ANALYSIS OF NKX3.1 TARGET GENES IN PROSTATE CANCER

By

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To my parents, sisters and lovely wife

for their enormous support, love and encouragement

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LIST OF ABBREVIATIONS

5-Azacytidine ..... Azacyt

Acetylated histone H3 ..... Ac-H3

Acetylated histone H4 ..... Ac-H4

American Cancer Society ..... ACS

Androgen receptor ..... AR

Benign prostatic hyperplasia ..... BPH

Bladder epithelium ..... BLE

Bladder mesenchyme ..... BLM

Chromatin immunoprecipitation ..... ChIP

Co-immunoprecipitation ..... Co-IP

Cyclin-dependent kinase ..... CDK

Dithiothreitol ..... DTT

DNA methyltransferases ..... DNMT

Early B cell factor ..... EBF

Electromobility shift assays ..... EMSA

Haploinsufficiency ..... HI

Heterochromatin protein-1 ..... HP1

High-grade PIN ..... HGPIN

Histone acetyl transferases ..... HATs

Histone deacetylase 1 ..... HDAC1

Histone deacetylase inhibitors ..... HDACi

Histone deacetylases ..... HDACs

Histone methyl transferases ..... HMTs

Human intelectin ..... hIntL

Hypoxia inducible factor 1 ..... HIF-1

Loss of heterozygosity ..... LOH

Lysine specific demethylase 1 ..... LSD1

Methyl binding proteins ..... MBDs

P300/CBP-associated factor ..... P/CAF

Position-effect variegation ..... PEV

Prostate cancer ..... PCa

Prostate-derived Ets factor ..... PDEF

Prostatic intraepithelial neoplasia ..... PIN

Retinoblastoma protein ..... Rb

Ribosomal highly basic 23-kDa protein RPL13A

RNA Interference ..... RNAi

Serial analysis of gene expression ..... SAGE

Serum response factor ..... SRF

Smooth muscle gamma-actin ..... SMGA

Sonic hedgehog ..... Shh

Standard deviation ..... SD

Suberoyl anilide bihydroxamide ..... SAHA

Testosterone replacement ..... TR

Trichostatin A ..... TSA

Tumor suppressor gene ..... TSG

Untranslated region ..... UTR

Urogenital epithelium ..... UGE

Urogenital mesenchyme ..... UGM

Wilms tumor-aniridia-genitourinary-mental retardation ..... WAGR

CHAPTER I

INTRODUCTION

PROSTATE CANCER

Epidemiology:

Prostate cancer (PCa) is the most common cancer next to skin cancer, in American men. The American Cancer Society (ACS) estimates that during 2007 about 218,890 new cases of prostate cancer will be diagnosed in the United States. Approximately, about 1 man in 6 will be diagnosed with prostate cancer during his lifetime. Prostate cancer is the second leading cause of cancer deaths next to lung cancer in American men. ACS estimates that 27,050 men in the United States will die of prostate cancer in 2007 comprising about 10% of cancer-related deaths in men.

Multiple risk factors (Figure 1) have been proposed to play role in the development of prostate cancer which include aging, environmental factors, familial inheritance and level of steroid hormones (Abate-Shen and Shen, 2000). Aging is the most significant risk factor for the development of prostate cancer. The chance of having prostate cancer rises rapidly after age 50 and about two thirds (2/3) of all prostate cancers are found in men over the age of 65. The incidence of prostate cancer in the United States is significantly higher than in Asian and African countries. Furthermore in the United States, African-American men are more likely to suffer from prostate cancer than white American men. African-American men present with advanced stage prostate cancer lesions predisposing them to higher death rates.

Dietary and environmental factors play pivotal role in prostate carcinogenesis (Carter and Coffey, 1990).

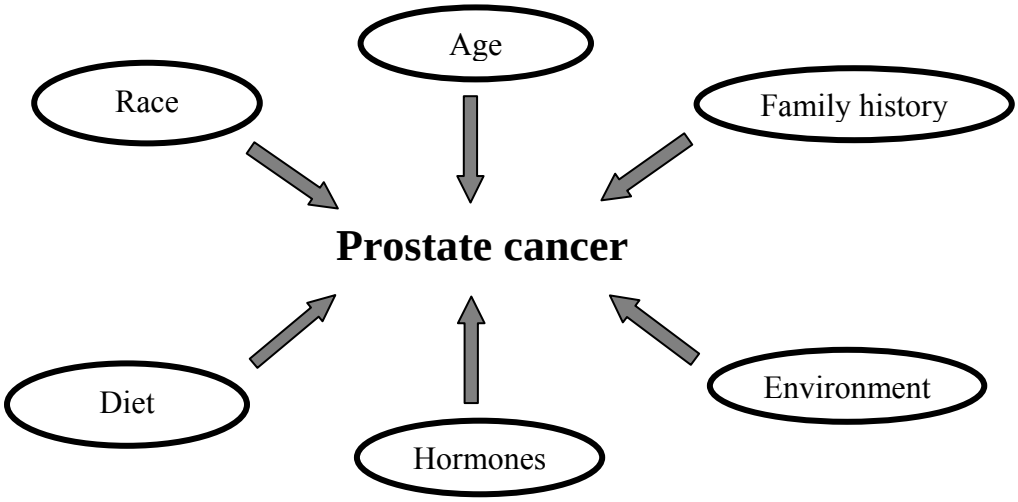


Figure 1. Schematic of prostate cancer risk factors: Age, family history, race, environmental factors including diet and levels of androgens are some of the suggested risk factors for prostate cancer.

Prostate cancer runs in families and hereditary factors account for ~10% of prostate cancers and are often associated with early onset of disease (Carter et al., 1992). Several studies have suggested an association between breast and prostate cancer (Anderson and Badzioch, 1993; Tulinius et al., 1992), but the molecular mechanisms are still unknown. Additionally, age-related decrease in the ratio of androgen to estrogen might provide a risk factor for the initiation of prostate cancer (Prehn, 1999).

Prostate anatomy and development:

The prostate gland surrounds the urethra and is located posterior to the bladder. Although, the prostate gland produces important secretary proteins in the seminal fluid, it is not absolute for fertility. Adult human prostate is a small acorn shaped tissue with ductal-acinar histology. Morphologically, human prostate can be divided into three different zones (Figure 2): the peripheral zone, the transitional zone, and the central zone (McNeal, 1988). Benign prostatic hyperplasia (BPH), a non-malignant growth of prostate occurs mainly in the transitional zone while prostate carcinoma arises primarily in the peripheral zone.

During embryogenesis, prostate develops from urogenital sinus which is a derivative of hindgut. During gestation, primitive urogenital sinus is first separated from terminal hindgut through division of the cloaca by urorectal septum. The rostral, caudal and intermediate parts of the primitive urogenital sinus subsequently form urinary bladder, penile urethra and prostate respectively. The overall process of prostate development remains similar in both humans and mice but the maturation stage differs as the ductal morphogenesis occurs at puberty under the action of androgens in humans.

In mice, Nkx3.1 a homeobox gene is expressed in urogenital sinus epithelium prior to prostate development and is subsequently expressed in all stages of prostate development. Cunha, Hayward and colleagues have provided evidence that interactions between the epithelial and stromal components are essential for prostate development and differentiation utilizing tissue recombination technique (Cunha, 1972a; Cunha, 1972b; Cunha et al., 1996; Hayward et al., 1997). In the fetus,

testosterone stimulates budding of the prostate epithelium from urogenital sinus which produces growth factors like sonic hedgehog to activate underlying mesenchyme (Podlasek et al., 1999). On the other hand, paracrine signals of growth factors from mesenchyme are responsible for glandular morphogenesis and epithelial growth.

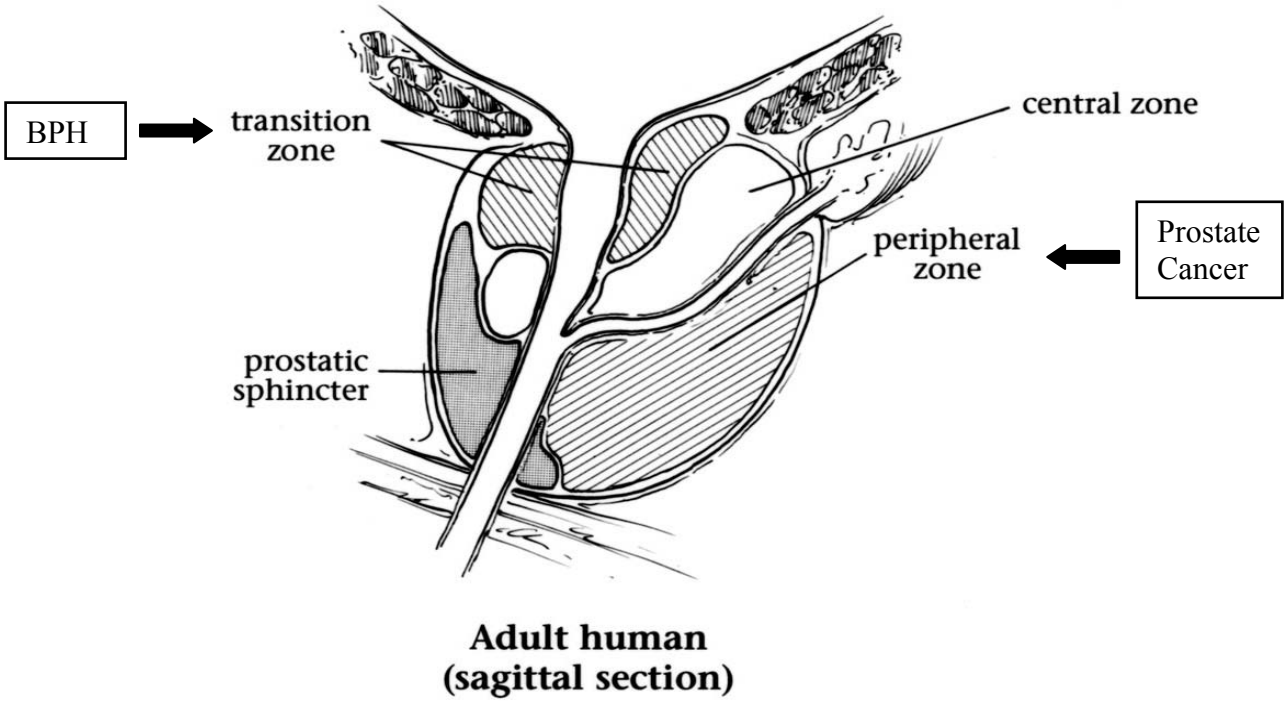


Figure 2. Morphological zones of prostate gland: Human prostate gland is subdivided into three morphological zones namely, central zone, transitional zone (BPH prone zone) and peripheral zone (cancer prone zone) *[adapted from McNeal 1969]*

The prostate consists of a fibromuscular stromal and a glandular epithelial compartment. Smooth muscle cells, fibroblasts and endothelial cells are the stromal components while the secretory luminal cells, basal cells and neuroendocrine cells are the epithelial components (Figure 3). Luminal epithelial cells produce prostatic secretory proteins. Prostate stem cells are suggested to be subpopulation of androgen independent basal cells.

