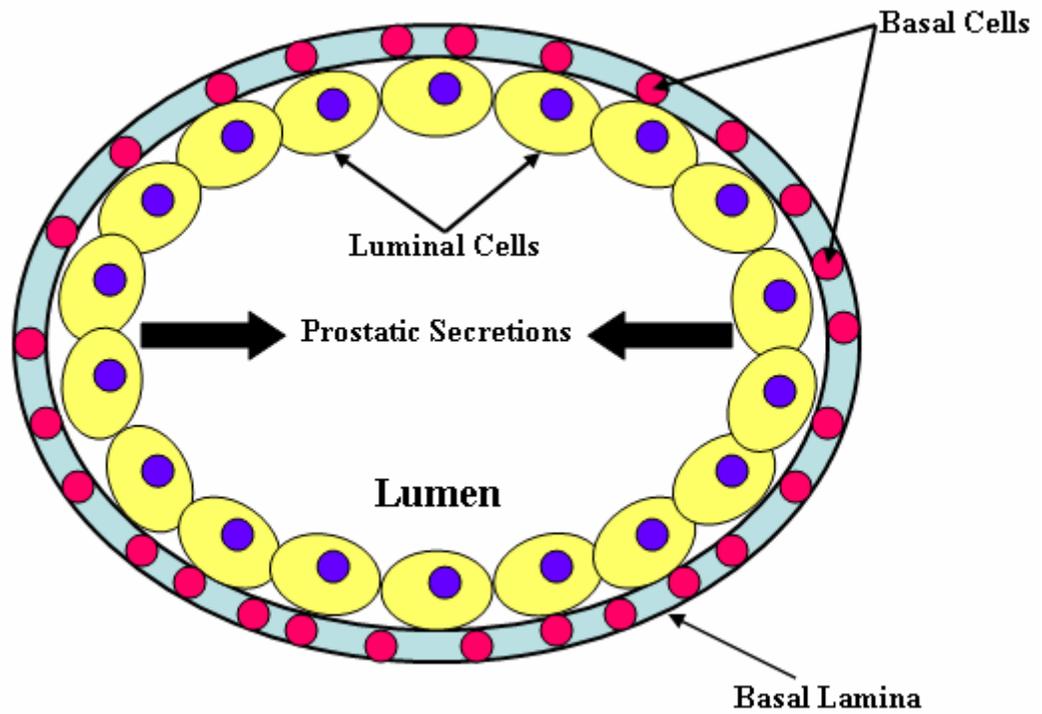


Figure 3. Schematic depiction of the cell types within a human prostatic duct.

lesions precedes the development of prostate carcinoma by at least ten years. Third, phenotypic changes associated with PIN lesions are also found in prostate carcinoma. For example, changes in the expression of certain markers of differentiation, such as E-cadherin, are seen in both PIN lesions and prostate carcinoma. Finally, architectural and cytological features of PIN lesions closely mimic that of prostate carcinoma. Yet, despite these similarities PIN lesions are distinguishable from their malignant counterparts. PIN lesions have an intact basement membrane and do not invade the stroma, unlike prostate carcinoma. Also, PIN lesions do not produce high levels of prostate-specific antigen (PSA), a member of the kallikrein family of serine proteases and whose elevation in blood serum levels can be indicative of prostate carcinoma. Histologically, the loss of basal epithelial cells in PIN lesions leads to complete loss of the basal layer noted in full-blown carcinoma leading to local invasion of surrounding stroma and seminal vesicles. A characteristic genotypic difference that marks the transition from PIN to prostate carcinoma is the loss of human chromosome regions 10q and 13q. Other genetic changes follow, including loss of human chromosome 17p, gain of chromosome 8q, and mutation or amplification of the *androgen receptor (AR)* gene. Androgen-independent cell growth resulting from genetic alterations of *AR* is a critical step as prostate carcinomas progress towards metastases. These metastases generally localize to the bone, where it is almost always fatal.

1.4 Genes involved in hereditary prostate cancers

Prostate cancer is not a member of a hereditary cancer syndrome (Porrka and Viskorpi, 2004), and only 10% of prostate cancers are believed to be hereditary. This makes the

analysis of familial prostate cancers difficult since the incidence of sporadic cases is frequent enough to obscure results amongst familial clusters (Bott et al., 2005). Despite these difficulties several susceptibility loci for hereditary prostate cancers (HPCs) have been reported, such as 1p36, 1q24-q25, 1q42.4-q43, 8p22-23, 16q23, 17p12-p13, 19q13, 20q13, and Xq27-q28. One or more of these regions may harbor high-penetrance prostate cancer susceptibility genes (Reviewed in Porrka et al., 2004). Table 1 displays a list of genes involved in both hereditary and spontaneous prostate cancers.

HPC1/RNASEL

The first reported prostate cancer susceptibility locus, HPC1, was found on human chromosome 1 (Smith et al., 1996). The HPC1 locus harbors *RNASEL*, an antiviral, pro-apoptotic, and interferon-activated RNase L. Prostate tumors carrying germline mutations of this gene also showed a loss of the wild-type allele and reduced *RNASEL* enzyme activity, suggesting a tumor suppressor role for the HPC1 gene product. Several truncating mutations segregate independently within individual “HPC1-families” (Carpten et al., 2002). *RNASEL* mutations are associated with increased risk for prostate cancer and early onset (before age 65). A single nucleotide polymorphism (SNP; Arg462Gln) reduces *RNASEL* enzymatic activity three-fold compared with the wild-type enzyme and this polymorphism has been linked to increased risk of prostate cancer (reviewed in Dong, 2006).

Table 1
Genes Involved in Prostate Cancer

A list of genes involved in hereditary and spontaneous human prostate cancers. The human chromosomal location and cellular function of each gene and its gene product are listed. Each gene is classified as an oncogene or tumor suppressor gene based on gain of function/loss of function studies. In addition, each gene is classified as homozygous or hemizygous based on the minimum number of alleles affected in human prostate tumor progression. Each gene is also listed as early or late indicating whether inactivation or overexpression occurs early or late during prostate cancer development.

<u>Gene</u>	<u>Human chromosome</u>	<u>Function</u>	<u>Onc./TS</u>	<u>inactivation</u>	<u>early/late</u>
HPC1/RNASEL	1q25	RNase	TS	homozygous	early
HPC2/ELAC2	17p12	DNA repair	TS	homozygous	early
MSR1	8p22	membrane glycoprotein	TS	homozygous	early
BRCA1	17p21	cell cycle control	TS	homozygous	early
BRCA2	13q12	DNA repair	TS	homozygous	early
NKX3A	8p21	transcription factor	TS	hemizygous	early
RB	13q14	cell cycle control	TS	homozygous	late
PTEN	10q23	phosphatase	TS	hemizygous	early/late
P53	17p13	transcription factor	TS	hemizygous	late
CDKN1B	12p12	CDK inhibitor	TS	hemizygous	late
CDKN2	9p21	CDK inhibitor	TS	homozygous	late
KLF6	10p13	transcription factor	TS	hemizygous	early
CDH1	16p22	cell-cell adhesion	TS	hemizygous	early/late
CTNNA1	5q22	cell-cell adhesion	TS	homozygous	early
CTNNB1	3p22	cell-cell adhesion	Onc	hemizygous	late
MYC	8q24	transcription factor	Onc	hemizygous	early
AR	Xq11	transcription factor	Onc	hemizygous	early
SRD5A2	2p23	androgen metabolism	Onc	hemizygous	early
BCL2	15q24	cell survival	Onc	hemizygous	late
GSTP1	12q13	ROS metabolism	TS	homozygous	early

CDK- cyclin-dependent kinase

ROS- reactive oxygen species

Onc.- oncogene

TS- tumor-suppressor gene

HPC2/ELAC2

The first cloned prostate cancer susceptibility gene, *ELAC2*, was isolated from the HPC2 locus. *ELAC2* is located at human chromosome 17p12 (Tavtigian et al., 2001) and encodes a protein involved in DNA interstrand-cross link repair. Two missense variants, Ser217Leu and Ala541Thr, do not seem to alter *ELAC2* enzymatic activity but are associated with increased risk for prostate cancer. More common polymorphisms appear to be associated with risk of prostate cancer in certain populations (reviewed in Simard et al., 2003), and it is estimated that such polymorphisms may be associated with 2% of all prostate cancers (Camp and Tavtigian, 2002).

MSR1

Another prostate cancer susceptibility gene identified in prostate cancer families is the *macrophage scavenger receptor 1 (MSR1)* gene, located at human chromosome 8p22-23. Segregating germline mutations in *MSR1* were reported in 13 prostate cancer families (Xu et al., 2002). *MSR1* expression is induced in macrophages by oxidative stress. Macrophage scavenger receptors are trimeric membrane glycoproteins that bind, internalize and process negatively charged macromolecules and most likely regulate reactive oxygen intermediates. Mutations in *MSR1*, especially truncating mutations, have been shown to be associated with prostate cancer risk in both familial and sporadic cases.

BRCA1/BRCA2

BRCA1 was believed initially to be linked to familial prostate cancers. *BRCA1* is located at human chromosome 17p21 and a linkage has been found between the 17p21-

22 region and hereditary prostate cancers. However, patients carrying inactivating *BRCA1* mutations were shown to be at minimally increased risk for prostate cancer (Thompson and Easton, 2002). Additionally, similar *BRCA1* mutations have not been found in high-risk prostate cancer families. Therefore, it is believed that any role *BRCA1* may play in prostate cancer is limited.

The *BRCA2* gene, on the other hand, has been consistently shown to play a role in prostate cancer. The *BRCA2* gene product functions in cell cycle control and DNA repair. The link between *BRCA2* and prostate cancer was first observed in breast-ovarian cancer families with *BRCA2* mutations (Friedensen, 2005). Founder mutations have been discovered in Ashkenazi Jews and certain Scandinavian populations, and these mutations are significantly associated with prostate cancer risk (Kirchhoff et al., 2004). *BRCA2* mutations are particularly significant in prostate cancers diagnosed at a younger age (early fifties; Edwards et al., 2003). Although *BRCA2* mutations have not been discovered in sporadic prostate cancers, they are estimated to contribute to 35% of familial prostate cancer cases.

Other susceptibility loci have been discovered, but the genes at these loci remain undefined. A susceptibility gene has not as yet been identified at human chromosome 17p22, the locus originally thought to be linked to *BRCA1*. Other susceptibility loci with yet-to-be identified genes include PCAP (1q42-q43), HPCX (Xq27-q28), CAPB (1p36), HPC20 (20q13), and 16q23.

1.5 Genes involved in sporadic prostate cancers

Sporadic cancers arise from genetic changes incurred by normal cells, and chromosomal loss is one of the most frequent genetic alterations in cancer (Dong, 2001). One of the most effective tools used to analyze chromosomal abnormalities is comparative genomic hybridization (CGH), a technique that detects losses and gains of chromosomal DNA. CGH studies have revealed two critical aspects important to understanding the genesis of sporadic cancers of the prostate (Visakorpi, 1995). First, chromosomal deletions are much more common than gains or amplifications. Secondly, chromosomal losses are detected very early in prostate carcinogenesis while gains and amplifications are observed in the later, hormone-refractory stages of prostate cancer progression. This indicates that inactivation of tumor suppressor genes, presumably carried by deleted chromosomal regions, is a critical early step in the development of prostatic neoplasia.

The loss of a chromosomal region may lead to one of several outcomes, each conferring a different set of consequences on the fate of the affected cell. A regional genetic loss may occur in one chromosome but it is repaired using the sister chromosomal region as a template and thus occurring without a quantitative loss of genetic material. On the other hand, loss of heterozygosity in this instance could reveal formerly recessive mutations that effectively inactivate a given gene. Another potential outcome is a hemizygous deletion with no repair, resulting in a quantitative genetic loss. In addition to revealing a potential recessive mutation, this type of LOH could also lead to

haploinsufficiency. Haploinsufficiency is defined as insufficient gene expression resulting from genetic loss of one allele independent of the genotype of the retained allele. Therefore, haploinsufficiency can induce a significant phenotypic effect even if a retained allele is wild-type. A third type of deletion is homozygous deletion, where both chromosomes are deleted for a given locus. Homozygous deletions completely abolish gene function.

Comparative genomic hybridization analysis has revealed several chromosomal regions commonly lost in prostate cancers, including 6q, 8p, 10q, 13q, 16q, and 18q as well as those regions commonly gained or amplified in prostate tumors such as 7p, 7q, 8q, and Xq (reviewed in Dong, 2001 and Porkka and Visakorpi, 2004). The chromosomal regions gained or lost in many prostate tumors have been mined for putative oncogenes or tumor suppressor genes that reside within the affected regions.

NKX3A

The most frequently deleted chromosomal region in prostate cancers using CGH analysis is 8p (reviewed in Dong, 2001). One study suggests that 8p deletions may be present in as many as 80% of prostate cancers (Cher, et al., 1996). A minimally deleted region at 8p21 has been defined, suggesting that the putative tumor suppressor gene is found at 8p21. The strongest candidate is *Nkx3.1*, a prostate-specific gene with homology to the *Drosophila* NK homeodomain transcription factor family (He et al., 1997). *Nkx3.1* is the focus of this thesis and will be discussed in greater detail below.

RBI

The second most frequently deleted chromosomal region (50%) in prostate cancer is 13q (Cooney et al., 1996). Three distinct sub-regions of allelic loss have been detected in prostate cancers, 13q14, 13q21-22, and 13q33 (Hyytinen et al., 1999). The strongest tumor suppressor candidate for the 13q14 loss is the retinoblastoma gene, *RBI*. The retinoblastoma gene product, pRb, regulates cell cycle progression by blocking entry into S-phase. pRb is also thought to regulate apoptosis in prostate cells, especially in response to androgen depletion (Bowen et al., 1998). Reintroduction of *RBI* into an Rb-negative prostate adenocarcinoma cell line, DU145, resulted in reduced tumorigenicity (Bookstein et al., 1990). Additionally, homozygous loss of *RBI* results in prostatic dysplasia and invasive prostate carcinoma in *RBI* knockout mice (Wang et al., 2000). Although *RBI* mutations have been found in localized and invasive prostate carcinomas (Ittmann, 1996), several other studies have shown that *RBI* mutations are rare in prostate cancer (reviewed in Elo and Visakorpi, 2001).

PTEN/MMAC1

Up to 50% of sporadic prostate cancers carry deletions of human chromosome 10q (reviewed in Bott et al., 2005), and cytogenetic and CGH analyses revealed that chromosomal region 10q23 was likely to be the location of a putative tumor suppressor gene. Subsequent studies identified the Phosphatase and Tensin homologue/Mutated in Multiple Advanced Cancers gene *PTEN/MMAC1* as the critical gene lost in patients with 10q23 deletions (Cairns et al., 1997). *PTEN* encodes a dual specificity phosphatase that functions as a lipid phosphatase, targeting phosphatidylinositol 3,4,5-triphosphate (PIP3).

Dephosphorylation of PIP3 results in the downregulation of the Akt/PKB signaling pathway. Akt/PKB kinase activity promotes cell survival, mitosis, and angiogenesis and inhibits apoptosis (Fernandez et al., 2002, Sun et al., 1999, Zhong et al. 2000). Several lines of evidence implicate PTEN in prostate carcinogenesis (reviewed in Abate-Shen and Shen, 2000). First, *PTEN* was mutated in the first four prostate cancer cell lines examined and is frequently lost in prostate cancer cell lines. Second, *PTEN* undergoes homozygous deletion in ~10% of primary prostate tumors. Third, *PTEN* alterations are more common in prostate adenocarcinomas than in primary prostate cancers. Fourth, immunohistochemical (IHC) staining shows reduced PTEN expression in primary prostate tumors and xenografts. Fifth, loss of PTEN at gene and expression levels has been reported in 25-33% of prostate adenocarcinomas and hormone-refractory carcinomas (Cairns et al., 1997). Finally, *PTEN* heterozygous mice develop prostatic epithelial hyperplasia and dysplasia (Di Cristofano et al., 1998), suggesting a role for haploinsufficiency, and *PTEN* deletions induce prostate cancer in conventional and conditional knockout mice (Wang et al., 2003). Despite a lack of reported *PTEN* mutations in primary prostate tumors, the evidence for a central role for *PTEN* in prostate cancer development is compelling.

P53

Allelic loss at human chromosome 17p13 occurs in 50% of advanced prostate cancers and metastases (Cher et al., 1996). The region deleted harbors the *p53* locus, and does not involve the closely linked *BRCA1* gene (Brooks et al., 1996). Loss of *p53* is perhaps not surprising since it is one of the most commonly inactivated genes in human

cancer. *p53* encodes a key regulator of the G1/S and G2/M transitions and plays a key role in the induction of DNA repair genes. Upon genetic insult, *p53* will either induce growth arrest and DNA repair or initiate apoptosis, depending on the extent of the stress. Wild-type *p53* protein degrades quickly in normal cells, but mutated *p53* alleles give rise to a more stable protein that accumulates in the nucleus. Nuclear accumulation of mutated forms of *p53* can generally be detected by IHC, and the induction of IHC-positive prostate cells correlates well with high-grade prostate cancers (reviewed in Bott et al., 2005). In fact, *p53* mutations can be detected in at least 20% of advanced prostate carcinomas (Bookstein et al., 1993) and maybe as many as 70% (Meyers et al., 1998); however, the overall *p53* mutation rate is lower in prostate cancers than in many other cancers such as colon, lung, brain, breast, and bladder where *p53* is mutated in over 50% of the cases studied. Indeed, Li-Fraumeni cancer syndrome patients, who harbor germline *p53* mutations, have a low incidence of prostate cancer (Kleihues et al., 1997), although it is possible they succumb to other tumors before developing prostate cancer. Given its association with advanced disease, *p53* mutation is more likely to be a compounding event that occurs late in the progression of prostate cancer.

CDKN1B (p27/kip1)

Deletions of human chromosome 12p12-13 are found in prostate cancers, suggesting that one or more a tumor suppressor genes reside in this region (Dong, 2001). *CDKN1B* is the best candidate, given its role in cell cycle regulation. *CDKN1B* encodes p27, a cyclin-dependent kinase (CDK) inhibitor that impedes the phosphorylation of pRb, thus blocking cell cycle progression. Loss of p27 expression occurs frequently in many

carcinomas, including breast, colon and prostate (reviewed in Macri and Loda, 1999). Additionally, p27 is inactivated by altered subcellular localization conferred by aberrant phosphorylation and/or ubiquitinylation (reviewed in Abate-Shen and Shen, 2000). Loss of p27 activity is frequently observed in advanced prostate cancers (Kibel et al., 2001), and p27 inactivation correlates with tumor grade (Guo et al., 1997). Mutations of *CDKN1B* are rare in human cancers (Bott et al., 2005), however several studies suggest that haploinsufficiency for *CDKN1B* is oncogenic (reviewed in Dong, 2006). *CDKN1B* homozygous nulls and hemizygous mice develop hyperplastic prostates and are predisposed to tumors in multiple tissues. Analyses of hemizygotes revealed that the remaining *CDKN1B* allele is neither mutated nor silenced. Interestingly, inactivation of one *PTEN* allele and one or both *CDKN1B* alleles accelerates spontaneous prostate tumor formation in mice; all *PTEN* +/-; *CDKN1B* -/- develop spontaneous tumors by three months of age (Di Cristofano, 2001).

CDKN2 (p16/ink4A)

Another CDK inhibitor frequently inactivated in prostate cancers is *CDKN2* (Jarrard et al., 1997). The *CDKN2* gene product, p16, also blocks pRb phosphorylation and inhibits cell cycle progression. Loss of p16 also facilitates the bypassing of senescence in many cell types, including prostate epithelial cells (Jarrard et al., 1999). Deletions of human chromosome 9p21, which harbors *CDKN2*, are found in 20-45% of prostate cancers. *CDKN2* mutations are rare in primary prostate cancers, but are found more commonly with advanced metastatic disease (reviewed in Bott et al., 2005). *CDKN2* is inactivated by either mutation or deletion in 40-60% of advanced prostate

carcinomas (reviewed in MacGrogan and Bookstein, 1997). Jarrard et al. (1997) suggest that p16 may be inactivated by deletion of one allele and silencing via methylation at the remaining allele. Oddly, increased p16 expression has been observed in some recurrent prostate cancers (Halvorsen et al., 2000) and in aggressive ovarian cancers (Dong et al., 1997). Reasons for this anomalous pattern of p16 expression are not obvious.

KLF6

KLF6 is a zinc-finger transcription factor that restricts cell proliferation in cell culture studies (Chen et al., 2003). Allelic loss and mutation of the remaining allele was detected in about half of primary prostate carcinoma samples (Narla et al., 2001), although other studies estimate the mutation frequency to be somewhat lower. Germline *KLF6* mutations are rare. However a germline *KLF6* polymorphism has been detected that confers an increased risk for prostate cancer in a study of 3,411 men (Narla et al., 2005). This polymorphism results in increased transcription of three alternatively spliced KLF6 isoforms that mislocalize to the cytoplasm and antagonize wild-type KLF6 function. Much work remains to elucidate what role KLF6 may play in prostate cancer development.

CDH1 (E-cadherin)

Allelic losses of human chromosome 16q, specifically 16q22-24, alerted investigators to a potential role for the cell adhesion protein E-cadherin (Latil et al., 1997). E-cadherin is a member of a transmembrane glycoprotein family that regulates cell-cell adhesion in a Ca²⁺-dependent manner. E-cadherin expression correlates with

epithelial differentiation whereas loss of E-cadherin expression is associated with epithelial de-differentiation and invasiveness of human carcinomas (Vleminckx et al., 1991). Decreased E-cadherin expression is associated with high-grade prostate carcinomas (Latil et al., 1997) and metastatic potential in primary prostate carcinomas (Richmond et al., 1997). Half of primary and metastatic prostate cancers showed reduced or absent E-cadherin IHC staining, while all benign and non-malignant tissues stained uniformly positive (Umbas et al., 1992). Somatic *CDHI* mutations have not as yet been detected. Reduced or complete loss of expression stems from 16q chromosomal loss, frequently seen in late-stage prostatic disease, or from gene silencing by hypermethylation of the *CDHI* promoter (Graff et al., 1995).

CTNNA1/CTNNB1 (α/β -catenins)

Alpha- and beta-catenins form part of the same cell adhesion mechanism with E-cadherin. Decreased α -catenin expression was observed in PC3 human prostate carcinoma cells and was linked to a homozygous deletion of the *CTNNA1* gene at human chromosome 5q (Ewing et al., 1995). Restoration of α -catenin expression in PC3 cells reduced their tumorigenicity in nude mice. Beta-catenin has another important role in addition to cell adhesion. Beta-catenin interacts with the Tcf and Lef transcription factors as part of the Wnt signaling pathway. Activating *CTNNB1* mutations stabilize β -catenin, leading to its nuclear accumulation, Tcf/Lef activation, and the promotion of cell growth. Such *CTNNB1* mutations are found in about 5% of prostate carcinomas (Voeller et al., 1998).

C-MYC

Gain of human chromosome 8q is the most common chromosomal aberration in hormone-refractory and metastatic prostate carcinoma detected by CGH (Visakorpi et al., 1995). Almost 90% of prostate adenocarcinomas and hormone-refractory carcinomas showed 8q gain, as opposed to only 5% of primary prostate tumors (Alers et al., 2000). The critical region of 8q has been identified as 8q24 (Cher et al., 1996), and *c-myc* is the likely oncogene involved. Myc is a transcription factor that is activated in many human cancers (reviewed in Pelengaris et al., 2002), promoting cell cycle progression and DNA replication. Primary tumors generally have only 8q gain, but metastases more commonly also have *c-myc* amplification (reviewed in Dong, 2006). Indeed, two commonly used prostate tumor cell lines, LNCaP and PC-3, have significant *c-myc* amplification and overexpression. Overexpression of myc in the mouse ventral prostate leads to lesions similar to PIN in humans (Zhang et al., 2000), and one overexpression study reported induction of prostate carcinoma (Ellwood-Yen et al., 2003).

AR

The prostate is an androgen-regulated organ, and this had led to long-standing interest in the role of androgen signaling in prostate cancer development. Androgens are essential for normal development of the prostate as well as its growth and maintenance. Androgen signaling is mediated by the androgen receptor (AR), a member of the ligand-dependent transcription factor superfamily. Androgens, namely testosterone and dihydrotestosterone, specifically bind and activate AR. Activated AR translocates to the nucleus where it targets gene regulation through specific DNA sequences known as

androgen response elements (ARE). Huggins and Hodges (1941) observed that androgen ablation by castration led to effective tumor regression by inducing massive apoptosis of androgen-dependent carcinoma cells. Androgen-ablation remains the most effective therapeutic tool for combating prostate carcinoma, although today androgens are largely inhibited by pharmaceutical agents such as flutamide and bicalutamide. Androgen ablation therapy, however, frequently leads to the recurrence of highly aggressive and metastatic prostate cancers that are androgen-independent. The transition to androgen independence is believed to result from growth selection for androgen-independent cells that coexist within heterogeneous prostate cancers prior to androgen deprivation (Issacs and Coffey, 1981). Initially, it was believed that androgen-independent prostate cancers were caused by loss of AR expression because AR is not expressed in many aggressive and metastatic rodent and human cancer cell lines (reviewed in Abate-Shen and Shen, 2000). However, it was shown subsequently that AR is expressed in primary prostate cancers, recurrent locally invasive tumors, and even in metastases. From these observations, it was evident that androgen-independent prostate carcinomas circumvented the requirement for androgens by a mechanism other than loss of AR expression. Other studies observed a number of genetic changes that resulted in aberrant androgen signaling. *AR* is amplified in about 30% of all hormone-refractory prostate cancers, although it is rarely amplified in untreated primary cancers. These results suggest that *AR* amplification is selected for following androgen withdrawal (reviewed in Elo and Visakorpi, 2001). However, almost all hormone refractory prostate tumors expressed abnormally high levels of AR even in the absence of *AR* amplification (Linja et al., 2001). The mechanism for AR overexpression in these tumors remains unknown.

Somatic *AR* mutations that result in aberrant androgen signaling have been identified in prostate tumor cells. *AR* is frequently mutated within the hormone-binding domain in both cell-lines and primary tumors, altering the androgen requirement for AR activation (Veldscholte et al., 1992, Elo et al., 1995). Mutations throughout the *AR* coding region, both in primary tumors and in hormone refractory cancers have also been noted (reviewed in Abate-Shen and Shen, 2000). Germline *AR* mutations are rare, but alleles carrying a truncation of a CAG microsatellite repeat have been linked to reduced efficacy of androgen ablation therapy and earlier onset of advanced cancers (Bratt et al., 1999).

Of related interest is the *SRD5A2* gene which encodes 5 α -reductase type II, the enzyme responsible for converting testosterone to its more active metabolite dihydrotestosterone. Certain *SRD5A2* polymorphisms result in increased 5 α -reductase activity and elevated dihydrotestosterone levels, both associated with increased risk of prostate cancer (Jaffe et al., 2000). Prostate cancers deficient in 5 α -reductase activity have not as yet been detected, and this enzyme is currently being investigated as a possible target for prostate cancer chemoprevention.

Bcl-2

Bcl-2 is an anti-apoptotic protein that inhibits the release of cytochrome c from the mitochondria, thus preventing the initiation of caspase cascades that bring about cell death. In normal prostates, Bcl-2 expression is restricted to basal cells but a significant number of PIN lesions and primary prostate cancers were shown to be positive for Bcl-2 expression, and all local and metastatic cancers post-androgen depletion stained positive for Bcl-2 (Colombel et al., 1993). This suggests that aberrant Bcl-2 expression is also

selected after androgen ablation. Bcl-2 overexpression is a characteristic of advanced and hormone-refractory prostate cancer, and is believed to help dramatically reduce apoptosis in these tumors (McDonnell et al., 1997). Bcl-2 overexpression also confers drug resistance to anti-cancer drugs such as cisplatin, paclitaxel, and methotrexate (Tu et al., 1995).

GSTP1

The most commonly altered gene in prostate cancer detected thus far is *glutathione S-transferase class pi (GSTP1)* (reviewed in Porkka and Visakorpi, 2004). Glutathione S-transferases catalyze the fusion of glutathione with harmful, electrophilic molecules and reactive oxygen species (ROS), protecting cells from these DNA-damaging carcinogens. Silencing of the *GSTP1* promoter by methylation has been detected in nearly 95% of prostate carcinomas and in 70% of PIN lesions, suggesting that *GSTP1* silencing is an early event in prostate tumorigenesis. Hypermethylation of the *GSTP1* promoter is a rare event in benign prostate tissues (reviewed in Bott et al., 2005). Mutations of *GSTP1* are also rare, but there are several *GSTP1* polymorphisms that have been linked with increased risk of prostate cancer (reviewed in Dong, 2006).

1.6 Cloning and characterization of Nkx3.1

Loss of heterozygosity at human chromosome 8p21 is an early and frequent genetic event in prostate carcinogenesis. LOH at 8p12-21 has been noted in approximately 60% of PIN lesions and 90% of prostate tumors (reviewed in Dong, 2001), and this observation prompted researchers to identify the gene or genes targeted by 8p12-

21 deletion. Capitalizing on patients carrying small deletions, the target region was eventually delimited to a 2 Mb region of chromosome 8p21-22. Several genes map to this region, including *N33*, *FEZ1/LZTS1*, and *EPB49*, and each was examined for its potential role in prostate tumorigenesis. None of these genes, however, presented themselves as obvious candidates.

The strongest candidate for the 8p target gene is *Nkx3.1*. *Nkx3.1* is a member of the NK subfamily of homeodomain genes, a group of genes defined by a 60-amino acid DNA-binding domain termed the homeodomain. NK family members have been implicated in cell fate specificity and organogenesis of many species, including *Drosophila*, in which they were first identified by Kim and Nirenberg (1989). *Nkx3.1* is one of two mammalian orthologues of *Drosophila NK-3*, the other being *Nkx3.2*.

A human *Nkx3.1* cDNA was cloned using a high-throughput cDNA sequencing approach in which 8,825 expressed sequence tag (EST) cDNA clones were sequenced from three libraries made from both normal and malignant prostate tissues (He et al., 1997). Nine copies of a particular EST were identified amongst the prostate ESTs, but not amongst over 275,000 non-prostate, tissue-specific ESTs that were also analyzed. The longest of these nine ESTs was used to probe a human genomic library and two normal prostate cDNA libraries. Sequence analysis of resulting clones revealed an open reading frame encoding a 234-amino acid protein that included a homeodomain with 77% identity to the *Drosophila NK-3* homeodomain. The human *Nkx3.1* cDNA was cloned independently and concurrently by Prescott et al. (1998) using differential display and cDNAs generated from untreated prostate cancer cells (LNCaP) and cells that were treated with a synthetic, metabolically stable androgen analog (mibolerone).

Murine *Nkx3.1* was initially cloned from a genomic library probed with a human cDNA fragment isolated from a prostate carcinoma library (Bieberich et al., 1996 and Sciavolino et al., 1997). Sequencing of the isolated mouse clone revealed an open reading frame that included a homeodomain sharing 100% amino acid identity with the homeodomain encoded by the 1.6-kb human cDNA probe. Sequence analysis of the mouse genomic clone revealed that the *Nkx3.1* coding region is divided into two exons. Exon 1 harbors the first 96 amino acid residues, with the remaining residues (97-234), including the entire homeodomain, are located within exon 2. A murine *Nkx3.1* cDNA was identified by screening an embryonic cDNA library (Sciavolino et al., 1997). Sequence analysis of the *Nkx3.1* cDNA unearthed a short 5'- untranslated region (UTR) of only 24 nucleotides, and a 2.5-kb 3'-UTR. Further analysis showed the mouse clone was more closely related to the NK family than other homeodomain-containing genes. For example, the cloned mouse homeodomain carries a tyrosine at residue 54, a characteristic residue specific to *Drosophila* NK-2, -3, and -4 and their vertebrate orthologues (Stein et al., 1996). The mouse homeodomain shared the greatest homology with the homeodomain of *Drosophila* NK-3 (*bagpipe*) with identity at 47 of 61 residues (77%; Fig. 4). This compares to *Drosophila* NK-2 and NK-4 with which the mouse homeodomain shared only 60% and 52% identity, respectively. Additionally, the mouse homeodomain shared significant amino acid identity with residues at the extreme ends of the NK-3 homeodomain. These shared residues encompass helix III of the homeodomain and its amino-terminal arm, and is distinct from the analogous portions of the NK-2 or NK-4 homeodomains. The protein encoded by the mouse *Nkx3.1* cDNA was predicted to be 26 kDa, but following *in vitro* transcription/translation a protein with an apparent

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hNkx3 .1      MLRVPEPRPGEAKAEG----AAPPTPSKPLTS-FLIQDILRDGAQRQGG-RTSSQRQRDP
mNkx3 .1      MLRVAEPREPRVEAGGRSPWAAPPTQSKRLTS-FLIQDILRDRERHGGHSGNPQHSPDP
dNK-3         MLNM-----ESAGVS--AMAGLSKSLTTPFSINDILT-----RSNPETRRMS

hNkx3 .1      EPEPEPEPEG-----GRSRAGAQNDQLSTGPR-----AAPEEAETLAETEPE-----
mNkx3 .1      RRDSAPEPEPK-----AGGRGVAPEDPSI--R-----HSP--AETPTPEPSD-----
dNK-3         SVDSEPEPEKPKPSSDRERSASKSPPLCC--RDLGLYKLTQPKIQPSARQPSNYLQYYA

hNkx3 .1      -----RHLGSYL---LD---SENTSGALPRLPQTPKQ
mNkx3 .1      -----AHFETYL---LD---CEHNPGLASAPQVTKQ
dNK-3         AAMDNMNHHHQATGTSNSSAADYMQRKLAYFGSTLAAAPLDMRRCTSNDSDCDSPPLSSS

hNkx3 .1      P-----QKRSRAAFSHTQVIELERKFSHQKYL SAPERAHLAKNLKLTETQV
mNkx3 .1      P-----QKRSRAAFSHTQVIELERKFSHQKYL SAPERAHLAKNLKLTETQV
dNK-3         PSESPLSHDGGSLSRKRSRAAFSHAQVFELERFAQQRYS GPERSEMAKSLRLTETQV

hNkx3 .1      KIWFQNRRYKTKRKQLSSELGDL---EKHS--SLPALKKEEAFSRAASLVS-----
mNkx3 .1      KIWFQNRRYKTKRKQLSEDLGVL---EKNSPLSLPALKDDSLPSTSLVS-----
dNK-3         KIWFQNRRYKTKRKQIQQHEAALLGASKRVPVQVLVREDGSTTYAHMAAPGAGHGLDPAL

hNkx3 .1      -----VYNSYPY-----YPYLY-----CVGSWSP----
mNkx3 .1      -----VYTSYPY-----YPYLY-----CLGSWHP----
dNK-3         INIYRHQLQLAYGGLPLPQMMPFPYFYPOHKVQPQIPPTQSSSFVNASSASSSPVPIP

hNkx3 .1      -----AFW
mNkx3 .1      -----SFW
dNK-3         IPSAVRPQRTPCSPNGQMMSVESGAESVHSAEDVDENVEID

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Figure 4. An alignment of human and mouse Nkx3.1 and *Drosophila* bagpipe (NK-3). Shared amino acid residues are highlighted in red. All protein sequences retrieved from NCBI protein database and aligned using MAFFT version 5.853 (<http://timpani.genome.ad.jp/~mafft/server/>).

molecular weight of 38 kDa was obtained (Sciavolino et al., 1997). The difference between the predicted and apparent molecular weights is presumably due to a high content of proline residues (~15%) and possibly post-translational modification. Human *Nkx3.1* shares 100% identity to mouse *Nkx3.1* within the homeodomain, and 67% overall protein identity (Fig. 4). The similarities between the human and mouse cDNA sequences and genomic organization reveal that *Nkx3.1* is highly conserved between these two species.

Tanaka et al. (2000) mapped murine *Nkx3.1* to the central region of chromosome 14, a region that is syntenic to human chromosome 8. Interestingly, the *NK-2* (*tinman*) homolog *Nkx2.6* also maps to this region of mouse chromosome 14. The relative position and direction of transcription of *Nkx3.1* and *Nkx2.6* are the same as both *NK-2* and *NK-3* in *Drosophila*. Genomic Southern blot analysis seems to suggest that this relationship between *Nkx3.1* and *Nkx2.6* is conserved in humans, other primates, and chickens.

He et al. (1997) used a 20-kb genomic *Nkx3.1* clone as a probe for fluorescence *in situ* hybridization and mapped human *Nkx3.1* to chromosome 8p21. Allelic deletions of chromosome 8p21 are observed in prostate cancer tissues so frequently (up to 80%) that this loss is believed to be an early genetic event in prostate tumor formation. These observations led researchers to focus on *Nkx3.1* expression and function as it might elucidate how loss of 8p21 contributes to prostate cancer formation.

1.7 Nkx3.1: Roles in development

Homeodomain proteins, including NK-family members, play critical roles in body patterning and development during embryogenesis (Krumlauf, 1994). Consequently,

much of the early work on *Nkx3.1* concentrated on profiling its expression pattern in developing mice. Universally, two main expression patterns emerged. The earliest expression of *Nkx3.1* occurs in developing somites (Tanaka et al., 1999; Schneider et al., 2000). The vertebral column is formed from epithelial cells of somites that transition to mesenchymal cells to form the sclerotome. The sclerotome is the progenitor cell population of the axial skeleton. These cells migrate and organize around the notochord and subsequently differentiate into chondroblasts which form a cartilaginous matrix that is ultimately replaced with ossified bone to form vertebrae. Early in embryogenesis, at 7.5 days post-coitum (d.p.c.), *Nkx3.1* is expressed in paraxial mesoderm near the neural fold (Tanaka et al., 1999). *Nkx3.1* was expressed segmentally within the first nine somites at 8.5 d.p.c, and expression proceeds caudally with somite maturation such that *Nkx3.1* is expressed only in the most caudal somites by 10.5 d.p.c.

Herbrand et al. (2002) examined the role of *Nkx3.1* specifically in vertebral development. Both murine *bagpipe* homologs, *Nkx3.1* and *Nkx3.2* are expressed in newly formed somites as they form the sclerotome, but *Nkx3.1* expression ceases shortly after sclerotome formation, while *Nkx3.2* expression persists even in mature chondrocytes. *Nkx3.2*-deficient mice die perinatally and exhibit a profound skeletal phenotype characterized by a reduced vertebral column, most notably in the cervical vertebrae, and cranial bone defects. These skeletal defects are believed to cause breathing problems that result in postnatal death. This contrasts with *Nkx3.1*-deficient animals, which do not show overt skeletal abnormalities (Bhatia-Gaur et al., 1999; Schneider et al., 2000; Tanaka et al., 2000). Herbrand et al. showed that neither *Nkx3.1* nor *Nkx3.2* exert control over the other's expression. Double null mutant mice, lacking

both alleles for both *Nkx3.1* and *Nkx3.2* died between 12.5 and 17.5 d.p.c., although the cause of death was not apparent. Skeletal analysis of the double null mutant embryos revealed a shorter vertebral column than those seen in *Nkx3.2*^{-/-} mice, suggesting that both *Nkx3.1* and *Nkx3.2* act in a common pathway and contribute to vertebral formation and that *Nkx3.2* can supplant the need for *Nkx3.1* in axial skeletal formation.

The second pattern that emerges from developmental expression studies occurs in the developing male urogenital system. Late in embryogenesis, beginning at 14.5 d.p.c., *Nkx3.1* is expressed in the urogenital sinus and in the developing testis (Sciavolino et al., 1997). Within the male urogenital sinus, *Nkx3.1* is restricted to the rostral and caudal ends of the urogenital sinus. At 15.5 d.p.c., *Nkx3.1* expression is seen specifically in the basal epithelial layer rostral urogenital sinus (Bhatia-Gaur et al., 1999). By 17.5 d.p.c., *Nkx3.1* expression becomes restricted to the epithelium of the outgrowing ventral, dorsolateral and anterior prostatic buds, specifically at the distal ends of these prostatic buds, the area of the most active morphogenesis and this expression pattern persists into postnatal development. *Nkx3.1* is thus the earliest known marker of prostatic epithelium. At the caudal end of the urogenital sinus, *Nkx3.1* is expressed in the epithelial buds of the bulbourethral gland at 16.5 d.p.c.

Bhatia-Gaur et al. (1999) further underscored the importance of *Nkx3.1* in prostate development using a tissue recombination assay. The requirement of specific epithelial-mesenchymal interactions for prostate formation is well established and can be recapitulated in tissue recombinants. Only urogenital mesenchyme (UGM) will give rise to prostate tissues, even when combined with non-urogenital epithelium (such as bladder epithelium). *Nkx3.1* was expressed in all tissue recombinants derived from UGM, even

those from non-urogenital epithelium (bladder), but not in recombinants formed from bladder mesenchyme. All tissues derived from urogenital mesenchyme expressed *Nkx3.1*, even those using non-urogenital (bladder) epithelium. Conversely, tissue recombinants derived from non-urogenital (bladder) mesenchyme did not express *Nkx3.1* even in recombinants derived using urogenital epithelium. Thus, *Nkx3.1* expression parallels the known mesenchymal-epithelial requirements for prostate tissue formation in a tissue recombination assay. *Nkx3.1* was expressed early in prostate tissue formation, when secretory ducts first begin to form. Additionally, recombinants formed with epithelium from mice that lack androgen receptor (*Tfm*) initially form prostatic ducts, but ultimately fail to mature and express secretory proteins. *Nkx3.1* expression in these recombinants waned with time compared to recombinants formed with wild-type epithelium, such that *Nkx3.1* was eliminated in most of the *Tfm* tissue recombinants by four weeks of age. These results indicate that while functional androgen receptors are not required for initial *Nkx3.1* expression in developing prostate tissues, androgen receptors are required for maintenance of *Nkx3.1* expression in maturing prostate tissues. This study points to the necessity of *Nkx3.1* in the development of mature, functional prostate tissue.

Several groups independently generated *Nkx3.1* mutant mice in an effort to study the effect of *Nkx3.1* on normal prostate development (Bhatia-Gaur et al., 1999; Schneider et al., 2000; Tanaka et al., 2000). *Nkx3.1* ^{-/-} adult males had complete urogenital systems and are viable and fertile, but displayed severe morphological defects in the prostate gland and bulbourethral gland. This indicates that *Nkx3.1* cooperates with other regulatory genes to direct prostate development because prostates were still present even

with loss of *Nkx3.1*. All prostatic lobes were present in the mutant mice, but prostatic ductal formation was reduced by 60-75%. This reduction in ductal tips is evident as early as 10-11 days of age, when ductal branching is normally complete. Consistent with these findings, several major prostatic secretory proteins such as prostatic spermine-binding protein (SBP) were reduced or eliminated in homozygous mutant mice (Bhatia-Gaur et al, 1999). The overall sizes and weights of homozygous mutant prostates were similar to wild-type mice. However, epithelial hyperplasia and dysplasia was observed in both nullizygous and hemizygous mice and this may account for the reduced structure yet similar weights of the mutant and wild-type prostates. This dysplasia and hyperplasia was more severe and was present earlier in nullizygotes than in hemizygotes, and this condition worsened with time. Frank tumors did not develop in any of these animals by two years of age (Tanaka et al., 2000). The prostatic luminal epithelial cells of both hemizygous and nullizygous mice demonstrated a four- to six-fold increase in proliferating cells compared to wild-type. Therefore, the loss of a single *Nkx3.1* allele results in increased epithelial cell proliferation within the prostate. Also, the bulbourethral glands were smaller and had an altered cellular composition in the nullizygous mutants. Bulbourethral glands are primarily composed of mucin-producing cells, but the mutant bulbourethral gland exhibited a 15-fold reduction in these cells and an 11-fold increase in ductal cells. Additionally, the mutant bulbourethral gland secreted novel proteins, such as p20, a salivary gland secretion related to common salivary protein 1 (CSP1). Hyperplasia or dysplasia was not observed in the bulbourethral glands of *Nkx3.1* nullizygotes. In sum, results with genetically engineered mice clearly implicate

Nkx3.1 in proper prostate and bulbourethral gland development and function, and suggest that loss of *Nkx3.1* function may predispose prostate epithelium to tumor development.

During development, *Nkx3.1* expression was also observed in other tissues; however, there was little agreement between researchers about sites of *Nkx3.1* expression other than the somites, testis and prostate. These discordant results are likely due to varying detection methods (Northern blot, RT-PCR, *in situ* hybridization, etc.) employing different *Nkx3.1* probes with varying sensitivities and backgrounds. Among the other tissues that express *Nkx3.1* during development are kidney, thyroid, seminal vesicles, dorsal aorta, brain, lung alveoli, tongue epithelium, and Rathke's pouch (pituitary gland) (Sciavolino et al., 1997; Bhatia-Gaur et al., 1999; Tanaka et al., 1999; Schneider et al., 2000). In both human and mouse adults, *Nkx3.1* expression is tightly restricted to the luminal epithelial cells of all three prostatic lobes and the testis, albeit at less than 1% of the expression level seen in the prostate (Bieberich et al., 1996; He et al., 1997).

Several observations establish *Nkx3.1* as a target of androgen signaling. *Nkx3.1* mRNA levels declined precipitously but not completely after castration, prior to the onset of post-castration atrophy (Bieberich et al., 1996). Among several cultured human prostate tumor cell lines examined, *Nkx3.1* expression was only noted in the androgen-responsive human prostate carcinoma cell line LNCaP (He et al., 1997; Prescott et al., 1998). *Nkx3.1* was not detected in androgen receptor-negative prostate cell lines DU145 and PC-3. Expression of *Nkx3.1* in LNCaP cells was observed only in the presence of androgens (mibolerone, R1881) and in a dose-dependent manner. Treatment with androgens failed to stimulate *Nkx3.1* expression in DU145 and PC-3 cells. Prescott et al. (1998) also showed that androgens induce *Nkx3.1* expression through direct

transcriptional up-regulation by the androgen receptor. These findings were bolstered by the observation of Zhu et al. (1999) that the androgen receptor antagonist flufenamic acid dramatically reduced the expression of *Nkx3.1* and other androgen-regulated genes such as *PSA* and *hK2*. These results indicate that androgens are required for maintenance of *Nkx3.1* expression in adults.

In developing urogenital tissues, *Nkx3.1* is first expressed at 15.5 d.p.c., before the prostate is androgen-responsive (Takeda and Chang, 1991). This coupled with the earlier observations that *Nkx3.1* is expressed at low levels after castration of adult males (Bieberich et al., 1996) and is expressed in developing somites which do not respond to androgens suggested the existence of an androgen-independent mechanism for stimulating *Nkx3.1* expression. A study by Kos et al. (1998) reported that *Sonic hedgehog* (*Shh*) signals from the notochord induced expression in newly formed somites. Later, Schneider et al. (2000) generated *Nkx3.1/Sonic hedgehog* compound mutant knockout mice and observed a loss of *Nkx3.1* expression in somites and urogenital tissues. This suggests that *Shh* is required for *Nkx3.1* expression in somites and urogenital sinus, and that distinct regulatory domains control *Nkx3.1* expression in different tissues. Chen et al. (2005a) delineated the regulatory elements responsible for the temporal and spatial expression patterns of *Nkx3.1* by examining a beta-galactosidase reporter construct linked to genomic sequences 20kb upstream and 12kb downstream of the *Nkx3.1* start codon. They showed that the key regulatory element that directs *Nkx3.1* expression in somites and in testis is a promoter region that lies from 3kb to 5kb upstream of the *Nkx3.1* start codon. In contrast, a distinct regulatory element located 5kb downstream of the *Nkx3.1* start codon was found to direct *Nkx3.1* expression in the

prostate and bulbourethral gland. Therefore, distinct regulatory elements mediate *Nkx3.1* expression in different tissues, yet it remains uncertain whether Shh directs *Nkx3.1* expression via either of these regulatory elements.

1.8 Functional analysis of Nkx3.1

1.8.1 DNA-binding properties

Since the *Nkx3.1* homeodomain shares 77% amino acid identity with the homeodomain of *Drosophila NK-3*, and because many of the shared residues are known to determine the binding specificity of other homeodomain proteins (Damante et al., 1996), it was anticipated that the consensus Nkx3.1 DNA recognition sequence would be similar to that of other NK-family members. Steadman et al. (2000) conducted an iterative immunoselection and amplification assay (SAAB) with bacterially-expressed Nkx3.1 to define the consensus Nkx3.1 DNA-binding sequence. These studies revealed that Nkx3.1 preferentially bound a 5'-TAAGTA-3' consensus sequence. These results clearly distinguished the DNA-binding specificity of Nkx3.1 from other homeodomain proteins like Msx1, which typically bind a 5'-TAAT-3' core and from other NK-family members, such as Nkx2.1 (5'-CAAGTG-3') and Nkx2.5 (5'-TNAAGTG-3'). Nkx3.1 bound the 5'-TAAGTA-3' sequence specifically and with an estimated two-fold greater affinity than Nkx2.1 (5'-CAAGTG-3') and Msx1 (5'-TAATTG-3') binding sites. The calculated dissociation constant (K_d) for Nkx3.1 bound to its consensus sequence was 20 nM, which is significantly lower than those seen with other homeodomain-containing proteins and other classes of DNA-binding proteins (Table 2).

Table 2**Comparative DNA-Binding Affinities**

A comparison of dissociation constants (K_d) for various members of the major transcription factor classes. A lower dissociation constant translates into a higher affinity for the cognate DNA-binding site. Nkx3.1 has a relatively low affinity for its derived consensus DNA-binding site (5'-TAAGTA-3') in comparison to other homeodomain-containing proteins.

<u>Protein</u>	<u>DNA-binding domain</u>	<u>K_d (nM)</u>	<u>Reference</u>
Nkx3.1	HD	20.00	Steadman et al., 2000
Cut	HD	8.00	Harada et al., 1996
Oxt2	HD	2.20	Briata et al., 1999
NHF-6	HD	2.00	Lannoy et al., 1998
Antp	HD	1.70	Affolter et al., 1990
Hox2-C	HD	1.60	Corsetti et al., 1992
MyoD	bHLH	8.80	Spinner et al., 2002
Tal/E47	bHLH	3.60	Ghosh et al., 2001
PAS	bHLH	2.70	Chapman-Smith et al., 2003
Deadpan	bHLH	2.60	Winston et al., 1999
CEBP α	LZ	3.20	Olivier et al., 1994
myc	LZ	0.44	Jung et al., 2005
Sp1	ZF	0.47	Letovsky and Dynan, 1989
NGFI-A	ZF	0.44	Swirnoff and Milbrandt, 1995
Sp2	ZF	0.23	Moorefield and Horowitz, 2005
NGFI-C	ZF	0.14	Swirnoff and Milbrandt, 1996
Egr3	ZF	0.14	Swirnoff and Milbrandt, 1997

HD- homeodomain

bHLH- basic helix-loop-helix

LZ- leucine zipper

ZF- zinc-“finger”

It is not currently known whether Nkx3.1 can bind DNA as monomer or as part of a multimeric complex. *In vitro* studies suggest that Nkx2.5 can bind DNA as a monomer, or as a homodimer or heterodimer with other NK2 proteins, namely Nkx2.3 and Nkx2.6 (Kasahara et al., 2001). The Nkx2.5 homeodomain was found to be critical for dimerization. Specifically, two amino acid residues within the Nkx2.5 homeodomain, Lys57 and Arg58 (relative to the homeodomain), were indispensable for dimerization. As shown in Table 3, Nkx3.1 possesses both of these key dimerization residues as do most, but not all NK-2 and NK-3 family members. Contrastingly, these two critical residues are conspicuously absent from many NK-1 family members, Nkx6.x proteins and most Hox homeodomain proteins. It should also be noted that Nkx2.5 Arg58 also been shown to be necessary for interactions with the zinc-“finger” protein GATA-4 (Kasahara et al., 2001). This would suggest that most NK-2 and NK-3 proteins, including Nkx3.1, have the capacity to bind DNA as homodimers and possibly as heterodimers with other NK family members.

To date, no endogenous binding sites for Nkx3.1 within the promoter of physiological target genes have been identified. The *NK-3* promoter is believed to harbor sites by which *NK-3* represses its own expression (Kim et al., 1998), so by extension many speculated that *Nkx3.1* may be itself an Nkx3.1 target gene. However, Herbrand et al. (2002) reported no increase or decrease in the activity of a beta-galactosidase reporter under the control of the *Nkx3.1* promoter in *Nkx3.1*-deficient mice compared to wild-type mice. An examination of the proximal 1kb of the *Nkx3.1* promoter reveals no obvious Nkx3.1 consensus binding elements (Jiang et al., 2004; Jiang et al., 2005). Taken

Table 3**Alignment of Various Homeodomain Helix III Sequences**

An alignment of Helix III sequences from various homeodomain-containing proteins (homeodomain residues 43-60). Lys57 and Arg58 (relative to the first amino acid of the homeodomain) are required for Nkx2.5 homodimerization and dimerization with other NK-2 proteins. Most NK-2 and NK-3 family members share these two residues (underline and bold), while most NK-1 and Hox family member do not. All protein sequences retrieved from NCBI protein database.

<u>Organism</u>	<u>Protein</u>	<u>Helix III Sequence</u>
Drosophila	NK-2	ATQVKIWFQNRRYKSK <u>RGD</u>
Drosophila	NK-3	ETQVKIWFQNRRYKT <u>KRKQ</u>
Human	Nkx2.1	PTQVKIWFQNHRYK <u>MKRQA</u>
Human	Nkx2.3	STQVKIWFQNRRYK <u>CKRQR</u>
Human	Nkx2.5	STQVKIWFQNRRYK <u>CKRQR</u>
Human	Nkx2.8	PTQVKIWFQNHRYKL <u>KRAR</u>
Human	Nkx3.1	ETQVKIWFQNRRYKT <u>KRKQ</u>
Human	Nkx3.2	ETQVKIWFQNRRYKT <u>KRRQ</u>
Human	MSX1	ETQVKIWFQNRRAK <u>AKRLQ</u>
Xenopus	POU1	KEVVRVWFQNRQKE <u>KRMT</u>
Zebrafish	Nkx5.1	ETQVKIWFQNRN <u>KWKRQL</u>
Drosophila	NK-1	ETQVKIWFQNRRTKWKKQQ
Mouse	Nkx1.1	ETQVKIWFQNRRTKWKKQN
Mouse	NkX2.6	STQVKIWFQNRRYKSKSQR
Human	Nkx6.1	ESQVKVWFQNRRTKWRK <u>KH</u>
Human	Nkx6.2	ESQVKVWFQNRRTKWRK <u>RH</u>
Human	HoxA9	RRQITWFQNRVKEK <u>KVL</u>
Human	HoxB9	ERQVKIWFQNR <u>RMKM</u> KMN
Human	Hox11	DAQVKTWFQNRRTKWRR <u>QT</u>
Human	HoxB13	ERQVKIWFQNR <u>RMKM</u> KIN
Human	Otx2	ESRVQVWFKNRR <u>AKCR</u> QQQ
Human	HNF-1	EVRVYNW <u>FANRR</u> KEEAFRH

together, these results strongly suggest that *Nkx3.1* does not autoregulate. Other potential Nkx3.1 target genes include *smooth muscle gamma actin (SMGA)* (Carson et al., 2000; Fillmore et al., 2002) in visceral mesoderm tissues and *prostate cancer gene 1 (PCAN1)* (Cross et al., 2004) and *prostate-specific antigen* in prostatic epithelial tissues (Chen et al., 2002; Chen et al., 2005). None of these candidates, however, have been confirmed *in vivo* as targets of Nkx3.1 regulation.

1.8.2 Nkx3.1 transcriptional potential

All Nk proteins contain a 23-amino acid region (termed the TN domain) that is similar to a domain (termed the engrailed homology domain-1 (eh1)) identified in other homeoproteins (Smith and Jaynes, 1996). Homeoproteins carrying eh1 domains function as transcriptional repressors via the recruitment of co-repressors of the Groucho/TLE family (Jimenez et al., 1997). The *Drosophila* orthologue of Nkx3.1, NK-3, has been shown to form complexes with Groucho, as well as at least one histone deacetylase, and such complexes are competent to repress transcriptional activity (Choi et al., 1999). Surprisingly, the NK-3 homeodomain and not the NK-3 TN domain was shown to be indispensable for Groucho interactions. Although Nkx3.1 has not been shown to directly interact with Groucho/TLE repressors or histone deacetylases, it is widely assumed that Nkx3.1 also acts as a transcriptional repressor. Whether the Nkx3.1 TN domain binds Groucho/TLE proteins *in vivo* remains to be determined, however Korkmaz et al. (2000) cloned four variant Nkx3.1 cDNAs which resulted in amino-terminal in-frame deletions that abrogated the putative TN domain. It is worth mentioning that although these Nkx3.1 variants have been cloned from prostate cells, obvious splice junctions or cryptic

exons that might explain the origin of these variants have yet to be identified in genomic DNA. Thus, whether these variant cDNAs reflect alternatively spliced mRNAs or are artifacts of reverse transcription remains to be determined. Steadman et al. (2000) sought to determine the functional consequence of the binding of Nkx3.1 to its consensus sequence, 5'-TAAGTA-3'. This sequence was multimerized and used to generate reporter genes with three tandem repeats in both sense (5'-TAAGTA-3') and antisense (5'-ATGAAT-3') orientations. In this synthetic context Nkx3.1 appeared to function as a transcriptional repressor since it decreased basal levels of transcriptional activity three-fold for both sense and antisense reporter genes. This result is similar to those reported for Nkx3.2, which acted as a repressor in a cell culture assay (Murtaugh et al., 2001) using a synthetic reporter gene. Interestingly, Nkx3.2 required both the domains amino-terminal to the homeodomain (which harbors the eh-1 domain) and residues carboxy-terminal to the homeodomain to actualize its full repressor potential.

Using a yeast-two hybrid approach, Chen et al. (2002) screened for novel Nkx3.1-interacting proteins within a human cDNA expression library prepared from the prostate. They identified prostate-derived Ets factor (PDEF) as a potential partner of Nkx3.1, and these proteins were shown form a specific physical complex when over-expressed in LNCaP prostate tumor cells. These complexes between these proteins were later shown to require the homeodomain and carboxy-terminus of Nkx3.1 and the Ets domain and preceding linker region of PDEF (Chen et al., 2005b). Co-expression of Nkx3.1 repressed PDEF-directed *PSA* transcription, and *trans*-repression by Nkx3.1 was dependent on the same domains shown to be required for physical interactions with

PDEF. Consistent with these results, the ectopic expression of Nkx3.1 in LNCaP cells was also shown to reduce endogenous *PSA* transcription by 25%.

There is some evidence however, that Nkx3.1 can act a transcriptional activator. Carson et al. (2000) examined the expression of *smooth muscle gamma-actin (SMGA)* as a marker of smooth muscle cell differentiation, and found that Nkx3.1 cooperates with serum response factor (SRF) in CV-1 fibroblasts. Nkx3.1 and SRF functioned synergistically to activate *SMGA* as much as five-fold over either SRF or Nkx3.1 alone. This synergistic transactivation required adjacent Nkx3.1 and SRF binding sites in the *SMGA* promoter. It was also shown that SRF DNA-binding activity was greatly enhanced by the binding of Nkx3.1 to DNA. The residues carboxy-terminal to the Nkx3.1 homeodomain contained a potent transcriptional repression domain in this context, similar to the findings with Nkx3.2 (Murtaugh et al., 2001). Loss of this repressor domain resulted in a 15-fold increase in *SMGA* activity was observed in the absence of SRF, suggesting some latent Nkx3.1 activator activity. Fillmore et al. (2002) later reported that Nkx3.1 was capable of activating human *SMGA* 20-fold in the absence of SRF, indicating that Nkx3.1 can act as a transcriptional activator in certain contexts.

Some indirect evidence that suggests Nkx3.1 functions as both a transcriptional activator and repressor comes from expression profiling conducted on *Nkx3.1*-deficient mice (Magee et al., 2003; Ouyang et al., 2005). Microarray analysis comparing *Nkx3.1* nullizygous, hemizygous and wild-type mice, all having undergone a castration/testosterone replacement regiment, revealed 57 significantly altered genes. Of these putative *Nkx3.1* targets, 29 were positively regulated by *Nkx3.1* and 28 were negatively regulated by *Nkx3.1*. This result coincides neatly with the findings of Ouyang

et al. (2005). They reported that a microarray comparison of older (>12 months) *Nkx3.1*-deficient mice to wild-type controls revealed 638 differentially expressed genes. Of these, 299 were up-regulated in the mutants, indicating that these genes are negatively regulated by *Nkx3.1*. The remaining 339 genes were positively regulated by *Nkx3.1*. These results suggest that *Nkx3.1* functions can function as either a transcriptional activator or repressor, likely depending on promoter context.

1.8.3 Post-translational modifications

Homeodomain proteins are known to be highly regulated by phosphorylation during development (Krause and Gehring, 1989). Kim et al (1998) identified a novel family of kinases that differentially interact with NK proteins. Three homeodomain-interacting serine/threonine protein kinases (HIPKs) were ultimately identified. HIPK2 enhanced the DNA-binding activity of NK-3 although this was independent of its phosphorylation by HIPK2. NK-3-mediated transcriptional repression was also enhanced by HIPK2 coexpression, and this activity was dependent on HIPK2 kinase activity. NK-3 was shown to be a target of HIPK2 kinase activity, and there is speculation that *Nkx3.1* is phosphorylated by HIPKs, although this has not yet been definitively shown.

