THE ROLE OF NKX3.1 IN THE INITIATION AND PROGRESSION OF PROSTATE CANCER

By

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To my loving husband, Graham, for his endless love, support & encouragement

To my parents and brother, who made me believe all things were possible

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Akt	Protein Kinase B
Aldh2	Aldehyde dehydrogenase 2 family
AP	Anterior prostate
AR	Androgen receptor
ARE	Androgen response element
ARR	Androgen response region
Asns	Asparagine synthetase
Atf3	Activating transcription factor 3
bHLH-Z	Basic helix-loop-helix leucine-zipper
BPH	Benign prostate hyperplasia
BRCA1	Breast cancer 1 susceptibility gene
BRCA2	Breast cancer 2 susceptibility gene
BUG	Bulbourethral gland
CARN	Castration-resistant Nkx3.1-expressing cells
Casp3	Caspase 3
CAT	Chloramphenicol acetyltransferase
CBP	CREB (cAMP response element-binding) binding protein
Ceacam1	Carcinoembryonic antigen-related cell adhesion molecule 1 casp8
Cflar	casp8 and fadd-like apoptosis regulator
CZ	Central zone
DAB	3,3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DHT	Dihydrotestosterone
DKO	$p53^{-/-}$;$Arf^{-/-}$ double knockout
DP	Dorsal prostate
Dpc	Days postcoitum
DTT	Dithiothreitol
ER	Estrogen receptor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gro	Groucho
GRO	Global Run-On
GWAS	Genome-wide association studies
HDAC1	Histone deacetylase 1
HGPIN	High-grade prostate intraepithelial neoplasia
Hk2	Hexokinase 2
HIPK2	Homeodomain-interacting protein kinase 2
HPC1	Hereditary prostate cancer 1

HPC3	Hereditary prostate cancer 3
HPCX	Hereditary prostate cancer X
IGFBP-3	Insulin-like growth factor binding protein
Itpr2	Inositol 1,4,5-trisphosphate receptor, type 2
LGPIN	Low-grade prostate intraepithelial neoplasia
LNCaP	Human prostate adenocarcinoma cells derived from the left supraclavicular lymph node metastasis
LOH	Loss of heterozygosity
LP	Lateral prostate
Mad1	Max dimerization protein 1
Mad3	Max-associated protein 3
Mad4	Max-associated protein 4
MB	Myc Box
MEF	Mouse embryonic fibroblasts
Miz1	Myc-interacting Zn finger protein-1
Mnt	Max binding protein
MOI	Multiplicity of infection
Mt2	Metallothionein-2
MT2A	Metallothionein 2A
Mt3	Metallothionein-3
Mxi1	Max-interacting protein 1
Myc	v-myc myelocytomatosis viral oncogene homolog
MycCap	Prostate cancer cells derived from a c-Myc transgenic mouse
Nedd4L	Neural precursor cell expressed, developmentally down-regulated 4-like
Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
Nkx3.1	Nk3 homeobox 1
NTD	Amino-terminus domain
OHT	4-hydroxytamoxifen
p19ARF	Cyclin-dependent kinase inhibitor 2A
p15Ink4b	Cyclin-dependent kinase inhibitor 2B
p300	E1A binding protein p300
PB	Probasin
PDEF	Prostate epithelial-specific Ets transcription factor
PEI	Polyethylenimine
pHH3	Phospho-histone H3
PI3K	Phosphoinositide 3-kinase
PIN	Prostate intraepithelial neoplasia
Pnpt1	Polyribonucleotide nucleotidyltransferase 1
Prcka	Protein kinase C protein kinase C, alpha

Prdx6	Peroxiredoxin 6
PSA	Prostate-specific antigen
Pten	Phosphatase and tensin homolog
PZ	Peripheral zone
QSOX1	Quiescin Q6 sulfhydryl oxidase 1
Rb	Retinoblastoma 1
RT-PCR	Real time polymerase chain reaction
SCID	Severe combined immunodeficiency
SEER	Surveillance, Epidemiology and End Results Program
Sept9	MLL septin-like fusion protein
Seq	Sequencing
shRNA	Short-hairpin RNA
siRNA	Small interfering RNA
SMGA	Smooth muscle gamma-actin
SMA	Smooth muscle actin
SNP	Single nucleotide polymorphism
Sp1	Specificity protein 1
SRF	Serum response factor
TAD	Transactivation domain
Tip48	48 kDa TATA box-binding protein-interacting protein
Tip49	49 kDa TATA box-binding protein-interacting protein
TN	Tinman
TRRAP	Transformation/transcription domain-associated protein
TSA	Tyramide signal amplification
TSG	Tumor suppressor gene
Txnip	Thioredoxin-interacting protein
TZ	Transitional zone
Ugcg	UDP-glucose ceramide glucosyltransferase
UGE	Urogenital sinus epithelium
UGM	Urogenital sinus mesenchyme
UGS	Urogenital sinus
Utrn	Utrophin
VEGF-C	Vascular endothelial growth factor C
VP	Ventral prostate
VSV-G	Vesicular stomatitis virus glycoprotein
YFP	Yellow Fluorescent protein

CHAPTER I

INTRODUCTION

The Prostate

Prostate Function

The prostate is a male accessory sex gland responsible for supporting and promoting male fertility and insemination. The prostate is composed of small exocrine glands that produce a thin, slightly alkaline fluid, rich in proteins and compounds such as acid phosphatase, citric acid, prostate specific antigen (PSA), cholesterol, zinc and calcium (Aumuller and Seitz, 1990). The alkalinity of prostatic secretions support sperm survival during insemination by neutralizing the acidity of the vaginal tract (Aumuller and Seitz, 1990).

Human Prostate Anatomy

The human prostate gland surrounds the prostatic urethra and sits in front of the rectum, below the urinary bladder (Figure 1). Three separate and distinct fascial layers (prostatic, endopelvic and denonvilliers') and a band of fibromuscular stroma cover the prostate (Ayala et al., 1989; Raychaudhuri and Cahill, 2008). Originally, the prostate was classified into diverse lobes based on studies of fetal prostate glands, however the current

and most widely accepted classification divides the unilobular prostate into four glandular zones: transitional, peripheral, central and fibromuscular (Figure 1) (Lowsley, 1912; McNeal, 1981, 1988).

The transitional zone (TZ) represents 5-10% of the glandular prostate. It surrounds the prostatic urethra and is the site of benign prostatic hyperplasia (BPH) and 20% of prostate carcinomas (McNeal, 1978). The cone-shaped central zone (CZ) comprises 25% of glandular volume. This zone surrounds the ejaculatory ducts and is located between the base of the bladder and the verumontanum (Cohen et al., 2008). Although only 2.5% of tumors originate in this zone, the resulting tumors tend to be more aggressive and more likely to invade the seminal vesicles (Cohen et al., 2008). The peripheral zone (PZ) constitutes 70% of the prostate and is located at the back of the prostate gland, close to the rectum. This zone is responsible for approximately 75% of prostate adenocarcinomas (McNeal, 1969). The final zone, anterior fibromuscular stroma, is a thick, non-glandular tissue that contributes to sphincter function.

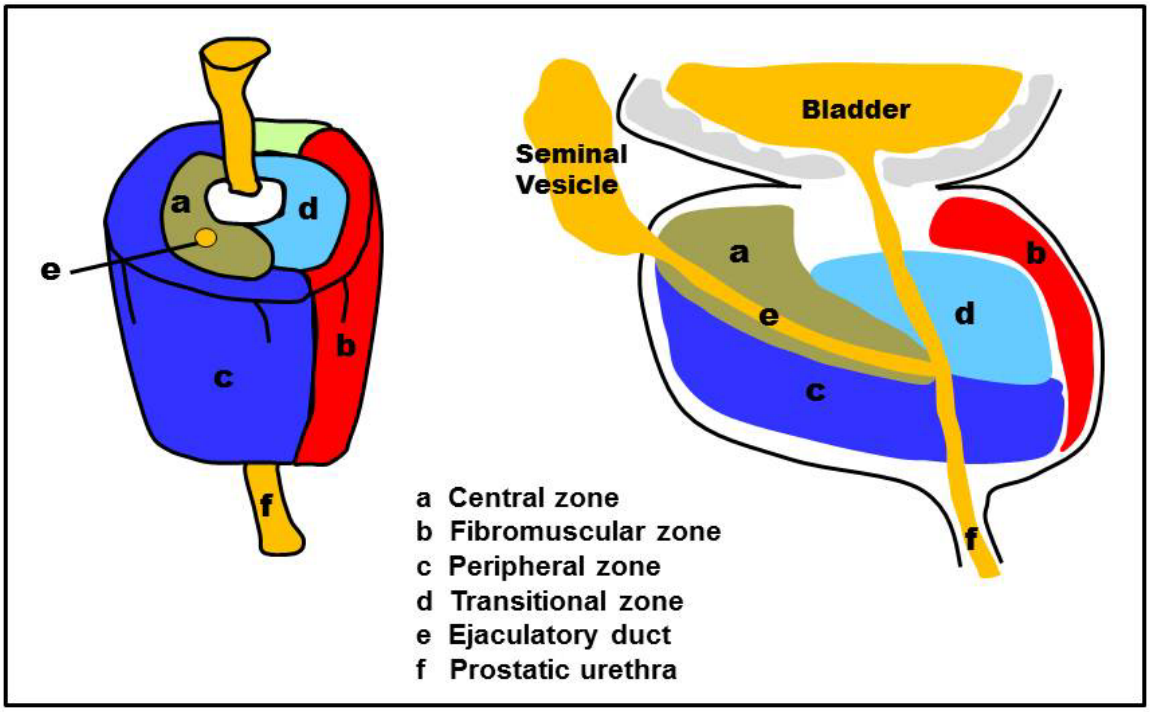

Figure 1. Zonal anatomy of the human prostate. The prostate is subdivided into 4 zones: central zone (a), fibromuscular zone (b), peripheral zone (c) and transitional zone (d).

Murine Prostate Anatomy

In contrast to the human prostate, the murine prostate is composed of multiple lobes arranged at the base of the bladder, surrounding the urethra (Figure 2). These lobes are named after their anatomical position and include the anterior prostate (AP), ventral prostate (VP), dorsal prostate (DP) and lateral prostate (LP) (DP and LP are referred to as the DLP). The ductal morphology, shape, histology and secretion patterns of each lobe are unique (Cunha et al., 1987). For example, histologically the AP displays extensive epithelial-infolding, while the DLP has less infolding and the VP shows minimal infolding (Cunha et al., 2004). Analogy between murine lobular structures and zonal structures of the human prostate remain elusive. In spite of the anatomical differences, studies have shown that organogenesis and steroid responsiveness of both murine and human prostates are similar (Meeks and Schaeffer, 2011).

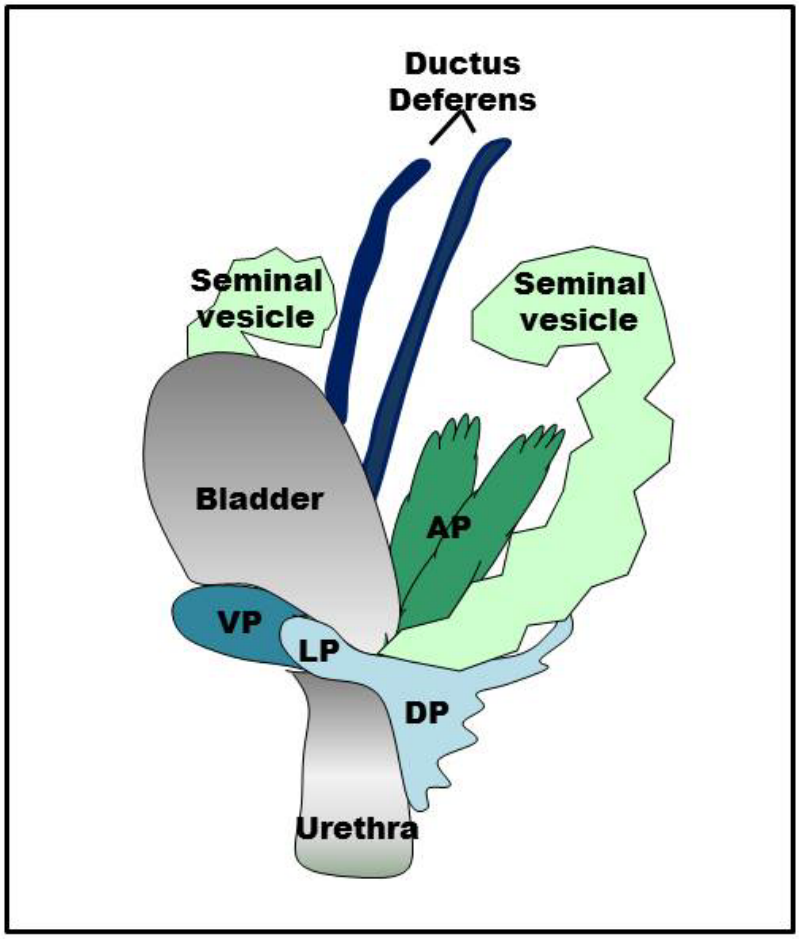

Figure 2. Adult murine prostate structure (lateral view). The murine prostate consists of 4 distinct lobes: anterior prostate (AP), dorsal prostate (DP), lateral prostate (LP) and ventral prostate (VP). [Modified from (Sugimura et al., 1986)].

Prostate Development

The prostate originates from the urogenital sinus (UGS), an ambisexual endodermal tube derived from the hindgut that also develops into the prostatic urethra and bulbourethral glands (BUGs) (Staack et al., 2003). The UGS is composed of both epithelial (UGE) and mesenchymal (UGM) cells. Intimate interactions between the epithelial and mesenchymal tissue are essential for prostate development. At approximately 8 weeks gestation in humans and 13.5 days postcoitum (dpc) in mice, fetal testicular androgens are produced and engage the UGM androgen receptor (AR) (Cooke et al., 1991; Shannon and Cunha, 1983; Staack et al., 2003; Takeda et al., 1985). Androgen regulated signals from the UGM stimulate the epithelium to initiate prostatic budding around week 10 in the human fetus and 17 dpc in mice (Staack et al., 2003; Sugimura et al., 1986; Timms, 2008). The prostatic epithelial buds elongate into cords and undergo extensive ductal outgrowth, branching and canalization into the surrounding mesenchymal cells (Sugimura et al., 1986) As canalization ensues, the epithelium differentiates into luminal and basal epithelial cells while the UGM differentiates into prostatic smooth muscle and interfascicular fibroblasts (Cunha et al., 2004; Hayward et al., 1996a; Hayward et al., 1996b; Hayward and Cunha, 2000).

Postnatal, the human prostate undergoes minimal size increase and differentiation. However, during puberty, testicular androgen levels increase and trigger an exponential increase in prostatic growth, from 2 grams to 20 grams (Berry et al., 1984; Hayward and Cunha, 2000). The mature prostate increases in size with age.

Cell Types in Prostatic Ducts

There are three morphologically and functionally distinct epithelial cell types that together construct a mature prostate: luminal, basal and neuroendocrine (Figure 3). Luminal epithelial cells are the dominant cell type within the prostate. These tall columnar cells are identified by expression of AR, cytokeratin 8 and 18, and cell surface marker CD57 (Cunha et al., 1987; Liu et al., 1997). These cells are dependent on androgens and produce prostatic secretory proteins including PSA and prostatic acid phosphatase, which are secreted in seminal fluid. Basal epithelial cells are small, flat and form a continuous layer along the basement membrane. These non-secretory, androgen-independent cells express cytokeratin 5 and 14, p63 (homolog of the p53 tumor suppressor) and cell surface marker CD44 (Liu et al., 1997; Signoretti et al., 2000; Tran et al., 2002). The last cell type, neuroendocrine cells, represents a very small fraction of the prostatic duct and are sparsely scattered throughout the basal layer. These cells are morphologically indistinguishable from basal cells but release neuropeptides (ie. synaptophysin) and express secretory proteins (ie. chromogranin A) (Abate-Shen and Shen, 2000; Matusik et al., 2008).

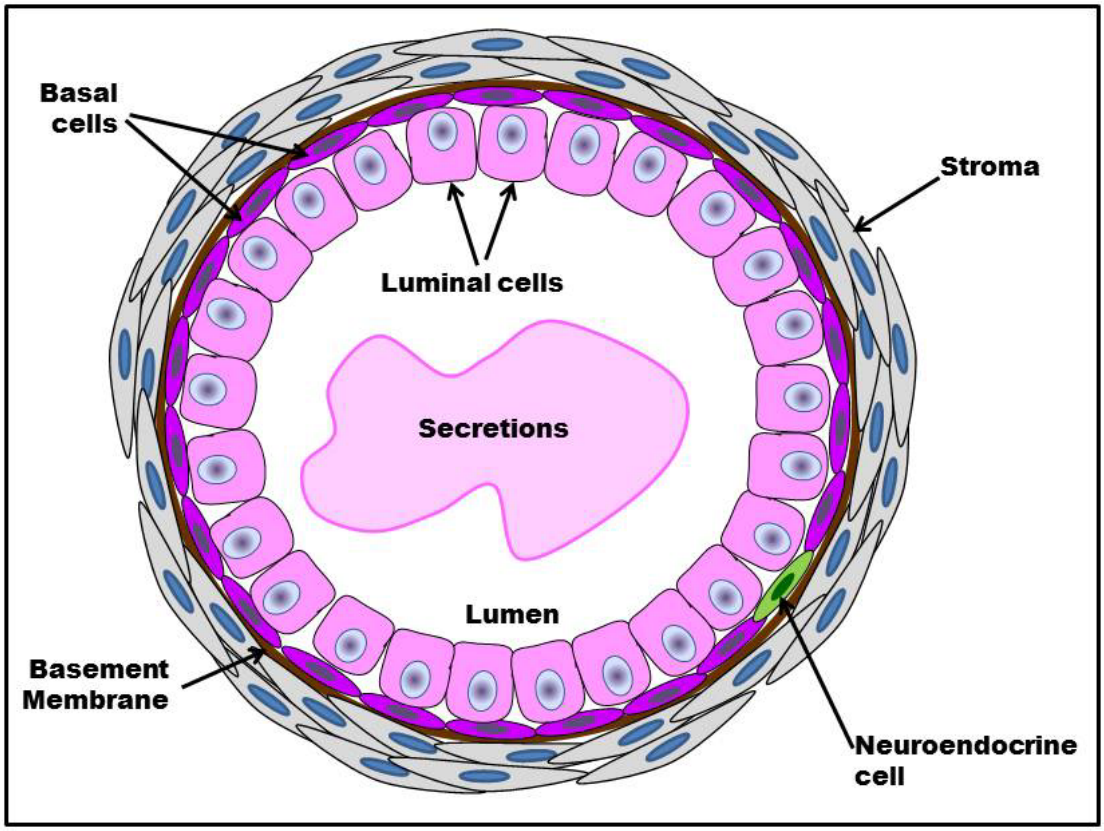

Figure 3. Cell types within mature prostatic ducts. The prostatic duct includes three types of epithelial cells (luminal, basal and neuroendocrine). A continuous basement membrane layer separates epithelial cells from stromal cells. Prostatic secretions are excreted into the lumen of the prostatic duct.

Prostate Stem Cells

Stem cells are classified according to their self-renewal capacity and potential to differentiate into multiple cellular lineages. In general, stem cells divide asymmetrically producing one daughter cell that retains the parental stem cell properties while the other daughter becomes an intermediate (transit amplifying) cell population with limited proliferative potential. As transit amplifying cells mature they terminally differentiate into a specific cell type. Stem cells are limited in number and are supported within a defined niche that maintains balance between quiescence and self-renewal (Lang et al., 2009).

Murine castration results in prostatic involution and loss of luminal epithelial cells. Re-administration of androgen causes the residual basal cells to proliferate and regenerate luminal and neuroendocrine cells. Based on these observations, Isaacs and Coffey proposed that pluripotent stem cells for the entire prostate resided in the basal layer and gave rise to progenitor cells that terminally differentiated into luminal and neuroendocrine cells (Isaacs and Coffey, 1989). The origin of prostate stem cells, basal or luminal has become a topic of controversy. Several candidate stem/transient cell populations have been identified suggesting that a variety of stem cells exist within the prostatic duct. Basal candidates include p63, CD133, Sca1+, CD117; luminal candidates include castration-resistant Nk3 homeobox 1 (Nkx3.1)-expressing cells (CARNs) (Leong et al., 2008; Richardson et al., 2004; Signoretti et al., 2005; Signoretti et al., 2000; Wang et al., 2009).

Initial studies of p63 showed that expression was essential for basal cell differentiation but not required for luminal or neuroendocrine differentiation (Kurita et al., 2004). An independent study showed that p63 expression was required for normal prostate development and maintained commitment to the prostate cell lineage, including secretory cells (Signoretti et al., 2005; Signoretti et al., 2000). CD133-expressing cells were shown to have an increased proliferative potential and formed fully differentiated prostate-like acini when grafted in immunocompromised mice (Richardson et al., 2004). Sca1+ cells were also indicated as prostatic stem cells. Sca1+ cells isolated from the proximal region of the prostate regenerated functional prostatic ducts *in vivo* (Xin et al., 2005). More recently, CD117 was identified as a prostate stem cell marker. *In vitro* studies showed that CD117+ cells gave rise to lumen-containing colonies (Leong et al., 2008). In prostate regeneration studies, CD117+ grafts produced functional prostate glands with ductal branching and luminal and neuroendocrine cells which were retained following serial transplantation (Leong et al., 2008). Remarkably, authors showed that a single CD117+ cell regenerated functional prostates (Leong et al., 2008).

In 2009, Wang et al. provided evidence for the existence of a luminal stem cell population, CARNs. As the name insinuates, CARNs are rare androgen-independent luminal cells that express Nkx3.1. In prostate regeneration studies, genetically labeled CARNs displayed bipotentiality and self-renewal capacity (Wang et al., 2009). Single-cell CARN transplantation reconstituted prostate ducts composed of basal, luminal and neuroendocrine cells (Wang et al., 2009).

Prostate Cancer

Incidence & Risk Factors

Prostate cancer is the second leading cancer among American men and a leading cause of cancer-related mortality (Howlader N, 2010). It was estimated that approximately 240,890 new diagnoses would be made and approximately 33,720 men would die from prostate cancer in the United States in 2011 (Howlader N, 2010). Furthermore, it is estimated that approximately 1 in 6 men will be diagnosed with prostate cancer within their lifetime (Howlader N, 2010). Several risk factors are suggested to play a role in prostate cancer development including, but not limited to, age, genetics and steroid hormones.

Age

Age is the most significant risk factor in prostate cancer; a sharp increase in incidence with age is a hallmark of this disease. Men 65 and older hold the highest risk. According to a study by the National Cancer Institute Surveillance, Epidemiology and End Results (SEER) Program, 60% of all newly diagnosed prostate cancer cases and almost 80% of all deaths occur in men 70 years of age and older (Howlader N, 2010).

Genetics

Genetic mapping and linkage studies of prostate cancer have identified genetic mutations that confer a Mendelian pattern of inheritance (Gann, 2002). It is estimated that hereditary factors account for 10-40% of prostate cancers and are typically associated

with early onset (Gann, 2002). A number of genetic cancer susceptibility genes have been identified including hereditary prostate cancer 1 (HPC1), hereditary prostate cancer x (HPCX), hereditary prostate cancer 3 (HPC3), breast cancer 1 susceptibility gene (BRCA1) and breast cancer 2 susceptibility gene (BRCA2) (Berry et al., 2000a; Berry et al., 2000b; Bock et al., 2001; Cunningham et al., 2003; Farnham et al., 2005; Gronberg et al., 1997a; Gronberg et al., 1997b; Liede et al., 2004; Smith et al., 1996; Thompson and Easton, 2002; Xu et al., 1998). HPC1 and BRCA2 were considered high penetrance genes and accounted for 4-13% and 5%, respectively, of hereditary prostate cancers examined (Colloca and Venturino, 2011). Furthermore, men harboring a mutation in BRCA2 had a 20% increased risk of developing aggressive prostate cancer (Edwards et al., 2010; Edwards et al., 2003; Kote-Jarai et al., 2011). Genome-wide association studies (GWAS) have identified over 40 single nucleotide polymorphisms (SNPs) linked to increased prostate cancer (Goh et al., 2012). These SNPs have the potential to offer clinical advantages such as risk prediction, treatment efficacy and toxicity.

Steroid Hormones

Signaling between steroid hormones and their receptors plays a critical role in all stages of prostate tumorigenesis. AR maintains strong activity in the absence of circulating androgens and is frequently mutated in advanced cancers. The tumor's resilience to retaining AR signaling suggests that it plays a role in tumor maintenance and progression. Androgens are required for the growth, development and maintenance of the prostate and have been associated with increased prostate cancer risk. Chronic testosterone treatment

in five different rat models resulted in increased prostate cancer incidence (Hoover et al., 1990; Noble, 1977; Pollard and Luckert, 1985, 1986, 1987; Pour and Stepan, 1987). Dihydrotestosterone (DHT), a potent androgenic metabolite, has also been associated with prostate cancer and BPH. Testosterone is converted to DHT by the enzyme 5α-reductase (Bruchovsky and Wilson, 1968; Farnsworth and Brown, 1963a, b). Clinical studies with 5α-reductase inhibitors, in men of average age, showed a 25% reduction in prostate cancer risk (Andriole et al., 2004a; Andriole et al., 2004b; Bosland and Mahmoud, 2011; Goodman et al., 2004; Thompson et al., 2003).

Estrogen has also been shown to play a role in tumorigenesis of several organs including the prostate (Liehr, 2000). A significant percentage of men age 60 years and older have decreased serum testosterone levels compared to middle aged men (Araujo et al., 2004; Liu et al., 2007). Although testosterone levels decrease, estrogen levels remain steady or sometimes increase. The shift in the testosterone:estrogen ratio (high estrogen and low testosterone) has been implicated in prostate cancer development. The enzyme aromatase, expressed in human and rodent prostates, catalyzes the conversion of testosterone to estrogen. Prostate tumors were shown to have increased aromatase expression and enzymatic activity (Ellem et al., 2004; Hiramatsu et al., 1997; Matzkin and Soloway, 1992; Stone et al., 1987b; Tsugaya et al., 1996). Furthermore, when Noble rates were treated with a combination of estradiol and testosterone, prostate cancer incidence increased to 90-100% (Bosland et al., 1995).

Molecular Genetics of Prostate Cancer

The etiology of human prostate cancer is not well understood. Disease progression is hypothesized to involve the accumulation of genetic mutations and/or epigenetic changes which influence morphological alterations. In this multi-step model of progression normal prostate glands are transformed to prostatic intraepithelial neoplasia (PIN) which advances to invasive carcinoma and ultimately metastasis (Figure 4). PIN is characterized by increased cellular proliferation, hyperchromatia, nuclear enlargement, nucleolus enlargement and preservation of the basal layer and basement membrane. There are four major architectural patterns for PIN: flat, cribriform, tufting and micropapillary, however these patterns do not dictate clinical significance (Bostwick and Cheng, 2012; Bostwick et al., 2004; Bostwick and Qian, 2004; Epstein, 2009). PIN is subcategorized into low-grade (LGPIN) and high-grade (HGPIN); HGPIN is distinguishable from LGPIN by the presence of prominent nucleoli and progressive disruption of the basal cell layer. HGPIN also displays molecular abnormalities that overlap with carcinoma including cytologic changes, increased cellular growth, chromosomal aberrations and biomarker expression (Table 1, Figure 5) (Bostwick et al., 1996; Bostwick and Qian, 2004; Nagle et al., 1992). Consequently, HGPIN is recognized as the precursor lesion to locally invasive adenocarcinoma. Microinvasion through the basement membrane by transformed luminal epithelial cells symbolizes the progression from HGPIN to invasive carcinoma. More aggressive lesions invade the neighboring seminal vesicles or metastasize to other sites (primarily bone and lymph node) (Abate-

Shen and Shen, 2000). These more aggressive tumors usually account for prostate cancer-related mortality.

Several chromosomal alterations have been identified at various stages throughout the progression of prostate cancer (Table 2). The most frequent chromosomal alterations are loss of 8p, 10q, 13q, 16q and gains of 7p, 7q, 8q and Xq (Abate-Shen and Shen, 2000; Elo and Visakorpi, 2001; Mazzucchelli et al., 2004; Nelson et al., 2003; Porkka and Visakorpi, 2004). Tumor susceptibility genes associated with these chromosomal aberrations include Nkx3.1, Pten (phosphatase and tensin homolog), Rb (retinoblastoma 1), c-Myc (v-myc myelocytomatosis viral oncogene homolog) and AR.

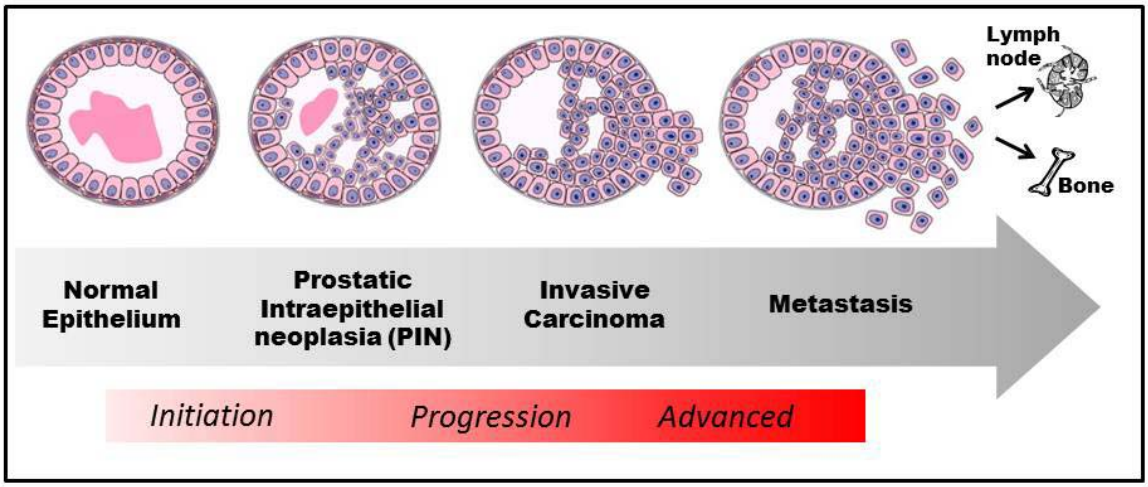

Figure 4. Multi-step model of prostate cancer progression. Transformation of normal prostate luminal epithelium results in PIN development, followed by invasive carcinoma and eventually metastasis. Prostate cancer cells typically metastasize to the lymph node and bone. [Adapted from (Abate-Shen and Shen, 2000)].