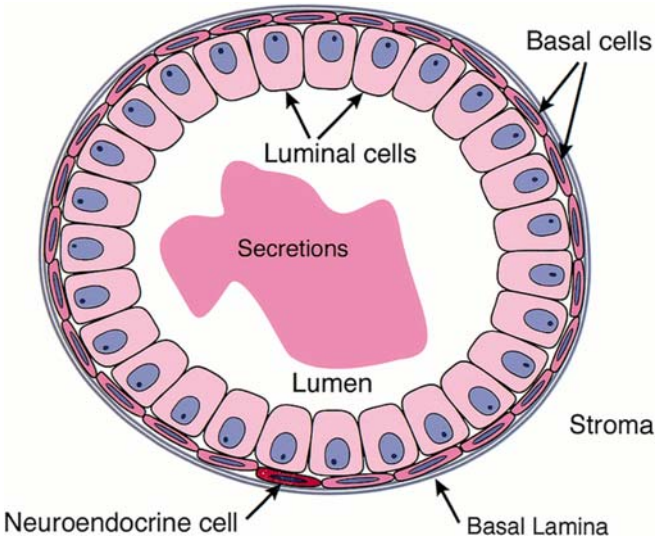


Figure 3. Schematic of cell types in prostate gland: Human prostatic duct epithelial compartment contains three different types of epithelial cells luminal (secretory) epithelium, basal epithelium and neuroendocrine cells. *(Adapted from Abate and Shen 2000)*

A number of regulatory genes play significant role in the normal development of prostate summarized in (Table 1).

Table 1. Candidate genes involved in prostate development

Classification	Genes
Nuclear hormone receptor	Androgen receptor (AR)
Homeodomain transcription factor	Nkx3.1 HoxD13
Forkhead box transcription Factor	FoxA1 FoxA2
Secreted signaling factors	Sonic hedgehog (Shh) BMP-4
Growth factors	FGF7 FGF10 TGFß1

Molecular genetics of prostate cancer:

The development and progression of prostate cancer is complex and believed to involve sequential accumulation of multiple genetic changes. Although ~ 10% tumors can have inherited genetic mutations (Table 2), essentially all the tumors acquire genetic alterations during the course of tumorigenesis. The multistep model for prostate tumor progression is depicted in (Figure 4). Several oncogenes are activated in the process of carcinogenesis by different mechanisms such as gene amplification, translocations, or point mutations. Many tumor suppressor genes are also inactivated by the loss of heterozygosity (LOH) or inactivation of the other allele by a mutation (Dong, 2006). Recently, haploinsufficiency at tumor suppressor loci and epigenetic changes including DNA hypermethylation or histone modifications have also been proposed as mechanisms in the progression of prostate cancer. Some of the multiple genes involved in the initiation / progression of prostate cancer are summarized in (Table 3).

Table 2. Genes implicated in familial prostate cancer

Gene or locus	Chromosomal locus	Mode of inheritance
HPC1	1q24-25	AD
PCAP	1q42.2-43	AD
CAPB	1p36	AD
HPCX	Xq27-28	X-linked/AR
HPC20	20q13	AD
ELAC2/HPC2	17p11	AD

Prostate Cancer- Multi step model

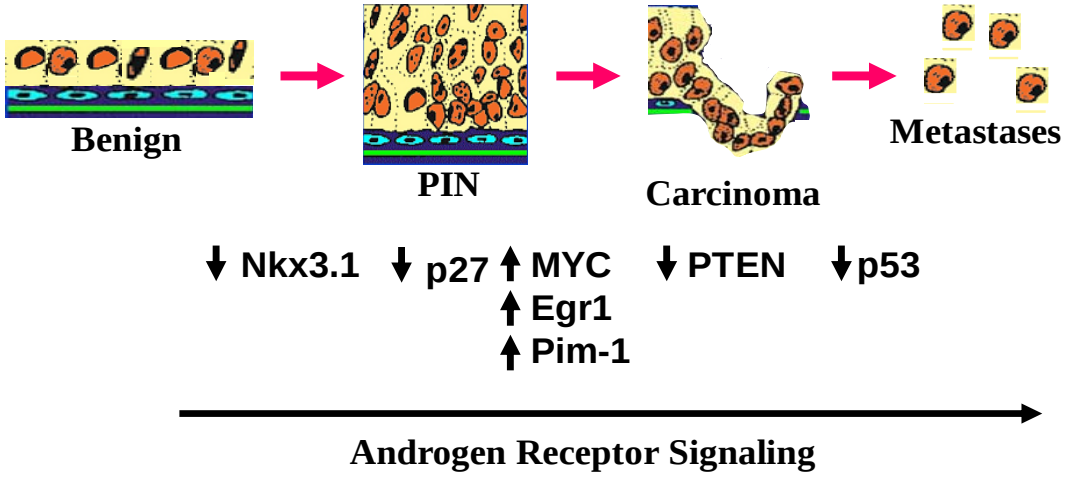


Figure 4. Multi-step model for prostate tumor progression: Multiple tumor suppressor genes such as Nkx3.1, p27, PTEN and p53 are lost while many oncogenes such as MYC, Egr1 and Pim1 are activated during the progression of prostate cancer. Androgen signaling plays a pivotal role during prostate cancer progression.

Table 3. Genes involved in prostate cancer initiation and progression

Prostate Cancer stages	Genes	Comments
Initiation & progression to carcinoma	NKX3.1	Putative prostate tumor suppressor gene, 8p21 chromosomal region is deleted in prostate cancer, Mice lacking one or both alleles develop PIN lesions
	PTEN	Frequent deletion of human gene 10q23, Heterozygote mice develop hyperplasia and dysplasia
	Rb, p27	Cell cycle regulatory proteins, Rb and p27 are often lost in prostate cancer
	Myc	Amplified in some cancers, co-operates with RAS or other oncogenes
	ATBF1	16q22 region is frequently deleted in sporadic prostate cancers
	KLF5, KLF6	Haploid loss of 13q22 has been reported in prostate cancer
	E-cadherin, Integrins and c-CAM	Cell adhesion molecules, reduced expression in PIN and carcinoma
Advanced carcinoma & metastasis	AR	AR signaling plays crucial role, frequently mutated or amplified in prostate cancer
	p53	Higher mutation rates in metastatic tumors than in primary cancer
	IGF1, TGFβ1, EGF	Growth factors, promote prostate epithelium growth and invasion
	Bcl2	Key regulator of apoptosis, overexpressed in androgen independent prostate tumors

Nkx subfamily of homeodomain proteins:

Homeodomain proteins are a family of transcription factors characterized by a 60-amino acid domain (homeodomain) that binds to certain regions of DNA. The homeodomain was first discovered in *Drosophila* and has been shown to play a significant role in determining the anterior-posterior axes of both invertebrates and vertebrates. Structurally, the homeodomain folds into three α helices, the latter two folding into a helix-turn-helix conformation which is characteristic of transcription factors that bind to DNA in the major groove of the double helix (Otting et al., 1990). The third helix is the recognition helix where amino acids make contact with the bases of the DNA (Figure 5).

Homeobox genes including NK subfamily genes are expressed either widely or in a tissue specific manner and are critical in specifying cell fates, development and differentiation in many species. Several studies in Drosophila have provided invaluable insights about functional relevance of NK homeobox genes. Particularly, NK-3 was identified as the bagpipe gene which is required for visceral mesoderm development (Kim and Nirenberg, 1989) while NK-2 (tinman) is required for the development of cardiac mesoderm (Azpiazu and Frasch, 1993). In amniotes, these functional relationships are well conserved.

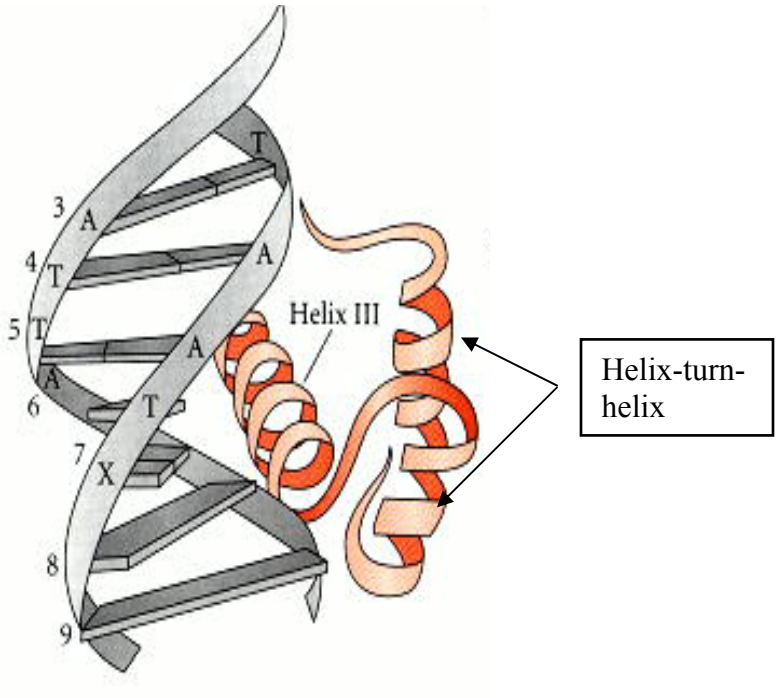


Figure 5. Homeodomain (Helix-turn-helix) & DNA interactions: Note that homeodomain contains three α helices; two of them form a helix-turn-helix conformation while the third helix (recognition helix) makes contact with the DNA *(Adapted from book developmental biology, 6th edition by Scott Gilbert, Sinauer Associates, Inc publisher, 2000).*

Nkx3.1 gene structure:

Human and mouse Nkx3.1 share ~ 60% overall amino acid homology and 100% homology within the homeodomain. Mouse *Nkx3.1* is located at chromosome 14, syntenic with chromosome 8p in humans. The mouse *Nkx3.1* gene (Figure 6) contains two coding exons, a short 5' untranslated region (UTR) and long 3' UTR which contains consensus sequences involved in post-transcriptional regulation (Sciavolino and Abate-Shen, 1998). The homeobox is located in the second exon which is identical to the homeodomain of NK-3. Nkx3.1 protein contains a TN motif (similar to eh1 domain identified in other homeoproteins) that interacts with Gro /TLE co-repressors and hence responsible for repressor activity of Nkx3.1. In humans, alternatively spliced forms of the Nkx3.1 gene have been reported which encode variants of the N-terminal coding region upstream of homeodomain (Korkmaz et al., 2000).