Gelmann et al. (2002) hypothesized that previously identified polymorphic *Nkx3.1* protein (Voeller et al., 1997) may be differentially phosphorylated *in vivo*. The R52C variation disrupted a putative protein kinase C (PKC) binding site. These workers reported that *Nkx3.1* was phosphorylated by PKC *in vitro* and *in vivo* at serine 48, and that the polymorphic protein was phosphorylated three-fold less the wild-type protein. The phosphorylation of *Nkx3.1* at Ser48 reduced *Nkx3.1* DNA-binding capacity *in vitro*,

however, both proteins were capable of co-stimulating *SMGA* in concert with SRF, indicating no significant functional difference between wild-type and polymorphic proteins in this transcriptional context. Recently, Li et al. (2006) reported that Nkx3.1 is regulated *in vivo* by the protein kinase casein kinase 2 (CK2). CK2 phosphorylation stabilized Nkx3.1 in LNCaP cells and prevented the ubiquitination and subsequent degradation of Nkx3.1 via the 26S proteasome. These results indicate that Nkx3.1 is indeed a phosphoprotein *in vivo* and that Nkx3.1 function and stability is regulated by phosphorylation. This is consistent with other NK family members, such as Nkx2.1 and Nkx2.5 whose function and stability are regulated by phosphorylation *in vivo* (Aurisicchio et al., 1998; Kasahara and Izumo, 1999).

1.9 Nkx3.1 and prostate cancer

A number of genetic events have been implicated in prostate cancer initiation and progression to adenocarcinoma. The consistent loss of specific chromosomal regions strongly suggests that tissue-specific tumor suppressor genes are crucial in the initiation of prostate carcinoma. Obviously, those on chromosome 8p are of greatest interest because loss of heterozygosity within the human chromosome region 8p21-22 is among the most commonly described genetic aberrations in prostate cancer (reviewed in Bott, 2005).

Early work with *Nkx3.1* suggested that its role in the initiation of prostate cancer might be minimal. Once *Nkx3.1* was mapped to a minimally deleted region at 8p21, Voeller et al. (1997) sequenced retained *Nkx3.1* alleles derived from 48 radical prostatectomy cancer specimens and three metastases. One specimen revealed a biallelic

deletion of chromosome 8p21, and therefore lacked *Nkx3.1* sequences. The remaining fifty specimens were shown to lack tumor-specific mutations within *Nkx3.1* coding sequences. A polymorphism at nucleotide position 154 (C154T) was discovered in 20% of tumor samples, however since this polymorphism was also noted in 19% of normal prostate specimens as well as female peripheral blood samples it is not preferentially retained in cancer specimens. Genetic analysis of this polymorphism suggests that a single copy of the polymorphic allele can increase the risk of prostatic enlargement, benign prostatic hyperplasia, and aggressive forms of prostate cancer, but not overall prostate cancer risk (Rodriguez Ortner et al., 2006). Xu et al. (2000) conducted sequenced the *Nkx3.1* coding region in five prostate cancer cell lines and did not detect mutations but did note the C154T polymorphism first reported by Voeller et al. (1997). Because the genotyping conducted by Voeller et al. (1997) relied on bulk tissue samples, Ornstein et al. (2001) and Kim et al. (2002) sought to obtain samples for sequencing with minimal contamination by using laser capture microdissection to isolate malignant prostate cells and HGPIN cells respectively, but they too did not detect mutations within the *Nkx3.1* coding region. Recently however, Zheng et al. (2006) sequenced the *Nkx3.1* coding and regulatory regions of 159 hereditary prostate cancer probands and identified twenty-one germ-line variants, including the C154T polymorphism first reported by Voeller et al. (1997). One rare germ-line variant, 1454 A/G, co-segregated completely with prostate carcinoma in a family with an affected father and three affected sons, but not with an unaffected brother. The 1454 A/G variant encodes an amino acid change at Nkx3.1 residue 164, which lies within the Nkx3.1 homeodomain. This threonine to alanine substitution is predicted to destabilize the homeodomain via its inability to form a

critical hydrogen bond needed for proper conformation of Helix III. This destabilization was shown to be correlated with reduced DNA-binding activity *in vitro*. Interestingly, a mutation in *Nkx2.5* at the same homeodomain residue was found in a family with hereditary atrial septum defects and atrioventricular block (Kasahara et al., 2004). Although this germ-line mutation in a prostate cancer family has been discovered, tumor-associated somatic *Nkx3.1* mutations have yet to be reported.

Expression analysis of *Nkx3.1* in tumor samples also suggested a minimal role for *Nkx3.1* in prostate tumorigenesis. Initially, Xu et al. (2000) used RT-PCR analysis to show similar levels of *Nkx3.1* expression in both normal (96%) and tumor (98%) tissues. Expression of *Nkx3.1* was significantly reduced in cells undergoing morphological changes after androgen ablation therapy, but not in cells resistant to the withdrawal of androgens. Ornstein et al. (2001) characterized *Nkx3.1* gene expression in benign and malignant human prostate tissues by *in situ* hybridization. *Nkx3.1* mRNA was detected in the secretory epithelial cells, but not in basal cells of the twenty-five sample examined. They observed similar expression levels between benign and malignant tissues and across the tumor progression spectrum, indicating that *Nkx3.1* mRNA is expressed in all prostate tumor stages. Korkmaz et al. (2004) matched *Nkx3.1* mRNA levels to prostate cancer tissue specimens and failed to detect a correlation between *Nkx3.1* mRNA levels and prostate tumor grade or clinical stage.

Despite these early findings, there are several lines of evidence that implicate *Nkx3.1* as the candidate tumor suppressor gene associated with 8p loss. First is the fact that *Nkx3.1* maps to the two-megabase minimally deleted region at 8p21 (Voeller et al.,

1997; He et al., 1997). This region undergoes allelic imbalance in as much as 80% of PIN lesions and prostate carcinomas (Dong, 2001).

Secondly, studies of *Nkx3.1* mutant mice reveal that *Nkx3.1* functions to maintain mature and fully differentiated prostate epithelium (Bhatia-Gaur et al., 1999; Schneider et al., 2000; Tanaka et al., 2000). *Nkx3.1* nullizygotes display severe morphological defects in the prostate including reduced ductal branching, hyperplasia and dysplasia. *Nkx3.1* mutant prostates also show abnormal secretory protein expression concomitant with abnormal ductal development.

Thirdly, *Nkx3.1* mice develop PIN-like lesions that closely resemble human PIN (Bhatia-Gaur et al., 1999; Abdulkadir et al., 2002). Abdulkadir et al. (2002) generated conditional *Nkx3.1*-deficient mice by mating mice with floxed *Nkx3.1* alleles with mice that express Cre recombinase under control of the *prostate-specific antigen (PSA)* promoter. The *PSA* promoter is activated in a prostate-specific manner only after puberty, so that the prostate is fully developed. This was a significant advance over previous *Nkx3.1* transgenic mice (Bhatia-Gaur et al., 1999, Schneider et al., 2000, Tanaka et al., 2000) because these mice had pronounced developmental abnormalities that confounded any examination of *Nkx3.1* loss in normal, mature prostate. Cre recombinase was expressed in all lobes of the prostate in mice as young as 10 weeks. Histological examination of the conditional *Nkx3.1*-deficient prostates revealed focal epithelial hyperplasia and PIN lesions. The murine PIN lesions shared many common characteristics of human PIN lesions such as enlarged, misshapen nuclei, prominent nucleoli, E-cadherin expression and high Ki-67 labeling indices indicating areas of active proliferation. Most notably, PIN lesions were observed in mice in which only one *Nkx3.1*

was targeted by Cre recombinase, suggesting that loss of a single *Nkx3.1* allele is sufficient to cause PIN formation. The conditional loss of *Nkx3.1* proves that *Nkx3.1* loss, and not abnormal development leads to PIN formation in *Nkx3.1* mutant mice. In all cases PIN lesions were more common and more severe in nullizygous mice than in heterozygous animals. PIN lesions also showed a disrupted basal layer, a hallmark of human PIN lesions, indicated by IHC staining of a basal cell marker, high-molecular-weight cytokeratin. IHC analysis of PIN lesions of heterozygous mice showed loss of *Nkx3.1* expression in 92% of the lesions examined, suggesting that the loss of a single *Nkx3.1* allele results in loss of *Nkx3.1* protein expression and that loss of *Nkx3.1* protein expression is associated with PIN lesion formation. While it is important to reiterate that frank carcinomas were not observed in *Nkx3.1* mutant mice, this model closely approximates loss of chromosome 8p21-22 in human prostate cancer development. Indeed, Kim et al. (2002) employed wild-type and PIN-like tissue xenografts to demonstrate the neoplastic potential of murine PIN-like lesions in *nude* mice. *Nkx3.1*-deficient PIN-like xenografts underwent several successive stages of neoplastic progression and grew in a disorganized manner compared to wild-type tissues.

Fourthly, *Nkx3.1* cooperates with other tumor suppressor genes to suppress prostate tumor formation. Because the PIN lesions formed in *Nkx3.1* mutant mice do not progress into overt prostate carcinomas, additional genetic events are required for prostate tumor progression. By generating compound *Nkx3.1;Pten* mutant mice, Kim et al. (2002) found that these mice develop high-grade PIN lesions (HGPIN)/early carcinoma by six months of age. These lesions were marked by poorly differentiated cells with prominent and multiple nucleoli, increased nuclear/cytoplasmic ratios, and high

proliferative indices. Cooperativity between *Nkx3.1* and *Pten* was apparent from an increased incidence in HGPIN/early carcinoma lesions in *Nkx3.1*^{-/-};*Pten*^{+/-} and *Nkx3.1*^{+/-};*Pten*^{+/-} prostates compared to prostates from *Nkx3.1*^{+/+};*Pten*^{+/-} mice. None of these mice developed invasive prostatic adenocarcinoma before twelve months of age, perhaps because they generally succumb to lymphomas and other non-prostate tumors. IHC analysis revealed complete loss of Nkx3.1 protein expression in all lesions from compound heterozygotes, although *Nkx3.1* mRNA was readily detected in these lesions. In some cases, mislocalization of Nkx3.1 to the cytoplasm was observed. Nkx3.1 expression was also lost in LGPIN lesions in *Pten* heterozygotes, although these mice are genotypically wild-type for *Nkx3.1*. Loss of Nkx3.1 function led to increased Akt kinase activation in prostate tissues in compound mutant mice as early as two months of age and increased Akt kinase activity preceded lesion formation. Akt kinase activation may be the potential molecular link between *Nkx3.1* and *Pten* in tumorigenesis of the prostate. Tissue recombinants resulting from the prostate epithelium of compound *Nkx3.1*;*Pten* heterozygous and the urogenital sinus mesenchyme of wild-became neoplastic when transplanted in *nude* mice (Shen et al., 2003). They also observed invasive prostate adenocarcinoma in 84% of compound *Nkx3.1*;*Pten* heterozygous mice older than twelve months compared to wild-type littermate controls. Prostate adenocarcinoma was not reported in compound mutant mice younger than one year of age, highlighting the critical dependence on aging for disease progression. The reported prostate adenocarcinomas were highly proliferative, invaded the surrounding stroma and displayed a marked inflammatory response. These prostate adenocarcinomas were prone to metastasize to the iliac lymph nodes and form prostatic-like ducts filled with secretory material, similar

to lymph node metastases in human patients with advanced prostate cancer. Also, the dorsolateral prostate was the lobe most likely to develop adenocarcinoma; this is significant because the rodent dorsolateral prostate most closely corresponds anatomically to the human peripheral zone where most human prostate cancers occur. The prostates of compound *Nkx3.1;Pten* heterozygous mice were more resistant to androgen ablation by castration than organs of wild-type mice, and contained highly proliferative high-grade PIN lesions that were unaffected by androgen withdrawal.

Nkx3.1 also cooperates with the cyclin-dependent kinase inhibitor *p27^{kip1}* to suppress the formation of prostate cancers (Gary et al., 2004). *p27* expression is lost in many human prostate cancers, and the loss of single *p27^{kip1}* allele is sufficient to generate PIN lesions in mice. *Nkx3.1; p27^{kip1}* double nullizygous mice developed extensive PIN lesions with increased incidence compared to *Nkx3.1* or *p27^{kip1}* single nullizygous mice; 10% of double nullizygous mice develop PIN by 24 weeks of age. Interestingly, although *Nkx3.1* and *p27^{kip1}* single hemizygous mice develop PIN lesions, compound *Nkx3.1; p27^{kip1}* hemizygous mice do not show an increase in the number or severity of PIN lesions. This indicates cooperativity between *Nkx3.1* and *p27^{kip1}* where at least one allele of each gene is retained. Throughout a 36-week observation period, overt carcinomas were not reported.

Loss of *Nkx3.1* expression has also been shown to cooperate with oncogene-induced prostate tumorigenesis. Microarray-based expression profiling of transgenic mice overexpressing the *myc* oncogene revealed the loss of *Nkx3.1* expression in resulting prostate adenocarcinomas (Ellwood-Yen et al., 2003). *Myc* transgenic mice developed PIN-like lesions that variably expressed *Nkx3.1* that progressed to

adenocarcinoma in concert with the loss of Nkx3.1 expression. These results suggest that *myc* gain and *Nkx3.1* loss might be critical cooperating events in prostate tumor formation.

Lastly, Nkx3.1 protein expression studies conducted on human prostate tissues as well as *Nkx3.1* hemizygous mice (Abdulkadir et al., 2002) showed a significant discordance between *Nkx3.1* mRNA and protein levels. *Nkx3.1* mRNA levels in human prostate cancer tissues were similar to levels seen in adjacent normal human prostate tissue. However, Nkx3.1 protein levels were drastically reduced in the human prostate cancer tissues compared to normal controls. Likewise, *Nkx3.1* hemizygous mice expressed *Nkx3.1* mRNA at comparable levels to wild-type mice, but showed markedly reduced Nkx3.1 protein expression compared to wild-type controls. Bowen et al. (2000) developed an antiserum raised against purified recombinant Nkx3.1 and used this reagent to examine Nkx3.1 expression in human tissues by immunohistochemistry (IHC). Staining of a tissue microarray containing samples that span the spectrum of prostate tumor progression revealed three distinct staining patterns. Many samples stained uniformly, others showed heterogeneous staining, typically with malignant cells that did not express Nkx3.1 and adjacent normal cells that stained positive, still others lacked staining entirely. A significant reduction in Nkx3.1 expression was observed in advanced prostate cancers, defined as hormone-refractory or metastatic tumor samples. In particular, 78% of metastatic samples lacked Nkx3.1 expression and a further 12% exhibited reduced Nkx3.1 expression relative to normal prostatic tissue. In comparison, Nkx3.1 expression was detected more frequently in nonmalignant prostate conditions such as benign prostate hyperplasia (BPH). Five per cent of BPH samples exhibited

complete loss of Nkx3.1 expression and a further 11% showed reduced Nkx3.1 expression. Interestingly, PIN lesions exhibited complete loss of staining in 20% of samples and reduced staining in a further 35% of samples, consistent with the notion that PIN lesions represent a preneoplastic condition in human prostate epithelial tissues. In this study, the correlation of Nkx3.1 expression with prostate tumor progression was significantly better than the correlation between the Gleason score (used to grade tumors pathologically) and tumor progression, raising the possibility that Nkx3.1 protein expression might be useful as a prognostic marker for human prostate cancers. These results by Bowen et al. were bolstered by the findings of Asatiani et al. (2005) who used two-color fluorescence to quantify Nkx3.1 protein levels in normal and malignant human prostate cells. Using histone H1 to normalize their results, they found that Nkx3.1 expression is significantly reduced in prostate cancer cells and in high-grade PIN lesions, indicating that loss of Nkx3.1 expression occurs prior to malignant transformation. It should be noted however, that at least one study (Korkmaz et al., 2004) reported that *Nkx3.1* mRNA levels were highly correlated to Nkx3.1 protein levels in human prostate carcinoma samples and that there was no association between Nkx3.1 expression and prostate tumor grade or clinical stage.

In summation, the observations that *Nkx3.1* maps to a human chromosomal region that frequently undergoes loss of heterozygosity, that *Nkx3.1* is required to establish and maintain a mature and fully differentiated phenotype, that *Nkx3.1* mutant mice develop PIN lesions, that *Nkx3.1* cooperates with other known tumor suppressor genes to inhibit prostate tumor formation, and that Nkx3.1 protein expression is lost or reduced in

prostate tumors samples as well as *Nkx3.1* hemizygous mice strongly suggest a role for *Nkx3.1* in prostate tumor development.

Although human chromosomal region 8p21 undergoes loss of heterozygosity at a high frequency in human prostate cancers as well as PIN (Emmert-Buck et al., 1995; Kindich et al., 2006), the remaining *Nkx3.1* allele is not inactivated by mutation (Voeller et al., 1997; Xu et al., 2000; Ornstein et al., 2001; Kim et al., 2002) suggesting that *Nkx3.1* haploinsufficiency is sufficient to promote tumorigenesis. This suggestion is supported by the observation that *Nkx3.1* hemizygous mice develop PIN lesions, indicating that loss of a single *Nkx3.1* allele is sufficient to induce PIN formation (Abdulkadir et al., 2002). The mechanism(s) by which *Nkx3.1* is rendered haploinsufficient has been a matter of much speculation. One leading hypothesis is that the retained *Nkx3.1* allele is silenced by promoter methylation. Conflicting studies by Lind et al. (2005) and Asatiani et al. (2005) address this possibility. Lind et al. did not detect methylation of the *Nkx3.1* promoter in twenty human prostate carcinoma samples using methylation-specific PCR and bisulphate genomic sequencing. Asatiani et al., on the other hand, reported significantly higher *Nkx3.1* promoter methylation at three specific sites (-921, -903 and -47) in malignant cells compared to adjacent normal cells from twenty-two human prostate samples. Asatiani et al. also reported that the number of sites found to be methylated when combined with 8p21 LOH status was highly predictive of low *Nkx3.1* expression. While more work needs to be done to discern the extent to which methylation of retained *Nkx3.1* alleles silences *Nkx3.1* expression, it seems likely that promoter methylation is at least one mechanism that may explain *Nkx3.1* haploinsufficiency.

Magee et al. (2003) used micorarray-based expression profiling to describe the functional consequences of *Nkx3.1* haploinsufficiency and to identify targets that are deregulated when *Nkx3.1* expression is lost or reduced. These workers hypothesized that *Nkx3.1* controls the transition of prostatic epithelial cells from a proliferative program to differentiation in a dose-sensitive manner, and that disruption of *Nkx3.1* would lead to aberrant proliferation and failure to differentiate. To test this hypothesis, testosterone was withdrawn from nullizygous and hemizygous *Nkx3.1* mice via castration and then replaced with testosterone-laden pellets implanted subcutaneously. *Nkx3.1* mRNA was rapidly expressed in both wild-type and hemizygous mice following testosterone replacement (TR), although hemizygotes had quantitatively reduced levels of transcripts compared to wild-type controls. *Nkx3.1* protein expression followed a similar pattern, with detectable levels observed at 24 hours post-TR and persisting beyond 2 weeks. To discern the role of *Nkx3.1* on growth arrest in the regenerating prostate, expression of the cell proliferation maker Ki-67 was measured by qRT-PCR in wild-type and nullizygous prostates. Ki-67 expression peaked in wild-type mice at three days post-TR and was virtually absent after seven days of treatment when the amplifying luminal cells have terminally differentiated. Interestingly, Ki-67 expression levels in luminal epithelial cells from both hemizygous and nullizygous *Nkx3.1* mice was elevated at seven days post-TR, with a five-fold increase over wild-type observed for hemizygous mice and a twelve-fold increase for nullizygous mice. By fourteen days post-TR, the extended proliferation phase had resulted in hyperplasia in the *Nkx3.1*-deficient prostates. This seems to indicate that loss of one or both *Nkx3.1* alleles alters the timing of cell cycle withdrawal for amplifying luminal epithelial cells which in turn leads to hyperplasia. These

observations regarding cell proliferation *in vivo* coincide with a report by Kim et al. (2002) that showed that Nkx3.1 overexpression in human PC3 and rat AT6 prostate cells (both Nkx3.1-negative) reduced cellular proliferation, anchorage-independent growth, and tumorigenicity compared to cells that overexpressed a Nkx3.1 homeodomain mutant. Target genes were identified by Magee et al using microarray probes generated from total RNA harvested from wild-type, hemizygous and nullizygous *Nkx3.1* mutant mice that had undergone castration-testosterone replacement as well as normal mice. Fifty-seven genes were identified as significantly different between wild-type and *Nkx3.1*^{-/-} prostates. Some of these genes, such as *intelectin* and *probasin* are positively regulated by *Nkx3.1* and showed lower expression levels in *Nkx3.1*-deficient mice while other genes like *angiopoietin 2* and *elafin-like II* are repressed by *Nkx3.1* and thus had higher levels of expression in the absence of *Nkx3.1*. Markers of prostatic luminal epithelial differentiation such as *probasin* and *β-micro-seminoprotein*, a major prostatic secretory product, were identified as positively regulated by *Nkx3.1*, consistent with the role of *Nkx3.1* as a mediator of differentiated luminal epithelium. For some positively regulated genes, loss of one *Nkx3.1* had little or no effect on expression levels. For example, *probasin* and *Riken clone 2210008A03* were expressed at 72% and 87% in heterozygotes compared to wild-type mice. Other positively regulated genes, such as *intelectin*, were sensitive to loss of even one *Nkx3.1* allele, with expression virtually abolished in both *Nkx3.1*^{+/-} and *Nkx3.1*^{-/-} prostates. Interestingly, negatively regulated *Nkx3.1* target genes did not appear to be dose sensitive. Expression of genes like *angiopoietin 2* in heterozygotes closely resembles that of wild-type mice, with activation only observed in *Nkx3.1*^{-/-} mice. Two models, termed graded expression and stochastic expression, were

offered by Magee et al to explain their microarray results. The graded expression model holds that transcriptional regulation of target genes increases proportionally in response to increasing levels of one or more transcription factors. The stochastic model, on the other hand, holds that transcription is binary, and transcription factors regulate the probability that a target gene is “on” or “off”. For both dose-sensitive and –insensitive positively regulated *Nkx3.1* target genes, heterogeneous expression was observed in prostate tissues by *in situ* hybridization, indicating that *Nkx3.1* gene dosage influences the stochastic probability rather than the absolute level of target gene activity in a given cell. Magee et al. speculate that it is the loss of dose-sensitive target gene expression that leads to the extended proliferative phase seen in *Nkx3.1*-deficient mice, thus predisposing these proliferating cells to additional genetic insults that facilitate the progression from PIN to carcinoma.

In addition to regulating the terminal differentiation of luminal epithelial cells, *Nkx3.1* is also thought to play a role in regulating levels of reactive oxygen species. Ouyang et al. (2005) compared microarray-based gene expression profiles of wild-type and *Nkx3.1* nullizygous mice, and identified 638 genes that were differentially expressed in the anterior prostates of these two groups. Among the deregulated genes was a subset known to protect against oxidative damage. Expression analysis showed that the de-oxidant enzymes *Gpx2* and *Prdx6* were down-regulated and the pro-oxidant enzyme *sulphydryl oxidase 6 (Qscn6)* was activated in *Nkx3.1*-deficient prostates. These results were validated by RT-PCR, *in situ* hybridization and immunohistochemistry analysis. Anti-oxidant enzymes such as *Gpx2* and *Prdx6* covert potentially harmful free radicals to less harmful metabolites, and perturbation of this system leads to oxidative DNA damage.

Indeed, in concert with the de-regulation of Gpx2 and Prdx6 expression *Nkx3.1*-deficient prostates had a five-fold increase in oxidative DNA damage by twelve months of age compared to wild-type littermates. Increased oxidative DNA damage was detectable before the onset of PIN formation, suggesting the damage was direct result of *Nkx3.1* loss. Also, this increase in oxidative damage was unique to *Nkx3.1*-deficient mice and not other mice that develop PIN lesions such as *myc* transgenic mice. The strong link between aging and oxidative DNA damage, suggests that *Nkx3.1* loss may increase the effect and rate of aging on the prostate.

Loss of *Nkx3.1* expression has also been linked to the etiology of a tumor that develops outside of the prostate gland. Testicular germ cell tumors (TGCT) are the most common malignancy among adolescents and young men in Western industrialized countries. TGCTs develop from premalignant, non-invasive carcinomas *in situ* (CIS). Skotheim et al. (2003) examined expression of *Nkx3.1* protein in normal testis, CIS, and invasive TGCT. They found that all normal testis and CIS tested expressed *Nkx3.1*, but that only 9% of TGCTs examined expressed *Nkx3.1*. They also noted that *Nkx3.1* expression tended to be inversely correlated with metastasis, with 17% of non-metastatic TGCTs expressing *Nkx3.1* versus none of the metastatic TGCTs. Quantitative RT-PCR reveal that all TGCTs had reduced levels of *Nkx3.1* mRNA compared to normal testis, indicating that loss of *Nkx3.1* protein is at least in part due to reduced *Nkx3.1* transcript levels. Loss of *Nkx3.1* expression in testicular germ cell tumors is thought to be due to epigenetic factors because chromosome 8p21 is not a region with common loss of heterozygosity in TGCTs and because *Nkx3.1* mutations have not been reported in this tumor type.

1.10 Functional interactions between homeodomain proteins and zinc-“finger” proteins

Homeodomain proteins, like other DNA-binding transcription factors, are thought to regulate specific target genes through collaborations with other cofactors. These interactions, however, are particularly important for homeodomain proteins due to the relatively common DNA-binding sites of the homeodomain coupled with the highly specific patterns of gene expression required for cell differentiation and development. Since most homeodomain proteins bind sequences with a TAAT/G core motif (Gehring et al., 1994) and these motifs are abundantly distributed throughout the genomes of eukaryotic organisms (Walter and Biggin, 1996), identification of the cofactors involved in modulating homeodomain function will be vital to identifying target genes and understanding the role of homeodomain proteins in biological processes.

There exists a modest, but growing corpus of evidence that homeodomain proteins collaborate with zinc-“finger”-containing transcription factors to regulate gene expression. The evidence for these interactions spans the phylogenetic spectrum, suggesting that these interactions may be well-conserved and more common than originally thought. It is well established that the yeast mating-type switching gene *HO* is regulated by a functional interaction between the zinc-“finger” protein Swi5 and the homeodomain protein Pho2 (Brazas and Stillman, 1993; McBride et al., 1997). Swi5 and Pho2 have been shown to mutually increase each other’s affinity for binding sites within the *HO* promoter to synergistically activate *HO*. The interaction with Pho2 and activation of *HO* was dependent on a 24-amino acid domain immediately amino-terminal to the Swi5 zinc-“fingers”. Physical contact with Swi5 is mediated through a central

portion of Pho2 that serves as protein-protein interactive surface for not only Swi5, but Pho4 and Bas1 as well (Bhoite et al., 2002).

In *Drosophila*, the homeodomain protein *ems* (empty spiracles) coordinates head segmentation and anterior differentiation through a functional interaction with the zinc-“finger” protein *btd* (buttonhead). *Btd* is a probable orthologue of Sp-proteins and many functions of *btd* can be supplanted by the expression of human Sp1 (Wimmer et al., 1993; Schock et al., 2000). Proper *Drosophila* head development also relies on additional homeodomain/zinc-“finger” protein interactions. Mahaffey (2005) reported that the zinc-“finger” proteins *disco* (disconnected) and *disco-r* (disco-related) coordinated target gene expression by the homeodomain proteins *Dfd* (deformed) and *Scr* (Sex combs reduced) in post-oral head development in *Drosophila*. In this model, zinc-“finger” proteins establish specific regions in the *Drosophila* embryo that coordinate which homeodomain proteins can function to specify individual segment identity (Robertson et al., 2004).

Homeodomain/zinc-“finger” protein interactions have also been shown to be important in vertebrates. The GATA family of zinc-“finger” transcription factors is involved in several documented functional interactions with homeodomain proteins. The carboxy-terminal zinc-“finger” of GATA-4 and GATA-5 physically interacts with the hepatocyte nuclear factor-1 (HNF-1) homeodomain to synergistically activate the human *lactase-phlorizin hydrolase (LPH)* promoter, a marker of intestinal differentiation (van Wering et al., 2004; van Wering et al., 2002). GATA-6 and Nkx2.1 cooperate to activate *surfactant protein C (sp-C)* expression through a physical interaction between the GATA-6 zinc-“fingers” and the Nkx2.1 homeodomain (Liu et al., 2002). Interestingly, neither GATA-6 nor Nkx2.1 could independently activate *sp-C*, so activation can only

arise from the cooperative interaction between GATA-6 and Nkx2.1. Additionally, GATA-4 was unable to interact with Nkx2.1 to activate *sp-C*, indicating that the combinatorial interactions between homeodomain-containing proteins and zinc-“finger” proteins are partner-specific. Another Nkx family member, Nkx2.5, one of the mammalian homologs of *tinman*, is required for normal heart development (Lyons et al., 1995). Targeted *Nkx2.5* disruption in mice leads to embryonic death due to cardiac morphogenetic defects. *Atrial natriuretic factor (ANF)* is a cardiac transcriptional target of both Nkx2.5 and GATA-4, both of which are two of the earliest markers of cardiac differentiation (reviewed in Charron and Nemer, 1999), and therefore provided a unique target to study potential functional cooperation between Nkx2.5 and GATA-4. Nkx2.5 cooperates with GATA-4 to synergistically activate *ANF*, and again this synergy is dependent on physical interactions between the zinc-“fingers” of GATA-4 and the homeodomain of Nkx2.5 (Durocher et al., 1998). The other cardiac GATA factor, GATA-6 was unable to interact with Nkx2.5 activate *ANF*, providing yet another example of the specificity of combinatorial homeodomain-/zinc-“finger”-protein interactions.

The Sp-subfamily of zinc-“finger” transcription factors has also been implicated in several interactions with homeodomain proteins. The Sp/XKLF family of transcription factors are implicated in the regulation of a wide range of mammalian genes from ubiquitously-expressed housekeeping genes to tissue-specific genes (reviewed in Philipsen and Suske, 1999). These regulated genes govern the spectrum of cellular processes from cell cycle control and growth to differentiation. Sp1 has been shown to cooperate with the Abd-B subfamily of *Hox* genes to coordinate the expression of the

TGF- β family member, *bone morphogenetic protein-4* (*Bmp-4*), related to autopod limb development (Suzuki et al., 2003). Specifically, either HoxA-13 or HoxD-13 could cooperate with Sp1 to activate *Bmp-4*, but another Abd-B family member, Hox-A11 was unable to stimulate *Bmp-4* activity with Sp1. Sp1, Sp3 and Sp4 physically complexed with the homeodomain protein Crx to synergistically activate the photoreceptor-specific gene *opsin* (Lerner et al., 2005). Importantly, the zinc-“fingers” of Sp1, Sp3 and Sp4 were required to interact with Crx.

Specifically, Sp-proteins have been shown to physically and functionally interact with Nkx proteins. In particular, Sp1 and Sp3 have been shown to cooperate with the Nkx family member Nkx2.1 to activate rat *Clara cell secretory protein* (*CCSP*) (Toonen et al., 1996). Sp1 and Sp3 bound a GC-box *in vivo* located just upstream of an Nkx2.5 response element (NKE) located in the proximal *CCSP* promoter. The Sp-binding site and NKE are both located in a promoter region (-75 to +38) known to confer tissue-specific *CCSP* expression.

Functional interactions between homeodomain-containing proteins and zinc-“finger” transcription factors are indeed an increasingly apparent mechanism by which tissue-specific gene regulation is modulated. Additional interactions between homeodomain proteins and zinc-“finger” proteins must be identified and analyzed in order to better understand how homeodomain proteins regulate target genes in a specific fashion.

1.11 Conclusions and perspectives

Nkx3.1 is homeodomain-containing transcription factor that is expressed early in the development of the prostate gland and is believed to play an important role in the differentiation of prostatic epithelia. Because *Nkx3.1* maps to a human chromosomal region that undergoes loss of heterozygosity in upwards of 60% of PIN lesions and 90% of prostate cancers, and because the loss of a single *Nkx3.1* allele leads to the formation of precancerous neoplastic lesions in the prostate, *Nkx3.1* is thought to play a critical role in early prostate cancer formation. Given the relatively low DNA-binding specificity of homeodomain-containing proteins, it is likely that most if not all homeodomain proteins rely on cofactors to specifically regulate target genes. The Sp-family of zinc-“finger” transcription factors are known to functionally interact with homeodomain proteins, and Nkx family members specifically, to modulate tissue-specific gene expression. My goal for the studies reported herein was to determine whether combinatorial interactions between Nkx3.1 and one or more Sp-family members play a role in the regulation of transcription in prostate-derived cells. In the following chapters I will present the biochemical, molecular genetic and functional evidence that demonstrates that Nkx3.1 does indeed interact differentially with Sp-family members to regulate the activity of the human *prostate-specific antigen* promoter in prostate-derived cells. I will also document studies that I have performed in an attempt to (1) define portions of Nkx3.1 that dictate its sub-cellular localization and (2) identify Nkx3.1 target genes.

CHAPTER II

Materials and Methods

2.1 Cell culture

COS-1, LNCaP, and DU145 cells were obtained from the Duke Comprehensive Cancer Center Cell Culture Facility (Duke Univ. Medical Center, Durham, NC). Cells were cultured in Dulbecco's modified Eagle's medium or RPMI 1640 (GIBCO, Inc., Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA) and 50 µg/ml Pipracil in humidified incubators under 5% CO₂. Sf9 cells were obtained from Invitrogen, Inc. (Carlsbad, CA) and cultured in spinner flasks at 27 °C in Grace's insect media supplemented with 10% heat inactivated fetal bovine serum, 0.1% Pluronic F-68, and 10 µg/ml Gentamycin (GIBCO).

2.2 Plasmids

2.2.1 Expression constructs

The construction and characterization of pCMV4-Sp1/flu, pBSK-Sp1/flu, pCMV4-Sp3/flu, pBSK-Sp3/flu, pCMV4-Sp2/flu and pBSK-Sp2/flu has been described (Udvardia et al., 1993; Udvardia et al., 1995; Moorefield et al., 2004). An Sp4 expression construct, pCMV4-Sp4/flu, carrying an amino-terminal ten amino acid epitope from *Influenza* hemagglutinin was prepared using the PCR, a human Sp4 cDNA as template (a generous gift of Dr. Richard Tsika, Univ. of Missouri-Columbia, Columbia, MO) and the following primers: 5'-CAG ATC TAT GTA TCC TTA GAT GTG CCA GAC TAC GCT TCA GCA AAG ATG AGC GAT CAG AAG AAG GAG GAG-3' and 5'-GGT CAG AAT TCT TCC ATG TTG GTT GAA ACA TTG GG-3'. These and all other oligonucleotides used in this study were obtained from Invitrogen, Inc. The resulting

PCR product was cloned in pCR-Blunt II-TOPO (Invitrogen) to generate TOPOSp4/flu and then transferred to pCMV4 to generate pCMV4-Sp4/flu. An analogous Sp5 expression construct, pCMV4-Sp5/flu, carrying an amino-terminal epitope tag was prepared as above except for the substitution of the following PCR primers: 5'-CAG ATC TAT GTA TCC TTA CGA TGT GCC AGA CTA CGC TTC AGC AAA GAT GAG CGA TCA GAA GAA GGA GGA G-3' and 5'-CAG ATC TTC ATA GGT CCC GCG GAT TCT CCC GCT TC-3'. The murine Sp5 cDNA employed as template was obtained from Dr. Rosa S. P. Beddington (National Institute for Medical Research, London, UK). TOPO-Sp2 Δ Zn, carrying the *trans*-activation domain of Sp2, was prepared via the PCR using pBSK-Sp2/flu as template and the following primers: 5'- GGG CCA CCA TGA GCG ATC CAC AGA CCA GCA TGG CTG CC-3' and 5'- GGG TTA AAC GTG CTT CTT CTT GCC CTG CTC TCC-3'. The resulting PCR product was cloned in pCR-Blunt II-TOPO (Invitrogen), generating TOPO-Sp2 Δ Zn. A murine Nkx3.1 cDNA was a generous gift from Dr. Michael M. Shen (Center for Advanced Biotechnology and Medicine, Piscataway, NJ) and has been described (Sciavolino et al., 1997). A mammalian expression vector (pcDNA3-hNkx3.1) carrying a human Nkx3.1 cDNA was a generous gift from Dr. Charles J. Bieberich (Univ. of Maryland-Baltimore County, Baltimore, MD) and has been described (Chen et al., 2002). Nkx3.1 amino-terminal variant expression constructs pcDNA3.1-Nkx3.1v1, pcDNA3.1-Nkx3.1v2, pcDNA3.1-Nkx3.1v3 and pcDNA3.1-Nkx3.1v4 were generated via the PCR using pcDNA3.1-hNkx3.1 as the template and the following 5'-phosphorylated primers: pcDNA3.1-Nkx3.1v1: 5'-CGC GCC GTC CCG CAG GAT GTC CTG-3' and 5'-CCG GAG GAG GCC GAG ACG CTG GC-3'; pcDNA3.1-Nkx3.1v2: 5'-CGG CTC CGG AAC CCT

GAG CAT-3' and 5'-GAG CCA GAG CCA GAG GGA GGA CGC-3'; pcDNA3.1-Nkx3.1v3: 5'-CGC TTT CGC CTC CCC GGG CCG CGG-3' and 5'-CCG GAG GAG GCC GAG ACG CTG GC-3'; pcDNA3.1-Nkx3.1v4: 5'-CGC CTC CCC GGG CCG CGG CTC CGG-3' and 5'-GAG ACG CTG GCA GAG ACC GAG CC-3'. Resulting PCR products were cleaved with Dpn I to destroy template DNA, DNAs were self-ligated and used to transform *E. coli*. Plasmids derived from resulting transformants were sequenced to identify clones, termed pcDNA3.1-Nkx3.1v1, pcDNA3.1-Nkx3.1v2, pcDNA3.1-Nkx3.1v3 and pcDNA3.1-Nkx3.1v4, carrying the desired deletions.

A GST-fusion construct carrying the zinc-“finger” domain of Sp2, pGEX1N-Sp2Zn, was prepared via the PCR using pBSK-Sp2/flu as template and the following primers: 5'-GGG GGA TCC CAT GTG CCA CAT CCC CGA CTG TGG CAA GAC GTT CCG-3' and 5'-GGG GAA TTC TTA CAA GTT CTT CGT GAC CAG GTG GG-3'. The resulting PCR product was cleaved with Bam HI and Eco RI and sub-cloned in pGEX1N (Pharmacia, Inc., Piscataway, NJ). A GST-fusion construct, pGEX2T-mNkx3.1 was prepared via the PCR using a murine Nkx3.1 cDNA (pcDNA3-mNkx3.1) as template and the following primers: 5'- GGG GGA TCC CAT GCT TAG GGT AGC GGA GCC C-3' and 5'-GGG GAA TTC CTA CCA GAA AGA TGG ATG CCA GCT G-3'. The resulting PCR product was cleaved with Bam HI and Eco RI and sub-cloned in pGEX2T. Partial GST-mNkx3.1 expression constructs were prepared using pcDNA3-mNkx3.1 as template and the following PCR primer pairs: pGEX(1-87): 5'-GGG GGA TCC CAT GCT TAG GGT AGC GGA G-3'; 5'-GGG GAA TTC TTA GGG GCT ATG CCG GAT ACT TGG TGG-3'; pGEX(1-128): 5'-GGG GGA TCC CAT GCT TAG GGT AGC GGA G-3'; 5'-GGG GAA TTC TTA GGA GCG CTT CTG TGG CTG CTT

GGT G-3'; pGEX(1-216): 5'-GGG GGA TCC CAT GCT TAG GGT AGC GGA G-3'; 5'-GGG GAA TTC TTA ACG GAG ACC AAG GAG GTA CTG GGC-3'; pGEX(129-216): 5'-GGG GGA TCC ATG CAG AAG CGC TCC CGG GCC GCC TTC-3'; 5'-GGG GAA TTC TTA ACG GAG ACC AAG GAG GTA CTG GGC-3'. Resulting PCR products were cleaved with Bam HI and Eco RI and sub-cloned in pGEX2T. pGEX-FSH15 has been described (Murata et al., 1994).

The *Renilla*-fusion vectors pcDNA3.1-NhRL and pcDNA3.1-ChRL were a generous gift from Dr. Sanjiv S. Gambhir (Univ. of California-Los Angeles, Los Angeles, CA) and are derivatives of vectors that have been described (Paulmurugan et al., 2002). pNhRL-Zn carries the zinc-“fingers” of Sp2 fused downstream of the amino-terminal 229 amino acids of *Renilla* luciferase and was prepared via the PCR using pBSK-Sp2/flu as template and the following PCR primers: 5'-GGG GGA TCC GGA GGA GGT GGT TCA GGA GGT GGA GGT AGC TGC CAC ATC CCC GAC TGT GGC AAG ACG TTC CG-3' and 5'-GGG CTC GAG TTA CAA GTT CTT CGT GAC CAG GTG GG-3'. The resulting PCR product was cleaved with Bam HI and Xho I and sub-cloned in pcDNA3.1-NhRL. pHD-ChRL carries the homeodomain of human Nkx3.1 fused upstream of the carboxy-terminal 82 amino acids of *Renilla* luciferase and was prepared using pcDNA-hNkx3.1 as template and the following PCR primers: 5'-GGG GCT AGC ATG CTC AGG GTT CCG GAG CCG-3' and 5'-GGG GGA TCC CCA AAA AGC TGG GCT CCA GCT GC-3'. The resulting PCR product was cleaved with Nhe I and Bam HI and sub-cloned in pcDNA3.1-ChRL. pChRL-HD carries the homeodomain of human Nkx3.1 fused downstream of the carboxy-terminal 82 amino acids of *Renilla* luciferase and was prepared in two steps. An initial round of PCR was

performed using pcDNA-hNkx3.1 as template and the following primers: 5'-GGG GGA TCC GGA GGA GGT GGT TCA GGA GGT GGA GGT AGC ATG CTC AGG GTT CCG GAG CCG-3' and 5'- GGG CTC GAG TTA CCA AAA AGC TGG GCT CCA GCT GC-3'. The resulting PCR product was cleaved with Bam HI and Xho I and sub-cloned in pcDNA3.1, generating pcDNA3.1-NkxHD. A second round of the PCR was performed using pcDNA3.1-ChRL as template and the following primers: 5'-GGG GCT ACG ATG AAG CCC GAC GTC GTC CAG ATT GTC-3' and 5'-GGG GGA TCC CTG CTC GTT CTT CAG CAC GCG-3'. The resulting PCR product was cleaved with Nhe I and Bam HI and cloned in pcDNA3.1-NkxHD such that the Nkx3.1 homeodomain is downstream of the carboxy-terminal 82 amino acids of *Renilla* luciferase, generating pChRLHD.

The mammalian expression vector pDXTAT was prepared via the PCR using pTAT-HA (a generous gift from Dr. Steven F. Dowdy, Univ. of California-San Diego, San Diego, CA; Nagahara et al., 1998) and the following PCR primers: 5'- GGG GAG CTC ATG CGG GGT TCT CAT CAT CAT CAT C-3' and 5'-GGG GGT CGA CGG AGC CAG CAT AGT CTG GGA C-3'. The resulting PCR product was cleaved with Sac I and Acc I and sub-cloned in pBK-CMV (Stratagene, Inc., LaJolla, CA), generating pDXTAT. Derivatives of pDXTAT carrying full-length and partial human Nkx3.1 cDNAs were prepared via the PCR using pcDNA3.1-hNkx3.1 as template and the following PCR primers: pDXTAT(Nkx3.1):5'- GGG GAA TTC ATG CTC AGG GTT CCG GAG C-3'; 5'-GGG CTC GAG TTA CCA AAA AGC TGG GCT CCA GCT G-3'; pDXTAT(1-90):5'- GGG GAA TTC ATG CTC AGG GTT CCG GAG C-3'; 5'-GGG CTC GAG TTA CAG CGT CTC GGC CTC CTC CGG-3'; pDXTAT(1-123):5'-GGG

GAA TTC ATG CTC AGG GTT CCG GAG C-3'; 5'-GGG CTC GAG TTA CGG CTG CTT AGG GGT TTG GGG-3'; pDXTAT(1-183): 5'-GGG GAA TTC ATG CTC AGG GTT CCG GAG C-3'; 5'-GGG CTC GAG TTA CTG CTT TCG CTT AGT CTT ATA GCG-3'; pDXTAT(124-183): 5'-GGG GAA TTC ATG CAG AAG CGC TCC CGA GCT GCC-3'; 5'-GGG CTC GAG TTA CTG CTT TCG CTT AGT CTT ATA GCG-3'; pDXTAT(124-234): 5'-GGG GAA TTC ATG CAG AAG CGC TCC CGA GCT GCC-3'; 5'-GGG GAA TTC ATG CTC AGG GTT CCG GAG C-3'. Resulting PCR products were cleaved with Eco RI and Xho I and sub-cloned in pDXTAT. Analogous pDXTAT constructs without the HIV TAT nuclear localization signal were prepared using pDXTAT(Nkx3.1), pDXTAT(1-90), pDXTAT(1-123), pDXTAT(1-183), pDXTAT(124-183), and ; pDXTAT(124-234) as templates and the following 5'-phosphorylated PCR primers: 5'-GAC GAT GAC GAT AAG GAT CGA TGG GGA TCC-3' and 5'-GGG ACG TCA TAT GGA TAG CCG GAC ATG GT-3'. Resulting PCR products were cleaved with Dpn I to destroy template DNA, self-ligated, and used to transform *E. coli*. Plasmids prepared from resulting transformants were sequenced to identify clones harboring the desired deletions, creating pDXTAT-less(Nkx3.1), pDXTAT-less(1-90), pDXTAT-less (1-123), pDXTAT-less (1-183), pDXTAT-less (124-183), and pDXTAT-less (124-234).

Site-directed mutagenesis was employed to create a single amino acid change within the homeodomains of human (Q173E) and murine (Q174E) Nkx3.1. Briefly, pcDNA3.1-mNkx3.1 and pcDNA3.1-hNkx3.1 were used as templates for the PCR using Platinum Pfx DNA Polymerase (Invitrogen) and the following 5'-phosphorylated primers (mutations are indicated in bold): murine Nkx3.1, 5'-GAA CCA TAT TTT GAC TTG

GGT TTC GG-3' and 5'-GAG AAC AGA CGC TAT AAG ACC AAG CG-3'; human Nkx3.1, 5'-GAA CCA TAT CTT CAC TTG GGT CTC CG-3' and 5'-GAG AAC AGA CGC TAT AAG ACT AAG CGA AAA G-3'. Resulting PCR products were cleaved with Dpn I to destroy template DNA, self-ligated, and used to transform *E. coli*. Plasmids prepared from resulting transformants were sequenced to identify clones harboring the desired mutations, creating pcDNA3.1-mNkx3.1mut and pcDNA3.1-hNkx3.1mut.

Enhanced Yellow Fluorescent Protein (pEYFP-C1) fusion proteins containing human wild-type and mutant Nkx3.1 expression constructs, pEYFP-C1-Nkx3.1 and pEYFP-C1-Nkx3.1mut, were generated via the PCR using pcDNA3.1-hNkx3.1 and pcDNA3.1-hNkx3.1mut as templates and the following primers: 5'-GGG GGT ACC ATG CTC AGG GTT CCG GAG CCG-3', and 5'-CCC CTC GAG TTA CCA AAA AGC TGG GCT CCA GCT GCC-3'. The amplified cDNAs were subsequently digested with Bam HI and Xho I and sub-cloned "in-frame" at the Bgl II and Sal I sites of pEYFP-C1 to generate the mammalian expression plasmids pEYFP-C1-Nkx3.1 and pEYFP-C1-Nkx3.1mut. EYFP-C1 fusion proteins containing partial human Nkx3.1 proteins, pEYFP-C1-Δ90, pEYFP-C1-Δ123 and pEYFP-C1-Δ123v1 were generated following PCR that used pcDNA3.1-Δ90, pcDNA3.1-Δ123 and pcDNA3.1-Δ123v1 as templates and the following primers: pEYFP-C1-Δ90: 5'-GGG GAA TTC GGA TGC TCA GGG TTC CGG AGC CG-3' and 5'-GGG GGA TCC TTA CAG CGT CTC GGC CTC CTC CGG-3'; pEYFP-C1-Δ123 and pcDNA3.1-Δ123v1: 5'-GGG GAA TTC GGA TGC TCA GGG TTC CGG AGC CG-3' and 5'-GGG GGA TCC TTA CGG CTG CTT AGG GGT TTG GGG-3'. The amplified cDNAs were subsequently sub-cloned "in-frame" at the

Eco RI and Bam HI sites of pEYFP-C1 to generate the mammalian expression plasmids pEYFP-C1- Δ 90, pEYFP-C1- Δ 123 and pEYFP-C1- Δ 123v1. EYFP-C1 fusion proteins containing wild-type and mutant human Nkx3.1 homeodomains (residues 124-183), pEYFP-C1-HD and pEYFP-C1-HDmut were generated following PCR that used pcDNA3.1-hNkx3.1 and pcDNA3.1-hNkx3.1mut as templates and the following primers: 5'-GGG GAA TTC ATG CAG AAG CGC TCC CGA GCT GCC-3' and 5'-GGG GGA TCC TTA CTG CTT TCG CTT AGT CTT ATA GCG-3'. The amplified cDNAs were subsequently cloned in frame at the Eco RI and Bam HI sites of pEYFP-C1 to generate the mammalian expression plasmids pEYFP-C1-HD and pEYFP-C1-HDmut. EYFP-C1 fusion proteins containing partial human Nkx3.1 homeodomains, pEYFP-C1- Δ 144HD and pEYFP-C1- Δ 164HD were generated following PCR that used pcDNA3.1-hNkx3.1 as the template and the following primers: pEYFP-C1- Δ 144HD: 5'- GGG AAG CTT ATG CAG AAG CGC TCC CGA GCT GCC-3' and 5'-GGG GGA TCC TTA CTT GAA GGA CTC CAA CTC GAT CAC-3'; pEYFP-C1- Δ 164HD: 5'- GGG AAG CTT ATG CAG AAG CGC TCC CGA GCT GCC-3' and 5'-GGG GGA TCC TTA CTC GAA CTC CAA GAA CCG GTC CAC-3'. The amplified cDNAs were subsequently cloned in frame at the Hind III and Bam HI sites of pEYFP-C1 to generate the mammalian expression plasmids pEYFP-C1- Δ 144HD and pEYFP-C1- Δ 164HD.

2.2.2 Transcriptional reporter constructs

A chloramphenicol acetyltransferase (CAT) reporter gene governed by a minimal portion of the *Adenovirus* Major Late promoter, Δ 53MLP-CAT, (a generous gift of Dr. Adrian R. Black, Roswell Park Cancer Institute, Buffalo, NY) was employed in

transcription experiments to normalize for plate-to-plate variations in transfection efficiency. The transcriptional activity of this construct has been shown to not be regulated by Sp family members (Moorefield et al., 2004; Spengler et al., 2005). A second *Adenovirus*-derived reporter plasmid carrying this same promoter fragment linked to *Renilla* luciferase was prepared by annealing the following oligonucleotide and its complement, 5'-GGG CTC GAG GTT CAC AAT TTT CTG GTG GTG GGC TAT AAA AAA AGC TTG GG-3', digestion with Xho I and Hind III, and cloning into phRL-basic (Promega, Inc., Madison, WI), generating phRL-Δ53MLP. A firefly luciferase reporter construct (PSA-Lux) carrying a 5.3-kbp portion of the *PSA* promoter was a generous gift of Dr. Charles J. Bieberich and has been described (Chen et al., 2002). PSA-Lux constructs lacking various portions of the *PSA* promoter were prepared via the PCR using PSA-Lux as template, Platinum Pfx DNA Polymerase (Invitrogen), 5'-GAA TGC CAA GCT TGG GGC TGG GGA G-3' as a 3'-primer and the following 5'-primers: Bst EII (-4243 to +1): 5'-GGG TCT AGA CCA AAT CTT GTA GGG TGA CCA GAG-3'; Eco RV (-4075 to +1): 5'-GGT CTA GAC AAG CCT CGA TCT GAG AGA GAT ATC ATC-3'; Apa I (-2858 to +1): 5'-GGG TCT AGA CCT GAT GAA CAC CAT GGT GTG TAC AGG-3'; Sex AI (-1729 to +1): 5'-GGG TCT AGA GGC TGG CCT CGA ACT CCT GAC CTG G-3'. Resulting PCR products were cleaved with Xba I and Hind III and sub-cloned in pGL3-basic (Promega), generating pBstEII-Lux, pEcoRV-Lux, pApaI-Lux, and pSexAI-Lux. Site-directed mutagenesis was employed to inactivate putative Nkx3.1-binding sites located at -4973 (site 1) and -4922 (site 2) of the *PSA* promoter. Briefly, PSA-Lux was used as template for two PCR reactions using Platinum Pfx DNA Polymerase (Invitrogen) and the following 5'-phosphorylated primers

(mutations are indicated in bold): (site 1 mutation), 5'- **CCA** CGT ATG CTT GCA CTG CTG AAT GCT TGG G-3' and 5'-ACA CGG CAC TCC CCA GAG CCA GG-3; (site 2 mutation), 5'-**CCA** CGC TGA AGA TTA ACC CTG ACA CAT CCC-3' and 5'-CAG ATG CTC ATC TCA TCC TCA CAG-3'. Resulting PCR products were cleaved with Dpn I to destroy template DNA, DNAs were self-ligated and used to transform *E. coli*. Plasmids derived from resulting transformants were sequenced to identify clones, termed PSA-Lux mut1 and PSALux mut2, carrying the desired mutations. A firefly luciferase reporter construct carrying mutations within both putative Nkx3.1-binding sites, PSA-Lux DbImut, was prepared via the PCR using PSA-Lux mut1 as template, Platinum Pfx DNA Polymerase (Invitrogen), and the previously described site 2 primer pair. The resulting PCR product was sub-cloned and characterized as described above. Except where indicated, all PCR reactions employed Deep Vent polymerase (New England Biolabs, Inc., Beverly, MA). The integrity of all constructions was confirmed by dideoxy-sequencing using Sequenase version 2.0 DNA polymerase following a protocol supplied by the manufacturer (Amersham Life Science Inc., Arlington Heights, IL).

2.2.3 Baculovirus stocks

An human Nkx3.1 cDNA carrying a carboxy-terminal ten amino acid epitope tag derived from *Influenza* hemagglutinin was generated via the PCR using pcDNA3-hNkx3.1 as the template, Deep Vent DNA Polymerase (New England Biolabs, Inc.) and the following primers: 5'-GGG GAA TTC ATG CTC AGG GTT ACG GAG CCG-3' and 5'-GGG GGA TCC TTA CGC ATA ATC TGG CAC ATC ATA AGG GTA CCA AAA AGC TGG GCT CCA GCT GC-3'. The resulting amplified, epitope-tagged cDNA

was sub-cloned into pCR-Blunt_{II}-TOPO (Invitrogen) at the Eco RI and Bam HI sites and subsequently sub-cloned into pVL-1392 (Pharming, San Diego, CA) to create pVL-1392-Nkx3.1-flu. Recombinant Nkx3.1-flu was transferred to the viral DNA backbone via homologous recombination using proprietary reagents (BaculoGold; Pharmingen, Inc., San Diego, CA). Sf9 cells (3.0×10^6) were plated in T-25 flasks with 5 ml Grace's supplemented media and incubated at 27 °C for 15 min. Adherent cells were then transfected with 0.5 µg of linearized BaculoGold DNA and 2.0 µg of pVL1392-Nkx3.1-flu according to the manufacturer's protocol and transfected cells were cultured for 5 days at 27 °C. Baculoviral stocks were amplified by infecting 2.5×10^6 Sf9 cells plated in a T-25 flask for 5 days, followed by the infection of 7.0×10^6 Sf9 cells plated in a T-75 flask for 5 days, and finally by infecting 7.5×10^7 Sf9 cells seeded in a spinner flask for 5 days. The resulting Nkx3.1-flu baculoviral stock was harvested by centrifuging the contents of the infected spinner flask at 1000 RPM for 5 min at 4 °C and collecting the supernatant.

2.3 Transient transfections

Transient transfections were performed using SuperFect transfection reagent (Qiagen Inc., Hilden, Germany) per the manufacturer's protocol. To analyze levels of transcription in the absence of histone deacetylase activity, transfected cells were treated for 24 hours with 100-300 nM Trichostatin A (TSA; Sigma-Adrich, St. Louis, MO). Cell extracts were prepared for luciferase and CAT assays 48 h after transfection.

2.4 Luciferase assays

The Dual-Luciferase Reporter Assay System (Promega) was employed to quantify luciferase activity precisely as recommended by the manufacturer. Two days post-transfection, cell extracts were prepared from PBS-washed cells followed by a 15 min incubation in Passive Lysis Buffer (200 μ l/well). Cell debris was cleared by centrifugation at 14,000 RPM for 5 min. Reactions containing 100 μ l of extract and 100 μ l of Luciferase Assay Reagent II were mixed and luminescence was detected in a Lumat LB 9507 luminometer (EG&G Berthold, Bad Wildbad, Germany). Results were normalized to the abundance of Δ 53MLP-CAT (see below) or Δ 53MLP-Renilla activity.

2.5 Chloramphenicol acetyltransferase (CAT) assays

Chloramphenicol acetyltransferase (CAT) assays were performed using a liquid scintillation assay as previously described (Gorman et al., 1982). Extracts from DU145 cells were prepared two days post-transfection by collecting PBS-washed cells and resuspension in Passive Lysis Buffer. Resuspended cells were then transferred to microfuge tubes and freeze/thawed three times in a dry ice/ethanol and 37 °C baths. Cell debris was removed by centrifugation at 14,000R RPM for 5 min, and CAT assays were performed in scintillation vials. Extracts (25 μ l) were added to a cocktail containing 75 μ l 0.1 M Tris pH 7.8 and 100 μ l of 0.5 μ Ci/ml 3 H-Acetyl CoA (ICN) in 2mg/ml chloramphenicol dissolved in 0.1 M Tris pH 7.8. Reactions were vortexed gently and overlaid with 3 ml Econofluor-2 non-aqueous scintillation fluid (Packard Instrument Company, Inc., Meridian, CT). The abundance of 3 H-chloramphenicol in samples was

quantified at least three times for 30 sec each over a four-hour time course, and the mean counts per minute were used to normalize for plate-to-plate differences in luciferase activity in transient transfection experiments.

2.6 Expression and purification of GST-fusion proteins

2.6.1 Expression of GST-fusion proteins

BL21 CodonPlus competent bacterial cells (Stratagene, Inc., La Jolla, CA) transformed with GST-expression constructs were cultured in Terrific Broth media (Mediatech, Inc., Herndon, VA) overnight at 37 °C with 250 rpm agitation. Cells were diluted 1:10 with fresh Terrific Broth and cultured until OD=0.5 (approximately 1-1.5 hrs). Protein expression was induced via the addition of isopropyl-1-thio- β -D-galactopyranoside (IPTG; Sigma) at a final concentration of 0.2 mM and cells were cultured for an additional three hrs at 37 °C and 250 rpm. Cells were collected by centrifugation at 3000 rpm for 10 min, pellets were resuspended in 9 ml PBS containing 10 μ g/ml PMSF, pepstatin A, and leupeptin and 1 mM EDTA and cells were lysed by sonication. Debris was removed by centrifugation at 10,000 for 10 min at 4 °C, and Triton X-100 was added to a final concentration of 1%. The resulting cell pellet was suspended in 1.5% N-lauroylsarcosine (Sarkosyl; Sigma), 25 mM triethanolamine, 1 mM EDTA pH 8.0 and incubated on ice for 10 min. The suspension was centrifuged at 10,000 for 20 min at 4 °C and raised to 1% Triton X-100 and 1 mM CaCl₂.

2.6.2 Purification of GST-fusion proteins for protein/protein-binding assay

Glutathione-agarose beads (Sigma) were added to cleared bacterial extracts, and the mixture was rocked overnight at 4 °C. Beads were then washed three times with PBS and resuspended in one volume of NETN buffer (20 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40). To quantitate amounts of GST-fusion proteins bound to beads, 25 µl aliquots of bead-bound proteins were boiled in Laemmli sample buffer, eluted proteins were resolved on acrylamide gels in parallel with Bovine Serum Albumin (BSA) standards, and gels were stained with Coomassie Brilliant Blue.

2.6.3 Purification of GST-fusion proteins for protein/DNA-binding assay

Glutathione-agarose beads (Sigma) washed three times with Column Wash Buffer (150 mM NaCl, 2.5 mM KCl, 10 mM NaH₂PO₄, 2 mM KH₂PO₄, 1% Tween 20, 1% Triton X-100, 10 mM DTT, 250µM PMSF) and were added to cleared bacterial extracts and the mixture was rocked overnight at 4 °C. The beads were then loaded into a 5 mL plastic disposable column at 4 °C (Pierce, Rockford, IL) and washed six times with ice-cold Column Wash Buffer and twice with ice-cold Tris/PMSF (50 mM Tris pH 8.0, 250µM PMSF). Proteins were eluted from the beads with 2 mL ice-cold Tris/PMSF plus reduced glutathione (Sigma; 5 mg/ml). Excess glutathione was removed using Centricon YM-30 centrifugal filter devices (Millipore, Inc. Billerica, MA). Eluants were applied to Centricon filters and centrifuged at 5000 rpm for 60 min at 4 °C. To recover filter-bound proteins, filters were washed with 400 µl of ice-cold Tris/PMSF, inverted, and proteins were collected by centrifugation at 1000 rpm for 2 min at 4 °C. Concentrated, desalted proteins (500 µl each) were diluted with 500 µl ice-cold Storage Buffer (50 mM Tris pH

8.0, 20% glycerol, 0.5 mM PMSF, 1 mM DTT, 1 µg/ml leupeptin, 1 µg/ml pepstatin) and assayed for Nkx3.1 by Western blotting using anti-Nkx3.1 (T-19; Santa Cruz) as described below.