Table 1. Common features identified among prostate HGPIN lesions

Characteristics of HGPIN Lesions
Apoptotic Bodies
Chromatin Clearing
Increased Cellular Proliferation
Loss of Basal Epithelial Cells
Mitotic Figures
Nuclear Enlargement
Nucleolar Enlargement

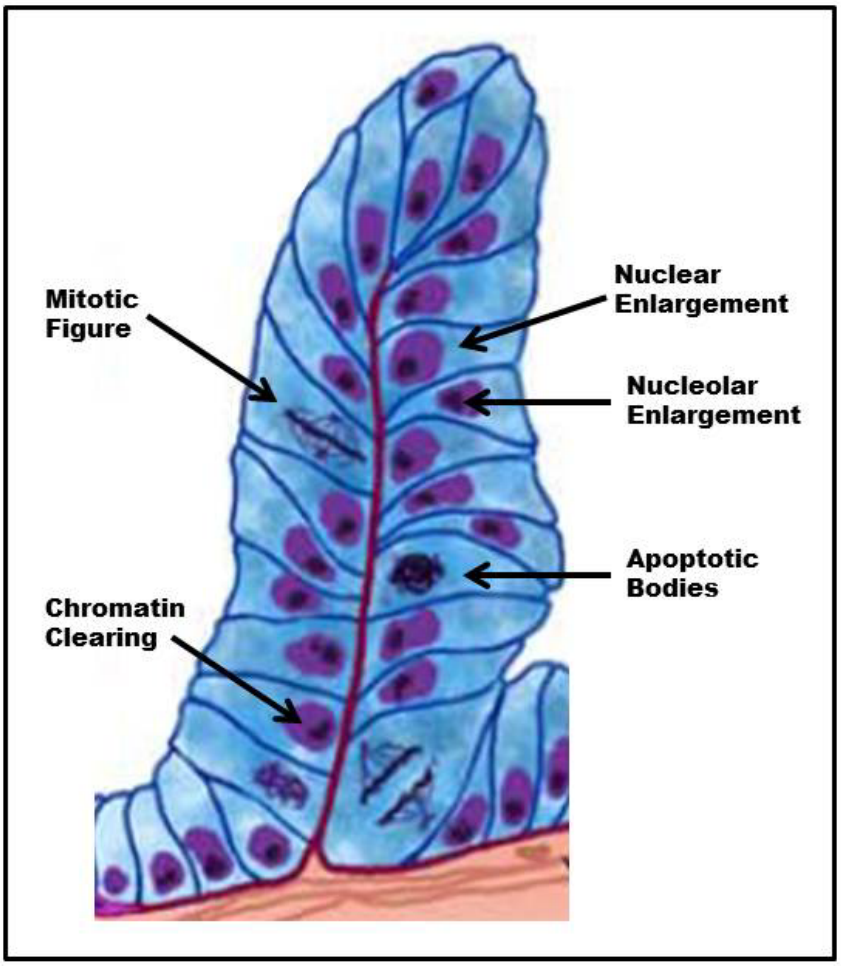

Figure 5. Morphological features of HGPIN lesions. Characteristic changes of HGPIN lesions include nuclear enlargement, nucleolar enlargement, increased mitotic figures, apoptotic bodies and chromatin clearing (loss of homogenously distributed chromatin). [Modified from (Iwata et al., 2010)].

Table 2. Susceptibility genes associated with prostate cancer

GENE	LOCATION	FUNCTION	ALLELIC ALTERATION	CANCER STAGE
Nkx3.1	8p21	transcription factor	deletion, LOH	initiation
c-Myc	8q24	transcription factor	amplification	initiation
Pten	10q23	phosphatase	deletion, LOH	progression
p27	12p13	cyclin-dependent kinase inhibitor	deletion, LOH	progression
Rb	13q14	cell cycle regulator	deletion	progression
p53	17p13	transcription factor/apoptotic regulator	deletion, mutations	advanced carcinoma, metastasis
AR	Xq11-12	nuclear hormone receptor	amplification, mutations	advanced carcinoma, metastasis, castration-resistance

Models of Prostate Cancer

Non-mouse Models

Human prostate cancer is a complex and clinically unpredictable disease. Tumor lesions are multifocal and heterogeneous (genetically and morphologically) making it difficult to obtain homogenous biopsy tissue for analysis. Recent techniques such as laser capture and microdissection have partially addressed this issue, however additional efforts are needed to elucidate the molecular pathways involved in prostate cancer initiation and progression. This information will aid in identifying molecular markers of disease progression and development of more effective treatments.

Canine

Animal models of prostate cancer have made significant contributions in defining molecular mechanisms involved in prostate tumorigenesis. Next to humans, canines are the only animals known to spontaneously develop HGPIN and adenocarcinoma (Aquilina et al., 1998; Waters et al., 1998). Furthermore, canine prostate cancer is age dependent, displays heterogeneity and metastasizes to the bone (Waters et al., 1998). Despite the large degree of similarity to human prostate cancer, there are several limitations to the canine model including low incidence, long latency, high cost, long gestation, and inability to genetically mutate.

Rat

Rats have also been used to model human prostate cancer. Several rat strains spontaneously develop prostate tumors but these lesions have long latency and lack metastasis (Jeet et al., 2010). Genetic manipulation is feasible with this model but induced prostate tumor lesions display significant variability. Despite the inconsistencies observed, rat models have contributed to understanding stromal-epithelial interactions in prostate cancer and the role of hormones in carcinogenesis (Bosland et al., 1995; Hoover et al., 1990; Lamb and Zhang, 2005; Noble, 1977; Pollard and Luckert, 1985, 1986, 1987; Pour and Stepan, 1987).

Mouse Models

Mouse models of prostate cancer are frequently used and have made significant contributions in identifying molecular mechanisms involved in disease initiation and progression. Similar to other animal models, there are distinct differences between mice and humans. First, mice do not spontaneously develop prostate cancer. Second, the human prostate has one lobe, while the mouse prostate consists of four lobes. Third, mice have a relatively short lifespan. Finally, cancer metastasis has a tendency to initiate from mesenchymal cells in mice compared to epithelial cells in humans (Valkenburg and Williams, 2011). Despite these differences, human and mouse genomes are ~95% identical with structurally similar genes that are easy to manipulate (de Jong and Maina, 2010). More importantly, genetically engineered mice display a high incidence of tumor

development and progression. Furthermore, mice are small, easy to handle and typically produce large litters making generation within a reasonable amount of time attainable. Thus, mice provide significant advantages to other animal models (Table 3).

Nkx3.1 Germline Knockout

Various techniques have been employed to evaluate the role of specific genes in prostate tumorigenesis. Germline knockout models are genetically engineered mice that harbor an inactivated or deleted gene. These models enable researchers to investigate the function of tumor suppressor genes (TSG). Creation of a germline knockout involves homologous recombination in embryonic stem cells and results in global expression of the mutation during embryogenesis. Inactivation or deletion of genes essential for organism growth, development and survival causes embryonic or premature lethality, a major caveat to this technique. Nevertheless, germline mutant mouse models have highlighted the role of many TSGs such as Nkx3.1, p27 and Pten in early stages of prostate tumorigenesis (Table 3).

Nkx3.1 is a transcription factor with tumor suppressing activity. Human Nkx3.1 maps to chromosomal region 8p21, a region that is frequently lost in prostate cancer. Using homologous recombination, mice lacking Nkx3.1 were generated by deleting the gene's second exon corresponding to the homeodomain. $Nkx3.1^{-/-}$ mice developed normally and were fertile, however males experienced difficulty forming copulatory plugs as they aged (Abdulkadir et al., 2002; Bhatia-Gaur et al., 1999). Abnormal phenotypes were only observed in the prostate and minor salivary glands (Schneider et al., 2000). The prostates

of Nkx3.1 mutant mice were smaller in size and displayed impaired ductal morphogenesis and defects in prostatic secretory protein production (Schneider et al., 2000; Tanaka et al., 2000). Loss of Nkx3.1 led to an extended proliferative phase in the luminal epithelial cells (Magee et al., 2003). Furthermore, $Nkx3.1^{-/-}$ prostate glands developed epithelial hyperplasia and dysplasia that advanced with age (Abdulkadir et al., 2002; Bhatia-Gaur et al., 1999; Schneider et al., 2000; Tanaka et al., 2000). Notably, heterozygous expression of Nkx3.1 resulted in a similar, but less severe phenotype compared to homozygous mutant mice, indicating dosage sensitivity (haploinsufficiency). Although $Nkx3.1^{-/-}$ mice developed LGPIN, the lesions failed to progress to invasive carcinoma (Bhatia-Gaur et al., 1999; Schneider et al., 2000). These results indicate that allelic deletion of Nkx3.1 plays a role in tumor initiation but alone is incapable of progressing to prostate adenocarcinoma.

Nkx3.1 Conditional Knockout

The conditional gene knockout model is a derivative of the germline knockout technique. Unlike germline mutants, conditional knockout models yield tissue-specific gene deletion. One significant advantage of this technique is the ability to investigate the function of embryonic lethal genes. For conditional mutagenesis, the target gene is flanked (floxed) by two loxP sites (short DNA recognition sequences) and introduced into mice. The floxed strain is bred with transgenic mice expressing tissue-specific Cre-recombinase, resulting in recombination between loxP sites and deletion of the floxed gene.

To evaluate the consequences of prostate-specific deletion of Nkx3.1 and more closely mimic loss observed in humans, conditional Nkx3.1 mutant mice were developed. Prostate-specific Cre-expression was driven under the control of the human PSA promoter. PSA is an androgen regulated serine protease expressed in human prostatic luminal epithelial cells. A 6 kb fragment of the PSA promoter, containing two androgen response elements (ARE) was used to generate these mice (Abdulkadir et al., 2002; Cleutjens et al., 1997). Conditional deletion of Nkx3.1 occurred in the luminal epithelium of mature mice and resulted hyperplasia and LGPIN (Abdulkadir et al., 2002). These data are consistent with observations in the Nkx3.1 germline mutants, corroborating Nkx3.1's role in tumor initiation.

c-Myc Conditional Overexpression

Conditional mouse models that overexpress a target gene have also been developed. These models employ mechanisms to activate tissue-specific expression of a target gene. One such gene that has been studied using this technique is c-Myc. c-Myc is a transcription factor that plays a critical role in development and is frequently amplified in prostate cancer. In 2003, two c-Myc transgenic models were generated using promoters with different strengths: Lo-Myc and Hi-Myc. Lo-Myc mice utilized the rat probasin promoter (PB) to drive low expression of the human c-Myc transgene (Ellwood-Yen et al., 2003). The PB promoter contains two distinct AR binding sites, ARBS-1 and ARBS-2 (collectively named androgen response region or ARR) and drives high levels of transgene expression to all lobes of the mouse prostate (Gingrich et al., 1996). On the

other hand, Hi-Myc mice comprised the ARR_2PB promoter bearing two additional ARRs linked to the PB promoter (Ellwood-Yen et al., 2003; Kasper et al., 1994). Mice from both transgenic mice developed PIN followed by invasive carcinoma, although the Lo-Myc mice showed longer latency (Ellwood-Yen et al., 2003). Lesions from these mice displayed increased proliferation, apoptosis and microvessel density but did not progress to metastatic disease (Ellwood-Yen et al., 2003). In these mice, castration at early stages of prostate tumor development resulted in complete regression, but this was not recapitulated in older mice. Since c-Myc expression is driven under the control of androgen regulated promoters, it is unknown if tumor regression resulted from androgen ablation or decreased c-Myc transgene expression. Interestingly, Nkx3.1 protein expression was decreased in PIN lesions and completely lost in tumors from both Lo-Myc and Hi-Myc mice. Microarray analysis of these tumors also showed decreased Nkx3.1 mRNA expression. This suggests that Nkx3.1 loss is a favorable mutation for c-Myc-initiated tumors and implies cooperation between these transcription factors.

To determine if low levels of c-Myc could transform mouse prostate epithelial cells, Iwata et. al. developed Super-Lo c-Myc mice. To drive prostate-specific gene expression of human c-Myc, the authors used an Nkx3.1 cis-acting region (Iwata et al., 2010). The resultant low levels of Myc expression in Super-Lo Myc mice produced LGPIN lesions with increased proliferation and apoptosis (Iwata et al., 2010).

Our laboratory generated a transgenic c-Myc mouse model, PBCre:Z-Myc, that displayed focal c-Myc expression in the prostate luminal epithelium (Kim et al., 2009; Roh et al.,

2006). These mice carried a single copy transgene which included a CMV enhancer/β-actin promoter and a latent c-Myc transgene. c-Myc expression in these mice resulted in mild pathology up to 2 years of age; most prostates displayed normal histology and a small percentage showed LGPIN.

Table 3. Selected mouse models of prostate cancer

MODEL	PRENEOPLASTIC LESIONS	CARCINOMA LESIONS
Nkx3.1$^{-/-}$	hyperplasia, dysplasia	NO
PSA-Cre;Nkx3.1$^{f/f}$	hyperplasia, dysplasia, LGPIN	NO
Pten$^{+/-}$	hyperplasia, HGPIN	NO
PBCre;Pten$^{f/f}$	HGPIN	adenocarcinoma
Pten$^{+/-}$;Nkx3.1$^{+/-}$	hyperplasia, HGPIN	adenocarcinoma, lymph node metastasis
ARR$_2$PB-Myc (Hi-Myc)	HGPIN	adenocarcinoma
PBCre4;Z-Myc	LGPIN	NO
PBCre;Z-Myc;Pten$^{f/+}$	HGPIN	adenocarcinoma
PBCre;Z-Myc; Pten$^{f/+}$;p53$^{f/+}$	HGPIN	adenocarcinoma
p53$^{-/-}$;Rb$^{-/-}$	hyperplasia, HGPIN	adenocarcinoma, neuroendocrine cancer, lymph node metastasis, lungs, liver, adrenal glands

Prostate Regeneration

While genetically engineered mice have proven to be useful in studying the molecular mechanisms of prostate tumorigenesis, generation is time consuming, taking a minimum of 18-24 months. Tissue recombination is a technique that has been used for over 30 years to study mesenchymal-epithelial interactions that drive urogenital tract development. This system proficiently enables proliferation and regeneration of prostatic glands with tubular branching morphology (Cunha, 2008). To date, this technique has been used to research urogenital development, the role of steroid hormones in the developing urogenital system, benign and malignant disease progression, the function of embryonic lethal genes in cancer development and epithelial plasticity (Hayward, 2002). Tissue recombinants recapitulated the histopathologic features of intact transgenic mice and provide a suitable model for investigating prostate carcinogenesis (Ishii et al., 2005). In general, tissue recombinants are made by combining epithelial and fetal mesenchymal cells and grafting underneath the renal capsule of a rodent host (Figure 6). A large degree of variability exists in the preparation of tissues for recombinants including species of epithelia and mesenchyme, cellular composition (single cell suspensions or tissue pieces) and genetic manipulation of cellular components. Although the renal capsule is the principal and most tolerable site for grafting, other areas include subcutaneous, intra-optical, chick chorioallantoic membrane and orthotopic (Hayward, 2002). When studying metastasis and androgen ablation, intraductal grafting is optimal. Grafting tissue recombinants *in vivo* under the renal capsule for 4 weeks recapitulated the

development, differentiation and functional expression of adult intact mouse prostates (Cunha, 2008). Homotypic grafts (epithelial and stromal cells from the same organ) induced and supported normal growth, but heterotypic grafts (epithelial and stromal cells from different organs) might induce a different developmental program (Figure 7) (Cunha, 2008). Following a period of growth and development, the host animal is sacrificed and the graft is removed and subjected to further analysis.

The flexibility offered by tissue recombination augments the advantages to using this model. First and foremost, tissue recombination is cost-effective and requires less time than generating a transgenic model. One prostate can be used to create several recombinants, which instantly increases the sample size. Cells can be manipulated by retroviral or lentiviral infection before grafting, which allows one to evaluate tumorigenesis initiated from a small mutant population. Furthermore, natural interactions between mesenchyme and epithelial cells facilitate regenerated prostate growth.

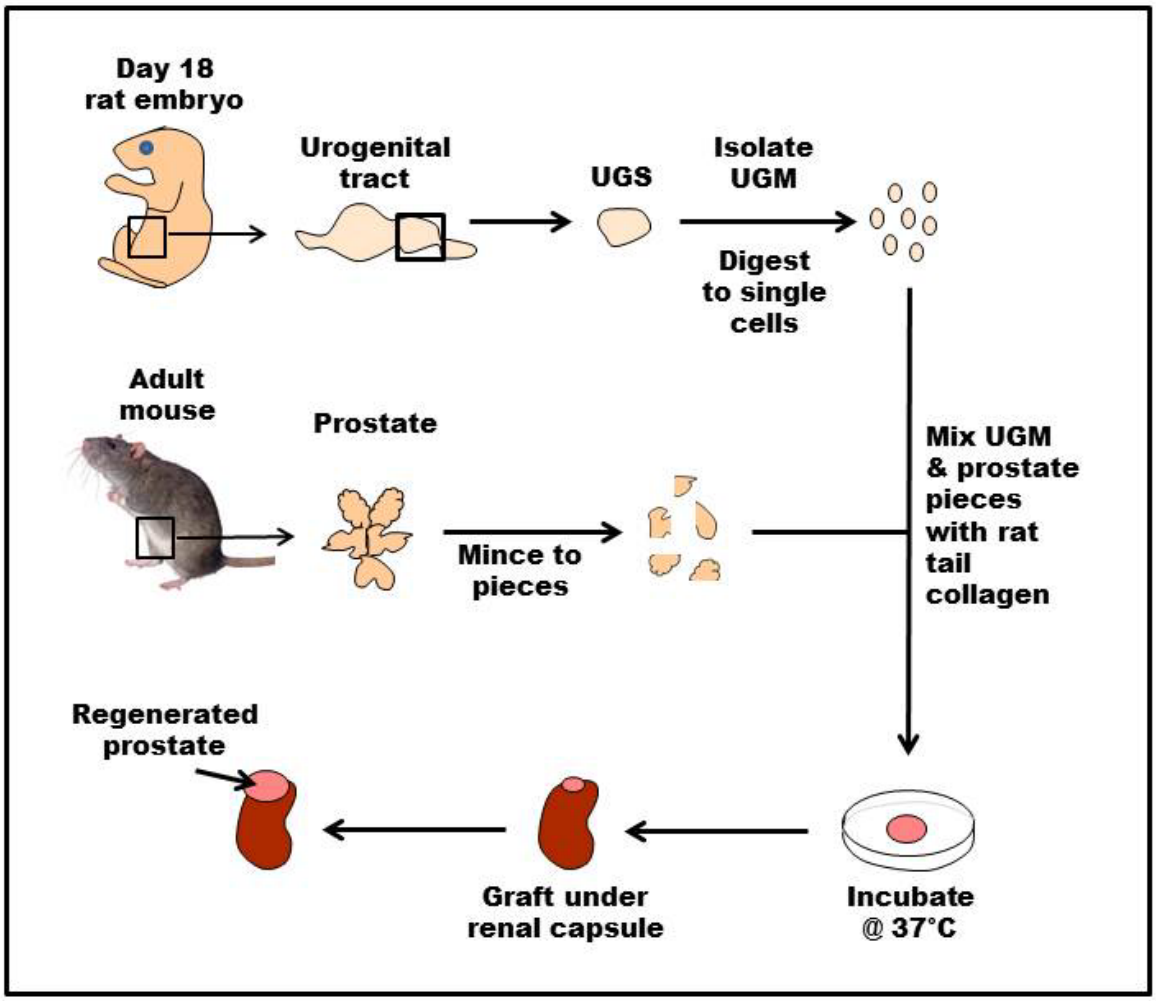

Figure 6. Prostate tissue regeneration methods. Fetal rat UGM (single cells or pieces) and adult prostate (single cells or pieces) are combined with collagen and grafted under the renal capsule of immunocompromised mice. Tissue recombinants are allowed to grow for ~6-10 weeks.

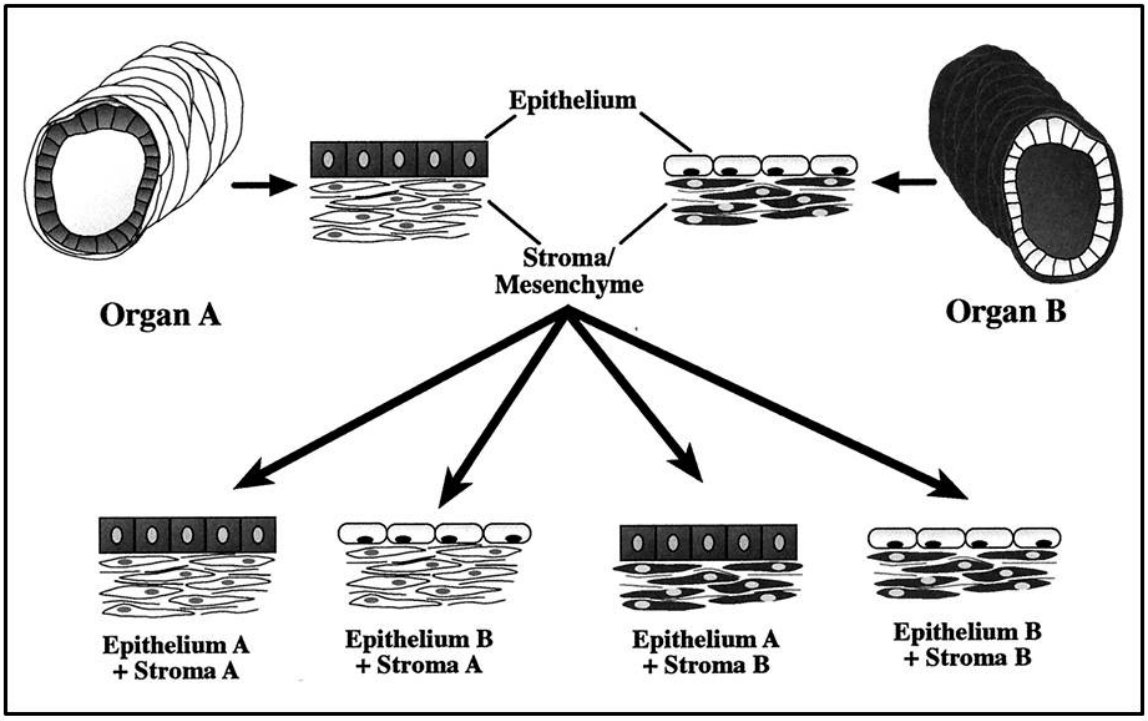

Figure 7. Homotypic and heterotypic prostate tissue recombinants. Stromal and epithelial tissues are separated and isolated. Recombinants can be made using homotypic (stromal and epithelial cells from the same organ) or heterotypic (stromal and epithelial cells from different organs) combinations. [Adapted from (Hayward, 2002)].

Homeobox Genes

Homeobox genes are a class of transcription factors that regulate spatial and temporal patterns during development and differentiation. The homeobox, a 180 nucleotide DNA sequence, was originally identified in Drosophila (McGinnis et al., 1984; Scott and Weiner, 1984), but later discovered to be well-conserved among all metazoa including sponges, vertebrates, humans and plants (Gehring et al., 1994). The homeobox encodes a 60 amino acid structure known as the homeodomain; a motif responsible for sequence-specific DNA binding. Structurally, the homeodomain consists of three well-defined α-helices that fold into a helix-turn-helix motif and a flexible fourth helix which elongates helix III. Three areas within the homeodomain contact DNA: helix III/IV (the recognition helix) binds to the major groove; the loop connecting helix I and II establishes contact with the backbone and the N-terminal arm binds specific residues in the minor groove (Gehring et al., 1994).

In 1989, four homeogenes from Drosophila were discovered by Nirenberg and Kim and designated NK1-4 (Kim and Nirenberg, 1989). Subsequent mutational analyses led to these genes being renamed to describe their mutant Drosophila phenotypes: NK1, slouch (slou); NK2, ventral nervous system defective (vnd); NK3, bagpipe (bap) and NK4, tinman (tin) (Holland, 2001). The NK cluster is now recognized to contain a total of seven highly conserved genes (Holland, 2001). The expression pattern of these genes

vary, however all seven genes play critical roles in cell fate specification and developmental differentiation in many species.

Structure of Nkx3.1

There are three members within the NK3 gene class: Nkx3.1, Nkx3.2 and Nkx3.3, which all contain a tyrosine residue at position 54 within the homeodomain. Human and murine Nkx3.1 genes were identified among prostate-specific expressed sequence tag databases and designated the first mammalian homolog of Drosophila NK3 (Bieberich et al., 1996; He et al., 1997; Sciavolino et al., 1997). Human Nkx3.1 maps to chromosomal region 8p21 and murine Nkx3.1 to chromosome 14. There is approximately 60% homology between the human and murine genes however the homeodomain structure exhibits 100% homology. The murine Nkx3.1 gene consists of a short 5' untranslated region (UTR), two exons and a long 3' UTR (Figure 8A) (Sciavolino et al., 1997). Exon 1 encodes a tinman (TN) motif, a 10 amino acid peptide that played a role in transcriptional repression of NK homeogenes (Muhr et al., 2001). Exon 2 encodes the signature homeodomain (Figure 8B). Posttranslational modifications of alter the proteins stability. N-terminal threonine phosphorylation prolonged the hald-life of Nkx3.1, while ubiquitination at the C-terminus marked the protein for proteosomal degradation (Li et al., 2006; Markowski et al., 2008).

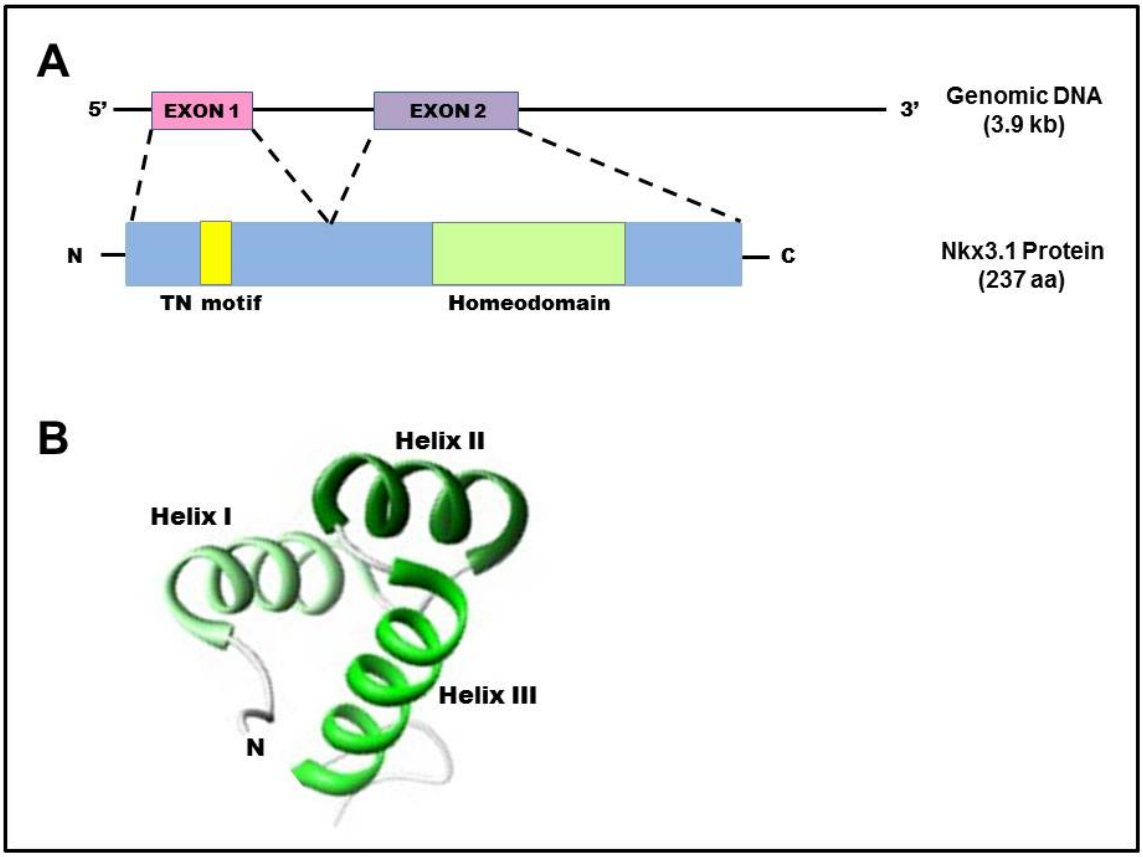

Figure 8. Diagram of mouse Nkx3.1 DNA and protein. (A) Genomic DNA includes a short 5' UTR and long 3' UTR, and two exons. The Nkx3.1 protein contains a TN motif near the N-terminus and a homeodomain motif. (B) Representation of the three well-defined α-helices of the Nkx3.1 homeodomain.

Transcriptional Activity of Nkx3.1

Nkx3.1 functions as a transcription factor through its homeodomain motif which facilitates sequence specific binding to DNA. Binding site selection and electron mobility shift assays identified that Nkx3.1 preferentially binds the TAAGTA consensus sequence (Steadman et al., 2000). Recent Nkx3.1 Chromatin immunoprecipitation coupled with massively parallel sequencing (ChIP-seq) analysis of adult mouse prostate performed in our laboratory redefined the Nkx3.1 consensus. This *in vivo* binding motif, AAGTW, is similar but not identical to the original binding consensus discovered *in vitro* (Anderson P, 2012).