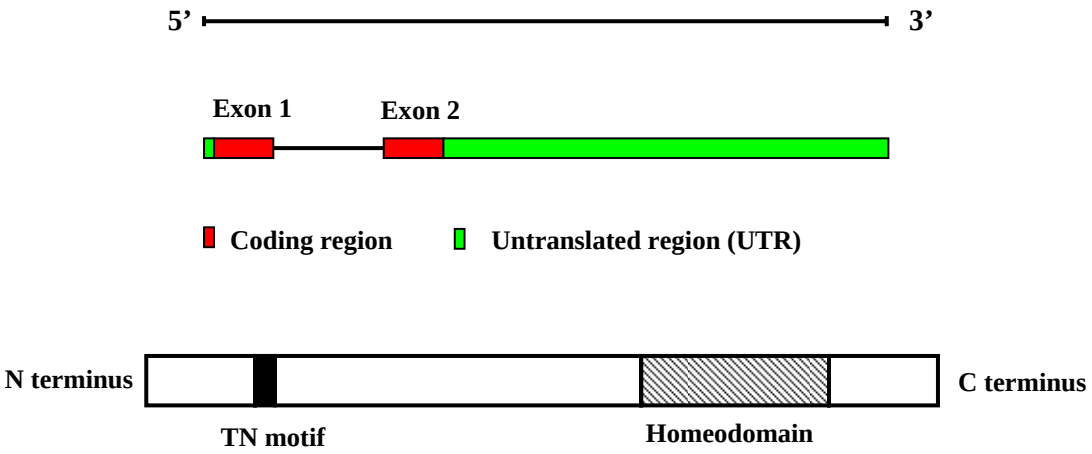


Figure 6. Schematic representation of mouse Nkx3.1 genomic DNA and protein: Nkx3.1 gene contains two exons with short 5' UTR and long 3' UTR. Homeodomain is located in the second exon. TN motif is responsible for the co-repressive activity.

Transcriptional activities of Nkx3.1:

In general, homeodomain proteins can bind at DNA consensus sequences containing a "TAAT" core (Catron et al., 1993). Steadman identified "TAAGTA/G" as a consensus sequence for human NKX3.1 by binding site selection assays (Steadman et al., 2000). By electromobility shift assays (EMSA) Steadman demonstrated that NKX3.1 was preferentially bound to the TAAGTA consensus rather than the binding site for Nkx2.1 (CAAGTG) or Msx1 (TAATTG). However, it remains unclear whether Nkx3.1 protein binds to DNA as a monomer or dimer.

Interestingly, previous studies have provided evidence that Nkx3.1 has a wider transcriptional potential and that can act as a transcriptional repressor as well as an activator. Most probably, this could be a promoter-specific process mediated through the differential recruitment of co-repressors / co-activators protein complexes containing Nkx3.1 at the specific promoter regions (Figure 7).

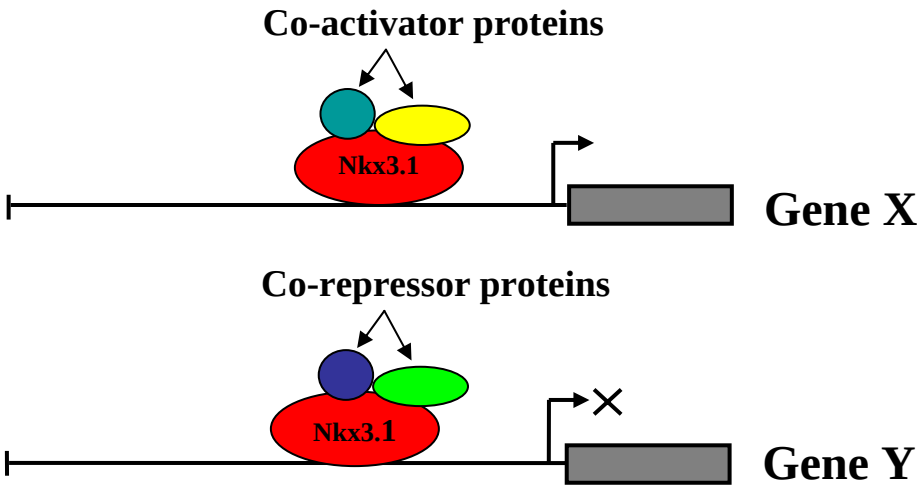


Figure 7. Schematic depiction of Nkx3.1 associated transcriptional activity: Nkx3.1 can form complex with either co-activator (top) or co-repressor (bottom) proteins on specific promoters causing gene activation (Gene X) or repression (Gene Y) respectively.

Several *in vitro* studies indicate that Nkx3.1 acts as a transcriptional repressor. Steadman and colleagues demonstrated that human NKX3.1 can repress activity of a synthetic reporter containing a trimerized NKX3.1 consensus site upstream of a basal promoter (Steadman et al., 2000). These observations are consistent with the reports regarding Nkx3.2 which also behaved as a transcriptional repressor in transient transfection experiments in cell culture and retroviral expression studies in chick embryo (Murtaugh et al., 2001). Choi et al., demonstrated the interaction of NK-3 with Groucho and HIP1K (serine-threonine kinase) by co-immunoprecipitation assays in Hela cells (Choi et al., 1999). He further proposed that Nk-3, Groucho and HIP1K proteins most likely exist in a complex with histone deacetylase HDAC1 constituting a single co-repressor complex (Choi et al., 1999). These studies indicate that Nkx3.1 represses transcriptional activity through recruitment of Gro/TLE co-repressor proteins. Chen et al., showed that transcriptional repressor activity of Nkx3.1 can be modulated by its interaction with PDEF (prostate-derived Ets factor) proteins (Chen et al., 2002). By using a yeast-two hybrid approach, Chen and colleagues isolated PDEF as a novel Nkx3.1 interacting protein within a human cDNA expression library prepared from the prostate. They demonstrated that PDEF can increase transcriptional activity of the PSA promoter and that activity was repressed when Nkx3.1 was co-expressed with PDEF (Chen et al., 2002; Oettgen et al., 2000). Recently, Nkx3.1 has been shown to negatively regulate the transcriptional activity of Sp-family members in prostate-derived cells (Simmons and Horowitz, 2006).

By contrast, some studies indicate that Nkx3.1 can act as a transcriptional activator. Carson and colleagues examined the expression of smooth muscle gamma-actin (SMGA) as a marker of smooth muscle differentiation and found that Nkx3.1 co-operates with serum response factor (SRF) in CV-1 fibroblasts (Carson et al., 2000). Authors demonstrated that Nkx3.1 and SRF can synergistically activate SMGA reporter activity. This synergistic transactivation required adjacent Nkx3.1 and SRF binding sites in the SMGA promoter. By using EMSA and footprinting analysis they further demonstrated that binding of Nkx3.1 to promoter DNA increased recruitment of SRF to the adjacent sites, resulting into increased transcriptional activity (Carson et al., 2000). This study also revealed that the C-terminus of Nkx3.1 protein possess co-repressor activity in addition to a TN motif. The deletion of C-terminus resulted into ~ 15 fold increase in transcriptional activity of SMGA promoter in the absence of SRF suggesting that the C-terminus was responsible for co-repressor activity (Carson et al., 2000). Later studies (Filmore et al., 2002) also confirmed these findings supporting the notion that Nkx3.1 can act as a transcriptional activator in certain contexts.

Recent gene expression profiling analysis in *Nkx3.1*-mutant mice has identified a subset of positively regulated as well as a subset of negatively regulated genes by Nkx3.1 (Magee et al., 2003). Magee and colleagues compared Nkx3.1 target gene expression in Nkx3.1 wild type (+/+), heterozygous (+/-) and knockout (-/-) animals by utilizing a castration-testosterone replacement paradigm. They identified 57 significantly altered genes; some of which were up-regulated while others were down-regulated in *Nkx3.1*-deficient mice compared to WT mice (Magee et al., 2003).

Another independent gene expression profiling study identified several dysregulated (up-regulated/down-regulated) anti-oxidant and pro-oxidant enzymes in *Nkx3.1*-mutant mice (Ouyang et al., 2005). Taken together, these studies provide indirect evidence that Nkx3.1 can act as either a transcriptional activator or repressor, probably depending upon promoter context.

Nkx3.1 and androgen receptor signaling:

The development, normal growth, and maintenance of the prostate gland are dependent on the function of androgens which act through activation of the androgen receptor (AR), a member of nuclear hormone receptor family of transcription factors. Following the pioneering work of Huggins and Hodges in the early 1940's androgen ablation and anti-androgen therapy has become the cornerstone of treatment for prostate cancer patients. However, despite the initial response in ~ 80-85 % of patients, androgen-independent tumors eventually emerge, leaving hormone therapy and complete androgen blockade ineffective (Laufer et al., 1999).

Several lines of evidence support the concept that Nkx3.1 is an androgen regulated gene. Nkx3.1 mRNA levels decline precipitously after castration, prior to the post-castration atrophy (Bieberich et al., 1996). Nkx3.1 expression is detected only in androgen-responsive human prostate cancer cell line LNCaP but not in the androgen-receptor deficient prostate cancer cell lines such as DU145 and PC-3 (He et al., 1997; Prescott et al., 1998). Additionally, Nkx3.1 expression is induced with androgen treatment in a dose-dependent fashion in LNCaP cells. Prescott and colleagues further demonstrated that androgen-induced Nkx3.1 expression was

directly through transcriptional up-regulation of the AR (Prescott et al., 1998). Later, Zhu and colleagues observed that Nkx3.1 expression was dramatically reduced by treatment with AR antagonist agents such as flufenamic acid (Zhu et al., 1999).