2.7 Cell extracts

2.7.1 Non-denatured whole cell extracts

Cells were scraped from tissue culture plates or decanted from spinner flasks and pelleted at 2500 RPM for 5 min at 4 °C. Pellets were washed with PBS and then resuspended in ten cell pellet volumes (CPV) of ice-cold EBC buffer (50 mM Tris pH 8.0, 120 mM NaCl, 0.5% NP-40, 100 mM NaF, 200 µM Na-orthovanadate and 10 µg/ml Pepstatin A, PMSF, and Leupeptin), and incubated for 60 min at 4 °C with agitation. Extracts were cleared of debris by centrifugation at 14,000 RPM for 30 min at 4 °C, and protein concentrations were determined using the Coomassie Plus Protein Assay Reagent Kit (Pierce) and BSA standards.

2.7.2 Nuclear extracts

Nuclear extracts were prepared using a method described by Lee, et al. (1988). Cells were rinsed with PBS, scraped from plates and collected by centrifugation at 1000 rpm for 10 min at 4 °C. The volume of the cell pellet was estimated then resuspended in an equal volume of hypotonic buffer A (10 mM Hepes pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF). Swollen cells were incubated on ice for 15 min, passed through a 25-gauge needle, and nuclei were collected by centrifugation at 3,000 rpm for 5 min at 4 °C. The cell pellet volume was again estimated and resuspended in

hypertonic buffer C (20 mM Hepes pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF) at approximately 1 ml/0.3 ml pellet volume, and nuclei were stirred for 5 min at 4°C. Extracts were subsequently cleared of debris by centrifugation at 14,000 rpm, 5 min at 4°C.

2.8 Column filtration chromatography

Non-denatured whole cell extracts were prepared from twenty 100-mm tissue culture plates of LNCaP cells that were cultured at 37 °C in RPMI 1640 culture media under 5% CO₂. Extracts from these twenty dishes were prepared in a total of 6 ml of EBC buffer as described above. To fractionate whole cell lysates, a HiPrep Sephacryl S-300 High Resolution 26/60 chromatography column (26/60; Amersham) with a bed volume of 320 ml containing 47 µm particles of a cross-linked copolymer of allyl dextran and N,N-methylenebisacrylamide was employed. This resin is designed for the separation of proteins in the range of 1,500-10 kDa. The column was calibrated using 3.9 ml of a 1:2 dilution of distilled water and Start Buffer (0.05 M Na₂HPO₄, 0.15 M NaCl, pH 7.2) and the Molecular Weight Marker Kit for Gel Filtration Chromatography (Sigma; includes molecular weight marker proteins of 669, 443, 150, 66, and 29 kDa). Prior to applying cell lysates, the column was pre-run at 21 psi of pressure in running buffer (50 mM Tris, 100 mM NaCl, 0.02% Sodium Azide) at a flow rate of 1 ml/min for 60 min. The entire 6 ml volume of lysate was applied to the column, run at 21 psi at a flow rate of 1 ml/min, and eluted proteins were collected in 3 ml aliquots every 3 minutes for an additional 105 minutes using the Biologic LP Chromatography system (BioRad). Elutions were immediately stored on ice. Collected fractions were concentrated using

Centricon YM-30 centrifugal filter devices (Millipore). One ml of each fraction was applied to the Centricon filter and centrifuged at 5000 rpm for 60 min at 4 °C. To recover filter-bound proteins, filters were inverted and proteins were collected by centrifugation at 1000 rpm for 2 min at 4 °C. Concentrated, fractionated proteins (50 µl each) were assayed for Nkx3.1 by Western blotting using anti-Nkx3.1 (T-19; Santa Cruz) as described below.

2.9 Protein/DNA-binding assays

2.9.1 Standard protein/DNA-binding assay

Oligonucleotides were radiolabeled and utilized in protein/DNA binding assays as previously described (Udvadia et al., 1992). Recombinant Sp-family members were prepared from baculovirus-infected Sf9 cells as previously described (Moorefield et al., 2004; Kennett et al., 1997). Briefly, 100 ng of recombinant human Nkx3.1 (a generous gift from Dr. Charles J. Bieberich) and/or 1 µl of baculovirus-infected Sf9 whole cell lysate was incubated for 20 min at 4 °C with 50,000 CPM of radiolabeled oligonucleotide probe, and protein/DNA complexes were resolved on 4.8% polyacrylamide gels at 250V for 1.5 h. In some experiments, protein/DNA complexes were challenged with 5 µl of anti-Nkx3.1 antibodies (T-19, N-15, L-15; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) prior to resolution on acrylamide gels. The following oligonucleotides and their complements were used in protein/DNA-binding assays (cognate binding sites are shown in bold): Nkx: 5'-AGA CGG ATC CTA TGC GCG ATT TTT **TAA GTA** GTT TTT CAG TAG CTA TCT GCA GGC GT-3'; Mut: 5'-AGA CGG ATC CTA TGC GCG ATT TTT **TCC ACA** GTT TTT CAG TAG CTA TCT GCA GGC GT-3'; NkxSp-5: 5'-GTA

CCT CGA GTA TAA GTA TAT GTG GGC GGG ACT AAG GAT CCG CGG-3';
NkxSp-10: 5'-GTA CCT CGA GTA TAA GTA TAT ATA TAG TGG GCG GGA
CTA AGG ATC CGC GG-3'.

2.9.2 LNCaP fraction “mixing” experiments

Recombinant Nkx3.1 was purified from *E. coli* as described above. Recombinant Nkx3.1 (200 ng or 1 µg) was incubated with 13 µl of concentrated or unconcentrated LNCaP cell extract fractions for 10 min at room temperature before supplementation with poly dI/dC (0.067 µg/ µl) and binding buffer (10 mM Tris pH 7.5, 50 mM NaCl, 5% glycerol, 5 mM MgCl₂, 1 mM EDTA, and 1 mM ZnCl₂) and radiolabeled probe (Nkx; 5 x 10⁴ cpm). Reactions were incubated for 10 min on ice and resulting protein/DNA complexes were resolved on 4.8% polyacrylamide gels at 250V for 1.5 h and visualized by autoradiography.

2.9.3 Phosphatase treatment

For treatment with lambda phosphatase, non-denatured LNCaP whole cell extracts (80 µl) were prepared as described above and incubated with 10 µl lambda phosphatase (400 units; New England Biolabs) and 10 µl 10x lambda phosphatase reaction buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM DTT, 0.1 mM EGTA, and 0.01 % Brij 35) and 1 mM PMSF for one hour at 30 °C. For treatment with Antarctic phosphatase, non-denatured LNCaP whole cell extract (80 µl) were incubated with 10 µl Antarctic phosphatase (50 units; New England Biolabs) in 10 µl 10x lambda phosphatase reaction buffer (50 mM Bis-Tris-Propane-HCl pH 6.0, 1 mM MgCl₂, 0.1 mM ZnCl₂) and

1 mM PMSF for one hour at 37 °C. Dephosphorylated LNCaP cell extracts were subsequently used in standard protein/DNA-binding assay as described above or incubated with recombinant Nkx3.1 (200 ng or 1 µg) for 10 min at room temperature before supplementation with poly dI/dC, binding buffer and radiolabeled probe (Nkx; 5 x 10⁴ cpm). Reactions were incubated for 10 min on ice and resulting protein/DNA complexes were resolved on 4.8% polyacrylamide gels at 250V for 1.5 h and visualized by autoradiography.

2.9.4 Deoxycholate treatment

For treatment with deoxycholate, non-denatured LNCaP whole cell extracts were prepared as described above and incubated with 1% sodium deoxycholate for 20 min on ice. NP-40 was added to the reaction (1.2% final concentration) after deoxycholate treatment. Deoxycholate-treated LNCaP extracts were subsequently used in standard protein/DNA-binding assays as described above or incubated with recombinant Nkx3.1 (200 ng or 1 µg) for 10 min at room temperature before supplementation with poly dI/dC, binding buffer and radiolabeled probe (Nkx; 5 x 10⁴ cpm). Reactions were incubated for 10 min on ice and resulting protein/DNA complexes were resolved on 4.8% polyacrylamide gels at 250V for 1.5 h and visualized by autoradiography.

2.10 *In Vitro* transcription/translation and protein/protein-binding assays

2.10.1 *In vitro* transcription/translation

In vitro transcribed/translated proteins were produced using pBSK-Sp1/flu, pBSK-Sp2/flu, pBSK-Sp3/flu, TOPO-Sp4, TOPO-Sp5, TOPO-Sp2ΔZn, or pcDNA3.1-

Nkx3.1 as template, a coupled reticulocyte lysate system (TNT; Promega) and a proprietary cocktail of radiolabeled amino acids (³⁵S-Translabel; ICN Pharmaceuticals, Costa Mesa, CA). Radiolabeled proteins were then employed in *in vitro* protein/protein-binding assays as described below.

2.10.2 *In vitro* protein/protein-binding assays

In vitro protein/protein-binding assays were performed by incubating 5 µg of bead-bound GST-fusion proteins with 10 µl [³⁵S]-L-methionine labeled (Tran ³⁵S-label; ICN) *in vitro* translated proteins in 400 µl NETN (20 mM Tris, pH 8.0, 100 mM NaCl, 6 mM MgCl₂, 1 mM EDTA, 0.5 mM NP-40, 1 mM DTT, 8% glycerol, and 1 mM PMSF) for 60 min at 4 °C with gentle rocking. Beads were washed four times with NETN and proteins were eluted by boiling in Laemmli sample buffer. Eluted proteins were resolved on denaturing acrylamide gels and visualized by autoradiography.

2.11 Antibodies

Affinity purified anti-Nkx3.1 (T-19; sc-15022; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) is a goat polyclonal antibody prepared against the amino-terminus of human Nkx3.1. Anti-Nkx3.1 (T-19) was used at a dilution of 1:1000 for Western blotting and 1:50 for chromatin immunoprecipitations. Affinity purified anti-Nkx3.1 (N-15; sc-15021; Santa Cruz Biotechnology, Inc.) is a goat polyclonal antibody prepared against the amino-terminus of human Nkx3.1. Anti-Nkx3.1 (N-15) was used at a dilution of 1:1000 for Western blotting and 1:50 for chromatin immunoprecipitations. Affinity purified anti-Nkx3.1 (L-15; sc-15025; Santa Cruz Biotechnology, Inc.) is a goat

polyclonal antibody prepared against the amino-terminus of mouse Nkx3.1. Anti-Nkx3.1 (L-15) was used at a dilution of 1:1000 for Western blotting and 1:50 for chromatin immunoprecipitations. Sc-2768 (Santa Cruz Biotechnology, Inc.) is a horseradish peroxidase-conjugated rabbit anti-goat IgG antibody and was used as a secondary antibody in Western blotting at a dilution of 1:5000. Affinity purified anti-HA (12CA5; Roche, Inc., Indianapolis, IN) is a mouse monoclonal antibody that recognizes a ten amino acid epitope (NH₂-YPYDVDPDYAS-CO₂H) derived from the *Influenza* hemagglutinin protein (HA). Anti-HA was used at a dilution of 1:1000 for Western blotting. All antibodies were used at a dilution of 1:5 for the identification of proteins in protein/DNA binding assays. For indirect immunofluorescence experiments, anti-HA (12CA5) was used at a dilution of 1:500. Alexa Fluor 594 anti-mouse secondary antibody and Alexa Fluor 488 goat anti-mouse antibody were obtained from Molecular Probes, Inc. (Eugene, OR) and were used at a dilution of 1:500 for indirect immunofluorescence experiments.

2.12 Western blotting

PBS-washed cells were pelleted at 1000 rpm for 10 min, resuspended in ten cell pellet volumes of Western sample buffer (2% SDS, 60 mM Tris pH 6.8, 100 mM DTT), passed through a 22.5 gauge needle, and boiled for 5 min. Debris was cleared by centrifugation at 14,000 rpm for 10 min at 4 °C, and protein concentrations were determined using the Coomassie Plus Protein Assay Reagent Kit (Pierce, Rockford, IL). Proteins were resolved on denaturing 10% polyacrylamide gels, and transferred to PVDF membranes (Millipore) at 20 V for 20 min using a semidry transfer apparatus (Transblot-SD, BioRad, Inc., Hercules, CA). Membranes were blocked with 5% fat-free dried milk

in TBS-T (2.42 g/L Tris pH 7.6, 8 g/L NaCl, supplemented with 1% Tween-20) for 60 min to overnight. Following blocking, membranes were incubated with primary antibodies diluted in TBS-T containing 5% milk for 60 min with gentle rocking. Membranes were then washed four times for 15 min each with TBS-T, and incubated with horseradish peroxidase-conjugated secondary antibodies for 30 min with gentle rocking. Anti-mouse (NA 9310; Amersham Pharmacia Biotech, Inc., Arlington Heights, IL) or anti-goat (Santa Cruz) secondary antibodies were diluted 1:10,000 or 1:5,000, respectively, in TBS-T containing 5% milk prior to incubation with membranes. Membranes were washed with TBS-T once for 15 min followed by four subsequent washes for 5 min each. Antigen-antibody complexes were detected using Western Blot Chemiluminescent Detection Reagents (ECL; Amersham).

2.13 DNA sequencing

Double-stranded DNA sequencing was performed using Sequenase version 2.0 T7 DNA polymerase (USB, Cleveland, OH). Plasmid DNAs (5 μ g) were denatured by incubation at 37 °C for 30 min in 20 μ l dH₂O plus 2 μ l (2 M NaOH, 2 mM EDTA). Reactions were neutralized and DNAs were precipitated with 2.2 μ l 3 M NaOAc and 65 μ l absolute ethanol. Annealing reactions were performed by resuspending denatured DNAs in 1.5 μ l dH₂O, 1 μ l Sequenase buffer, and 200 ng of primer, and incubating at 37 °C for 30 min. Annealed DNAs was extended by adding 0.5 μ l 0.1 M DTT, 1.0 μ l labeling mix (diluted 1:5 in dH₂O), 1.0 μ l Sequenase DNA polymerase (diluted 1:8 in Sequenase dilution buffer), and 0.5 μ l (5 μ Ci) [α -³⁵S]dATP (Perkin Elmer Life and Analytical Sciences, Inc., Boston, MA) and incubation at room temperature for 5 min.

To terminate extension reactions, 1.75 μ l of the extension reaction was added to 1.3 μ l each of four dideoxy termination mixtures. Termination reactions were incubated at 37 °C for 5 min, and then stopped by the addition of 2 μ l FDE (90% deionized formamide, 20 mM EDTA, bromophenol blue (0.2%), xylene cyanol (0.2%)). Sequencing reactions were boiled for 2 min, resolved on acrylamide gels at 70W for 2 hrs in 0.5 x TBE (5.45 g/L Tris (pH 8.0) 2.78 g/L Boric acid, and 0.51 g/L EDTA). Resulting sequencing reaction were visualized by autoradiography.

2.14 Indirect immunofluorescence

2.14.1 Indirect immunofluorescence

One day prior to transfection, 2×10^5 COS-1 cells were plated onto glass cover slips in six-well plates and incubated at 37 °C. Cells were cultured for 48 h following transfection and all subsequent procedures were performed at room temperature. Cover slips were transferred to 60 mm tissue culture dishes and cells were washed for five min with phosphate-buffered saline (PBS) on a rotating platform. Cells were fixed in 2% paraformaldehyde/PBS for 15 min and then washed for 5 min with PBS. Cells were subsequently permeabilized with 0.5% Triton X-100/PBS for 60 min, and then incubated for an additional 60 min in 1% fetal bovine serum/PBS. Cells were incubated with primary antibodies (diluted 1:500 in 1% BSA/PBS) for 60 min, and then washed three times for 5 min each in PBS on a rotating platform. Cells were incubated with secondary antibodies (diluted 1:500 in 1% BSA/PBS) and (DAPI; diluted 1:50,000 in 1% BSA/PBS) for 60 min, and then washed three times for 5 min each in PBS on a rotating platform. Following a single 5 min wash in dH₂O, cover slips were mounted on glass

microscope slides using Vectashield Mounting Media (Vector Labs, Burlingame, CA), sealed with clear nail polish and viewed using a Nikon TE-200 inverted epifluorescence microscope equipped with appropriate optics and filter blocks at a magnification of 100X under oil immersion. Results were recorded with a digital camera (SPOT, Jr.; Diagnostics Instruments, Sterling Heights, MI) and proprietary software using the manufacturer's instructions.

2.14.2 *In Situ* nuclear matrix preparation and analysis

Cells were analyzed *in situ* by direct and indirect immunofluorescence following the solubilization and preparation of nuclear matrices essentially as described by Javed et al. 2000. COS-1 cells (2×10^5) were plated on sterile glass coverslips in each well of 6-well plates and cultured at 37 °C overnight in DMEM culture media supplemented with heat-inactivated FBS, and 50 µg/ml Pipracil at 37°C under 5% CO₂. The following day, some wells were transfected with expression vectors carrying GFP-fusion constructs (EYFP-hNkx3.1, EYFP-Δ90, EYFP-Δ123, EYFP-Δ123v1, EYFP-HD, EYFP-Δ144HD, EYFP-Δ164HD or EYFP-HD mutant; 2 µg per well), others were transfected with mammalian Nkx3.1 expression vectors (pDXTAT-less-Nkx3.1, pDXTAT-less-Δ90, pDXTAT-less-Δ123, pDXTAT-less-Δ183, pDXTAT-less-Δ124-183, or pDXTAT-less-Δ124-234; 2µg per well) using SuperFect transfection reagent (Qiagen) as described above, and cultured for 24-48 hours at 37 °C under 5% CO₂. Plates were placed on ice, washed twice with ice cold PBS, and cells were solubilized in CSK buffer (100 mM NaCl, 0.3 M sucrose, 10 mM Pipes, 3 mM MgCl₂, 1 mM EGTA, 0.5% Triton X-100, pH 6.8) containing RNase inhibitors (2 mM vanadyl ribonucleoside complex or 40 U/ml RNasin). Following removal of CSK buffer chromatin was digested at 30 °C for 60 min via

incubation of nuclei in Digestion Buffer (50 mM NaCl, 0.3 M sucrose, 10 mM Pipes, 3 mM MgCl₂, 1 mM EGTA, 0.5% Triton X-100, pH 6.8) containing 50 U/ml RNase-free DNase I (Roche). Following removal of Digestion Buffer, nuclei were incubated for 10 min on ice in Stop Solution (Digestion Buffer containing 250 mM NH₄SO₄). All nuclei were subsequently fixed with 2% paraformaldehyde at room temperature for 15 min, washed with PBS, and stained with DAPI (4',6-diamidino-2-phenylindole) in 0.5% Triton X-100/PBS. Cells transfected with “TAT-less” constructs were incubated with primary antibodies (diluted 1:500 in 1% BSA/PBS) for 60 min, washed three times for 5 min each in PBS on a rotating platform, incubated with secondary antibodies (diluted 1:500 in 1% BSA/PBS) and (DAPI; diluted 1:50,000 in 1% BSA/PBS) for 60 min, and then washed three times for 5 min each in PBS on a rotating platform. Coverslips were washed with PBS, then dH₂O, and mounted using Vectashield mounting media. Fluorescence was imaged using a Nikon TE-200 inverted epifluorescence microscope equipped with appropriate optics and filter blocks at a magnification of 100x under oil immersion. Results were recorded with a digital camera (SPOT, Jr.: Diagnostics Instruments, Inc., Sterling Heights, MI) and proprietary software using the manufacturer’s instructions.

2.15 Chromatin immunoprecipitation

Twenty 100 mm tissue culture plates of LNCaP cells, cultured at 37 °C in RPMI 1640 culture media under 5% CO₂, were washed twice in PBS at room temperature. Room temperature, sterile PBS was added (9 ml per plate) followed by 1 ml of Formaldehyde crosslinking solution (11% formaldehyde [37% stock with 10%

methanol], 50 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 500 μ M EGTA) and cells were incubated for two hours at 4 °C. Crosslinking reactions were terminated by adding glycine to a final concentration of 125 mM and rocking plates gently for 5 min at room temperature. Cells were washed once with ice-cold PBS and collected by gentle scraping in 2 ml ice-cold PBS per plate. Cells were pooled and pelleted by centrifugation at 2,000 RPM at 4 °C. Pellets were resuspended in 6 ml of Cell Lysis Buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% NP-40, 0.5 mM PMSF, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin) and incubated on ice for 5 min. Nuclei were collected subsequently by centrifugation at 2,000 RPM at 4 °C and extracted with 2 ml High Salt Lysis Buffer (1x PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mM PMSF, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin) on ice for 5 min. Extracts were sonicated (Misonix XL2020, Misonix, Inc., Farmingdale, NY) on ice at power setting 3, three times for 20 second intervals and cleared by centrifugation at 14,000 RPM for 15 min at 4 °C. Supernatants were transferred to 1.5 ml microfuge tubes and pre-cleared with a 1:1 slurry of Protein A Sepharose (PAS; Zymed, Inc., San Francisco, CA) equilibrated with High Salt Lysis Buffer at a dilution of 1:20 for 30 min at 4 °C with gentle rocking. Pre-cleared extracts were collected by centrifugation at 14,000 RPM for 5 min at 4 °C. Supernatants were transferred to clean 1.5 ml microfuge tubes (500 μ l extract per tube) and incubated with 2 μ g (20 μ l) primary anti-Nkx3.1 antibody (L-15, N-15, or T-19) overnight at 4 °C with gentle rocking. Extracts were incubated with 50 μ l of 1:1 PAS slurry for two hours at 4 °C with gentle rocking. Beads were harvested by centrifugation at 14,000 RPM for 20 seconds at 4 °C and washed twice with 600 μ l High Salt Lysis Buffer and then four times with 600 μ l Wash Buffer (100 mM Tris pH 8.0, 500 mM LiCl, 1% NP-40, 1%

sodium deoxycholate, 0.5 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin). Bead pellets were subsequently resuspended in 400 µl Elution Buffer (100 mM NaHCO₃, 1% SDS) and incubated overnight at 67 °C to reverse crosslinks. Supernatants were isolated by centrifugation at 14,000 RPM for 5 min at room temperature and extracted once with 500 µl phenol/chloroform/isoamyl alcohol (25:24:1). The aqueous phases were transferred to clean 1.5 ml microfuge tubes and extracted once with 500 µl chloroform/isoamyl alcohol. Extracted aqueous phases were transferred to clean 1.5 ml microfuge tubes and DNA was precipitated with 1 ml ice-cold ethanol/sodium acetate solution (30:1 5 M NaOAc pH 5.2) and centrifugation at 14,000 RPM for 20 min at 4 °C. Genomic DNA pellets were air dried at room temperature for 30 min and resuspended in 40 µl TE (10 mM Tris pH 8.0, 1 mM EDTA). DNA isolates were treated with 1 µl Mung Bean nuclease (10 units; New England Biolabs) in 1x Mung Bean Nuclease Reaction Buffer (50 mM sodium acetate pH 5.0, 30 mM NaCl, 1 mM ZnSO₄) for 30 min at 30 °C and then purified using the Wizard[®] SV Gel and PCR Clean-Up System (Promega,) precisely as recommended by the manufacturer, and eluted in 50 µl dH₂O. DNA isolates were subsequently subcloned into Sma I-digested pBlueScript SK+ (Stratagene, La Jolla, CA) and used to transform *E. coli*. Plasmids prepared from resulting transformants were selected by blue/white screening and used as templates for PCR with the following primers: T7: 5'-GTA ATA CGA CTC ACT ATA GGG-3'; T3: 5'- AAT TAA CCC TCA CTA AAG GG-3'. The resulting PCR products were visualized by agarose gel electrophoresis to identify clones harboring genomic DNA. Selected clones were sequenced as described above and BLASTN was employed to identify sub-cloned sequences.

CHAPTER III
**Nkx3.1 Antagonizes Sp-mediated Transcriptional Activity in
Prostate-Derived Cells**

(a portion of this chapter appeared in S.O. Simmons and J.M. Horowitz, Nkx3.1 binds and negatively regulates the transcriptional activity of Sp-family members in prostate-derived cells, *Biochem. J.* 393: 397-409, 2006)

Nkx3.1, a human orthologue of the *Drosophila* gene *bagpipe* (*NK-3*), is expressed largely within the prostate gland (Bhatia-Gaur et al., 1999; Sciavolino et al., 1997). *Nkx3.1* is expressed in the developing mouse rostral urogenital sinus as early as 15.5 days post coitum and in all ductal derivatives in adult animals, suggesting that *Nkx3.1* is partly responsible for the development and differentiation of prostate tissue. *Nkx3.1* has also been implicated as a prostate tumor-suppressor gene, as its loss is associated with prostate tumorigenesis in both humans and rodents. *Nkx3.1* maps to a region of the human genome, 8p21, which is subject to deletion in up to 75% of human prostate cancers (He et al., 1997). Expression of Nkx3.1 protein is lost in 20% of patients with prostatic intraepithelial neoplasia (PIN), an early prostatic lesion, and the frequency of Nkx3.1-negative epithelial cells increases with disease progression (Bowen et al., 2000). For example, Nkx3.1 protein expression is absent in 34% of hormone refractory prostate carcinomas and 78% of metastatic prostate specimens. The maintenance of Nkx3.1 protein in at least some prostate tumors has led to speculation that haploinsufficiency may deregulate the proliferative potential of prostatic epithelia, and this proposition has been sustained by analyses of *Nkx3.1* “knockout” animals. Mice hemizygous or nullizygous for Nkx3.1 exhibit prostatic epithelial hyperplasia and dysplasia that increases in severity with age, defects in prostate ductal branching, and a reduction in the overall size of the ventral prostate (Bhatia-Guar et al., 1999; Tanaka et al., 2000; Schneider et al., 2000). Animals carrying a single conditional allele of *Nkx3.1* have also been shown to develop preinvasive lesions that are phenotypically similar to human PIN. Interestingly, PIN-like lesions that develop in these mice are devoid of Nkx3.1 protein indicating that the wild-type allele has been lost or silenced (Abdulkadir et al., 2002).

Although *Nkx3.1*-deficient animals have been followed for years, frank cancer has not as yet been observed suggesting that loss of *Nkx3.1* is not sufficient to induce prostate tumorigenesis. Offspring from inter-crosses between mice hemi- or nullizygous for *Nkx3.1* and the *Pten* tumor-suppressor gene exhibit greatly increased frequencies and severity of PIN suggesting that these genes cooperate in the suppression of prostate tumorigenesis (Kim et al., 2002).

Although it is widely believed that homeodomain proteins play an essential role in the specification of the body plan, it remains largely unclear how these transcriptional regulators promote development and differentiation. Most homeodomain proteins recognize similar consensus DNA sites containing the core motif 5'-TAAT/G-3' and as such it is likely that they direct the expression of tissue-specific genes via combinatorial interactions with additional transcription factors. Using a DNA binding-site selection strategy, Steadman et al. (2000) defined the Nkx3.1 consensus-binding site to be 5'-TAAGTA/G-3' and showed that expression of Nkx3.1 reduced the transcriptional potential of a reporter gene carrying multimers of this sequence. Although such artificial reporter constructs are unlikely to precisely mimic Nkx3.1 target genes, these data suggested that Nkx3.1 may function as a transcriptional repressor in prostate cells. Based on the expression of *Nkx3.1* in vascular mesoderm, Carson et al. (2000) identified the smooth muscle γ -*actin* gene as being a target of Nkx3.1 function. These workers showed that Nkx3.1 formed a physical complex with serum response factor (SRF) and that each of these proteins bound adjacent sites within a proximal portion of the γ -*actin* promoter. Interactions between these proteins were shown to occur via their respective DNA-binding domains, and co-expression of Nkx3.1 and SRF led to synergistic *trans-*

activation of γ -actin transcription. In contrast to these results, Chen et al. (2002) reported that Nkx3.1 forms a physical complex with a prostate-derived Ets factor (PDEF) and that Nkx3.1 blocked the capacity of PDEF to activate the *prostate-specific antigen (PSA)* promoter in human prostate cells. Physical interactions between PDEF and Nkx3.1 were later shown to require portions of their respective DNA-binding domains and sequences immediately downstream (Chen et al., 2005). Additional studies appear to corroborate that Nkx3.1 may function as a transcriptional repressor as NK-3 has been shown to down-regulate its own transcription and to associate with Groucho and histone deacetylase 1 (HDAC1), proteins previously shown to repress gene expression (Kim et al., 1998; Choi et al., 1999). In summary, Nkx3.1 has been shown to function as both an activator and repressor of transcription although few target genes have been identified and its precise role in the specification of prostate development is far from clear.

A number of disparate observations suggest that homeodomain proteins may regulate gene expression at least in part via their physical and functional interaction with zinc-“finger”-containing transcription factors. It is well established that the yeast mating-type switching gene *HO* is regulated by SWI5, a transcription factor with a three zinc-“finger” DNA-binding domain, and PHO2 a homeodomain protein (Brazas et al., 1995). SWI5 and PHO2 form physical complexes in solution and ternary complexes with DNA, binding adjacent sites within the *HO* promoter. PHO2 interacts with one of the SWI5 zinc-“fingers” and each protein facilitates the binding of the other to DNA. A *Drosophila* homeodomain protein, termed *empty spiracles (ems)*, specifies head segmentation and is required for proper differentiation of the anterior portion of the fly. These functions of *ems* have been shown to be dependent on the activities of a zinc-

“finger” transcription factor termed *buttonhead* (*btd*), a likely orthologue of the mammalian Sp-family of transcription factors (Schöck et al., 2000). Indeed, many functions of *btd* can be supplanted by the expression of human Sp1. *Btd* and *ems* were shown to form protein complexes that are dependent on the presence of the *btd* zinc-“finger” region, and proper differentiation of the fly head was shown to require the *btd* *trans*-activation and DNA-binding domains. Analogous collaborations between homeodomain- and zinc-“finger”-containing transcription factors also appear to occur in mammals as two recent reports have documented physical and functional interactions between Sp proteins and members of the Abd-B sub-family of *Hox* genes as well as Crx, a homeodomain factor required for photoreceptor-specific gene expression (Suzuki et al. 2003; Lerner et al., 2005). Analyses of “knockout” mice appear to corroborate these findings as nullizygoty for Sp1, Sp3, or Sp4 can lead to global, as well as tissue-specific, developmental abnormalities (Marin et al., 1997; Supp et al., 1996; Bouwman et al., 2000; Göllner et al., 2001a; Göllner et al., 2001b). Finally, placental-specific gene expression in humans has been shown to be dependent on physical and functional interactions between AP-2 γ , a zinc-“finger” protein, and Dlx-3, a homeodomain protein and orthologue of the *Drosophila Distal-less* gene (Peng and Payne, 2002). AP-2 γ and Dlx-3 bind adjacent DNA sequences and cooperate in the activation of genes such as β -*hydroxysteroid dehydrogenase/isomerase*. To determine if combinatorial interactions between Nkx3.1 and one or more Sp-family members play a role in the regulation of transcription in prostate-derived cells, I undertook a series of biochemical, molecular genetic and functional studies using the *prostate specific antigen (PSA)* promoter as a target gene. In this chapter, I report that: (i) a subset of Sp-family members stimulate

PSA transcription in prostate-derived cells, and co-expression of human or mouse Nkx3.1 negatively-regulates Sp-mediated transcription, (ii) Nkx3.1 antagonizes *trans*-activation by Sp proteins via Trichostatin A-sensitive and –insensitive mechanisms, (iii) a distal portion of the *PSA* promoter is required for Nkx3.1 to negatively-regulate Sp-mediated transcription in prostate-derived cells, (iv) Nkx3.1 DNA-binding activity is not required to antagonize Sp-mediated transcription, and (v) two non-contiguous portions of Nkx3.1 are sufficient to block Sp-mediated transcription.

3.1 Nkx3.1 antagonizes Sp-mediated transcriptional activity

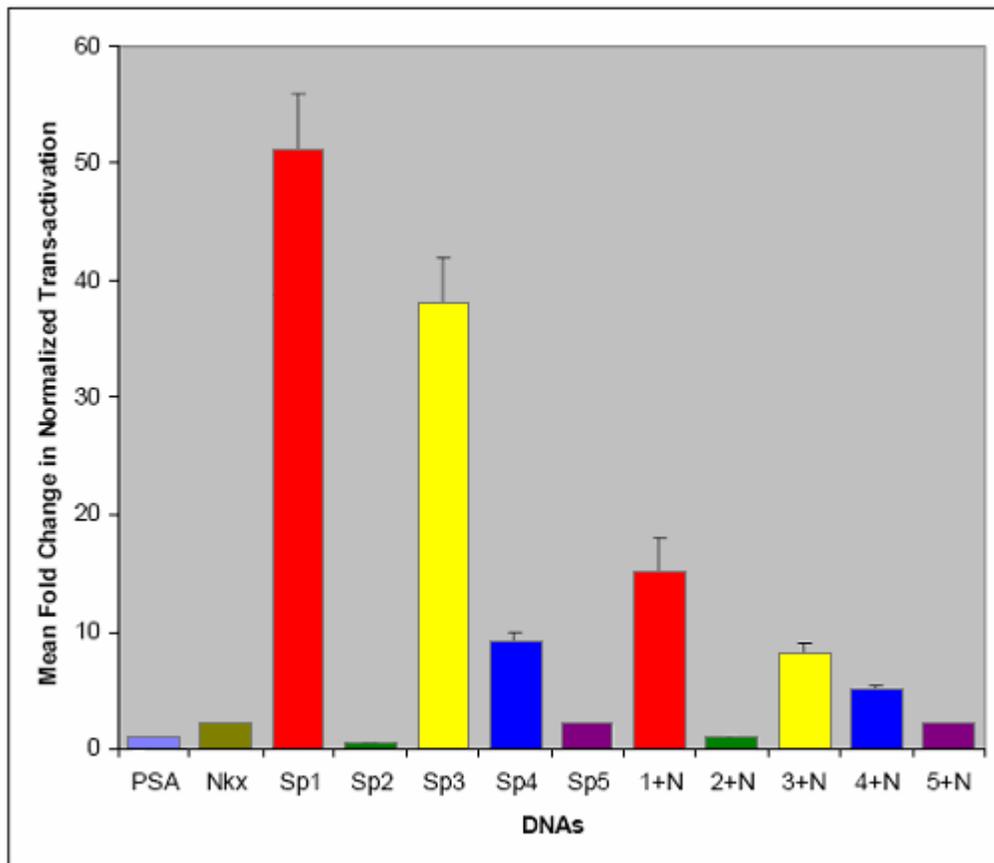
At the time I began this research only two publications had addressed the question of whether Nkx3.1 functions as a transcriptional activator or repressor. In one study, Carson et al. (2000) demonstrated that Nkx3.1 acted as a transcriptional activator of the avian *smooth muscle gamma actin (SMGA)* promoter when coexpressed with serum response factor (SRF) in CV-1 fibroblasts. Nkx3.1 collaborated with SRF to synergistically activate *SMGA* as much as eight-fold more than either SRF or Nkx3.1 alone. In contrast, Steadman et al. (2000) reported that expression of Nkx3.1 reduced the transcriptional potential of a reporter gene carrying multimers of an Nkx3.1 consensus DNA-binding sequence. As I began my studies, Nkx3.1 target genes had yet to be reported. Dr. Charles Bieberich (University of Maryland, Baltimore County) was kind enough to share his unpublished results indicating that human *prostate-specific antigen (PSA)* was a likely Nkx3.1 target gene in prostatic epithelial cells.

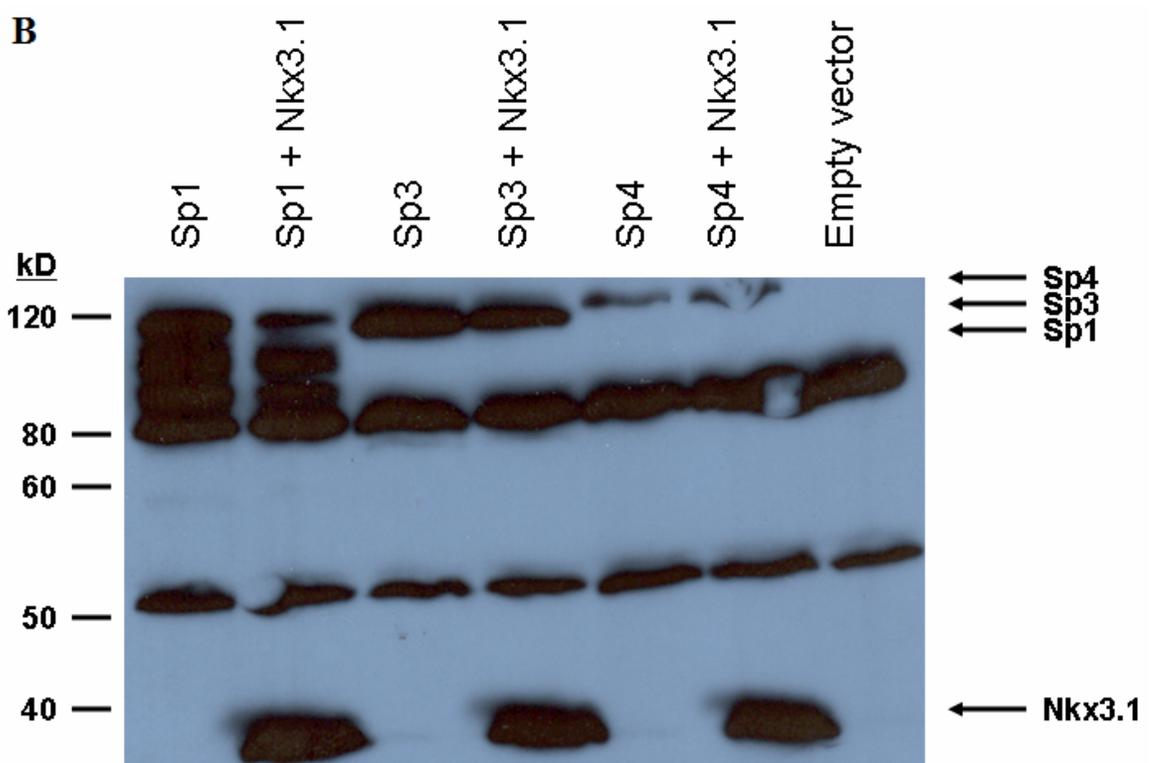
At the outset I wished to establish whether all or only a subset of Sp-family members regulates *PSA* transcription and whether Nkx3.1 regulates *PSA* activity. I first compared the capacity of various Sp-family members to stimulate the activity of a luciferase reporter construct whose expression is directed by a 5.3 Kbp human DNA fragment carrying the *prostate-specific antigen (PSA)* promoter (PSA-lux; Chen et al., 2002). I then asked whether the co-expression of Nkx3.1 affected Sp-mediated *PSA* transcription. Human DU145 prostate tumor cells were transiently transfected with PSA-lux as well as mammalian expression constructs for human Sp-family members and mouse Nkx3.1, and cell extracts were analyzed for firefly luciferase activity. The

resulting values were normalized to the abundance of *Renilla* luciferase activity elicited by a minimal adenovirus major late promoter. As illustrated in Fig. 5A, co-transfection of PSA with Sp1 or Sp3 resulted in a dramatic (38-51-fold) increase in levels of *PSA* transcription. In contrast with these results, transfection with equimolar amounts of Sp4 led to a more modest 9-fold increase in PSA-directed luciferase activity and cells receiving Sp2 or Sp5 exhibited little or no change in *PSA* transcription. Having established levels of transcription induced by Sp-family members, DU145 cells were subsequently co-transfected with mouse Nkx3.1 and each Sp-family member and resulting levels of *PSA* transcription were quantified. Co-expression of mNkx3.1 with Sp1, Sp3, or Sp4 reduced Sp-mediated *PSA* transcription two- to five-fold whereas co-expression of mNkx3.1 with Sp2 or Sp5 had little or no effect. The transient expression of mNkx3.1 alone did not appreciably alter basal levels of *PSA* transcription. Analogous results were obtained in parallel studies with two additional human prostate epithelial cell lines, PC-3 and LNCaP (data not shown). To confirm that decreased Sp-mediated *PSA* activity was due to repression by Nkx3.1 and not due to decreased Sp-protein expression or stability, I analyzed extracts made from DU145 cells transiently transfected with expression constructs for Sp1, Sp3 and Sp3 with and without an expression construct for Nkx3.1 by Western blot analysis. As shown in Fig. 5B, coexpression of Nkx3.1 did not affect the expression levels or stability of Sp1, Sp3 or Sp4. I conclude from these transcription assays that a subset of Sp-family members activates *PSA* transcription, and that Nkx3.1 antagonizes the activation exerted by this subset of Sp-family members on *PSA* transcription.

Figure 5. *Trans*-activation of the human *PSA* promoter by Sp-family members and *trans*-repression by mNkx3.1. *A*, effect of Sp-family members and Nkx3.1 on *PSA* transcription. Human DU145 prostate cells were transiently transfected with mNkx3.1 alone (500 ng; denoted *Nkx*), with Sp1-5 alone (500 ng), or with mNkx3.1 (500 ng) and an Sp-family member (500 ng; denoted by Sp-family member + N, *e.g.*, *I+N*). Basal levels of *PSA* transcription were set to equal to one and plotted are the mean fold changes in *PSA trans*-activation normalized to an internal firefly luciferase control following 48 hours of cultivation post-transfection (see Chapter 2, Materials and Methods). Empty expression vector DNA was included in control reactions to maintain constant input DNA concentrations (2 µg per plate). Data are derived from at least five independent plates of transfected cells. Error bars indicate standard deviation (S.D.) values. *B*, Western immunoblot of human Sp1, Sp3, Sp4 and Nkx3.1 protein expressed DU145 cells. Non-denatured extracts were prepared from DU145 cells transiently transfected with mammalian expression constructs carrying epitope-tagged Sp1 (lane 1), Sp1 and Nkx3.1 (lane 2), Sp3 (lane 3), Sp3 and Nkx3.1 (lane 4), Sp4 (lane 5), Sp4 and Nkx3.1 (lane 6), or empty pCMV4 (lane 7). Extracts were boiled in Laemmli sample buffer, resolved by SDS-PAGE, and transferred to nitrocellulose. Filters were incubated with anti-HA monoclonal antibody (12CA5) (1:1000) and then with HRP-conjugated anti-mouse secondary antibody (1:10000). Arrows indicates the positions of Sp1, Sp3, Sp4 and Nkx3.1.

A





3.2 Nkx3.1 represses Sp-mediated transcription via TSA-sensitive and-insensitive mechanisms

NK-3, the *Drosophila* orthologue of Nkx3.1 has been reported to function as a transcriptional repressor in conjunction with Groucho and HDAC1 (Choi et al, 1999). To determine whether the reduction in Sp-mediated *PSA* transcription by Nkx3.1 is dependent on histone deacetylase activity I performed a similar series of transient transfections and quantified *PSA* transcription in cells treated for 24 hrs with Trichostatin A (TSA), a potent inhibitor of HDAC activity. Consistent with the notion that the basal expression of *PSA* is dampened by histone deacetylases, DU145 cells transfected with PSA-Lux alone and treated with TSA exhibited a 10-fold increase in transcription (Fig. 6). TSA-treated cells co-transfected with PSA-Lux and Sp1, Sp3, or Sp4 exhibited a five- to 15-fold stimulation of Sp-mediated transcription above that noted without TSA treatment, whereas TSA had little or no effect on the induction of *PSA* transcription by Sp2 or Sp5. In partial accord with previously reported results, treatment with TSA diminished the capacity of mNkx3.1 to negatively-regulate the activity of Sp-family members (Choi et al., 1999). However, Sp1-, Sp3- and Sp4-directed *PSA* transcription still remained limited in TSA-treated cells when co-expressed with mNkx3.1. Given the results reported thus far I conclude that (1) *PSA* transcription is stimulated by a subset of Sp-family members in prostatic epithelia, (2) Nkx3.1 antagonizes the transcriptional activities of this same subset of Sp-family members, and (3) treatment with TSA reduces, but does not eliminate, the capacity of Nkx3.1 to negatively-regulate Sp-mediated

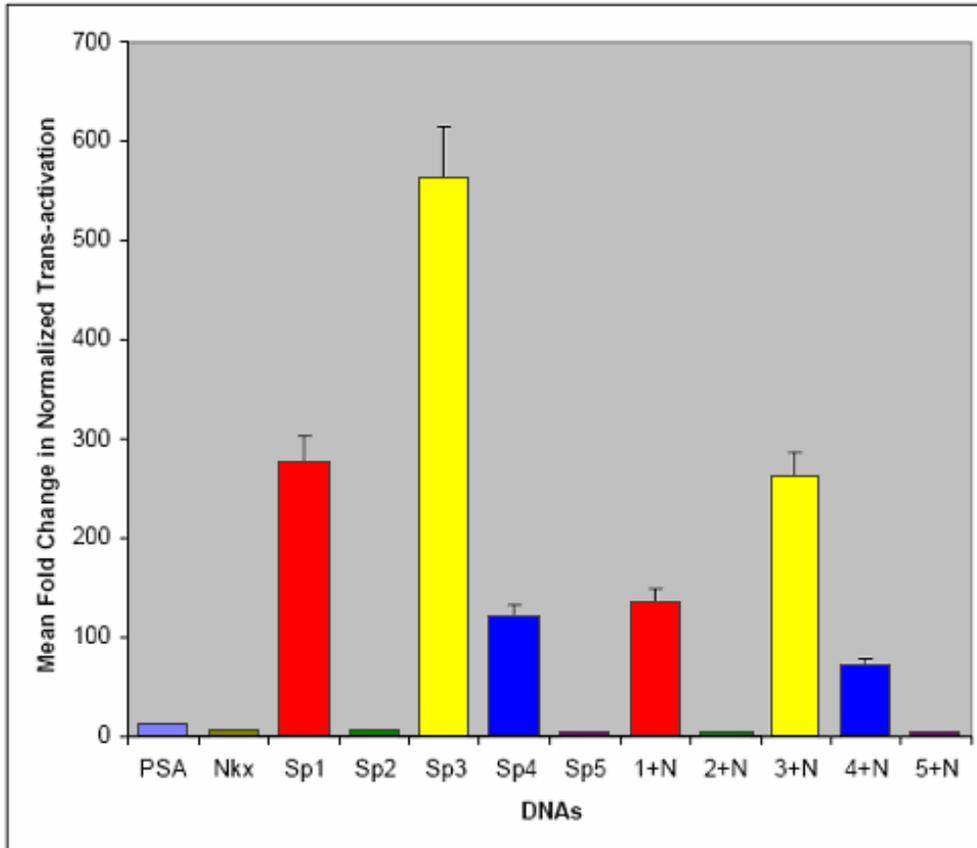


Figure 6. Nkx3.1 represses Sp-mediated transcription via TSA-sensitive and-insensitive mechanisms. Effect of Sp-family members and Nkx3.1 on *PSA* transcription following treatment with Trichostatin A. DU145 cells were transfected and processed as in *A* except that cells were treated with 100 nM Trichostatin A 24 hrs after transfection. Basal levels of *PSA* transcription were 10-fold greater than that shown in *A* and therefore were set equal to 10. Data are derived from at least five independent plates of transfected cells. Error bars indicate S.D. values.

transcription. This latter conclusion raised the intriguing possibility that Nkx3.1 may antagonize Sp function via both TSA-sensitive and –insensitive mechanisms.

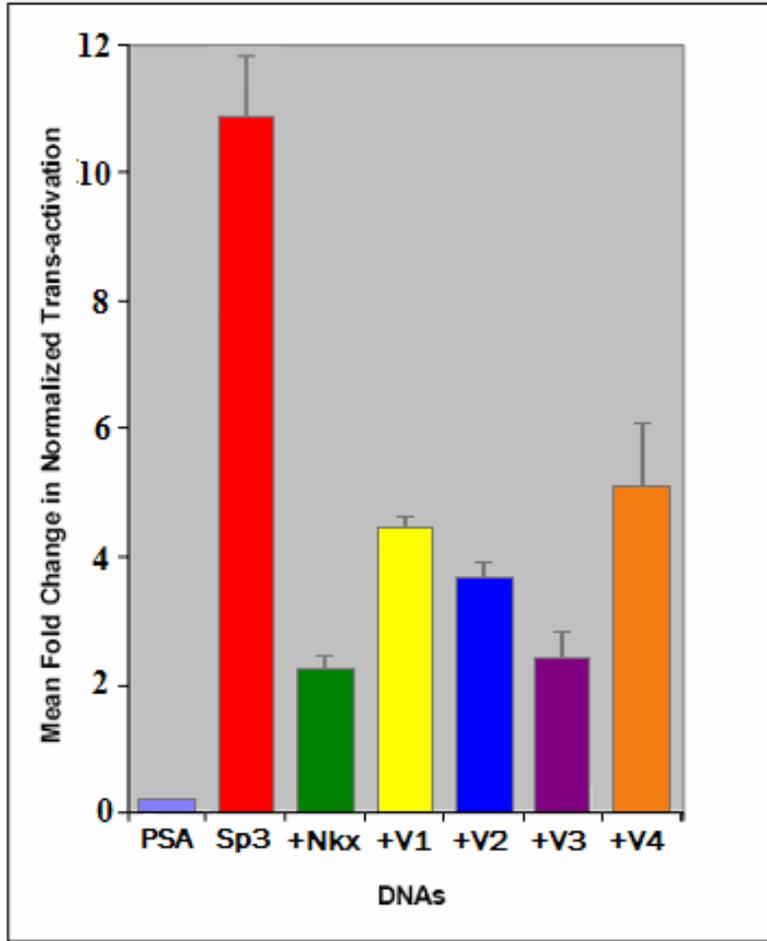
3.3 Putative alternately-spliced Nkx3.1 variants exhibit diminished capacity to antagonize Sp-mediated transcription

All Nkx proteins contain a conserved 11-amino acid peptide motif known as the TN domain (Lints et al., 1993). Muhr et al. (2001) reported that the TN domain is necessary for Groucho-dependent repression by Nkx proteins and that the TN domain was sufficient for Groucho recruitment. This suggests that all Nkx proteins, including Nkx3.1, have some transcriptional repressor potential, although it has not been shown that Nkx3.1 interacts with Groucho co-repressors via its TN domain. Interestingly, Korkmaz et al. (2000) reported the isolation of four human Nkx3.1 cDNAs that appear to result from alternative splicing. These authors noted that each of these novel cDNAs results in the “in-frame” deletion of 44 to 75 amino acid residues, and three of the four lack the Nkx3.1 TN domain (Fig. 7B). Although the structure of these cDNAs is consistent with alternative splicing, obvious *Nkx3.1* splicing signals or cryptic exons that might explain the origin of these variant cDNA have yet to be identified in genomic DNA. Thus, whether these variant cDNAs reflect alternatively spliced mRNAs or are artifacts of reverse transcription remains to be determined.

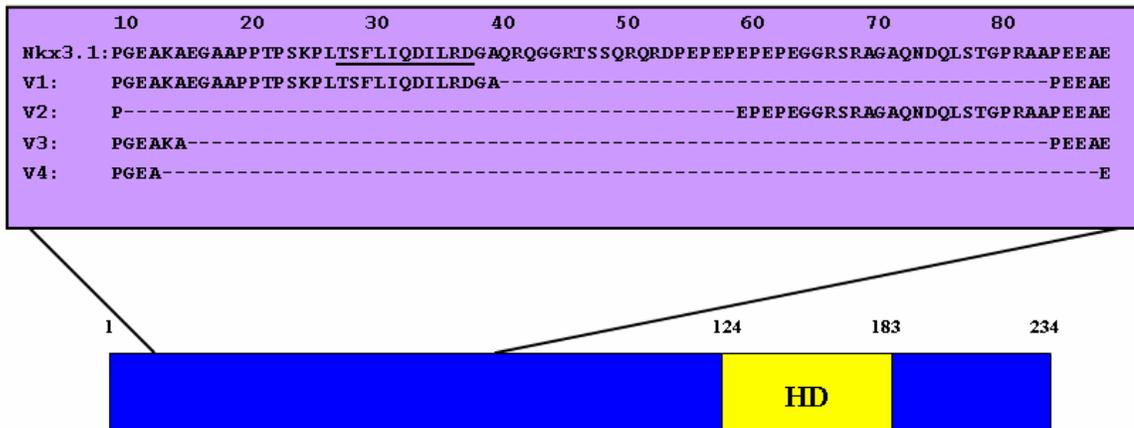
To assess whether the deletions carried by variant Nkx3.1 cDNAs have an impact on trans-repression of Sp proteins, DU145 cells were transiently transfected with expression constructs for Sp3 and human Nkx3.1 or amino-terminal Nkx3.1 variant proteins and *PSA* transcription was quantified. Consistent with earlier experiments using

Figure 7. Transcriptional regulation of *PSA* promoter by Sp3 and wild-type Nkx3.1 or amino-terminal Nkx3.1 variants. *A*, Transcriptional response of *PSA* promoter in DU145 cells following the ectopic expression of Sp3, human Nkx3.1 (hNkx), or amino-terminal Nkx3.1 variants (V1, V2, V3, and V4) depicted in *B*. Shown are changes in mean fold *trans*-activation of each construction relative to its basal activity (set equal to 1). Data are derived from three independent plates of cells transfected with each construction. Error bars indicate S.D. values. *B*, schematic diagram of hNkx3.1 and amino-terminal Nkx3.1 variants. The predicted translations of the amino termini of human Nkx3.1 and Nkx3.1 variant proteins V1, V2, V3, and V4 are shown in purple box. The Nkx3.1 TN domain is underlined. The homeodomain is indicated by a yellow box.

A



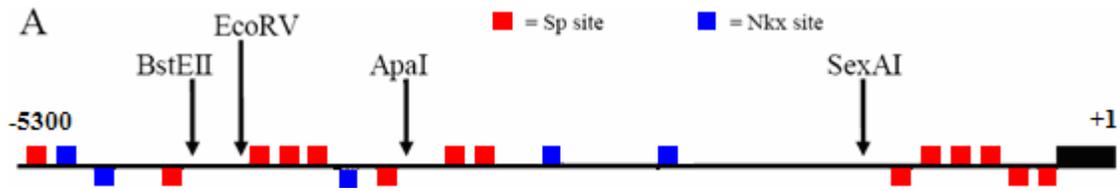
B



mouse Nkx3.1, human Nkx3.1 repressed Sp3-mediated *PSA* transcription (Fig 6A). In contrast to these results, Nkx3.1 variant proteins V1, V2, and V4 displayed a diminished capacity to repress Sp3-mediated transcriptional activity, confirming that residues within the Nkx3.1 amino terminus play a role in regulating Sp3 transcriptional activity. Variant V1 contains an intact TN domain, while variant V3, which showed comparable trans-repression activity to wild-type Nkx3.1, does not possess a TN domain. These results suggest that the Nkx3.1 TN domain is not required to antagonize Sp-mediated transcription in DU145 cells and imply that the association of Groucho with the Nkx3.1 TN domain does not play an important role in the *trans*-repression of Sp proteins. This conclusion is entirely consistent with the observation that Nkx3.1 antagonizes Sp-mediated transcription via TSA-sensitive and –insensitive mechanisms.

3.4 A distal portion of the human *PSA* promoter is required for Nkx3.1 to suppress Sp-mediated transcription in prostate-derived cells

Computer-assisted sequence analyses identified numerous predicted Sp-binding sites within the *PSA* promoter, as well as five predicted Nkx3.1 binding sites (Fig. 8A). To map portions of the *PSA* promoter that are required for Nkx3.1 to suppress Sp-mediated transcription I took advantage of a number of convenient restriction enzyme sites to create a series of nested deletions (Fig. 8A). The basal activities of each of these deleted constructs were compared to the full-length *PSA* promoter in DU145 cells, and for their capacity to be regulated by the ectopic expression of Sp3 and/or hNkx3.1. Sp3-mediated transcription appears to be dependent on two regions of the *PSA* promoter: deletion of sequences upstream of the BstE II site reduced Sp3-directed transcription



B

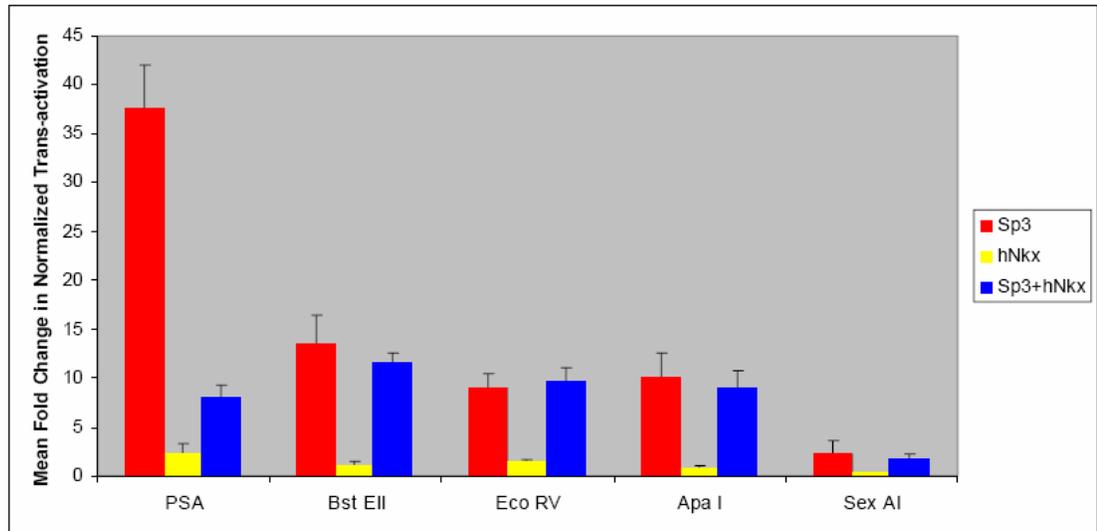


Figure 8. Sensitivity of *PSA* promoter deletion mutants to *trans*-activation by Sp3 and *trans*-repression by hNkx3.1. *A*, schematic diagram of the human *PSA* promoter (5300 bp; drawn 5' to 3'). Restriction sites used to generate deletion mutants are indicated as are predicted Nkx3.1- (blue boxes) and Sp- (red boxes) binding sites. Predicted protein-binding sites on the “sense” strand are shown above the line, those on the “antisense” strand are shown below the line. *B*, transcriptional response of wild-type and deleted *PSA* promoter constructs following the ectopic expression of Sp3, human Nkx3.1 (hNkx), or Sp3 and hNkx3.1 in DU145 cells. Shown are changes in mean fold *trans*-activation of each construction relative to its basal activity (set equal to 1). Data are derived from five independent plates of cells transfected with each construction. Error bars indicate S.D. values.

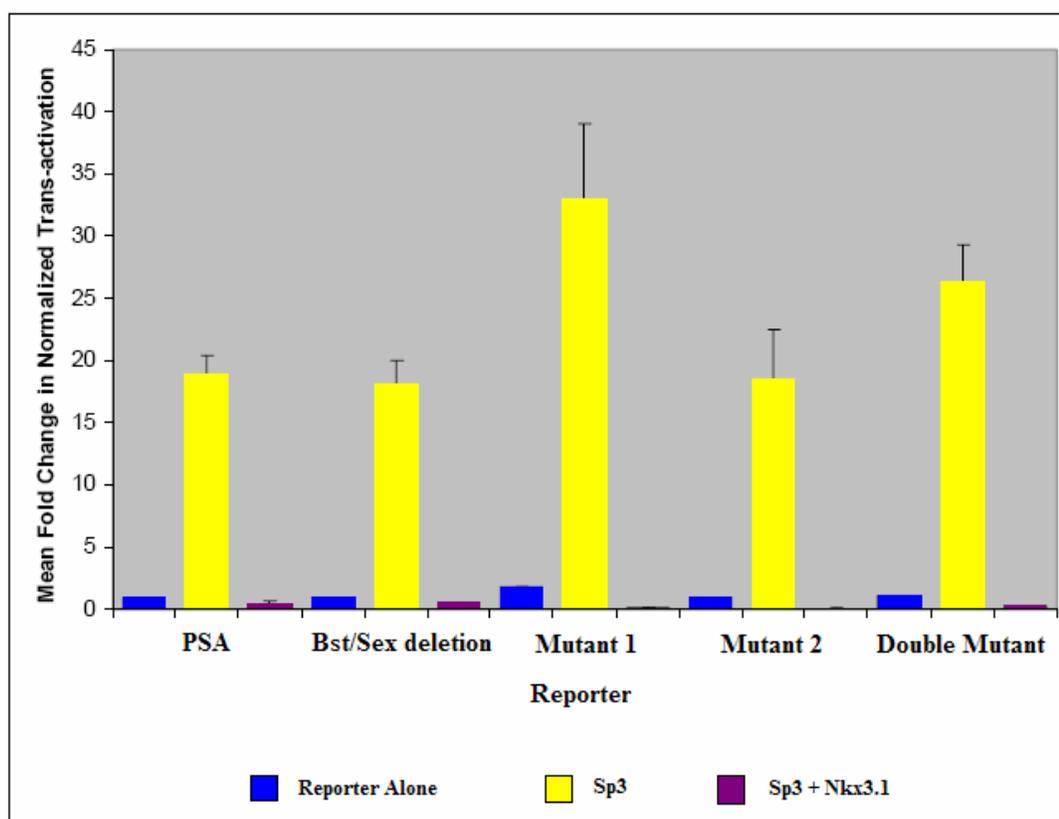
more than three-fold, and a further five-fold reduction in transcription was noted following the deletion of sequences upstream of the SexA I site (Fig. 8B). Little difference was noted between the full-length and deleted promoter constructs with respect to their sensitivity to hNkx3.1 when expressed alone. In contrast, the capacity of hNkx3.1 to down-regulate Sp3-directed transcription was completely dependent on sequences upstream of the BstE II site. To confirm this result, *PSA* promoter sequences between the Bst EII and Sex AI restriction sites were deleted and the resulting construct was examined for its sensitivity to Sp3 and hNkx3.1. As shown in Fig. 9A, the transcriptional response of this internally-deleted promoter construct was identical to that of the wild-type *PSA* promoter. Thus, the distal end of the *PSA* promoter carries one or more elements required for the antagonism of Sp-mediated transcription by Nkx3.1.