Analogous with other NK family members, Nkx3.1 has been shown to be a transcriptional repressor and activator. Homeodomain proteins interact with various co-factors which guide these transcription factors to specific promoters within the genome. Interactions with certain co-factors influence repression, while interactions with others induce transcriptional activation. Using a reporter construct with three tandem Nkx3.1 consensus sequences, human Nkx3.1 repressed transcriptional activity of a synthetic promoter in a prostate cancer cell line (Steadman et al., 2000). *In vitro* and *in vivo* studies in prostate models demonstrated that Nkx3.1 directly repressed expression of quiescin Q6 sulfhydryl oxidase 1 (Qsox1), a pro-oxidant enzyme (Ouyang et al., 2005; Song et al., 2009).

Accumulating evidence suggests that Nkx3.1 recruits a co-repressor complex containing homeodomain interacting-protein kinase 2 (HIPK2), Groucho (Gro) and histone

deacetylase 1 (HDAC1). Chloramphenicol acetyltransferase (CAT) reporter assays demonstrated that HIPK2, a serine/threonine kinase, enhanced DNA-binding and repressor activity of NK3 (Kim et al., 1998). Gro family proteins are transcriptional co-repressors that display DNA-binding dependent repression. *In vitro* binding assays using deletion mutations showed that Gro interacted with the conserved Nkx TN motif (Muhr et al., 2001). Additionally, Nkx repressor activity was dependent on TN domain recruitment of Gro proteins (Muhr et al., 2001). Using co-immunoprecipitation assays, Choi et al. demonstrated that NK3 was in complex with Gro and HIPK2 in transfected HeLa cells (Choi et al., 1999). Furthermore, NK3 and Gro directly interacted with HDAC1 and resulted in decreased promoter acetylation (Choi et al., 1999). A subsequent study showed that Nkx3.1 regulated p53 activity by interacting with HDAC1 (Lei et al., 2006). Other work demonstrated that human Nkx3.1 repressed vascular endothelial growth factor C (VEGF-C) transcription by recruiting HDAC1 to the promoter in prostate cancer cells (Zhang et al., 2008). In addition, previous studies in our laboratory showed that human Nkx3.1 expression level was correlated to HDAC activity at select target promoters (Mogal et al., 2007). These studies suggest that Nkx3.1 activity is mediated by physical interaction with co-factors, and together the complex facilitates and promotes gene repression.

Nkx3.1 repression activity has also been shown to indirectly affect the expression of target genes by modulating transcriptional activators required for other proteins. A yeast-two hybrid screen seeking Nkx3.1-interacting proteins identified prostate-derived Ets

factor (PDEF), a member of the Ets family, as a potential partner (Chen et al., 2002). PDEF, independently and in synergy with AR, enhanced activation of the PSA promoter (Oettgen et al., 2000). Reporter gene assays performed in LNCaP cells using transient expression of Nkx3.1 and PDEF resulted in abrogation of PDEF-mediated PSA promoter activation (Chen et al., 2002). Therefore, Nkx3.1 repressed PSA expression by preventing PDEF from activating the promoter. In support of this mechanism, Nkx3.1 also opposed specificity protein 1 (Sp1)-mediated transcription of the PSA promoter in prostate-derived cells (Simmons and Horowitz, 2006).

Despite the overwhelming amount of data establishing Nkx3.1 as a transcriptional repressor, studies also implicate Nkx3.1 as an activator. Carson et al. showed that Nkx3.1 interacted with serum response factor (SRF) and resulted in enhanced expression of smooth muscle γ actin (SMGA) (Carson et al., 2000). In CV-1 fibroblasts, co-expression of Nkx3.1 and SRF resulted in synergistic transactivation of the SMGA promoter (Carson et al., 2000). Furthermore, DNA binding and DNAse I footprinting experiments showed that Nkx3.1 facilitated binding of SRF to the SMGA promoter, which might be accomplished by conformational changes promoting enhanced binding of SRF (Carson et al., 2000). In addition, prostate epithelial-specific SMGA activation depended on the interaction between Nkx3.1 and SRF (Filmore et al., 2002). Lastly, Nkx3.1 indirectly activated expression of insulin-like growth factor binding protein 3 (IGFBP-3) (Muhlbradt et al., 2009).

Gene expression profiling has extended our understanding of Nkx3.1 regulation by identifying subsets of genes that are positively or negatively regulated. Magee et al. analyzed the expression of Nkx3.1 target genes in wild type, heterozygous and knockout mice (Magee et al., 2003). Authors also subjected cohorts of mice to castration-testosterone replacement treatment to assess androgen-dependent Nkx3.1 target gene expression (Magee et al., 2003). Comparing Nkx3.1-deficient prostates to wild type, these studies found 57 significantly changed genes, both upregulated and downregulated (Magee et al., 2003). In addition, nearly all target genes analyzed were simultaneously regulated by androgens and a number of positively regulated genes exhibited Nkx3.1 dosage-sensitivity (Magee et al., 2003). An independent study performed in wild type and knockout mice found that Nkx3.1 differentially regulated 638 genes, 299 were upregulated and 339 were downregulated (Ouyang et al., 2005). Interestingly, a number of genes involved in oxidative stress, antioxidant and pro-oxidant enzymes, were dysregulated (Ouyang et al., 2005). Recent ChIP-seq analysis performed in our laboratory demonstrated that out of 282 direct Nkx3.1 target genes, 153 were suggested to be upregulated and 129 repressed (Anderson P, 2012).

Overall, it appears that Nkx3.1 can execute transcriptional activation and repression. Several studies show Nkx3.1 recruits specific co-factors to induce transcriptional activity. Therefore, the presence or absence of these co-factors and/or competition between co-factors for interaction with Nkx3.1 might dictate activity. Tissue and cellular content are also presumed to influence Nkx3.1 activity.

The Role of Nkx3.1 in Development

As previously mentioned, homeobox genes, including Nkx3.1, play a critical role in cell development and differentiation. During murine prostate organogenesis, Nkx3.1 expression has been observed in a wide range of tissues. In early development, Nkx3.1 was expressed in the paraxial mesoderm and played a role in somitogenesis (Kos et al., 1998; Tanaka et al., 1999). Initially Nkx3.1 transcripts were expressed in the ventral of somites but following differentiation expression was restricted to the sclerotome. Explant culture studies indicated that Nkx3.1 expression depended on neural tube, notochord or sonic hedgehog protein expression (Kos et al., 1998). In situ hybridization showed that Nkx3.1 was co-expressed with vascular endothelial smooth muscle probes, and described to be among the caudal region of the dorsal aorta (Sciavolino et al., 1997; Tanaka et al., 1999). As development ensued, Nkx3.1 expression was transiently extended to additional sites including: tongue, teeth, Rathke's pouch, kidney, brain and hair follicles (Bieberich et al., 1996; Chen et al., 2005; Sciavolino et al., 1997; Tanaka et al., 1999; Treier et al., 1998). Postnatal, Nkx3.1 was largely restricted to tongue epithelium, small salivary glands, prostate, BUG and testis (Bieberich et al., 1996; Chen et al., 2005; Sciavolino et al., 1997; Tanaka et al., 1999). Although Nkx3.1 was expressed throughout embryogenesis in many organs, Nkx3.1-null mice do not display phenotypes in most of these tissues (Tanaka et al., 2000). The most reasonable explanation for the lack of a discernible phenotype is redundancy between Nkx3.1 and other NK family members. These family members contain similarly conserved domains and therefore have the

potential to regulate similar genes. Nkx3.1 null mice only exhibit phenotypes in the salivary glands and urogenital tissues. Histological analysis of the palatine glands showed decreased ductal branching, severe epithelial hyperplasia and abnormal secretion (Tanaka et al., 2000).

Generally, Nkx3.1 is recognized for its role in prostate development. Its significance is highlighted by developmental defects in the prostate (reduced ductal branching, decreased production of secretory proteins, epithelial hyperplasia and dysplasia) and BUG (changes in cellular differentiation, decreased production of secretory proteins) of mutant mice (Abdulkadir et al., 2002; Bhatia-Gaur et al., 1999; Tanaka et al., 2000). Prior to prostate and BUG formation, 15 dpc, Nkx3.1 RNA expression was observed in the lateral UGE (Bhatia-Gaur et al., 1999). As prostatic buds emerged from the rostral end of the UGE, Nkx3.1 expression was localized to the leading edge of outgrowing prostatic ducts (Bhatia-Gaur et al., 1999; Sciavolino et al., 1997). Following ductal canalization, Nkx3.1 was restricted to the luminal epithelium of the mature prostate. Similarly, at the caudal end of UGE, where the epithelial buds of the BUGs developed, Nkx3.1 was expressed at high levels (Bhatia-Gaur et al., 1999). Nkx3.1 continued to be expressed during BUG morphogenesis and in the mature epithelium (Bhatia-Gaur et al., 1999).

Although the morphology of mice and human prostates differ, prostate organogenesis among these species is similar. In the adult mouse, expression of Nkx3.1 is restricted to the prostate luminal epithelial cells and BUG, both ductal tissues (Bhatia-Gaur et al.,

1999). In humans, Nkx3.1 is detected in the prostatic epithelium and isolated clusters of urethral transitional epithelium, small bronchial mucous glands and testis (Bowen et al., 2000).

Nkx3.1 in Prostate Cancer

Decreased Nkx3.1 Expression in Human Prostate Cancer

Decreased expression of Nkx3.1 facilitates the progression of prostate cancer and is therefore recognized as a tumor suppressor. Immunohistochemical analysis in human neoplastic prostate epithelium indicated that Nkx3.1 expression was lost during early stages of prostate cancer and loss was correlated to tumor progression (Bowen et al., 2000). Specifically, Nkx3.1 protein expression was lost in 5% of BPH, 20% of HGPIN, ~40% carcinomas and ~80% metastasis (Bowen et al., 2000). An independent study by Asantiani et al. also showed that Nkx3.1 protein was decreased in pre-invasive and invasive cancer cells (Asatiani et al., 2005). These studies detail the gradual decrease of Nkx3.1 expression throughout human prostate cancer progression. Although the mechanism for complete loss of Nkx3.1 protein has not been elucidated, it is clear that there is selective pressure for Nkx3.1 protein loss during prostate cancer.

Nkx3.1 Mutant Mice Develop Pre-neoplastic Lesions

Mice with targeted deletion of Nkx3.1 developed prostatic epithelial hyperplasia that progressively displayed histological features resembling human PIN (Abdulkadir et al., 2002; Bhatia-Gaur et al., 1999; Kim et al., 2002a). Serial transplantation of PIN lesions

from Nkx3.1$^{-/-}$ mice resulted in progressively severe histopathological alterations, further supporting the critical role of Nkx3.1 in prostate tumorigenesis (Kim et al., 2002a). In addition, mice with conditional deletion of Nkx3.1 alleles in the mature prostate exhibited the same PIN phenotype (Abdulkadir et al., 2002). Nkx3.1 heterozygous mutant mice displayed reduced Nkx3.1 protein expression and developed prostatic epithelial dysplasia and hyperplasia comparable, but less severe, to homozygous Nkx3.1 mutant mice (Abdulkadir et al., 2002; Bhatia-Gaur et al., 1999). Furthermore, these heterozygous mutant mice developed LGPIN as a consequence of age (Abdulkadir et al., 2002; Bhatia-Gaur et al., 1999). Altogether these heterozygous mice closely recapitulate human prostate tumorigenesis.

Cooperation with Other Genetic Mutations Promotes Prostate Cancer Progression

Nkx3.1 loss in mutant mice results in LGPIN but is not sufficient for progression to invasive carcinoma. Although loss of Nkx3.1 does not induce carcinoma, it increased the susceptibility of mice with compound mutations to the development of invasive adenocarcinoma. For example, mice with loss of Nkx3.1 and Pten developed an increased incidence of HGPIN by 6 months of age (Kim et al., 2002b). Histologically, prostates from these compound mutant mice displayed multifocal lesions that contained poorly differentiated cells with prominent and multiple nucleoli, mitotic figures and an increased nuclear/cytoplasmic ratio (Kim et al., 2002b). Over time, these mutant mice developed invasive adenocarcinoma, and a few resulted in metastasis to the iliac lymph nodes (Abate-Shen et al., 2003).

Using a transgenic mouse model, our laboratory showed that Nkx3.1 cooperated with the cell cycle inhibitor p27kip1 (Gary et al., 2004). Compound mutant mice showed increased proliferation and decreased apoptosis compared to single mutant mice (Gary et al., 2004). These mice also demonstrated an increased incidence of PIN (Gary et al., 2004).

In addition to studies using compound mutant mice with Nkx3.1 deletions, other mouse models have provided evidence supporting the role of Nkx3.1 in prostate tumor initiation. Transgenic mice with prostate-specific c-Myc overexpression showed loss of Nkx3.1 protein and mRNA expression (Ellwood-Yen et al., 2003). Notably, loss of Nkx3.1 was gradual and correlated with tumor progression, suggesting selective pressure for Nkx3.1 loss (Ellwood-Yen et al., 2003). In the transgenic adenocarcinoma of mouse prostate (TRAMP) model, decreased Nkx3.1 protein was noticed as early as 6 weeks of age and further decreased with age (Bethel and Bieberich, 2007).

The accumulation of these murine studies indicates that Nkx3.1 loss can cooperate with other genes to promote prostate tumor initiation. They also highlight the potential for Nkx3.1 mutant mice to develop additional mutations that advance PIN lesions to invasive carcinoma.

Chromosomal Alterations

A number of genetic and epigenetic changes have been associated with the initiation and progression of prostate cancer. Furthermore, several chromosomal alterations have been described and attributed to play a role in specific stages of prostate cancer development.

In particular, human Nkx3.1 maps to chromosome 8p21, a region that undergoes loss of heterozygosity (LOH) in PIN and prostate cancer at a high frequency (Asatiani et al., 2005; Emmert-Buck et al., 1995; He et al., 1997; Vocke et al., 1996).

To date, no somatic Nkx3.1 mutations have been discovered in human prostate cancer, however germline mutations have been described to correlate with hereditary disease. Zheng et al. discovered 21 germ-line variants among a cohort of hereditary prostate cancer samples (Zheng et al., 2006). Interestingly, one of these polymorphisms, T164A, affected the stability of the Nkx3.1 homeodomain and resulted in decreased binding of Nkx3.1 to its cognate DNA binding sequence (Zheng et al., 2006). Recent GWAS identified SNPs in Nkx3.1 that represented genetic markers for prostate cancer susceptibility (Akamatsu et al., 2010; Eeles et al., 2009; Eeles et al., 2008). An allele carrying one such SNP in the 5' UTR decreased the binding affinity of Nkx3.1 to the Sp1 transcription factor, and caused altered target gene expression. Furthermore, this variant resulted in decreased transcriptional activity of the Nkx3.1 promoter, thus reduced Nkx3.1 expression (Akamatsu et al., 2010).

Haploinsufficiency at Nkx3.1 Facilitates Prostate Tumorigenesis

Haploinsufficiency at Nkx3.1 is commonly observed in prostate cancer and has been suggested as a mechanism for tumorigenesis. Usually, one normal copy of a gene is sufficient to maintain regular function, but some genes exhibit dosage sensitivity and require both copies. Haploinsufficiency refers to circumstances when one gene copy is insufficient for normal cellular function. Tumor suppressor haploinsufficiency has been

proposed as a mechanism for clonal expansion that results in pre-neoplastic and neoplastic phenotypes (Quon and Berns, 2001). Gene targeted studies in mice demonstrated that haploinsufficiency at the Nkx3.1 locus resulted in prostate luminal epithelial hyperplasia and LGPIN (Abdulkadir et al., 2002; Magee et al., 2003). These heterozygous mutant mice closely recapitulated what is observed in human prostate tumorigenesis. Further analysis of Nkx3.1-deficient luminal epithelial cells showed that they failed to properly exit the transient proliferating state and expanded the population of cells that were sensitive to subsequent mutations (Magee et al., 2003).

Gene expression analysis identified a collection of dosage-sensitive Nkx3.1 targets, which provides a potential mechanism for tumor initiation from loss of one Nkx3.1 allele (Magee et al., 2003). Functional studies conducted in our laboratory showed that haploid loss of Nkx3.1 influenced differential changes in histone acetylation and resulted in inactivation of gene targets involved in tumor suppression (Mogal et al., 2007). An alternative study using human samples, suggested that DNA methylation combined with LOH contributed to low Nkx3.1 protein expression (Asatiani et al., 2005).

c-Myc

Myc Gene Family

The Myc gene family consists of five transcription factors, c-Myc, N-Myc, L-Myc, B-Myc and S-Myc. The first member, c-Myc, was identified as a cellular homolog of the

viral oncogene (v-myc) of the avian myelocyomatosis retrovirus (Vennstrom et al., 1982). Subsequent studies discovered additional homologous sequences, N-Myc and L-Myc, in neuroblastoma cells and a small cell lung tumor, respectively (Nau et al., 1985; Schwab et al., 1983). B-Myc and S-Myc were isolated from a rat genomic library and classified as Myc genes based on sequence homology to previous members (Ingvarsson et al., 1988; Kuchino et al., 1989; Sugiyama et al., 1989).

Despite the structural similarity, the functions of these proteins vary. B-Myc and S-Myc suppress transformation, while c-Myc, N-Myc and L-Myc are implicated in cellular transformation (Resar et al., 1993). c-Myc is ubiquitously expressed. Rapidly proliferating cells express high levels of c-Myc, whereas normal quiescent cells engage several mechanisms to restrict protein accumulation and activity. Consequently, c-Myc proteins are quickly degraded after induction (Salghetti et al., 1999; Thomas and Tansey, 2011). N-Myc is primarily expressed in pre-B cells, kidney, forebrain, hindbrain and intestine (Nesbit et al., 1999). L-Myc is expressed in the developing kidney, brain, neural tube and lung (Nesbit et al., 1999). The bulk of research efforts have focused on the function of c-Myc, N-Myc and L-Myc; this dissertation concentrates on c-Myc (henceforth, Myc).

Gene & Protein Structure of Myc

Myc is a basic helix-loop-helix transcription factor located on human chromosome 8q24. The gene contains four promoters and three exons that encode two protein products, p67

(Myc 1) and p64 (Myc 2) (Figure 9A) (Wierstra and Alves, 2008). Myc 2 is the major protein product and consists of 439 amino acids. Alternative translation at exon one produces the Myc 1 protein which contains an additional 14 N-terminal amino acids. No differences exist in the subcellular localization, stability and post-translational modification of these protein products, but there are differences in their regulation, transcriptional activity and function. For example, high cell density *in vitro* resulted in a 5-10 fold induction of Myc 1 (Hann et al., 1992). Overexpression of Myc 1 inhibited cell growth, whereas Myc 2 stimulated cell growth and proliferation (Hann et al., 1994).

The Myc protein is composed of an N-teminal transactivation domain (TAD), a central region and a C-terminal basic-helix-loop-helix leucine-zipper (bHLH-LZ) motif (Figure 9B). The protein includes four regions with highly conserved sequences known as Myc Boxes (MBs). MBI and MBII are located in the transactivation domain between amino acids 45-65 and 128-143, respectively. (Evan et al., 1992; Stone et al., 1987a). MBI contains two phosphorylation sites (Ser 62 and Thr 58) shown to play a role in transactivation and transformation (Gupta et al., 1993; Henriksson et al., 1993). MBII has been associated with several functions including apoptosis, co-transformation, inhibiting differentiation and autoregulation (Evan et al., 1992; Stone et al., 1987a). MBIII resides in the central region and plays a role in transformation, transcriptional repression and inhibition of apoptosis (Herbst et al., 2005). MBIV is located within the C-terminal domain and regulates DNA binding, transformation, apoptosis and G2 cell cycle arrest (Cowling et al., 2006). In addition to MBIV, the basic motif is responsible

for recognizing DNA sequences, while the neighboring HLH-LZ motif is required for heterodimerization.

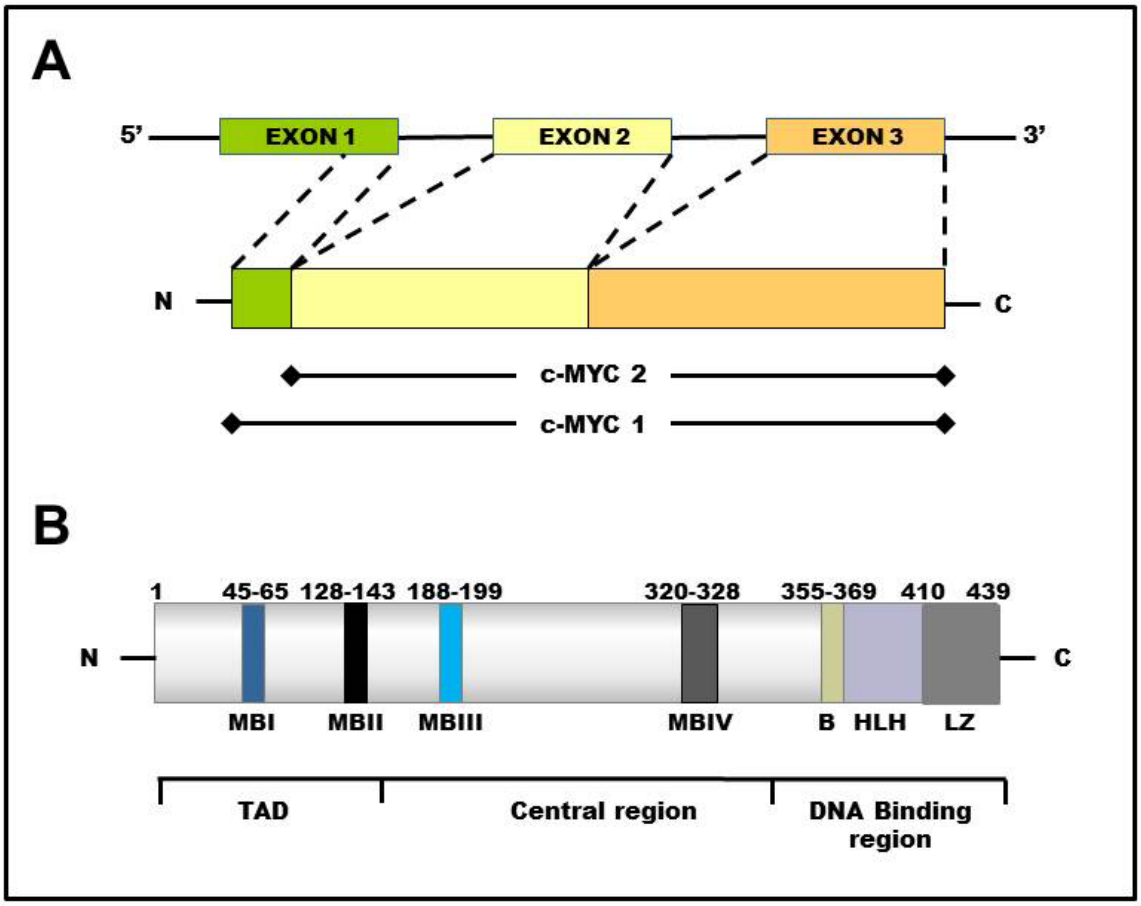

Figure 9. Diagram of Myc DNA and protein. (A) Genomic DNA includes three exons that encode two protein products, Myc 1 and Myc 2. (B) Schematic of Myc protein which includes four conserved Myc box regions (MBI-IV), a basic domain (B) and helix-loop-helix leucine-zipper motif (HLH-LZ).

Transcriptional Regulation

Myc activates and represses a multitude of target genes. Several proteins bind to specific regions within the Myc protein, however activity requires heterodimerization with protein partner Max via the bHLH-LZ domain (Figure 10A) (Blackwood and Eisenman, 1991). The Myc/Max heterodimer activates transcription by binding to Ebox DNA elements. Once bound, co-activators and protein complexes are recruited to the Ebox to promote transcription activation (Figure 10B). Co-activators include TRAAP (transformation/transcription domain-associated protein); Tip48 (48 kDa TATA box-binding protein-interacting protein); Tip49 (49 kDa TATA box-binding protein-interacting protein) and histone acetyltransferases such as CBP (CREB binding protein) and p300 (E1A binding protein p300) (McMahon et al., 1998; Vervoorts et al., 2003). Conversely, Myc is recruited to promoters via interactions with other proteins that repress transcriptional activity (Figure 10C). For instance, when the Myc/Max heterodimer interacted with Miz1 (Myc-interacting Zn finger protein-1), growth signals were suppressed and senescence was induced (van Riggelen et al., 2010). In addition, p19ARF (cyclin-dependent kinase inhibitor 2A) bound Myc and inhibited transcriptional activation (Datta et al., 2004).

Myc transcriptional activity is tightly regulated by a number of mechanisms. One such mechanism is via Max-interacting factors; Max forms dimers with numerous proteins independent of Myc including Mad1 (max dimerization protein 1), Mad3 (max-associated protein 3), Mad4 (max-associated protein 4), Mxi1 (max-interacting protein

1), and Mnt (max binding protein) (Hurlin and Huang, 2006). The resulting dimers localize to sites of ongoing transcription and compete with Myc-Max for the same Ebox DNA elements to enforce transcriptional repression. HDAC complexes are also recruited to facilitate transcriptional repression. Overall, Max-Myc complexes predominate in proliferating cells, whereas Max-Mnt and Max-Mad complexes are dominant in quiescent and differentiating cells, respectively (Hurlin and Huang, 2006).

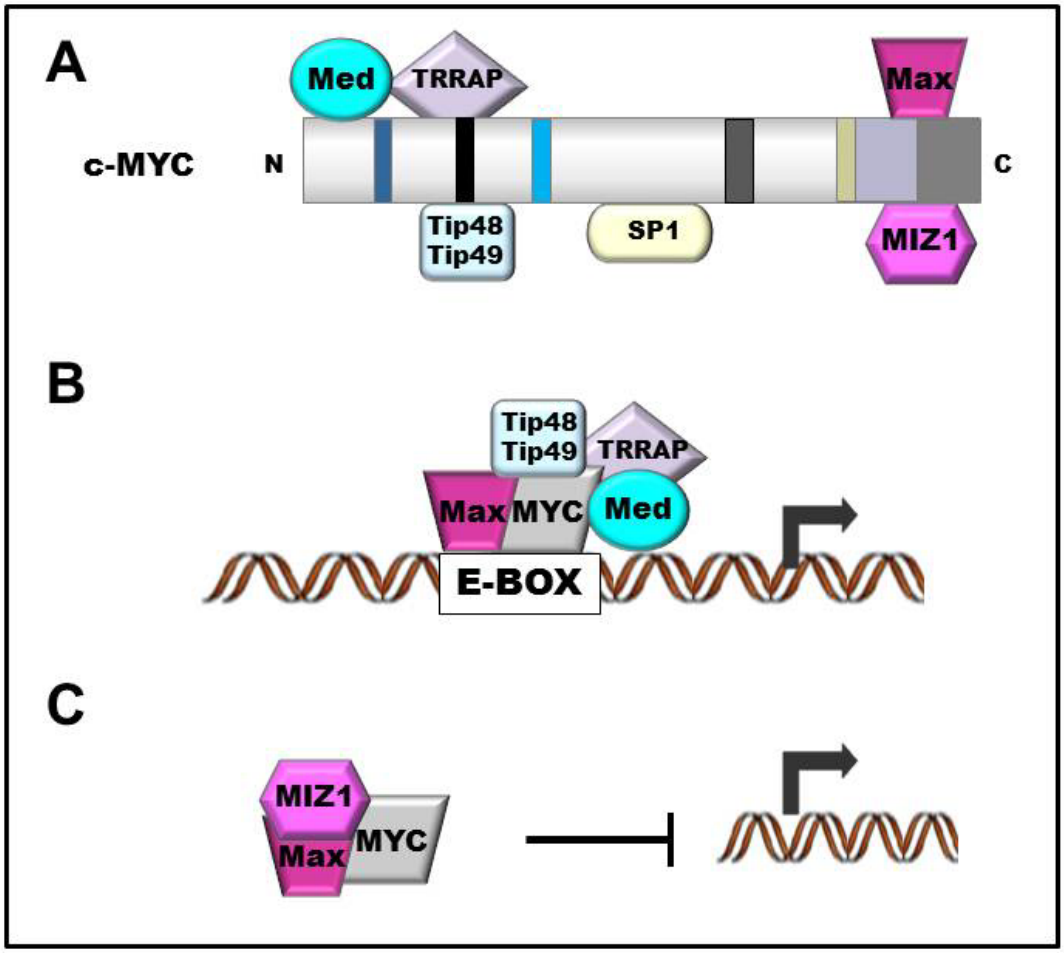

Figure 10. Myc-interacting proteins and transcriptional activity. (A) Myc-interacting proteins (Mediator, Med; Tip48/Tip49; TRRAP, Max, Miz1, Sp1) and their respective Myc binding locations. (B) Myc/Max bound with co-activators that facilitates transcriptional activation. (C) Myc/Max bound with Miz1 co-repressor, preventing transcriptional activation.

Myc & Human Prostate Cancer

Myc is a powerful regulator of several cellular processes including cell cycle progression, proliferation, metabolism, apoptosis, stem cell self-renewal, and transformation. Myc overexpression and activation is one of the most frequently observed occurrences among a wide spectrum of human malignancies. Myc is located at chromosomal region 8q24, an area that is frequently amplified in prostate cancer (Jenkins et al., 1997; Qian et al., 1997). Overexpression of mRNA and protein are also common (Buttyan et al., 1987; Fleming et al., 1986; Gurel et al., 2008). In a study comparing Myc expression in human prostate tissue, Myc was increased in 76% of PIN, 81.6% of adenocarcinoma and 68% of metastatic lesions (pelvic lymph node, soft tissue or bone). In one mouse model, prostate-specific overexpression of Myc resulted in PIN lesions that progressed to invasive carcinoma (Ellwood-Yen et al., 2003). In another model, PBCre;Z-Myc, focal overexpression of Myc resulted in a mild phenotype, with no overt pathology in most mice (Kim et al., 2009). However cancer developed when PBCre;Z-Myc mice were bred with Pten mutant mice. *In vitro* viral vector transduction of Myc transformed primary cells (benign human and murine epithelial) and resulted in tumor development in tissue recombination assays (Wang et al., 2010; Williams et al., 2005). Recent work indicated that Myc activation caused the increased nucleolar size and numbers observed in early prostate cancer lesions (Koh et al., 2011). These data suggest that Myc overexpression is an early event in prostate tumorigenesis.