Interestingly, Lei and colleagues demonstrated that NKX3.1 can negatively regulate androgen receptor (Lei et al., 2006). Overexpression of NKX3.1 inhibited mRNA and protein levels of AR in cell cultures while increased AR levels were noted in NKX3.1 knockout mice prostates (Lei et al., 2006). Furthermore, Lei and colleagues were able to demonstrate that Nkx3.1 can repress AR transcription by luciferase reporter assays. In conclusion, this study provided evidence that NKX3.1 and AR form a signaling feedback loop in which NKX3.1 is the negative modulator of AR and keeps AR level and AR-controlled pathways in check for normal prostate cell proliferation and survival (Lei et al., 2006).

Nkx3.1 in prostate development:

Homeodomain proteins, including NK-family members play a critical role in development and differentiation (Krumlauf, 1994). During embryogenesis, Nkx3.1 is expressed in a wide range of tissues outside urogenital system including hippocampus, cerebral cortex, dorsal aorta, tongue, teeth, arteries of the kidney and the dorsal part of Rathke's pouch (pituitary gland). However, Nkx3.1 null mutant mice do not show any phenotype in most of these nonurogenital tissues (Schneider et al., 2000; Sciavolino et al., 1997; Tanaka et al., 2000).

During organogenesis, formation of the prostate occurs through epithelial budding from the urogenital sinus. Nkx3.1 is expressed specifically in the prostate

and bulbourethral glands but not in the seminal vesicles, bladder or urethra (Bhatia-Gaur et al., 1999). Within the urogenital system, Nkx3.1 is first detected in lateral aspects of the urogenital sinus epithelium at 15.5 dpc, before prostate formation (Bhatia-Gaur et al., 1999). Nkx3.1 is expressed in the region from which prostatic epithelial buds emerge and subsequently prostatic buds undergo ductal branching and outgrowth into the surrounding mesenchyme. Nkx3.1 is expressed in all stages of differentiation of the prostate, before canalization of the prostatic duct, NKx3.1 is expressed uniformly in all epithelial cells but later its expression is restricted to the luminal epithelium.

Bhatia-Gaur further underscored the importance of Nkx3.1 in the development of prostate by using tissue recombination. Nkx3.1 was expressed specifically in tissue recombinants generated from prostate inducing mesenchyme (UGM) and bladder epithelium (BLE) in which Nkx3.1 is normally not expressed but not in tissue recombinants generated from bladder mesenchyme (BLM) and urogenital epithelium (UGE) which does normally express Nkx3.1(Bhatia-Gaur et al., 1999). Further, this study revealed that functional androgen receptor is not required for the initial Nkx3.1 expression in the developing prostate; however it is required for the maintenance of Nkx3.1 expression throughout maturation.

Several studies have supported the requirement of Nkx3.1 in prostate ductal morphogenesis and epithelial differentiation. Nkx3.1 null mutant mice are viable and fertile suggesting that prostate is not absolute for fertility (Bhatia-Gaur et al., 1999; Schneider et al., 2000; Tanaka et al., 2000). Overall prostate size and weights of Nkx3.1 null mutant mice remained same when compared with the wild type mice.

However, Nkx3.1 null prostates showed reduced ductal branching morphogenesis and decreased secretory protein production (Bhatia-Gaur et al., 1999). A similar phenotype was observed in the bulbourethral glands. In conclusion, these observations suggest that Nkx3.1 plays a role in early postnatal ductal morphogenesis as well as in the production of secretory proteins in mature prostate.

Nkx3.1 in prostate cancer:

During the course of tumorigenesis, normal prostate epithelial cells can progress towards malignancy by acquiring a series of mutations (Figure 4). Prostate cancer like most of other cancers arises from a precursor lesion called as prostatic epithelial neoplasia (PIN). Upon additional genetic hits, PIN lesions often progress to locally invasive disease and ultimately metastasis. Each stage of prostate cancer is associated with chromosomal alterations and shows characteristic morphological and histological changes.

Several chromosomal regions (8p21, 10q and 12q) are commonly lost in human prostate cancer suggesting involvement of many tumor suppressor genes in prostate tumor initiation and progression. In particular, loss of heterozygosity (LOH) at human chromosome 8p21-22 is most commonly described genetic alteration in prostate cancer (Bott et al., 2005). NKX3.1 is the leading candidate gene located in the human 8p21 region (Dong, 2001; Voeller et al., 1997). PTEN and $p27^{kip1}$ are the other candidates at regions 10q and 12q respectively (Abate-Shen and Shen, 2000; Di Cristofano and Pandolfi, 2000). PTEN and $p27^{kip1}$ tumor suppressors are lost in many cancers while loss of NKX3.1 is specific to prostate cancer.

Several studies have been conducted to identify mutations in the coding region of Nkx3.1 (Kim et al., 2002; Ornstein et al., 2001; Voeller et al., 1997; Xu et al., 2000). No mutations were found however, a polymorphism at nucleotide position 154 (C154T) was reported by several groups (Voeller et al., 1997; Xu et al., 2000). Recently, Zheng and colleagues resequenced the Nkx3.1 coding and regulatory regions of 159 probands of hereditary prostate cancer families and identified 21 germ-line variants, including the previously described C154T polymorphism (Zheng et al., 2006). This study showed that the germ-line variants were significantly linked to the risk of having hereditary prostate cancer. Nevertheless, prostate cancer associated somatic mutations have yet to be reported.

Apart from mutational analysis, several mRNA and protein expression studies for Nkx3.1 have also been performed (Asatiani et al., 2005; Bowen et al., 2000; Korkmaz et al., 2004; Xu et al., 2000). An initial NKX3.1 mRNA expression analysis by Xu and colleagues reported increased expression of NKX3.1 in prostate cancers compared with adjacent normal tissue (Xu et al., 2000). However, Bowen and colleagues (Bowen et al., 2000) examined NKX3.1 protein expression in tumor samples with different histological grades and found that NKX3.1 protein was lost in PIN lesions (~20%), invasive (~ 40%) and metastatic (~75%) prostate tumor tissues but not in benign prostatic hyperplasia (BPH) lesions (5%). This study also revealed that loss of NKX3.1 protein expression was strongly correlated with prostate tumor progression. These findings were further supported by another study in which the authors found a significant loss of NKX3.1 expression in preinvasive and invasive tumor tissues compared to normal surrounding prostate tissue (Asatiani et al., 2005).

Along with the above mentioned human studies, there are several lines of evidence that support the critical role of Nkx3.1 as a tumor suppressor in prostate. First and foremost is the fact that *Nkx3.1*-mutant mice develop PIN lesions that closely resemble human PIN lesions (Abdulkadir et al., 2002; Bhatia-Gaur et al., 1999). In mice, deletion of the Nkx3.1 gene leads to developmental defects in the prostate gland, including defects in ductal branching morphogenesis, prostatic secretions and epithelial hyperplasia and dysplasia (Bhatia-Gaur et al., 1999; Schneider et al., 2000; Tanaka et al., 2000). However, because of severe developmental defects these Nkx3.1 transgenic models were not ideal to explore the precise role of Nkx3.1 in prostate cancer. Abdulkadir et al (2002) generated a conditional *Nkx3.1*-deficient mouse model by using Cre- and loxP mediated recombination to delete Nkx3.1 gene in the prostates of adult transgenic mice. Conditional deletion of one or both alleles of Nkx3.1 resulted the development of PIN lesions which showed histological resemblance to PIN lesions from human patients (Abdulkadir et al., 2002). Notably, *Nkx3.1*-mutant mice develop PIN lesions but do not progress to carcinoma. Kim and colleagues successfully employed serial passage of tissue recombination to promote PIN lesions to progressive neoplastic lesions (Kim et al., 2002).

Although loss of Nkx3.1 alone is not sufficient for the development of prostate cancer, Nkx3.1 can cooperate with other tumor suppressor genes to suppress prostate tumor formation. In particular, Nkx3.1 has been shown to cooperate with PTEN which is also lost in human prostate cancer (Dong, 2001; Kim et al., 2002). The compound Nkx3.1; Pten mutant mice develop severe high-grade PIN (HGPIN)

lesions by 6 months of age than single mutants suggesting strong cooperativity between Nkx3.1 and Pten (Kim et al., 2002). This cooperativity between Nkx3.1 and Pten has been shown to be mediated through the PI3Kinase-Akt signaling pathway, whose activation occurs because of loss of function of Pten (Cantley, 2002; Di Cristofano and Pandolfi, 2000). Nkx3.1 can also cooperate with cyclin-dependent kinase inhibitor $p27^{kip1}$ to suppress prostate tumorigenesis (Gary et al., 2004). Nkx3.1; $p27^{kip1}$ double nullizygous mice developed extensive PIN lesions with increased incidence compared to Nkx3.1 or $p27^{kip1}$ single nullizygous mice. Interestingly, authors observed the most cooperativity with complete loss of at least one of the two genes because compound heterozygous mice exhibited a similar phenotype (not more severe) to single heterozygous mutants (Gary et al., 2004). There is also evidence that tumor suppressor gene *Nkx3.1* and the oncogene *Myc* can cooperate and that loss of Nkx3.1 along with gain of Myc may play critical roles in prostate tumor progression (Ellwood-Yen et al., 2003).

HAPLOINSUFFICIENCY

Origin and definition:

In diploid organisms, two functional copies of all genes are present, with the exception of genes on the X and Y chromosomes and imprinted genes. A single normal allele is often sufficient to maintain the normal function of a cell; however, some genes or gene functions are very sensitive to gene dosage level. Haploinsufficiency (HI) represents that special circumstance in which one working

allele is insufficient to support normal cellular function. Curt Stern first applied the term 'haploinsufficiency' in his study of dosage effects on the *cubitus interruptus* allele of Drosophila (Stern and Schaeffer, 1943). Despite the long history of haploinsufficiency in genetics, translation of this concept to tumor suppressor genes has been slow due to the lack of experimental evidence as well as a perceived conflict between concepts of haploinsufficiency and the original Knudson two hit hypothesis.

During the course of cancer development, a normal cell can progress towards malignancy by acquiring a series of mutations. These include mutations that activate proto-oncogenes (gain of function) or that inactivate recessive tumor suppressor genes (loss of function). According to the prevailing paradigm for tumor suppressor gene function, Knudson's two-hit model (Figure 8A), tumor suppressor genes are recessive and both alleles must be inactivated for tumorigenesis (Knudson, 1985). However, several recent studies have challenged the generality of this model and supported evidence for haploinsufficiency as another model (Figure 8B). Haploinsufficient tumor suppressor genes lead to accelerated tumorigenesis without the requirement for inherited mutation of one allele. Haploinsufficiency can be detrimental to various regulatory processes in cell biology such as cell cycle regulation, apoptosis and DNA synthesis or repair (Cook et al., 1998) and hence, it plays a significant role in many human disorders including cancer and developmental defects (Seidman and Seidman, 2002).