3.5 Nkx3.1 DNA-binding activity is not required to down-regulate Sp-mediated transcription

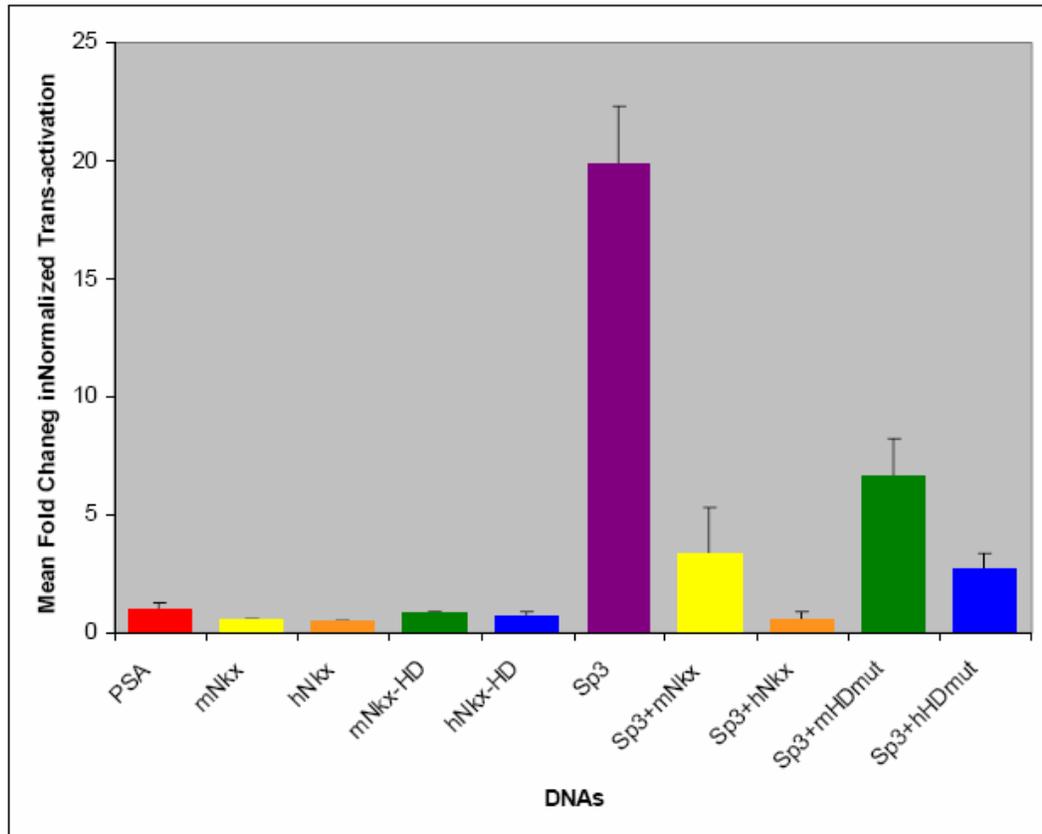
Computer-assisted analyses identified two putative Nkx3.1-binding sites (5'-TAAGTG-3', located on the promoter "sense" strand and termed site 1; 5'-TAAGTG-3', located on the "anti-sense" strand and termed site 2) within the distal segment of the *PSA* promoter required for the suppression of Sp-mediated transcription by hNkx3.1. To determine if one or both of these putative DNA-binding sites is/are required for the negative regulation of Sp-mediated transcription, each was mutated (5'-TcctTG-3') via site-directed mutagenesis. Resulting mutated promoter constructs were employed in transient transfection experiments in comparison with the parental wild-type *PSA* promoter. The basal transcription activity of each mutated promoter construct was

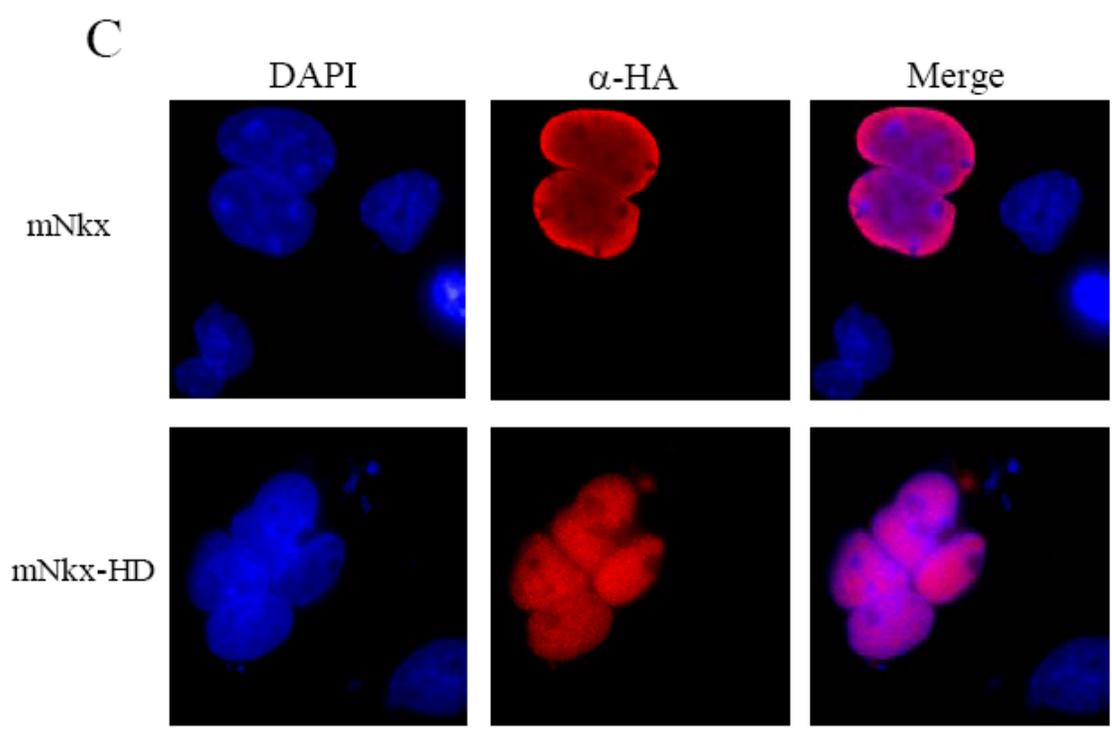
Figure 9. Transcriptional regulation of wild-type and mutated *PSA* promoter constructs by Sp3 and wild-type Nkx3.1 or DNA binding-deficient homeodomain mutants. *A*, DU145 cells were transfected with promoter constructs alone, in conjunction with Sp3, or with Sp3 and hNkx3.1. Mutated promoter constructs are: deletion of sequence in between the unique Bst EII and Sex AI sites within the human *PSA* promoter (labeled *Bst/Sex deletion*), mutation of most distal putative Nkx3.1-binding site (site 1; labeled *Mutant 1*), mutation of other putative Nkx3.1-binding site (site 2; labeled *Mutant 2*), or both sites mutated (labeled *Mutant 1+2*). Shown are changes in mean fold *trans*-activation relative to basal levels of *PSA* transcription (set equal to 1). Each bar represents results compiled from a minimum of six plates of transfected cells. Error bars indicate S.D. values. *B*, *trans*-repression by human and mouse Nkx3.1 proteins as well as derivatives carrying homeodomain mutations that ablate DNA-binding activity. DU145 cells were transfected with the human *PSA* promoter alone, in conjunction with wild-type mouse (*mNkx*) or human (*hNkx*) Nkx3.1 expression vectors or derivatives carrying homeodomain mutations (*mNkx-HD* and *hNkx-HD*), Sp3, or Sp3 and wild-type or mutated Nkx3.1 expression vectors. Shown are changes in mean fold *trans*-activation relative to basal levels of *PSA* transcription (set equal to 1). Each bar represents a minimum of six plates of transfected cells. Error bars indicate S.D. values. *C*, indirect immunofluorescence analysis of ectopically expressed wild-type and mutated Nkx3.1 proteins. COS-1 cells were transiently transfected with expression vectors encoding mNkx3.1 or the DNA-deficient homeodomain mutant employed in *A*. Nuclei were stained with DAPI and ectopically expressed proteins were detected with an anti-epitope tag (HA) antibody and Alexa Fluor 594 anti-mouse secondary antibody.

A



B





similar to that of wild-type *PSA*, and each was induced 18- to 36-fold following the transient expression of Sp3 (Fig. 9A). Interestingly, the ablation of either or both putative Nkx3.1-binding sites had little or no effect on the capacity of hNkx3.1 to antagonize Sp-mediated transcription as the induced transcription of wild type and mutated *PSA* promoters were each reduced at least 15-fold following co-expression of hNkx3.1 (Fig. 9A).

Given that two predicted Nkx3.1 binding sites within the distal portion of the *PSA* promoter were not required for down-regulation of Sp-mediated transcription, I reasoned that Nkx3.1 might regulate Sp-mediated *PSA* transcription by binding to partially degenerate DNA sequences that would not have been identified by my computer-assisted screen. To determine if a functional Nkx3.1 DNA-binding domain is required to antagonize Sp-mediated transcription, a single amino acid substitution (Q₁₇₄E) was introduced via site-directed mutagenesis within helix III of the human and mouse Nkx3.1 homeodomains. As previously shown for several other homeodomain proteins, this amino acid substitution inactivates DNA-binding activity due to the loss of a critical DNA contact at residue 54 of the 60 amino-acid homeodomain (Saadi et al., 2001; Hanes et al., 1991; Hanes et al., 1989; Triesman et al., 1989; Kim et al., 2003). I then assessed the capacities of these homeodomain mutants to negatively-regulate Sp-mediated transcription in parallel with their wild-type counterparts. Consistent with the notion that DNA-binding activity is not required for transcriptional repression by Nkx3.1, mutation of the human and mouse Nkx3.1 homeodomains resulted in only a modest reduction (approximately two-fold) in their activity as inhibitors of Sp-mediated transcription (Fig. 9B). To confirm that these mutated homeodomain constructs are karyophilic and localize

to nuclei at least as efficiently as wild-type Nkx3.1, I utilized indirect immunofluorescence to monitor the subcellular localization of wild type and mutated proteins. Transient expression of wild-type human or mouse Nkx3.1 in COS-1 cells led to protein expression throughout the nucleus, with much of the protein accumulating in perinuclear deposits (Fig. 9C and data not shown). The human and mouse homeodomain mutants were equally karyophilic, yet cells carrying these proteins exhibited a more uniform, granular distribution of Nkx3.1 within the nucleoplasm (Fig. 9C and data not shown). Whether this somewhat subtle difference in sub-nuclear distribution accounts for the relatively modest decline in Nkx3.1-directed transcriptional repression remains to be determined. I conclude from studies with Nkx3.1 homeodomain mutants that direct interactions between the homeodomain and DNA are not absolutely required for the suppression of Sp-mediated transcription.

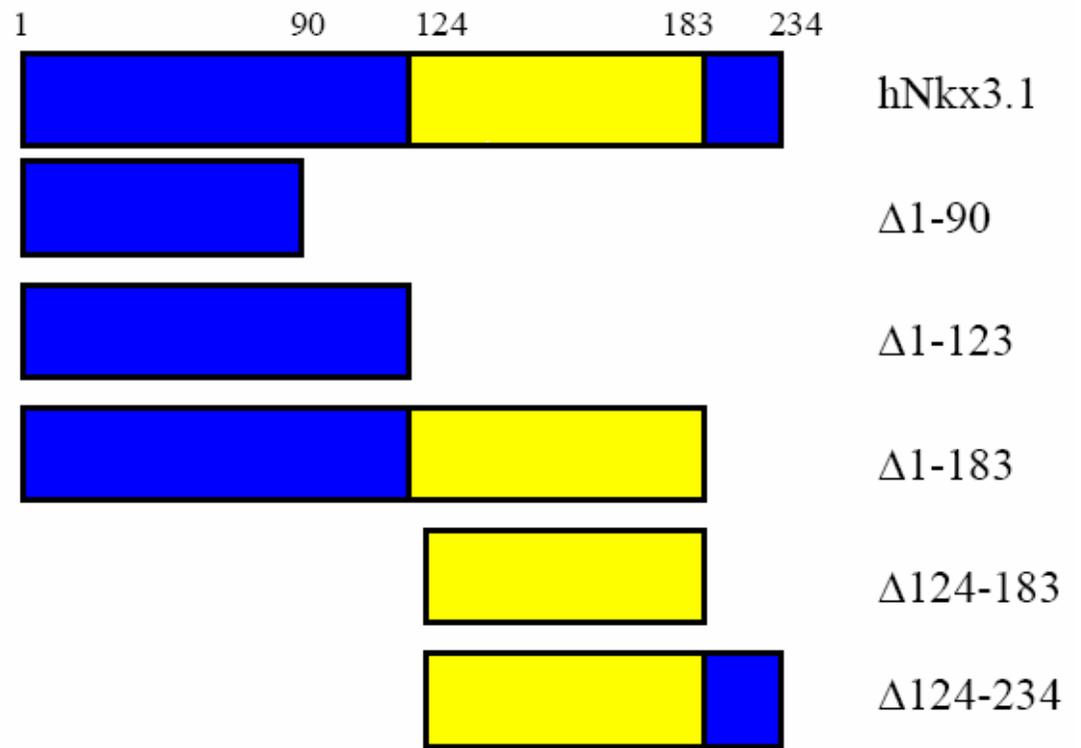
3.6 Two non-contiguous portions of Nkx3.1 are each sufficient to antagonize Sp-mediated transcription

To determine which portions of Nkx3.1 are required to inhibit Sp-mediated transcription, a series of expression vectors carrying partial hNkx3.1 cDNAs were constructed (Fig. 10A). To ensure that the partial hNkx3.1 proteins elicited by these cDNAs would localize to the nucleus, each cDNA was linked “in-frame” to a nuclear localization sequence derived from the HIV TAT protein and ectopically-expressed proteins were detected using an antibody prepared against a linked epitope tag. As illustrated in Fig. 10B, each partial hNkx3.1 construct elicited stable, karyophilic proteins. Each was then ectopically-expressed in DU145 cells and their effects on Sp3-

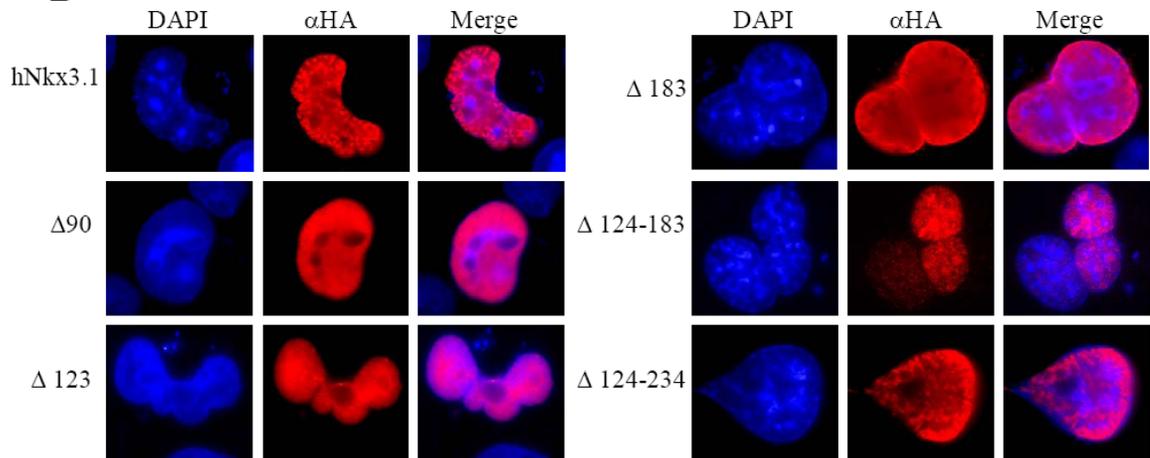
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Figure 10. *Trans*-repression of Sp-mediated transcription by two non-contiguous portions of Nkx3.1. *A*, schematic diagram of hNkx3.1 and truncated derivatives expressed in mammalian cells. The homeodomain is indicated by a yellow box as are amino acid endpoints for hNkx3.1 deletions. *B*, indirect immunofluorescence analysis of ectopically expressed wild-type and truncated hNkx3.1 proteins. COS-1 cells were transiently transfected with expression vectors encoding hNkx3.1 or the truncated mutants diagrammed in *A*. Nuclei were stained with DAPI and ectopically expressed proteins were detected with an anti-epitope tag (HA) antibody and Alexa Fluor 594 anti-mouse secondary antibody. *C*, *trans*-repression by wild-type and truncated hNkx3.1 derivatives. DU145 cells were transfected with the human *PSA* promoter alone, in conjunction with Sp3, or with Sp3 and expression vectors encoding wild-type or truncated hNkx3.1 mutants. Shown are changes in mean fold *trans*-activation relative to basal levels of *PSA* transcription (set equal to 1). Each bar represents a minimum of six plates of transfected cells. Error bars indicate S.D. values.

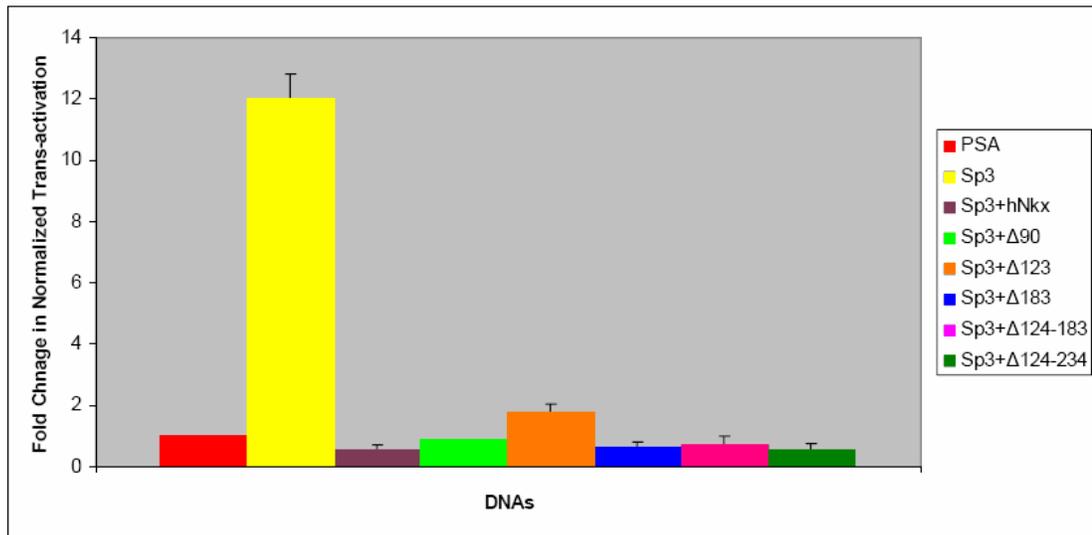
A



B



C



transcription was compared to that of wild-type hNkx3.1. As shown in Fig. 10C, transcriptional repression of *PSA* by Nkx3.1 deletions pDXTAT(Δ 1-90), pDXTAT(Δ 1-183), pDXTAT(Δ 124-183), and pDXTAT(Δ 124-234) ranged from 14- to 23-fold and was comparable to that of wild-type Nkx3.1 (23-fold). pDXTAT(Δ 1-123) was somewhat less potent as a negative regulator of Sp3, reducing *PSA* transcription seven-fold. Based on these results, at least two non-contiguous portions of Nkx3.1 appear to be sufficient for the negative regulation of Sp-mediated transcription. One region resides within the amino-terminal 90 amino acids of Nkx3.1 (*i.e.*, pDXTAT(Δ 1-90)), the other within the Nkx3.1 homeodomain (*i.e.*, pDXTAT(Δ 124-183)).

In this chapter, I provide evidence that Nkx3.1 functionally interacts with Sp-family members to regulate the expression of a prostate-specific gene. First I showed that a subset of Sp-family members activate the human *PSA* promoter in prostate-derived cells and that co-expression of Nkx3.1 suppressed the activation exerted by this same set of Sp-family members on *PSA* activity. Second, I used Trichostatin A, a potent inhibitor of histone deacetylase activity, to show that Nkx3.1 antagonizes Sp-mediated transcription through TSA-sensitive and -insensitive mechanisms, suggesting that Nkx3.1 represses Sp-directed transcriptional activity, at least in part, by HDAC recruitment. Third, I identified a 1 kbp distal portion of the human *PSA* promoter that is required for the antagonism of Sp-mediated transcription by Nkx3.1. Fourth, using mutagenesis of the *PSA* promoter as well as the Nkx3.1 homeodomain I showed that Nkx3.1 DNA-binding activity is not required for Nkx3.1 to repress Sp-mediated transcription. This latter result indicates that Nkx3.1 may affect Sp-mediated transcription directly, perhaps by protein/protein interactions, rather than via a

mechanism that includes its binding to DNA. Fifth, I show that two non-contiguous portions of Nkx3.1, one located within the first 90 amino-terminal amino acids of Nkx3.1 and the second located within the Nkx3.1 homeodomain are each sufficient to antagonize Sp-mediated transcription. In the following chapter I will demonstrate that Nkx3.1 physically interacts with Sp-family members, both *in vitro* and *in vivo*, and that portions of Nkx3.1 that are sufficient to antagonize Sp-mediated transcription are also sufficient to physically complex with Sp-proteins.

CHAPTER IV

Nkx3.1 Forms Specific Protein Complexes with Sp-family members

(a portion of this chapter appeared in S.O. Simmons and J.M. Horowitz, Nkx3.1 binds and negatively regulates the transcriptional activity of Sp-family members in prostate-derived cells, *Biochem. J.* 393: 397-409, 2006)

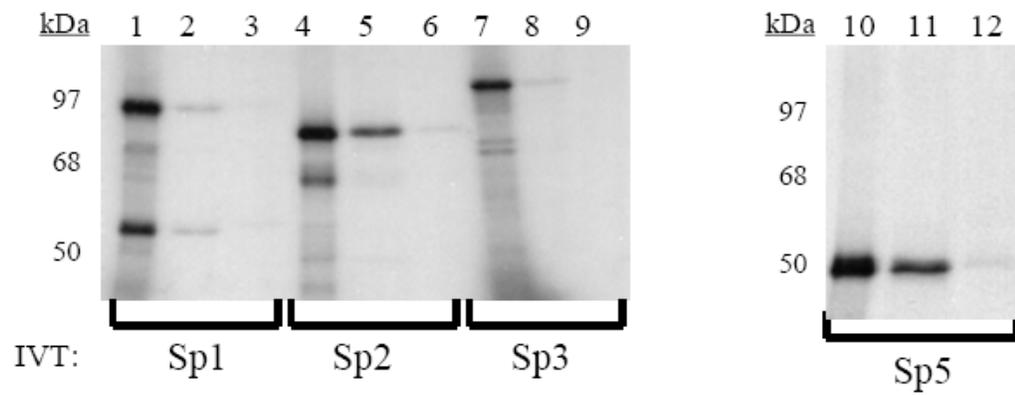
In a number of systems homeodomain-containing proteins collaborate with zinc-“finger”-containing transcription factors to bind and regulate target genes. In many instances functional collaborations between these proteins result from physical interactions between the homeodomain-containing and zinc-“finger” proteins. Often the highly-conserved DNA-binding domains of these two classes of transcription factors are involved in complex formation (van Wering et al., 2004; van Wering et al., 2002; Liu et al., 2002; Durocher et al., 1997). In the previous chapter I reported that Nkx3.1 antagonizes Sp-mediated *trans*-activation of the human *PSA* promoter and I showed that two non-contiguous portions of Nkx3.1, one located within the first 90 amino-terminal residues of Nkx3.1 and the other located within the Nkx3.1 homeodomain, were sufficient to suppress Sp-mediated transcription. In this chapter, I report that : (i) Nkx3.1 forms specific protein complexes with Sp-family members *in vitro* via portions of Nkx3.1 required for transcriptional suppression *in vivo*, (ii) the DNA-binding domains of Nkx3.1 and Sp-proteins physically interact *in vivo*, (iii) physical interactions between the DNA-binding domains of Nkx3.1 and a subset of Sp-proteins do not disrupt their capacities to bind DNA, and (iv) DNA-binding sites for both Nkx3.1 and Sp-proteins are required for the formation of ternary protein/DNA complexes.

4.1 Nkx3.1 forms specific protein complexes with Sp-family members *in vitro*

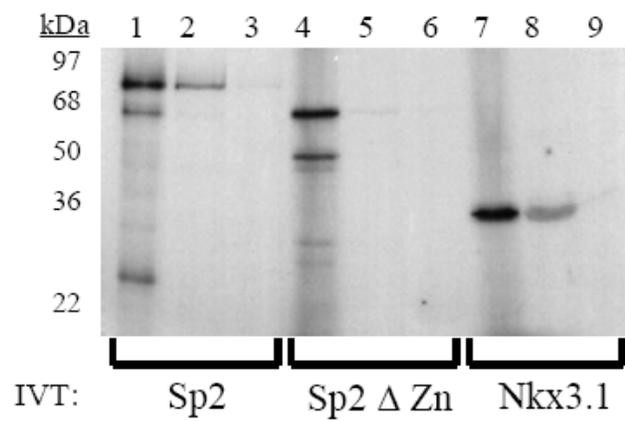
Previous studies indicate that homeodomain-containing proteins (*e.g.*, Nkx2.5) can form physical complexes with zinc-“finger” proteins (*e.g.*, GATA-4), and that such complexes collaborate in the regulation of target genes (Durocher et al., 1997). Given evidence presented in the previous chapter that (i) the transcriptional activity of a subset of Sp-family members is negatively regulated by Nkx3.1 and (ii) antagonism of Sp-mediated transcription by Nkx3.1 does not require DNA-binding activity, I reasoned that the formation of physical complexes between Nkx3.1 and Sp proteins might account, at least in part, for *trans*-repression. To determine whether Nkx3.1 can physically associate with Sp-family members, I performed a series of *in vitro* protein/protein-binding assays using bacterially expressed GST-fusion proteins prepared from a mouse Nkx3.1 cDNA and radiolabeled Sp proteins prepared in reticulocyte extracts. A full-length GST-Nkx3.1 fusion protein was bound to glutathione-Sepharose beads, incubated with equivalent amounts of radiolabeled Sp proteins, and loosely adherent proteins were removed by successive washes. Bead-bound radiolabeled proteins were resolved subsequently on acrylamide gels. As shown in Fig. 11A, each Sp-family member bound to GST-mNkx3.1 but not to a negative control fusion protein derived from a *Schistosoma* surface antigen (GST-FSH15). Interestingly, although each Sp-family member surveyed bound mNkx3.1 specifically, Sp2 and Sp5 (lanes 4-6 and 10-12) were reproducibly bound more efficiently than Sp1 or Sp3 (lanes 1-3 and 7-9).

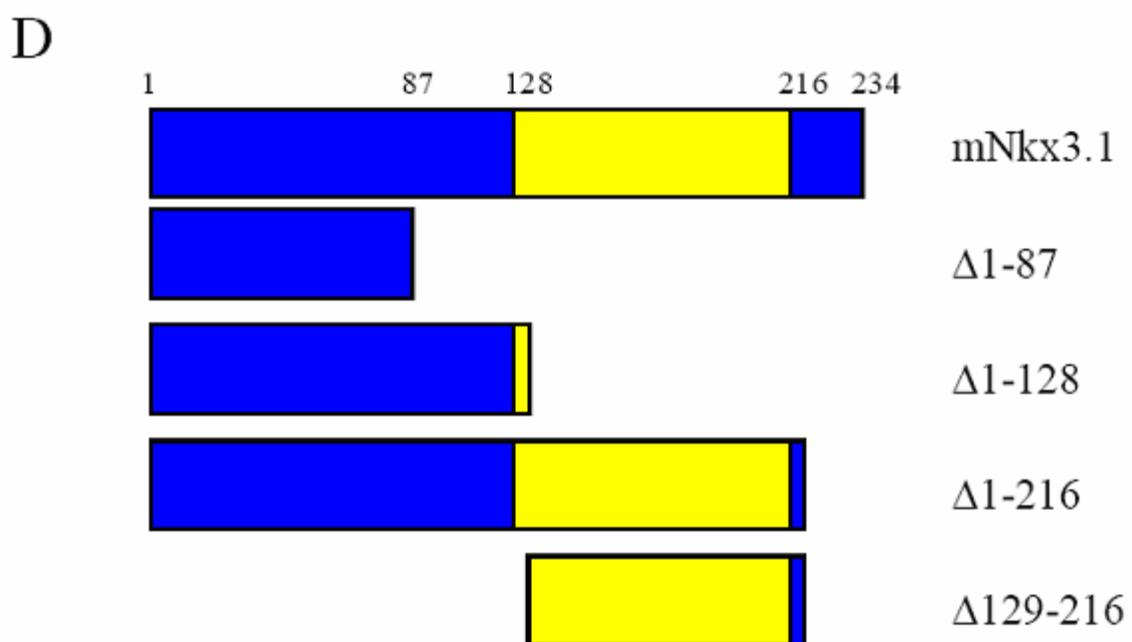
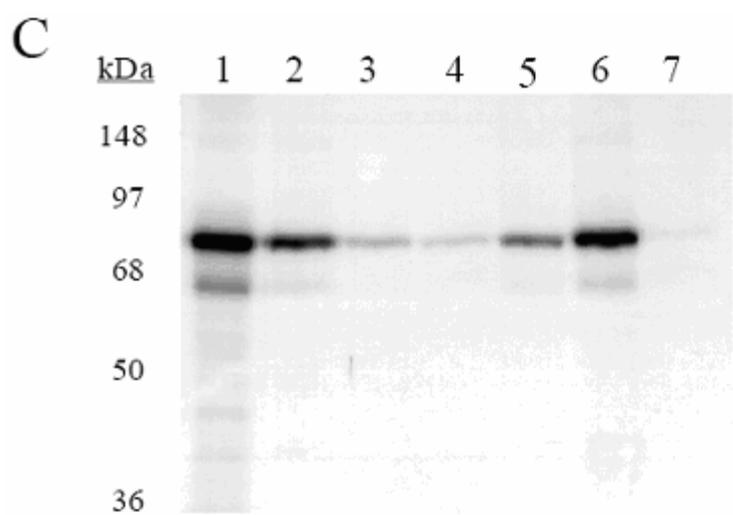
Figure 11. *In vitro* protein/protein-binding assay. *A*, specific binding of Nkx3.1 by Sp-family members. *In vitro* translated, radiolabeled Sp proteins were synthesized in reticulocyte lysates and incubated with a GST-fusion protein prepared using a full-length mNkx3.1 cDNA (lanes 2, 5, 8, and 11) or a control fusion protein (GST-FSH; lanes 3, 6, 9, and 12). *In vitro* translated proteins (10% of input) were resolved alone (lanes 1, 4, 7, and 10) as controls and are indicated at the *bottom* of each gel. Molecular weight markers are indicated on the *left*. *B*, the DNA-binding domain of Sp-family members is necessary and sufficient for complex formation with Nkx3.1. A full-length GST-mNkx3.1 fusion protein (lanes 2 and 5), GST-FSH (lanes 3 and 6), or a GST-fusion prepared from the Sp2 DNA-binding domain (pGEX1N-Sp2Zn; lanes 8 and 9) were challenged with *in vitro* translated, radiolabeled Sp2 protein (lanes 1-3), an Sp2 derivative lacking the DNA-binding domain (lanes 4-6), or mNkx3.1 (lanes 7-9). *In vitro* translated proteins (10% of input) were resolved alone (lanes 1, 4, and 7) as controls and are indicated at the *bottom* of each gel. Molecular weight markers are indicated on the *left*. *C*, two non-contiguous regions of Nkx3.1 carry binding sites for Sp-family members. *In vitro* translated, radiolabeled Sp2 was synthesized in reticulocyte lysates and incubated with a full-length mNkx3.1 GST-fusion protein (lane 2), or derivatives carrying various portions of mNkx3.1 as depicted in *D*. Lane 3, amino acids 1-87; Lane 4, amino acids 1-128; Lane 5, amino acids 1-216; Lane 6, amino acids 129-216. Sp2 (10% of input) is resolved as a control in lane 1, and GST-FSH was employed as a control binding protein in lane 7. Molecular weight markers are indicated on the *left*. *D*, schematic diagram of mNkx3.1 and truncated derivatives employed as GST-fusion proteins in *C*. The homeodomain is indicated by a yellow box as are amino acid endpoints for Nkx3.1 deletions.

A



B





4.2 The Nkx3.1 homeodomain physically interacts with the zinc-“fingers” of Sp-family members

To identify regions of Sp proteins that are required for the formation of complexes with Nkx3.1, I employed the PCR to generate a partial Sp2 cDNA that encodes the Sp2 *trans*-activation domain (Sp2 Δ Zn) and subcloned this PCR fragment into an *in vitro* transcription vector. Radiolabeled Sp2 and Sp2 Δ Zn proteins were generated by *in vitro* transcription/translation and employed in protein/protein-binding assays with GST-mNkx3.1 or GST-FSH15 (Fig. 11B). Consistent with previous protein-binding assays, full-length Sp2 bound to GST-mNkx3.1 (lanes 1-3) whereas an Sp2-derived protein carrying only its *trans*-activation domain did not (Sp2 Δ Zn; lanes 4-6). These results suggested that the Sp2 DNA-binding domain carries at least one site required for physical interactions with Nkx3.1. To determine if the Sp2 DNA-binding domain is both necessary and sufficient to bind Nkx3.1, I prepared another GST-fusion protein (GST-Sp2Zn) that carries the entirety of the Sp2 DNA-binding domain and performed protein/protein-binding assays with *in vitro* translated and radiolabeled mNkx3.1. In contrast to results obtained with Sp2 Δ Zn, Nkx3.1 bound efficiently and specifically to the Sp2 DNA-binding domain (Fig. 11B, lanes 7-9). I conclude from these results that Sp-family members form specific protein complexes with Nkx3.1 *in vitro*, and that the DNA-binding domain of Sp-family members is necessary and sufficient for these physical interactions.

To delimit region(s) of mNkx3.1 required for the formation of physical complexes with Sp-family members, I used the PCR to generate a series of partial cDNAs encoding the regions depicted in Fig. 11D. GST-fusion proteins were prepared from these partial

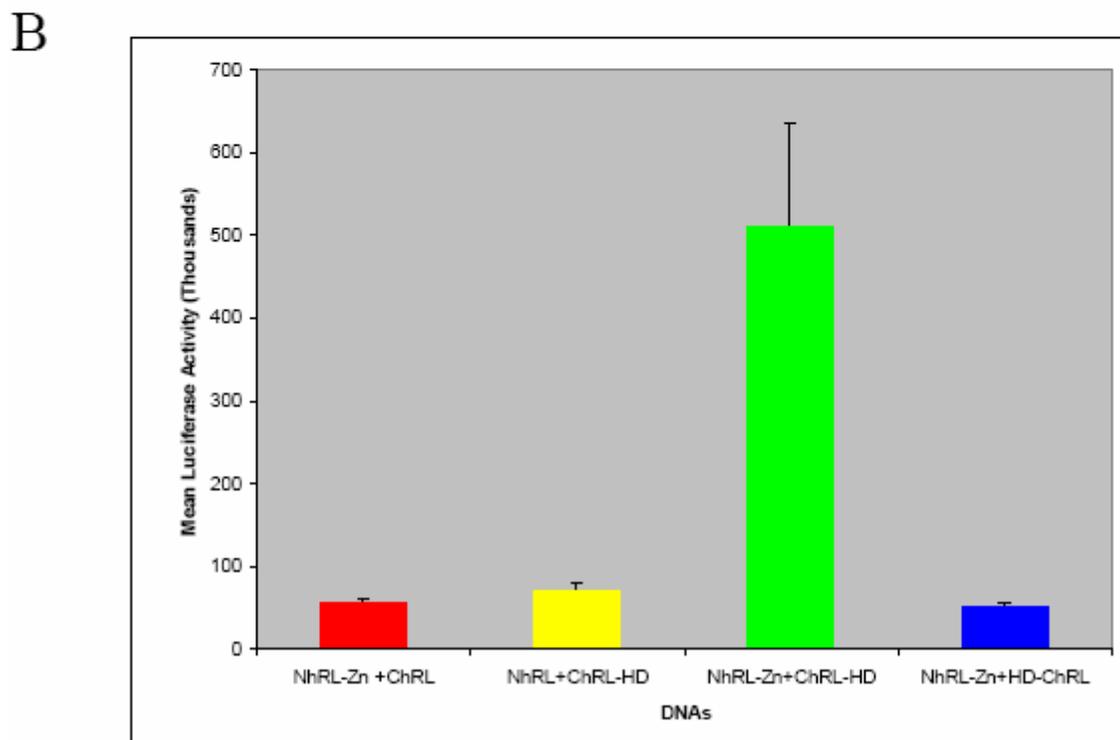
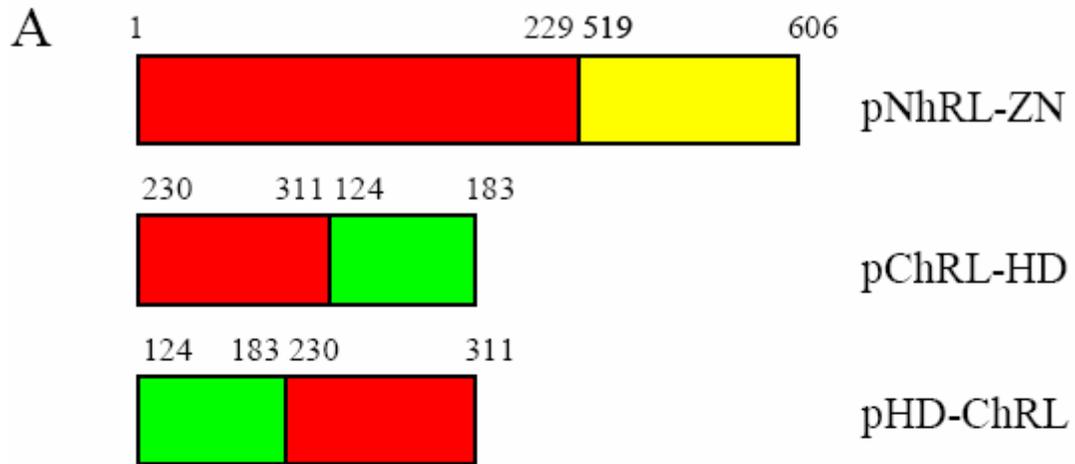
mNkx3.1 cDNAs, and subsequently incubated with radiolabeled full-length Sp2 protein produced by *in vitro* transcription/translation. Interestingly, at least two independent portions of mNkx3.1 were noted to be sufficient to bind Sp2 *in vitro*. The Nkx3.1 homeodomain appears to carry a major site of protein/protein interaction as a partial fusion protein comprised of this portion of mNkx3.1 (Δ 129-216) bound Sp2 as efficiently as full-length mNkx3.1 (Fig. 11C, lane 5). Yet another binding site for Sp proteins appears to reside within the amino-terminal 87 amino acids of mNkx3.1 as a GST-fusion protein carrying this portion of mNkx3.1 (Δ 1-87; Fig. 11C, lane 3) bound Sp2, albeit relatively weakly. Based on results from *in vitro* protein/protein-binding assays I conclude that Nkx3.1 and Sp-family members form stable, specific protein complexes largely through interactions between their respective DNA-binding domains. At least *in vitro*, a second relatively minor site of protein interaction is specified by amino acids within the amino-terminal 87 residues of mNkx3.1. Interestingly, these same two non-contiguous portions of Nkx3.1 that are sufficient to bind Sp2 *in vitro* are also the same two Nkx3.1 domains sufficient to block Sp-mediated transcription (Fig. 10C).

4.3 The DNA-binding domains of Nkx3.1 and Sp-proteins physically interact *in vivo*

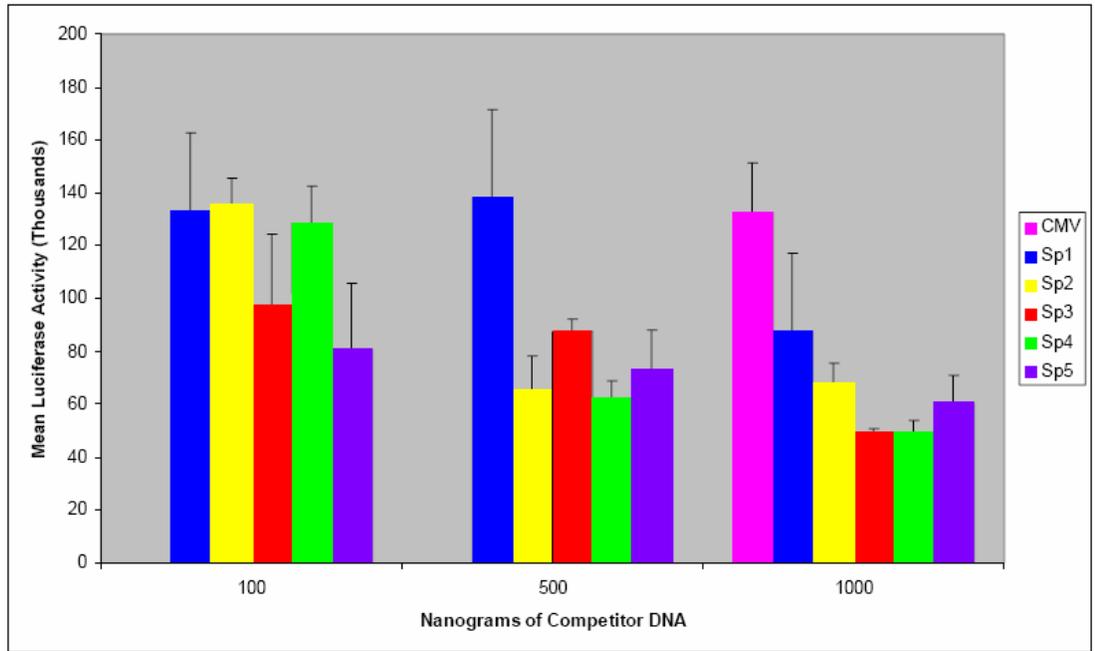
To extend my protein/protein-binding results I wished to determine whether physical complexes between Sp-proteins and Nkx3.1 occurred *in vivo* and were specified by their respective DNA-binding domains. To address these issues I employed a highly sensitive mammalian “two-hybrid” system that facilitates detection of protein/protein complexes via the reconstitution of *Renilla* luciferase activity when partial luciferase

domains are brought together in close proximity (Paulmurugan et al., 2002). I subcloned the DNA-binding domain of Sp2 (amino acids 519-606) into a mammalian expression vector, creating pNhRL-ZN, such that the Sp2 zinc-“fingers” were linked “in-frame” with the amino-terminal 229 residues of *Renilla* luciferase (Fig. 12A). Similarly, the human Nkx3.1 homeodomain (amino acids 124-183) was subcloned into a mammalian expression vector, creating pChRL-HD, such that it was linked “in-frame” with the carboxy-terminal 82 residues of *Renilla* luciferase (Fig. 12A). COS-1 cells were transfected with one or both of these constructs and then assayed for *Renilla* luciferase activity. As shown in Fig. 12B, extracts prepared from control cells co-expressing partial *Renilla* fusion proteins and their complimentary “empty” vectors resulted in luciferase levels modestly above the background detected in extracts prepared from mock transfected cells. In contrast, the co-expression of NhRL-Zn and ChRL-HD resulted in 10-fold higher levels of luciferase activity. The specificity of these *in vivo* interactions between the Nkx3.1 and Sp2 DNA-binding domains is underscored by one further experiment shown in Fig. 12B. NhRL-Zn was co-expressed with a third construct (pHD-ChRL) in which the Nkx3.1 homeodomain is sub-cloned upstream of the carboxy-terminal 82 amino acids of *Renilla* luciferase (Fig. 12A). This *Renilla*-fusion protein features a free amino-terminal end of the Nkx3.1 homeodomain whereas this end is tethered to the carboxy-terminus of *Renilla* luciferase in ChRL-HD. Similar to the aforementioned control assays, extracts prepared from cells co-expressing NhRL-Zn and

Figure 12. The DNA-binding domains of Nkx3.1 and Sp proteins form complexes in mammalian cells. *A*, schematic diagram of mammalian “two-hybrid” fusion proteins. The amino-terminal 229 amino acids of *Renilla* luciferase were fused to the DNA-binding domain (amino acids 519-606) of Sp2, creating pNhRL-ZN. The carboxy-terminal 82 amino acids of *Renilla* luciferase were fused to the human Nkx3.1 homeodomain (amino acids 124-183), creating pChRL-HD. An analogous construct carrying the human Nkx3.1 homeodomain upstream of the *Renilla* carboxy-terminus created pHD-ChRL. The Sp2 DNA-binding domain is indicated by a yellow box, and the Nkx3.1 homeodomain is indicated by a green box. Amino acid endpoints are indicated above each schematic drawing. *B*, reconstitution of luciferase activity *in vivo*. COS-1 cells were transfected with 0.5 µg pNhRL-ZN and 0.5 µg pChRL, an empty control vector, or 0.5 µg of fusion constructs carrying the human Nkx3.1 homeodomain (pChRL-HD and pHD-ChRL). As an additional control, 0.5 µg of pChRL-HD was transfected with 0.5 µg of pNhRL, the corresponding empty control vector. An irrelevant plasmid was included in all transfections to maintain constant input DNA concentrations (2 µg per plate). Plotted are mean values of *Renilla* luciferase activity with each bar representing results compiled from six or more plates of transfected cells. Error bars indicate S.D. values. *C*, competition for reconstitution of *Renilla* luciferase activity by wild-type Sp-family members. COS-1 cells were transfected with 0.5 µg of pNhRL-ZN and 0.5 µg of pChRL-HD as well as increasing amounts (100-1000 ng) of expression vectors carrying wild-type Sp-family members or 1 µg of empty expression vector (pCMV4). Where appropriate, an irrelevant plasmid was included in transfections to maintain constant input DNA concentrations (2 µg per plate). Plotted are mean values of *Renilla* luciferase activity with each bar representing results compiled from six or more plates of transfected cells. Error bars indicate S.D. values.



C



HD-ChRL resulted in minimal levels of luciferase activity. Western blots prepared from transfected cells indicated that each *Renilla*-fusion protein was expressed at equivalent levels *in vivo* (data not shown). Thus, the reconstitution of *Renilla* luciferase activity appears to require orientation-specific interactions between *Renilla*-fusion proteins carrying the Nkx3.1 homeodomain and Sp2 zinc-“fingers”.

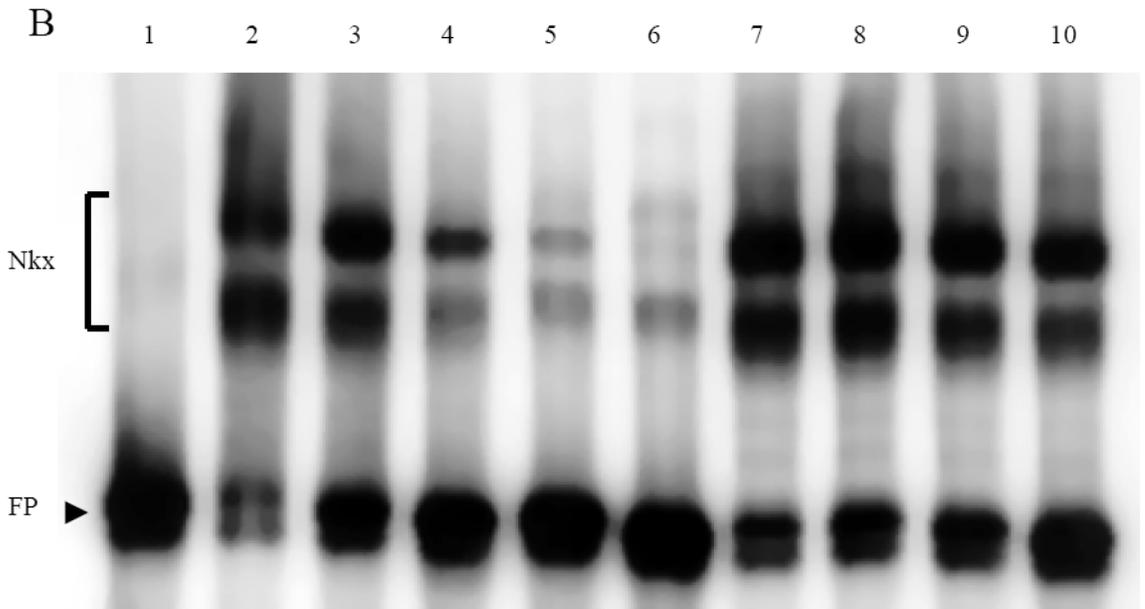
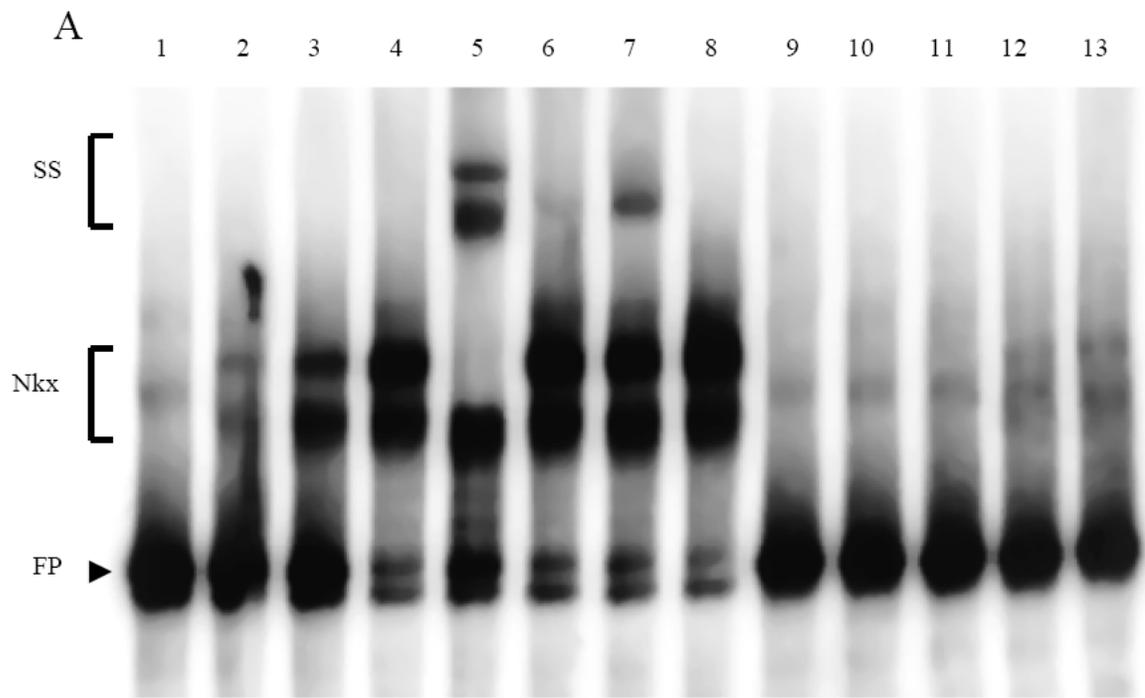
To confirm the physiological relevance of these *in vivo* protein/protein interactions, I performed a competition experiment in which NhRL-Zn and ChRL-HD were co-expressed in COS-1 cells in conjunction with increasing amounts of mammalian Sp-expression vectors or 1000 ng of empty expression vector (pCMV4). Should the interactions between *Renilla*-fusion proteins be physiologically relevant, I predicted that the co-expression of wild-type Sp proteins should prevent the reconstitution of *Renilla* luciferase activity by displacing NhRL-Zn. Consistent with this prediction, co-expression of wild-type Sp-proteins led to a dose-dependent decrease in *Renilla* luciferase activity (Fig. 12C). In contrast, little or no reduction in luciferase activity was noted in extracts prepared from cells receiving empty expression vector. I conclude from these experiments that the DNA-binding domains of Sp-family members and Nkx3.1 interact specifically *in vivo*.

4.4 Recombinant human Nkx3.1 binds its cognate DNA-binding sequence specifically *in vitro*

The data presented thus far indicate that Nkx3.1 antagonizes Sp-mediated transcription via TSA-sensitive and –insensitive mechanisms, and that Nkx3.1 and Sp-family members physically interact via their respective DNA-binding domains. Given

these results I reasoned that Nkx3.1 might negatively regulate Sp-family members, at least in part, via competition for their capacity to bind DNA. This mechanism of *trans*-repression would be expected to be TSA-insensitive and dependent on the affinity of Nkx3.1 for a given Sp DNA-binding domain. To address this possibility I developed an Nkx3.1 protein/DNA-binding (“gel-shift”) assay and assessed the capacity of Sp-family members to bind DNA following incubation with recombinant Nkx3.1 protein. As a first step I incubated 10-200 ng of bacterially expressed human Nkx3.1 protein with a radiolabeled oligonucleotide probe carrying a consensus DNA-binding site (WT; 5'-TAAGTA-3') or an analogous probe carrying nucleotide substitutions (Mut; 5'-TCCACA-3') that were expected to prevent the formation of hNkx3.1 protein/DNA complexes. As illustrated in Fig. 13A, reactions in which the wild-type probe was incubated with increasing amounts of hNkx3.1 led to the formation of two novel protein/DNA complexes (denoted “Nkx”; lanes 2-4). These novel protein/DNA complexes were barely detectable when hNkx3.1 was incubated with the mutated probe (lanes 10-13), consistent with the proposition that recombinant hNkx3.1 binds DNA specifically in these *in vitro* assays. To confirm the identity of the proteins comprising these complexes, “gel-shift” reactions were challenged with three anti-Nkx3.1 antibodies and resulting antigen/antibody complexes were resolved through acrylamide gels. Two anti-Nkx3.1 antibodies, T19 and N15, were prepared against human Nkx3.1 protein whereas a third, L15, was prepared against mouse Nkx3.1. Consistent with the antigenic specificities of the antibodies employed, addition of T19 or N15 to Nkx3.1 “gel-shift” reactions led to the generation of “supershifted” protein/DNA complexes (denoted “SS”; Fig. 13A, lanes 5 and 7) whereas L15 did not (Fig. 13A, lane 6). As one further test of

Figure 13. Protein/DNA-binding assays with recombinant human Nkx3.1 protein. *A*, recombinant hNkx3.1 binds DNA specifically *in vitro*. Radiolabeled oligonucleotide probes carrying consensus (WT; lanes 1-8) or mutated (Mut; lanes 9-13) Nkx3.1-binding sites were incubated with or without varying amounts of bacterially expressed hNkx3.1. Lanes 1 and 9, probe alone; lanes 2 and 10, 10 ng hNkx3.1; lanes 3 and 11, 50 ng hNkx3.1; lanes 4-7, and 12, 100 ng hNkx3.1; lanes 8, and 13, 200 ng hNkx3.1. Protein/DNA complexes were challenged with anti-Nkx3.1 antibodies in lanes 5 (T-19), 6 (L-15), and 7 (N-15). Nkx3.1 protein/DNA complexes are indicated by *Nkx*, antibody-bound “super-shifted” complexes are indicated by *SS*, and free probe is indicated by *FP*. *B*, competition experiment using wild-type and mutated oligonucleotides. A radiolabeled oligonucleotide probe carrying a consensus Nkx3.1-binding site (WT) was incubated alone (lane 1) or with 100 ng of recombinant hNkx3.1 protein (lanes 2-10) and challenged with increasing amounts of unlabeled wild-type (WT; lanes 3-6) or mutated (Mut; lanes 7-10) oligonucleotides. Molar excess of unlabeled DNA: lanes 3 and 7, 10x; lanes 4 and 8, 50x; lanes 5 and 9, 200x; lanes 6, and 10, 1000x. Nkx3.1 protein/DNA complexes are indicated by *Nkx*, and free probe is indicated by *FP*.

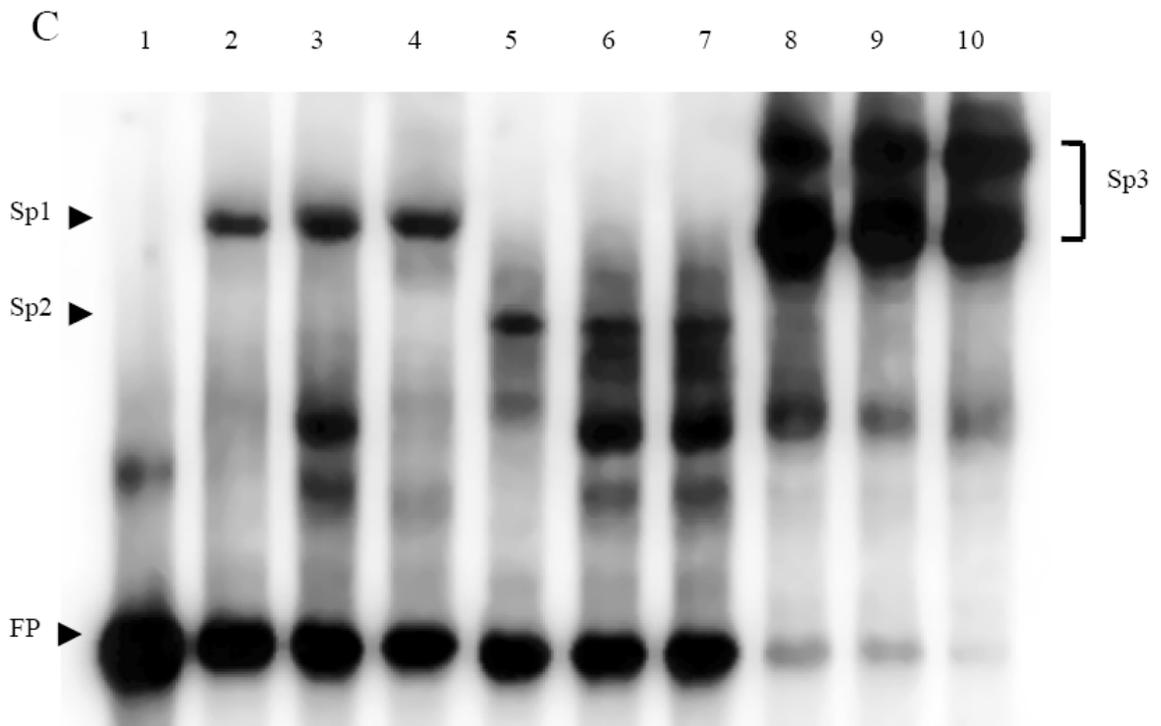
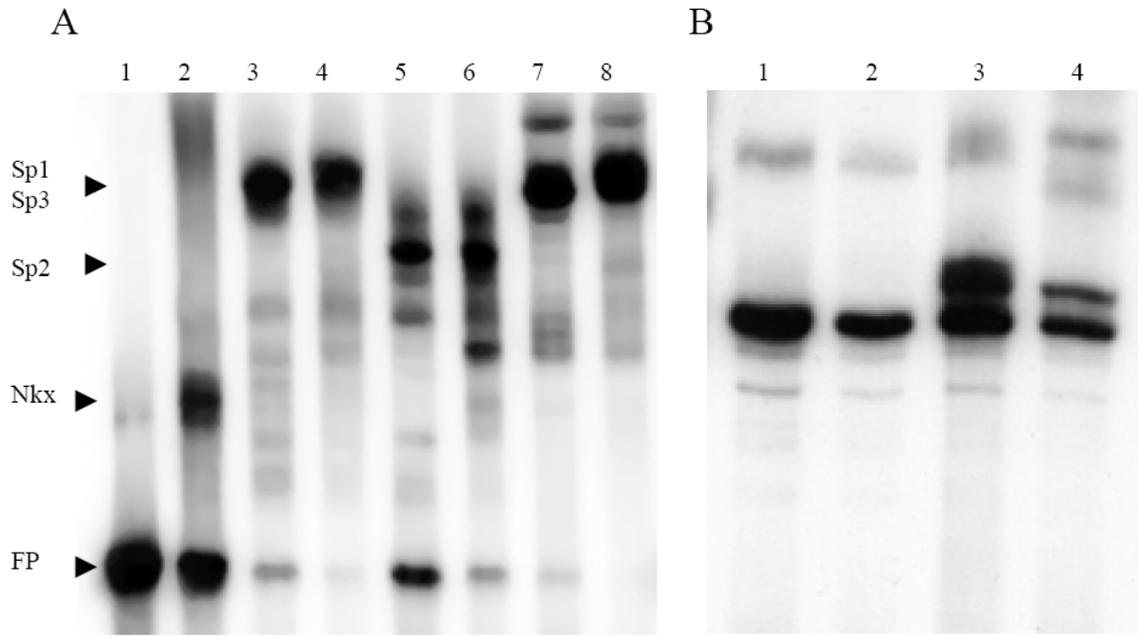


DNA-binding specificity, I performed a competition experiment in which increasing amounts of unlabeled wild-type or mutated oligonucleotides were added to standard “gel-shift” assays preloaded with radiolabeled wild-type probe. Inclusion of a 50-fold molar excess of unlabeled wild-type oligonucleotides (Fig. 13B, lane 4) significantly diminished the abundance of Nkx3.1 protein/DNA complexes, whereas the abundance of such complexes was unaffected by the inclusion of up to a 1000-fold molar excess of mutated oligonucleotides (Fig. 13B, lanes 7-10). I conclude from these results that recombinant hNkx3.1 binds its cognate DNA-binding sequence specifically *in vitro*.