Nkx3.1 & Myc in Prostate Tumorigenesis

Concurrent loss of 8p21 (Nkx3.1) and gain of 8q24 (Myc) are frequent observations in human prostate cancer and associated with increased genomic instability and poor prognosis (Locke et al., 2012; Sato et al., 1999). Kindich et al. analyzed human prostate carcinoma samples and found that the ratio of Nkx3.1 to Myc gene copy number was significantly correlated with tumor stage (Kindich et al., 2006). Furthermore, Nkx3.1 mRNA and protein expression was decreased in Myc models of prostate cancer (Ellwood-Yen et al., 2003). These studies suggest that loss of Nkx3.1 and Myc overexpression might coordinate tumor progression.

Goal of Dissertation

The goal of this dissertation research was to investigate the cooperation between loss of Nkx3.1 and Myc overexpression in promoting prostate tumorigenicity. Based on previously published data and functional knowledge of the proteins, the hypothesis that loss of Nkx3.1 cooperates with Myc overexpression to promote tumorigenesis through regulation of shared target genes was proposed. This hypothesis was tested using the following specific aims:

Aim 1: To determine the ability of Nkx3.1 loss and Myc overexpression to synergistically promote malignant transformation *in vivo.*

Aim 2: To identify the mechanism associated with the regulation of Nkx3.1 and Myc shared target genes in prostate cancer.

CHAPTER II

GENERATION OF A CONDITIONAL MOUSE MODEL WITH CONCURRENT LOSS OF NKX3.1 AND MYC OVEREXPRESSION

Introduction

For nearly 20 years genetically engineered mouse models have been exploited to define molecular mechanisms involved in human prostate cancer (Kasper, 2005; Lamb and Zhang, 2005). Human prostate tumorigenesis involves selective clonal expansion of individual cells that acquire genetic mutations conferring growth advantages. Extensive efforts have been made to identify mutations driving late stages of prostate cancer. However, there is limited work focused on the specific genes and molecular mechanisms contributing to tumor initiation and early intraepithelial neoplastic growth (Ashida et al., 2004).

One gene that plays a critical role in human and mouse prostate cancer initiation is the homeodomain transcription factor, Nkx3.1. In a recent study, Nkx3.1 was identified as an important factor for proper prostatic stem cell maintenance (Wang et al., 2009). It has also been demonstrated that Nkx3.1 expression is decreased in human prostate cancers and protein loss correlated with disease progression (Bowen et al., 2000). Additionally, a germ-line mutation that impaired Nkx3.1 DNA binding ability has been associated with

hereditary prostate cancer (Zheng et al., 2006). Furthermore, GWAS identified a functional variant in Nkx3.1 that resulted in reduced gene expression and increased prostate cancer susceptibility (Akamatsu et al., 2010).

Several transgenic models of prostate cancer have illustrated the tumor suppressive role of Nkx3.1. Nkx3.1 mutant mice develop prostatic epithelial hyperplasia, dysplasia and LGPIN lesions, however these lesions do not progress to invasive carcinoma (Abate-Shen et al., 2003; Abdulkadir et al., 2002; Kim et al., 2002b; Wang et al., 2003). Subsequent studies showed that loss of a single Nkx3.1 allele resulted in clonal expansion of proliferating cells and increased the population of target cells for subsequent genetic mutations (Magee et al., 2003). Therefore, Nkx3.1 acts as a critical gatekeeper, preventing other genetic insults from initiating prostate tumorigenesis (Song et al., 2009). Despite the fact that Nkx3.1 loss facilitates tumor initiation, additional genetic mutations are necessary for lesion progression.

One potential co-factor of Nkx3.1 is the oncogenic transcription factor Myc. Myc has been shown to be involved in early development of prostate tumorigenesis and disease progression (Gurel et al., 2008; Iwata et al., 2010; Nesbit et al., 1999; Sato et al., 1999). Clinical studies suggest that loss of Nkx3.1 and overexpression of Myc are strongly correlated in human prostate carcinoma (Kindich et al., 2006; Locke et al., 2012). Thus, cooperation between Nkx3.1 and Myc might be a critical event in prostate cancer. Development of a mouse model that closely mimics human prostate cancer, driven by loss of Nkx3.1 and Myc overexpression could clarify the cooperative approach of these

two genes and illuminate how they, collectively, drive tumorigenesis. Two Myc-driven mouse models resulted in different interpretations of the interaction among Nkx3.1 and Myc in prostate cancer (Ellwood-Yen et al., 2003; Iwata et al., 2010). To address these differences and delineate the relationship between Nkx3.1 and Myc, a new transgenic mouse model exhibiting prostate specific loss of Nkx3.1 and Myc overexpression was generated.

Results

Generation of PBCre;Z-Myc;Nkx3.1$^{f/f}$ Trigenic Mice

To define the functional role of Nkx3.1 loss and Myc overexpression in prostate tumorigenesis, PBCre;Z-Myc;Nkx3.1 trigenic mice were generated. Promoters commonly used to drive prostate-specific transgene expression such as PB, modified ARR_2PB and PSA are themselves Nkx3.1 target genes. Therefore, mating Nkx3.1-deficient mice with PB-Myc mice would result in decreased Myc transgene expression due to the absence of Nkx3.1 (Figure 11). To circumvent this complication, Cre/loxP mediated recombination was used to concurrently delete Nkx3.1 and overexpress Myc in the mouse prostate epithelium. Specifically, PBCre;Z-Myc mice, expressing a latent Cre-activatable Z-Myc allele, were bred with Nkx3.1$^{f/f}$ mice (Abdulkadir et al., 2002; Kim et al., 2009). Thus, prostate-specific Cre expression resulted in concurrent deletion of Nkx3.1 and activation of Myc expression (Figure 12).

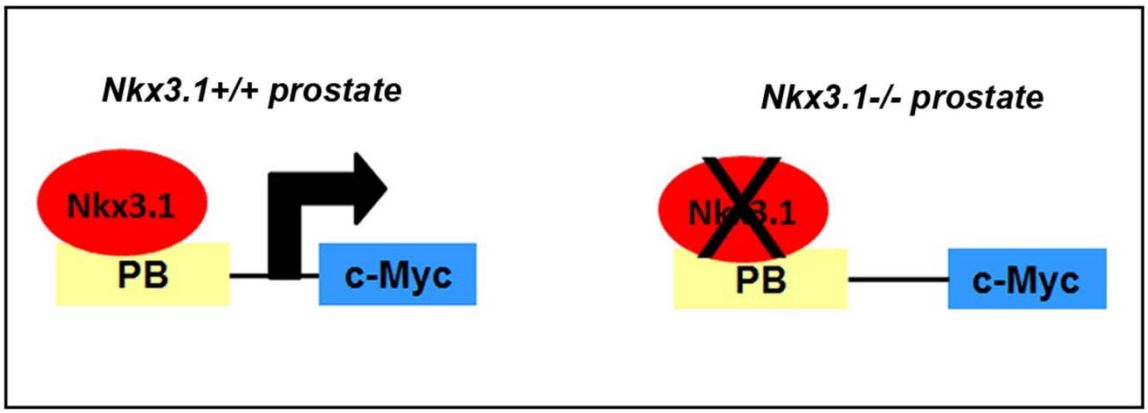

Figure 11. Nkx3.1 is required to drive prostate-specific transgene expression. Prostate-specific promoters such as PB, ARR_2PB and PSA (represented as PB in yellow box) are Nkx3.1 targets. Prostates with loss of Nkx3.1 protein (such as $Nkx3.1^{-/-}$) results in a decrease in promoter activation and therefore a reduction in transgene expression.

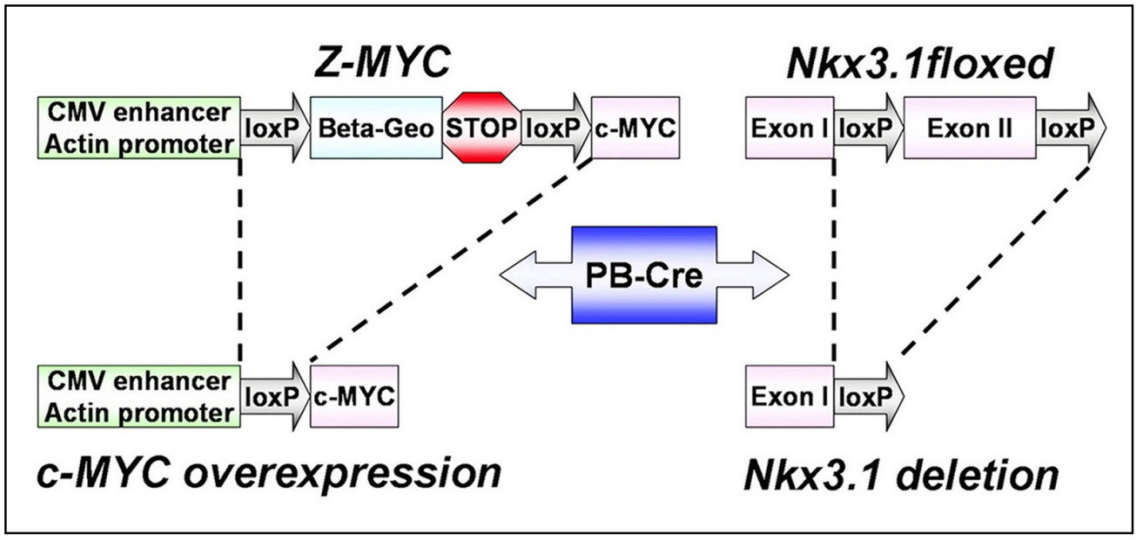

Figure 12. Generation of transgenic mice with prostate-specific deletion of Nkx3.1 and Myc overexpression. Diagram for concurrent overexpression of Myc and deletion of Nkx3.1 in the prostates of transgenic mice. The Z-Myc construct contains a latent Myc allele under the control of the CMV enhancer/actin promoter. The floxed allele of Nkx3.1 has loxP sites flanking exon 2, which encodes the Nkx3.1 homeodomain. Cre-recombinase is under the control of the prostate-specific PB promoter (PBCre; shown in the blue box). When PBCre is expressed, the Myc transgene will be activated and the Nkx3.1 gene will be concurrently deleted.

PBCre Successfully Targets Nkx3.1 for Deletion and Activates Myc

Previous conditional Nkx3.1 knockout mice used the PSA-Cre transgene to mediate prostate-specific recombination of Nkx3.1 (Abdulkadir et al., 2002). This model, however, employed an alternative prostate-specific promoter, PBCre. PBCre is a strong promoter that delivers high levels of Cre expression and high penetrance in prostate luminal epithelial cells (Wu et al., 2001). To confirm that PBCre efficiently recombined the floxed Nkx3.1 alleles and the Z-Myc transgene, immunohistochemical analysis was used to evaluate protein expression. Nkx3.1 immunostaining showed decreased expression in the prostates of PBCre;Z-Myc;Nkx3.1$^{f/+}$ and complete loss in PBCre;Z-Myc;Nkx3.1$^{f/f}$ (Figure 13). Despite the differences in Nkx3.1 expression, Myc was activated to a similar extent in the prostates of all transgenic mice bearing the Z-Myc transgene. Furthermore, Myc immunohistochemical analysis of PBCre;Z-Myc (also denoted PBCre;Z-Myc;Nkx3.1$^{+/+}$), PBCre;Z-Myc;Nkx3.1$^{f/+}$ and PBCre;Z-Myc;Nkx3.1$^{f/f}$ mice illustrated focal staining exclusive to the prostate luminal epithelium (Figure 14), consistent with previous studies (Kim et al., 2009).

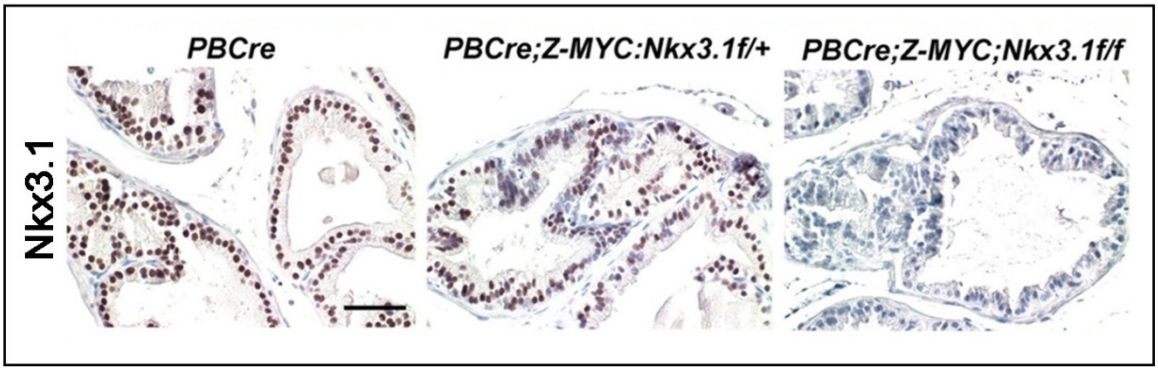

Figure 13. PBCre expression results in deletion of floxed Nkx3.1 alleles. PBCre, PBCre;Z-Myc;Nkx3.1$^{f/+}$ and PBCre;Z-Myc;Nkx3.1$^{f/f}$ prostates were stained with anti-Nkx3.1 to examine protein expression (brown). PBCre;Z-Myc;Nkx3.1$^{f/+}$ exhibit reduced expression and PBCre;Z-Myc;Nkx3.1$^{f/f}$ show complete loss of Nkx3.1. Scale bar, 50 um.

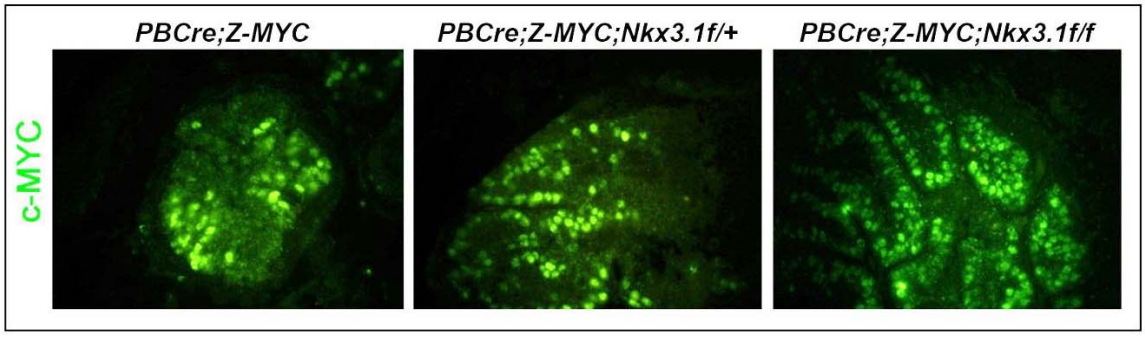

Figure 14. Myc is focally expressed in Nkx3.1/Myc prostates. PBCre;Z-Myc;Nkx3.1$^{+/+}$, PBCre;Z-Myc;Nkx3.1$^{f/+}$ and PBCre;Z-Myc;Nkx3.1$^{f/f}$ prostates were stained with anti-Myc to examine protein expression (green fluorescence).

Myc Overexpression Does Not Directly Regulate Nkx3.1

Prevailing Myc-driven mouse models of prostate cancer have suggested a relationship between Myc and Nkx3.1. In Hi-Myc and Lo-Myc models generated by Ellwood-Yen et al. (discussed in Chapter I), Nkx3.1 mRNA and protein levels were decreased in the PIN to carcinoma transition (Ellwood-Yen et al., 2003). In contrast, Iwata et al. argued that Myc overexpression directly induced loss of Nkx3.1 in PIN lesions (Iwata et al., 2010). Iwata et al. reported that Myc-expressing cells within PIN lesions from Hi-Myc, Lo-Myc and Super-Lo-Myc (discussed in Chapter I) had reduced Nkx3.1 expression (Iwata et al., 2010). If Myc directly suppressed Nkx3.1 expression, deleting the Nkx3.1 allele would have no added advantage to Myc-overexpression in prostate cells, nor permit evaluation of Nkx3.1 and Myc cooperation.

To address this paradox, expression of Nkx3.1 and Myc was examined in PBCre;Z-Myc;Nkx3.1$^{+/+}$ prostates using double immunohistochemical staining. These prostates expressed wild type Nkx3.1 alleles and conditionally activated Myc. While some Myc-overexpressing cells showed evidence of Nkx3.1 downregulation, a number of cells co-expressing Myc and Nkx3.1 were observed (Figure 15A). Immunostaining of serial sections for Myc and Nkx3.1 confirmed these results and showed Myc-overexpressing cells in benign tissue expressed robust levels of Nkx3.1 (Figure 15B). Thus, in this mouse model, Myc overexpression does not immediately lead to downregulation of Nkx3.1 protein expression. These results are consistent with previous observations in PBCre;Z-Myc mice which showed that Myc overexpression sensitizes cells to

transformation, but does not by itself lead to significant prostatic pathology, contrary to the case in Hi-Myc and Lo-Myc mice (Ellwood-Yen et al., 2003; Iwata et al., 2010; Kim et al., 2009). Differences in background strains or Myc expression levels might account for these differences (Iwata et al., 2010). In summary, these data indicate that the PBCre;Z-Myc;Nkx3.1 model is well suited to test whether Nkx3.1 deletion will modify Myc-initiated prostate tumorigenesis *in vivo*.

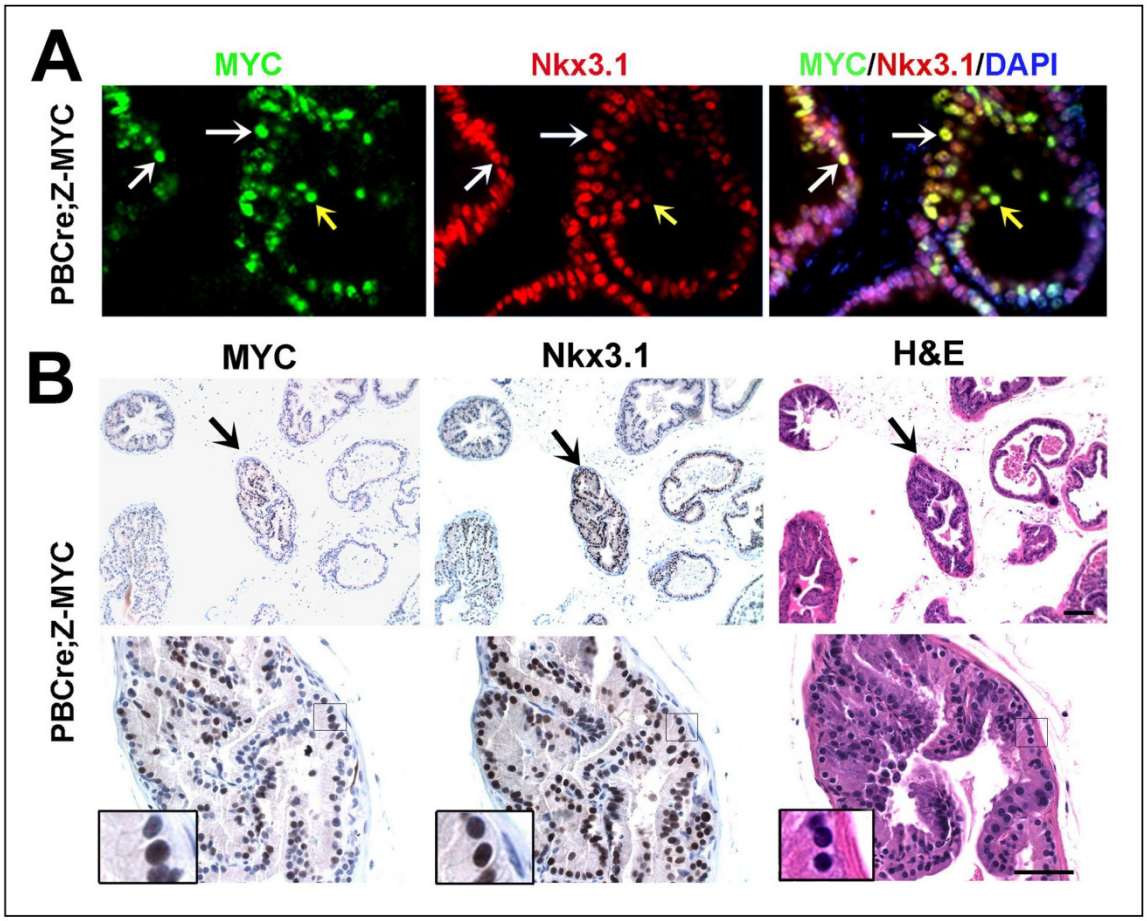

Figure 15. Myc overexpression does not directly suppress Nkx3.1 expression in prostate epithelial cells. (A) Double immunofluorescence for Myc (green) and Nkx3.1 (red) performed on a 57-wk old PBCre;Z-Myc;Nkx3.1$^{+/+}$ transgenic mouse prostate illustrates cells co-expressing Myc and Nkx3.1 (white arrows) as well as a few Myc-expressing cells with decreased Nkx3.1 (yellow arrows). Sections were counter-stained for DNA with DAPI (blue). (B) Adjacent prostate sections from a 37-wk old PBCre;Z-Myc;Nkx3.1$^{+/+}$ transgenic mouse were stained for Myc (brown) or Nkx3.1 (brown) or H&E. Myc and Nkx3.1-stained sections were counterstained with hematoxylin. Arrows point to a gland overexpressing Myc while retaining robust Nkx3.1 expression. Scale bars, 50 µm.

Discussion

Concomitant Nkx3.1 loss and Myc gain contributes to human prostate cancer progression, aggression, poor prognosis and relapse (Kindich et al., 2006; Locke et al., 2012). Accumulating evidence supporting a strong relationship between loss of Nkx3.1 and Myc overexpression in human prostate cancer reinforces the need for a representative preclinical model. Therefore, a transgenic mouse model with prostate specific loss of Nkx3.1 and Myc gain was developed. This model is advantageous to prevailing models for several reasons. First, the model mimics hallmarks of human prostate cancer including: sporadic tumor initiation, heterogeneity and multifocality. In this mouse model, Myc is sporadically and focally overexpressed and primarily results in normal pathology. This implies that Myc overexpression, in the absence of additional mutations is not sufficient to directly transform prostate luminal epithelia. But, the addition of another mutation, like Nkx3.1 loss, may advance transformed cells to HGPIN or invasive carcinoma. Nkx3.1/Myc mutant mice sequentially accumulate genetic alterations, which closely parallel human tumorigenesis. Other transgenic mouse models induce systematic mutations throughout entire cellular populations, resulting in overt neoplastic transformation.

Previous models of prostate cancer have supported cooperation between allelic loss of Nkx3.1 and Myc-overexpression in driving initiation and progression (Ellwood-Yen et al., 2003; Iwata et al., 2010). Unfortunately these models show conflicting results

regarding the interaction between Nkx3.1 and Myc in prostate cancer. In one model, Nkx3.1 loss and Myc overexpression are distinct events that cooperate to promote prostate cancer (Ellwood-Yen et al., 2003; Song et al., 2009). The other model suggests that Nkx3.1 loss is coincident with Myc overexpression, implying they coordinate regulation in a single pathway (Iwata et al., 2010). In this chapter, it was demonstrated that Myc overexpression does not induce Nkx3.1 loss. PBCre;Z-Myc;Nkx3.1$^{+/+}$ prostates clearly show cells that co-express both Nkx3.1 and Myc. Therefore, Nkx3.1 loss and Myc gain are independent incidents in this model. Importantly, PBCre;Z-Myc;Nkx3.1 mutant mice are well suited to test whether deletion of Nkx3.1 will cooperate with Myc-initiated prostate tumorigenesis *in vivo*. Further characterization and evaluation of the cooperation among Nkx3.1 and Myc in PBCre;Z-Myc;Nkx3.1$^{f/f}$ prostates will be described in Chapter III.

Finally, prostate-specific transgene expression is uncoupled from androgen regulation in this model. Prostate cancer proliferation is heavily dependent on androgens and as a result the standard clinical treatment is androgen ablation. Current transgenic models demonstrating an association between Nkx3.1 and Myc utilize androgen responsive promoters to drive Myc transgene expression (Ellwood-Yen et al., 2003; Iwata et al., 2010) As a result, androgen dependence cannot be assessed because castration results in regression of the tumor and silencing of the initiating oncogene. Nkx3.1/Myc mutant mice utilize the CMV enhancer/β-Actin promoter to drive prostate-specific transgene expression. Thus, once Cre-recombinase is activated, Nkx3.1 is deleted and Myc is

constitutively expressed under the CMV/β-actin promoter. Unlike other transgenic mice, these mice provide a better model to test how Nkx3.1/Myc initiated tumors respond to hormone ablation.

In conclusion, PBCre;Z-Myc;Nkx3.1 mice are a new prostate cancer model with concomitant and conditional deletion of Nkx3.1 and Myc overexpression. Myc overexpression and loss of Nkx3.1 are independent events capable of promoting prostate tumorigenesis. Furthermore, Myc overexpression sensitizes the cells to an additional mutational event, such as loss of Nkx3.1. This model replicates the focal, heterogeneous and selective profile reflected in human prostate cancer and well-suited to evaluate the relationship between Nkx3.1 loss and Myc overexpression.

Materials and Methods

Animals

Z-Myc, PBCre and Nkx3.1$^{f/f}$ mice have been previously described (Abdulkadir et al., 2002; Roh et al., 2006; Wu et al., 2001). To obtain mice with prostate-specific deletion of Nkx3.1, PBCre;Nkx3.1$^{+/+}$ were bred with Nkx3.1$^{f/f}$ mice to obtain PBCre;Nkx3.1$^{f/+}$ mice. PBCre;Nkx3.1$^{f/+}$ males were subsequently mated with Nkx3.1$^{f/+}$ females. To generate trigenic mice, Z-Myc;Nkx3.1$^{f/+}$ female mice were bred with PBCre;Nkx3.1$^{f/+}$ to obtain PBCre;Z-Myc;Nkx3.1$^{+/+}$, PBCre;Z-Myc;Nkx3.1$^{f/+}$, PBCre;Z-Myc;Nkx3.1$^{f/f}$ offspring and

littermate controls. All animal care and experiments were carried out according to Vanderbilt Institutional Animal Care and Use Committee approved protocols.

Immunohistochemical staining

Tissues were prepared for histological analysis as described (Abdulkadir et al., 2001). Briefly, paraffin-embedded sections (6μm thick) were de-paraffinized with xylene and rehydrated in decreasing concentrations of ethanol, incubated in Target Retrieval Solution, Citrate pH 6.0 (DAKO) and subjected to antigen retrieval in a pressure cooker for 15 min. Sections were incubated in 1% hydrogen peroxide and 30% methanol diluted in PBS for 30 min to quench endogenous peroxidase activity. Sections were washed with PBS and blocked with 5% BSA diluted in PBS for 1 hr. Primary antibody Nkx3.1 (1:1000, a gift from Dr. Charles Bieberich) was diluted in 1% BSA, applied to sections and incubated overnight at 4°C in a humidified chamber. Unbound antibody was removed by washing with PBS. HRP-conjugated secondary IgG antibody was applied for 1 hr at room temperature in a humidified chamber followed by PBS washes. Bound antibody was visualized by incubation with DAB (3,3'-diaminobenzidine) according to manufacturer's protocol (SIGMAFAST tablets, Sigma-Aldrich). Sections were washed in water, counterstained with hematoxylin and mounted.

Myc immunofluorescence & Nkx3.1/Myc double immunofluorescence staining

Tissues were prepared for histological analysis as described (Abdulkadir et al., 2001) and subjected to a protocol similar to immunohistochemical staining. Paraffin-embedded sections (6μm thick) were de-paraffinized with xylene and rehydrated in decreasing

concentrations of ethanol and incubated for 30 min in peroxidase blocking solution (citric acid, disodium hydrogen phosphate 2 hydrate, sodium azide) and 1.5% methanol diluted in PBS. Sections were washed in PBS and subjected to antigen retrieval in Tris-EDTA buffer (pH 8) in a pressure cooker for 7 min. Sections were blocked in 1% BSA diluted in PBS for 1 hr. Primary antibody Myc (1:15000, Santa Cruz) was diluted in 1% BSA, applied to sections and incubated at 4°C for 3 days in a humidified chamber. Unbound antibody was removed by washing with PBS. HRP-conjugated secondary IgG antibody was applied for 1 hr at room temperature in humidified chamber followed by PBS washes. Primary antibody was amplified using Tyramide Signal Amplification (TSA) (green fluorescein, Perkin Elmer) for 10 min. For double staining, sections were washed and incubated at 4°C overnight with 2nd primary antibody, Nkx3.1 (1:1000, gift from Dr. Charles Bieberich). Sections were washed in PBS and incubated in fluorophore-conjugated secondary IgG antibody Alexa Fluor 594 (red fluorescein, Molecular Probes). Nuclear stain (4',6-diamidino-2-phenylindole, DAPI) and sample mounting was performed using vectashield mounting medium (Vector Laboratories).

CHAPTER III

LOSS OF NKX3.1 COOPERATES WITH MYC OVEREXPRESSION

Introduction

Human prostate tumorigenesis develops through a series of defined steps: formation of prostate intraepithelial neoplasia (PIN), invasion into the surrounding stroma, metastasis via lymph nodes or blood vessels and/or castration resistance. Expression of Nkx3.1, a prostate specific transcription factor, is commonly attenuated in mouse and human prostate tumors (Abate-Shen et al., 2008; Abdulkadir, 2005). Nkx3.1 mutant mice develop PIN but do not develop invasive carcinoma (Abdulkadir et al., 2002; Kim et al., 2002a). Studies have shown that Nkx3.1 cooperates with additional mutations to promote tumor progression (Abate-Shen et al., 2003; Lei et al., 2006). For example, deletion of a single allele of the Pten TSG promoted the progression of premalignant lesions in $Nkx3.1^{-/-}$ and $Nkx3.1^{+/-}$ mice to invasive carcinoma with metastasis (Abate-Shen et al., 2003; Kim et al., 2002b). Conversely, in a conditional Pten deletion model of prostate cancer, Nkx3.1 expression was uniformly lost and reconstituted Nkx3.1 expression by lentiviral-mediated gene transfer into Pten-deficient cells potently suppressed tumorigenicity (Lei et al., 2006; Wang et al., 2003). Thus a wealth of

evidence suggests that reductions in Nkx3.1 protein are permissive for prostate cell transformation.