Models of tumor suppression

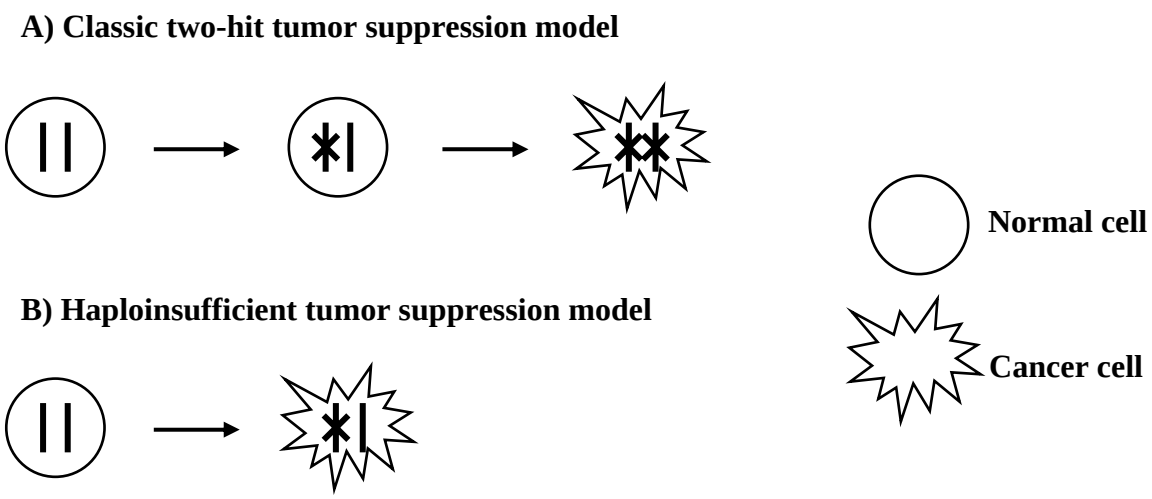


Figure 8. Models of tumor suppressor genetics: A) Classic two-hit model for tumor suppression implies that both alleles must be inactivated for tumorigenesis. B) Haploinsufficient tumor suppression model implies that even a loss of single allele is sufficient for tumorigenesis.

Haploinsufficiency and tumorigenesis:

The incidence of human cancer depends upon the number and frequency of rate limiting mutations and the size of the target cell population (Quon and Berns, 2001). It is estimated that 4-8 rate limiting mutations are required for the development of most human cancers. With these considerations, cancer is expected to arise at a low frequency (~1 in 10^{-21} cells). However, the odds for developing cancer during one's lifetime (~1 in 3) are much higher (Quon and Berns, 2001). Hence, there must be additional factors which increase the human cancer risk. These include epigenetic changes such as promoter hypermethylation that can silence tumor suppressor genes or genomic instability that can increase the overall mutation rate (Cahill et al., 1999; Jaenisch and Bird, 2003). Haploinsufficiency at tumor suppressor loci has certainly been considered as one of the phenomena that increase overall cancer risk, with different tumor suppressor genes showing varying degrees of haploinsufficiency (Cook and McCaw, 2000; Quon and Berns, 2001).

Several tumor suppressor genes including *p53* (*Trp53)*, *p27^{kip1}(Cdkn1b)*, *Dmp1* and *Nkx3.1* encode transcription factors that demonstrate haploinsufficiency (Abdulkadir et al., 2002; Bhatia-Gaur et al., 1999; Cook and McCaw, 2000; Fero et al., 1998; Inoue et al., 2001; Venkatachalam et al., 1998). The first definitive evidence for haploinsufficiency for tumor suppression was provided for p27^{kip1} (Fero et al., 1998). Mice homozygous for loss-of-function mutations in p27^{kip1} suffer from multi-organ hyperplasia. Fero demonstrated that p27^{kip1} heterozygous mice developed tumor similar to p27^{kip1} homozygous with a penetrance of 32 % (Fero et al., 1998). Further, when treated with a carcinogen or irradiation, p27^{kip1} heterozygous mice

developed more tumors than wild type mice bur fewer than homozygous mutants. Notably, the wild type allele of $p27^{kip1}$ was expressed in these tumors, suggesting haploinsufficiency for $p27^{kip1}$ (Fero et al., 1998).

Venkatachalam et al (1998), analyzed mice carrying one mutant allele of p53. Almost, all $p53^{+/-}$ mice developed tumors or died by 2 years of age whereas in wild-type mice only 20% mice died or developed tumors. The wild-type p53 allele was retained in many tumors occurring in $p53^{+/-}$ mice (Venkatachalam et al., 1998). This study provided supporting evidence for haploinsufficiency for p53 including the facts that p53 transcripts were unmutated after sequencing, intact p53 protein expression was observed, p53 dependent transcriptional responses were intact, and lastly, irradiation induced increase in apoptosis was observed in $p53^{+/-}$ mice but not in $p53^{-/-}$ mice (Venkatachalam et al., 1998). Recently, p21 one of the key p53 target genes has been found to be haploinsufficient for tumor development (Jackson et al., 2003). Mice heterozygous and homozygous for a mutation of p21 were more prone to tumor formation after irradiation and tumors from heterozygous mice retained the wild-type allele of p21. Intriguingly, tumors from p21 homozygous null mice metastasized while those from heterozygous did not suggesting a less severe phenotype and haploinsuffciency for p21 (Jackson et al., 2003).

Inoue et al (2001) investigated the effects of Dmp1 haploinsufficiency in Dmp1 mutant mice. Dmp1 is a transcription factor which induces Arf gene transcription by binding to its promoter. Both homozygous and heterozygous Dmp1 mutant mice were significantly more tumor prone than wild-type mice both spontaneously and after irradiation (Inoue et al., 2001). Furthermore, tumors in

$Dmp1^{+/-}$ mice retained expression of the wild-type allele again providing evidence for haploinsufficiency.

Several studies have shown that haploid deletion of tumor suppressor gene Nkx3.1 is frequently associated with prostate cancer and prostatic intraepithelial neoplasia (PIN) (Bova et al., 1993; He et al., 1997; Macoska et al., 1995). Consistent with the observations of LOH in human PIN lesions and prostate cancer, mice in which a single Nkx3.1 allele is conditionally deleted in adulthood developed prostatic hyperplasia and PIN lesions (Abdulkadir et al., 2002). Interestingly, the hyperplastic lesions of these mice as well as those of conventional heterozygous mice retained Nkx3.1 protein expression from the remaining wild type allele providing evidence for haploinsufficiency for Nkx3.1 (Abdulkadir et al., 2002; Bhatia-Gaur et al., 1999). Magee et al (2003) investigated the molecular mechanism of haploinsufficiency in Nkx3.1 heterozygous mice and proposed a model of dosage sensitive and stochastic Nkx3.1 target gene regulation as a potential mechanism (Magee et al., 2003). Dosage-sensitive and stochastic Nkx3.1 target gene regulation is discussed later in a separate section.

Many other genes also demonstrate haploinsufficiency in tumorigenesis including genes involved in cell cycle regulation and maintenance of genomic stability (Santarosa and Ashworth, 2004) (Table 4).

Table 4. Genes that demonstrate haploinsufficiency in tumorigenesis

Gene	Function	Associated Cancer syndrome
APC	Regulation of β-catenin, microtubule binding	Colorectal cancer, FAP
Arf	Stabilizes P53	Melanoma
ATM	DNA integrity/repair	Ataxia telangectasia
BRCA1/2	DNA repair/transcription	Breast, ovarian other cancers
Dmp1	Transcription factor	Gastrointestinal cancer
H2AX	DNA repair	
MAD2	Mitotic checkpoint	
MSH2	DNA mismatch repair	HNPCC, endometrial cancers
NF1	GAP for p21 ras proteins	Neurofibromatosis type I
Nkx3.1	Transcription factor	Prostate cancer
P27	Cell cycle control checkpoint	AML
P53	Transcription factor, apoptosis, response to DNA damage	Soft tissue sarcoma, osteosarcoma, Li-Fraumeni Syndrome
PTEN	Dual-specificity phosphatase	Breast, thyroid, endometrial Ca
Rb	Modulation of cell cycle	Retinoblastoma, osteosarcoma
SMAD4	Signal transduction	Pancreatic, colorectal cancer
TGFβ1	Growth Factor	

Haploinsufficiency and developmental defects:

Haploinsufficiency of transcription factors may cause some developmental anomalies especially if the transcription factor is critical for development or organogenesis. Haploinsufficiency of members of several gene families including Homeobox genes, T-box genes, HMG box genes, Paired box genes and Zinc finger genes produce more than 30 human syndromes which are summarized in Table 5 (Seidman and Seidman, 2002).

Table 5. Transcription factor haploinsufficiency and developmental syndromes

Gene	Class	Developmental defects / syndromes
PITX3 NKX2.5 NKX2.1 SHOX	Homeobox	- Rieger syndrome (Malformation of anterior chambers of eye, muscular dystrophy - Heart defects including atrial septal defects and atrioventricular nodal defects - Choreoathetosis, pulmonary dysfunction - Deformities of radius, ulna and carpal bones
TBX1 TBX5 TBX3	T-box	- DiGeorge syndrome - Holt-Oram syndrome (Atrial septal defect and hand anomalies) - Ulnar-mammary syndrome (delayed puberty, obesity, ulnar ray defects)
HFN1B / 1A SOX 9/ 10	HMG box	- Familial glomerulocystic kidney disease and diabetes (maturity onset diabetes in young) - Deafness, aganglionic megacolon, dwarfism
PAX2 PAX8 PAX6	Paired box	- Renal and optic coloboma - thyroid dysgenesis - Aniridia type II
WT1 GATA3	Zn finger	- WAGR syndrome (Wilms tumor-aniridia-genitourinary-mental retardation) - Hypoparathyroidism, sensorineural deafness renal dysplasia
FOXC2 FOXC1	Forkhead	- Lymphedema-distichiasis syndrome - Defects of eye, glaucoma

HAPLOINSUFFICIENCY AT THE NKX3.1 LOCUS: DOSAGE-SENSITIVE, STOCHASTIC GENE REGULATION

Nkx3.1 regulates cell cycle exit during luminal cell regeneration:

Nkx3.1-deficient mice (*Nkx3.1*$^{+/-}$ and *Nkx3.1*$^{-/-}$) develop prostatic hyperplasia and PIN lesions over time which mimic the PIN lesions in humans (Abdulkadir et al., 2002; Bhatia-Gaur et al., 1999). These observations suggested that Nkx3.1 may establish or maintain luminal epithelia in a growth-arrested state and that loss of one or both alleles of Nkx3.1 may lead to proliferation of luminal cells. Magee et al., (2003) further extended these observations and analyzed the dosage-sensitive phenotype of *Nkx3.1*-deficient mice by utilizing the castration-testosterone replacement (TR) model as shown in figure 9 (Magee et al., 2003).