4.5 Physical interactions between the DNA-binding domains of Nkx3.1 and Sp-proteins do not disrupt their capacity to bind DNA

To determine whether physical interactions between the DNA-binding domains of Nkx3.1 and Sp-proteins preclude DNA-binding activity, two double-stranded oligonucleotides were synthesized that carry consensus Nkx3.1 and Sp-binding sites separated by five (SpNkx-5) or ten (SpNkx-10) base pairs. These dual binding-site oligonucleotides were subsequently challenged with bacterially expressed hNkx3.1 protein, recombinant Sp-proteins expressed in insect cells via baculovirus infection, or mixtures of these proteins. The functional properties of these baculovirus-produced Sp-proteins have been characterized extensively (Moorefield et al., 2004). When each protein preparation was assayed alone, Nkx3.1 (lane 2) as well as Sp1 (lane 3), Sp2 (lane 5), and Sp3 (lane 7) each bound the SpNkx-5 probe (Fig. 14A). Incubation of Nkx3.1 and Sp1 (lane 4) or Sp3 (lane 8) proteins with SpNkx-5 resulted in the formation of a

Figure 14. Protein/DNA-binding assays with recombinant human Nkx3.1 and Sp proteins. *A*, protein/DNA-binding assays with recombinant hNkx3.1 and human Sp-family members. A radiolabeled oligonucleotide probe (NkxSp-10) carrying consensus Nkx3.1- and Sp2-binding sites separated by 10 bps was incubated alone (lane 1) or with 100 ng of recombinant hNkx3.1 (lanes 2, 4, 6, and 8) and baculovirus extracts carrying recombinant Sp1 (lanes 3 and 4), Sp2 (lanes 5 and 6), or Sp3 (lanes 7 and 8) proteins. Nkx3.1 protein/DNA complexes are indicated by *Nkx*. *Sp1*, *Sp2*, and *Sp3* indicate their respective protein/DNA complexes, and free probe is also indicated (*FP*). *B*, extended electrophoresis of protein/DNA-binding assays with recombinant hNkx3.1 and human Sp3 proteins. A radiolabeled NkxSp-5 probe was incubated with baculovirus extracts carrying recombinant Sp3 protein (lanes 1-4) and 100 ng of recombinant hNkx3.1 (lanes 3 and 4). Protein/DNA complexes were challenged with anti-hNkx3.1 antibody T-19 in lanes 2 and 4. *C*, protein/DNA-binding assays with recombinant hNkx3.1 and human Sp-family members using a probe carrying a consensus Sp-binding site. A radiolabeled probe carrying a consensus Sp2-binding site was incubated alone (lane 1) or with baculovirus extracts carrying recombinant Sp1 (lanes 2-4), Sp2 (lanes 5-7), or Sp3 (lanes 8-10) proteins and 100 ng of recombinant hNkx3.1 (lanes 3, 4, 6, 7, 9, and 10). Protein/DNA complexes were challenged with anti-hNkx3.1 antibody T-19 in lanes 4, 7, and 10. *Sp1*, *Sp2*, and *Sp3* indicate their respective protein/DNA complexes, and *FP* indicates free probe.



novel complex with a slightly reduced mobility compared to Sp1 (lane 3) and Sp3 (lane 7) protein/DNA complexes. Interestingly, mixtures of Nkx3.1, Sp2, and SpNkx-5 did not result in the formation of an analogous novel complex (lane 6). Identical results were obtained for each of these protein mixtures in parallel “gel-shift” assays performed with the SpNkx-10 oligonucleotide (data not shown). To verify that the novel complexes I observed result from the formation of ternary protein/DNA complexes, I challenged “gel-shift” reactions with an anti-Nkx3.1 antibody and then employed an extended period of electrophoresis to resolve closely migrating protein/DNA complexes (Fig. 14B). For this experiment, recombinant Sp3 protein was added to each “gel-shift” reaction (Fig. 14B, lanes 1-4) whereas recombinant Nkx3.1 was added only to reactions resolved in lanes 3 and 4. As shown in Fig. 14B, inclusion of an anti-Nkx3.1 antibody depleted the abundance of a novel complex formed by reactions prepared with both hNkx3.1 and Sp3 (compare lanes 3 and 4). Addition of this same antibody to a control “gel-shift” reaction (Fig. 14B, lane 2) did not affect the abundance of a protein/DNA complex formed by recombinant Sp3 protein. I conclude from these results that interactions between the DNA-binding domains of Nkx3.1 and Sp1 or Sp3 do not compete for their association with DNA, at least under the experimental conditions I have employed. In contrast, interactions between Sp2 and Nkx3.1 appear to compete for Nkx3.1 DNA-binding activity.

4.6 DNA-binding sites for both Nkx3.1 and Sp-proteins are required for ternary complex formation

Although binding sites for Nkx3.1 and Sp-proteins were carried by oligonucleotides in the previous protein/DNA-binding assays, it remained unclear whether the formation of ternary complexes resulted from protein/protein or protein/DNA interactions. To determine if ternary protein/DNA complexes require cognate DNA-binding sites for Nkx3.1 and Sp-proteins, I performed a series of “gel-shift” assays using oligonucleotide probes containing binding sites for Nkx3.1 or Sp-family members. As shown in Fig. 14C, Sp1, Sp2, and Sp3 bound a probe containing a single Sp-binding site (lanes 2-10) and inclusion of hNkx3.1 did not result in the formation of protein/DNA complexes with decreased mobility (lanes 3, 6, and 9). Inclusion of anti-Nkx3.1 antibodies in parallel reactions (lanes 4, 7, and 10) confirmed the lack of ternary complex formation. Identical results were obtained using an oligonucleotide probe that carries a binding site for Nkx3.1, but not Sp-family members (data not shown). I conclude from these results that DNA-binding sites for both Nkx3.1 and Sp-proteins are required for ternary complex formation. That is, neither Sp-proteins nor Nkx3.1 can tether the other to DNA at least under the experimental conditions I have employed. Taken together with results illustrated in Figs. 13A and 13B, I also conclude that Nkx3.1 and Sp1 or Sp3 can occupy DNA-binding sites separated by as little as five base pairs. Further experiments will be required to determine why Sp2 and hNkx3.1 did not form ternary complexes under similar experimental conditions.

The results in this chapter show that Nkx3.1 forms specific physical complexes with members of the Sp-family of transcription factors. Based on results from *in vitro* protein/protein-binding assays I showed that Nkx3.1 and Sp-family members form stable protein complexes largely through interactions between their respective DNA-binding domains, although a second relatively minor site of protein interaction is specified by amino acids within the amino-terminal 87 residues of mNkx3.1 *in vitro*. I then demonstrated that the DNA-binding domains of Sp-family members and Nkx3.1 interact specifically *in vivo* using a sensitive mammalian “two-hybrid” system. Next, I described the development of an Nkx3.1 protein/DNA-binding assay which I used to show that interactions between the DNA-binding domains of Nkx3.1 and a subset of Sp-family members do not compete for their association with DNA. Finally, I showed that DNA-binding sites for both Nkx3.1 and Sp-proteins are required for ternary complex formation and that that Nkx3.1 and Sp1 or Sp3 can occupy DNA-binding sites separated by as little as five base pairs. Taken together with results from the previous chapter, these protein/protein- and protein/DNA-binding results indicate that portions of Nkx3.1 required for physical interactions with Sp-proteins *in vitro* are also sufficient to antagonize Sp-mediated transcription *in vivo*. Thus, I have shown that there is a direct correlation between the formation of Nkx3.1/Sp protein complexes and the antagonism of Sp-mediated transcription. In the next chapter, I will describe several studies that examine Nkx3.1 subcellular localization and demonstrate that Nkx3.1 is specifically targeted to the nuclear matrix and binds condensed chromatin during mitosis.

CHAPTER V
**Nkx3.1 Binds the Nuclear Matrix and Associates with
Condensed Chromatin throughout Mitosis**

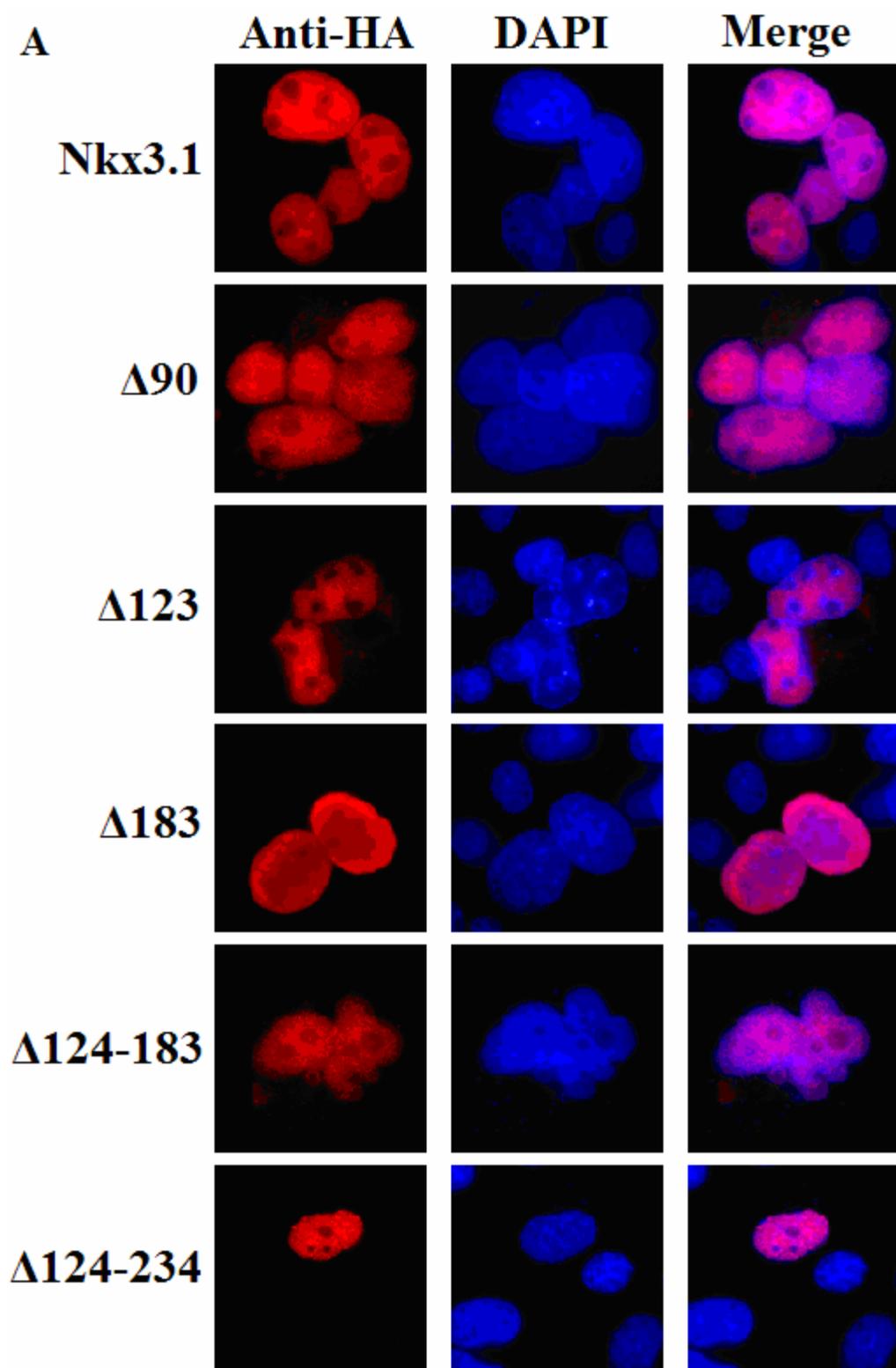
The results presented in Chapter IV indicate that Nkx3.1 forms specific physical complexes with Sp proteins largely via interactions between their respective DNA-binding domains. For some Nkx3.1/Sp complexes, such as those between Nkx3.1 and Sp1 or Sp3, these interactions appear to preserve the capacity of each protein to bind to their consensus binding sites *in vitro*. Indeed, using an *in vitro* protein/DNA-binding assay I showed that DNA-binding sites for both Nkx3.1 and Sp proteins are required for ternary complex formation. In contrast to these results, complexes involving Nkx3.1 and Sp2 appear to compete for interactions of Nkx3.1 with DNA. In addition to defining the Nkx3.1 homeodomain as a site of protein/protein interactions with Sp proteins I identified a portion of the Nkx3.1 amino-terminus as harboring a second site of Nkx3.1/Sp interactions. Capitalizing on these observations, I created a series of expression vectors carrying mutated Nkx3.1 cDNAs and showed that the Nkx3.1 amino-terminus and homeodomain independently antagonize Sp-mediated transcription in prostate-derived cells. Consequently, my results indicate that there is a direct correlation between portions of Nkx3.1 required for protein/protein interactions and *trans*-repression of Sp-mediated transcription.

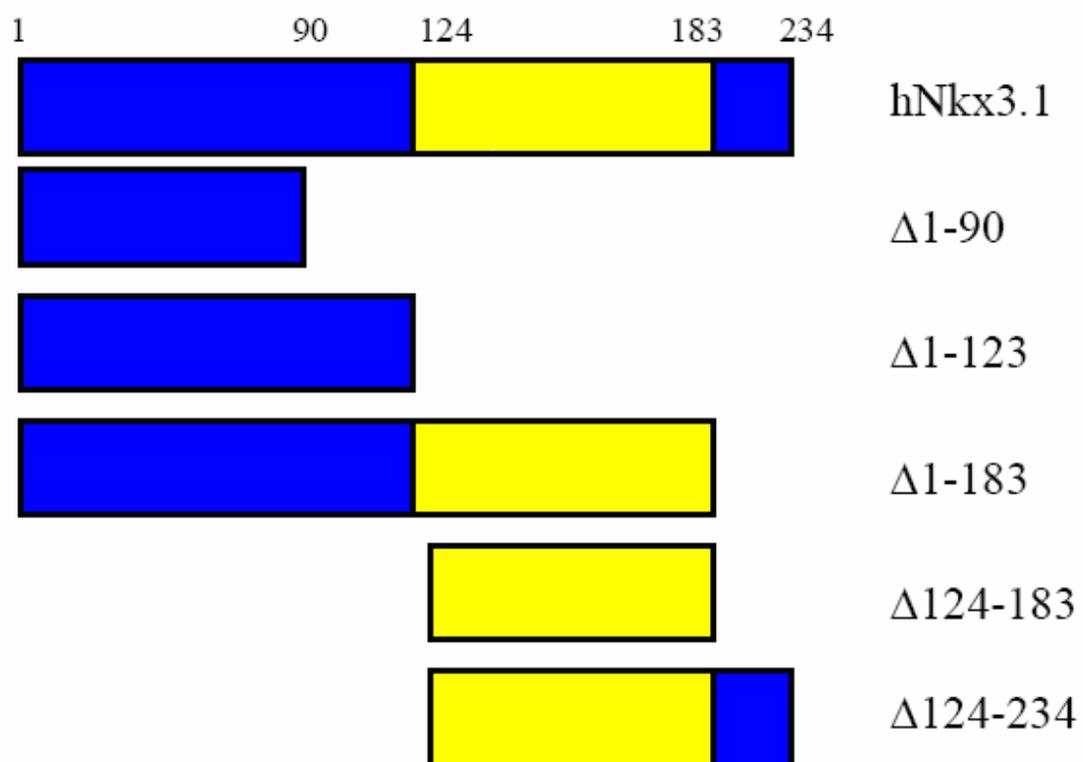
A number of investigators have reported that homeodomain protein function can be regulated by alterations in subcellular localization (Mann and Abu-Shaar, 1996; Parker et al., 2000; Baas et al., 2000; Fei et al., 2000). Since little information was available regarding (1) the subcellular localization of Nkx3.1 nor (2) domains of Nkx3.1 that regulate its subcellular localization, I embarked on a series of studies that focused on these issues.

5.1 The Nkx3.1 homeodomain carries at least one nuclear localization signal

To identify portions of Nkx3.1 that regulate its subcellular localization, expression vectors encoding wild-type human Nkx3.1 as well as the Nkx3.1 deletion mutants employed in Chapter III were synthesized and ectopically-expressed in COS-1 cells (Fig. 15B). In contrast to the expression vectors employed in Chapter III, these Nkx3.1 constructions lacked the heterologous nuclear localization sequence derived from the HIV TAT protein and thus their subcellular localization relied on signals intrinsic to Nkx3.1 itself. Transiently-transfected cells were fixed with paraformaldehyde, and Nkx3.1 proteins were detected 48 hours post-transfection by indirect immunofluorescence using an antibody prepared against a linked epitope tag (HA). Consistent with results reported for other homeodomain-containing proteins, wild-type Nkx3.1 localized exclusively to non-nucleolar portions of the cell nucleus (Fig. 15A). Surprisingly, each partial-Nkx3.1 protein also appeared to be specifically targeted to nuclei of transfected cells suggesting that Nkx3.1 may encode more than one nuclear localization signal (NLS; Fig. 15A). Multiple NLSs are not uncommon in homeodomain-containing proteins (Parker et al., 2000; Hessabi et al., 2000), although their functional significance is as yet uncertain. To determine if Nkx3.1 might encode multiple NLSs I performed computer-assisted search for such sequences within the human and mouse Nkx3.1 proteins (predictNLS; <http://cubic.bioc.columbia.edu/predictNLS/>). This search identified two predicted NLS sequences (NH₂-PQKRSRAA-CO₂H, NH₂-NRRYKTK-CO₂H) that are conserved within each protein and both of which are encoded within the Nkx3.1 homeodomain. An alternative interpretation of the subcellular localization

Figure 15. Nkx3.1 contains at least at least one nuclear localization signal. *A*, indirect immunofluorescence analysis of ectopically expressed wild-type and truncated hNkx3.1 proteins. COS-1 cells were transiently transfected with expression vectors encoding hNkx3.1 or the truncated mutants diagrammed in *B*. Nuclei were stained with DAPI and ectopically expressed proteins were detected with an anti-epitope tag (HA) antibody and Alexa Fluor 594 anti-mouse secondary antibody.



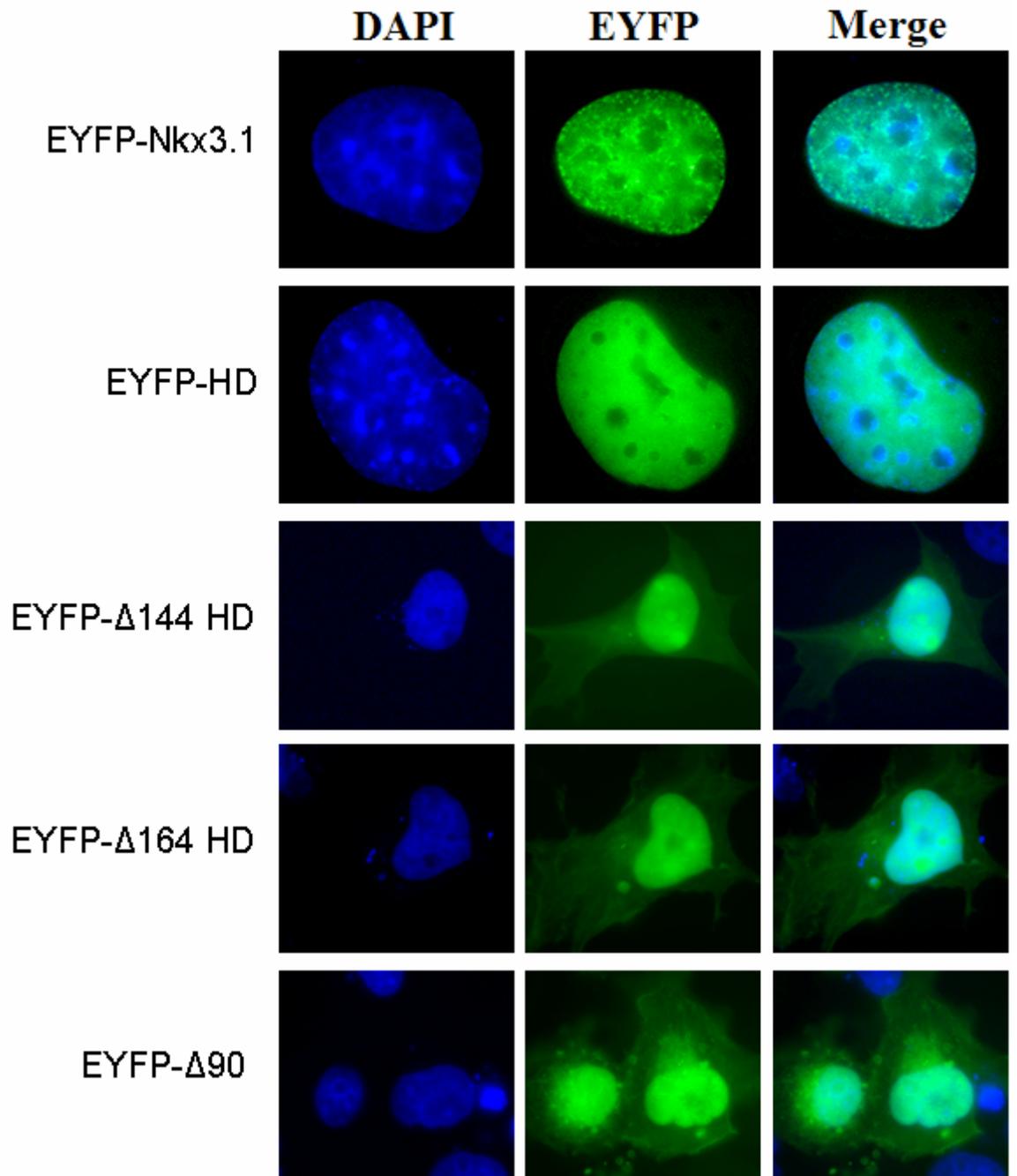
B

results presented in Fig. 15A is that each of the partial-Nkx3.1 proteins are simply small enough to diffuse into the nuclei of transfected cells. Proteins smaller than 60 kDa are believed to have unfettered access to the cell nucleus by passive diffusion through nuclear pores (Allen et al., 2000). Indeed, given their relatively small size (8-23 kDa) each of the partial-Nkx3.1 proteins would be predicted to gain nuclear access via this mechanism. Yet, such diffusible proteins would also be expected to be detected in the cytoplasm assuming that my indirect immunofluorescence assay is sufficiently sensitive.

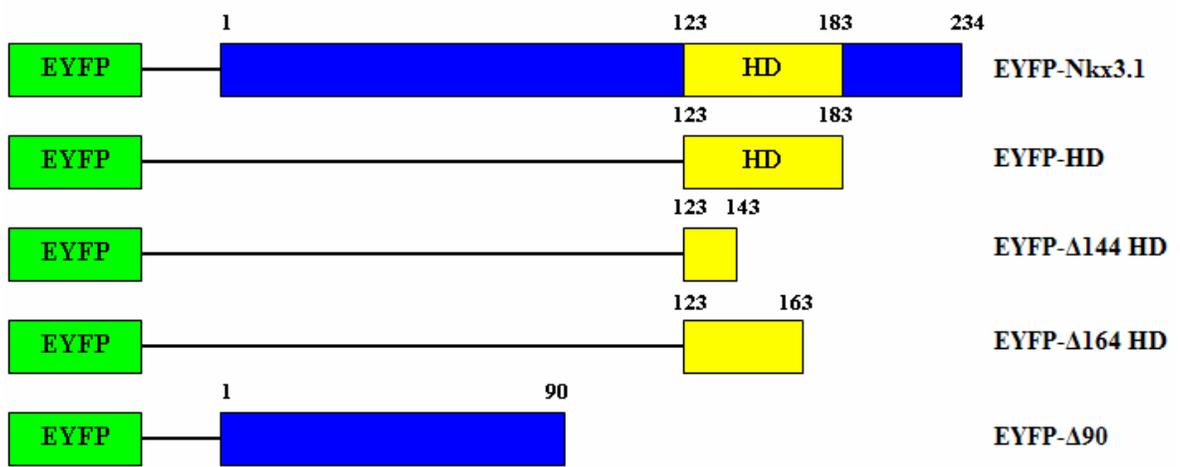
To ascertain if Nkx3.1 carries more than one NLS I created a series of enhanced yellow fluorescent protein (EYFP) - fusion proteins prepared with portions of Nkx3.1 that would be predicted to carry nuclear targeting sequences based on computer analyses and the data presented in Fig. 15A. Given the increased sensitivity offered by direct fluorescence, I reasoned that EYFP-Nkx3.1 fusions would indicate whether particular portions of Nkx3.1 were actively transported to the nucleus or whether they gained nuclear access via passive diffusion. Accordingly, a human Nkx3.1 cDNA was sub-cloned “in-frame” downstream of the EYFP coding region, creating EYFP-Nkx3.1. Consistent with results using indirect immunofluorescence, COS-1 cells expressing a full-length human EYFP-Nkx3.1 fusion protein exhibited brightly stained nuclei (EYFP-Nkx3.1; Fig. 16A). Similar results were obtained with an EYFP-fusion protein prepared with the entirety of the Nkx3.1 homeodomain (EYFP-HD; Fig. 16A), suggesting that the homeodomain carries at least one functional NLS that is sufficient to target this protein to the nucleus. This result is quite satisfying since most, if not all, homeodomains encode at least one NLS (Hessabi et al., 1997; Parker et al., 2000; Komuves et al., 2000; Fei et al., 2000; Chatelain et al., 2006). In contrast to these results, EYFP-fusion proteins that

Figure 16. The Nkx3.1 homeodomain carries at least one nuclear localization signal.
A, Direct immunofluorescence analysis of ectopically expressed wild-type and truncated hNkx3.1 proteins. COS-1 cells were transiently transfected with expression vectors encoding hNkx3.1 or the truncated mutants diagrammed in *B*. Nuclei were stained with DAPI.

A



B



lacked the carboxy-terminal 20 (EYFP- Δ 164 HD) or 40 (EYFP-Δ144 HD) amino acids of the Nkx3.1 homeodomain exhibited staining of nuclei and cytoplasm, consistent with their passive diffusion throughout transfected cells. Each of these partial homeodomain constructs lack a carboxy-terminal, computer-predicted NLS (NH₂-NRRYKTK-CO₂H) yet carry an amino-terminal, computer-predicted NLS (NH₂-PQKRSRAA-CO₂H). Given their subcellular distribution, I conclude that this putative amino-terminal NLS is insufficient to direct nuclear localization. Indeed, these results suggest that a carboxy-terminal, computer-predicted NLS (NH₂-NRRYKTK-CO₂H) is likely to be functional and sufficient for translocation of the Nkx3.1 homeodomain to the nucleus. Alternatively, deletion of the carboxy-terminal 20 amino acids may distort the Nkx3.1 homeodomain sufficiently such that an NLS encoded elsewhere is prevented from directing nuclear localization. Additional experiments will be required to explore these alternative interpretations. Finally, an EYFP-fusion protein that carries the amino-terminal 90 amino acids of human Nkx3.1 exhibited bright staining throughout transfected cells. This stands in marked contrast with the aforementioned indirect immunofluorescence results (Fig. 15A) indicating that the amino-terminal 90 amino acids were sufficient to confer nuclear localization. I presume that the increased sensitivity of direct immunofluorescence accounts for these discordant results, and tentatively conclude that the Nkx3.1 amino-terminus does not encode an independent NLS.

5.2 Nkx3.1 binds to the nuclear matrix of transfected COS cells

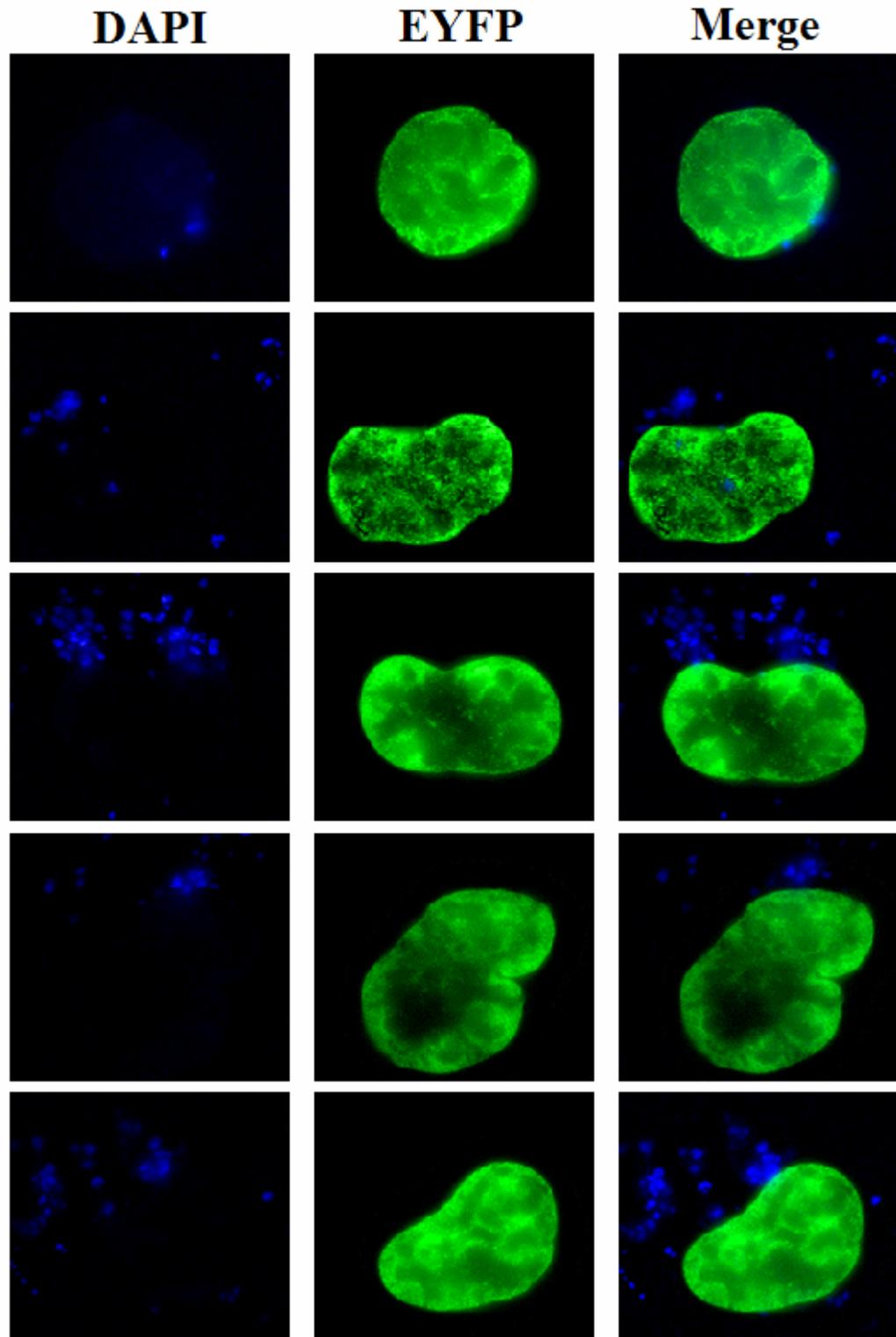
While these subcellular localization studies were underway several members of the Sp-family of proteins were shown to be constituents of the nuclear matrix

(Moorefield, et al 2006; He, et al, 2005). The nuclear matrix is an insoluble, filamentous structure that has been proposed to form a scaffold for active chromatin and to mediate the actions of regulatory pathways that modulate transcription factor function (Merdes and Cleveland, 1998; Zhang et al., 1994; Mancini et al., 1999; Bidwell et al., 1998). A wide variety of transcriptional regulators as well as proteins involved in DNA repair and replication have been shown to associate with the nuclear matrix (reviewed in Loidl and Eberharter, 1995; Martelli et al., 1996). Yet, few homeodomain proteins have been identified within this subcellular compartment (Kim et al., 1996; Dickinson et al., 1997), and the protein sequences that mediate their localization are largely uncharacterized.

I reasoned that Nkx3.1 might also be associated with the nuclear matrix, either directly via its encoding a nuclear matrix targeting signal (NMTS) or perhaps indirectly by virtue of its association with Sp proteins. Once again I took advantage of the EYFP-Nkx3.1 fusion protein employed in earlier subcellular localization studies. This construct was transiently transfected in COS-1 cells, and following incubation for 24-48 hours transfected cells were solubilized with Triton X-100 *in situ*. Cells were treated *in situ* with DNase I and chromatin was removed by extraction with ammonium sulfate. Nuclear matrices were then fixed with paraformaldehyde and stained with DAPI to determine the extent of chromatin extraction. This technique has previously been used to isolate nuclear matrices for both wide-field and confocal microscopy (Javed et al., 2000; Zaidi et al., 2001; Li et al., 2005; Moorefield et al., 2006). A representative experiment is shown in Fig. 17. Here, nuclear matrices prepared from five transfected COS-1 cells were examined for the expression of EYFP-Nkx3.1 (middle column) and for their DNA content as revealed by DAPI staining (left column). Images in the left and middle

Figure 17. Nkx3.1 binds the nuclear matrix of COS-transfected cells. Direct immunofluorescence analysis of ectopically expressed EYFP-hNkx3.1 fusion protein. COS-1 cells were transiently transfected with expression vectors encoding hNkx3.1. Cells were then solubilized with Triton X-100 then treated with DNase I. Chromatin was extracted using 2M ammonium sulfate. The remaining nuclear matrices were fixed with paraformaldehyde and stained with DAPI to insure that the chromatin extraction was thorough.

DNase I-treated

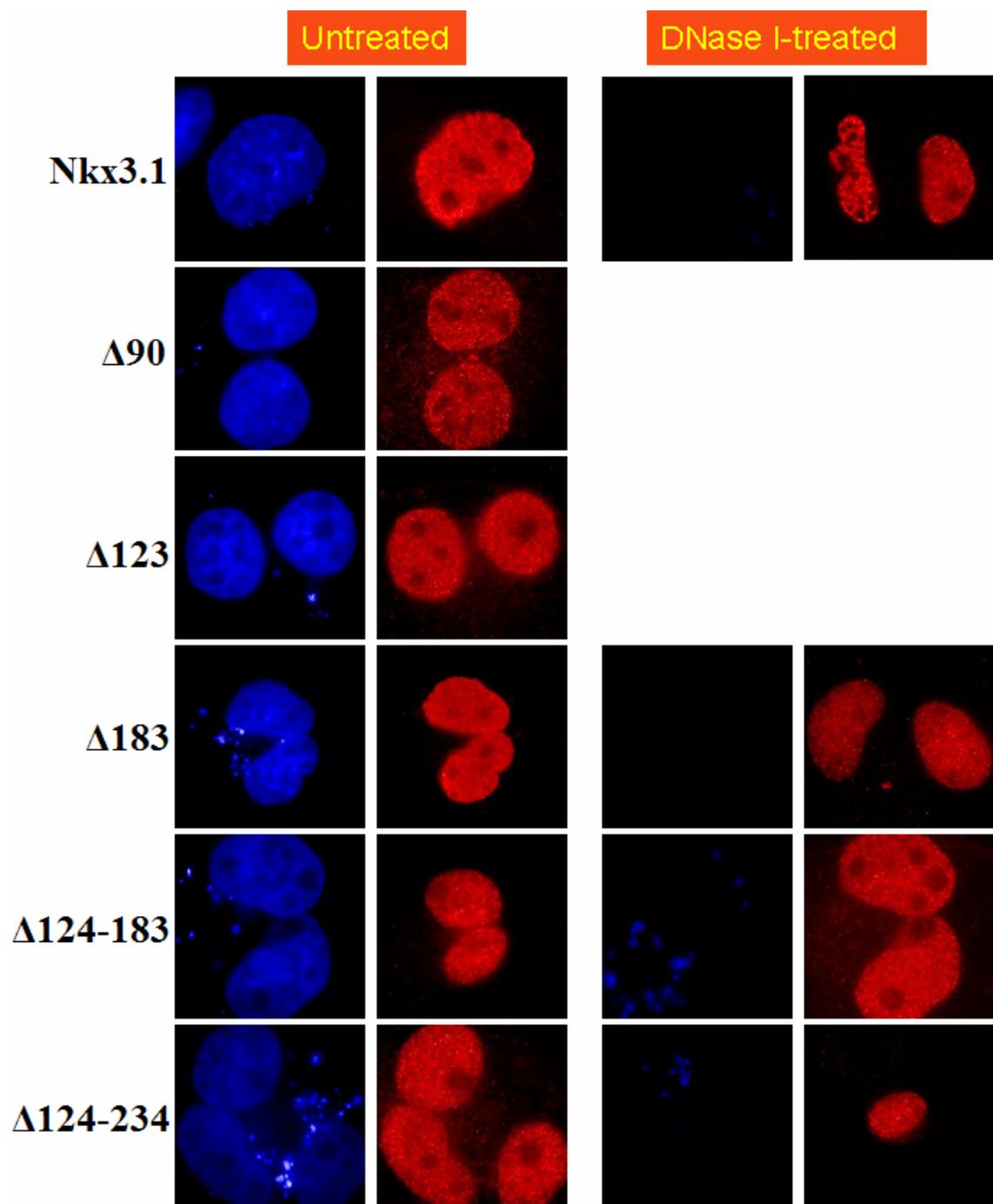


columns appear merged in the right-most column. As indicated in Fig. 17, the nuclear matrices prepared from these five cells each exhibit strong staining for EYFP-Nkx3.1 yet each is devoid of chromatin. In contrast, control matrices prepared from cells expressing EYFP alone were not similarly stained and antisera against known matrix-associated proteins, such as NumA and Lamin B1, stained matrices prepared in parallel (data not shown and Moorefield, et al 2006). I conclude from these results that Nkx3.1 can associate with the nuclear matrix in a chromatin-independent fashion.

5.3 The Nkx3.1 homeodomain contains a nuclear matrix targeting sequence

To identify sequences required for association of Nkx3.1 with the nuclear matrix, COS-1 cells were transiently transfected full-length and partial human Nkx3.1 expression constructs and nuclear matrices were prepared as outlined above. Parallel cultures of transfected cells that were not treated with DNase I were prepared as controls. Nkx3.1 proteins were visualized by indirect immunofluorescence microscopy with an antibody against a linked epitope tag (HA). Consistent with results presented in Fig. 15A, each Nkx3.1 expression construct elicited proteins that localized to nuclei of transfected cells. Interestingly, only a subset of ectopically-expressed partial Nkx3.1 proteins associated with nuclear matrices (Fig. 18). Expression of partial proteins carrying the amino-terminal 90 ($\Delta 90$) or 123 ($\Delta 123$) amino acids of Nkx3.1 did not associate with nuclear matrices despite their translocation to nuclei via passive diffusion (as documented in section 5.1). In contrast, all other partial Nkx3.1 proteins ($\Delta 183$, $\Delta 124-183$, and $\Delta 124-234$) associated with the nuclear matrices of transfected cells. Careful inspection of the amino acids shared by these matrix-associated partial proteins reveals that each carries

Figure 18. A nuclear matrix targeting sequence lies within the Nkx3.1 homeodomain. Indirect immunofluorescence analysis of ectopically expressed wild-type and truncated hNkx3.1 proteins. COS-1 cells were transiently transfected with expression vectors encoding hNkx3.1 or the truncated mutants. Cells were then solubilized with Triton X-100 then treated with DNase I. Chromatin was extracted using 2M ammonium sulfate. The remaining nuclear matrices were fixed with paraformaldehyde and stained with DAPI and ectopically expressed proteins were detected with an anti-epitope tag (HA) antibody and Alexa Fluor 594 anti-mouse secondary antibody.



the Nkx3.1 homeodomain (amino acids 124-183), suggesting that this domain encodes one or more nuclear matrix targeting sequences (NMTS).

To confirm results from indirect immunofluorescence analyses of nuclear matrices, I analyzed a series of EYFP-Nkx3.1 partial fusion proteins for their capacity to associate with the nuclear matrix. Three EYFP-fusion proteins were prepared using amino acids derived from the amino terminus of human Nkx3.1. One, $\Delta 90$, carries the amino-terminal 90 amino acids of Nkx3.1 whereas another, $\Delta 123$, carries the amino-terminal 123 amino acids of Nkx3.1. A third is an amino-terminal variant of $\Delta 123$, termed $\Delta 123v1$ that was constructed based on a previous report documenting the cloning of several alternatively-spliced human Nkx3.1 cDNAs that result in “in-frame” deletions of portions of the Nkx3.1 amino-terminus (Korkmaz et al., 2000). This particular variant lacks amino acids 40-83, and was employed here to compare its subcellular localization properties with that of $\Delta 123$ (see schematic in Fig.19B). A final EYFP-fusion protein was prepared with the entirety of the human Nkx3.1 homeodomain. Consistent with indirect immunofluorescence results, $\Delta 90$ and $\Delta 123$ EYFP-fusion proteins did not associate with nuclear matrices prepared from transfected cells despite their capacity to passively diffuse to cell nuclei (Fig. 19A). Surprisingly, the $\Delta 123v1$ partial Nkx3.1 amino-terminal variant exhibited a completely distinct subcellular localization pattern. As shown in Fig. 19A, cells expressing EYFP- $\Delta 123v1$ exhibited brightly fluorescent nuclei and this fusion protein readily associated with the nuclear matrix. Thus, deletion of amino acids 40-83 results in an Nkx3.1 variant protein that appears to be actively transported into nuclei where it associates with nuclear matrices. Additional experiments

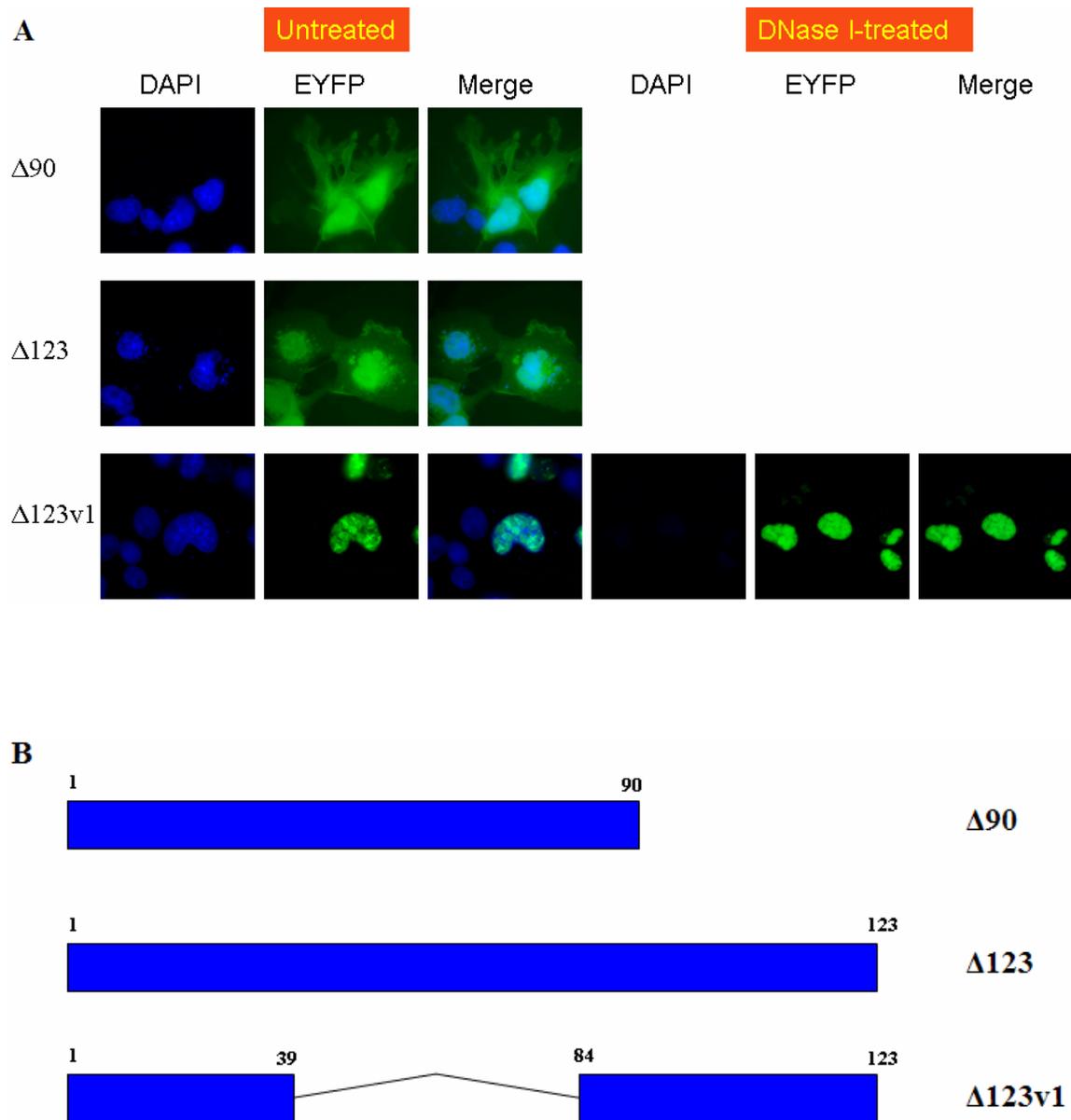


Figure 19. EYFP- $\Delta 123v1$ readily associates with the nuclear matrix. *A*, Direct immunofluorescence analysis of ectopically expressed truncated hNkx3.1 proteins. COS-1 cells were transiently transfected with expression vectors encoding truncated mutants diagrammed in *B* fused with EYFP. Cells were then solubilized with Triton X-100 then treated with DNase I. Chromatin was extracted using 2M ammonium sulfate. The remaining nuclear matrices were fixed with paraformaldehyde and stained with DAPI.

will need to be performed to reveal the signals that determine the subcellular localization of this Nkx3.1 variant. It is worth mentioning that although this Nkx3.1 variant and others have been cloned from prostate cells, obvious splice junctions or cryptic exons that might explain the origin of these variants have yet to be identified in genomic DNA. Thus, whether these variant cDNAs reflect alternatively spliced mRNAs or are artifacts of reverse transcription remains to be determined. Consistent with indirect immunofluorescence results, cells expressing an EYFP-fusion protein prepared with the Nkx3.1 homeodomain exhibited bright nuclear staining and nuclear matrices prepared from such cells were also fluorescently labeled (Fig. 20). Thus, the Nkx3.1 homeodomain indeed appears to be necessary and sufficient to facilitate the association of Nkx3.1 with the nuclear matrix. Additional experiments will be necessary to determine the precise amino acids within the homeodomain that define the Nkx3.1 NMTS and to ascertain whether one or more independent NMTSs may reside within this domain.

5.4 An intact Nkx3.1 homeodomain is required for nuclear matrix attachment

Given that the Nkx3.1 homeodomain carries at least one NMTS, it became of interest to determine whether an intact, functional Nkx3.1 homeodomain is required for nuclear matrix attachment. To address this question I prepared an EYFP-fusion protein with an Nkx3.1 homeodomain carrying a point mutation (Q173E) that has previously been shown to abrogate DNA-binding activity (Schier and Gehring, 1992; Damante et al., 1994). This mutated construction (EYFP-HD mutant) or a wild-type EYFP-homeodomain fusion were transiently expressed in COS-1 cells from which nuclear

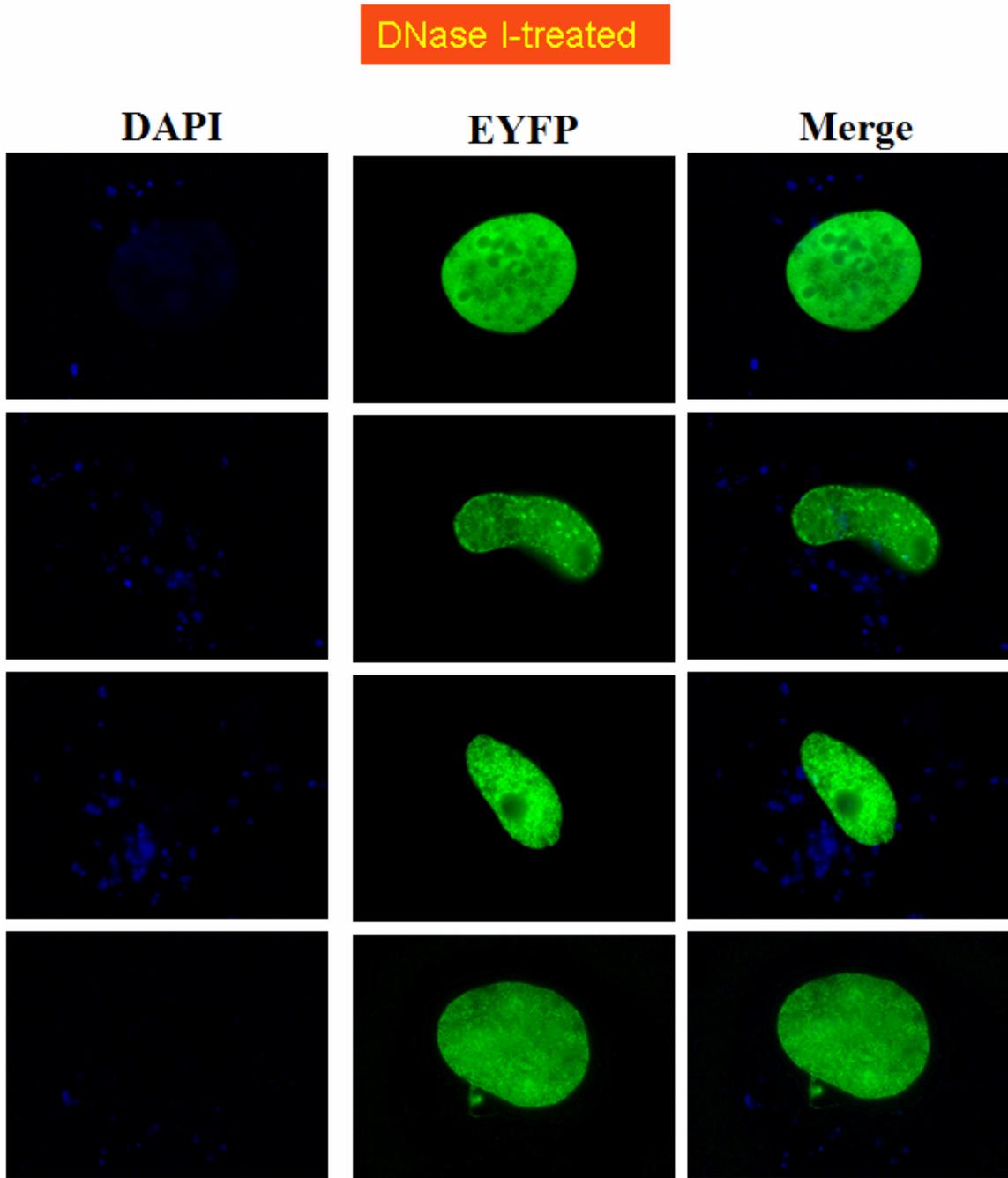


Figure 20. The Nkx3.1 homeodomain is sufficient to bind the nuclear matrix of COS-transfected cells. Direct immunofluorescence analysis of ectopically expressed EYFP-hNkx3.1 homeodomain fusion protein (EYFP- HD). COS-1 cells were transiently transfected with an expression vector encoding EYFP- HD. Cells were then solubilized with Triton X-100 then treated with DNase I. Chromatin was extracted using 2M ammonium sulfate. The remaining nuclear matrices were fixed with paraformaldehyde and stained with DAPI.

matrices were prepared subsequently. Parallel control cultures were also prepared for comparison. As shown in Fig. 21, cells expressing EYFP-HD mutant exhibited a spectrum of fluorescent phenotypes. The nuclei of some cells were homogeneously-stained (top two rows of images) in a fashion similar to that of cells expressing a wild-type EYFP-homeodomain fusion protein (for example, compare these images with those in Fig. 20). Yet, others carried what appeared to be one or more brightly-fluorescent protein aggregates in addition to modestly-stained nuclei. Still others carried one or more extremely large, brightly-fluorescent protein aggregates. I presume that these distinct phenotypes reflect the synthesis of varying amounts of the mutated EYFP-fusion protein and the subsequent aggregation of mis-folded homeodomains. Regardless, these results indicate that a functional homeodomain is not required to localize exclusively to the nucleus.

Consistent with previous results, the wild-type EYFP-Nkx3.1 homeodomain fusion protein associated with nuclear matrices prepared from transiently-transfected cells (data not shown and Fig. 20). In contrast to these results, only a subset of nuclear matrices prepared from cells transfected with the homeodomain point mutant exhibited nuclear matrix staining. Unlike nuclear matrices prepared from cells expressing a wild-type Nkx3.1 homeodomain, all nuclear matrices fluorescently-stained with the mutated homeodomain fusion protein also showed large protein aggregates (Fig. 22). In rare instances some of these nuclear matrices also showed modest homogeneous staining reminiscent of that produced by the wild-type homeodomain (for example, see images in the second and fourth rows of Fig. 22). Given these results, I conclude that an Nkx3.1 homeodomain that is competent to bind DNA appears to facilitate nuclear matrix

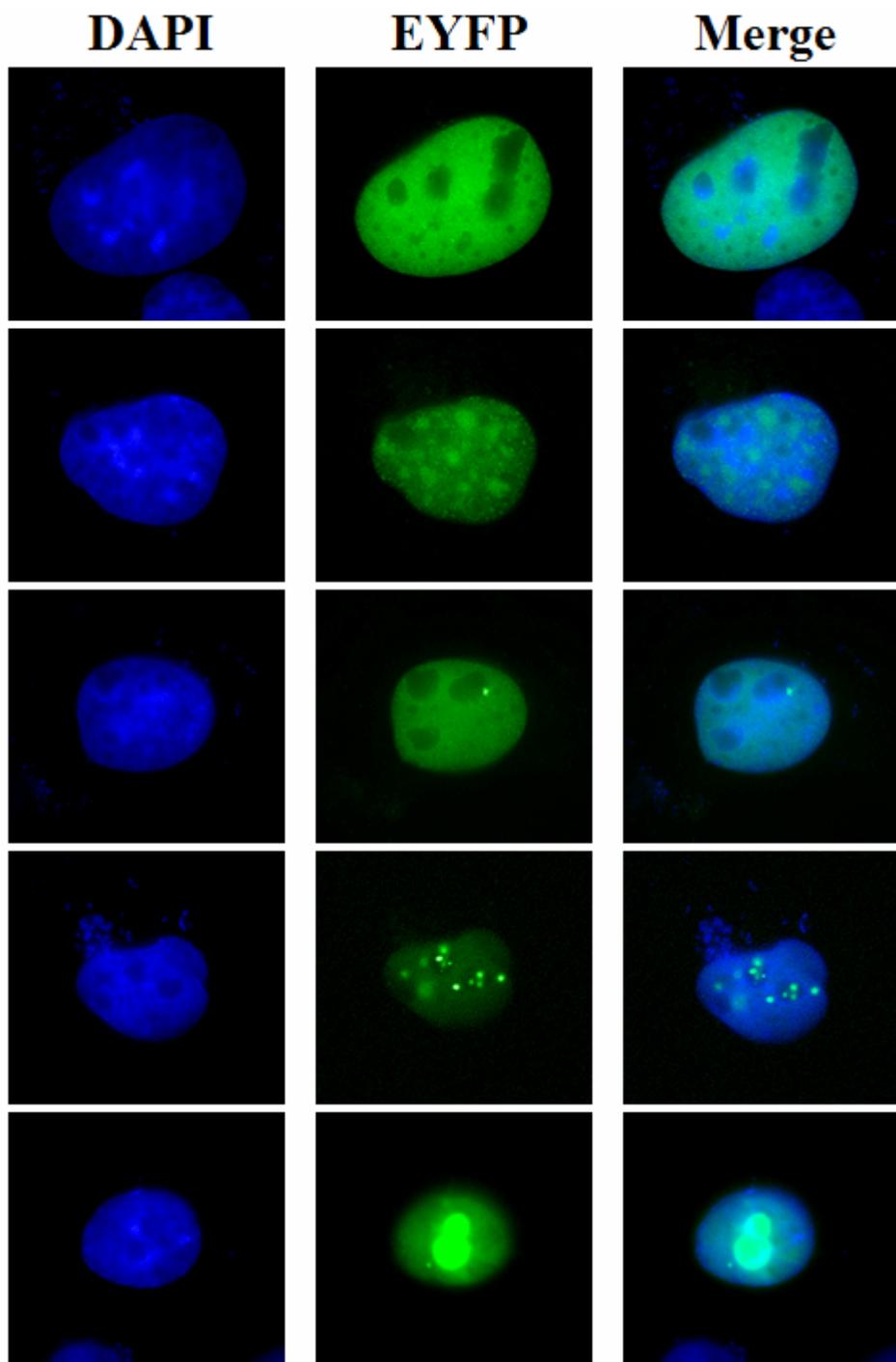
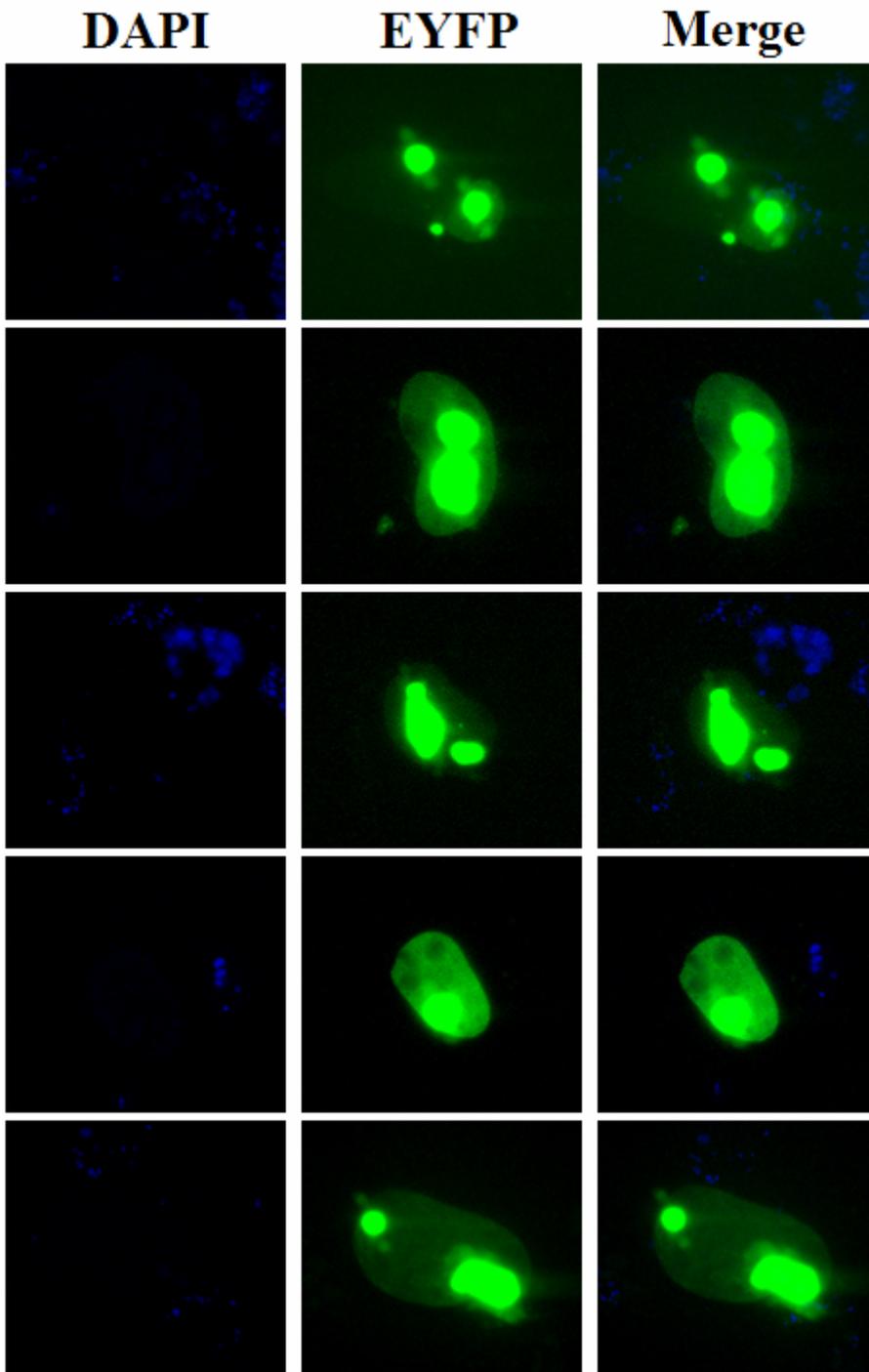


Figure 21. Nkx3.1 homeodomain mutant forms protein aggregates in COS-transfected cells. Direct immunofluorescence analysis of ectopically expressed EYFP-hNkx3.1 homeodomain mutant fusion protein (EYFP- HD mutant). COS-1 cells were transiently transfected with an expression vector encoding EYFP- HD mutant. Nuclei were stained with DAPI.

Figure 22. A functionally intact Nkx3.1 homeodomain is required to bind the nuclear matrix of COS-transfected cells. Direct immunofluorescence analysis of ectopically expressed EYFP-hNkx3.1 homeodomain mutant fusion protein (EYFP- HD mutant). COS-1 cells were transiently transfected with an expression vector encoding EYFP- HD mutant. Cells were then solubilized with Triton X-100 then treated with DNase I. Chromatin was extracted using 2M ammonium sulfate. The remaining nuclear matrices were fixed with paraformaldehyde and stained with DAPI.

DNase I-treated



association. Moreover, the association of EYFP-HD mutant proteins with the nuclear matrix is likely due to the insolubility of aggregated protein. With this said, it is worth pointing out that I cannot rule-out the possibility that mutated homeodomain proteins may retain some affinity for the nuclear matrix. The matrices results suggest that proper conformation of the Nkx3.1 homeodomain is required for association with the nuclear matrix, although it is possible that the point mutation at Q173 disrupts a critical primary protein sequence required for nuclear matrix attachment independent of secondary and tertiary protein structure.