As mentioned in Chapter II, the combination of Nkx3.1 loss and Myc overexpression is frequently observed in human prostate tumors and shown to contribute to increased genomic instability, tumor progression, increased tumor stage and poor prognosis (El Gammal et al., 2010; Locke et al., 2012; Tsuchiya et al., 2002). This suggests that Nkx3.1 and Myc are cooperating factors in prostate cancer. To model the potential cooperation between Nkx3.1 and Myc, PBCre;Z-Myc;Nkx3.1 mice were generated (described in Chapter II). In these mice, expression of PBCre concurrently deletes floxed Nkx3.1 alleles and activates a latent Myc transgene (Figures 12-14). This chapter demonstrates that Nkx3.1 and Myc cooperate and promote prostate carcinogenesis.

Results

Nkx3.1 Loss Cooperates with Myc to Promote Prostate Tumorigenesis

Prostate tumorigenesis was characterized in cohorts of conditional Nkx3.1/Myc mutant mice. Prostate-specific deletion of Nkx3.1 using the PBCre line resulted in epithelial hyperplasia and dysplasia (Figure 16), similar to PSA-Cre conditional mice (Abdulkadir et al., 2002). Focal prostate expression of Myc in PBCre;Z-Myc;Nkx3.1$^{+/+}$ mice resulted in mild pathology (Figure 17) as reported earlier (Kim et al., 2009). Notably, cooperation between Nkx3.1 loss and Myc overexpression was evident as early as 15 weeks of age in

PBCre;Z-Myc;Nkx3.1$^{f/+}$ and PBCre;Z-Myc;Nkx3.1$^{f/f}$ mice, 62% and 67% developed focal HGPIN lesions, respectively (Figure 17). Strikingly, 70% of the PBCre;Z-Myc;Nkx3.1$^{f/f}$ HGPIN lesions developed microinvasive cancer (Figure 17 and 18). Over time, the density of focal HGPIN lesions increased to more than 5 foci per section in PBCre;Z-Myc;Nkx3.1$^{f/f}$ mice (Figure 17A). As shown in Figure 19, proliferation was significantly increased in PbCre;Z-Myc;Nkx3.1$^{f/f}$ prostates, while apoptosis showed no significant change.

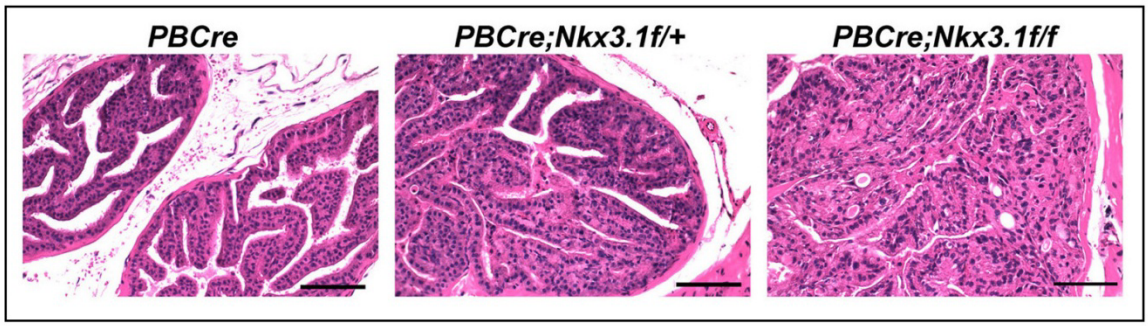

Figure 16. Conditional loss of Nkx3.1 results in prostate epithelial hyperplasia and dysplasia. Hemotoxylin & eosin-stained sections of anterior prostates from 35-week old PBCre (also denoted PBCre;Nkx3.1$^{+/+}$), PBCre;Nkx3.1$^{f/+}$ and PBCre;Nkx3.1$^{f/f}$ mice. Epithelial hyperplasia and dysplasia is evident in both PBCre;Nkx3.1$^{f/+}$ and PBCre;Nkx3.1$^{f/f}$ prostates. Scale bar 100µm.

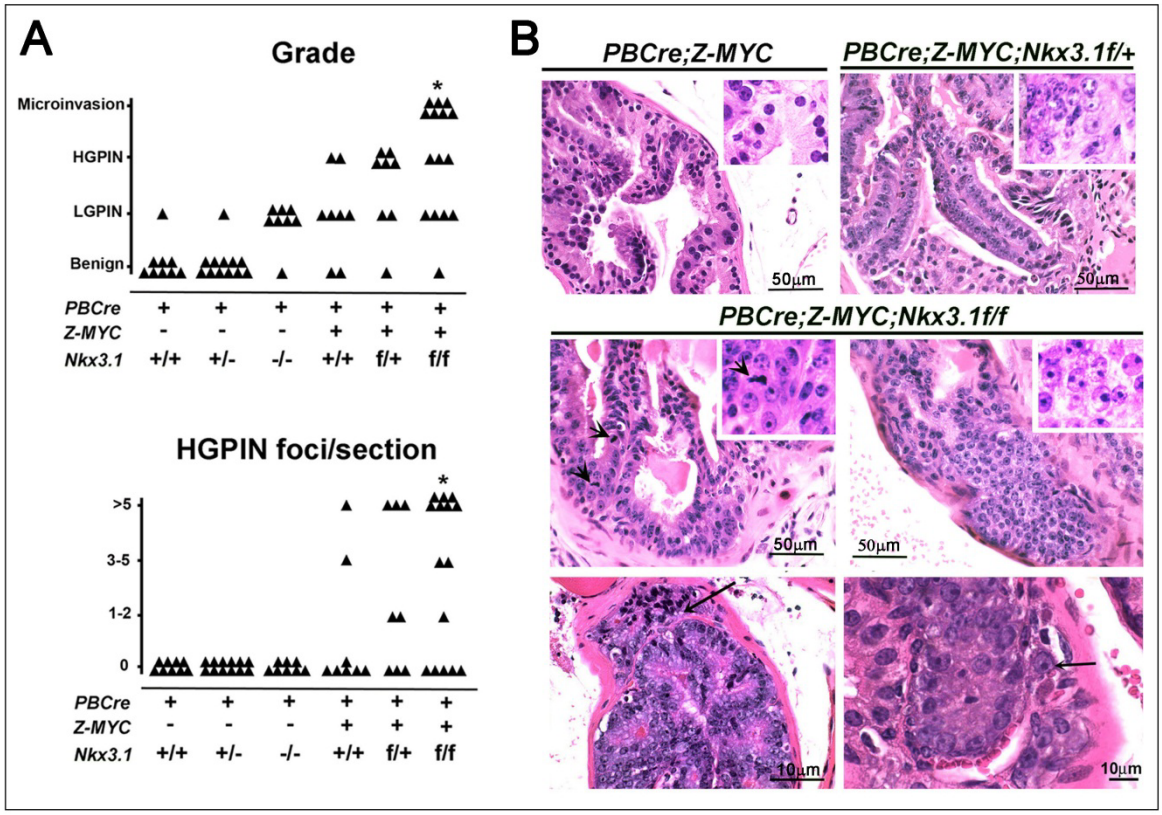

Figure 17. Pathology of compound Nkx3.1/Myc mutant mice. (A) Graph summarizing prostate histology of all Nkx3.1/Myc mutant mice, (top) each triangle represents one prostate, (bottom) each triangle represents HGPIN foci per prostate. Chi-square test, * $p<0.01$ for PBCre;Z-Myc;Nkx3.1$^{f/f}$ relative to PBCre;Z-Myc;Nkx3.1$^{+/+}$. (B) Histological characterization of prostate lesions in Nkx3.1/Myc mutant mice. H&E stained sections of adult prostates show HGPIN lesions in PBCre;Z-Myc;Nkx3.1$^{f/+}$ and PBCre;Z-Myc;Nkx3.1$^{f/f}$ mice. Insets show higher magnifications. Short black arrows denote mitotic figures while long black arrows indicate areas of possible microinvasion.

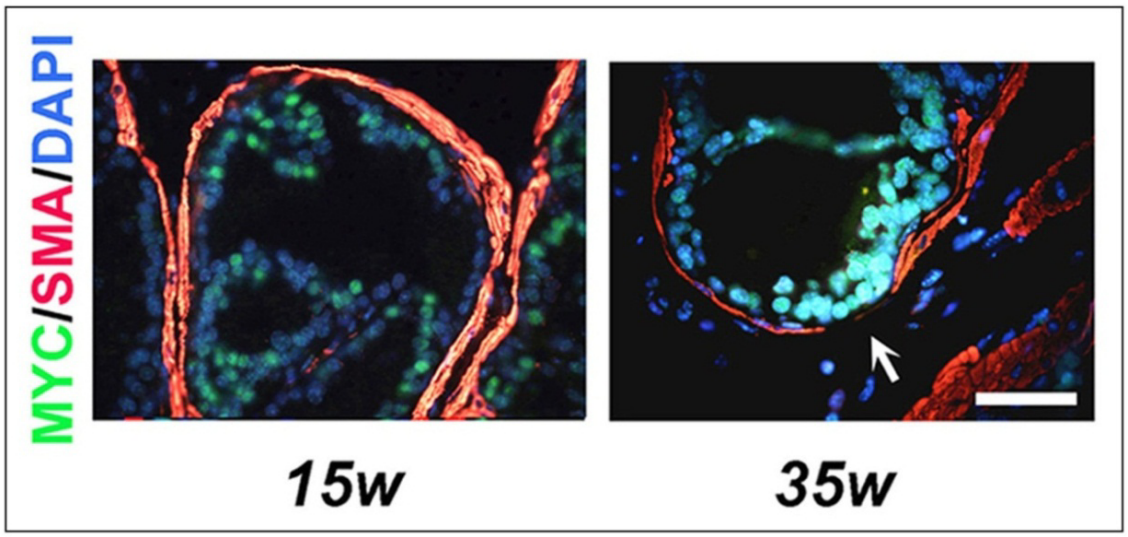

Figure 18. PBCre;Z-Myc;Nkx3.1$^{f/f}$ mice develop microinvasive cancer. Myc (green fluorescence) and smooth muscle actin (SMA, red fluorescence) double staining in a 35 week old PBCre;Z-Myc;Nkx3.1$^{f/f}$ mouse show focal disruption in SMA (white arrow) indicating microinvasion. Scale bar, 50μm.

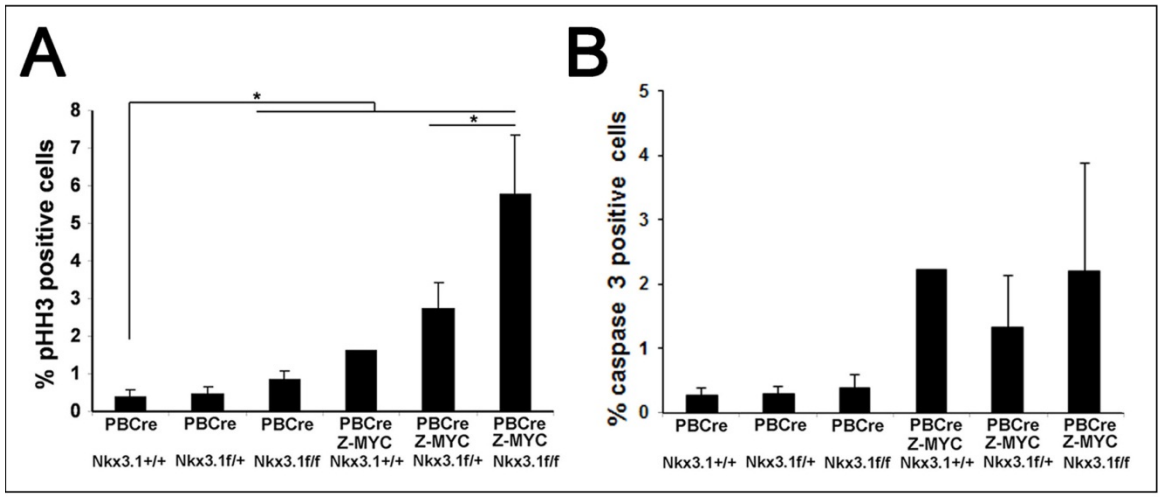

Figure 19. Nkx3.1/Myc mutant mice have increased proliferation but not apoptosis. (A) Proliferation was analyzed using phospho-histone H3 (pHH3), a mitotic marker. Double staining with Myc/pHH3 was performed to quantitate proliferation. Proliferation is significantly increased in PBCre;Z-Myc;Nkx3.1$^{f/f}$ prostates. (B) Apoptosis was assessed by double staining prostates with Myc/activated caspase-3. No significant difference was observed apoptosis among Myc-expressing prostates. N=3-4 mice per group, except PBCre;Z-Myc;Nkx3.1$^{+/+}$ (N=2) which has been previously characterized (Kim et al., 2009). *$p<0.05$.

Nkx3.1 and Myc Co-regulate Tumorigenesis in Regenerated Prostate Tissue Grafts

To extend the *in vivo* studies, prostate regeneration was performed using two tissue recombination models. Prostates regenerated by tissue recombination can recapitulate the phenotypes of transgenic mice (Ishii et al., 2005), with the added advantages of cost-effectiveness and reduction in the time required to generate animals by complex breeding. In the first recombinant model, adult transgenic mouse prostate tissue pieces were combined with fetal rat UGM and rat-tail collagen (Figure 20A). Tissue recombinants were grafted orthotopically into the anterior prostates of adult severe combined immunodeficiency (SCID) mice. Six or 10 weeks later, regenerated prostate grafts were harvested. Regenerated glands were distinguished from host prostate tissue by Myc expression and/or loss of Nkx3.1 expression (Figure 20D). Histological examination of grafts showed that PBCre;Z-Myc;Nkx3.1$^{+/+}$ and PBCre;Nkx3.1$^{f/f}$ grafts were either histologically normal or developed epithelial hyperplasia and LGPIN; grafts from PBCre;Z-Myc;Nkx3.1$^{f/f}$ mice contained HGPIN lesions with microinvasive cancer (Figure 20B-E).

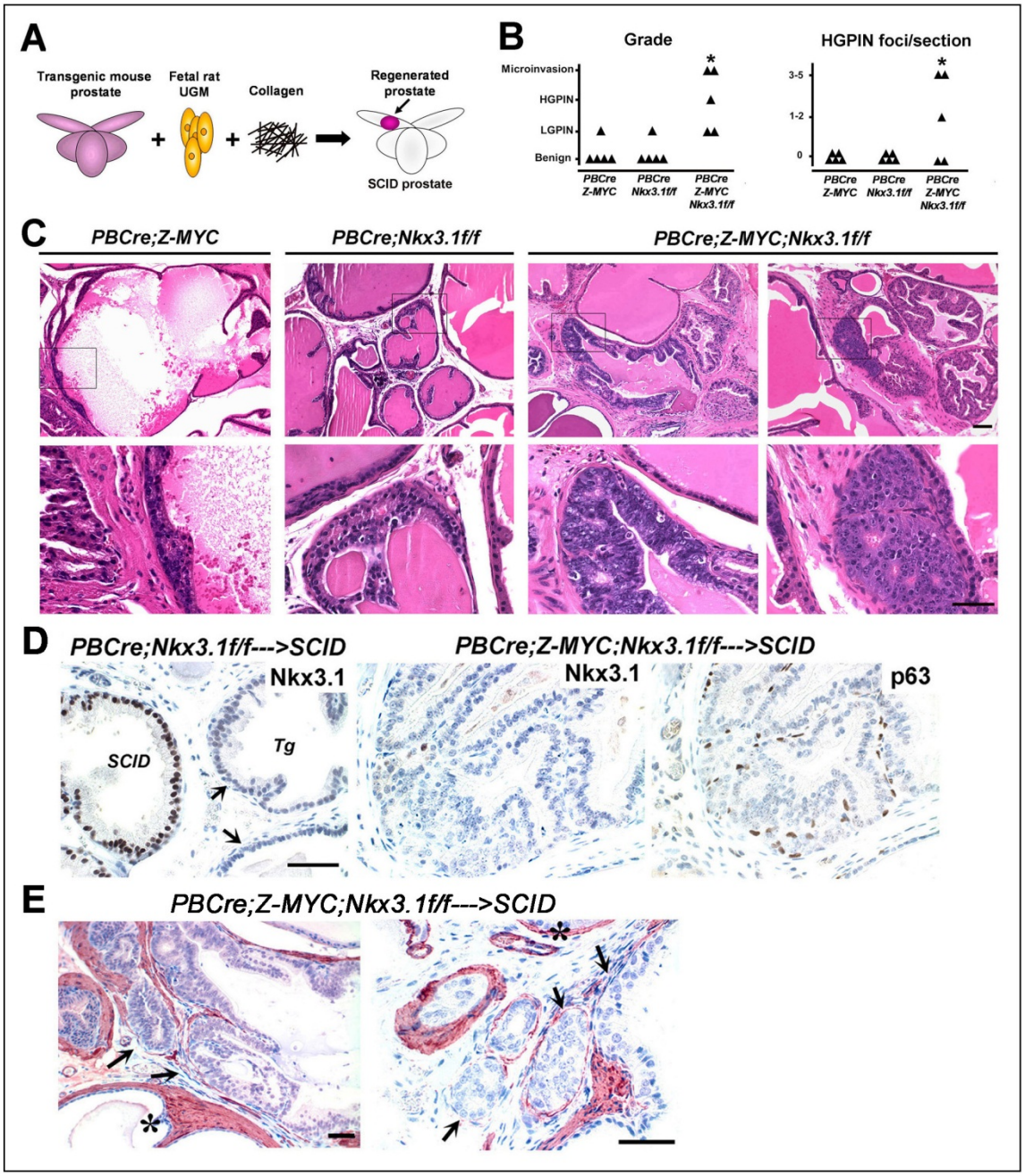
A
Transgenic mouse prostate
Fetal rat UGM
Collagen
Regenerated prostate
SCID prostate
B
Grade
Microinvasion
HGPIN
LGPIN
Benign
PBCre Z-MYC
PBCre Nkx3.1f/f
PBCre Z-MYC Nkx3.1f/f
HGPIN foci/section
3-5
1-2
0
C
PBCre;Z-MYC
PBCre;Nkx3.1f/f
PBCre;Z-MYC;Nkx3.1f/f
D
PBCre;Nkx3.1f/f--->SCID
PBCre;Z-MYC;Nkx3.1f/f--->SCID
Nkx3.1
SCID
Tg
p63
E
PBCre;Z-MYC;Nkx3.1f/f--->SCID

Figure 20. Nkx3.1 and Myc co-regulate prostate tumorigenesis. (A) Scheme for prostate regeneration by tissue recombination to recapitulate phenotypes of transgenic mice. Transgenic prostate pieces were recombined with rat UGM and collagen, and grafted orthotopically in anterior prostate of SCID mice. (B) Graph summarizing pathology of regenerated prostates. (left) each triangle represents one prostate, (right) each triangle represents HGPIN foci per prostate. Chi-square test, *$p<0.05$ for PBCre;Z-Myc;Nkx3.1$^{f/f}$ relative to others. (C) Histological characterization of regenerated prostates. H&E stained sections of 10 week old grafts show mostly normal glands with areas of hyperplasia and dysplasia consistent with LGPIN in PBCre;Z-Myc;Nkx3.1$^{+/+}$ and PBCre;Nkx3.1$^{f/f}$, while PBCre;Z-Myc;Nkx3.1$^{f/f}$ grafts show multiple foci of HGPIN. (D) Loss of Nkx3.1 (brown staining) in regenerated grafts. Host prostate tissue has robust Nkx3.1 expression but transgenic tissue (Tg) shows protein loss, indicated by arrows. p63 staining marks basal cells in HGPIN lesion from PBCre;Z-Myc;Nkx3.1$^{f/f}$ regenerated prostates. (E) Focal loss of SMA (indicated by black arrows) identifies sites of microinvasion in 10 week PBCre;Z-Myc;Nkx3.1$^{f/f}$ grafts. * benign glands with a normal thick stromal layer. Scale bars, 50μm.

In the second regeneration model, tissue recombination combined with lentiviral-mediated gene transfer was employed (Xin et al., 2003). $Nkx3.1^{+/+}$ or $Nkx3.1^{-/-}$ dissociated adult prostate cells were infected with either YFP-control or YFP-Myc-expressing lentivirus (Wang et al., 2010) (Figure 21A,B). Transduced cells were recombined with fetal rat UGM and collagen and grafted under the renal capsule of adult male SCID mice. At 6 weeks, grafts were isolated and Myc expression was confirmed by immunohistochemistry (Figure 21C). Histologically, $Nkx3.1^{+/+}$;Myc grafts showed hyperplastic prostatic glands and a few focal PIN lesions (Figure 21C). On the other hand, $Nkx3.1^{-/-}$;Myc grafts demonstrated HGPIN lesions characterized by prominent nucleoli, high mitotic activity and apoptotic figures (Figure 21C). Further analysis of $Nkx3.1^{-/-}$;Myc glands showed loss of SMA and p63, characteristics of microinvasion and neoplastic progression, respectively (Figure 21C). The $Nkx3.1^{-/-}$;Myc glands had an increased proliferative index, while apoptosis was not changed (Figure 21D). The regenerated grafts complement the phenotypes observed in the intact transgenic prostates and confirm cooperation between Nkx3.1 and Myc.

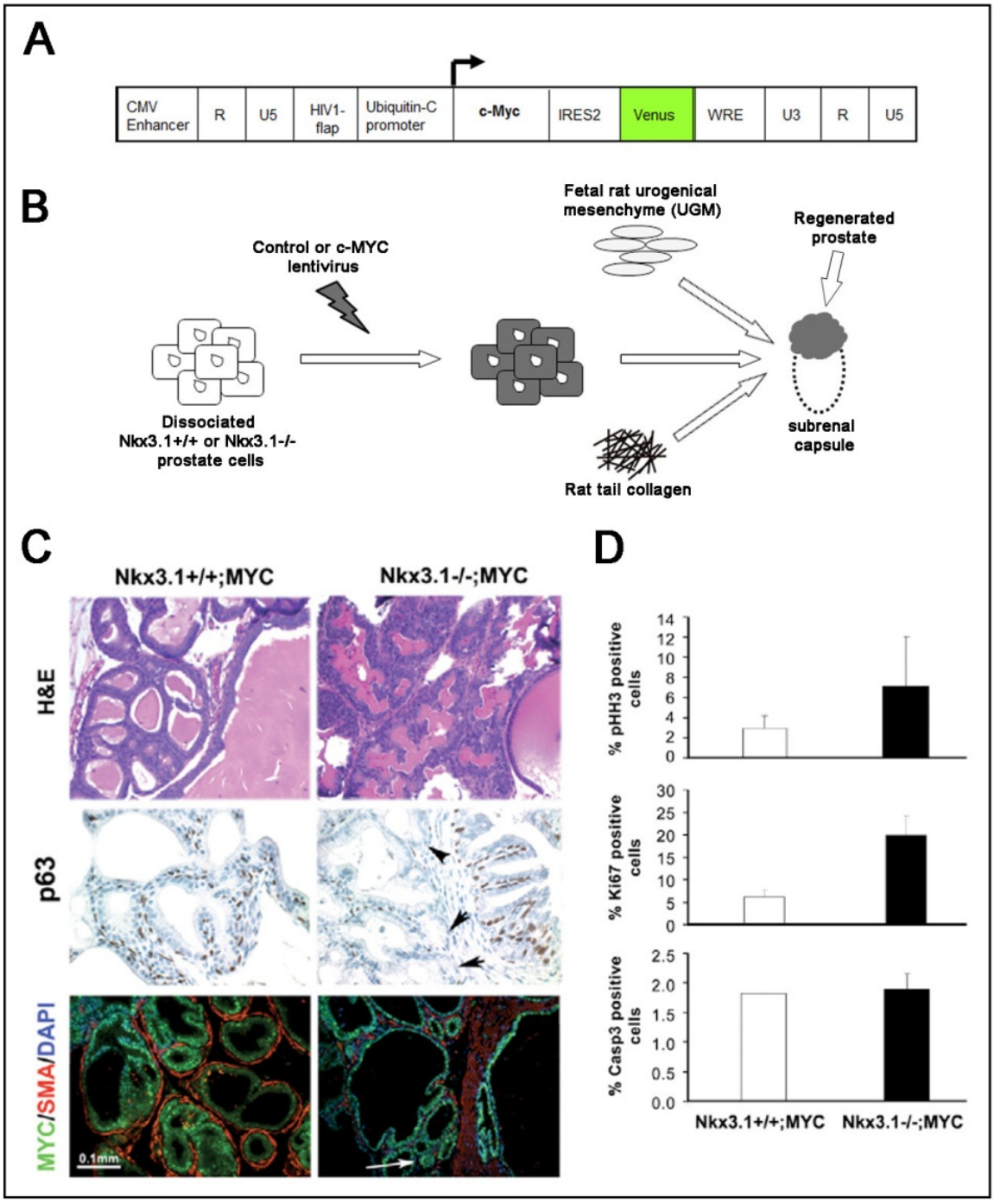
A
CMV Enhancer
R
U5
HIV1-flap
Ubiquitin-C promoter
c-Myc
IRES2
Venus
WRE
U3
R
U5
B
Control or c-MYC lentivirus
Fetal rat urogenical mesenchyme (UGM)
Regenerated prostate
Dissociated Nkx3.1+/+ or Nkx3.1-/- prostate cells
subrenal capsule
Rat tail collagen
C
Nkx3.1+/+;MYC
Nkx3.1-/-;MYC
H&E
p63
MYC/SMA/DAPI
0.1mm
D
% pHH3 positive cells
% Ki67 positive cells
% Casp3 positive cells
Nkx3.1+/+;MYC
Nkx3.1-/-;MYC

Figure 21. Cooperativity between Myc and Nkx3.1 loss in vivo by prostate regeneration. (A) Schematic of lentivirus containing Myc and YFP. (B) Diagram for subrenal prostate tissue regeneration. $Nkx3.1^{+/+}$ or $Nkx3.1^{-/-}$ dissociated adult prostate cells are infected with either YFP-control or YFP-Myc-expressing lentivirus. Transduced cells are recombined with fetal rat UGM and collagen and grafted under the renal capsule of adult male SCID mice. (C) Histology, focal loss of basal cells (p63) and microinvasion (focal loss of SMA) indicate progression in $Nkx3.1^{-/-}$;Myc grafts. (D) Double staining with Myc was used to quantitate proliferation and apoptosis in Myc-expressing glands. Increased proliferation (phospho-histone H3 and Ki67 staining) is observed in $Nkx3.1^{-/-}$;Myc grafts compared to $Nkx3.1^{+/+}$;Myc grafts. Apoptosis, assessed by activated caspase-3 staining, is unchanged. N=3 grafts per group.

Discussion

Prostate tumorigenesis involves sequential accumulation of genetic mutations. Mutations in Nkx3.1 and Myc are frequently and simultaneously detected in human prostate tumor samples (Kindich et al., 2006; Locke et al., 2012). Prevailing attempts have been made to delineate the relationship between Nkx3.1 and Myc; however, whether they in fact cooperate to promote tumorigenesis remains unknown. PBCre;Z-Myc;Nkx3.1$^{f/f}$ mice were used to examine the cooperation between Nkx3.1 loss and Myc gain. These compound mutant mice exhibit focal Myc activation and conditional deletion of Nkx3.1. Myc activation and deletion of Nkx3.1 individually result in mild pathology. Similar to previous studies, PBCre;Z-Myc;Nkx3.1$^{+/+}$ mice largely result in histologically normal glands, with a small percentage developing LGPIN (Kim et al., 2009). Additionally, PBCre;Nkx3.1$^{f/f}$ prostates develop hyperplasia and dysplasia. Conversely, concurrent loss of Nkx3.1 and Myc gain in PBCre;Z-Myc;Nkx3.1$^{f/+}$ and PBCre;Z-Myc;Nkx3.1$^{f/f}$ mice develop HGPIN, indicative of cooperation. These lesions show atypical cells characterized by prominent nucleoli, hyperchromatia, and mitotic figures. Notably, several of these HGPIN lesions demonstrate microinvasive cancer. PBCre;Z-Myc;Nxk3.1$^{f/f}$ prostates also show a significant increase in proliferation with no difference in apoptosis relative to controls. The net increase in proliferation further supports the cooperation between Nkx3.1 loss and Myc overexpression.

In addition to the transgenic model, two independent prostate tissue regeneration studies were performed. Both studies support the cooperative phenotype observed in the transgenic prostates. These results confirm that loss of Nkx3.1 and overexpression of Myc are independent events that cooperate to promote tumorigenesis.

In summary, these studies present three models with Nkx3.1 deletion and Myc activation. Focal Myc expression cooperates with Nkx3.1 loss and results in HGPIN lesions with microinvasive cancer. Given that genetic mutations affecting Nkx3.1 and Myc expression are regularly observed in prostate cancer, these models provide valuable tools to further analyze the molecular mechanisms associated with these tumors. They also provide a means to identify molecular targets driving tumorigenesis.

Materials and Methods

Animals

Z-Myc, PBCre, Nkx3.1$^{+/+}$, Nkx3.1$^{-/-}$ and Nkx3.1$^{f/f}$ mice have been described (Abdulkadir et al., 2002; Roh et al., 2006; Wu et al., 2001). The generation of PBCre;Nkx3.1$^{+/+}$, PBCre;Nkx3.1$^{f/+}$ PBCre;Nkx3.1$^{f/f}$ mice have been described in Chapter II. The generation of trigenic PBCre;Z-Myc;Nkx3.1$^{+/+}$, PBCre;Z-Myc;Nkx3.1$^{f/+}$, PBCre;Z-Myc;Nkx3.1$^{f/f}$ have also been described in Chapter II. All animal care and experiments were carried out according to Vanderbilt Institutional Animal Care and Use Committee approved protocols.