Magee and colleagues castrated *Nkx3.1*$^{+/+}$, *Nkx3.1*$^{+/-}$ and *Nkx3.1*$^{-/-}$ mice (8 weeks old), subsequently implanted sustained release testosterone pellets for 14 days after castration and analyzed mRNA and protein expression pattern of Nkx3.1 and Ki67 as a luminal cells proliferation marker (Magee et al., 2003). The Ki67 profiles revealed that *Nkx3.1*$^{-/-}$ and *Nkx3.1*$^{+/-}$ (lesser extent) luminal epithelium cells failed to exit the transient proliferation state. In contrast to wild type luminal cells, *Nkx3.1*$^{-/-}$ and *Nkx3.1*$^{+/-}$ luminal epithelium cells were proliferating for at least one week after testosterone replacement. This extended proliferation resulted into the luminal hyperplasia which was evident in *Nkx3.1*$^{-/-}$ prostates by 14 days post-TR (Magee et al., 2003). In spite of the delay in terminal differentiation, *Nkx3.1*-deficient cells ultimately did undergo growth arrest following TR (Magee et al., 2003).

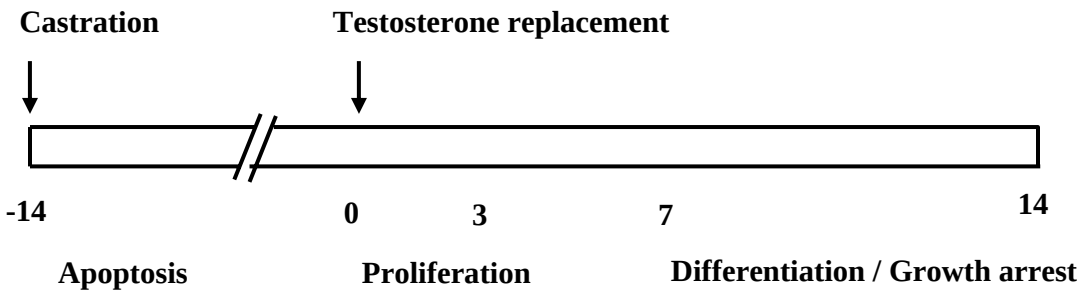


Figure 9. Schematic of Castration-testosterone replacement model for prostate growth and differentiation: After castration *Nkx3.1*-mutant mice prostates undergo apoptosis in 14 days. After testosterone treatment, prostates cells undergo proliferation (3-7 days), differentiation (7-14 days) and ultimately growth arrest *(Adapted from Magee et al., 2003).*

Dosage sensitivity of Nkx3.1 target genes:

Androgens play a pivotal role in the growth and maintenance of the prostate and perturbations in androgen signaling may lead to the development of prostate cancer. Magee et al., (2003) provided evidence that *Nkx3.1*-deficient luminal epithelial cells undergo an extended phase of transient proliferation. This observation implied that Nkx3.1 and androgens cooperatively regulate gene expression during prostate regeneration. Magee and colleagues performed gene expression profiling studies on *Nkx3.1*$^{+/+}$, *Nkx3.1*$^{+/-}$ and *Nkx3.1*$^{-/-}$ mice after castration-TR and identified 57 genes that were significantly altered in *Nkx3.1*$^{-/-}$ mice compared to wild type mice. Interestingly, hierarchical clustering of Nkx3.1 target genes identified discrete clusters of positively (activated) and negatively (repressed) regulated genes by Nkx3.1. Positively regulated genes (e.g., *probasin*, *intelectin*) showed much lower expression in *Nkx3.1*$^{+/-}$ and *Nkx3.1*$^{-/-}$ mice compared to the expression in wild type mice whereas negatively regulated genes (e.g., *angiopoietin 2*, *sushi-repeat protein X*) showed higher expression in *Nkx3.1*$^{+/-}$ and *Nkx3.1*$^{-/-}$ mice as shown in Figure 10 (Magee et al., 2003). Notably, the expression of all Nkx3.1 regulated genes was influenced by androgen status supporting the notion that Nkx3.1 is an androgen target and its expression is regulated by androgens.

Upon further analysis, Magee and colleagues observed a spectrum of dosage-sensitivity among the Nkx3.1-activated genes (Figure 10A), genes like *Probasin* and *Riken clone* appeared relatively insensitive to the Nkx3.1 dosage (similar expression levels were observed in *Nkx3.1*$^{+/+}$ and *Nkx3.1*$^{+/-}$ mice) while genes like *Intelectin* and *Pdzk1* appeared highly dosage-sensitive even to the loss of single allele of Nkx3.1

(Note that expression levels in *Nkx3.1*$^{+/-}$ mice were similar to the levels in *Nkx3.1*$^{-/-}$ mice).

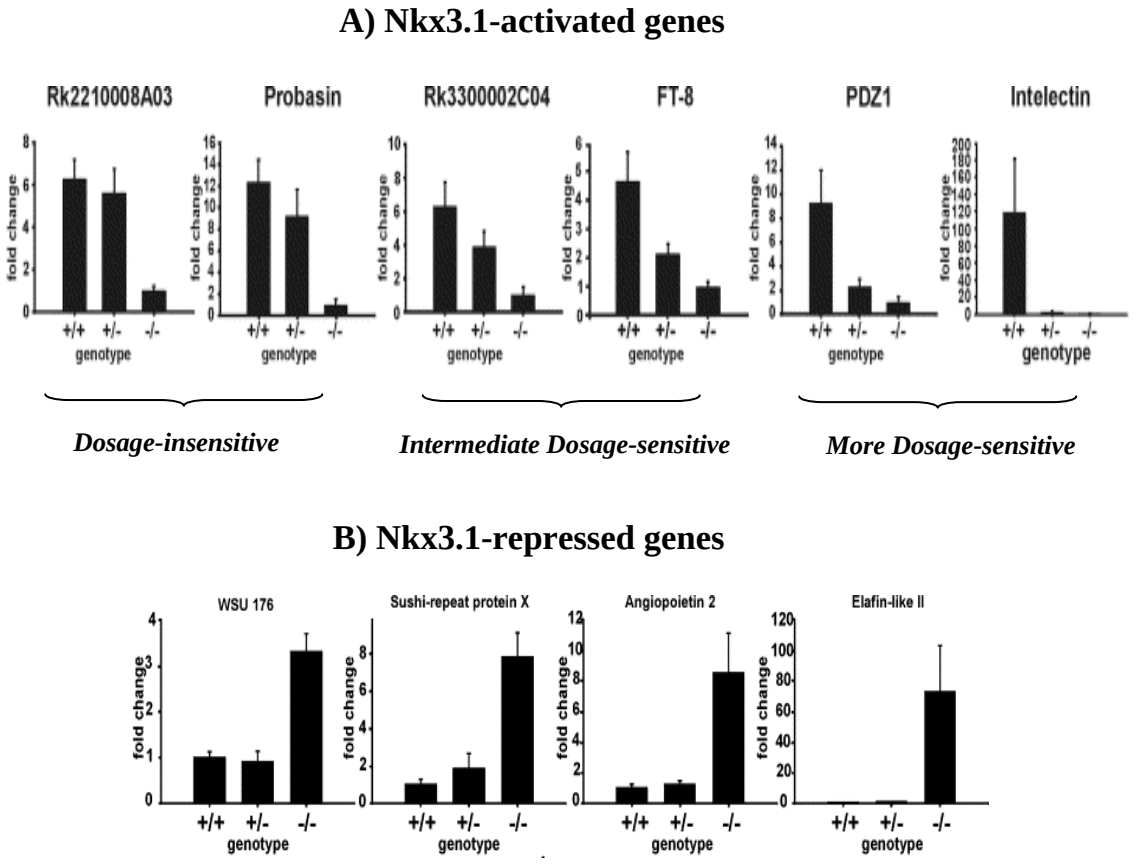


Figure 10. Androgen regulated Nkx3.1 target gene qRT-PCR expression profile: A) <u>*Nkx3.1-activated genes*</u> show a spectrum of dosage-sensitivities. Genes such as *Probasin* and *Riken* clone are relatively insensitive while genes such as *Intelectin* and *Pdzk1* are more sensitive to the Nkx3.1 dosage. B) <u>*Nkx3.1-repressed genes*</u> such as *Angiopoietin 2* and *Elafin-like II* do not show dosage-sensitivity. *(Adapted and modified from Magee et al., 2003)*

Stochastic expression of Nkx3.1-activated target genes:

Transcriptional regulation is very complex and two types of transcriptional models are commonly accepted (Figure 11). The first transcriptional model is the most popular view and implies that transcription is a graded process with promoter activity directly proportional to the levels of one or more transcription factors (e.g., promoter activity increases uniformly in all cells in response to increasing levels of transcription factor dosage). This model is commonly referred as the "rheostatic" or "rate" or "graded" model (Figure 11A).

As opposed to the graded model, there is a lot of supporting evidence for the "stochastic" or "binary" or "probabilistic" model for transcriptional activation (Fiering et al., 2000). According to this model, genes exist in either "on" or "off" state and transcription factor dosage regulates the probability of a gene occupying either active or inactive state as shown in Figure 11B (Fiering et al., 2000). Numerous studies have demonstrated that cells within a homogeneous population do not always respond with a uniform induction of gene expression even though they are subjected to identical stimuli. The expression of ApoB in the avian liver was found to be responsive to estrogen and this induction was achieved by recruitment of hepatocytes from a silent to an expression state (Lin et al., 1986). Van Roon et al (1989) observed that induction of different enzymes involved in the ornithine cycle such as carbamoyl synthetase, phosphoenolpyruvate carboxykinase and arginase by glucocorticoids was stochastic in cultures of fetal hepatocytes (van Roon et al., 1989). The expression of various serum proteins including albumin appeared heterogeneous in hepatocytes and further, hepatocytes were recruited to the expressing pool in a stochastic manner upon

activation (Michaelson, 1993). Cytokine induction by antigen presentation in clonal populations of T cells was also reported as a stochastic process (Bucy et al., 1994). Newlands and colleagues examined expression of both muscle specific transgenes and endogenous genes in the individual nuclei of multinuclear myofibers and found that the genes were expressed in only subset of nuclei even though all were part of a single cell providing additional evidence for the stochastic regulation (Newlands et al., 1998). Lastly, Riviere and colleagues demonstrated that individual alleles of IL-4 gene can be regulated independently and stochastically within the same nucleus (Riviere et al., 1998).