5.5 Helix III of the Nkx3.1 homeodomain is required for matrix attachment

Few nuclear matrix targeting sequences have been defined, and to date there is little consensus among defined NMTSs. To precisely delimit amino acids within the Nkx3.1 homeodomain that specify nuclear matrix attachment, I employed two EYFP-fusion proteins with carboxy-terminal deletions of the human Nkx3.1 homeodomain. As diagrammed in Fig. 23B, Nkx3.1 homeodomain proteins lacking either twenty (EYFP- Δ 164 HD) or forty (EYFP- Δ 144 HD) carboxy-terminal amino acid residues were ectopically expressed in COS-1 cells. Consistent with results reported earlier (Fig. 16A), both homeodomain deletions were distributed throughout transfected cells indicating that they lack sequences targeting them to particular subcellular compartments. Also consistent with results reported earlier (Fig. 16A), the wild-type homeodomain fusion protein readily associated with nuclear matrices. In contrast, nuclear matrices prepared from cells expressing EYFP- Δ 164 HD and EYFP- Δ 144 HD were not fluorescently-labeled (Fig. 23A). These results suggest that, in addition to encoding an NLS, the

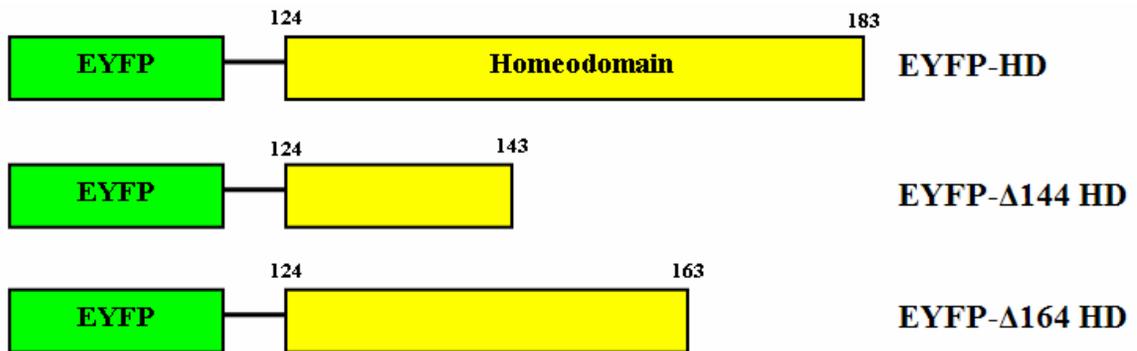
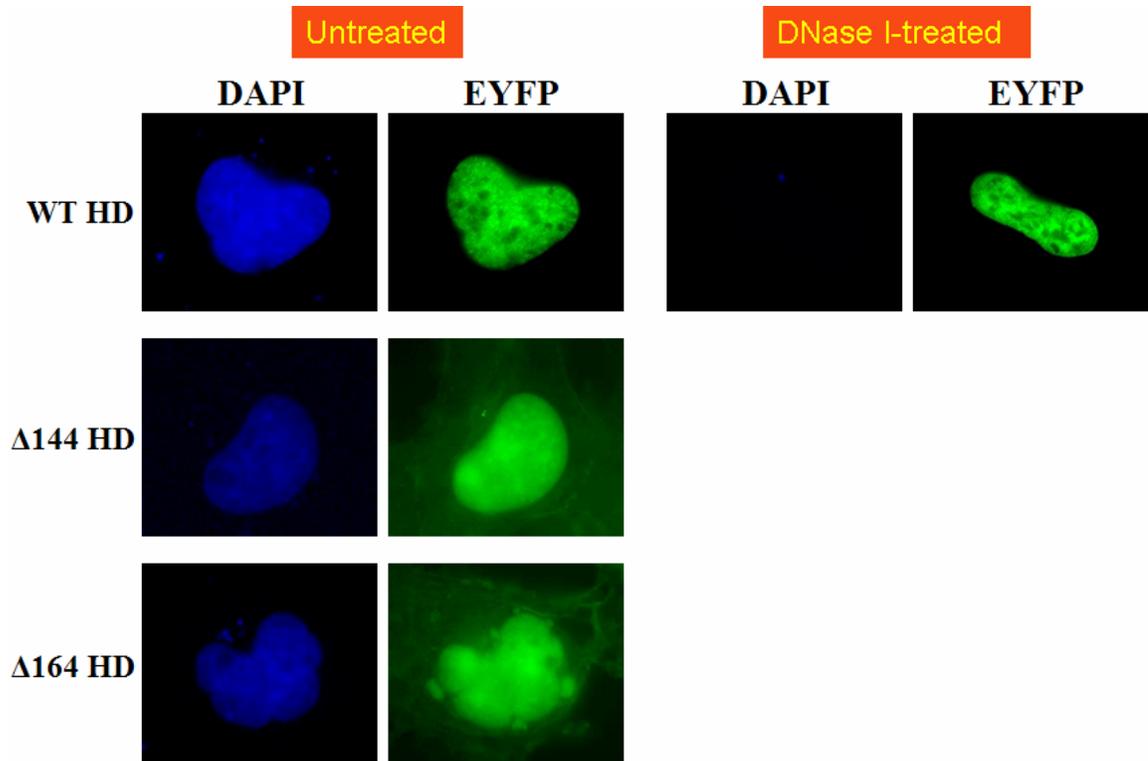


Figure 23. Helix III of the Nkx3.1 homeodomain is required for matrix attachment.
A, Direct immunofluorescence analysis of ectopically expressed truncated hNkx3.1 homeodomain proteins. COS-1 cells were transiently transfected with expression vectors encoding the Nkx3.1 homeodomain or truncated mutants diagrammed in *B* fused with EYFP. Cells were then solubilized with Triton X-100 then treated with DNase I. Chromatin was extracted using 2M ammonium sulfate. The remaining nuclear matrices were fixed with paraformaldehyde and stained with DAPI.

carboxy-terminal twenty amino acid residues (residues 164-183) of the Nkx3.1 homeodomain also may encode an NMTS. These amino acids (164-183) roughly correspond to Helix III of the Nkx3.1 homeodomain. Alternatively, deletion of amino acids 164-183 may distort the Nkx3.1 homeodomain sufficiently such that an NMTS encoded elsewhere is blocked from directing nuclear matrix association. To address these alternatives I made several attempts to prepare an EYFP-fusion protein that carries the carboxy-terminal 20 amino acids of the Nkx3.1 homeodomain. Should this portion of the homeodomain encode an NLS and NMTS, as suggested by experiments presented in Figs. 15A and 22A, then this fusion protein would be expected to be (1) efficiently transported to the nucleus and (2) associated with proteins in this subcellular compartment. Unfortunately, all attempts to amplify this portion of the Nkx3.1 cDNA failed and therefore preparation of this fusion protein was not possible. Additional efforts in the future will be required to address this critical issue.

5.6 Nkx3.1 remains bound to condensed chromatin throughout mitosis

While in the process of analyzing Nkx3.1 nuclear localization and matrix attachment, I had the opportunity to note the subcellular localization of Nkx3.1 in mitotic cells. When the nuclear envelope breaks down in prometaphase soluble proteins from the nucleoplasm join cytoplasmic proteins within the confines of the plasma membrane. During telophase, the nuclear envelopes of each daughter cell are re-established and the process of nuclear importation of karyophilic proteins begins. Swanson and McNeil (1987) showed that larger macromolecules (> 40 kDa) are excluded from condensed mitotic chromosomes and from newly formed, post-mitotic interphase nuclei.

Additionally, a wide variety of transcription factors, such as Oct-1, Ets-1, c-fos, Sp proteins, and Ikaros have been shown to be specifically excluded from mitotic chromatin, and this is consistent with the observation that transcription is blocked in mitotic cells (Segil et al., 1991; Fleischman et al., 1993; Komura and Ono, 2005; He and Davie, 2006; Dovat et al., 2002). Interestingly, factors such as TBP, Hox1.1, GAGA, p300, and UBF appear to remain associated with mitotic DNA, and the mechanisms that determine which proteins are to be excluded remain to be determined (Chen et al., 2002; Schulze et al., 1987; Kellum et al., 1995; Zaidi et al., 2003; Chen et al., 2005). In COS-1 cells expressing an epitope-tagged human Nkx3.1 cDNA, mitotic cells stained with an anti-HA antibody showed that Nkx3.1 remained bound to condensed mitotic chromatin (Figs. 23 A and B). In contrast, parallel cultures of cells transfected with expression constructs encoding various Sp-family members showed that Sp proteins are excluded from mitotic chromatin (data not shown and Fig. 25). Given these data it is tempting to speculate that Nkx3.1 may play a role in chromatin condensation or, organization during mitosis or perhaps function to stabilize chromosomes as they are distributed to daughter cells.

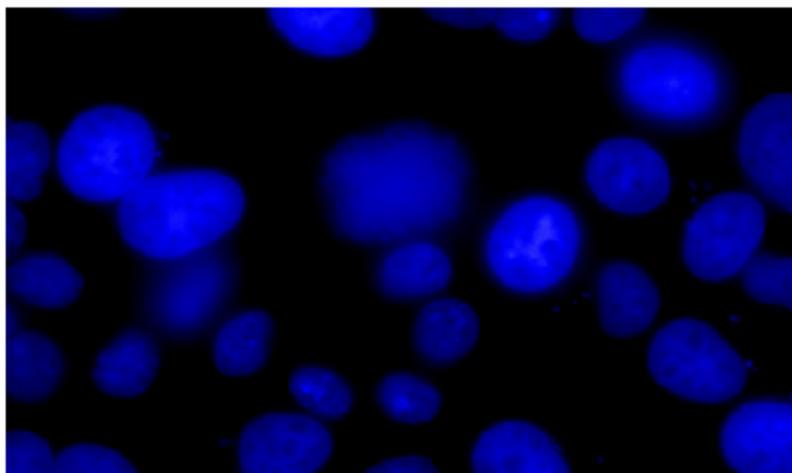
5.7 The Nkx3.1 homeodomain is sufficient for mitotic chromatin inclusion

To confirm the association of Nkx3.1 with mitotic chromosomes an EYFP- fusion protein prepared with the entirety of the Nkx3.1 homeodomain was transiently expressed in COS-1 cells, and the distribution of Nkx3.1 was observed in live mitotic cells. Consistent with the results obtained by indirect immunofluorescence, EYFP-HD was observed to be bound uniformly to condensed mitotic chromosomes. Four representative mitotic cells are depicted in Fig. 26. The top panel presents a prometaphase nucleus and

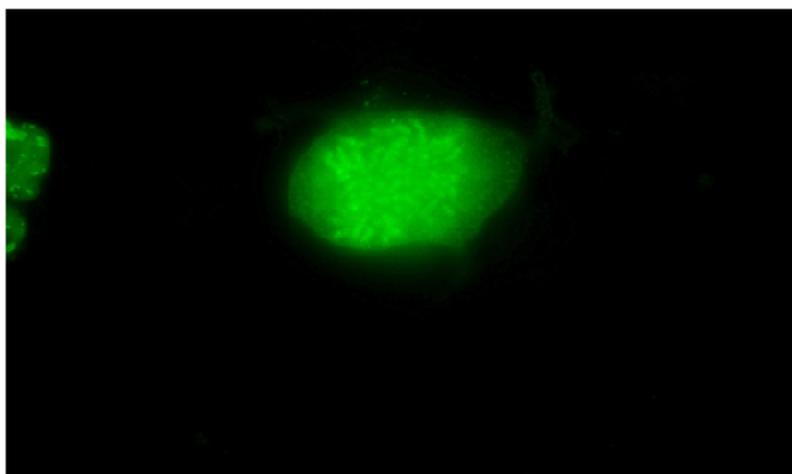
Figure 24. Nkx3.1 remains bound to condensed chromatin throughout mitosis. *A* and *B*, Indirect immunofluorescence analysis of ectopically expressed hNkx3.1 protein. COS-1 cells were transiently transfected with an expression vector encoding hNkx3.1. Nuclei were stained with DAPI and ectopically expressed proteins were detected with an anti-epitope tag (HA) antibody and Alexa Fluor 488 goat anti-mouse secondary antibody.

A

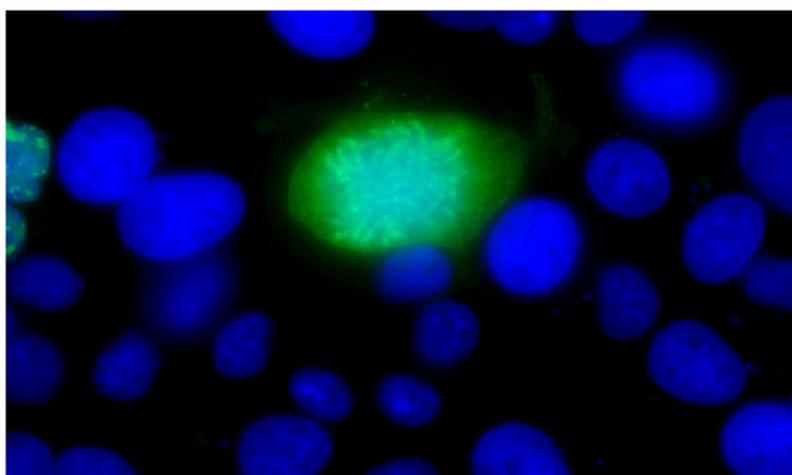
DAPI



Anti-HA

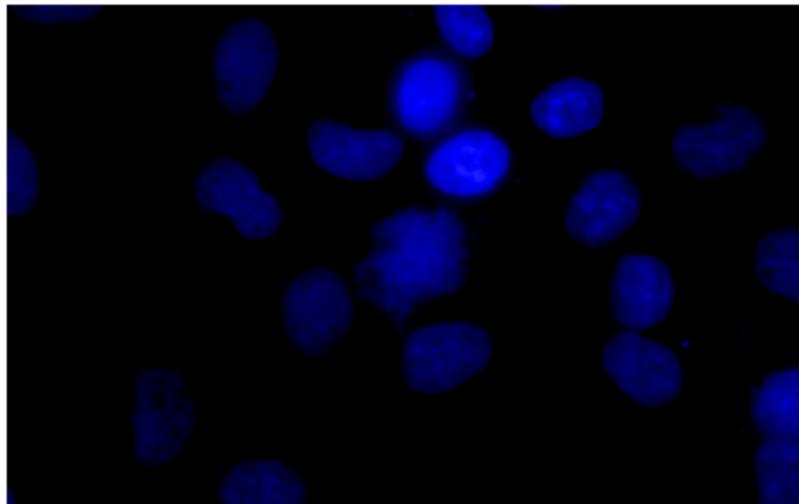


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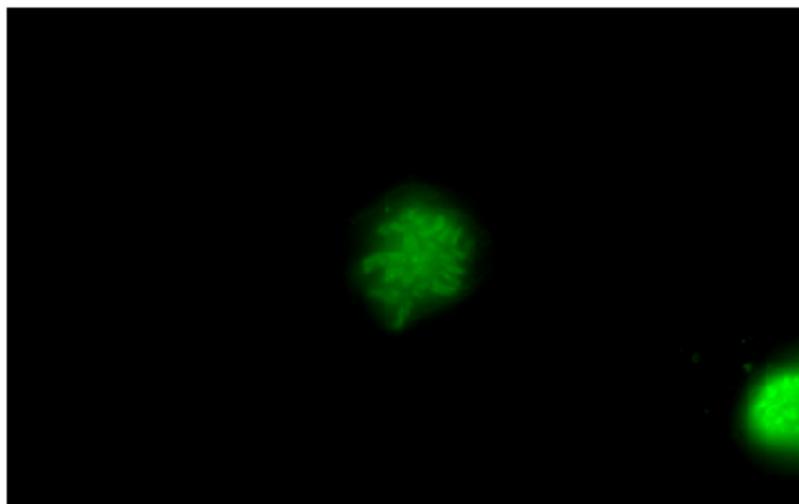


B

DAPI



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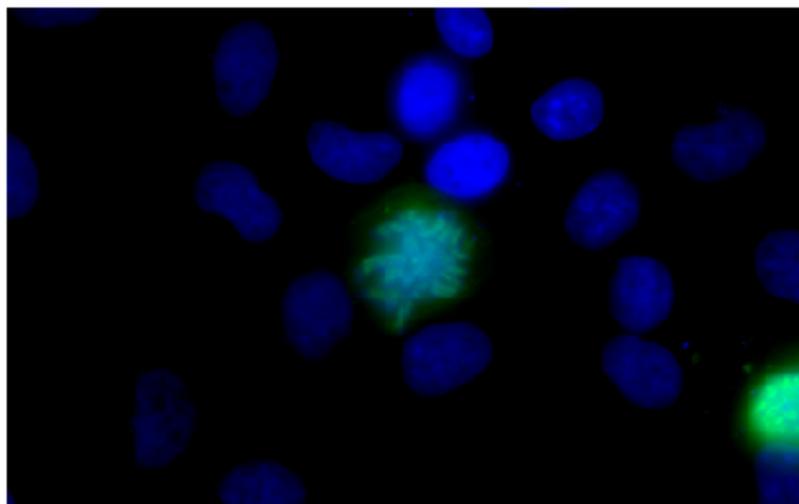
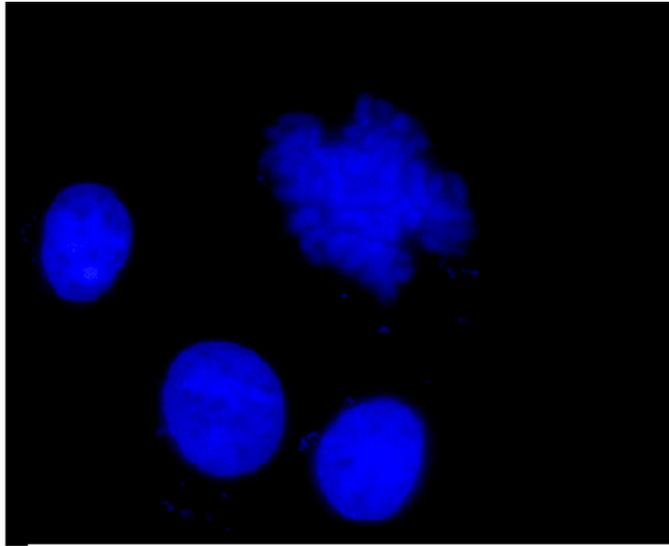
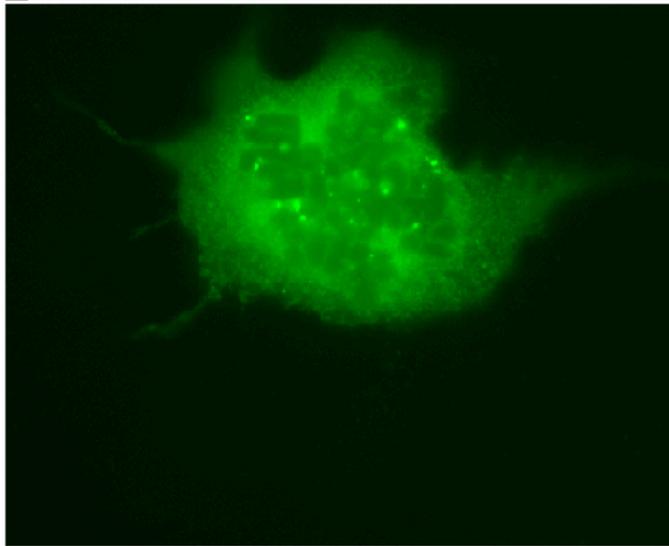


Figure 25. Sp2 is excluded from the nucleus during mitosis. Indirect immunofluorescence analysis of ectopically expressed Sp2 protein. COS-1 cells were transiently transfected with an expression vector encoding Sp2. Nuclei were stained with DAPI and ectopically expressed proteins were detected with an anti-epitope tag (HA) antibody and Alexa Fluor 488 goat anti-mouse secondary antibody.

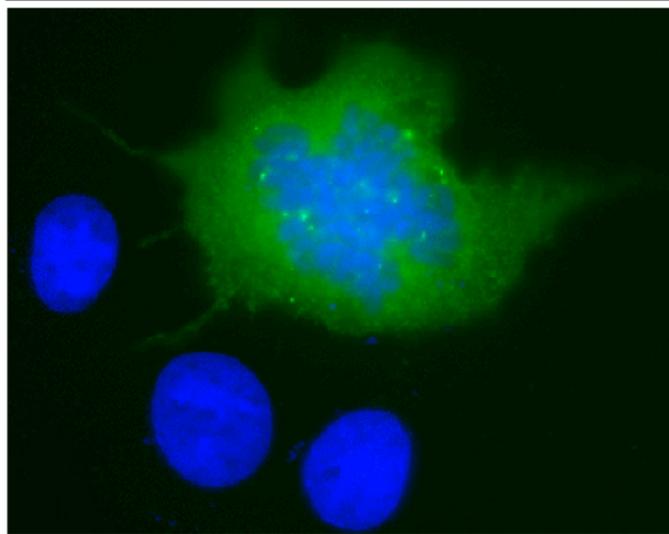
DAPI



Anti-HA



Merge



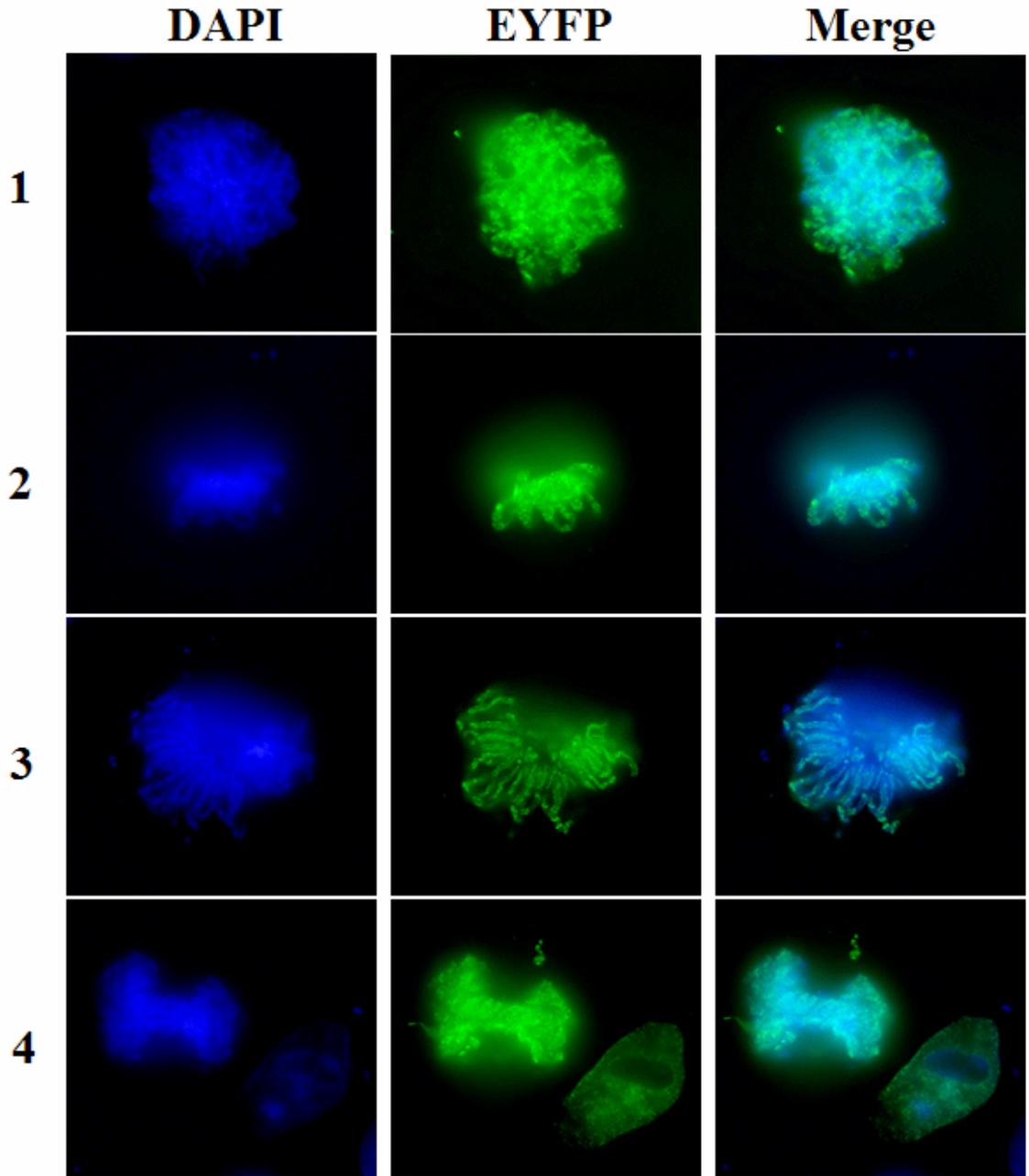


Figure 26. The Nkx3.1 homeodomain is sufficient for mitotic chromatin inclusion. Direct immunofluorescence analysis of ectopically expressed EYFP-hNkx3.1 homeodomain fusion protein (EYFP- HD). COS-1 cells were transiently transfected with an expression vector encoding EYFP- HD. Several mitotic stages are represented: early prophase (1), late prophase (2), metaphase (3), and late anaphase (4).

all chromosomes appear to be stained uniformly at this stage of mitosis. The second panel shows a late prometaphase nucleus, in which the chromatin is further condensed. The third panel reflects a typical metaphase cell and fully condensed chromosomes begin to be aligned along the metaphase plate. The bottom panel shows an early anaphase cell in which sister chromatids have begun to separate to opposite spindle poles. These results would appear to confirm that Nkx3.1 is associated with condensed mitotic chromosomes, and indicates that the homeodomain is sufficient for inclusion in mitotic chromatin. It is also worth mentioning that although it has been widely assumed that Nkx3.1 is a DNA-binding protein in mammalian cells results presented here provide the first experimental evidence that Nkx3.1 binds DNA *in vivo*. Interestingly, similar results were not obtained with an analogous EYFP-fusion protein that carries an Nkx3.1 homeodomain with an amino acid substitution at amino acid 173 (Q173E; data not shown). As mentioned above, this mutation has been shown to block the association of homeodomains with DNA, thus underscoring the need for a functional Nkx3.1 homeodomain to associate with mitotic chromatin.

Results reported in this chapter outline my efforts to characterize the subcellular localization of Nkx3.1. These studies showed that Nkx3.1 is a nuclear protein and contains at least one nuclear localization signal (NLS), likely within the carboxy-terminal 20 amino acids of the homeodomain. I have also showed that Nkx3.1 associates with the nuclear matrix likely via a nuclear matrix targeting sequence (NMTS) that may be coincident with the NLS within Nkx3.1 homeodomain. I showed further that a functionally intact Nkx3.1 homeodomain is not required for nuclear localization but is required for association with the nuclear matrix. In addition to these results I showed that

Nkx3.1 is associated with mitotic chromatin throughout most, if not all, of mitosis and that a functionally intact Nkx3.1 homeodomain is sufficient for inclusion within mitotic chromosome. Further studies will be required to identify the nuclear matrix components bound by the Nkx3.1 homeodomain, and to determine whether matrix-attached Nkx3.1 is transcriptionally active. Additional studies will also be required to delineate what role, if any, Nkx3.1 may play mitosis.

CHAPTER VI
Regulation of Nkx3.1 DNA-binding Activity and a Genome-wide Search for Nkx3.1 Target Genes

In chapter 4, I detailed the use of an *in vitro* DNA-binding assay to determine if the DNA-binding activities of Nkx3.1 and/or Sp-proteins are affected by the formation of Nkx3.1/Sp protein complexes. I concluded that complexes between the Nkx3.1 homeodomain and the zinc-“fingers” of Sp1 and Sp3 did not affect their respective DNA-binding activities. In contrast to these results, Sp2 appeared to neutralize Nkx3.1 DNA-binding activity in parallel assays. To extend these results beyond assays incorporating recombinant proteins, I sought to assess the DNA-binding activity of Nkx3.1 and Nkx3.1-containing protein complexes in prostate-derived cells. Surprisingly, at the time these studies were initiated a search of the literature did not reveal a single report documenting Nkx3.1 DNA-binding activity in prostate cell extracts. My initial goal was to determine whether endogenous Nkx3.1 and Sp proteins could be detected as binary and/or ternary protein/DNA complexes in extracts prepared from prostatic epithelia. The detection of such complexes would corroborate evidence from my *in vitro* DNA-binding assays, and strengthen correlative evidence supporting a functional link between Nkx3.1 and Sp-family members. Ultimately, I also hoped to take advantage of insights gained from such DNA-binding assays to perform a genome-wide screen for Nkx3.1-target genes via a series of chromatin immunoprecipitation (ChIP) experiments.

One of the most difficult challenges in the characterization of Nkx3.1 function has been the scarcity of authenticated target genes. For those few genes that have been suggested to be physiologically relevant targets of Nkx3.1, DNA-binding sites within the promoters of these genes have yet to be defined. Chromatin immunoprecipitation (ChIP) is a powerful technique to map physical interactions between a protein of interest and a given target gene. Such studies can determine precisely which portions of a gene are

bound by a DNA-binding protein, and changes in protein/DNA interactions as a function of cell-cycle progression can also be obtained when ChIP studies are performed in synchronized cell populations. Finally, ChIP analyses offer the opportunity to catalogue the spectrum of sequences targeted by a given protein when assays are broadened to genome-wide screens for putative target genes. In this chapter, I describe my efforts to develop an assay to detect Nkx3.1 DNA-binding activity in extracts prepared from a variety of eukaryotic sources. I also chronicle my efforts to identify novel Nkx3.1 target genes using a ChIP approach in prostate-derived cells.

6.1 The ectopic expression of Nkx3.1 in insect and mammalian cells does not lead to the detection of Nkx3.1 DNA-binding activity in cell extracts

When I began these experiments there were few reports characterizing Nkx3.1 DNA-binding activity. Sciavolino et al. (1997) reported that *in vitro* translated mouse Nkx3.1 protein exhibited NK-like DNA-binding, and Steadman et al. (2000) identified a consensus Nkx3.1 DNA-binding sequence (5'-TAAGTA/G-3') using a bacterially-expressed, maltose-binding protein (MBP)-human Nkx3.1 fusion protein. Finally, Carson et al. (2000) used bacterially-expressed Nkx3.1 protein to determine that Nkx3.1 bound a putative Nkx3.1-binding site within the proximal *SMGA* promoter. To assess the DNA-binding properties of Nkx3.1 within the context of eukaryotic cells, I began by developing an *in vitro* Nkx3.1/DNA-binding assay using Nkx3.1 expressed in Sf9 insect cells following baculovirus infection. Our lab had successfully employed Sf9 cells and baculovirus expression to study the DNA-binding properties of Sp-family members (Kennett et al., 2002; Moorefield et al., 2004) as well as E2F, DP, and Rb-family proteins (Tao et al., 1997), and I anticipated that analogous studies with Nkx3.1 would be equally informative.

To generate a baculovirus stock carrying Nkx3.1, an HA-epitope tagged, full-length mouse Nkx3.1 cDNA was subcloned into a baculovirus transfer vector, and Sf9 cells were co-transfected with the transfer vector as well as linearized baculovirus genomic DNA. Viral supernatants were collected several days later and serially amplified. To determine if this viral stock elicited Nkx3.1 protein in infected cells, non-denatured, whole cell extracts were prepared from infected and uninfected Sf9 cells and analyzed by immunoblotting with a polyclonal anti-Nkx3.1 antibody (T-19). As shown in

Fig. 27, copious amounts of a protein of 36 kDa, the expected apparent molecular weight of Nkx3.1, were detected in infected Sf9 cells. A second Nkx3.1-related protein of slightly lessened mobility (ca. 40 kDa) is also detectable in Fig. 27, and this presumably indicates that at least a fraction of Nkx3.1 is post-translationally modified in Sf9 cells. It is worth noting that Nkx3.1-related proteins of less than 36 kDa were not detected in infected cell extracts, suggesting that Nkx3.1 is relatively stable in Sf9 cells and is not subject to extensive proteolysis. As would be expected, Nkx3.1-related proteins were not detected in uninfected cells extracts. I conclude from these results that the ectopic expression of Nkx3.1 in Sf9 cells leads to the synthesis of abundant amounts of stable, soluble protein.

To determine if baculovirus-expressed Nkx3.1 is competent to bind DNA, non-denatured extracts were prepared from infected Sf9 cells and incubated with radiolabeled oligonucleotide probes carrying either a consensus Nkx3.1 DNA-binding site (Nkx probe) or a mutated Nkx3.1 binding site (Mut probe). Resulting protein/DNA complexes and “free” probes were resolved on non-denaturing polyacrylamide gels and prepared for autoradiography. Bacterially-produced Nkx3.1 was analyzed in parallel as a positive control for these protein/DNA-binding assays. As expected, recombinant Nkx3.1 protein purified from *E. coli* bound a radiolabeled probe carrying a consensus Nkx3.1-binding site, giving rise to a single prominent protein/DNA complex. (Fig. 28, lane 2). A fraction of this complex was “super-shifted” upon inclusion of an anti-Nkx3.1 antibody (lane 3). Binding of bacterially-expressed Nkx3.1 to a radiolabeled probe carrying a mutated Nkx3.1-binding site was almost imperceptible and only apparent upon extended exposure times (compare lanes 2 and 6), underscoring the specificity of these protein/DNA

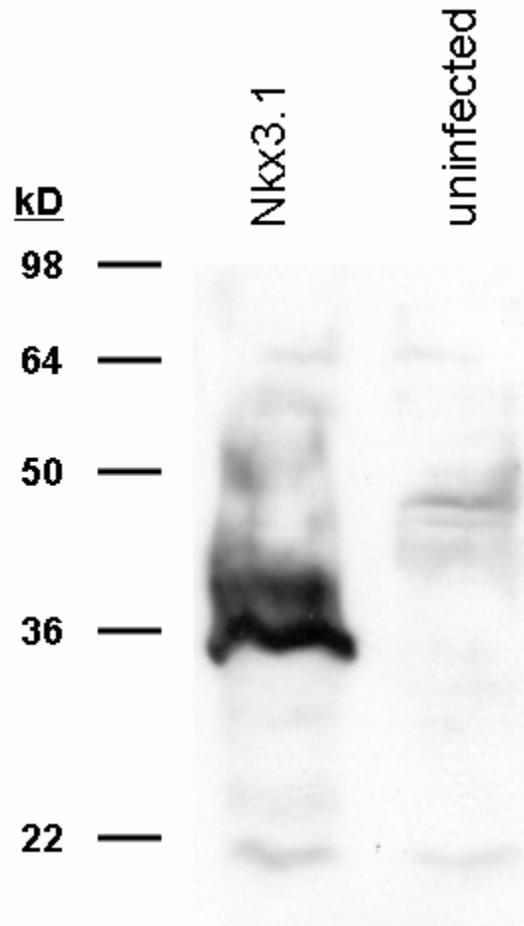


Figure 27. Ectopic Nkx3.1 expression in Sf9 cells. Western immunoblot of mouse Nkx3.1 protein expressed Sf9 insect cells. Non-denatured extracts were prepared from uninfected Sf9 cells (lane 2) and Sf9 cells infected with a recombinant mouse Nkx3.1 baculoviral stock (lane 1). Extracts were boiled in Laemmli sample buffer, resolved by SDS-PAGE, and transferred to nitrocellulose. Filters were incubated with anti-Nkx3.1 polyclonal antibody (T-19) (1:1000) and then with HRP-conjugated anti-goat secondary antibody (1:5000).

interactions. In contrast to these results, Nkx3.1 DNA-binding activity was not detected in extracts prepared from infected Sf9 cells. Indeed, infected cell extracts were as devoid of Nkx3.1 DNA-binding activity as uninfected control extracts (compare lanes 4 and 8).

Given results using extracts prepared from infected Sf9 cells, I reasoned that insect cells may not be a suitable setting for the detection of Nkx3.1 DNA-binding activity. Since Nkx3.1 expressed in *E. coli* bound DNA specifically in parallel assays, one could imagine that post-translational modification of Nkx3.1 or perhaps Nkx3.1-binding proteins in Sf9 cells prevent the formation of Nkx3.1 protein/DNA complexes. Therefore, as a second means to study Nkx3.1 DNA-binding activity in eukaryotic cells I transfected COS-1 cells with mammalian expression vectors carrying an HA-tagged human Nkx3.1 cDNA or an analogous construct carrying an amino acid substitution within the Nkx3.1 homeodomain that is predicted to block DNA-binding activity. Non-denatured whole cell extracts were prepared from COS-1 cells 48 hours post-transfection, and Nkx3.1 expression was verified by immunoblotting using a polyclonal anti-Nkx3.1 antibody (T-19). As shown in Fig. 29, copious amounts of wild-type and mutated Nkx3.1 proteins were detected in extracts prepared from transfected, but not control, cells. Next, transfected and control COS-1 extracts were incubated with radiolabeled oligonucleotide probes carrying consensus or mutated Nkx3.1-binding sites, and resulting protein/DNA complexes were resolved on non-denaturing polyacrylamide gels in parallel with analogous reactions loaded with bacterially-expressed Nkx3.1 protein. As expected, an abundant protein/DNA complex was formed in reactions

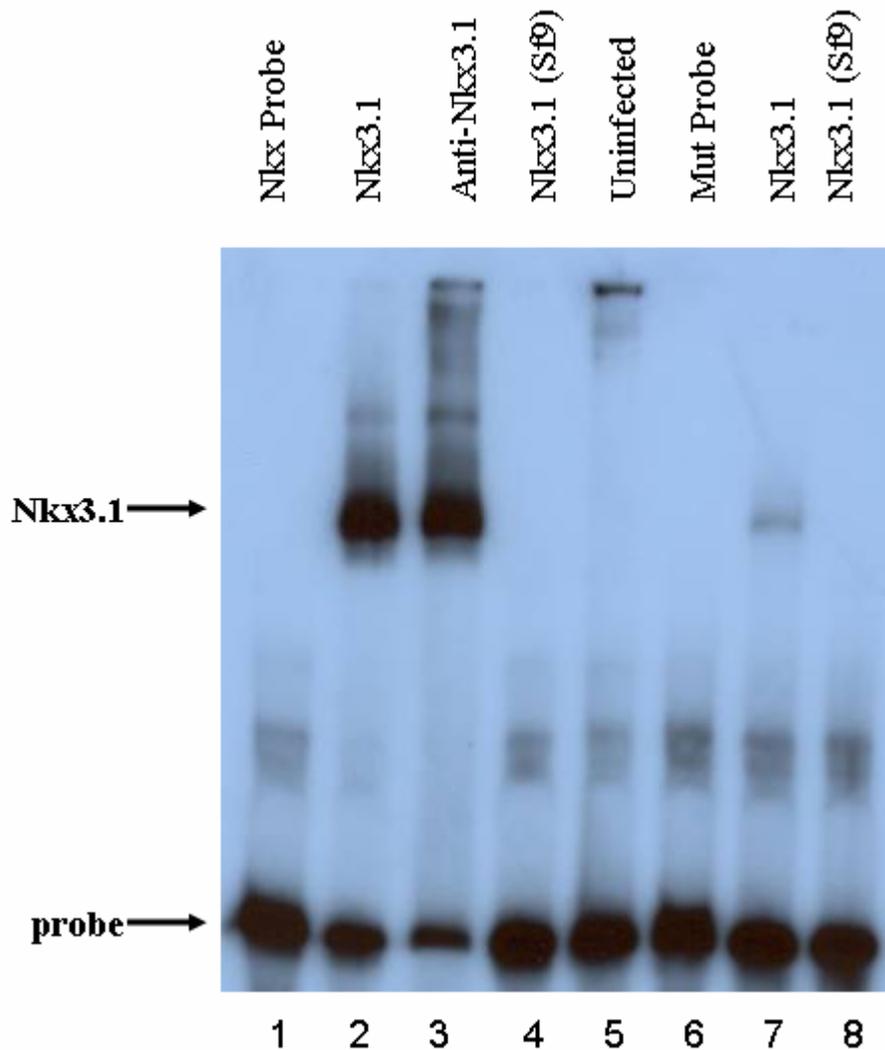


Figure 28. DNA-binding activity of Nkx3.1 expression in Sf9 cells. Non-denatured whole cell extracts were prepared from uninfected Sf9 cells (lane 5) and Sf9 cells infected with a recombinant mouse Nkx3.1 baculoviral stock (lanes 4 and 8). Sf9 extracts or recombinant Nkx3.1 purified from *E. coli* were incubated with a radiolabeled oligonucleotide probe carrying a consensus Nkx3.1 DNA-binding site (Nkx probe; lanes 1-5) or a mutated Nkx3.1 DNA-binding site (Mut probe; lanes 6-8). Protein/DNA complexes were resolved on a non-denaturing polyacrylamide gel and visualized by radiography. Arrows indicated the position of Nkx3.1/DNA complexes and free probe.

containing Nkx3.1 produced in *E. coli* and a consensus Nkx3.1 probe (Fig. 30, lane 2). A similar complex was not detected with a mutated oligonucleotide probe (lane 8). Consistent with results obtained in infected Sf9 cells, wild-type Nkx3.1 expressed in COS-1 cells did not form protein/DNA complexes with either radiolabeled probe (lanes 3 and 9). Similarly, protein/DNA complexes were not detected in COS-1 extracts from cells transfected with the Nkx3.1 homeodomain mutant nor were such complexes apparent in control cell extracts (lanes 5 and 11). Given that wild-type and mutated Nkx3.1 proteins were abundantly expressed in transfected cell extracts, I conclude that Nkx3.1 expressed in COS-1 cells is not competent to bind an oligonucleotide carrying a consensus Nkx3.1 DNA-binding site. It is worth reiterating that these negative results, as well as those obtained with extracts prepared from infected Sf9 cells were performed under experimental conditions that were sufficient to detect Nkx3.1 DNA-binding activity using protein synthesized in *E. coli*.

6.2 Nkx3.1 DNA-binding activity is not detectable in extracts prepared from LNCaP prostate epithelial cells

Considering the results I obtained from Sf9 and COS cells I reasoned that Nkx3.1 might not have exhibited *in vitro* DNA-binding activity because Nkx3.1 may be inhibited by post-translational modification or an inactivating cofactor(s) in these settings. Further, I hypothesized that prostate epithelial cells may represent the only appropriate environment to assess Nkx3.1 function. To address this hypothesis I prepared non-

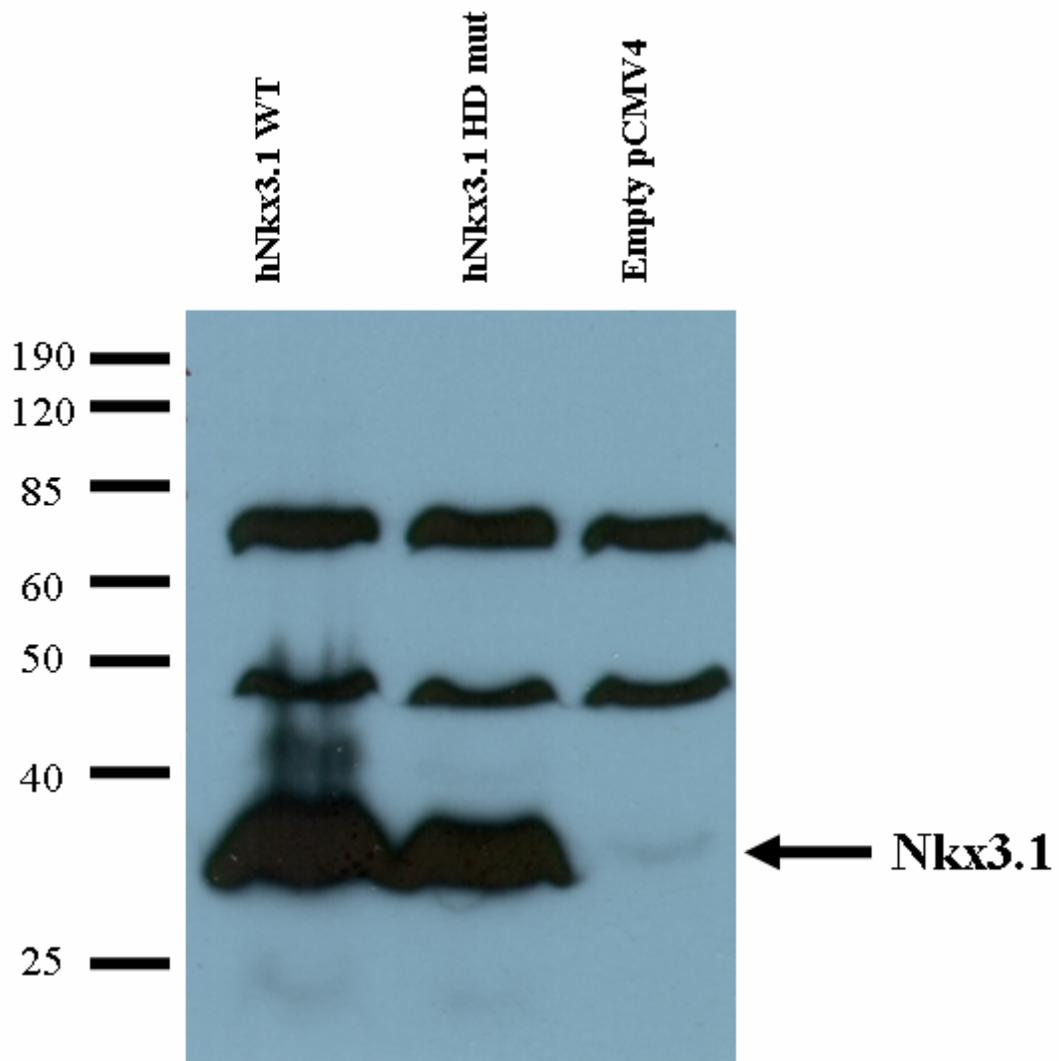
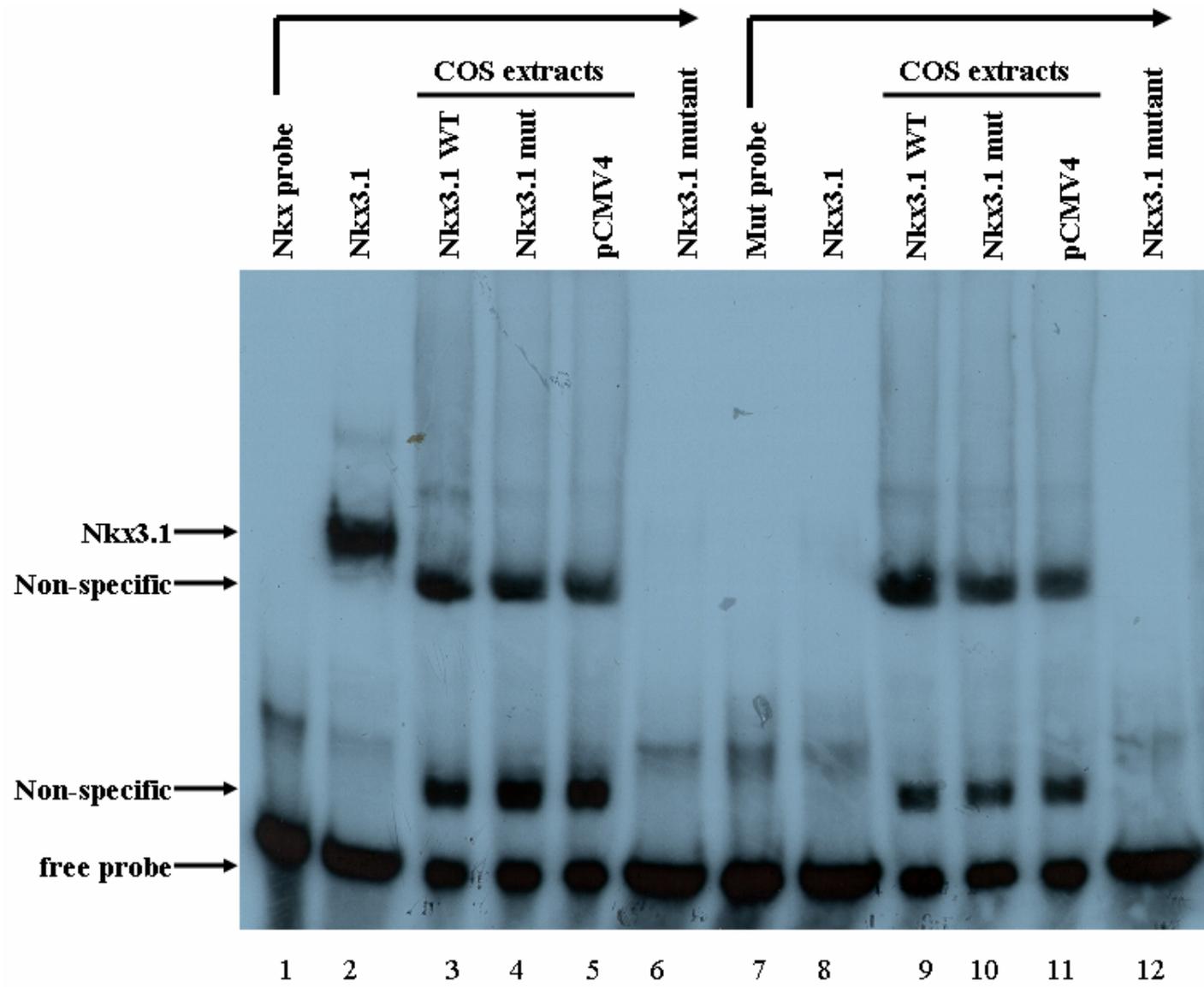


Figure 29. Ectopic Nkx3.1 expression in COS cells. Western immunoblot of human Nkx3.1 protein expressed COS cells. Non-denatured extracts were prepared from COS cells transiently transfected with mammalian expression constructs carrying wild-type human Nkx3.1 (lane 1), a human Nkx3.1 homeodomain point mutant (lane 2), or empty pCMV4 (lane 3). Extracts were boiled in Laemmli sample buffer, resolved by SDS-PAGE, and transferred to nitrocellulose. Filters were incubated with anti-Nkx3.1 polyclonal antibody (T-19) (1:1000) and then with HRP-conjugated anti-goat secondary antibody (1:5000). Arrow indicates the position of Nkx3.1.

Figure 30. DNA-binding activity of Nkx3.1 expression in COS cells. Non-denatured whole cell extracts were prepared from COS cells transiently transfected with mammalian expression constructs carrying wild-type human Nkx3.1 (lanes 3 and 9), a human Nkx3.1 homeodomain point mutant (lanes 4 and 10), or empty pCMV4 (lanes 5 and 11). COS extracts or recombinant wild-type Nkx3.1 (lanes 2 and 8) or a human Nkx3.1 homeodomain point mutant (lanes 6 and 12) purified from *E. coli* were incubated with a radiolabeled oligonucleotide probe carrying a consensus Nkx3.1 DNA-binding site (Nkx probe; lanes 1-6) or a mutated Nkx3.1 DNA-binding site (Mut probe; lanes 7-12). Protein/DNA complexes were resolved on a non-denaturing polyacrylamide gel and visualized by radiography. Arrows indicated the position of Nkx3.1/DNA complexes and free probe.



denatured whole cell extracts from untransfected LNCaP cells. LNCaP is a human prostate adenocarcinoma cell line that expresses endogenous Nkx3.1. Sequence analysis has showed that LNCaP cells carry two wild-type Nkx3.1 alleles (Voeller et al., 1997). LNCaP extracts were incubated with either the Nkx or Mut radiolabeled oligonucleotide probes and resulting protein/DNA complexes were resolved by non-denaturing PAGE. Once again, bacterially-expressed Nkx3.1 was employed as a positive control for these experiments. As expected, an abundant protein/DNA complex was formed in reactions containing Nkx3.1 produced in *E. coli* and a consensus Nkx3.1 probe (Fig. 31, lane 2). In contrast, Fig. 31 shows that a single protein/DNA complex was detected using LNCaP extracts using both the Nkx and Mut probes (lanes 4, 5 and 8). Thus, specific Nkx3.1/DNA-binding activity was not detected in extracts prepared from untransfected LNCaP cells.

Given that loss of Nkx3.1 function correlates with prostate tumor development I considered the possibility that prostate adenocarcinoma cells such as LNCaP might harbor functionally defective Nkx3.1 protein perhaps via the activity of an inhibitory factor that abrogates Nkx3.1 DNA-binding activity. To determine if Nkx3.1 may be bound by such an inhibitory factor I fractionated non-denatured, whole cell LNCaP extracts by size exclusion chromatography. This procedure resulted in thirty-six protein fractions ranging in size from >600kD to <30kD. Alternating fractions were analyzed by immunoblotting with a polyclonal anti-Nkx3.1 antibody (T-19) to determine if Nkx3.1 was detected in high molecular weight complexes. As shown in Fig. 32, Nkx3.1 was primarily detected in fractions 27 through 33, precisely where one would expect to identify a monomeric 36 kDa protein. These results suggested that Nkx3.1 was not

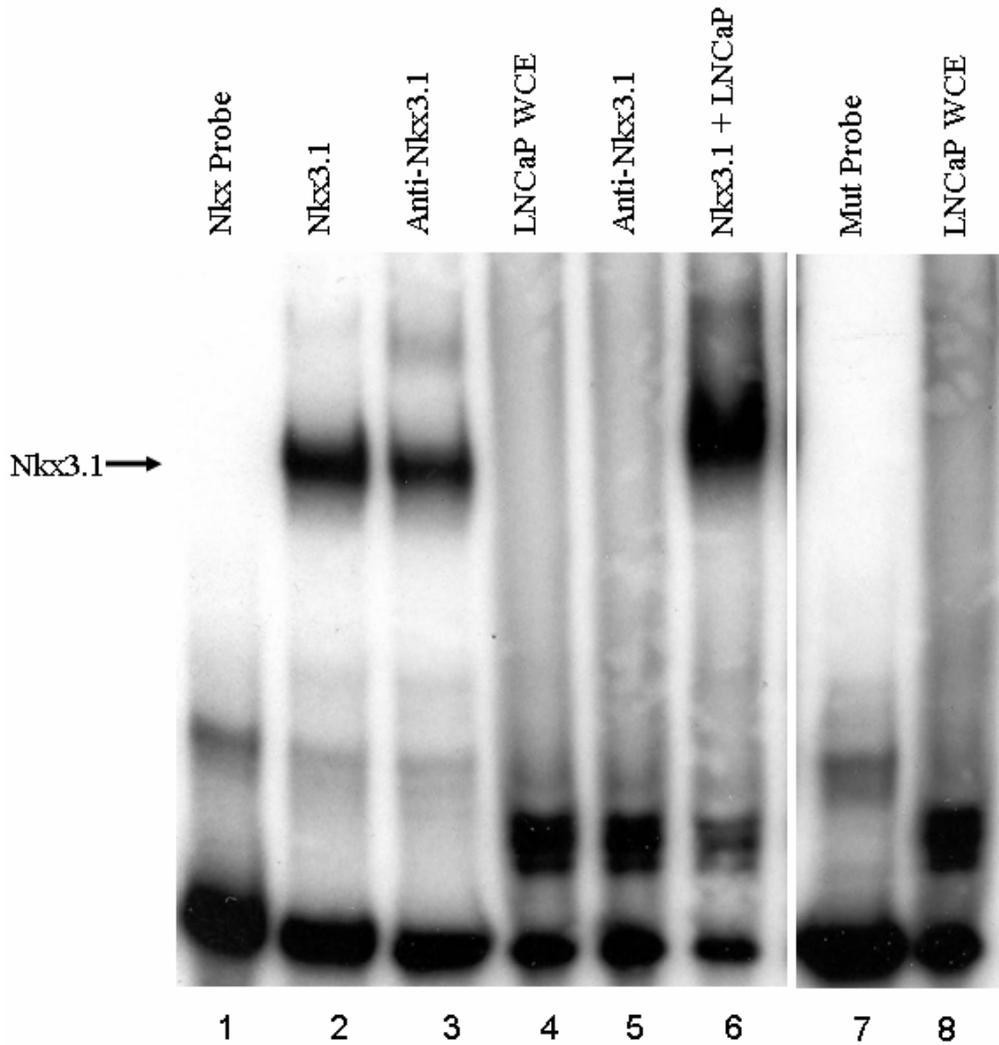


Figure 31. DNA-binding activity of endogenous Nkx3.1 expression in LNCaP cells. Non-denatured whole cell extracts were prepared from untransfected LNCaP cells. LNCaP extracts (lanes 4, 5 and 8) or recombinant wild-type Nkx3.1 (lanes 2 and 3) purified from *E. coli* were incubated with a radiolabeled oligonucleotide probe carrying a consensus Nkx3.1 DNA-binding site (Nkx probe; lanes 1-6) or a mutated Nkx3.1 DNA-binding site (Mut probe; lanes 7 and 8). Protein/DNA complexes were resolved on a non-denaturing polyacrylamide gel and visualized by radiography. Protein/DNA complexes were challenged with a polyclonal anti-Nkx3.1 antibody (T-19) (lanes 3 and 5). Arrows indicated the position of Nkx3.1/DNA complexes and free probe.

associated with a high molecular weight protein complex. I then assayed each fraction for its capacity to bind the Nkx or Mut oligonucleotide probes. Fig. 33 shows that DNA-binding activity was not detected in LNCaP whole cell extracts (A and B, lane 3) or in any LNCaP fraction, including those that contain endogenous Nkx3.1 (A, lanes 4-14, and B lanes 4-10). To determine if LNCaP fractions contain an enzyme that may block Nkx3.1 DNA-binding activity via post-translational modification, I then assayed each fraction for its capacity to impact the DNA-binding activity of Nkx3.1 protein purified from *E. coli*. Briefly, 1 μ g of purified recombinant Nkx3.1 protein was incubated for 20 minutes with a fixed volume of fractioned LNCaP extracts prior to incubation with a radiolabeled Nkx oligonucleotide probe. As shown in Fig. 34, LNCaP fractions did not diminish the DNA-binding activity of recombinant Nkx3.1 protein (A, lanes 4-14 and B, lanes 5-11) although highly concentrated LNCaP whole cell extracts did retard the migration of Nkx3.1 protein/DNA complexes (A, lane 3). I then sought to determine whether this slower migrating complex was the result of a specific Nkx3.1 binding protein within the LNCaP extracts or whether this reflected non-specific protein/protein interactions. As shown in Fig. 34B, a ten-fold dilution of the concentrated LNCaP extract did not retard the migration of Nkx3.1 protein/DNA complexes (compare lanes 3 and 4), suggesting that retardation is due to non-specific binding of “sticky” proteins found in concentrated LNCaP extracts.

Although LNCaP fractions did not affect the abundance of Nkx3.1 protein/DNA complex using recombinant protein as substrate, I wanted to confirm that this was not due to the dilution of LNCaP extracts during the fractionation process. That is, I was concerned that recombinant Nkx3.1 might have been in excess in these protein/DNA-

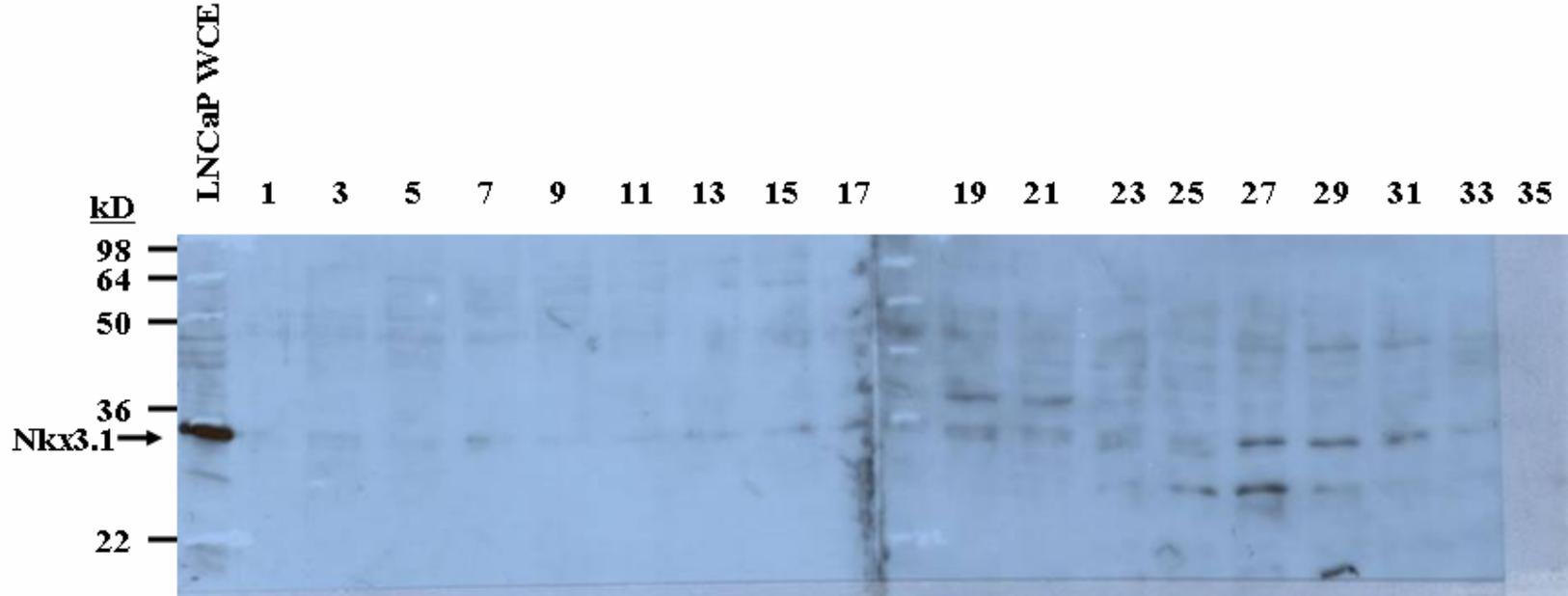
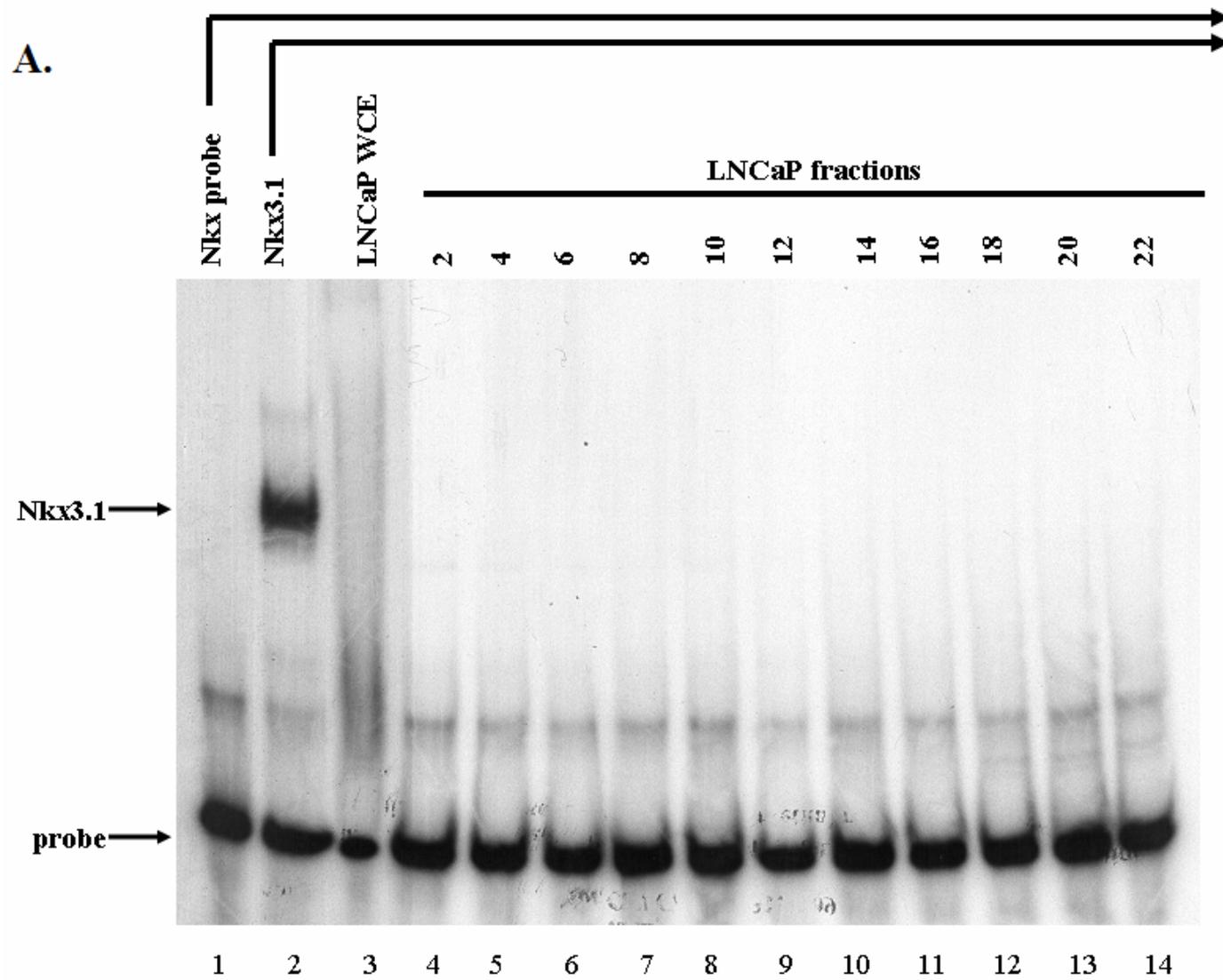


Figure 32. Nkx3.1 is endogenously expressed in LNCaP cells. Western immunoblot of sub-fractionated LNCaP cell extracts. Non-denatured extracts were prepared from untransfected LNCaP cells and were separated by size-exclusion chromatography. 20 μg of input LNCaP extract and 600 μl of each column chromatography fraction (approximately 0.2 $\mu\text{g}/\text{lane}$) were boiled in Laemmli sample buffer, resolved by SDS-PAGE, and transferred to nitrocellulose. Filters were incubated with anti-Nkx3.1 polyclonal antibody (T-19) (1:1000) and then with HRP-conjugated anti-goat secondary antibody (1:5000). Arrow indicates the position of Nkx3.1.