Immunohistochemical staining

Tissues were prepared for histological analysis as previously described in Chapter II. Primary antibodies include Nkx3.1 (1:1000, gift from Dr. Charles Bieberich), pHH3 (identifies mitotic cells) (1:500, Upstate), Ki67 (identifies cells undergoing active phases of the cell cycle) (1:50, Abcam), activated caspase 3 (identifies apoptotic cells) (1:500, Cell Signaling), SMA (1:2000, Sigma) and p63 (PIN cocktail, Biocare medical).

Double immunofluorescence staining

Tissues were prepared for histological analysis similar to the previously described method in Chapter II. Following antigen retrieval, sections were initially incubated in

Mouse on Mouse (M.O.M.) blocking reagent (Vector Laboratories) for 1 hr and subsequently blocked in 1% BSA diluted in PBS for 1 hr. The first primary antibody was Myc (1:15000, Santa Cruz) and the second primary antibody was SMA (1:2000, Sigma-Aldrich).

Proliferation and Apoptosis Assay

Following double immunohistochemical staining for pHH3, Ki67 and activated Caspase 3 combined with Myc, at least 500 Myc-positive cells (in prostates expressing Myc) per sample were counted and quantified. Number analyzed per group was 3-4 prostates.

Lentiviral preparation

Lentivirus construction and preparation was performed as previously described (Wang et al., 2010). Briefly, lentivirus constructs (Control or Myc) were co-transfected with VSV-G envelope glycoprotein, and HIV-1 packaging vector Δ8.9 (gift from Dr. David Baltimore, Caltech) in 293FT cells (Invitrogen) using PEI reagent (Sigma). Virus supernatant was collected at 48 and 72 hrs post transfection and filtered through a 0.45 μm filter. Viral supernatants were concentrated by ultracentrifugation using Centricon-70 filters (Millipore) according to the manufacturer's protocol. Concentrated virus was stored at -80°C. To titer, $1x10^5$ HT1080 cells were plated in a 6-well cell culture dish. The following day, cells were transduced with serial dilutions of virus and 8μg/mL

polybrene (Millipore). Three days post-transduction, cells were subjected to FACS analysis and analyzed for YFP-positive cells.

Prostate Regeneration by Tissue Recombination

Tissue regeneration was performed similar to previous reports (Wang et al., 2010; Xin et al., 2003). Rat UGS was dissected from 18-day embryonic fetuses of Sprague Dawley rats followed by 90 minute tryptic digestion (10mg/mL 1:250, Fisher Scientific) at 4°C (Hayward et al., 1998). Adult Nkx3.1$^{+/+}$ and Nkx3.1$^{-/-}$ prostates were dissected, minced and digested using collagenase (GIBCO) in DMEM supplemented with 10% FBS at 37°C for 90 min. Following digestion, cells were passed through 100μm nylon mesh (Fisher Scientific) and washed. Dissociated cells were counted using hemacytometer and viability was determined by trypan blue exclusion. 1x10^5 viable cells were transduced with concentrated lentivirus (control or Myc) at multiplicity of infection (MOI) =50, in the presence 8μg/mL polybrene, followed by centrifugation for 3 hrs (Xin et al., 2003). Transduced cells were thoroughly washed and recombined with rUGM in rat tail collagen (Hayward et al., 1998). Recombinants were incubated overnight and grafted beneath the renal capsule of adult male SCID mice. Grafts were collected 6 weeks post grafting.

For orthotopic tissue regeneration, 8-10 week old PBCre;Z-Myc;Nkx3.1$^{+/+}$, PBCre;Nkx3.1$^{f/f}$ or PBCre;Z-Myc;Nkx3.1$^{f/f}$ mouse prostate tissues and single cell rUGM were combined and incubated in collagen overnight. Recombinants were grafted under the capsule of one anterior prostate lobe of adult SCID mice and 6 or 10 weeks later, grafts were harvested.

Statistical Analysis

Student's t-test and Chi-squared test were used to determine statistical significance. Values were deemed significant at $p<0.05$. Quantitative variables were represented as means ± SD.

CHAPTER IV

NKX3.1 AND MYC CO-REGULATE SHARED TARGETS INVOLVED IN PROSTATE TUMORIGENESIS

Introduction

Classical studies showed that malignant transformation is crucially dependent on cooperative interactions between distinct oncogenic mutations (Fearon and Vogelstein, 1990; Knudson, 2001, 1985; Land et al., 1983b; Ruley, 1983). In a model of Ras/p53-mediated transformation, cooperativity between oncogenic mutations was shown to be mediated by the synergistic co-regulation of a class of targets called 'cooperation response genes' which are required for tumorigenicity (McMurray et al., 2008). Presently, it is unknown whether co-regulation of multiple cancer-related target genes by cooperating oncogenic mutations is a general process in carcinogenesis (Luo and Elledge, 2008). Furthermore, the potential mechanism(s) that drive specific oncogenes/TSGs to promote target gene cooperativity are unclear. In principle, for oncogenes/TSGs that encode transcription factors, direct co-regulation of target gene expression might provide a mechanistic explanation for cooperativity.

Using both transgenic mice and prostate regeneration models (described in Chapters II and III), Nkx3.1 was shown to cooperate with Myc overexpression and resulted in the development of multifocal HGPIN lesions with microinvasive cancer.

Nkx3.1 is a prostate-specific transcription factor that mediates transcriptional regulation to protect the prostate from tumor initiation (Kim et al., 2002a; Lei et al., 2006; Schneider et al., 2000). Myc, another transcription factor and known oncogene, is a potent regulator of cell growth, proliferation and thereby a contributing factor to tumorigenesis (Dang et al., 2006). To test the hypothesis that Nkx3.1/Myc co-regulate shared gene targets to promote prostate cancer progression, a combination of gene expression analyses, chromatin immunoprecipitation (ChIP) and immunohistochemistry assays were performed. In this chapter, Nkx3.1 and Myc are shown to coordinate the regulation of cancer-relevant genes that facilitate tumor progression.

Results

Network Analysis Identifies Genes Co-regulated by Nkx3.1 and Myc

ChIP-seq analysis was performed to identify Nkx3.1 binding sites within mouse prostate (Anderson P, 2012). Network analysis was conducted to identify potential relationships between Nkx3.1 target genes and target genes of other major transcription factors. Direct Nkx3.1 targets were queried using the GeneGO MetaCore database (Nikolsky et al., 2005), which builds gene networks among transcription factors. Remarkably, the top

ranked network ($p=3.94 \times 10^{-169}$) enriched in direct Nkx3.1 target genes was the proto-oncogene Myc (Figure 22A, Table 4). Specifically, 65 of the 282 identified direct Nkx3.1 targets were also direct Myc targets (Figure 22A, Table 4). More importantly, several of the co-regulated genes were among cancer-relevant pathways and significantly associated with relapse in human prostate cancer patients (Anderson P, 2012). These data suggest that alterations in Nkx3.1 and Myc might cooperate to modulate the expression of common targets relevant to tumor progression. Furthermore, it implies the intriguing possibility that loss of Nkx3.1 may promote prostate tumorigenesis by deregulating a set of cancer-related target genes that are also regulated by Myc.

The direct Myc targets identified in GeneGO analysis were based on published binding data (ie. ChIP-chip or ChIP-seq) derived from cell lines such as fibroblasts, lymphocytes and embryonic stem cells. To validate Myc binding of shared target genes identified by GeneGO in the prostate, ChIP-PCR was performed. Myc-CaP cells, prostate cancer cells derived from a c-Myc transgenic mouse (Watson et al., 2005) that expresses Myc but not Nkx3.1 (Figures 22B), were used to confirm Myc binding. Myc binding was confirmed in seven out of eight shared target genes tested, including Hk2 (hexokinase 2), Prdx6 (peroxiredoxin 6), Txnip (thioredoxin-interacting protein), Sept9 (MLL septin-like fusion protein), Mt2 (metallothionein-2), Mt3 (metallothionein-3) and Utrn (utrophin) (Figure 22B). Binding was also confirmed in LNCaP cells, which express Nkx3.1 and Myc (Anderson P, 2012).

Table 4. GeneGO MetaCore network analysis for the top three transcription factor networks enriched in direct Nkx3.1 target genes.

#	Network	Go Processes	Total nodes	Root nodes	p-value	zScore	gScore
1	SP1	response to chemical stimulus (43.6%; 2.506e-10), response to copper ion (7.7%; 1.245e-08), negative regulation of interleukin-12 biosynthetic process (5.1%; 1.352e-08), negative regulation of vitamin D biosynthetic process (5.1%; 2.695e-08), negative regulation of vitamin metabolic process (5.1%; 2.695e-08)response to chemical stimulus (43.6%; 2.506e-08)	80	79	3.55E-186	125.06	125.06
2	Myc	negative regulation of molecular function (21.1%; 5.612e-10), negative regulation of interleukin-12 biosynthetic process (5.6%;9.218e-09), negative regulation of vitamin D biosynthetic process (5.6%; 1.838e-08), negative regulation of vitamin metabolic process (5.6%; 1.838e-08), negative regulation of calcidiol 1-monooxygenase activity (5.6%;3.298e-08)	73	72	3.95E-169	119.31	119.31
3	HNF4α	response to hormone stimulus (20.8%; 1.038e-05), response to endogenous stimulus (20.8%; 2.783e-05), response to organic substance (26.4%; 3.025e-05), response to steroid hormone stimulus (15.1%; 3.301e-05), cellular amino acid and derivative metabolic process (15.1%; 7.787e-05)	55	54	8.69E-126	103.07	103.07

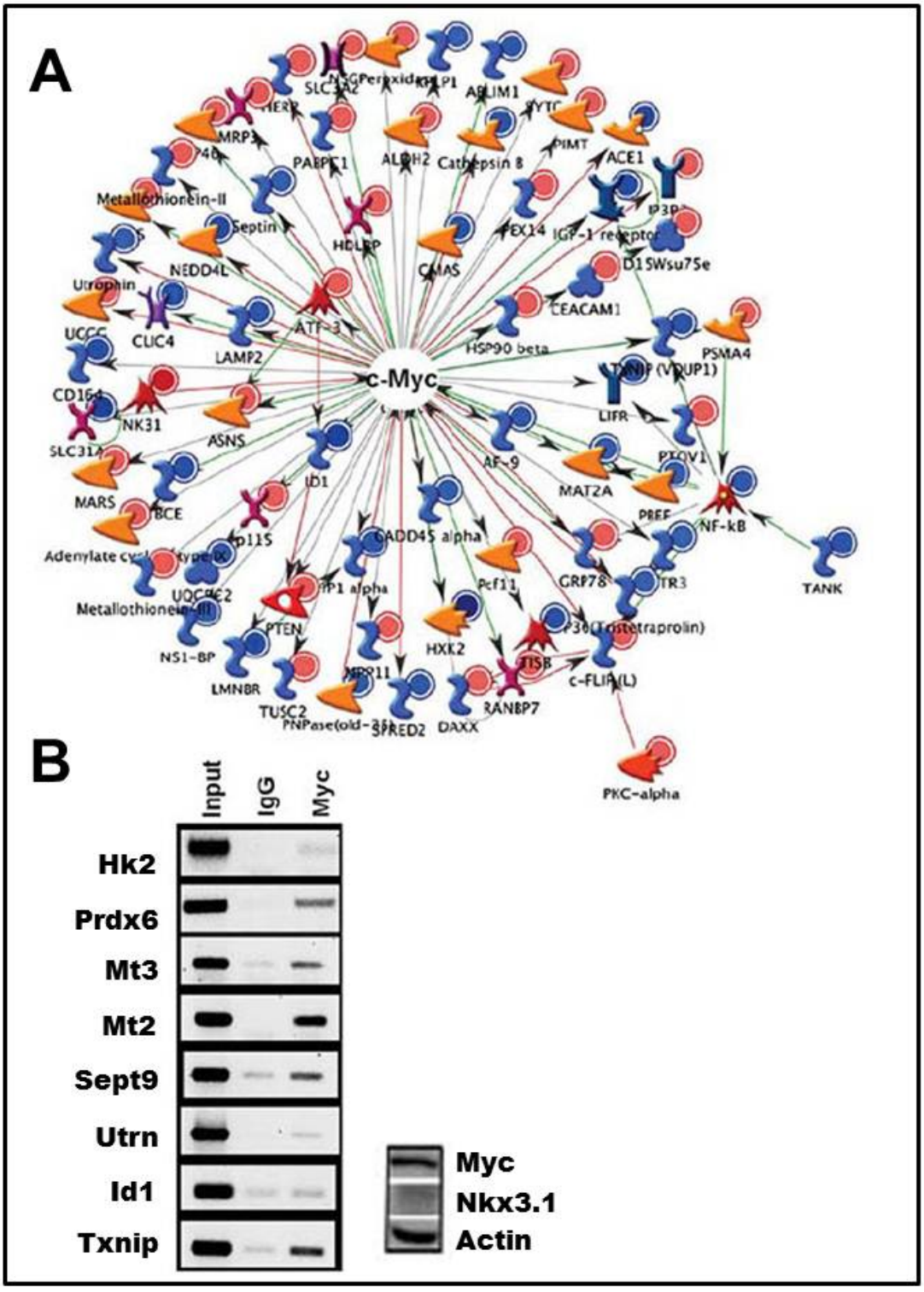
A
c-Myc
MRP3
PAEPE1
ALDH2
Cathepsin 8
PIMT
ACE1
Metallothionein-II
Septin
HDLBP
CMAS
IGF-1 receptor
D15Wsu75e
NEDD4L
Utrophin
CLIC4
ATF-3
CEACAM1
LAMP2
HSP90 beta
PSMA4
CD164
NK31
ASNS
LIFR
SLC31A
ID1
AF-9
MATZA
MARS
BCE
GADD45 alpha
NF-kB
TANK
GRP78
TR3
PTEN
Pcf11
HXK2
TISB
c-FLIP(L)
NS1-BP
LMNBR
TUSC2
RANBP7
DAXX
PKC-alpha
B
Input
IgG
Myc
Hk2
Prdx6
Mt3
Mt2
Sept9
Utrn
Id1
Txnip
Myc
Nkx3.1
Actin

Figure 22. Identification of a subset of direct Nkx3.1 target genes co-regulated by Myc. (A) Network Analysis using GeneGO MetaCore^{TM} software identifies a subset of direct Nkx3.1 target genes that are known to be bound by Myc ($p=3.94x10^{-169}$). Genes upregulated in the Nkx3.1 germline knockout prostates are shown as blue circles, while those downregulated are indicated in as red circles in the diagram. (B) ChIP-PCR validation of Myc binding to select shared Nkx3.1/Myc target genes in Myc-CaP mouse prostate adenocarcinoma cell line. These cell lines express Myc but not Nkx3.1 as shown in the inset western blot. Results are representative of at least two independent experiments.

Nkx3.1 Interacts with Myc

Nkx3.1 and Myc are both transcription factors that function by binding to target gene promoters. Network and ChIP analyses have shown that these proteins bind common gene promoters. As a result, studies were conducted to determine if Nkx3.1 and Myc physically interact. Co-immunoprecipitation assays performed in 293T cells showed that Myc and Nkx3.1 were in complex. To investigate if DNA was mediating the interaction, proteins were released from DNA by ethidium bromide treatment. Binding between Nkx3.1 and Myc was not abrogated by ethidium bromide treatment, indicating that the interaction was independent of DNA (Figure 23B). On the other hand, the Myc MBII deletion mutant showed severely reduced binding to Nkx3.1 (Figure 23C). These results suggest that Nkx3.1 interacts with the MBII domain of Myc, a region that plays an important role in transcriptional activity of the protein. Hence, binding of Nkx3.1 to the MBII domain has the potential to modulate Myc transcriptional activity.

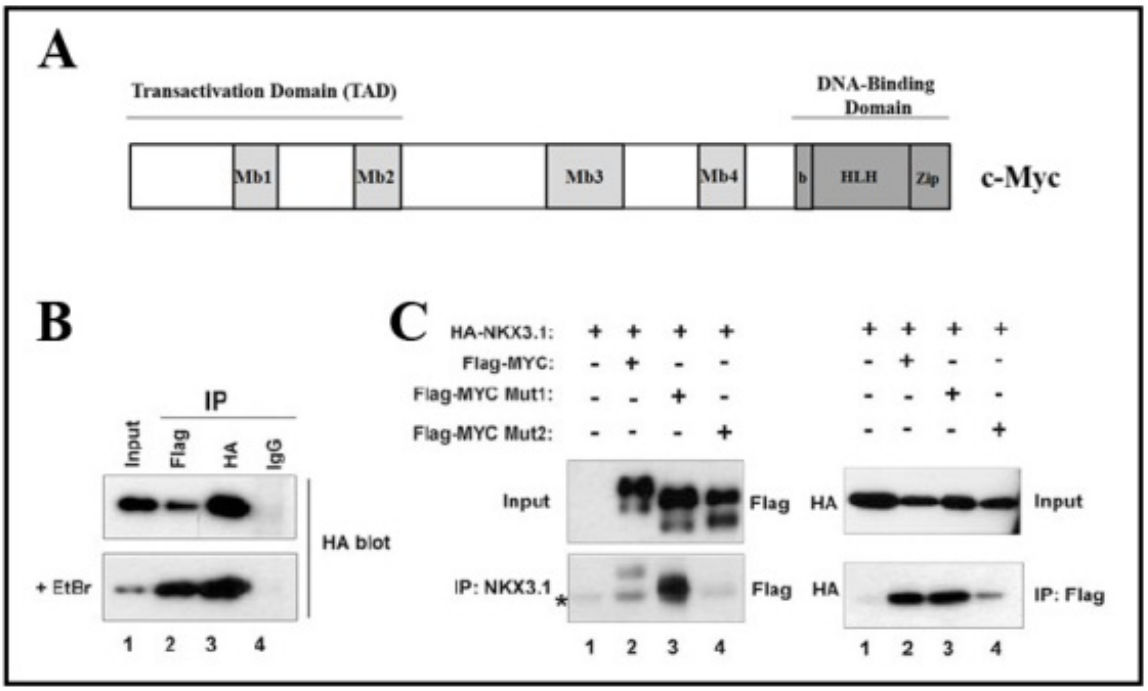

Figure 23. Nkx3.1 and Myc interact. (A) Structure of the Myc protein. Transactivation domain (TAD) which includes evolutionary conserved MBI-II; MBIII-IV; DNA binding domain, bHLH-LZ. (B) Nkx3.1 and Myc co-immunoprecipitation. Cell lysates from 293T cells expressing HA-Nkx3.1 and Flag-Myc were immunoprecipitated with the indicated antibodies and immunoblotted for HA. To release proteins from DNA, ethidium bromide (EtBr) was added to lysates for 30 minutes prior to immunoprecipitation and during washes. (C) Co-immunoprecipitation of HA-Nkx3.1 and Flag-Myc wild type (lane 2), Flag-Myc mutant 1 lacking Myc-box 1 (lane 3) or Flag-Myc Mutant 2 lacking Myc-box 2 (lane 4) in 293T cells. *indicates a non-specific band.

Nkx3.1 Opposes Myc Transcriptional Activity

To directly examine co-regulation of target genes by Nkx3.1 and Myc, $p53^{-/-}$;$Arf^{-/-}$ double knockout (DKO) mouse embryonic fibroblasts (MEF) expressing the MycER protein were used (Eilers et al., 1989). MycER is fusion protein containing a modified oestrogen receptor ligand-binding domain (ER) (Eilers et al., 1989; Littlewood et al., 1995). This steroid-activatable protein is constitutively expressed but only responsive to the synthetic steroid 4-hydroxytamoxifen (OHT). OHT activation of MycER induces proliferation, apoptosis and transformation in the same manner as wild type Myc (Alarcon et al., 1996; Eilers et al., 1989; Littlewood et al., 1995).

The ability of Nkx3.1 to modulate Myc-regulated activation/repression was explored using this inducible MycER system. Quantitative RT-PCR results demonstrated that Nkx3.1 opposed Myc-mediated activation of several cancer relevant targets such as Hk2 (a key regulator of glycolytic pathway); Igf1r (insulin-like growth factor 1 receptor; a tyrosine kinase receptor that activates PI3K and MAPK growth signaling cascades) and Nedd4L (neural precursor cell expressed, developmentally down-regulated 4-like; a ubiquitin ligase that regulates TGFβ signaling) (Figure 24A). Nkx3.1 also opposed Myc repression of genes such as Id1 (inhibitor of DNA binding 1; a dominant negative helix-loop-helix protein) (Figure 24A).

To determine if Nkx3.1 modulates endogenous Myc-mediated regulation of target genes in the prostate, LNCaP cells (endogenously expresses Nkx3.1 and Myc) were employed. When Nkx3.1 is decreased in LNCaP cells, Hk2 is upregulated (Figure 24C).

Furthermore, in LNCaP cells engineered to express the MycER protein, depletion of Nkx3.1 by siRNA enhanced tamoxifen-induced activation of Hk2 gene expression (Figure 24D). Overall, these results indicate that Nkx3.1 can modulate Myc's ability to regulate a subset of shared target genes.

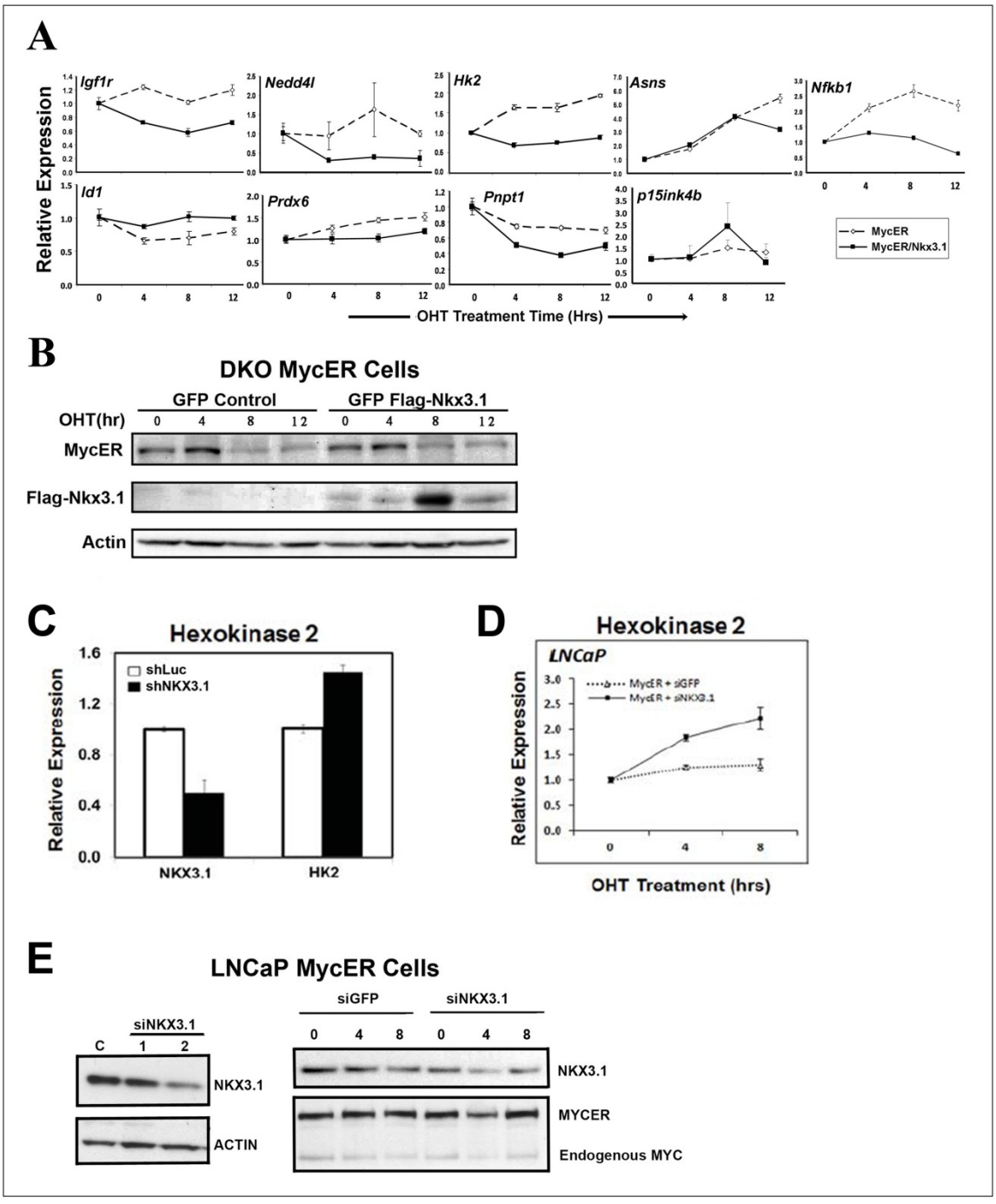
A
Relative Expression
Igf1r
Nedd4l
Hk2
Asns
Nfkb1
Id1
Prdx6
Pnpt1
p15ink4b
MycER
MycER/Nkx3.1
OHT Treatment Time (Hrs)
B
DKO MycER Cells
GFP Control
GFP Flag-Nkx3.1
OHT(hr)
0 4 8 12 0 4 8 12
MycER
Flag-Nkx3.1
Actin
C
Hexokinase 2
shLuc
shNKX3.1
Relative Expression
NKX3.1
HK2
D
Hexokinase 2
LNCaP
MycER + siGFP
MycER + siNKX3.1
Relative Expression
OHT Treatment (hrs)
E
LNCaP MycER Cells
siNKX3.1
C 1 2
NKX3.1
ACTIN
siGFP
siNKX3.1
0 4 8 0 4 8
NKX3.1
MYCER
Endogenous MYC

Figure 24. Nkx3.1 and Myc co-regulate expression of shared target genes. (A) Expression of selected Nkx3.1/Myc target genes was analyzed using quantitative RT-PCR in an inducible Myc system (MycER;$p53^{-/-}$;$Arf^{-/-}$ MEFs) where Myc is activated by OHT. The effect of concurrent Nkx3.1 expression on Myc target gene activation (Hk2, Igf1r, Nedd4l, Nfkb1, Asns, Prdx6) or repression (Id1, Pnpt1, p15Ink4b) was determined. (B) Expression of the induced proteins determined by western blot analysis in DKO-MycER cells transfected with Flag-tagged Nkx3.1-expressing lentivirus co-expressing GFP and treated with OHT. (C) Depletion of Nkx3.1 by shRNA in LNCaP cells leads to upregulation of Hk2 expression, compared to control shLuc (luciferase shRNA) treatment. (D) Depletion of Nkx3.1 by siRNA in LNCaP-MycER cells enhances activation of Hk2 gene expression by OHT, compared to control siGFP treatment. (E) (left) Expression of Nkx3.1 knockdown in LNCaP-MycER cells before OHT induction. "C" denotes control, 1 and 2 are different siRNA transfection conditions. (right) Expression of the indicated proteins determined by western blot analysis in LNCaP-MycER cells transfected with Nkx3.1 or GFP siRNA and treated with OHT. qRT-PCR results are mean $\pm$ s.d., representative of at least two independent experiments performed in triplicate.

Nkx3.1 and Myc Co-regulate Target Gene Expression

Gene expression microarray data analysis of Myc-grafts (high Myc/low Nkx3.1) and control grafts (low Myc/high Nkx3.1) showed that expression of 20 of 65 Myc/Nkx3.1 shared targets were significantly altered compared to only two of 65 randomly selected genes (FDR=0.02; Chi-square=166.9846, P<2.2e-16) (Figure 25A). Microarray data obtained from human prostate cancer cells (LNCaP, PC3 and DU145) following Myc knockdown by siRNA (Koh et al., 2011) was examined for evidence of shared target gene dysregulation following Myc depletion. Using a 1.4-fold cut-off, significant dysregulation of shared target genes compared to a randomly generated list of genes was observed in all three cell lines (Figure 25B). Concordance in the direction of target gene expression changes (ie. up or down) between the human cell line siMyc and Myc-overexpressing mouse prostate microarray data sets was also analyzed (Figure 25B). The concordance rate was high for the PC3 and DU145 cells (0.67 and 0.75 respectively), but lower (0.4) for LNCaP cells. The lower concordance rate in LNCaP cells may be related to the fact that, Myc knockdown in these cells led to a significant alteration in Nkx3.1 levels, thereby confounding the analysis.

The potential relevance of Nkx3.1/Myc co-regulated genes in human prostate cancer was also explored. Raw microarray and clinical data for 66 patients was downloaded from NCBI Gene Expression Omnibus (GSE21034) (Taylor et al., 2010). The patients were divided into two categories based on presence (32 patients) or absence (34 patients) of biochemical relapse. Using GSEA, an association between relapse and expression of

Nkx3.1/Myc shared target genes (FDR=0.036) was observed (Anderson P, 2012). In tumors from patients without relapse, significant upregulation of a subset of shared genes (Figure 25C) was detected. The specific genes driving this association were found in the leading edge of the gene set. Notably, nine of the 13 genes (70%) downregulated in the Myc-expressing (high Myc/low Nkx3.1) mouse prostate tissues were upregulated in tumors without relapse. These genes include Ugcg (UDP-glucose ceramide glucosyltransferase), Mt2A (metallothionein 2A), Itpr2 (inositol 1,4,5-trisphosphate receptor, type 2), Ceacam1 (carcinoembryonic antigen-related cell adhesion molecule 1), Aldh2 (aldehyde dehydrogenase 2 family), Cflar (casp8 and fadd-like apoptosis regulator), Prcka (protein kinase C, alpha) and Atf3 (activating transcription factor 3). These results suggest that suppression of these shared target genes in high Myc/low Nkx3.1 tumors may favor relapse.