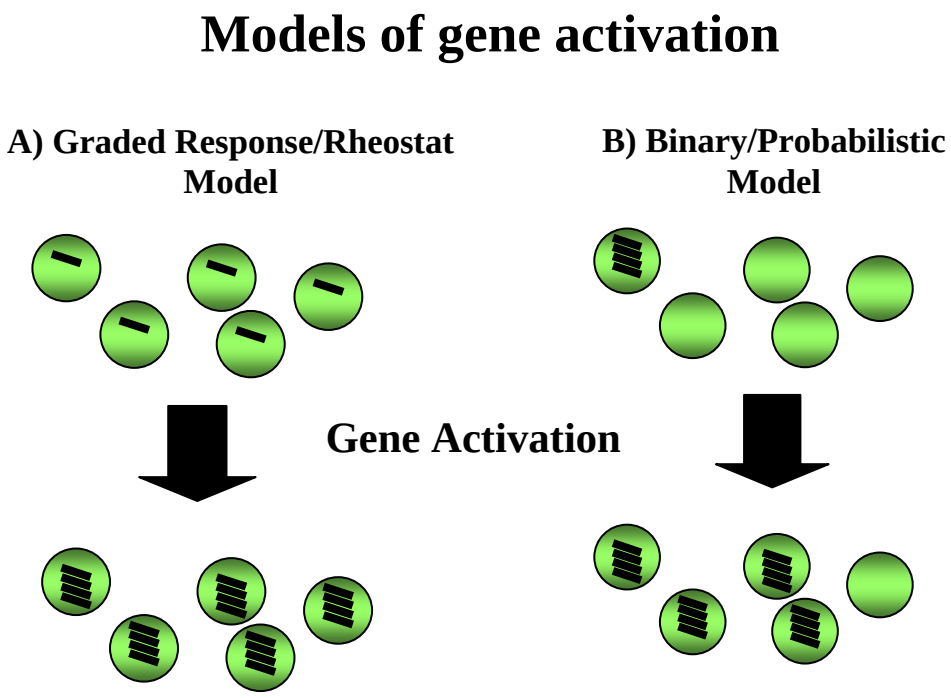


Figure 11. Models of transcriptional regulation: A) *Graded response(Rheostat)model* implies that promoter activity is directly proportional to the levels of one or more transcription factors supporting the uniform expression of a given gene at a cellular level B) *Stochastic (probabilistic) model* implies that genes exist in either "on" or "off" state and transcription factor dosage determines the probability of a given gene to occupy either state supporting the non-uniform expression pattern at a cellular level.

Although, most experimental data are consistent with either model of gene activation; the two models are not mutually exclusive and hence, both forms of regulation might be possible. To distinguish whether Nkx3.1 target genes are activated by a graded model or stochastic model, Magee and colleagues performed in situ hybridization experiments in *Nkx3.1*-mutant prostates for the two representative activated genes *probasin* (dosage-insensitive target) and *intelectin* (dosage-sensitive target). In situ hybridization analysis revealed the stochastic (non-uniform) expression patterns for both probasin and intelectin (Figure 12). Probasin was uniformly expressed in wild type and *Nkx3.1*$^{+/-}$ prostates, but *Nkx3.1*$^{-/-}$ prostates showed a heterogeneous population with probasin expressing and non-expressing cells (Magee et al., 2003). Similarly, in the case of intelectin wild type prostates, heterogeneous expression profile was observed while no expression was observed in *Nkx3.1*$^{+/-}$ and *Nkx3.1*$^{-/-}$ prostates (Figure 12). Notably, Nkx3.1 protein expression in wild type and heterozygous prostates showed a uniform pattern.

Based on these observations, Nkx3.1-activated target genes seem to be regulated by stochastic or binary promoters wherein Nkx3.1 gene dosage mainly influence the stochastic probability of either “on” or “off” state of target gene rather than the absolute level of target gene activity in a given cell.

Stochastic expression of Nkx3.1 target genes

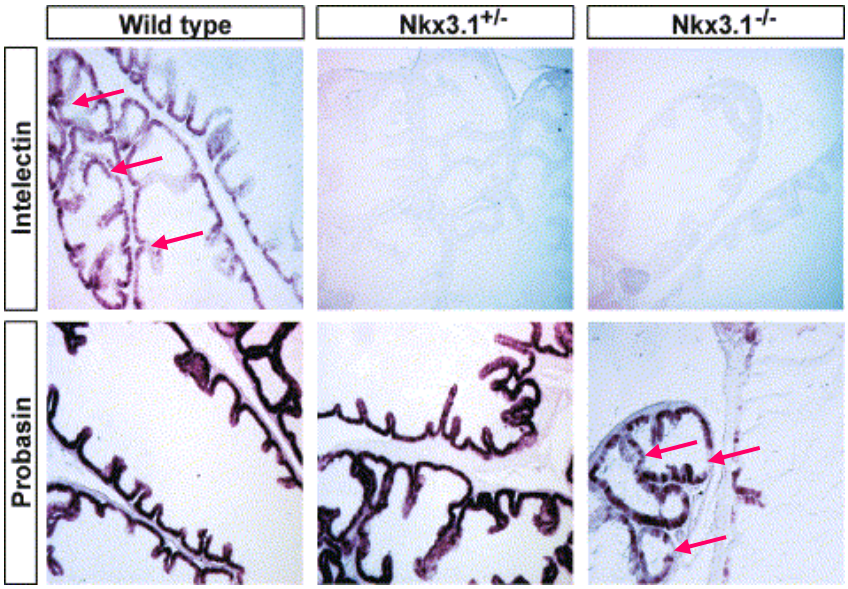


Figure 12. Stochastic expression pattern of Nkx3.1 target genes by in situ hybridization: Note the mosaic (heterogeneous) pattern of expression for intelectin in wild type and probasin in *Nkx3.1*$^{-/-}$ prostates. Note the arrow pointing cells do not express either intelectin or probasin *(Adapted and modified from Magee et al., 2003).*

Model of Nkx3.1 haploinsufficiency in prostate cancer:

The dosage sensitive and stochastic nature of Nkx3.1 target genes readily explains the null phenotype in some heterozygous cells and molecular mechanisms underlying the haploinsufficiency in prostate tumor initiation. Consistent with the stochastic model, Magee and colleagues observed that Nkx3.1 dosage affects the probability that a given target gene exists in either an "on" or "off" state. They proposed a mechanism wherein at the point of tumor initiation; loss of single allele of Nkx3.1 would enhance the probability of complete inactivation of a dosage-sensitive target gene in luminal epithelial cells in a stochastic manner, in turn extending the proliferative phase of luminal amplifying cells. These cells would undergo clonal expansion and provide a larger target cell population which could further acquire additional genetic mutations during prostate tumor progression (Magee et al., 2003).

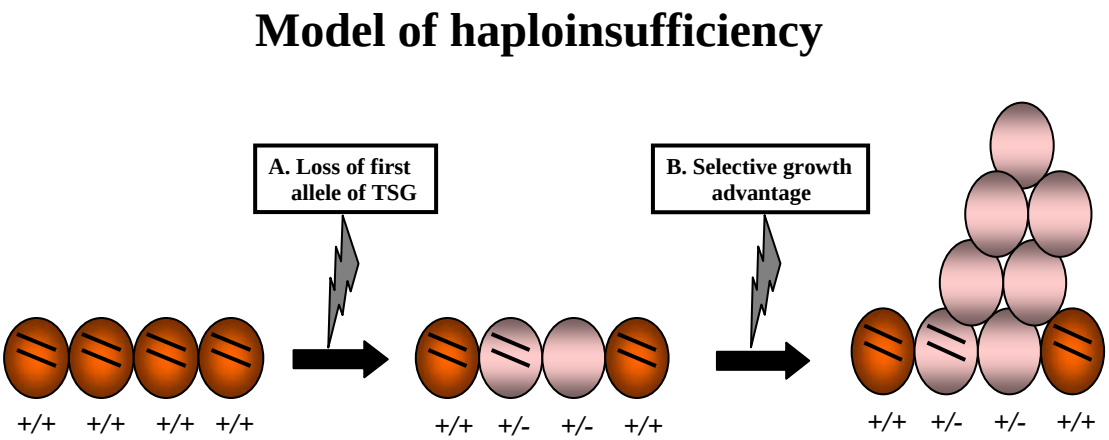


Figure 13. A model of haploinsufficiency in prostate tumor initiation: A) Loss of first allele of tumor suppressor gene (TSG) such as Nkx3.1 stochastically inactivates target gene expression (Black lines) in a subset of heterozygous cells. B) These cells would clonally expand and provide a larger reservoir of cells for additional mutations during tumorigenesis

NOISE (STOCHASTICITY) IN GENE EXPRESSION

Definition:

Genetically identical cells or organisms exhibit remarkable diversity even though they have identical histories of environmental exposure. Noise or variation in the process of gene expression often contributes to such a phenotypic variability (Raser and O'Shea, 2005). 'Noise' in gene expression can be defined as the measured level of variation in gene expression among identical population of cells. Mathematically noise can be calculated as the ratio of standard deviation (SD) to the mean of population.

Sources and measurement of noise:

Gene expression often involves a series of single molecule events and as each of these molecular events is subject to fluctuations, gene expression is best viewed as a stochastic process (Rao et al., 2002). Four potential sources of variation in gene expression have been proposed: 1) The inherent stochasticity of biochemical processes; 2) differences in the internal states of a population of cells such as cell cycle progression; 3) environmental differences; 4) random or directed genetic mutations (Raser and O'Shea, 2005).