Figure 33. DNA-binding activity of sub-fractionated LNCaP cell extracts. Non-denatured extracts were prepared from untransfected LNCaP cells and were separated by size-exclusion chromatography. Sub-fractionated LNCaP extracts (A, lanes 4-14; B, lanes 4-10), input LNCaP whole cell extract (A and B, lane 3) or recombinant wild-type Nkx3.1 (A and B, lane 2) purified from *E. coli* were incubated with a radiolabeled oligonucleotide probe carrying a consensus Nkx3.1 DNA-binding site (Nkx probe). Protein/DNA complexes were resolved on a non-denaturing polyacrylamide gel and visualized by radiography. Arrows indicated the position of Nkx3.1/DNA complexes and free probe.



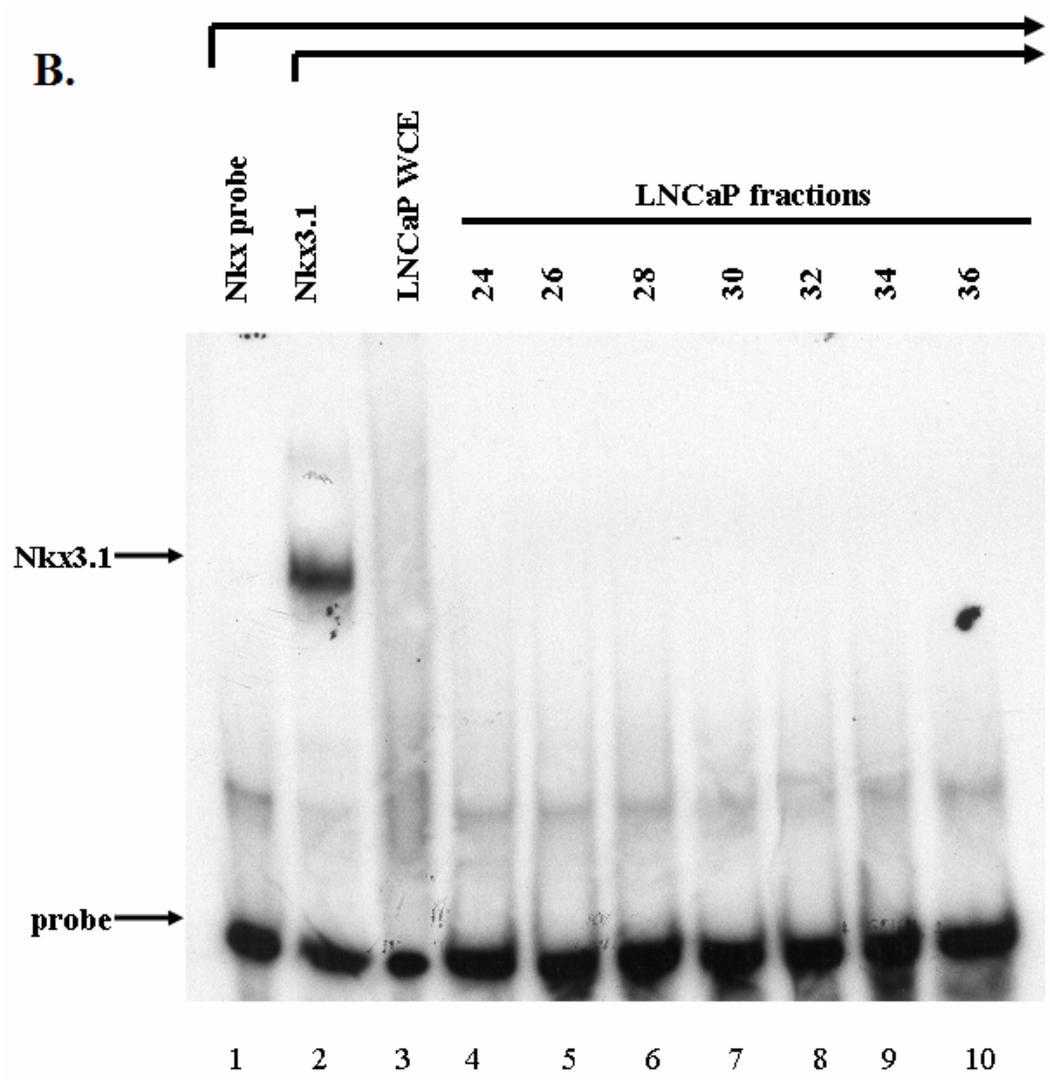
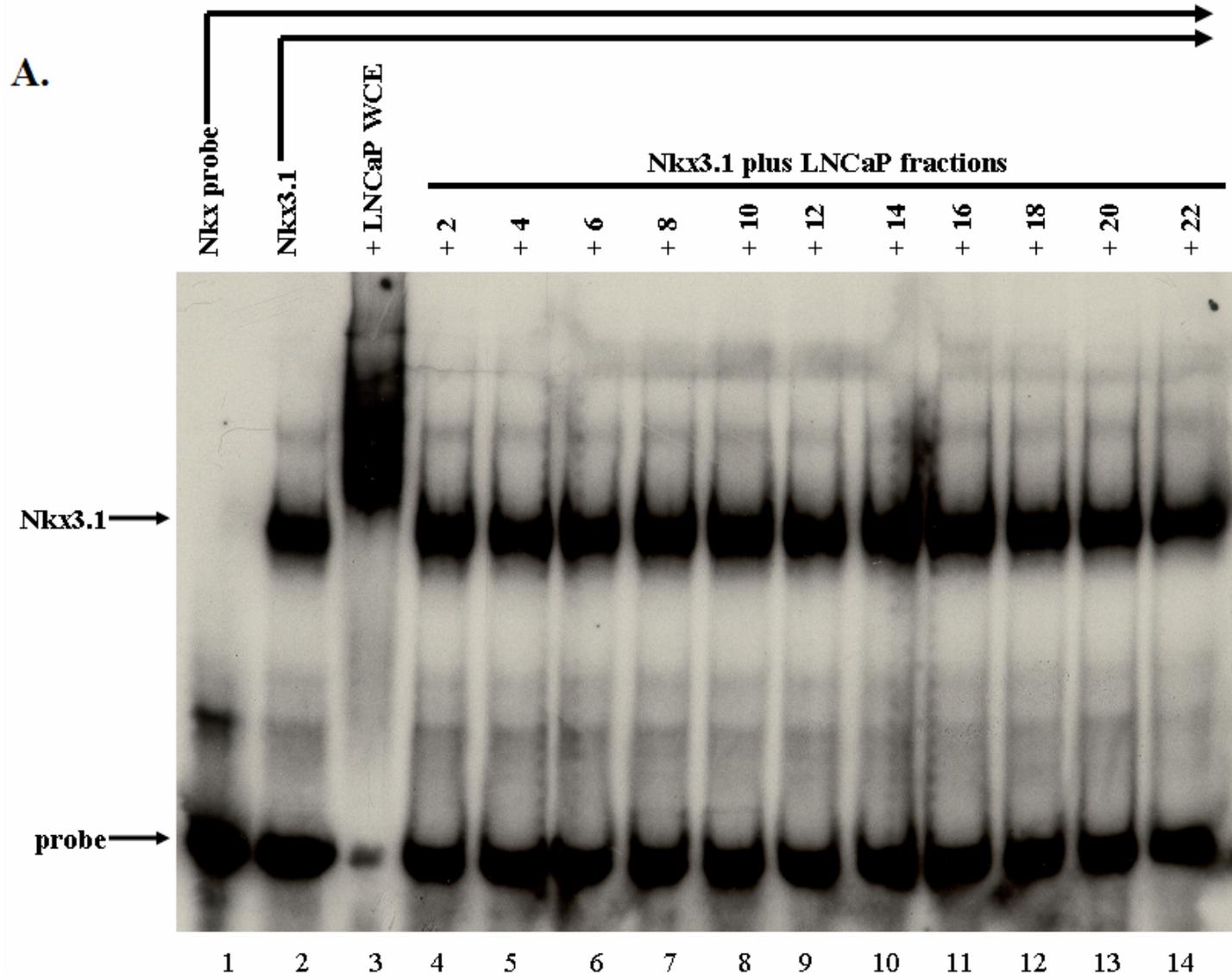
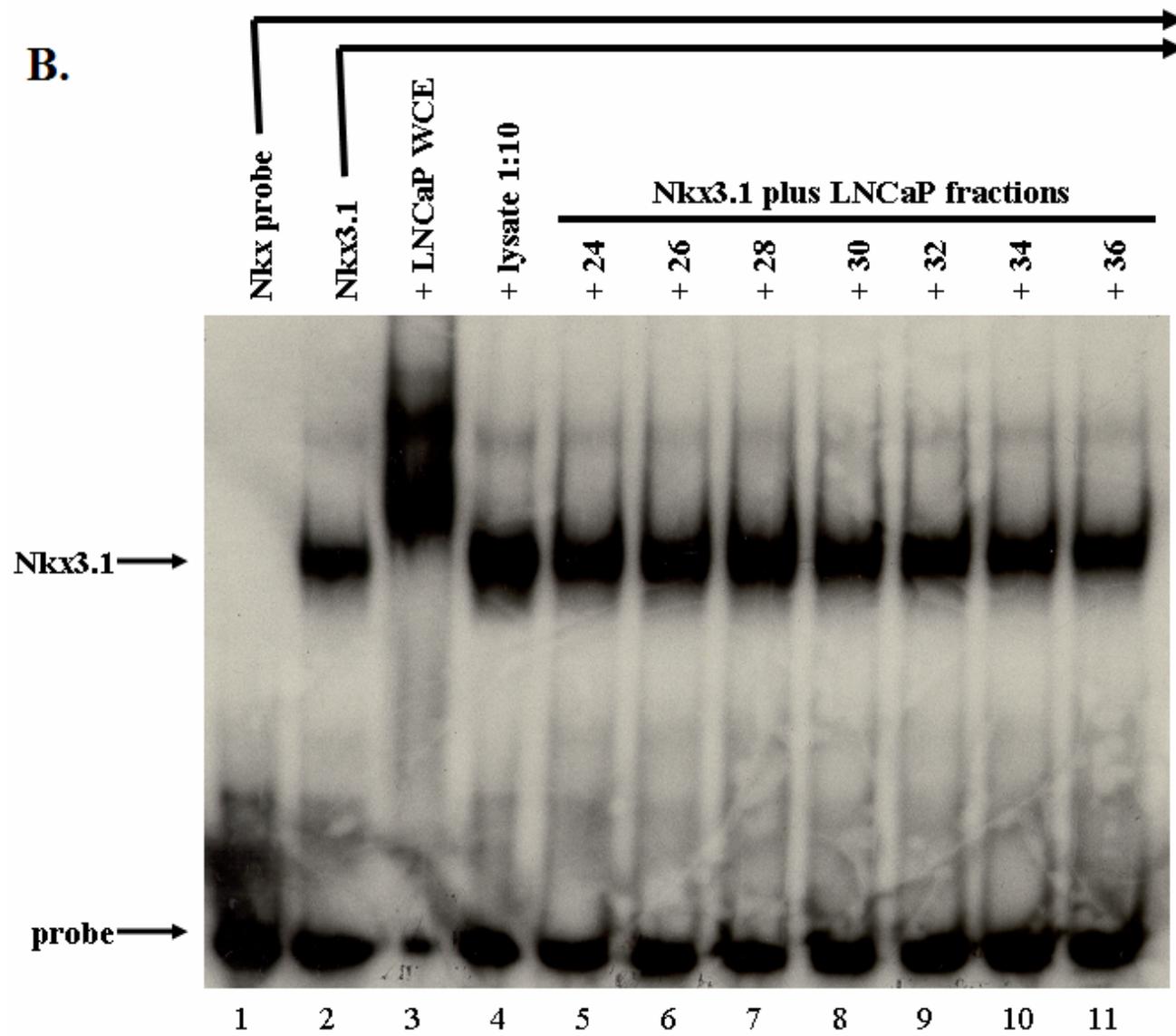


Figure 34. Recombinant Nkx3.1 DNA-binding activity is not perturbed by sub-fractionated LNCaP cell extracts. Non-denatured extracts were prepared from untransfected LNCaP cells and were separated by size-exclusion chromatography. Sub-fractionated LNCaP extracts (A, lanes 4-14; B, lanes 5-11), input LNCaP whole cell extract (A and B, lane 3), input LNCaP whole cell extract diluted 1:10 (B, lane 4) were incubated with 1 μ g recombinant wild-type Nkx3.1 purified from *E. coli* prior to incubation with a radiolabeled oligonucleotide probe carrying a consensus Nkx3.1 DNA-binding site (Nkx probe). Recombinant wild-type Nkx3.1 (A and B, lane 2) was used as a positive DNA-binding control. Protein/DNA complexes were resolved on a non-denaturing polyacrylamide gel and visualized by radiography. Arrows indicated the position of Nkx3.1/DNA complexes and free probe.



B.



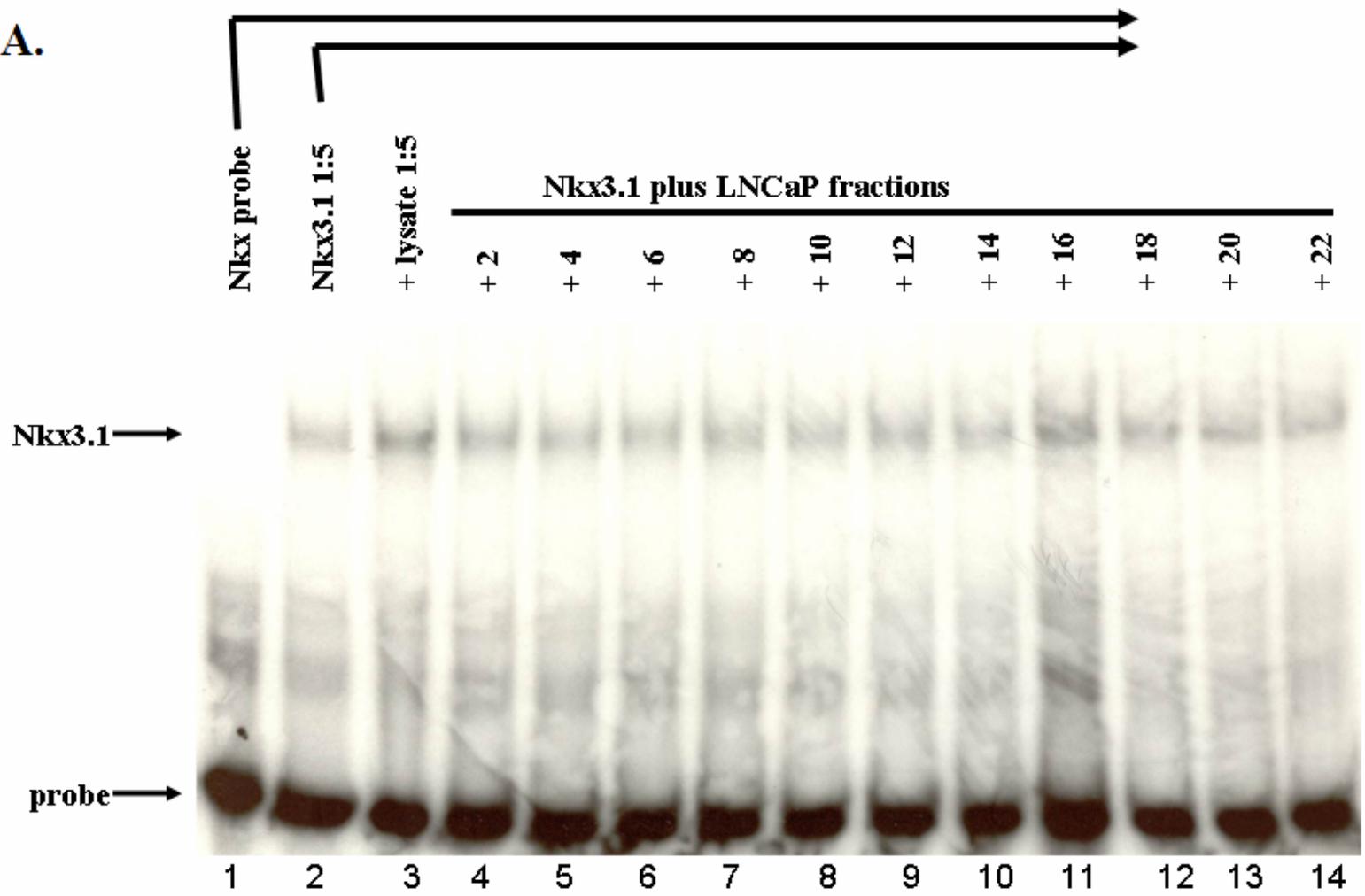
binding assays. To address this issue I conducted another series of Nkx3.1 protein/DNA-binding assays using five-fold less recombinant Nkx3.1 protein (200 ng) with fractionated LNCaP. Again, as shown in Fig. 35, addition of LNCaP extracts did not affect the abundance of Nkx3.1 protein/DNA complexes. I then decided to concentrate a subset of fractionated LNCaP extracts using Centricon™ filters that retained proteins larger than 30 kDa. The resulting concentrated LNCaP fractions were subsequently incubated with 200 ng recombinant control Nkx3.1 protein prior to incubation with the Nkx probe. Once again, these results proved negative (Fig. 36). I conclude from these experiments that Nkx3.1 protein/DNA-binding activity could not be detected within extracts prepared from LNCaP cells under conditions in which recombinant Nkx3.1 purified from *E. coli* was detected.

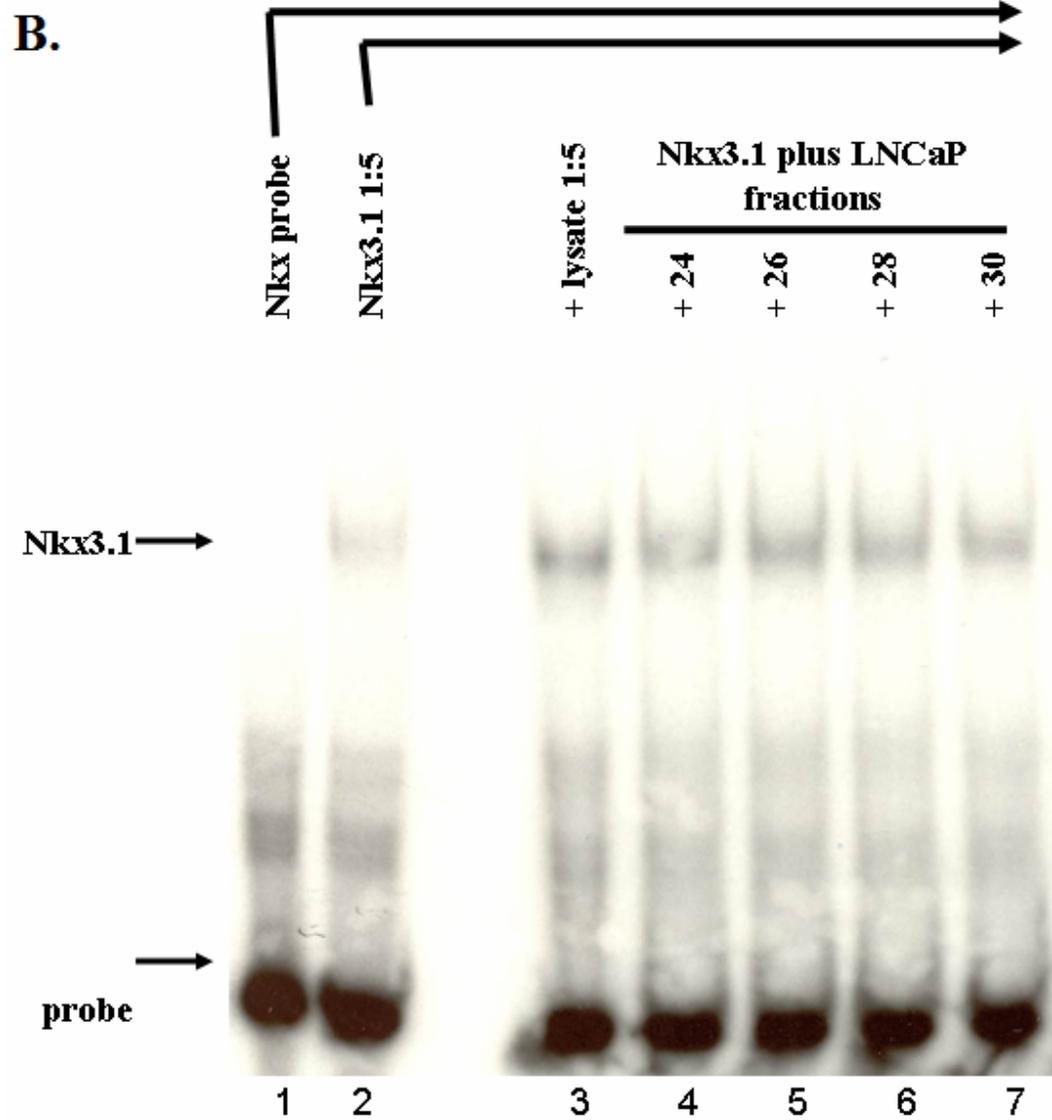
6.3 Mechanisms regulating Nkx3.1 DNA-binding activity *in vivo*

I subsequently proceeded to examine two possible mechanisms by which Nkx3.1 DNA-binding activity might be regulated in LNCaP cells. Given that homeodomain-containing proteins are known to be highly phosphorylated *in vivo* (Krause and Gehring, 1989) and that Nkx3.1 expressed in eukaryotic systems failed to bind an Nkx3.1 consensus sequence *in vitro*, I reasoned that an inhibitory phosphorylation event may be responsible for the absence of Nkx3.1 DNA-binding activity in LNCaP cells. To address this possibility, non-denatured LNCaP whole cell extracts were incubated with either Lambda phosphatase or Antarctic phosphatase prior to incubation with a radiolabeled Nkx probe and subsequent resolution by PAGE. Both Lambda and Antarctic phosphatase are commonly used to dephosphorylate serine, threonine and tyrosine

Figure 35. Recombinant Nkx3.1 DNA-binding activity is not perturbed by sub-fractionated LNCaP cell extracts. Non-denatured extracts were prepared from untransfected LNCaP cells and were separated by size-exclusion chromatography. Sub-fractionated LNCaP extracts (A, lanes 4-14; B, lanes 4-7), input LNCaP whole cell extract (A and B, lane 3), input LNCaP whole cell extract diluted 1:5 (B, lane 3) were incubated with 200 ng recombinant wild-type Nkx3.1 purified from *E. coli* prior to incubation with a radiolabeled oligonucleotide probe carrying a consensus Nkx3.1 DNA-binding site (Nkx probe). Recombinant wild-type Nkx3.1 (A and B, lane 2) was used a positive DNA-binding control. Protein/DNA complexes were resolved on a non-denaturing polyacrylamide gel and visualized by radiography. Arrows indicated the position of Nkx3.1/DNA complexes and free probe.

A.





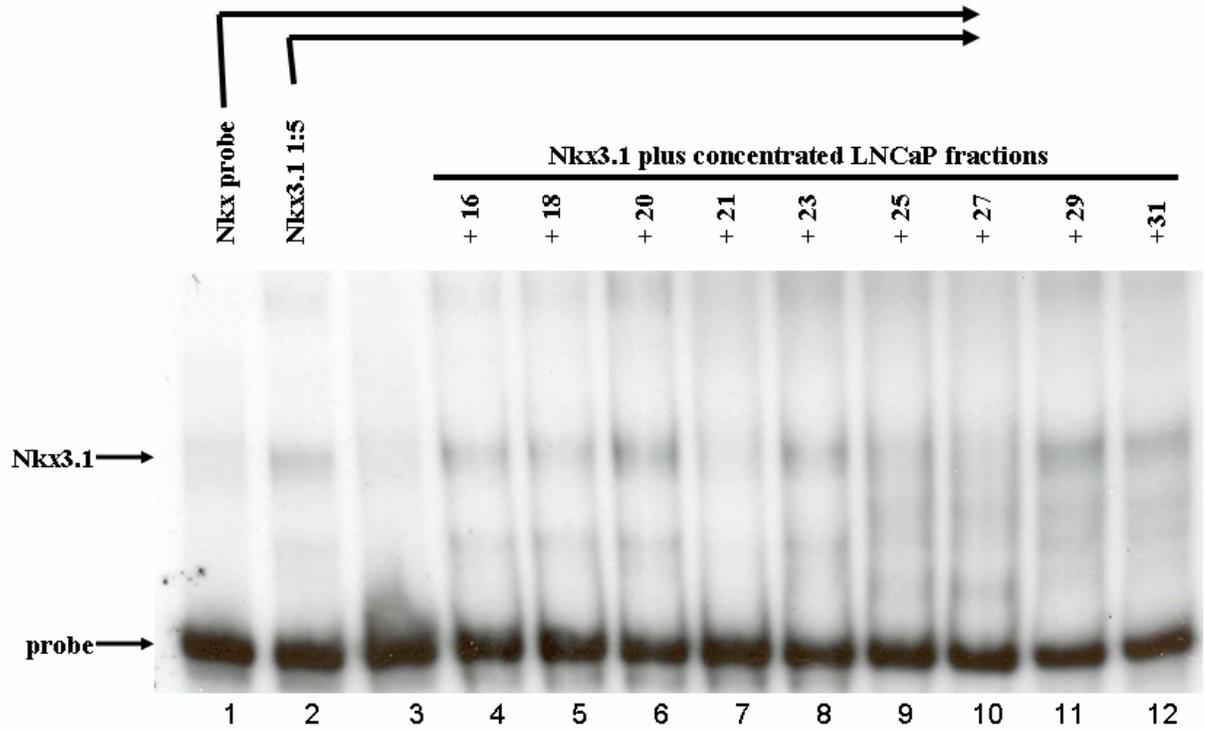


Figure 36. Recombinant Nkx3.1 DNA-binding activity is not perturbed by sub-fractionated LNCaP cell extracts. Non-denatured extracts were prepared from untransfected LNCaP cells and were separated by size-exclusion chromatography. An aliquot of each resulting fraction was concentrated by Centricon centrifugation. Concentrated sub-fractionated LNCaP extracts (lanes 4-12) and input LNCaP whole cell extract diluted 1:5 (lane 3) were incubated with 200 ng recombinant wild-type Nkx3.1 purified from *E. coli* prior to incubation with a radiolabeled oligonucleotide probe carrying a consensus Nkx3.1 DNA-binding site (Nkx probe). Recombinant wild-type Nkx3.1 (lane 2) was used as a positive DNA-binding control. Protein/DNA complexes were resolved on a non-denaturing polyacrylamide gel and visualized by radiography. Arrows indicated the position of Nkx3.1/DNA complexes and free probe.

residues. As shown in Fig. 37, specific Nkx3.1/DNA-binding activity was not detected in extracts prepared from LNCaP cells despite prior treatment with either phosphatase (lanes 5 and 7). Additionally, treatment with either phosphatase did not affect the formation of protein/DNA complexes by recombinant Nkx3.1 (lanes 4, 6, and 8). Thus, my inability to detect Nkx3.1 protein/DNA binding activity does not appear to be due to an inhibitory phosphorylation event.

Hosohata et al. (2003) reported the identification of a novel Nkx3.1-interacting protein (p44) that bound Nkx3.1 in the cytoplasm, but not the nucleus of LNCaP cells. Moreover, p44 expression was shown to be confined to the luminal epithelium of the prostate and correlated with prostate tumor development. These observations led me to speculate that perhaps p44 or other proteins play a role in blocking the DNA-binding activity of Nkx3.1, such that when whole cell extracts are prepared, p44 in the cytosol is mixed with functionally competent nuclear Nkx3.1 and prevents detection of specific Nkx3.1/DNA binding activity using these extracts. I theorized that aberrant regulation of Nkx3.1 by p44 or a similar protein might be a possible mechanism for Nkx3.1 inactivation in LNCaP cells. I used two approaches to address this possibility. First, I made nuclear extracts from COS-1 cells transfected with a mammalian expression vector carrying full-length human Nkx3.1 as well as from untransfected LNCaP cells. One would predict that such nuclear extracts would be free of potential “contamination” by cytosolic p44. These nuclear extracts were then assayed for DNA-binding activity and resolved by non-denaturing PAGE. As expected, Fig. 38 shows that recombinant Nkx3.1 purified from *E. coli* formed a specific complex with the Nkx radiolabeled oligonucleotide probe (lane 2). Yet, Nkx3.1 protein/DNA binding activity was not

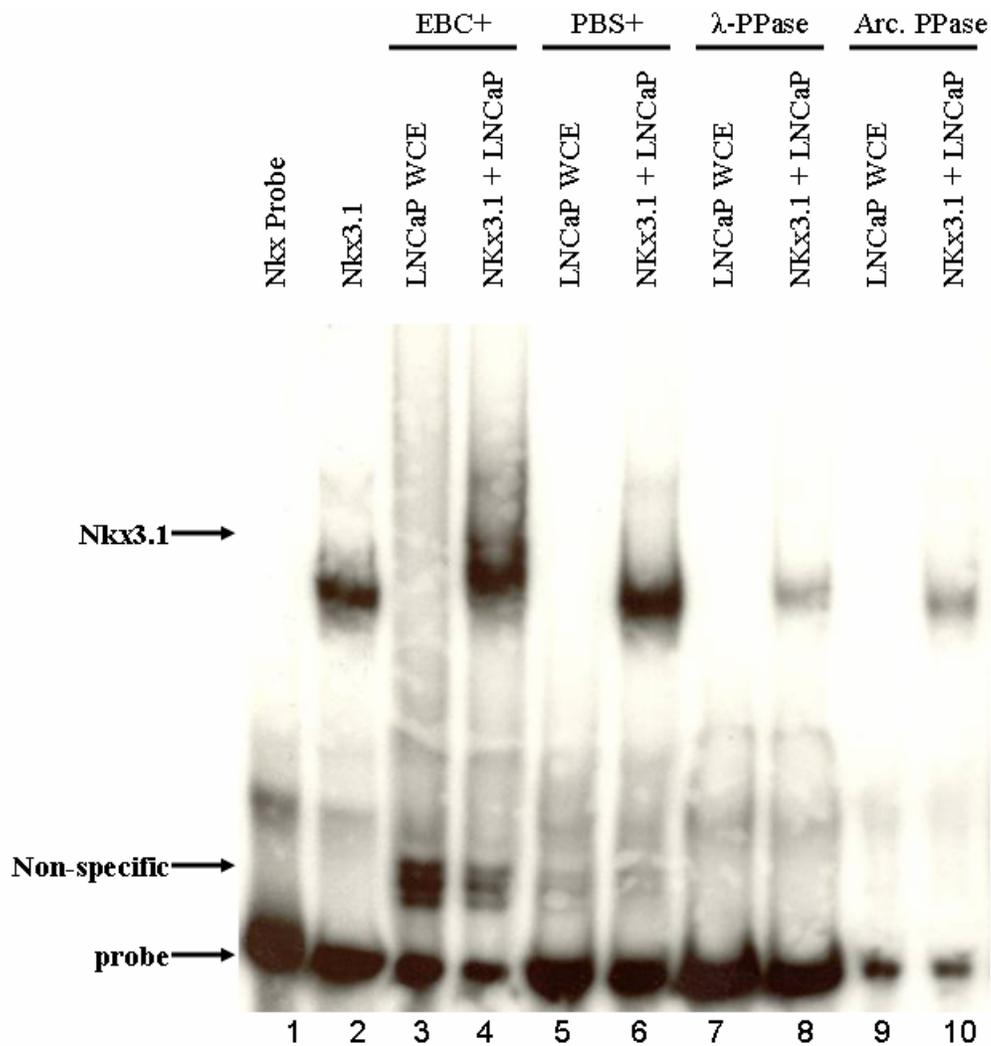


Figure 37. Endogenous Nkx3.1 DNA-binding activity is not activated by phosphatase treatment. Non-denatured extracts were prepared from untransfected LNCaP cells in either EBC+ or PBS+ buffers. LNCaP PBS+ extracts were subsequently treated with either λ -phosphatase (in 1X λ -phosphatase buffer supplemented with 2mM MnCl₂, 30° C, 1 hour) or Antarctic phosphatase (1X Antarctic phosphatase buffer, 37° C, 1 hour). LNCaP EBC+ extract (lane 3), LNCaP PBS+ extract (lane 5), LNCaP extract treated with λ -phosphatase, and LNCaP extract treated with Antarctic phosphatase were incubated with a radiolabeled oligonucleotide probe carrying a consensus Nkx3.1 DNA-binding site (Nkx probe) or with 200 ng recombinant wild-type Nkx3.1 purified from *E. coli* prior to incubation with the Nkx probe (lanes 4, 6, 8, 10, respectively). Recombinant wild-type Nkx3.1 (lane 2) was used as a positive DNA-binding control. Protein/DNA complexes were resolved on a non-denaturing polyacrylamide gel and visualized by radiography. Arrows indicated the position of Nkx3.1/DNA complexes and free probe.

detected with nuclear extracts prepared from transfected COS-1 cells or untransfected LNCaP cells (lanes 5-7; 11-12). To confirm these results I treated LNCaP whole cell extracts with increasing amounts of sodium deoxycholate to disrupt protein-protein interactions that might inhibit Nkx3.1 DNA-binding activity. As shown in Fig. 39, Nkx3.1 protein/DNA complexes were not detected despite prior deoxycholate treatment. Taken together, these results suggest that my failure to detect Nkx3.1 protein/DNA complexes using LNCaP extracts was not due to inhibitory protein-protein interactions. Thus, I was not able to obtain *in vitro* results in support of the conclusion that Nkx3.1 expressed in eukaryotic cells binds DNA *in vivo*. However, work described in Chapter V using fluorescently-tagged Nkx3.1-fusion proteins strongly suggested that Nkx3.1 does bind DNA *in vivo*. Perhaps oligonucleotides may not be suitable reagents to detect Nkx3.1 DNA-binding activity in eukaryotic cell extracts and/or that Nkx3.1 is capable of binding DNA only in the context of chromatin.

Figure 38. Endogenous Nkx3.1 DNA-binding activity is not detected in nuclear extracts *in vitro*. Nuclear extracts were prepared from untransfected LNCaP cells or from COS cells transiently transfected with a mammalian expression construct carrying wild-type human Nkx3.1. LNCaP nuclear extracts (lanes 4, 5, 10), transfected COS nuclear extracts (lanes 6 and 11) or recombinant wild-type Nkx3.1 (lanes 2 and 8) or a human Nkx3.1 homeodomain point mutant (lanes 3 and 9) purified from *E. coli* were incubated with a radiolabeled oligonucleotide probe carrying a consensus Nkx3.1 DNA-binding site (Nkx probe; lanes 1-6) or a mutated Nkx3.1 DNA-binding site (Mut probe; lanes 7-11). Protein/DNA complexes were challenged by a polyclonal anti-Nkx3.1 antibody (T-19) (lane 5). Protein/DNA complexes were resolved on a non-denaturing polyacrylamide gel and visualized by radiography. Arrows indicated the position of Nkx3.1/DNA complexes and free probe.

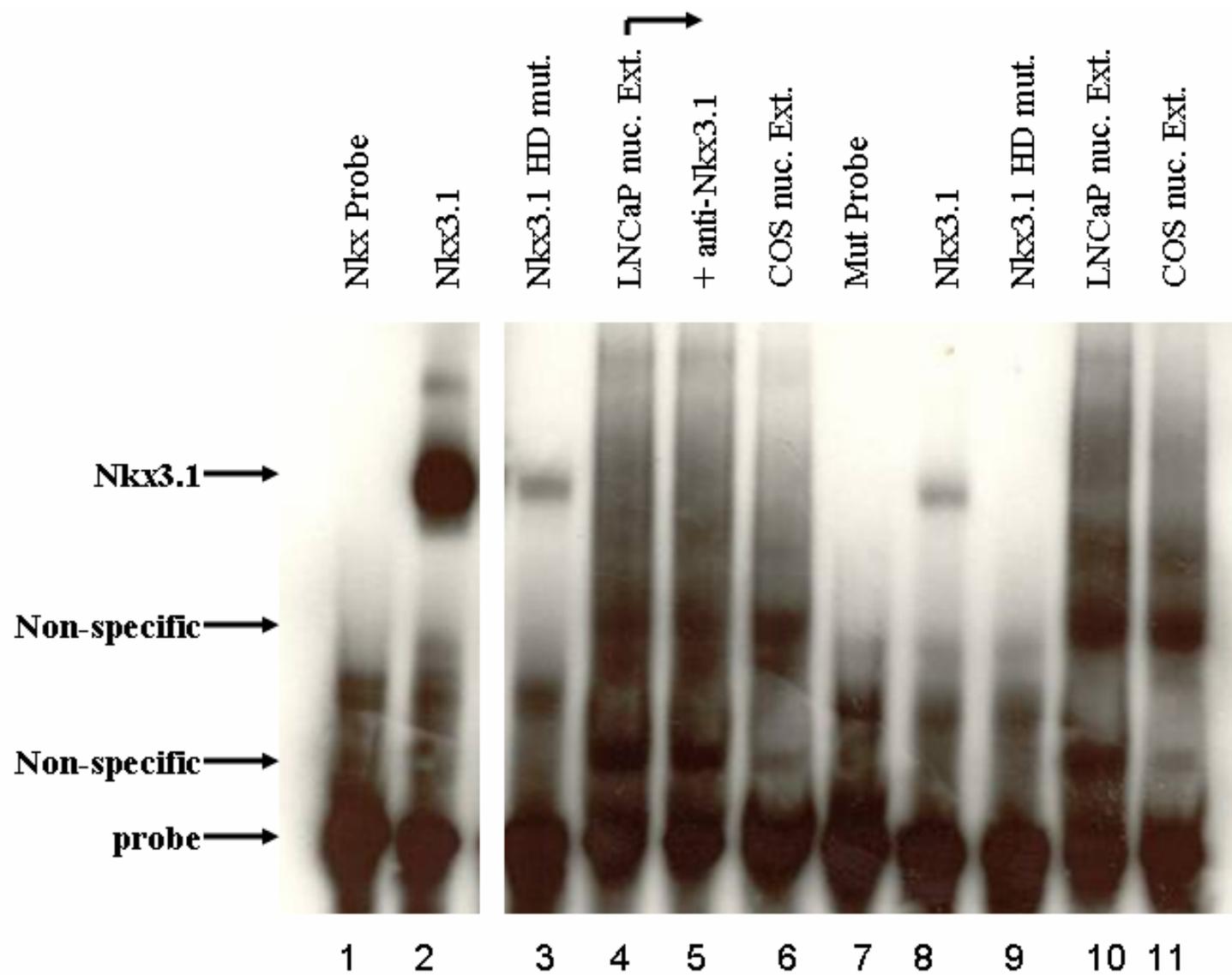
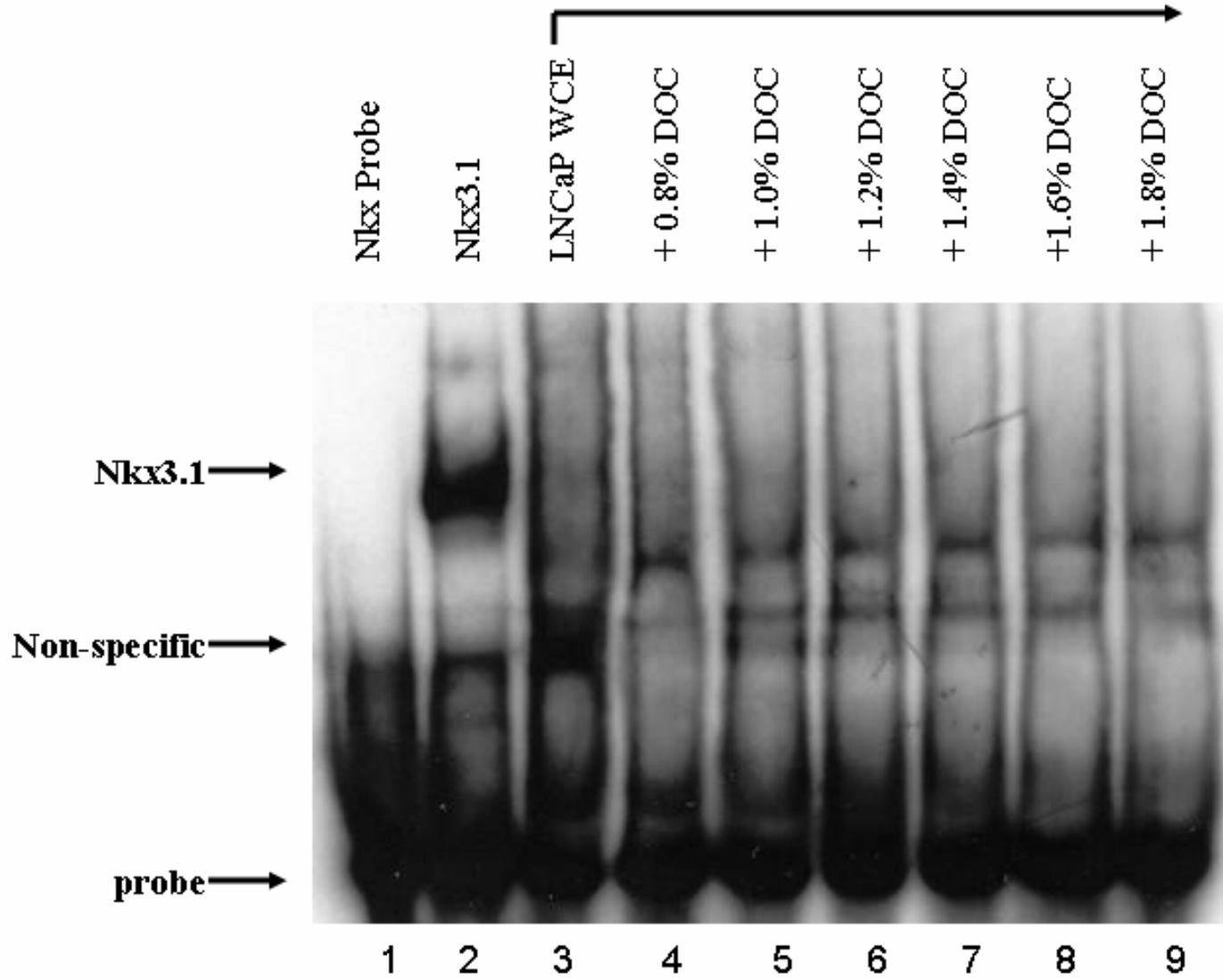


Figure 39. Endogenous Nkx3.1 DNA-binding activity is not activated by deoxycholate treatment. Non-denatured extracts were prepared from untransfected LNCaP cells. LNCaP extracts were subsequently treated with sodium deoxycholate (0.8%-1.8%) for 20 minute on ice. NP-40 was then added to a final concentration of 1.2%. Deoxycholate-treated extracts (lanes 4-9), untreated LNCaP extract (lane 3), or recombinant wild-type Nkx3.1 purified from *E. coli* were incubated with a radiolabeled oligonucleotide probe carrying a consensus Nkx3.1 DNA-binding site (Nkx probe). Protein/DNA complexes were resolved on a non-denaturing polyacrylamide gel and visualized by radiography. Arrows indicated the position of Nkx3.1/DNA complexes and free probe.



6.4 A genome-wide screen for Nkx3.1 target genes

The results I have presented thus far regarding Nkx3.1 DNA-binding activity are seemingly quite contradictory. In two instances Nkx3.1 appeared to be competent to associate with chromatin in mammalian cells. First, the ectopic expression of a wild-type human Nkx3.1 cDNA in COS-1 cells led to the detection of mitotic chromatin that was uniformly stained using an anti-epitope tag antibody and indirect immunofluorescence. Second, an EYFP-Nkx3.1 homeodomain fusion protein was also detected bound to the chromatin of mitotic cells. Since this latter result was obtained in live cells, the apparent association of Nkx3.1 with chromatin was clearly not an artifact of cell fixation or promiscuous antibody staining. One additional result also suggested that the interactions of Nkx3.1 with chromatin are likely to reflect physiologically relevant interactions: the staining of mitotic chromatin by the Nkx3.1 EYFP-homeodomain fusion protein was abrogated by a single amino acid substitution previously shown to destabilize homeodomain/DNA interactions. I was also able to show that Nkx3.1 expressed in *E. coli* was quite competent to bind DNA specifically *in vitro*. Yet, despite many attempts I was unable to detect *in vitro* Nkx3.1 DNA-binding activity using extracts prepared from cells that express endogenous Nkx3.1 or were induced to express Nkx3.1 via viral infection or transfection. Indeed, Nkx3.1 DNA-binding activity was not detected in extracts prepared from cells (COS-1) in which I had observed interactions between Nkx3.1 and mitotic chromatin *in situ*. How might these results be reconciled? One possibility is that Nkx3.1 may rely on interactions with one or more co-factors, perhaps other sequence-specific DNA-binding proteins or general chromatin-binding proteins, to bind DNA avidly. Should this be the case one would predict that Nkx3.1 target genes

might be catalogued via a genome-wide strategy termed chromatin immunoprecipitation (ChIP).

Although ChIP is a very versatile tool, it requires careful optimization of reaction conditions. The basic steps in this technique are fixation of proteins to DNA, sonication of DNA into manageable fragments, immunoprecipitation of protein/DNA complexes with an antibody against the factor of interest, and sequence analysis of resulting immunoprecipitated DNA. Briefly, living cells are treated with formaldehyde, a cross-linking agent that results in the covalent and reversible linkage between genomic DNA and associated proteins. The cross-linked chromatin is extracted after disruption of cell and nuclear membranes and fragmented by sonication. The chromatin associated with a factor of interest is then purified via immunoprecipitation, cross-links are reversed by treatment with glycine, and released genomic DNA is purified to remove contaminating protein and RNA. The ends of resulting DNA fragments are usually polished with T4 DNA polymerase to facilitate ligation of a universal oligonucleotide linker, and all DNA fragments in the sample are amplified via the polymerase chain reaction (PCR). As a control, an aliquot of the input chromatin DNA is processed using an identical procedure. The ChIP-enriched and control DNA can then be used in a variety of ways. Putative target genes may be identified by “shot-gun” cloning and sequencing of immunoselected DNA fragments. A suspected target gene may also be identified via the PCR using target gene-specific primers and immunoselected DNA fragments as template. Another relatively new technique (ChIP on chip) has been developed to quickly identify promoter elements isolated by ChIP analysis. ChIP on chip uses immunoselected DNA fragments labeled with different fluorescent dyes to hybridize onto a single DNA microarray

containing genomic DNA promoter sequences. This technique allows for high-throughput identification of targeted promoter elements.

In an attempt to identify Nkx3.1 target genes in prostate-derived cells, I initiated a series of ChIP studies using Nkx3.1-specific antisera. Only two prostate-derived cell lines, LNCaP and CWR22, express Nkx3.1 underscoring the near universal loss of Nkx3.1 expression in prostate cancers. LNCaP is an androgen-dependent prostate carcinoma cell line derived from a lymph node metastasis. LNCaP cells are somewhat difficult to culture, as they do not attach well to cell culture dishes, grow very slowly relative to many other prostate-derived cell lines, rapidly acidify the medium, and do not become confluent. Despite these difficulties LNCaP cells have been the “gold standard” for endogenous Nkx3.1 studies, therefore I chose this cell line for my ChIP experiments. An absolute requirement for immunoselecting Nkx3.1-DNA complexes isolated from LNCaP cells is an antibody capable of immunoprecipitating human Nkx3.1. For my experiments I chose a commercially available polyclonal anti-Nkx3.1 antibody (T-19). I had shown in previous studies that T-19 immunoprecipitated endogenous human Nkx3.1 efficiently from LNCaP whole cell extracts. I also used two additional polyclonal anti-Nkx3.1 antibodies (N-15 and L-15) that I had shown immunoprecipitated human Nkx3.1 in previous experiments with mixed results.

Despite several attempts I was unable to generate blunt-ended genomic DNA fragments suitable for cloning using T4 DNA polymerase. T4 DNA polymerase is often problematic for generating blunt-ended DNA fragments because it possesses both 5'- to 3'- DNA polymerase activity and 3'- to 5'- exonuclease activity. This exonuclease activity often proves counterproductive, as it can produce 5' overhangs that make blunt-

ended cloning considerably less efficient. As an alternative strategy I used Mung Bean nuclease which possesses both 5'- to 3'- and 3- to 5'- nuclease activity, but does not efficiently digest double-stranded DNA. This alternative strategy resulted in the recovery of blunt-ended fragments that were readily cloned into suitable vectors. As one further modification of the ChIP procedure, I chose not to use a linker-ligation, PCR-amplification approach to enrich immunoselected DNA fragments. Although PCR amplification is supposed to be an unbiased method to enrich ChIP DNA fragments, I was concerned that PCR amplification might bias my genome-wide screen. For example, long genomic fragments or those with high GC content might amplify inefficiently and thus be under-represented in the population of molecules that resulted following immunoprecipitation. As an alternative strategy I chose to use a sensitive cloning technique that would maximize my ability to isolate and identify un-enriched ChIP fragments: I used Sma I-digested pBluescript (pBSK+) to clone immunoselected genomic DNA fragments and identified potential positive clones by screening on bacterial plates containing IPTG and X-gal.

6.5 Characterization of immunoselected Nkx3.1-DNA complexes

Bacterial colonies carrying potential DNA fragments of interest were selected and cultured overnight, plasmid DNA was purified from small cultures, and further screened for inserts by the PCR using oligonucleotide primers that flank the multiple-cloning site of pBSK+. Resulting PCR products were visualized by agarose gel electrophoresis and staining with ethidium bromide. A control PCR reaction using empty pBSK+ as a template yielded a PCR product of about 160 bp. Of the 722 clones resulting from two

ChIP experiments using the T-19 anti-Nkx3.1 antibody and screened by PCR, approximately 94% proved to be false positives. That is, the majority of the bacterial colonies that I analyzed lacked human DNA although each appeared to be a “positive” based on my colorimetric screen. A sample of these false positives was analyzed by DNA sequencing to determine why they did not express β -galactosidase encoded by the uninterrupted pBSK+ multiple cloning site. Sequencing revealed that these false positives appeared due to single nucleotide deletions at the Sma I restriction site, disrupting the β -galactosidase reading frame. Although true “positives” were obtained relatively infrequently (6%; 42 of 772 clones contained novel DNA inserts), each was subsequently sequenced and analyzed (see Table 4). Following automated searches (BLASTN) of the human genome sequence, 36 of 42 “positives” were noted to contain human genomic DNA. Parallel ChIP experiments using the N-15 and L-15 anti-Nkx3.1 antibodies produced even higher rates of false positives (98.6% and 98.2%, respectively), which was not completely unexpected given the decreased efficiency with which these two antibodies precipitate Nkx3.1. Of 641 clones selected with the N-15 antibody, only nine contained novel DNA inserts. Sequence analysis of these clones showed that only two contained identifiable human genomic DNA. Only eight novel inserts were detected by PCR screening of 441 clones selected by the L-15 antibody. Sequence analysis of these clones showed that four of these contained identifiable human genomic DNA. In total, 1804 colonies were screened, producing 59 clones analyzed by DNA sequencing. Forty-two of the 59 clones contained novel human, genomic DNA inserts.

Overall, the ChIP screens yielded human DNA fragments that spring from many portions of the human genome and were classified into two general groups based on the

Table 4
Overall ChIP Results

LNCaP cells were used in four independent chromatin immunoprecipitation (ChIP) assays employing one of three polyclonal anti-Nkx3.1 antibodies: L-15, N-15, or T-19. The number of resulting bacterial colonies that were analyzed for DNA by PCR is indicated for each antibody employed. The number of PCR-“positive” clones that were determined to carry DNA fragments are indicated as well as the subset that were shown by sequence analysis to carry human genomic DNA. PCR-“positive” clones that did not carry human DNA were shown to carry bacterial DNA sequences of unknown origin.

<u>Antibody</u>	<u>Bacterial Colonies Screened by PCR</u>	<u>Number of Clones Carrying DNA Fragments</u>	<u>Number of Clones Carrying Human Genomic DNA</u>
L-15	441	8	4
N-15	641	9	2
T-19	722	42	36
Total	1804	59	42

frequency with which they were isolated. The first group includes sequences that were isolated only once. As shown in Table 5, ten sequences were retrieved from seven autosomes and both sex chromosomes and four of these fragments were recovered with the T-19 antibody. Clone T50 carries a 114 bp human genomic DNA fragment that maps to an intron within the *Neuregulin 1* locus on human chromosome 8p12. The possibility that Nkx3.1 may regulate *Neuregulin 1 in vivo* is intriguing since the *Neuregulin 1* gene product, NDF, is a ligand for the HER2, HER3 and HER4 family of receptor tyrosine kinases. While NDF expression is uniformly high in normal luminal prostate epithelium, NDF expression is significantly absent in prostate cancer specimens, suggesting that functional loss of the NDF/HER ligand/ receptor loop may be an early event associated with prostate tumorigenesis (Lyne et al., 1997).

Clone T71 carries a 500 bp DNA fragment from the psuedoautosomal region of human chromosomes Xp21 and Yp11. Although the psuedoautosomal sequence on the X-chromosome is not located near obvious genes, the Y-chromosomal sequence is located within 60 kb of several putative transcripts (expression sequence tags, ESTs) and is flanked by endogenous retroviral (ERV) insertions. ERV repeat sequences make up an estimated 8% of the human genome and are known to contribute to the regulation of certain human genes, such as *Down syndrome critical region 4 (DSCR4)* and *DSCR8* (Dunn et al., 2006). Although ERV repeat sequences contain strong promoter elements, it is not clear that these ERV repeats regulate genes located on human chromosome Yp11, or if Nkx3.1 plays a role in modulating regulation through ERV repeat sequences.

Two other fragments were selected using the T-19 antibody. The DNA fragment carried by clone T74 shared homology with several regions of the human genome and as

Table 5

Unique Human DNA Sequences Recovered by Chromatin Immunoprecipitation (ChIP)

Ten human genomic DNA fragments that were each isolated once by ChIP. Recovered sequences are identified by a letter that indicates the anti-Nkx3.1 antibody (L-15, N-15, or T-19) that was used to immunoselect that clone. The fragment length is listed in base pairs (bp). The chromosomal location and genomic contig for each clone is also listed. For each clone, nearby genes or informative chromosomal features are also listed where possible. Putative Nkx3.1 DNA-binding sites carried by each ChIP sequence are also indicated.

<u>ChIP Sequence</u>	<u>Fragment Size</u>	<u>Chromosome Location</u>	<u>Genomic Contig</u>	<u>Region or Gene Nearby</u>	<u>Putative Nkx3.1-Binding Sites</u>
L15	174 bp	6q16	AL731777	Cytochrome C pseudogene 90 kbp downstream	5'-TTAGTA-3', 5'-TAAGCA-3'
L91	61 bp	Xp21	AC078957	ChIP fragment is unique DNA flanked by 287 AT-rich and other repeat classes	5'-TAAATA-3'
L263	260 bp	10p13	AL157706	775 bp downstream of an ORF (Q9H098) encoding a conserved protein of 131 amino acids with coiled-coil domains; similar to a protein (O95990) that is down-regulated in renal cell carcinoma	5'-TAATTA-3', 5'-TAAGGA-3', 5'-AAAGTA-3', 5'-TTAGTA-3'
L501	93 bp	18q22	AC090393	ChIP fragment is unique DNA flanked by 285 AT-rich repeats	None
N35	84 bp	2q37	AC019068	70 kbp upstream of hypothetical gene (FLJ22527) and 16 kbp downstream of CpG island; gene is evolutionarily conserved, and predicted protein (AAX93149) has ATPase domain of AAA+ class	None
N412	52 bp	22p13 (near telomere)	AC147648	Flanked by two ESTs and 379 AT-rich repeats	None

		22p13 (near telomere)	AC145613	Flanked by two ESTs and 324 AT-rich repeats	
		16p11 (near centromere)	AC093091	No nearby features	
T50	114 bp	8p12	AF491780	Intron within Neuregulin 1 locus	5'-TAAATA-3', 5'-AAAGTA-3'
T71	80 bp	Xq21 Yp11.25	AL606475 AC012077	No nearby features Flanked by ERV1 repeats as well as 267 others Several ESTs 60 kbp downstream	5'-AAAGTA-3'
T74	39 bp			Homology with several regions; uncertain significance	None
T120	139 bp	10q25	AL157787	No nearby features	5'-TCAGTA-3', 5'-GAAGTA-3'

such a precise chromosomal mapping of this fragment was not possible. This DNA fragment is also extremely short (39 bp), further confounding precise mapping. Clone T120, on the other hand, is 139 bp in length and maps to human chromosome 10q25 but is not located near annotated genes.

Four unique DNA fragments were identified in the L-15 ChIP screen. Clone L263 is a 260 bp fragment that maps to human chromosome 10q13, 775 bp downstream of an open reading frame (Q9H098) encoding a conserved protein of 131 amino acids with coiled-coil domains. A similar protein (O95990) is down-regulated in renal cell carcinoma (Wang et al., 2000; Yamato et al., 1999). Clone L15 maps to human chromosome 6p16, 90 kb downstream of a *cytochrome C* pseudogene. Clone L91 maps to a chromosomal region of Xp21 flanked by nearly 300 AT-rich repeats and other classes of DNA repeats. Clone L501 maps to a region of human chromosome 18q22, which is also flanked by nearly 300 AT-rich repeats. The significance of AT-rich repeats is unclear, but there is some evidence that AT-rich genomic sequences are fragile sites (FRA) for chromosomal deletions and translocations (Finnis et al., 2005). Another role of these AT-rich sequences is genomic organization. AT islands are involved in the organization of genomic DNA on the nuclear matrix by acting as scaffold/matrix attachment regions, S/MARs. DNA duplexes of AT islands are unusually flexible and prone to base unpairing, which are crucial MAR attributes. Various AT islands show high binding affinity for isolated nuclear matrices and associate with the nuclear matrix *in vivo* (Woynarowski, 2004).