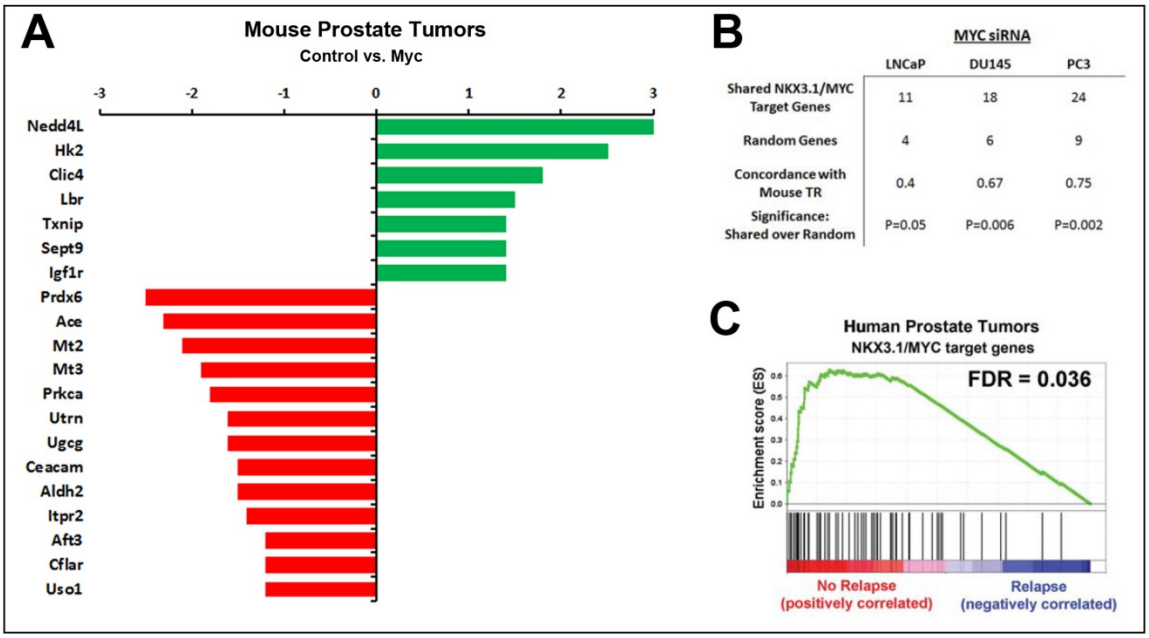

Figure 25. Dysregulation of shared Nkx3.1/Myc target genes in mouse and human prostate cancer cells. (A) Dysregulation of Nkx3.1/Myc targets in prostate regeneration model. Affymetrix arrays were used to compare gene expression between regenerated prostate epithelial cells transduced with Myc or control lentivirus. The Myc grafts contain HGPIN lesions with loss of Nkx3.1 and Myc overexpression. Using Significance Analysis of Microarrays and a 1.4 cut-off, 20 out of 65 Nkx3.1/Myc target genes were significantly altered in the HGPIN lesions, seven were significantly upregulated (green) and 13 were significantly downregulated (red). (B) Gene expression microarray data for LNCaP, PC3, and DU145 cells treated with siRNA targeting Myc (Koh et al., 2011) was examined for alterations in Nkx3.1/Myc target genes. There was significant alteration in the expression of shared Nkx3.1/Myc targets compared to a randomly generated list of genes. The concordance in the direction of gene expression changes due to Myc modulation in cell lines was determined. (C) GSEA analysis shows association between expression of Nkx3.1/Myc target genes and relapse in human prostate tumors. Gene expression arrays from tumors consisting of 32 tumors with relapse and 34 tumors without relapse were analyzed. False Discovery Rate (FDR) = 0.036.

To determine if the shared target genes were altered in Nkx3.1/Myc mutant mice, immunohistochemistry was employed to examine expression levels of Hk2, Nedd4L and Prdx6. In prostate tissue sections from PBCre;Z-Myc;Nkx3.1$^{f/f}$ mouse grafts, significant upregulation of Hk2 and Nedd4L and downregulation of Prdx6 proteins were observed in the HGPIN/microinvasive carcinoma lesions (Figure 26). Additional immunohistochemical staining showed coordinate target gene dysregulation within the same HGPIN lesion (Figure 27). These results support a role for these co-regulated target genes in prostate tumorigenesis.

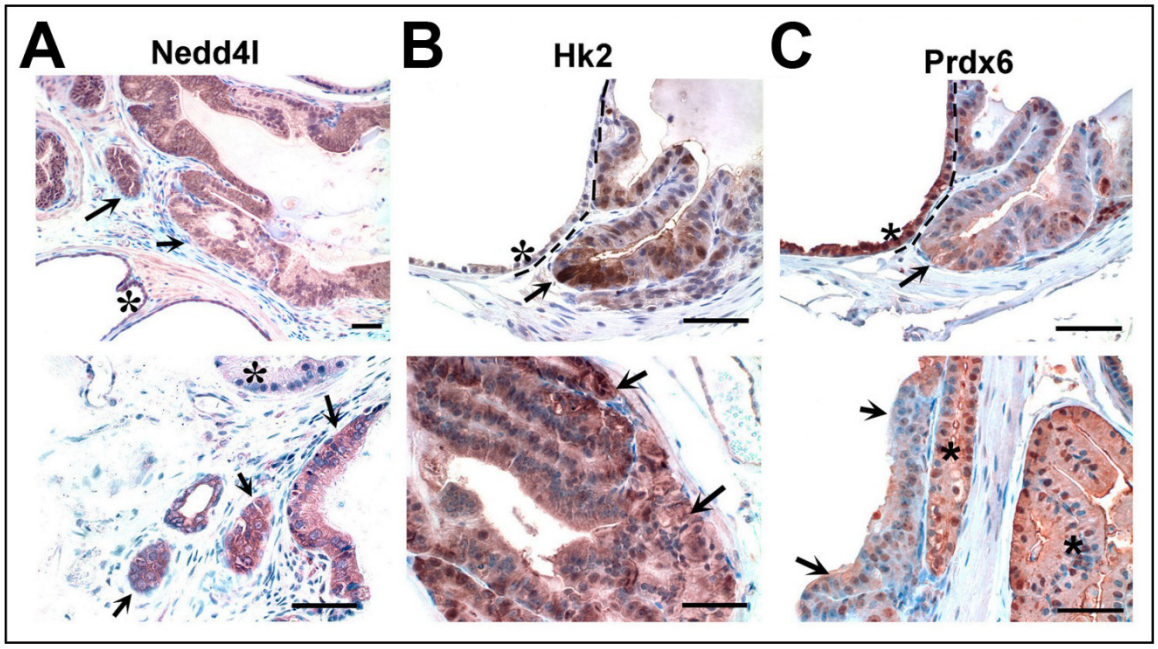

Figure 26. Nkx3.1 and Myc co-regulate target gene expression in early tumor lesions. 10 week PBCre;Z-Myc;Nkx3.1$^{f/f}$ regenerated prostates show modulated expression of shared target genes in HGPIN/cancer lesions by immunohistochemistry. Nedd4L (A) and Hk2 (B) are upregulated, whereas Prdx6 (C) is downregulated. Arrows indicate areas with increased (Nedd4L, Hk2) or decreased (Prdx6) expression.*indicates benign glands. Scale bars, 50mm.

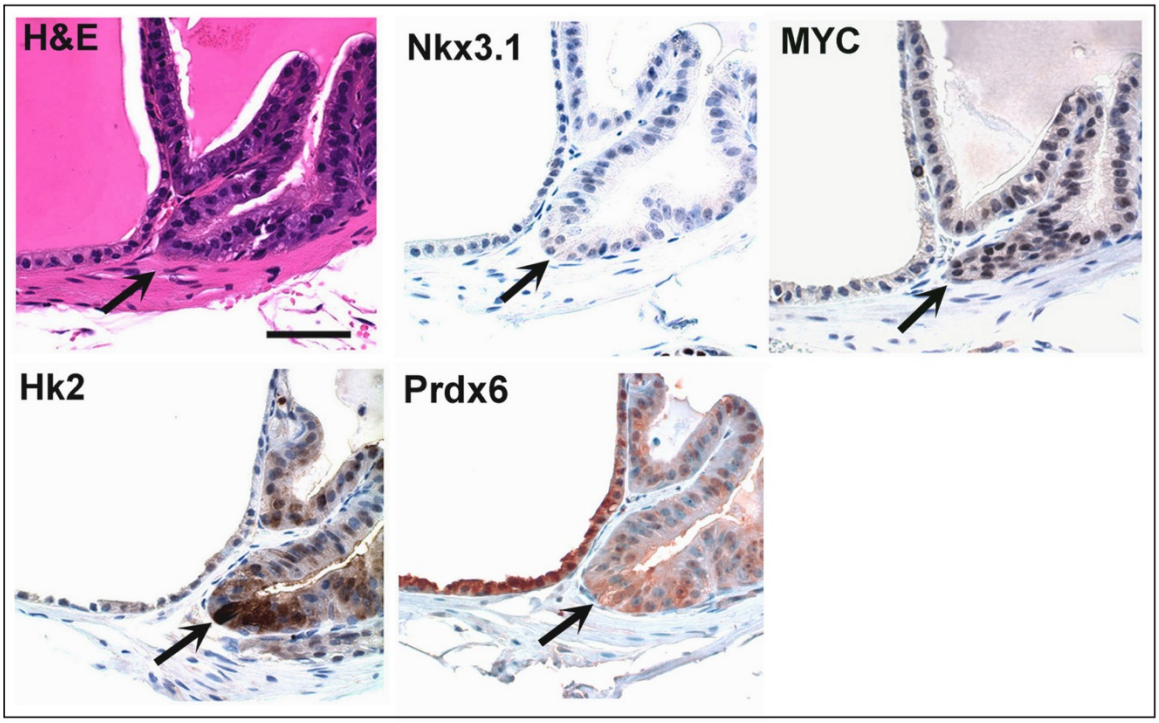

Figure 27. Nkx3.1/Myc regulation of target gene expression is correlated in vivo. Adjacent sections from 10-week PBCre;Z-Myc;Nkx3.1$^{f/f}$ prostate grafts show upregulation of Hk2 and downregulation of Prdx6 expression in HGPIN lesion with Myc overexpression and loss of Nkx3.1 (arrow indicates HGPIN lesion). H&E, Hematoxylin and eosin stain. Scale bars, 50mm.

Discussion

It is well accepted that multiple events (genetic or epigenetic) are required to promote tumorigenesis (Knudson, 2001; Krepkin and Costa, 2011; Land et al., 1983a, b; Luo and Elledge, 2008; McMurray et al., 2008; Peltomaki, 2012). Recent mathematical modeling suggests that cells harboring mutations in a single oncogene and tumor suppressor rapidly expand throughout a cell population (Krepkin and Costa, 2011). Furthermore, it was projected that the cooperation between a single oncogene and tumor suppressor played a significant role in driving early stages of tumorigenesis (Krepkin and Costa, 2011).

Interestingly, genomic analysis identified significant overlap between targets of the prostate tumor suppressor Nkx3.1 and oncogene Myc. Twenty five percent of the Nkx3.1 direct target genes identified by ChIP-seq and gene expression data (Anderson P, 2012) were also bound by Myc. More importantly, several of these "shared" Nkx3.1/Myc targets have been implicated in various aspects of tumorigenesis. For example, Hk2, a key glycolytic enzyme, is involved in promoting the "Warburg" effect and tumorigenesis in cancer cells; energy production in cancer cells is by glycolysis (Mathupala et al., 2009). The E3 ubiquitin ligase Nedd4L which is involved in targeting components of the TGFβ pathway for degradation was found in some studies to be upregulated in prostate cancer (Hellwinkel et al., 2011).

It is likely that dysregulation of multiple Nkx3.1/Myc target genes contributes to malignant transformation. Myc is a well-known regulator of proliferation, differentiation

and tumorigenesis (Dang et al., 2006). Thus, some of the consequences of Nkx3.1 loss in the prostate, including hyper-proliferation and de-differentiation (Abdulkadir et al., 2002; Bhatia-Gaur et al., 1999) may be achieved via direct dysregulation of the shared targets. In human prostate cancer, overexpression of Myc is an early and frequent event (Gurel et al., 2008; Wang et al., 2010). The expression of Nkx3.1 is also reduced in human prostate tumors (Bethel et al., 2006; Bowen et al., 2000) and an inherited Nkx3.1 gene variant with reduced expression was shown to be associated with increased risk of prostate cancer (Akamatsu et al., 2010).

These studies suggest that Nkx3.1 modulates Myc's transcriptional regulation of shared target genes. There are several mechanisms by which Nkx3.1 could modulate Myc transcriptional activity. For example, Nkx3.1 might affect recruitment of Myc to chromatin. On the other hand, binding of Nkx3.1 to promoter/enhancers of shared targets may affect Myc transactivation ability by preventing assembly of an active transcription complex. Further studies are needed to explore these possibilities.

Finally, although not all shared target genes are coordinately regulated by Myc and Nkx3.1, these analyses provide evidence for cooperation between Myc overexpression and Nkx3.1 loss in promoting cancer and dysregulating multiple shared target genes involved in tumorigenesis. The type of interaction identified between Myc and Nkx3.1, whereby two transcription factors converge to cross-regulate common target genes, may provide a mechanistic basis for some oncogene/tumor suppressor gene cooperativity.

Materials and Methods

Animals

Z-Myc, PbCre, Nkx3.1$^{+/+}$, Nkx3.1$^{-/-}$ and Nkx3.1$^{f/f}$ mice have been described (Abdulkadir et al., 2002; Roh et al., 2006; Wu et al., 2001). The generation of PbCre;Nkx3.1$^{+/+}$, PBCre;Nkx3.1$^{f/+}$ PBCre;Nkx3.1$^{f/f}$ mice have been described in Chapter II. The generation of trigenic PBCre;Z-Myc;Nkx3.1$^{+/+}$, PBCre;Z-Myc;Nkx3.1$^{f/+}$, PBCre;Z-Myc;Nkx3.1$^{f/f}$ have also been described in Chapter II. All animal care and experiments were carried out according to Vanderbilt Institutional Animal Care and Use Committee approved protocols.

Histology and Immunohistochemistry

Tissues were prepared for histological analysis as previously described in Chapter II. Primary antibodies include: Myc (1:5000, Santa Cruz), Nkx3.1 (1:1000, gift from Dr. Charles Bieberich, University of Maryland Baltimore County), Hexokinase II (1:50, Cell Signaling), Prdx6 (1:200, gift from Dr. Aron Fisher, University of Pennsylvania (Chatterjee et al., 2011)) and Nedd4L (1:400, Abcam).

Network Analysis

The 282 direct Nkx3.1 target genes were uploaded into the MetaCore^{TM} analytical suite (GeneGO, Inc., St. Joseph, MI), and analyzed as described previously (Nikolsky et al.,

2005). Networks centered on transcription factors were built such that sub-networks centered on transcription factors were generated and ranked by a P-value and a G-Score. The red circles indicate direct Nkx3.1 targets, gray arrows represent Myc target genes identified in ChIP assays, green arrows represent target genes induced by Myc and red arrows represent target genes repressed by Myc.

Gene Set Enrichment Analysis (GSEA)

Raw gene expression microarray data from human tumors was obtained with matching clinical data for 66 human prostate adenocarcinoma patients from Gene Expression Omnibus (GSE21034). The microarray data was processed and converted to Robust Multi-Array Analysis (RMA)-normalized expression values in Bioconductor using XPS. The patients were divided into two populations based on presence (Relapse, 32 patients) or absence (No Relapse, 34 patients) of biochemical relapse within five years. GSEA v2.07 (Subramanian et al., 2005) was used to test for expression changes in a gene set consisting of 65 Nkx3.1/Myc co-regulated genes. Leading edge analysis shows Nkx3.1/Myc target genes are upregulated in "No Relapse" and downregulated in "Relapse" patients.

Microarray analysis

Generation control and Myc-expressing reconstituted prostates using wild-type ($Nkx3.1^{+/+}$) prostates has been previously described in Chapter III and (Wang et al.,

2010). Total RNA was isolated with TRIzol (Invitrogen) and RNAeasy kit (Qiagen). RNA quality was checked on an Agilent Bioanalyzer. All samples used for microarray analysis have high quality score (RNA Integrity Number >7). RNA (1 μg) was reverse transcribed with T7–oligo(dT) primer and labeled with biotin using One Cycle Target Labeling kit (Affymetrix) following manufacturer's protocol. Three to four replicates of each group were prepared, labeled, and hybridized to Mouse Gene 1.0 ST v1.r4 arrays (Affymetrix) and scanned on GeneChip scanner 3000 (Affymetrix). Data was collected using GCOS software (Affymetrix) and pre-processed in Expression Console (version 1.1; Affymetrix) using the RMA-Sketch normalization model. Log2-transformed data was analyzed using the SAM algorithm for statistical analysis. The comparison made was control vs Myc overexpressing grafts.

Cell Culture and Transfections

p53$^{-/-}$*;Arf*$^{-/-}$ double knockout (DKO) MEFs expressing MycER (gift from Dr. Steve Hann, Vanderbilt University) were cultured in DMEM supplemented with 10% FBS, 100U/ml penicillin and streptomycin, and 50 ug/mL hygromycin B. $4.5x10^5$ cells were plated on 10 cm cell culture dishes. Approximately 16 hours post plating transfections were performed using PEI and 10 ug of either GFP control or FUGW-Nkx3.1 plasmid (kind gifts from Dr. Hong Wu, UCLA). 48 hrs post transfection, cells were maintained in 0.1% FBS for 36 hrs followed by activation of MycER with 10 μM OHT for 4,8, or 12 hrs. Cells were thoroughly washed with cold PBS and treated with trypsin followed by

centrifugation to collect cells. Cell pellets were divided in two, one used for immunoblotting and one used for RNA isolation, both described in detail below.

pBabe-Puro-MYC-ER plasmid (kind gift from Dr. Gerard Evan, UCSF) was used to generate a retrovirus and transduce LNCaP cells to develop LNCaP-MycER cells. LNCaP-MycER cells were maintained in RPMI supplemented with 10% FBS, 100U/ml penicillin and streptomycin and 1 ug/mL of puromycin. Approximately 16 hours post plating, two consecutive cell transfections were performed using Superfect (Invitrogen), with siNkx3.1 or siGFP duplex (Thermo Fisher). Following transfection, cells were cultured in media containing 0.1% FBS for 36h followed by activation of MycER with 100nM 4-OHT.

LNCaP si471 cells with stable shRNA knockdown of Nkx3.1 and LNCaP siLuc cells with Luciferase siRNA control (Bowen and Gelmann, 2010) (kind gifts from Drs. Cai Bowen and Edward Gelmann, Columbia University) were cultured in RPMI supplemented with 10% FBS, 100U/ml penicillin and streptomycin and 10 ug/mL of puromycin.

RNA Isolation and quantitative RT-PCR

Total RNA was isolated using TriZol (Invitrogen) and RNAeasy kit (Qiagen) according to manufacturer's protocol. Samples were dissolved in DEPC-treated milli-Q water and quantified by spectrophotometric readings at 260 nm (A_{260}). Purity of total RNA was

determined by the A_{260}/A_{280} and A_{260}/A_{230} ratio, and then integrity of RNA samples was confirmed by electrophoresis on 1% agarose gels. cDNA was synthesized by reverse transcription using 1ug RNA using primer cocktail (200 ng/µl oligodT and 50 ng/µl of random hexamer). Reaction mix contained 10 mM dNTP's, 0.1 M DTT, RNAsin and M-MLV reverse transcriptase (Gibco/BRL 200 units/µl). Reaction conditions were 68°C for 10 min and 42°C for 60 min. DEPC-treated milli-Q water was used to make final volume of 250 µl. cDNA samples were then boiled for 5 min and stored at -20°C. Real time quantitative PCR reaction was performed in Taqman (Applied Biosystems) using SYBR Green 2X master mix (Applied Biosystems). All PCR reactions were performed in triplicate and independently repeated at least two to three times. The following primers were used:

Name	**Primer Sequence**
Asns (Forward)	GGAGGACAGCCCCGATCT
Asns (Reverse)	CATGATGCTCGCTTCCAATATAAT
Hk2 (Forward)	CGCCGGATTGGAACAGAA
Hk2 (Reverse)	CCCGTCGCTAACTTCACTCACT
Id1 (Forward)	GCAAAGTGAGCAAGGTGGAGAT
Id1 (Reverse)	CCAGCTGCAGGTCCCTGAT
Igf1r (Forward)	TGGCGATTTAGAGAAACGAACA
Igf1r (Reverse)	TGTCACGATGCCGGTTACC
Nedd4L (Forward)	TGAGCAAGCTCACCTTCCA
Nedd4L (Reverse)	CCCGTGACAGTTGACGAAC
Nfkb1 (Forward)	GGATGACAGAGGCGTGTATTAGG

Nfkb1 (Reverse)	GTAGATAGGCAAGGTCAGAATGCA
p15Ink4b (Forward)	AGATCCCAACGCCCTGAAC
p15Ink4b (Reverse)	CCCATCATCATGACCTGGATT
Pnpt1 (Forward)	GCAGAATCCCCACAAACTACCT
Pnpt1 (Reverse)	TCGACTTGTAAGAACCTCTCTGTCA
Prdx6 (Forward)	TCTGGCAAAAAATACCTCCGTTA
Prdx6 (Reverse)	GCCCCAATTTCCGCAAAG

Chromatin Immunoprecipitation and PCR

Formaldehyde (Fisher Scientific) was added directly to cell culture media at a final concentration of 1%. Fixation was terminated after 10 minutes by the addition of glycine to a final concentration of 0.125 M for 5 minutes. Cells were thoroughly rinsed with cold PBS, scraped with 1mL cold PBS with protease inhibitors (1mM PMSF, 5ug/mL Aprotinin, 5ug/mL Pepstatin A) and collected by centrifugation. Samples were resuspended in 1mL of Low salt buffer (0.1% SDS, 1% Triton-x, 2mM EDTA pH 8, 20mM Tris pH 8, 150mM NaCl) with protease inhibitors (1mM PMSF, 10mM β-Glycerol Phosphare, 38ug/mL Aprotinin, 5ug/mL Leupeptin, 5ug/mL Pepstatin, 1mM NaF, 0.1mM $NaVO_4$, 1mM DTT) and sonicated on ice to an average length of 200–1,000bp. Protein concentration and DNA fragment size was checked using BCA protein assay and gel electrophoresis, respectively. Lysates were immunoprecipitated using protein A-agarose beads and Myc polyclonal antibody (4ug, Santa Cruz) or rabbit IgG

(4ug, Santa Cruz). Immune complexes were washed using low salt buffer, high salt buffer (0.1% SDS, 1% Triton-x, 2mM EDTA pH 8, 20mM Tris pH 8m 500mM NaCl), LiCl buffer (0.25mM LiCl, 1% NP40, 1% deoxycholate, 1mM EDTA pH 8, 10mM Tris pH 8) and TE. Crosslinking was reversed by addition of NaCl followed by incubation at 65°C for 4 hrs. Phenol-chlorofom was used for DNA extraction. Pellets were collected by microcentrifugation, resuspended in 100 µl of H_2O, and analyzed by using PCR. Total input samples were resuspended in 250µl of H_2O.

PCR reactions contained 0.5µl of ChIP DNA, designated primers, $MgCl_2$, dNTPs, 5X buffer, Taq DNA polymerase (supplier) in a total volume of 22µl. PCR products were run on a 2% agarose gel and analyzed by ethidium bromide staining. The following primers were used:

Name	**Primer Sequence**
Hk2 (Forward)	CTCAGAGGAAGAGACAGCTG
Hk2 (Reverse)	CTCCTTGACTGGGTCAGTG
Id1 (Forward)	TGGAGAGGTAGCTGAGAGTT
Id1 (Reverse)	GCTCTGATGTCCCATATGCT
Mt2 (Forward)	CTGGCCATATCCCTTGAGC
Mt2 (Reverse)	AGTTCTAGGAGCGTGATGGA
Mt3 (Forward)	TCTGCCCAACCTAGAAACCT
Mt3 (Reverse)	TAGCCTGGATTCTCCTCCAG
Prdx6 (Forward)	CCACTGACTTCCTATTTCCTAAGC
Prdx6 (Reverse)	CTCCTTTCTCCAGAGTAGTTGG
Sept9 (Forward)	GGCTGACAGAAGACCAAGATG

Sept9 (Reverse)	TGTGCCAATCCAGAATCACG
Txnip (Forward)	GGAATGGCTCCAACCAGATA
Txnip (Reverse)	CTCTCGGTGCTTTGACTTGT
Utrn (Forward)	CCATACTAAGGATGCAGGCATG
Utrn (Reverse)	CAGTGTAAGCTCCAAGACAGC

Co-immunoprecipitation

293T cells (ATCC) were plated to approximately 80% confluency the day before transfection. 10ug of total DNA was transfected into 293T cells using PEI transfection reagent. 48 hours post transfection, cells were washed with cold PBS, scraped with IP Lysis Buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.2% NP-40, 1X protease inhibitor cocktail (Roche)) and collected by centrifugation at 4°C. Protein concentrations were measured using the Bio-Rad D_C protein assay reagent. 500 ug of protein extracts were brought to a final volume of 500 uL with IP lysis buffer and incubated with 2ug of Flag antibody (Sigma), 20 uL of HA affinity matrix (Roche) or HRP-conjugated IgG control and incubated with 360° rotation at 4°C overnight. For Flag and IgG control samples, 40 uL of protein A/G plus agarose beads were added to IP lysates and incubated with 360° rotation at 4°C for 3 hrs. Cells were washed once with IP lysis buffer, three times with IP wash buffer (50 mM HEPES pH 7.4, 500 mM NaCl, 1 mM EDTA, 0.2% NP-40) and once more with IP lysis buffer. Beads were resuspended in 2X protein sample buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 4 mM

EDTA, 4% 2-mercaptoethanol, 0.1% bromophenol blue) and boiled for 5 min. 10-15 uL of sample were subjected to Western blot analysis. Plasmids used include: HA-tagged-NKX3.1 in pCDNA3.1 plasmid, control pCDNA3.1 and FLAG-tagged-MYC constructs (wild type, delta 20-48 MYC Box I mutant or delta 118-152 MYC Box II mutant (Wood et al., 2000)). For ethidium bromide (EtBR) treatment, 50 mg/ml of EtBR was added to the lysates and incubated on ice for 30 min. The same concentration of EtBR was used during the washing steps. For input, 4-10 % of lysates was used.

Western blotting

Whole cell lysates were prepared using RIPA extraction buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.1% SDS). Protein concentrations were measured using the Bio-Rad D_C protein assay reagent. Extracts containing 50 µg of protein were electrophoresed on a 10% SDS-polyacrylamide gel and blotted onto Hybond P PVDF membrane (GE Healthcare Bio-Sciences Corp). The blotted membrane was blocked with 5% fat-free dry milk for 1 h at room temperature or overnight at 4°C with rotation. Membranes were then incubated with primary for 2 hrs at room temperature or overnight at 4°C with rotation. The membrane was washed thoroughly and then incubated for 1 h at room temperature with an HRP-conjugated secondary IgG antibody (1:3000, Bio-Rad). The membrane was rinsed, treated with ECL reagent (PerkinElmer Life Sciences) for 1 min and exposed to x-ray film. The primary antibodies used include:

Nkx3.1 (1:4000, gift from Dr. Charles Bieberich), Myc (1:400, Santa Cruz), beta-actin (1:1000, Santa Cruz), Hemagglutinin (HA) Tag (1:1000, US Biological), Flag (1:1000, Sigma)

CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

Discussion

Human prostate cancer is perceived to undergo step-wise progression of normal prostate epithelium to PIN, invasive carcinoma and metastasis. The transition from PIN to carcinoma marks a major step in the progression of prostate cancer. This transformation involves increased protease activation resulting in disruption of the basement membrane and extracellular matrix, altered cellular adhesion and changes in cellular motility (Nagle et al., 1992). Identifying molecular mechanisms responsible for the initiation and progression of PIN lesions can offer insight to the development of new therapies aimed at prostate cancer prevention. A series of genetic and epigenetic alterations have been implicated to contribute to the development and transition of PIN lesions including loss of tumor suppressors (ie. Nkx3.1, Pten, p27), gain of oncogenes (ie. Myc, Pim1, Egr1), hypermethylation, histone modification, telomere shortening and gene fusion.

Nkx3.1 is a homeodomain transcription factor expressed in the luminal epithelium of the prostate. During embryogenesis, Nkx3.1 is one of the earliest markers of the developing prostate epithelium (Bhatia-Gaur et al., 1999). Functional studies indicate that Nkx3.1 plays a critical role in the prostate development and differentiation; loss results in defects

in prostate branching morphogenesis, epithelial cell differentiation and growth and production of prostatic secretory proteins (Abdulkadir et al., 2002; Bhatia-Gaur et al., 1999; Tanaka et al., 2000). Gene expression and ChIP studies demonstrate that Nkx3.1 regulates several genes including AR, Probasin, Prdx6, Qsox1, Hk2 and Nedd4L (Anderson P, 2012; Magee et al., 2003; Ouyang et al., 2005; Song et al., 2009; Tan et al., 2012).

This dissertation focused on understanding the role that Nkx3.1 plays in the initiation and progression of prostate cancer. Functional analysis of Nkx3.1 in human prostate tumor cell lines and mouse models have provided insight into its role in tumor initiation. Expression of Nkx3.1 conferres growth suppressive activity both *in vivo* and *in vitro* (Bhatia-Gaur et al., 1999; Kim et al., 2002a; Schneider et al., 2000; Tanaka et al., 2000). Overexpression of Nkx3.1 results in decreased tumor size, tumor growth and induces apoptosis (Kim et al., 2002a; Lei et al., 2006). Nkx3.1 has been demonstrated to engage with cell cycle and cell death machinery by recruiting HDAC1 and stabilizing p53 (Lei et al., 2006). Furthermore, haploinsufficiency or complete loss of Nkx3.1 results in cell cycle exit delays (Magee et al., 2003) and creates an environment susceptible to additional tumor promoting aberrations. Nkx3.1 loss has consistently been shown to result in PIN development (Abdulkadir et al., 2002; Kim et al., 2002a; Schneider et al., 2000); however, additional cooperating events are required for these PIN lesions to transition to advanced lesions (Abate-Shen et al., 2003; Kim et al., 2002b). These, along

with other findings not discussed in detail here, classify Nkx3.1 as a prostate tumor initiation "gate keeper."