Two unique DNA fragments were identified using the N-15 antibody. Clone N35 maps to human chromosomal region 2q37, 70 kb upstream of hypothetical gene

FLJ22527. The predicted FLJ22527 gene product (AAX93149) is a 199 amino acid protein with an AAA+ class ATPase domain. AAA+ class ATPase proteins are a large and functionally diverse group of enzymes that are able to induce conformational changes in a wide range of substrate proteins. The family's defining feature is a structurally conserved ATPase domain that assembles into oligomeric rings and undergoes conformational changes during cycles of nucleotide binding and hydrolysis (Hanson and Whiteheart, 2005). Clone N412 is another relatively short fragment (52 bp) that made precise chromosomal mapping imprecise; this DNA fragment maps to two distinct human chromosomes, 16p11 and 22p13. The region of homology at 16p11 was not near annotated genes whereas the region of homology at 22p13 was flanked by two ESTs and over 300 AT-rich repeats.

Importantly, none of the unique DNA fragments isolated using the T-19, L-15 and N-15 antibodies carried a consensus Nkx3.1 DNA-binding site. Four of these ten DNA fragments lacked recognizable Nkx3.1-like sequences and only two (L15 and L263) carried a minimal 5'-TAAG-3' core sequence identified as the consensus Nkx3.1 DNA-binding core *in vitro* (Steadman et al., 2000). While it is possible that these fragments are merely artifacts resulting from non-specific Nkx3.1 protein/DNA interactions, there are a number of possibilities that might explain why these fragments represent actual Nkx3.1 targets. One possibility is that Nkx3.1 binds a slightly different DNA sequence *in vivo* than *in vitro*. For example, Sciavolino et al. (1997) demonstrated that Nkx3.1 binds both the NK-2 5'-CAAG-3' core and the Msx1 5'-TAAT-3' core *in vitro* and thus it is possible that Nkx3.1 DNA-binding activity extends to a wider variety of sequences than might be expected. Another possibility is that Nkx3.1 interacts with DNA as part of a

complex with other DNA-binding proteins. Although DNA binding would be expected to be mediated by the Nkx3.1 homeodomain, interactions with other transcription factors may play a role in the selection and regulation of target genes and it is conceivable that such interactions significantly alter the Nkx3.1 consensus DNA-binding sequence (Nasiadka et al., 2000). Finally, it is possible that the isolated DNA fragments were obtained via interactions of Nkx3.1 with DNA that do not involve the homeodomain. That is, Nkx3.1 may have been tethered to DNA via an as yet unidentified DNA-binding protein.

The second group of immunoselected genomic DNA fragments were those isolated repeatedly, and which I have termed “repetitive” sequences. Such repetitive sequences constitute the majority of DNA fragments identified by ChIP and were collected only with the T-19 antibody. Fig. 40 shows an alignment of the 32 repetitive sequences identified by ChIP. After aligning the repetitive sequences, it became clear that the 32 repetitive sequences could be classified into three groups. Sixteen repetitive sequences were termed Group I repeats (Fig. 41). Most Group I repeats featured a perfect Nkx3.1 consensus DNA-binding site (5'-TAAGTA-3') flanked on either side by five thymidine residues. The significance of the poly-dT tracts that flank the Nkx3.1 DNA-binding site is not obvious; however, poly-dT tracts “flatten” the DNA helix such that it exhibits less intrinsic bending (Hagerman and Hagerman, 1996; Suter et al., 2000). The precise chromosomal locations of the Group I fragments were difficult to determine given their relatively short (56-61 bp) length. A BLASTN search utilizing the Group I sequences produced 118 results amongst human genomic sequences. The core 5'-

Figure 40. Alignment ChIP sequences isolated repeatedly. Thirty-two genomic DNA fragments immunoselected by ChIP using the T-19 antibody are aligned with common nucleotides highlighted in blue and yellow. Three primary sequence patterns emerge from an alignment of these sequences (shown in Figs.40-42). Sequences are identified by their clone designations and are indicated on the left.

	(1)	1	10	20	30	40	50	60	70	80	90	100	119		
T107	(1)	-----	-----	-----	-----	-----	AGACG	CGATCCTAT	CCCG	---ATTTT	TTAAGTAGTTTT	TCAG	---TAGCTATCTGCAGGCGT	GGGCT	
T429	(1)	-----	-----	-----	-----	-----	AGACGG	ATCCTATG	CG	CGATT	TTTTAAGTAGTTTT	TCAG	---TAGCTATCTGCAGGCGT	-----	
T48	(1)	-----	-----	-----	-----	-----	-----	TTGAG	CGGGATATT	GCTCTGTT	GCCAGTC	---TTGG	---AGTGCAGTGGCC	---	
T494	(1)	-----	-----	-----	-----	-----	AGACGG	ATCCTATG	CG	CGATT	TTTTAAGTAGTTTT	TCAG	---TAGCTATCTGC	GGCGT	
T501	(1)	-----	-----	-----	-----	-----	-----	TGTACT	TCGAGTATA	AAGTATATA	TATAGTGGG	---CGGGACT	TAAGGATCCGGC	---	
T534	(1)	TGGTGGCATGTGCCTGTAGTCTCAGCTACTCAGGAGGCTGAGGCAGGGGAATCG	TTG	ACC	CAGGA	GGTGC	AGGTTT	C	AGTGAG	CCGAGATT	GCACCA	CTCGC	CTCACCTGC	---	
T536	(1)	-----	-----	-----	-----	-----	AGACGG	ATCCTATG	ACGAC	GATTTTTT	TAAGT	AGTTTT	TCAG	---TA	
T544	(1)	-----	-----	-----	-----	-----	-----	GTA	CTCGAGTATA	AAGTATATA	TATAGTGGG	---CGGGACT	TAAGGATCCGGC	---	
T547	(1)	-----	-----	-----	-----	-----	AGACGG	ATCCTATG	ACGAC	GATTTTTT	CCACA	GTTTT	TCGC	---TAGCTATCTGCAGGCGT	
T554	(1)	-----	-----	-----	-----	-----	-----	GTA	CTCGAGTATA	AAGTATATA	TATAGTGGG	---CGGGACT	TAAGGATCCGGC	---	
T573	(1)	-----	-----	-----	-----	-----	-----	GTA	CTCGAGTATA	AAGTATATA	TATAGTGGG	---CGGGACT	TAAGGATCCGGC	---	
T584	(1)	-----	-----	-----	-----	-----	-----	GTA	CTCGAGTATA	AAGTATATA	TATAGTGGG	---CGGGACT	TAAGGATCCGGC	---	
T588	(1)	-----	-----	-----	-----	-----	AGACGG	ATCCTATG	CG	CGATT	TTTTAAGTAGTTTT	TCAG	---TAGCTATCTGC	GGCGT	
T592	(1)	-----	-----	-----	-----	-----	-----	GTA	CTCGAGTATA	AAGTATATA	TATAGTGGG	---CGGGACT	TAAGGATCCGGC	---	
T596	(1)	-----	-----	-----	-----	-----	AGACGG	ATCCTATG	CG	CGATT	TTTTAAGTAGTTTT	TCAG	---TAGCTATCTGC	GGCGT	
T603	(1)	-----	-----	-----	-----	-----	-----	GTA	CTCGAGTATA	AAGTATATA	TATAGTGGG	---CGGGACT	TAAGGATCCGGC	---	
T606	(1)	-----	-----	-----	-----	-----	AGACGG	ATCCTATG	ACGAC	GATTTTTT	TAAGT	AGTTTT	TCAG	---TA	
T609	(1)	-----	-----	-----	-----	-----	-----	GTA	CTCGAGTATA	AAGTATATA	TATAGTGGG	---CGGGACT	TAAGGATCCGGC	---	
T611	(1)	-----	-----	-----	-----	-----	AGACGG	ATCCTATG	CG	CGATT	TTTTCCACA	GTTTT	TCAG	---TAGCTATCTGC	
T615	(1)	-----	-----	-----	-----	-----	AGACGG	ATCCTATG	CG	CGATT	TTTTAAGTAGTTTT	TCAG	---TAGCTATCTGC	GGCGT	
T616	(1)	-----	-----	-----	-----	-----	AGACGG	ATCCTATG	CG	CGATT	TTTTAAGTAGTTTT	TCAG	---TAGCTATCTGC	GGCGT	
T620	(1)	-----	-----	-----	-----	-----	-----	GTA	CTCGAGTATA	AAGTATATA	TATAGTGGG	---CGGGACT	TAAGGATCCGGC	---	
T622	(1)	-----	-----	-----	-----	-----	AGACGG	ATCCTATG	CG	CGATT	TTTTAAGTAGTTTT	TCAG	---TAGCTATCTGC	GGCGT	
T624	(1)	-----	-----	-----	-----	-----	AGACGG	ATCCTATG	ACGAC	GATTTTTT	TAAGT	AGTTTT	TCAG	---TA	
T643	(1)	-----	-----	-----	-----	-----	-----	GTA	CTCGAGTATA	AAGTATATA	TATAGTGGG	---CGGGACT	TAAGGATCCGGC	---	
T670	(1)	-----	-----	-----	-----	-----	-----	TCTT	CACTGGG	GACCATC	ATTATTTA	AAAT	ATGGC	CTGAA	
T800	(1)	-----	-----	-----	-----	-----	AGACGG	ATCCTATG	ACGAC	GATTTTTT	CCACA	GTTTT	TCGC	---TAGCTATCTGCAGGCGT	
T805	(1)	-----	-----	-----	-----	-----	-----	TGTACT	TCGAGTATA	AAGTATATA	TATAGTGGG	---CGGGACT	TAAGGATCCGGC	---	
T807	(1)	-----	-----	-----	-----	-----	-----	TGTACT	TCGAGTATA	AAGTATATA	TATAGTGGG	---CGGGACT	TAAGGATCCGGC	---	
T808	(1)	-----	-----	-----	-----	-----	-----	TGTACT	TCGAGTATA	AAGTATATA	TATAGTGGG	---CGGGACT	TAAGGATCCGGC	---	
T813	(1)	-----	-----	-----	-----	-----	AGACGG	ATCCTATG	CG	CGATT	TTTTAAGTAGTTTT	TCAG	---TAGCTATCTGC	GGCGT	
T815	(1)	-----	-----	-----	-----	-----	AGACGG	ATCCTATG	CG	CGATT	TTTTAAGTAGTTTT	TCAG	---TAGCTATCTGC	GGCGT	
Consensus	(1)	-----	-----	-----	-----	-----	AGACG	ATCCTATG	ACGAC	GATTTTTT	ATAGTTTT	TCGG	TA	CTATCTGC	GGCGT

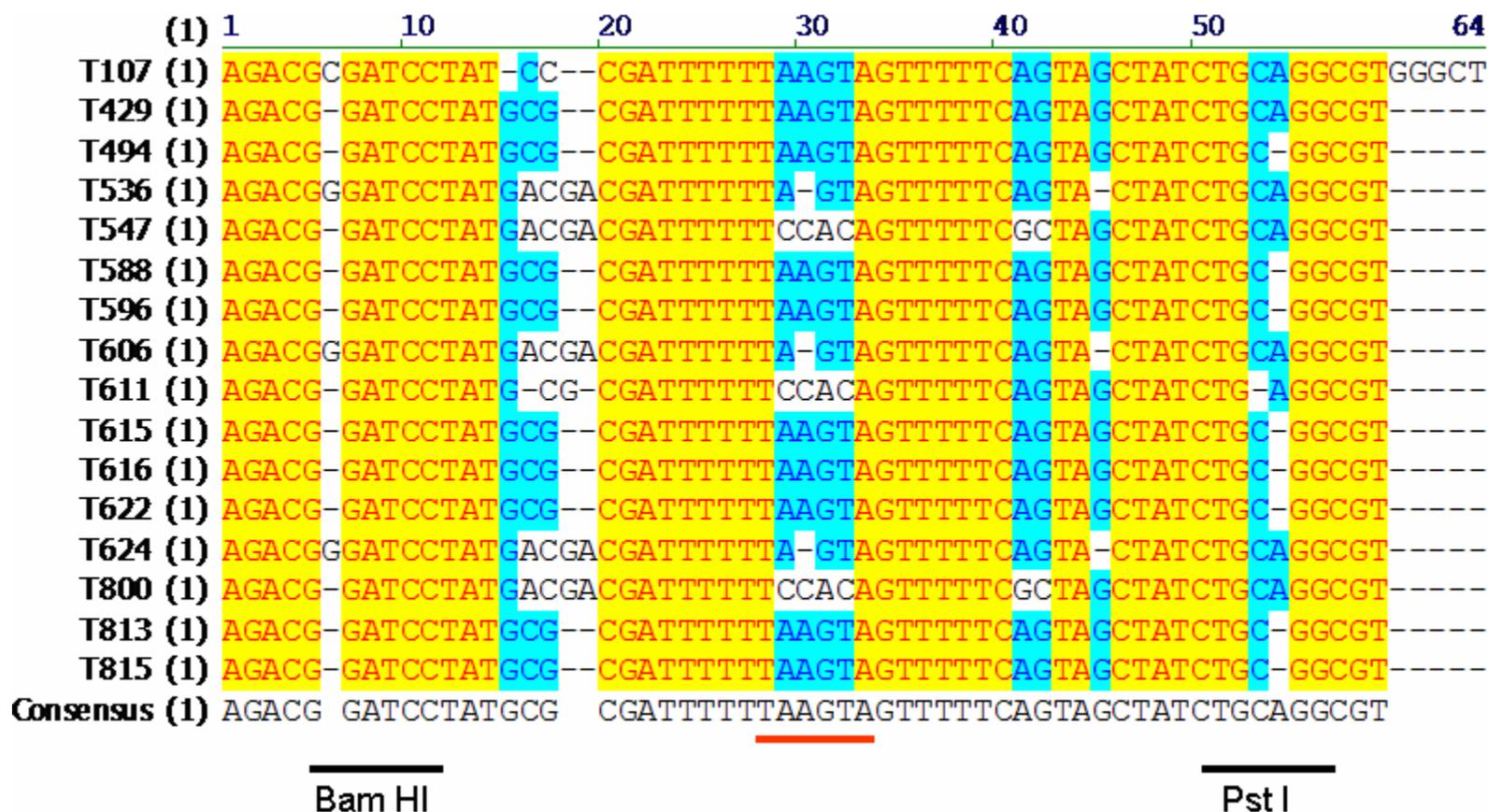


Figure 41. Alignment of Group I “repetitive” ChIP sequences. Sixteen repetitive ChIP sequences were termed Group I based on their common nucleotide sequences. Sequences shared by all sixteen isolates are highlighted in yellow. Sequences shared by a majority of the sixteen isolates are highlighted in blue. A perfect Nkx3.1 consensus DNA-binding site was identified in ten of the Group I sequences and is indicated by a red underline. Bam HI and Pst I restriction sites were identified in most Group I sequences and are identified below a computer-generated consensus sequence. Isolate designations are indicated on the left.

TTTTTTAAGTAGTTTTT-3' sequence that is the hallmark of Group I sequences was identified on human chromosomes 2, 3, 4, 6, 7, 8, 11, 12, 13, 14, 15, 17, 18, and X. Further analyses indicate that the Group I core sequence is often found within known genes. For example, the Group I core sequence was localized within the fifth intron of *A kinase anchor protein 11* on human chromosome 13. The Group I core was also located within introns of *C11orf14* on human chromosome 11 and *structural maintenance of chromosome 6-like 1* on human chromosome 2.

Thirteen additional “repetitive” sequences shared significant homology and were termed Group II repeats. As shown in Fig. 42, all Group II sequences had a perfect consensus Nkx3.1 DNA-binding site and a near-perfect Nkx3.1 binding site (5'-TAAGGA-3') separated by only 19 bp. The consensus Nkx3.1 DNA-binding site in Group II repeats was flanked on only one side by a poly-dTdA repeat, which like poly-dT tracts, also renders DNA “flat” and rigid (Muller et al., 2001). In contrast, the near-consensus Nkx3.1 binding site in Group II repeats is flanked by GC-rich tracts. Again the precise chromosomal location of the Group II sequences was difficult to determine due to fragment length (50-51 bp). A BLASTN search of Group II sequences amongst human genomic sequences produced 276 results. The consensus Nkx3.1 DNA-binding site and flanking poly-dTdA tract was localized on human chromosomes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 17, 21, and X. Many of the Group II sequences were not localized near annotated genes, the only notable exception being human chromosome 2. A group II Nkx3.1 DNA-binding site and flanking poly-dTdA tract is located 7 kb upstream of *MGC47799*, whose gene product, AAY24008, is a forkhead-associated protein.

Figure 42. Alignment of Group II “repetitive” ChIP sequences. Thirteen ChIP sequences were termed Group II based on their common nucleotide sequences. Sequences shared by all thirteen Group II isolates are highlighted in yellow. Sequences shared by a majority of the sixteen Group II isolates are highlighted in blue. Two Nkx3.1 consensus DNA-binding sites were identified in each of the Group II sequences and are identified by red underlines. Ava I and Bam HI restriction sites were identified in most Group II sequences and are identified below a computer-generated consensus sequence. Isolate designations are indicated on the left.

	(1)	1	10	20	30	40	51
T501	(1)	T	GTACCT	TCGAGTATAAAGTATATATATAGTGGGCGGGACTAAGGATCCGCGG			
T544	(1)	-	GTACCT	TCGAGTATAAAGTATATATATAGTGGGCGGGACTAAGGATCCGCGG			
T554	(1)	-	GTACCT	TCGAGTATAAAGTATATATATAGTGGGCGGGACTAAGGATCCGCGG			
T573	(1)	-	GTACCT	TCGAGTATAAAGTATATATATAGTGGGCGGGACTAAGGATCCGCGG			
T584	(1)	-	GTACCT	TCGAGTATAAAGTATATATATAGTGGGCGGGACTAAGGATCCGCGG			
T592	(1)	-	GTACCT	TCGAGTATAAAGTATATATATAGTGGGCGGGACTAAGGATCCGCGG			
T603	(1)	-	GTACCT	TCGAGTATAAAGTATATATATAGTGGGCGGGACTAAGGATCCGCGG			
T609	(1)	-	GTACCT	TCGAGTATAAAGTATATATATAGTGGGCGGGACTAAGGATCCGCGG			
T620	(1)	-	GTACCT	TCGAGTATAAAGTATATATATAGTGGGCGGGACTAAGGATCCGCGG			
T643	(1)	-	GTACCT	TCGAGTATAAAGTATATATATAGTGGGCGGGACTAAGGATCCGCGG			
T805	(1)	T	GTACCT	TCGAGTATAAAGTATATATATAGTGGGCGGGACTAAGGATCCGCGG			
T807	(1)	T	GTACCT	TCGAGTATAAAGTATATATATAGTGGGCGGGACTAAGGATCCGCGG			
T808	(1)	T	GTACCT	TCGAGTATAAAGTATATATATAGTGGGCGGGACTAAGGATCCGCGG			
Consensus	(1)		GTACCT	TCGAGTATAAAGTATATATATAGTGGGCGGGACTAAGGATCCGCGG			

 Ava I	 Bam HI
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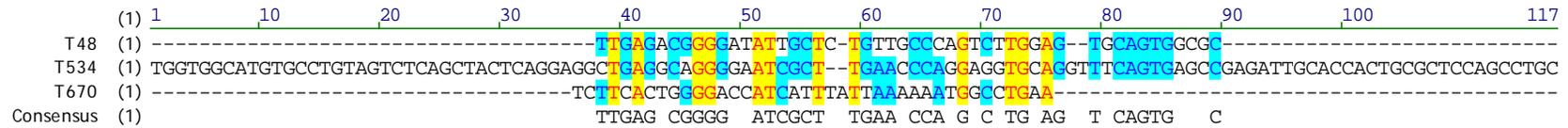


Figure 43. Alignment of Group III “repetitive” ChIP sequences. Three ChIP sequences were termed Group III based on their common sequences. Sequences shared by all three Group III isolates are highlighted in yellow. Sequences shared by a majority of Group III isolates are highlighted in blue. Obvious Nkx3.1 consensus DNA-binding sites were not identified in Group III sequences. A computer-generated consensus sequence is indicated below the three isolates and isolate designations are indicated on the left.

The remaining three “repetitive” sequences comprise Group III (Fig. 43). Group III sequences bear little resemblance to those of Group I or Group II, and Group III sequences lack consensus Nkx3.1 DNA-binding sites. Clone T534 was the longest of the Group III repeats (114 bp) and was localized to human chromosome 6, approximately 30 kb upstream of *SUPT3H*, whose gene product, Spt3, is a transcription factor (Yu et al, 1998). Clone T670 is the shortest Group III sequence (39 bp) and was localized to human chromosomes 3, 20 and 22, but was not juxtaposed near annotated genes on these chromosomes. The sequence represented by clone T48 was localized to sequences on human chromosomes 2, 3, 6, 7, 8, 9, 12, 16, 19, 21, and 22.

There are several reasons to believe that the “repetitive” sequences retrieved represent independent cloning events and are not due to clonal amplification during rescue or contamination. First, an alignment of the “repetitive” sequences clearly shows that the sequences are not identical in length or in sequence. Secondly, most “repetitive” sequences were isolated in two independent ChIP assays. Thirdly, only a subset of the retrieved sequences is “repetitive”. If the “repetitive” sequences were collected due to replication after bacterial transformation one would expect to see duplicates of nearly all retrieved sequences, but this is not the case. Indeed, ten unique sequences were retrieved compared to the thirty-two sequences that were classified into the three “repetitive” groups. Lastly, the “repetitive” sequences retrieved by ChIP assay are also found to be dispersed within the human genome suggesting that they truly represent low-copy repetitive DNA sequences.

In summary, a genome-wide screen for putative Nkx3.1 target genes yielded 42 clones containing novel, human genomic DNA. Ten of these clones harbored unique

genomic fragments, while the remaining 32 clones carried sequences that were isolated repeatedly and could be subdivided into three sequence classes. Amongst the ten unique isolates, several mapped within or near known genes and at least one (T50) mapped to a gene associated with prostate tumorigenesis, *Neuregulin-1*. Nkx3.1 consensus DNA-binding sequences were rarely associated with these DNA fragments, although sequences reminiscent of consensus sites were occasionally identified. Many of the recovered repetitive sequences mapped to locations that are within or near known genes, but their precise origin was difficult to determine due to their relatively short fragment length and because these repetitive sequences are dispersed throughout the human genome. The vast majority of these repetitive sequences carried one or more consensus Nkx3.1 DNA-binding sites. Thus, based on these ChIP results Nkx3.1 appears to bind specifically to (1) non-repetitive regions of chromatin within and near known genes as well as (2) at least two subtypes of repetitive genomic DNA. Further work must be performed to corroborate that the results from this genome-wide screen represent *in vivo* Nkx3.1 targets. The ChIP results presented here could most easily be authenticated using a ChIP-PCR strategy utilizing primers that flank the sequences isolated by ChIP. An independent ChIP assay would be conducted as previously described (see Chapter II, sec 2.15) except that the immunoselected genomic DNA fragments would be used as template for the PCR with primers that flanked a specific suspected genomic DNA fragment. If the candidate genomic DNA fragment is isolated by ChIP, then the PCR reaction should yield a single PCR product of predicted size, thus confirming that DNA region as a target of Nkx3.1 *in vivo*. As an initial step to validate at least one uniquely cloned ChIP isolate I designed two PCR primer pairs that flanked the T50 intronic

sequence from the human *Neuregulin* locus, but I was unable to establish the proper PCR conditions using control genomic DNA derived from LNCaP cells; therefore, I was unable to test the reproducibility of my ChIP experiments. More work should be done to extend these findings and to authenticate the genomic DNA fragments I isolated as targets of Nkx3.1 *in vivo*. Identifying the population of Nkx3.1-targeted genes will be vital in understanding how Nkx3.1 directs prostate development and how loss of Nkx3.1 triggers prostate tumor formation.

CHAPTER VII
Discussion

Homeodomain-containing transcription factors play a central role in the development of metazoans, orchestrating the development of the body plan, determining cell-fate, and stimulating organogenesis. Each is characterized by a conserved 60 amino acid DNA-binding domain that interacts with a characteristic DNA sequence (5'-TAAT/G-3') in the promoters of target genes. Given the variety of developmental outcomes that spring from their interaction with a common DNA-binding sequence, it is widely believed that homeodomain proteins identify and regulate target genes in collaboration with a constellation of additional DNA-binding proteins. Functional interactions between homeodomain-containing proteins and zinc-“finger” transcription factors are one mechanism by which tissue-specific gene regulation is modulated. My goal for the studies reported herein was to determine whether combinatorial interactions between Nkx3.1, a homeodomain protein linked to the development of the human and mouse prostate gland, and one or more Sp-family members play a role in the regulation of transcription in prostate-derived cells. I report that Nkx3.1 collaborates with Sp-family members to regulate the transcription of *prostate-specific antigen (PSA)* in prostate-derived cells. Nkx3.1 negatively-regulates the transcriptional induction of *PSA* by Sp-family members, and does so via TSA-sensitive and –insensitive mechanisms. Consistent with the notion that one or more promoter elements is required for Nkx3.1 to suppress Sp-mediated transcription, a distal segment of the *PSA* promoter was identified as being necessary for *trans*-repression. Additionally, I report that (1) Nkx3.1 and Sp-family members form specific protein complexes *in vitro* and *in vivo*, and (2) portions of Nkx3.1 that are required for complex formation with Sp-family members are also required to antagonize Sp-mediated transcription. I conclude that Nkx3.1 negatively

regulates the transcriptional activity of Sp-family members in prostate-derived cells and speculate that these interactions, and their deregulation in tumor cells, may have important implications for prostate cell proliferation and differentiation.

Prior to the initiation of my studies little was known about the sub-cellular localization of Nkx3.1, nor had Nkx3.1 target genes been identified. I used a variety of strategies to address sub-cellular localization and report that Nkx3.1 is karyophilic and associates with the nuclear matrix in interphase cells. Using a battery of partial-Nkx3.1 fusion proteins I was able to show that sequences within the Nkx3.1 homeodomain appear to specify both nuclear localization and nuclear matrix association. I also report that unlike many sequence-specific DNA-binding proteins, Nkx3.1 is associated with chromatin throughout most, if not all, of mitosis. I used chromatin immunoprecipitation to perform a genome-wide screen for Nkx3.1 target genes. These efforts led to the identification of a number of single-copy human genomic fragments as well as low-copy repetitive DNAs that may be targets of Nkx3.1.

Nkx3.1 antagonizes Sp-mediated transcription in prostate-derived cells

Given that functional interactions between homeodomain-containing proteins and zinc-“finger” transcription factors modulate tissue-specific gene regulation in many tissues, I speculated that combinatorial interactions between Nkx3.1 and one or more Sp-family members may play a role in the regulation of prostate-specific transcription. I chose to use the human PSA promoter as a substrate for these studies since previous studies had established it to be regulated by Nkx3.1 (Chen et al., 2002). A subset of Sp-

family members, namely Sp1, Sp3 and Sp4 activated *PSA* transcription in human prostate-derived cells, and co-expression of Nkx3.1 antagonized Sp-mediated transcription. It is unclear whether Nkx3.1 antagonizes Sp2- or Sp5-mediated transcription in prostate cells since neither Sp2 nor Sp5 activated *PSA* transcription, and target genes for these Sp proteins have yet to be identified. By using TSA, a potent inhibitor of histone deacetylases (HDACs), Nkx3.1 was shown to repress Sp-mediated transcription through TSA-sensitive and -insensitive mechanisms, suggesting that Nkx3.1 represses Sp-mediated transcription, at least in part, via interactions with HDACs. Since TSA treatment reduced, but did not eliminate the capacity of Nkx3.1 to negatively-regulate Sp-mediated transcription, there is at least one additional mechanism by which Nkx3.1 represses Sp-mediated transcription. Additionally, proteins derived from putative *Nkx3.1* mRNA splice variants were used to show that a protein motif termed the “TN domain”, found within the Nkx3.1 amino-terminus and thought to be a site for Groucho/TLE co-repressor recruitment, was not required to antagonize Sp-mediated transcription. This latter result implies that the association of Groucho/TLE proteins with the Nkx3.1 TN domain does not play an important role in the *trans*-repression of Sp proteins and is entirely consistent with the observation that Nkx3.1 antagonizes Sp-mediated transcription through interactions with HDACs. I also show that two non-contiguous portions of Nkx3.1, the homeodomain and a second domain within the Nkx3.1 amino-terminus, are each sufficient to antagonize Sp-mediated transcription.

Nkx3.1 DNA-binding activity is not required for *trans*-repression of Sp proteins

The capacity of Nkx3.1 to down-regulate *PSA* transcription induced by Sp-family members was found to be entirely dependent on a discrete 1.1 Kbp promoter fragment at the distal end of the human *PSA* promoter. This distal region has been shown to carry an androgen response element (ARE), yet this site is not likely to be involved in the down-regulation of Sp-mediated transcription by Nkx3.1 since I documented *trans*-repression in prostate-derived cells that do not express the androgen receptor, such as DU145. Computer-assisted analyses identified two putative Sp-binding sites within this distal portion of the *PSA* promoter, and their potential relevance for the induction of *PSA* or regulation by Nkx3.1 has yet been determined. Two putative Nkx3.1-binding sites within this distal segment of the *PSA* promoter were also identified in this computerized screen, yet their mutational inactivation had little effect on the capacity of Nkx3.1 to antagonize Sp-directed transcription. Given these mutational results and the capacity of homeodomain mutants to block Sp-mediated transcription, I speculate that Nkx3.1 associates indirectly with chromatin via one or more factors that bind the distal end of the *PSA* promoter (Fig. 44). According to this model, once tethered to this portion of the *PSA* promoter Nkx3.1 antagonizes Sp-mediated transcription via the recruitment of co-repressors and/or by direct interactions with Sp-family members that blocks their association with co-activators.

Elevated levels of PSA in patient blood serum are directly correlated with prostatic hyperplasia and prostate tumorigenesis, and quantitation of serum PSA levels is a clinically significant diagnostic tool. Here I demonstrate that *PSA* is a transcriptional

target for a subset of Sp-family members in prostate-derived cells, and co-expression of Nkx3.1 represses Sp-mediated transcription. Based on the data presented in this study one would predict that loss of Nkx3.1 protein expression, a common and early event in prostate tumorigenesis, would lead to unfettered Sp-mediated transcription and thus elevated levels of serum PSA. Whether increased rates of *PSA* transcription play an important role in the elevation of serum PSA levels remains to be determined. Given that Sp proteins regulate a wide variety of genes, loss of Nkx3.1 function and its consequent effects on *trans*-activation by Sp proteins might be predicted to lead to global perturbations in gene expression, deregulated cell growth and differentiation.

The Nkx3.1 homeodomain physically interacts with the zinc-“fingers” of Sp-family members

In vitro protein-binding assays reported here identified Sp-binding sites within two non-contiguous portions of Nkx3.1 and at least one binding site for Nkx3.1 within the zinc-“fingers” of Sp-family members. Similar to previous studies with SRF and PDEF, my studies indicate that the majority of Sp protein-binding activity resides within the Nkx3.1 homeodomain (Carson et al., 2000; Chen et al., 2002). These interactions are likely to be physiologically relevant as (1) I established that the Nkx3.1 homeodomain is necessary and sufficient for the formation of physical complexes with the zinc-“fingers” of Sp proteins *in vivo* using a mammalian “two-hybrid” approach and (2) such complexes were disrupted in a dose-dependent fashion by the co-expression of full-length Sp-family members. It is worth noting that comparable physical interactions have been reported between the homeodomain and zinc-“fingers” of Nkx2.5 and GATA-4, two factors

essential for cardiogenesis (Durocher et al., 1997). Unexpectedly, my mammalian “two-hybrid” results also suggested that the Nkx3.1 homeodomain and the zinc-“fingers” of Sp proteins may interact in an orientation-specific fashion. That is, protein/protein interactions were detected when the Nkx3.1 homeodomain was linked downstream, but not upstream, of the carboxy-terminal 82 amino acids of *Renilla* luciferase. These results are not due to problems inherent with *Renilla* reconstitution since the carboxy-terminal 82 amino acids reconstitute luciferase activity readily when fused downstream of control proteins, such as FKBP12 (S.O. Simmons and J.M. Horowitz, unpublished observations). Given that (1) the Nkx3.1 homeodomain resulted in background levels of luciferase activity when fused in this orientation and (2) homeodomains with “free” amino-termini are capable of folding into functional units (*e.g.*, capable of binding DNA; Kissinger et al., 1990), I speculate that Nkx3.1 and Sp proteins may interact in a specific, orientation-dependent manner.

The protein/protein-binding assays described in Chapter IV identified a second, independent Sp protein-binding site within the amino terminus (residues 1-90) of Nkx3.1. In *in vitro* studies this binding site appears to be relatively weak compared to the Sp-binding site carried by the Nkx3.1 homeodomain, and the Sp amino acids with which this site interacts have not as yet been determined. Interestingly, the amino-terminus of hNkx3.1 has been reported to be subject to alternative splicing resulting in five distinct isoforms carrying varying portions of the amino-terminal 90 amino acids (Korkmaz, et al, 2000). Thus, it is conceivable that Nkx3.1 isoforms may differ in their physical and functional interaction with Sp proteins or other zinc-“finger” proteins. Regardless of such potential differences, my results indicate that Sp-mediated transcription is

antagonized independently by the ectopic expression of the Nkx3.1 homeodomain or amino-terminus. It is worth pointing out that although Nkx3.1 bound all Sp-family members *in vitro*, Sp2 and Sp5 appeared to be bound more efficiently than Sp1, Sp3, and Sp4. Unfortunately, since Sp2 and Sp5 had little or no effect on *PSA* transcription I was unable to compare their sensitivity to regulation by Nkx3.1. Target genes of Sp2 or Sp5 have not as yet been identified, thus the functional consequence of the increased efficiency with which they interact with Nkx3.1 remains to be established.

Protein/DNA-binding assays indicate that Nkx3.1 does not antagonize the DNA-binding activity of Sp-family members. Indeed, Nkx3.1 and Sp1 or Sp3 proteins formed ternary complexes on oligonucleotides carrying cognate binding sites separated by as little as five base pairs. Additional protein/DNA-binding assays utilizing oligonucleotides carrying Nkx3.1- or Sp-binding sites indicated that, at least *in vitro*, neither Nkx3.1 nor Sp-proteins could tether the other to DNA. Taken together, these protein/DNA-binding assays suggest that physical interactions between DNA-bound Nkx3.1 and Sp proteins result in transcriptional regulation. However, I have also shown that (1) Nkx3.1 homeodomain mutants that are deficient in DNA-binding activity are still potent antagonists of Sp-mediated transcription and (2) partial Nkx3.1 proteins capable of binding Sp-family members are also quite potent inhibitors of Sp-mediated transcription. What is the mechanism by which Nkx3.1 negatively regulates Sp-mediated transcription? I speculate that Nkx3.1 regulates Sp-family members by attracting co-repressors to Sp protein/DNA complexes and/or by interfering with the interaction of co-activators necessary for Sp-mediated transcription (Fig. 44). Nk proteins contain a conserved 23-amino acid sequence, termed the TN domain, which in some instances can facilitate the

recruitment of histone deacetylases (HDACs) to DNA and the down-regulation of transcription (Muhr et al., 2001; Kim et al., 1998; Choi et al., 1999). Interestingly, a TN-like sequence in human Nkx3.1, amino acids 24-46, is located within an amino-terminal 90 amino acid region that I have shown binds to Sp-proteins *in vitro* and inhibits Sp-mediated transcription *in vivo*. I have also shown that the Nkx3.1 homeodomain harbors an Sp protein-binding site and this portion of Nkx3.2, a closely-related homeodomain-containing protein, has been shown to interact with HDAC1 and Smad proteins and recruit co-repressors, such as Sin3A (Kim et al., 2003). Yet another possibility is the influence of a family of homeodomain-interacting protein kinases (HIPKs) that function as co-repressors of transcription via binding the homeodomain of NK-3 proteins (Kim et al., 1998). Consistent with the notion that HDAC association plays a role in the antagonism of Sp-mediated transcription, treatment of prostate-derived cells with a potent HDAC inhibitor, Trichostatin A (TSA), resulted in significant, albeit incomplete relief from *trans*-repression by Nkx3.1. Whether Nkx3.1 also inhibits the association of Sp proteins with one or more co-activators, perhaps providing a TSA-insensitive mechanism for transcriptional regulation of Sp-family members, remains to be determined.

Nkx3.1 is associated with the nuclear matrix

Apart from its direct role as a regulator of Sp-mediated transcription in prostatic epithelium, the subcellular localization and chromatin immunoprecipitation studies reported herein suggest that Nkx3.1 may have additional roles in cell physiology. In

accord with a previous study (Korkmaz et al., 2000), I showed that Nkx3.1 is a nuclear protein and extended these observations via the identification of the carboxy-terminus of the homeodomain as the likely location of at least one nuclear localization signal. My subcellular localization studies also revealed that (1) Nkx3.1 associates with the nuclear matrix or transfected COS cells, (2) the Nkx3.1 homeodomain is sufficient for nuclear matrix attachment, and (3) the Nkx3.1 nuclear matrix targeting signal spans at least part of Helix III of the Nkx3.1 homeodomain. These results constitute the first report of any NK-family member associating with the nuclear matrix. The nuclear matrix provides an intranuclear scaffold for proteins involved in a variety of cellular processes including chromatin modification, DNA replication and repair, transcription, and RNA splicing (Mattout-Drubezki and Gruenbaum, 2003; Nickerson, 2001; Stein et al., 2000; van Wijnen et al., 1993). Although several sequence-specific DNA-binding proteins have been shown to be associated with the nuclear matrix, including the glucocorticoid receptor (DeFranco and Guerrero, 2000), SATB1 (Seo et al., 2005), Oct-1 (Kim et al., 1996) and AP-1 (van Wijnen et al., 1993), few homeodomain proteins have been shown to localize to this sub-nuclear fraction. Although the target proteins bound by any given nuclear matrix-associated transcription factor and the mechanisms that govern their association are far from certain, common patterns are being to emerge. A subset of nuclear matrix-associated transcription factors, such as Sp1 and Oct-1, shuttle between the nuclear matrix and soluble, non-matrix associated cellular fractions as a function of cell growth/differentiation status (Bagchi et al., 1995). This observation has led to speculation that the nuclear matrix may serve as a reservoir for transcription factors that are liberated from the nuclear matrix and then regulate gene expression upon activation

by one or more signal transduction pathways (Stein et al., 2000). For such factors the nuclear matrix may mask or otherwise inactivate DNA-binding activity, rendering them functionally inert. Whether Nkx3.1 shuttles to and from the nuclear matrix and whether matrix association negatively regulates Nkx3.1 DNA-binding activity remain open questions.

Nkx3.1 remains bound to condensed chromatin throughout mitosis

As a consequence of my studies of Nkx3.1 subcellular localization in interphase cells, the distribution of Nkx3.1 within a number of mitotic nuclei was also observed. Nkx3.1 was shown to be bound to condensed chromatin throughout most, if not all, stages of mitosis. Binding to condensed mitotic chromosomes was found to be dependent upon an intact Nkx3.1 homeodomain. A few DNA-binding proteins factors such as TBP, Hox1.1, GAGA, p300, and UBF appear to remain associated with mitotic DNA (Chen et al., 2002; Schulze et al., 1987; Kellum et al., 1995; Zaidi et al., 2003; Chen et al., 2005); however, many DNA-binding proteins such as Oct-1, Ets-1, c-fos, Sp proteins, and Ikaros are not bound to mitotic chromatin (Segil et al., 1991; Fleischman et al., 1993; Komura and Oro, 2005; He and Davie, 2006; Dovat et al., 2002), but rather are excluded near the onset of prophase and do not re-associate with chromatin until after the post-mitotic nuclei have been reestablished (*e.g.*, Sp2 is excluded from mitotic chromosomes illustrated in Fig. 25). The reasons why Nkx3.1 is not excluded from mitotic chromatin remain unclear. It is widely believed that transcription is silenced throughout mitosis, so it would follow that Nkx3.1 is unlikely to play a transcriptional role during this cell cycle

phase. One possibility is that Nkx3.1 may function to anchor chromosomes to the newly formed nuclear matrix after completion of mitosis in order to assist with reorganization in progeny nuclei. Chromatin structure during nuclear reorganization has been shown to be a critical factor for specified gene expression during development (Bickmore et al., 2004), so one could imagine that properly organized chromatin in newly formed nuclei might be a vital determinate for activation of prostate-specific genes for normal prostate development and maintenance of fully differentiated prostate phenotype. Conversely, disorganized chromatin due to the loss of a critical chromatin binding factor such as Nkx3.1 could lead to altered gene expression patterns that preclude the formation of fully differentiated prostate cells. Alternatively, one could imagine that Nkx3.1 plays a protective role during mitosis. For example, Nkx3.1 might prevent chromosome breakage and/or ensure that chromosomes segregate properly. One would expect such functions to be anti-oncogenic and thus they would be entirely consistent with Nkx3.1's role as a tumor-suppressor gene product.

Nkx3.1 DNA-binding activity

I have shown that Nkx3.1 expressed in *E. coli* is functional in an *in vitro* protein/DNA binding assay and Nkx3.1 synthesized in eukaryotic cells does not bind DNA under identical conditions. Why might this be? Several possible explanations are worth entertaining. I initially began my studies under the assumptions that either: (1) a factor or factors required by Nkx3.1 to bind DNA were absent in the eukaryotic cells that I examined, or (2) one or more factors that inhibit Nkx3.1 DNA-binding activity may

exist in eukaryotes. Given that Nkx3.1 is a mammalian protein, and many of my studies were performed in prostate cells (*e.g.*, LNCaP) that express endogenous Nkx3.1, the former possibility seemed highly unlikely. Consequently, I favored the possibility that one or more inhibitory mechanisms in eukaryotic cells abrogate *in vitro* Nkx3.1 DNA-binding activity. I could imagine any number of possible mechanisms by which *in vitro* DNA-binding activity might be inhibited. Such mechanisms, however, must be quite general since Nkx3.1 collected from a variety of cell types, ranging from insect cells to green monkey kidney fibroblasts to human prostatic epithelial cells, lacked *in vitro* DNA-binding activity. My studies addressed two possible inhibitory mechanisms: phosphorylation and protein/protein interactions. Treatment of LNCaP cell extracts with two phosphatases commonly employed to dephosphorylate proteins *in vitro* did not result in the restoration of Nkx3.1 DNA-binding activity, suggesting that DNA-binding by Nkx3.1 is not limited by this mechanism. This said, I cannot rule-out the possibility that one or more critical Nkx3.1 amino acids remained phosphorylated following treatment with phosphatases. It is also quite possible that Nkx3.1 DNA-binding activity may be blocked by other post-translational modifications (*e.g.*, acetylation, sumoylation, or glycosylation). In an attempt to liberate Nkx3.1 from one or more putative inhibitory proteins I treated LNCaP cell extracts with sodium deoxycholate, a potent ionic detergent and then neutralized these reactions with NP-40. Similar to results obtained with extracts treated with phosphatases, Nkx3.1 DNA-binding activity was not restored under these experimental conditions suggesting that protein-protein interactions are unlikely to account for the lack of Nkx3.1 DNA-binding activity in mammalian cell extracts.

Given these results, an alternative explanation is that Nkx3.1 DNA-binding activity is readily detected using Nkx3.1 purified from *E. coli* simply because the bacterial extracts had a much higher concentration of Nkx3.1 than extracts prepared from eukaryotic cells. Nkx3.1 has a much lower affinity for DNA *in vitro* than many other sequence-specific DNA-binding proteins, including a variety of homeodomain-containing proteins. Indeed, with a dissociation constant (K_d) of 20 nM (Steadman et al., 2000), Nkx3.1's affinity for DNA *in vitro* is approximately 100 times less than that of Sp2 and 10 times less than most other homeoproteins (Table 2). Thus, it is possible that the detection of stable protein/DNA complexes requires higher concentrations of Nkx3.1 than may be harvested from eukaryotic cells. Yet, this explanation seems unlikely for at least two reasons. First, I employed a sensitive protein/DNA-binding assay that is capable of detecting protein/DNA complexes at relatively low concentrations. Second, DNA-binding activity was detected using Nkx3.1 synthesized in *E. coli* at input concentrations (as assayed by Western blot; data not shown) comparable to those obtained in extracts from Nkx3.1-transfected COS-1 cells or LNCaP cells. Thus, Nkx3.1 protein concentration is unlikely to account for differences in Nkx3.1 DNA-binding activity in extracts prepared from bacterial or eukaryotic cells.

What then accounts for the apparent absence of Nkx3.1 DNA-binding activity in eukaryotic cell extracts? Given my sub-cellular localization results, a strong possibility is that the fraction of Nkx3.1 that is competent to bind DNA is that fraction bound to the nuclear matrix of eukaryotic cells. For example, protein/protein interactions that may stabilize the homeodomain may occur via the association of Nkx3.1 with the nuclear matrix. Such interactions are not unprecedented as a matrix-associated protein, NMP, was

shown to enhance the DNA-binding activity and oncogenic potential of NF- κ B in breast cancer cells (Raziuddin et al., 1997). I speculate that soluble Nkx3.1, that fraction of Nkx3.1 that may be recovered in non-denatured eukaryotic extracts, is simply not competent to bind DNA *in vivo* or *in vitro*. If the fraction of Nkx3.1 that is competent to bind DNA is associated exclusively with the nuclear matrix, then one would predict that this fraction would be eliminated along with insoluble cellular components that I routinely discarded during the preparation of whole cell extracts. Is there reason to suspect that nuclear matrix-associated Nkx3.1 is competent to bind DNA? Although I do not have direct evidence in support of this contention, I have shown that a functional Nkx3.1 homeodomain is required for association with (1) the nuclear matrix and (2) mitotic chromatin (Figs. 23 and 25). In trying to define mechanisms that limit Nkx3.1 DNA-binding activity in eukaryotic cells, I perhaps ignored the most fundamental distinction between prokaryotes and eukaryotes: a defined nucleus, and specifically, the nuclear matrix. If DNA-competent Nkx3.1 is sequestered by the nuclear matrix (insoluble fraction), then it is not surprising that soluble Nkx3.1 was found not to bind to its cognate DNA-binding site in *in vitro* assays. This would also explain why fractionated LNCaP extracts were found not to bind DNA themselves nor inhibit bacterially-produced Nkx3.1 from binding DNA. But why is it that soluble Nkx3.1 harvested from eukaryotic cells appears incompetent to bind DNA? I speculate that in eukaryotic, but not bacterial, cells soluble Nkx3.1 is modified such that it lacks the requirements for both matrix attachment and DNA-binding activity. An experiment that would test the notion that nuclear matrix-associated Nkx3.1 is competent to bind DNA would be to determine whether one can detect Nkx3.1 DNA-binding activity *in situ*. For

example, nuclear matrices could be isolated from COS-1 cells expressing wild-type Nkx3.1 or a derivative lacking a functional homeodomain, and then challenged with fluorescently-labeled oligonucleotide probes carrying either a consensus Nkx3.1 DNA-binding site or a mutated Nkx3.1 binding site (analogous to the oligonucleotide probes used in Chapter IV). If matrix-associated Nkx3.1 is competent to bind DNA, then one would expect that nuclear matrices prepared from cells that express wild-type Nkx3.1 should be bound by a fluorescently-labeled Nkx3.1 oligonucleotide probe, but not a mutated probe. In contrast, matrices prepared from cells expressing a homeodomain mutant would not be expected to bind either oligonucleotide probe.

A body of evidence indicates that nuclear matrix attachment and DNA-binding activity are not mutually exclusive. A number of matrix-associated proteins have been shown to bind chromatin “loops” through sequences termed “matrix attachment regions” (MARs), and proteins bound to chromatin via MARs can regulate gene expression as well as play a role in chromatin organization and chromosomal maintenance (Puvan Kumar et al., 2006; Visser et al., 2005). Matrix attachment regions are highly AT-rich repeat sequences commonly found in introns as well as proximal and distal sites flanking the 5' and 3' ends of genes (Boulikas, 1992). Given that Nkx3.1 clearly associates with the nuclear matrix and that a genome-wide screen for putative Nkx3.1 target genes yielded repetitive AT-rich sequences found within or near known genes, I speculate that Class I and Class II repetitive sequences (Figs. 40 and 41) may represent two classes of matrix attachment regions. Should this be the case, it follows that Nkx3.1 may function to tether chromatin to the nuclear matrix via such matrix attachment regions. Further studies will be required to determine what role, if any, Nkx3.1 plays in the regulation of

genes located near these putative MARs such as *A kinase anchor protein 11*, *C11orf14*, *structural maintenance of chromosome 6-like 1* and MGC47799 *in vivo*. Should Nkx3.1 regulate the transcriptional activity of MAR-associated genes, one could imagine that such matrix-chromatin interactions might provide for a global mechanism by which Nkx3.1 regulates prostate-specific transcription. Indeed, specific spatial organization of chromatin by the nuclear matrix has been shown to be important in gene expression of fibroblasts (Petrova et al., 2006). If this model proves correct, then one would predict global disruption of prostate-specific gene expression upon loss of Nkx3.1 function (Fig. 45).

Identification of putative Nkx3.1 target genes

One of the most difficult challenges in the characterization of Nkx3.1 function has been the scarcity of authenticated target genes. For those few genes that have been suggested to be physiologically relevant targets of Nkx3.1, DNA-binding sites within the promoters of these genes have yet to be defined. I theorized that identification of Nkx3.1 target genes *in vivo* would be invaluable in determining Nkx3.1's role in both prostate development and prostate tumorigenesis. I employed a genome-wide screen for putative Nkx3.1 target genes in LNCaP cells using a chromatin immunoprecipitation approach. I successfully cloned 42 novel human genomic fragments by co-immunoprecipitation with endogenous Nkx3.1. Of these, 10 isolates were cloned once only. Although none of the singly-isolated DNA fragments contained a consensus Nkx3.1 DNA-binding sequence, a majority carried sequences resembling Nkx3.1 cognate DNA-binding sequences. Most of

these mapped in or near known genes. Notably, one isolate (T50) mapped to an intron within the *Neuregulin 1* locus on human chromosome 8p12. The *Neuregulin 1* gene product, NDF, is a ligand for the HER2, HER3 and HER4 family of receptor tyrosine kinases. While NDF expression is uniformly high in normal luminal prostate epithelium, NDF expression is significantly absent in prostate cancer specimens, suggesting that functional loss of the NDF/HER ligand/ receptor loop may be an early event associated with prostate tumorigenesis (Lyne et al., 1997). Recently however, NDR has been shown to promote proliferation of prostate tumor cells through both androgen-dependent and – independent mechanisms (Mendoza et al., 2002; Gregory et al., 2005). The role of *Neuregulin* in prostate tumor formation is as yet uncertain, since evidence suggests it has roles in both promoting and suppressing prostate tumorigenesis. What needs to be determined is whether Nkx3.1 regulates NDR expression and if so, how the loss of Nkx3.1 may affect NDR expression and prostate tumor formation. Other than *Neuregulin*, I failed to identify additional Nkx3.1 targets genes that were prostate-specific or had obvious connections to prostate tumor formation. Additional ChIP experiments should be performed with steps taken to improve overall cloning efficiency. In retrospect, a linker-ligation, PCR-amplification approach to enrich immunoselected DNA fragments would probably have yielded many more clones containing genomic DNA. Undoubtedly, more positive clones will lead to the identification of Nkx3.1 target genes clearly involved in prostate physiology or tumor formation. Additionally, an alternative ChIP-PCR strategy should be employed to identify specific candidates, such as *PSA*, suspected of being targets of Nkx3.1 *in vivo*. Microarray-based expression profiling has generated an interesting list of putative Nkx3.1 genes that could be authenticated by a

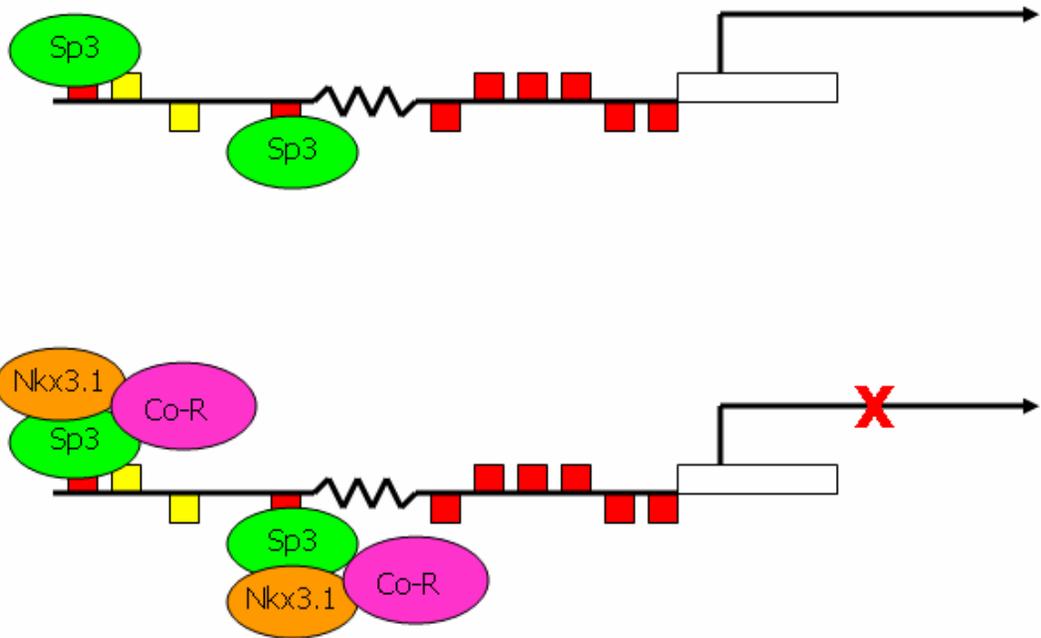
ChIP-PCR approach. Unraveling the mystery of Nkx3.1-directed gene expression would unquestionably produce invaluable clues to defining the role Nkx3.1 plays in both prostate development and prostate tumor formation.

The results reported here suggest that Nkx3.1 may have several important roles in the establishment and maintenance of mature prostate epithelium. Nkx3.1 is a homeodomain-containing protein and presumably functions primarily as a transcription factor. For those few genes that have been suggested to be physiologically relevant targets of Nkx3.1, DNA-binding sites within the promoters of these genes have yet to be defined. However indirect evidence of Nkx3.1 transcriptional function comes from comparative gene expression profiles of wild-type and *Nkx3.1* nullizygous mice. One such study lists over 600 putative gene targets identified by microarray analysis (Ouyang et al., 2005). Interestingly, the list included approximately even numbers of genes up- and down-regulated by Nkx3.1 in the mouse prostate. Here I provide evidence that Nkx3.1 functions to regulate the Sp-mediated transcription of a prostate-specific gene, not through a conventional direct DNA-binding mechanism, but rather through an indirect protein-protein interaction mechanism. In addition to antagonizing Sp-mediated transcription, I also show that Nkx3.1 attaches to the nuclear matrix and that it binds DNA sequences that may constitute matrix attachment regions *in vivo*. I propose that Nkx3.1 organizes chromatin through matrix attachment in manner that facilitates prostate-specific gene expression. Additionally I show that Nkx3.1 binds condensed chromatin throughout mitosis, and suggest that Nkx3.1 plays a role in post-mitotic reorganization. It is increasingly apparent that loss of Nkx3.1 expression plays a critical role in the initiation of prostate tumorigenesis. Although I was not able to specifically

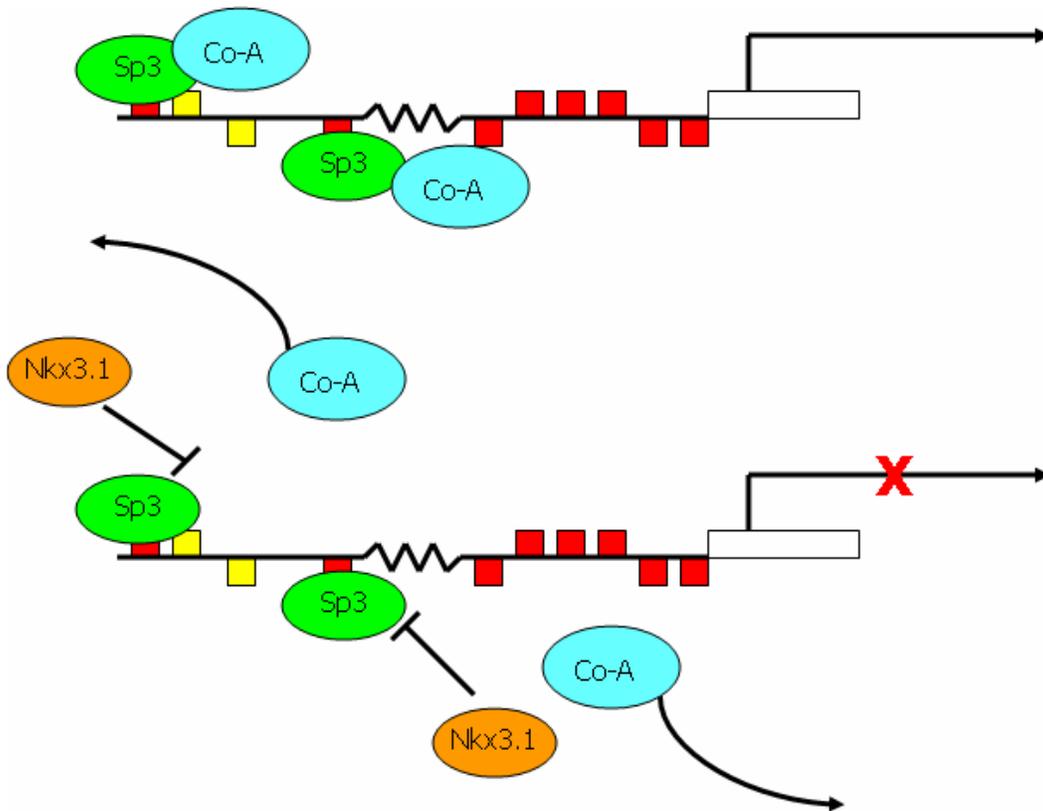
define the function(s) of Nkx3.1 whose loss triggers prostate tumor formation, I was able to unearth important clues about Nkx3.1 function that may ultimately lead to such a determination.

Figure 44. Two models for Nkx3.1 regulation of Sp-mediated transcription in prostate cells. *A*, Nkx3.1 recruits transcriptional co-repressor (Co-R) through direct contact with Sp-proteins or via another DNA-binding protein or *B*, Nkx3.1 disrupts Sp-protein association with required transcriptional co-activator (Co-A).

A



B



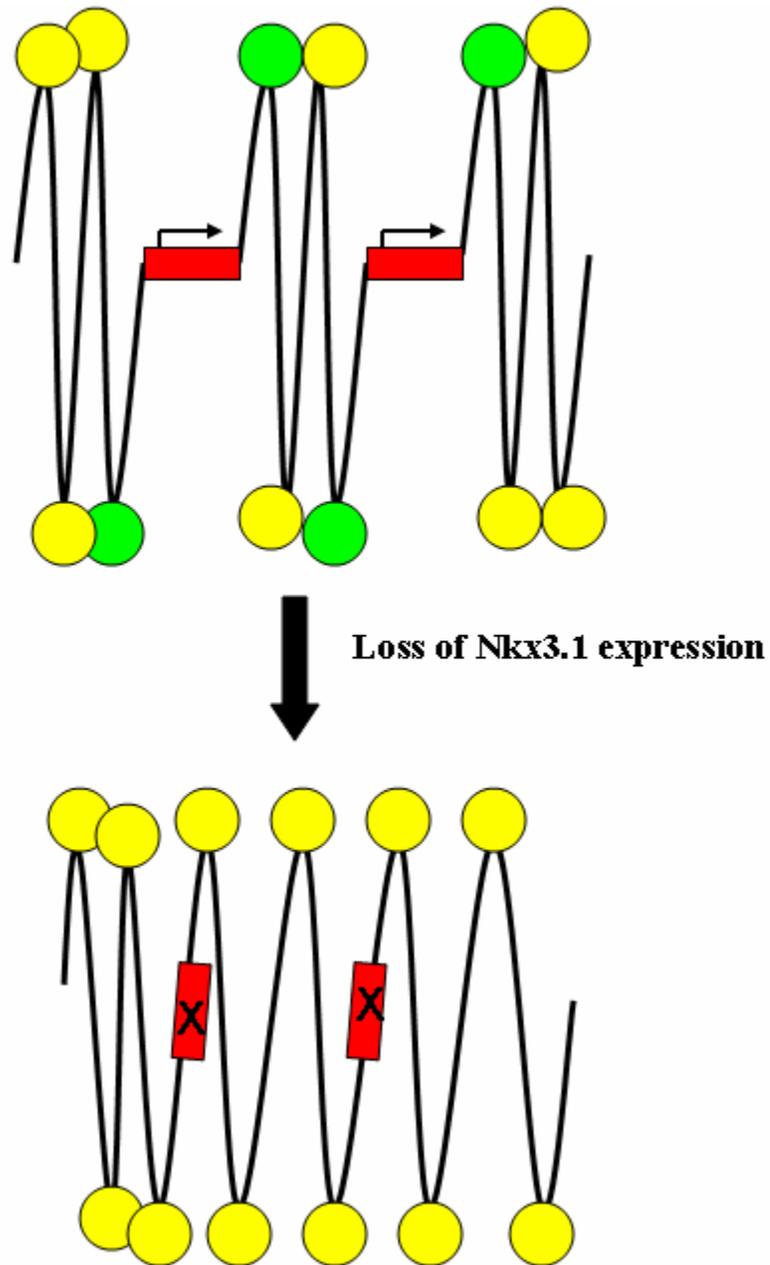


Figure 45. A model for transcriptional regulation by Nkx3.1 nuclear matrix attachment. Nkx3.1 (green circles) and other matrix-associated DNA-binding proteins (yellow circles) attach to the nuclear matrix of prostatic epithelial cells and bind chromatin via matrix attachment regions (MARs) to facilitate access to prostate specific genes (red boxes) by active transcriptional complexes. Loss of Nkx3.1 protein expression results in loss of Nkx3.1-specific chromatin-matrix attachment and deregulation of prostate-specific gene expression.

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