In human and mouse prostate cancer Myc has been suggested to cooperate with Nkx3.1 (Ellwood-Yen et al., 2003; Kindich et al., 2006). Myc is a helix-loop-helix transcription factor that plays a role in several biological processes including cellular proliferation, growth, apoptosis, differentiation blockage, self-renewal and transformation (Cole and Henriksson, 2006; Meyer and Penn, 2008). It is estimated that Myc regulates approximately 15% of all genes (Dang et al., 2006). Myc-mediated transcription of target genes is modulated through its interaction with multiple proteins which facilitates either activation or repression (Adhikary and Eilers, 2005). Given Myc's potent growth-promoting effects, expression and function is tightly regulated in normal, quiescent cells (Salghetti et al., 1999; Thomas and Tansey, 2011). Deregulation of Myc plays a role in several cancers and approximately 70% of prostate cancers (Koh et al., 2010). To further enhance the current knowledge of Nkx3.1's role in tumorigenesis, the cooperation between Nkx3.1 and the proto-oncogene Myc (both frequent observations in human prostate cancer which independently play a role in the development of early prostate tumor lesions) was examined (Abdulkadir et al., 2002; Gurel et al., 2008; Kim et al., 2002a; Schneider et al., 2000; Wang et al., 2010).

Results presented in this dissertation demonstrate that mice with concurrent Nkx3.1 deletion and Myc activation develop HGPIN lesions as early as 15 weeks of age. Further analysis indicates that PBCre;z-Myc;Nkx3.1$^{f/f}$ prostates have an increased proliferative

index, an increase in focal HGPIN density and show signs of microinvasion. The mechanism of cooperativity remains to be determined but a number of shared target genes are significantly dysregulated in mouse prostate tumors with Myc activation and Nkx3.1 loss. Initial studies presented in Chapter IV suggest that Nkx3.1 opposes Myc's regulation of a subset of genes.

Based on these studies, the following model to explain the cross-talk between Nkx3.1 and Myc in prostate tumorigenesis is proposed (Figure 28). The levels of Nkx3.1 and Myc vary in tumorigenesis, with the Myc:Nkx3.1 ratio increasing with progression. Data presented in this dissertation suggests that Nkx3.1 and Myc bind to and co-regulate a common subset of target genes relevant to prostate tumorigenesis. Furthermore, Nkx3.1 antagonizes Myc's transcriptional activity. Thus, as the levels of Myc increase and Nkx3.1 decrease, Myc's transactivation of pro-tumorigenic target genes (such as Hk2, Igf1R, Nedd4L) increases, thereby promoting tumorigenesis. In the case of anti-tumorigenic target genes (e.g. Ace, Mt2) that are repressed by Myc and activated by Nkx3.1, the converse is true, as the Myc:Nkx3.1 ratio increases, the expression of these target genes will decrease, enhancing tumorigenesis.

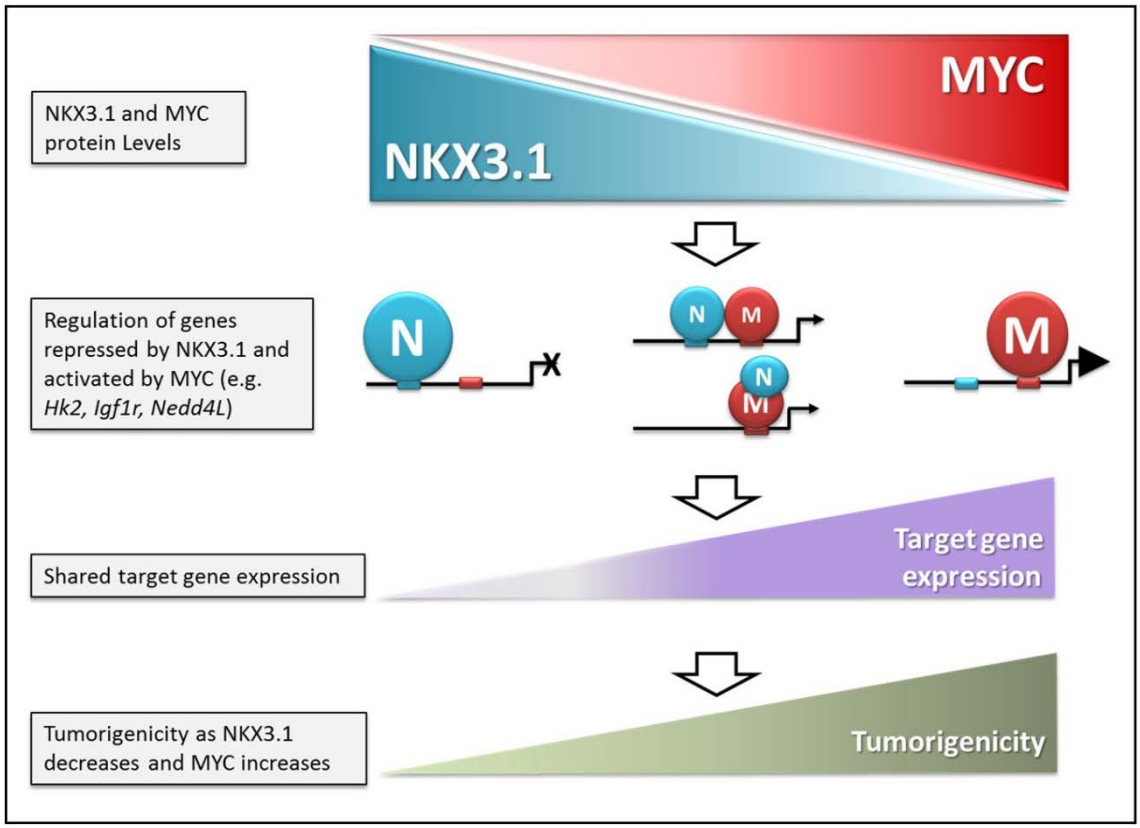

Figure 28. Model for co-regulation of prostate tumorigenesis by convergence of Nkx3.1 and Myc on common target genes. Nkx3.1 and Myc protein levels vary during tumorigenesis ranging from low Myc/high Nkx3.1 in benign tissue to high Myc/low Nkx3.1 in more advanced tumors. In benign tissue where Nkx3.1 proteins levels are high and Myc levels very low, shared target genes such as Hk2 are bound and repressed by Nkx3.1. In samples where both Nkx3.1 and Myc are expressed, Nkx3.1 can bind to its consensus DNA site as well as form a complex with Myc to dampen Myc's transcriptional activity and target gene expression. In advanced tumors, where Myc is highly expressed and Nkx3.1 expression lost, Myc binds and activates its target genes unopposed. Thus as tumorigenesis progresses, the Myc:Nkx3.1 ratio increases and the expression of pro-tumorigenic shared target genes (activated by Myc and repressed by Nkx3.1) increase. In the model depicted here, Nkx3.1 is shown as a transcriptional repressor and Myc as an activator of pro-tumorigenic target genes. However, the converse, where Nkx3.1 activates and Myc represses anti-tumorigenic target genes also fits the general model.

Future Directions

One of the features of PbCre;Z-Myc;Nkx3.1$^{f/f}$ transgenic mice is that the phenotype recapitulates the multistep model of carcinogenesis observed in human prostate cancer. The loss of Nkx3.1 promotes proliferation and results in hyperplastic epithelial cells that are sensitive to a subsequent tumorigenic mutation (Myc overexpression). Although mice with concurrent loss of Nkx3.1 and Myc overexpression demonstrate cooperation as evidenced by the development of HGPIN and microinvasion, the lesions did not progress to advanced invasive carcinoma by 35 weeks of age. This is more than likely due to the fact that the Z-Myc transgene is focally expressed resulting in a small percentage of Myc positive cells. Previous studies showed that young Z-Myc mice display ~18% of Myc positive epithelial cells; however, the frequency of Myc positive cells increases to ~43% by 1 year (Kim et al., 2009). Therefore, in the future, it would be interesting to evaluate the histology of prostates aged to 1 year. It is predicted that these aged lesions will show a more progressive phenotype including advanced invasive carcinoma.

It is likely that these advanced lesions will accumulate additional genetic mutations enhancing tumor progression. Nkx3.1 has been suggested as a crucial factor in Pten initiated prostate tumors (Lei et al., 2006; Song et al., 2009). Therefore, future studies should investigate advanced Nkx3.1/Myc lesions for the propensity of Pten loss and Akt activation.

Primary tumors are typically dependent on AR activity which is often used as a measure of disease progression. Consequently, androgen ablation is the primary treatment for prostate cancer. Nkx3.1 negatively regulated AR activity in murine and human prostate cancer cells whereby steady state levels of Nkx3.1 limited AR activity (Lei et al., 2006). PbCre;Z-Myc;Nkx3.1$^{f/f}$ regenerated prostate grafts display increased AR expression compared to benign tissue (Figure 29). These results imply that AR might be contributing to the proliferative phenotype observed in the PbCre;Z-Myc;Nkx3.1$^{f/f}$ prostates. It would be advantageous to determine the level of AR activity and cell cycle proteins that are facilitating enhanced cellular proliferation. In addition, AR can activate the PI3K/Akt pathway, leading to increased proliferation. Furthermore, if the Nkx3.1/Myc lesions are dependent on AR activity, castration of these mice should result in tumor regression. If these lesions respond to AR ablation, it would suggest that human tumors with an Nkx3.1-null/Myc overexpression profile, could respond well to hormone ablation therapy.

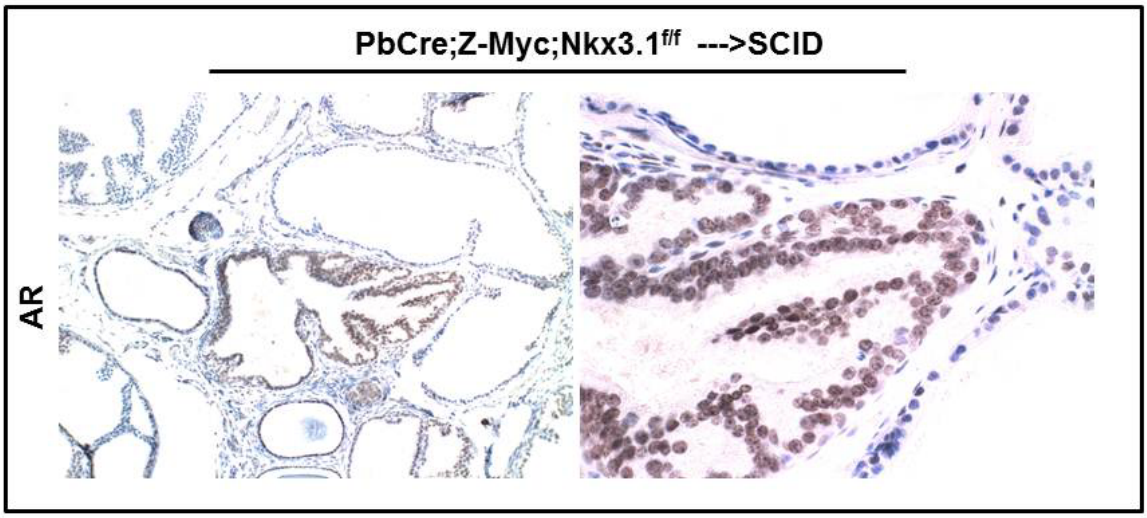

Figure 29. AR expression is overexpressed in PBCre;Z-Myc;Nkx3.1$^{f/f}$ prostates. Immunohistochemical staining with anti-AR shows increased protein expression in PbCre;Z-Myc;Nkx3.1$^{f/f}$ regenerated prostates compared to benign glands.

This work has demonstrated that Nkx3.1 and Myc cooperate to promote tumorigenesis by dysregulation of shared gene targets. A pertinent remaining question is how does Nkx3.1 modulate Myc's regulation of shared target genes? There are several potential mechanisms by which Nkx3.1 could interfere with Myc's transcriptional activity at the promoter of shared target genes including: hindering Myc from binding to its consensus, obstructing the association of co-factors/co-activators which disrupts the formation of a functional transcription initiation complex, preventing recruitment of RNA polymerase and/or blocking necessary histone modifications (Figure 30).

ChIP analysis could be performed to better understand the potential mechanism. Binding analysis by ChIP will determine if Nkx3.1 prevents binding or recruitment of components required for active Myc transactivation at specific promoters. One major pitfall of ChIP analysis is that the results are qualitative not quantitative. Hence, the results will only imply transcriptional activity. Global Run-On sequencing (GRO-seq), on the other hand, maps the position, orientation and amount of transcriptionally engaged RNA (Core et al., 2008). GRO-seq provides genome-wide interrogation of active transcription and will enable functional evaluation of the relationship between Nkx3.1 and Myc regulated gene expression. Moreover, it will allow quantitative assessment of transcriptional changes with high resolution in a Myc induced prostate cancer cell line when Nkx3.1 is present.

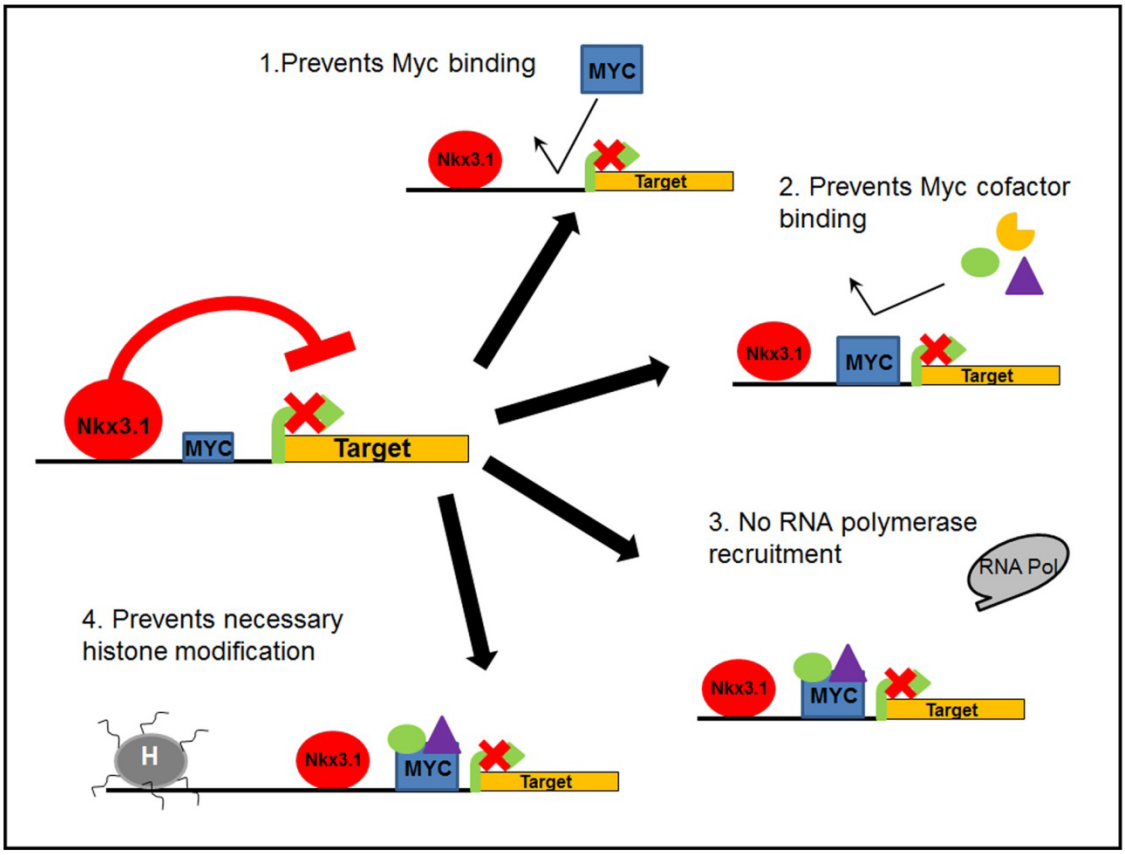

Figure 30. Proposed models of Nkx3.1 regulation of Myc. Model 1 suggests that the binding of Nkx3.1 to shared target promoters prevents Myc from binding to its consensus. Model 2 implies that both Nkx3.1 and Myc are able to bind shared target promoters; however the presence of Nkx3.1 prevents the binding or recruitment of essential Myc co-factors. Model 3 proposes that Nkx3.1, Myc and co-factors are able to bind; however Nkx3.1 interferes with the recruitment of RNA polymerase and formation of an active transcription initiation complex. For model 4, Nkx3.1 prevents histone modifications required for Myc regulated transcription of shared target gene expression.

Nkx3.1 was shown to interact with Myc within the MbII domain. Several groups have shown that the Myc pathway is an attractive cancer therapeutic target (Vita and Henriksson, 2006). Numerous strategies have been explored to target dysregulated Myc including inhibiting interactions essential for Myc's transcriptional activity (Vita and Henriksson, 2006). Results from this dissertation indicate that Nkx3.1 antagonizes Myc's transcriptional activity of shared target genes therefore this interaction might prove to be important in regulating the transcriptional activity of Myc in prostate carcinoma. To evaluate the impact of this interaction it is important to identify the specific residues responsible for binding. To begin investigating the key Myc amino acids that interact with Nkx3.1, preliminary results were obtained using co-immunoprecipitation, wild-type Nkx3.1 (HA-Nkx3.1) and Myc mutant-W136E, a point mutation in the highly conserved tryptophan residue in the MBII domain. Results concluded that the W136E point mutation does not prevent binding between Nkx3.1 and Myc (Figure 31). Additional binding assays using scanning Myc deletion mutants (Tworkowski et al., 2002) with wild-type Nkx3.1 and Nkx3.1 deletion mutants with wild type Myc should identify the responsible binding regions. If the amino acids responsible for the interaction between Nkx3.1 and Myc are identified, small molecules can be generated to mimic this interaction in an attempt to modulate Myc's transcriptional activity in prostate carcinoma.

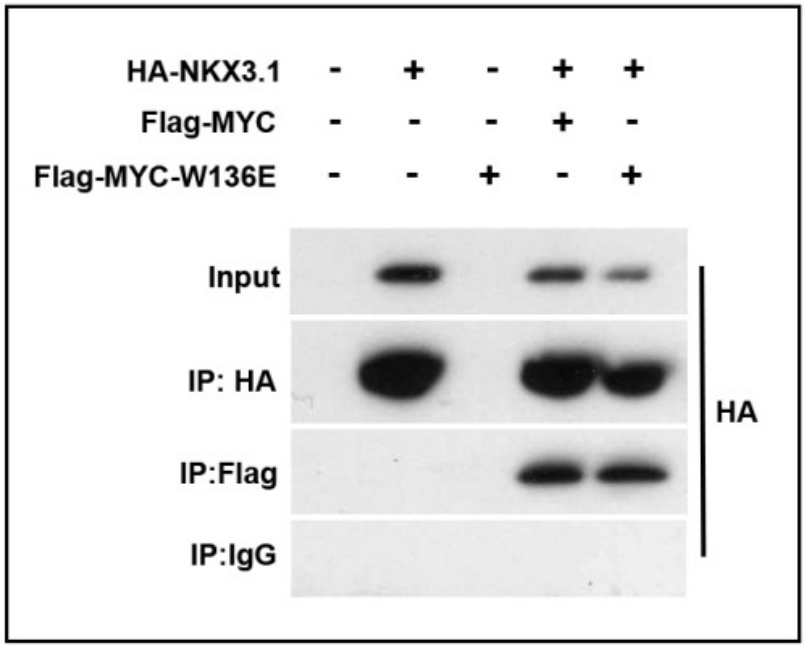

Figure 31. Mutant Myc-W136E does not prevent Nkx3.1/Myc interaction. Cell lysates from 293T cells expressing HA-Nkx3.1 and Flag-MYC or Flag-Myc-W136E were immunoprecipitated with the HA indicated antibodies and immunoblotted for HA.

Another possible way that Nkx3.1 could disrupt Myc's transcriptional activity is by competing with activators that bind to the MbII. Several critical Myc activators have been shown to bind to this region including Tip48, TBP (TATA binding protein), TRRAP and Bcl2 (B-cell lymphoma 2) (Jin et al., 2006; Maheswaran et al., 1994; McMahon et al., 1998; Wood et al., 2000). Pilot studies were conducted to examine the effect of Nkx3.1 expression on the Tip48- Myc complex. Tip48 is an AAA+ ATPase that plays a role in chromatin remodeling, transcription regulation and DNA damage repair (Huber et al., 2008). In addition to interacting with Myc, Tip48 expression has been shown to be regulated by Myc (Huber et al., 2008). In gastric cancer cells, expression of Tip48 influenced Myc promoter binding and cellular transformation (Li et al., 2010). Interestingly, Tip48 played a role in increasing the invasive properties of prostate tumor cells (Kim et al., 2005).

Co-immunoprecipitation assays in 293T cells unexpectedly demonstrated that Nkx3.1 and Tip48 interact independent of Myc (Figure 32). Tip48 has been previously shown to interact with HDAC1 and this interaction has been suggested to exert gene repression (Lee et al., 2010). HDAC1 is also known to interact with Nkx3.1 (Lei et al., 2006). So, one potential mechanism by which Nkx3.1 opposes Myc transactivation is by recruiting HDAC1 to the Tip48 complex, resulting in deacetylation of the promoter and decreased target activation (Figure 33). Further experiments are necessary to confirm these initial findings.

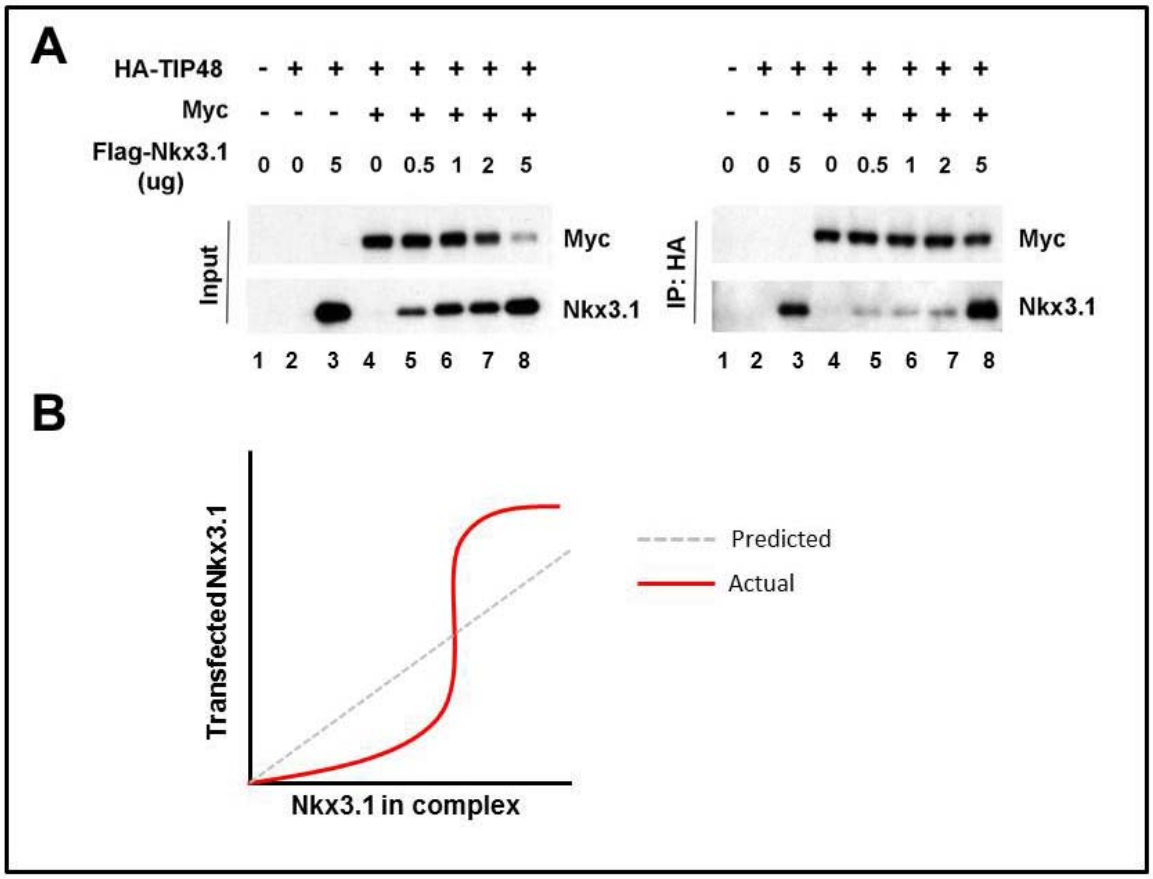

Figure 32. Nkx3.1 interacts with Tip48 and included in the Tip48/Myc complex. (A) NKX3.1, Tip48 and MYC co-immunoprecipitation. Co-immunoprecipitation of Mock (lane 1), HA-Tip48 (lane 2), HA-Tip48 and Flag-Nkx3.1 (lane 3), HA-Tip48 and MYC (lane 4) and HA-Tip48, MYC and increasing concentrations of Flag-NKX3.1 (lanes 5-8) in 293T cells. (B) Sigmoid binding stoichiometry interpreted from co-immunoprecipitation results. When 0.5-2ug of Nkx3.1 is transfected, a linear increase in Nkx3.1 is observed. But when 5ug of Nkx3.1 is transfected, there is a sharp increase in the amount of Nkx3.1 in complex with Tip48 and Myc.

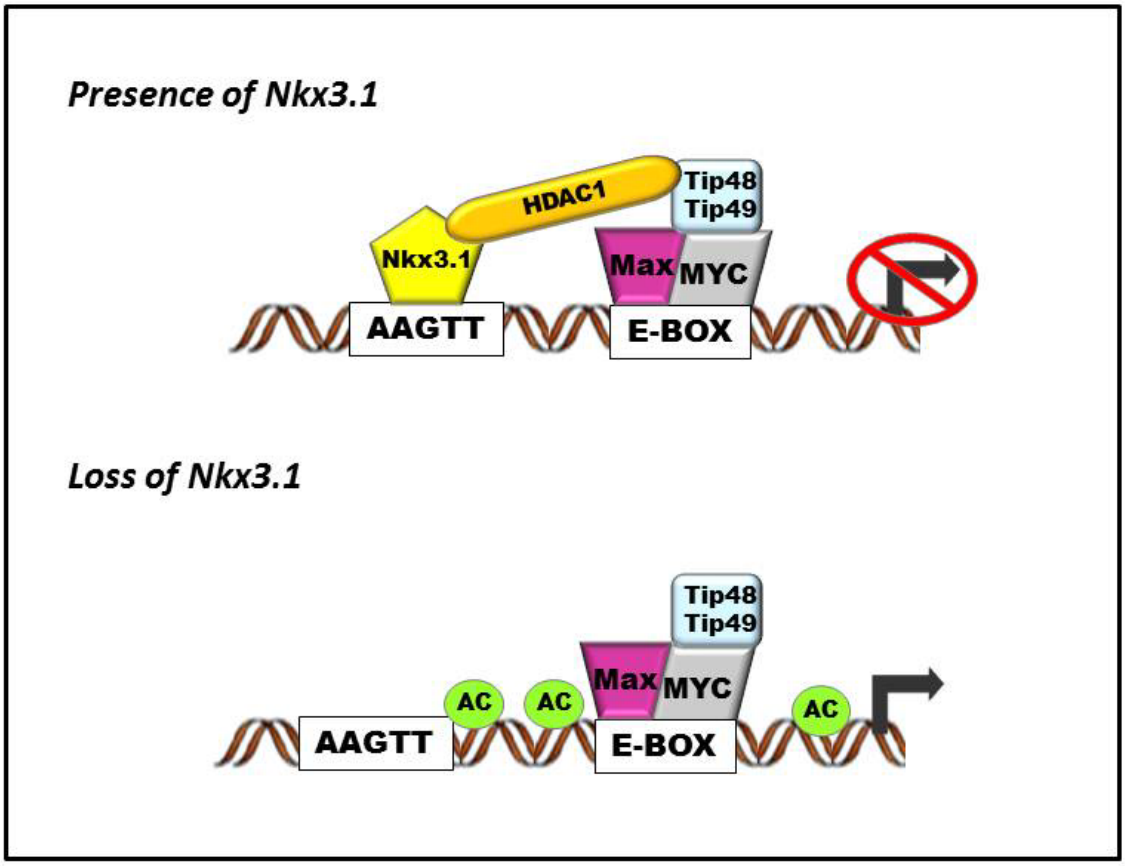

Figure 33. Updated Model for Nkx3.1 regulation of Myc. In the presence of Nkx3.1, Nkx3.1 recruits HDAC1 to the promoter of Nkx3.1/Myc shared gene targets. HDAC binds to both Nkx3.1 and Tip48 and antagonizes Myc regulated transcription. In transformed prostate cells, Nkx3.1 is lost allowing activation of Myc transcription.

Significance

One of the challenges in prostate cancer research has been the lack of accurate mouse models that recapitulate human prostate tumorigenesis. Therefore the primary goal of these studies was to generate a mouse model that mimics the multi-step progression of prostate cancer observed in humans. Combined loss of Nkx3.1 and Myc overexpression are frequently observed in human prostate cancer and correlated with increased tumor stage (Kindich et al., 2006; Locke et al., 2012; Sato et al., 1999). Furthermore, preclinical mouse models have suggested that loss of Nkx3.1 and Myc overexpression cooperate to drive prostate cancer progression and aggression (Ellwood-Yen et al., 2003; Iwata et al., 2010).

Given the potential significance of Nkx3.1 and Myc in prostate cancer, trigenic mice with prostate specific loss of Nkx3.1 and Myc overexpression were generated. Prostates from these mice displayed HGPIN lesions with microinvasive cancer. Mechanistic analysis identified a subset of Nkx3.1 and Myc shared target genes which were shown to be dysregulated in human and murine prostate tumor cells. In addition, these shared target genes were associated with human prostate cancer relapse. Altogether these studies propose that Nkx3.1 and Myc cross-regulate tumor relevant target genes to promote tumorigenesis.

The findings within this dissertation highlight the significance of Nkx3.1 loss and Myc overexpression in prostate tumorigenesis and propose a potential mechanism by which

these alterations promote tumorigenesis. More importantly, these studies identified target genes that might have prognostic or therapeutic use. Furthermore, the Nkx3.1/Myc mutant mice provide a suitable platform for therapeutic testing of these targets.

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