Recently, scientists have developed a two-reporter system with fluorescent protein (GFP) variants which allows the quantification of protein levels and detection of noise in living cells. This method involves quantification of expression of two equivalent, independent gene reporters placed in the same cell so that one can

distinguish between two types of noises: 'intrinsic noise' referring to sources that create differences between two reporters within the same cell and 'extrinsic noise', referring to sources that affect two reporters equally in any given cell but create differences between two cells (Figure 14).

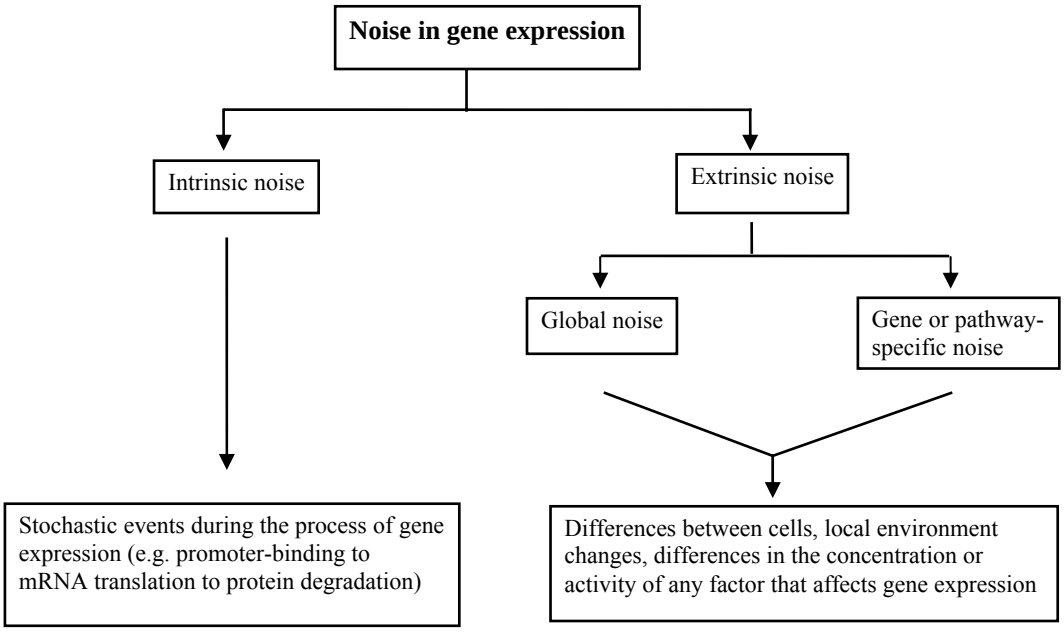


Figure 14. Noise in gene expression: Noise can be divided as intrinsic noise which occurs because of stochastic events during the process of gene expression or extrinsic noise which could be global affecting expression of all genes or gene /pathway specific.

Noise analysis:

To study the origin of noise in gene expression, McAdams and Arkin first proposed a stochastic model of gene expression in prokaryotes which implied that proteins are produced in random bursts and protein translation can amplify transcriptional noise (McAdams and Arkin, 1997). Numerous other models have

further validated these findings by analyzing the mechanisms that contribute the generation of noise in gene expression (Kierzek et al., 2001; Thattai and van Oudenaarden, 2001). As an experimental verification, van Oude-naarden and colleagues studied expression of GFP markers to quantify noise in the prokaryote *Bacillus subtilis* and provided evidence that transcription is the source for most of the noise during translation (Ozbudak et al., 2002).

Elowitz et al (2002) employed two-reporter method to detect noise in gene expression in *Escherichia coli* which quantified cyan and yellow fluorescent protein levels expressed from identical promoters on the same prokaryotic chromosome. Elowitz and colleagues identified noise in protein levels in a clonal population of *E. coli* due to the stochastic nature of gene expression (Elowitz et al., 2002).

Raser and O'Shea measured noise in cells of budding yeast, *Saccharomyces cerevisiae* (Raser and O'Shea, 2004). These studies revealed that the noise in protein level was due to the slow interconversion between inactive and active promoter states (Figure 15) and stochastic chromatin remodeling events (Raser and O'Shea, 2004). These studies further demonstrated that extrinsic noise was the predominant form of the detectable noise and it was global in nature (Raser and O'Shea, 2004).

Noise in biology:

Noise is not limited to gene expression but has a wider relevance in biology. Any individual in a population of living organisms is unique and indeed the genetic and environmental histories contribute to the cellular variability. Noise has many roles in biological functions which include generation of errors during DNA

replication leading to mutations and evolution, noise-driven divergence of cell fates, noise-induced amplification of signals and the maintenance of the quantitative individuality of cells (Rao et al., 2002). Hence, multiple cellular processes such as ion-channel gating, neural firing and cytoskeleton dynamics are influenced by noise (Allen and Stevens, 1994; van Oudenaarden and Theriot, 1999; White et al., 2000).

Significance of noise:

Noise in gene expression may confer a fitness advantage or disadvantage. Intrinsic noise can produce fluctuations in the relative expression of two alleles of the same gene in a heterozygote, resulting in cells that express no allele, either individual allele or both alleles (phenomenon of hybrid vigor). Intrinsic noise plays a role in haploinsufficiency where increased levels of noise leads to stochastic inactivation of target genes in a subset of heterozygous cells, in turn promoting tumorigenesis (Magee et al., 2003).

Furthermore, stochasticity in gene expression may play a role in differentiation in multicellular organisms or in survival in fluctuating environments for unicellular organisms such as during the lambda phage lysis-lysogeny switch and in mammalian olfactory neuronal receptor choice (Arkin et al., 1998; Serizawa et al., 2003).

Finally, noise can be advantageous if environmental conditions become more stressful, as noisy population may produce some members that could be better adapted to the new environment. In conclusion, stochasticity in gene expression is not

necessarily an obstacle for cellular behavior, but it may be an evolvable source of population diversity for better adaptation (Raser and O'Shea, 2004).

Control of noise:

Various mechanisms have been proposed that control noise in biological systems. Several lines of evidence suggest that control of noise is under evolutionary pressure. The simplest and most common noise attenuating regulatory mechanism is negative feedback. Becksei and colleagues constructed a negative feedback module in *E. coli* and demonstrated that variation in expression of GFP was reduced by the addition of the negative feedback using the tetracycline repressor (Becskei and Serrano, 2000).

Another type of feedback is integral feedback which is a part of negative feedback that uses internal memory states to attenuate noise. This type of mechanism is suggested in the case of bacterial chemotaxis in which integral feedback measures temporal changes in chemical concentrations rather than steady-state changes resulting into biased motion towards an attractant and thus early adaptation (Yi et al., 2000).

Gene dosage and parallel cascades reduce noise by increasing the likelihood of gene expression or establishing consensus from multiple signals (Cook et al., 1998; McAdams and Arkin, 1999). Other mechanisms including regulatory checkpoints in cell cycle events (Hartwell and Weinert, 1989) and kinetic proofreading in protein translation to correct possible errors (Rodnina and Wintermeyer, 2001) have also been studied.

Experimental evidence from *Bacillus subtilis* suggest that frequent transcription followed by inefficient translation results in lower noise in protein levels compared to infrequent transcription followed by efficient translation (Ozbudak et al., 2002). Similarly, studies from Raser and O'Shea in budding yeast suggest that frequent promoter activation events followed by inefficient transcription results in lower noise in mRNA levels than infrequent promoter fluctuations followed by efficient transcription as shown in Figure 15 (Raser and O'Shea, 2004). These studies underscore the importance of stochastic chromatin remodeling events in the generation of noise in gene expression.

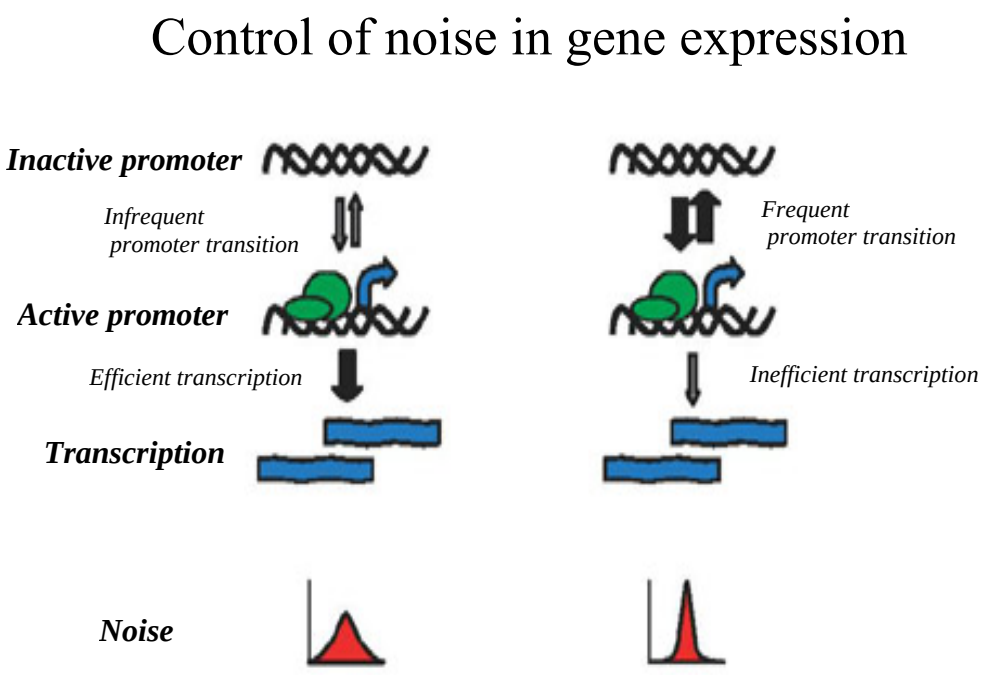


Figure 15. Control of noise in gene expression: Inactive to active promoter transitions and stochastic chromatin remodeling events play role in the control of noise. Frequent promoter transitions followed by inefficient transcription can attenuate noise whereas infrequent or slow promoter transitions followed by efficient transcription may increase noise in gene expression *(Adapted and modified from Raser et al., 2005).*

Epigenetics:

'Epigenetic' is the term used to describe mitotically and meiotically heritable states of gene expression that are not due to the changes in DNA sequence (Bird, 2002). Epigenetic changes are implicated in many aspects of cell biology (Figure 16) including cancer development, X-inactivation, genomic imprinting, position-effect variegation and developmental anomalies (Yoo and Jones, 2006). Two of the most studied epigenetic phenomena are DNA methylation and covalent modifications of histones.

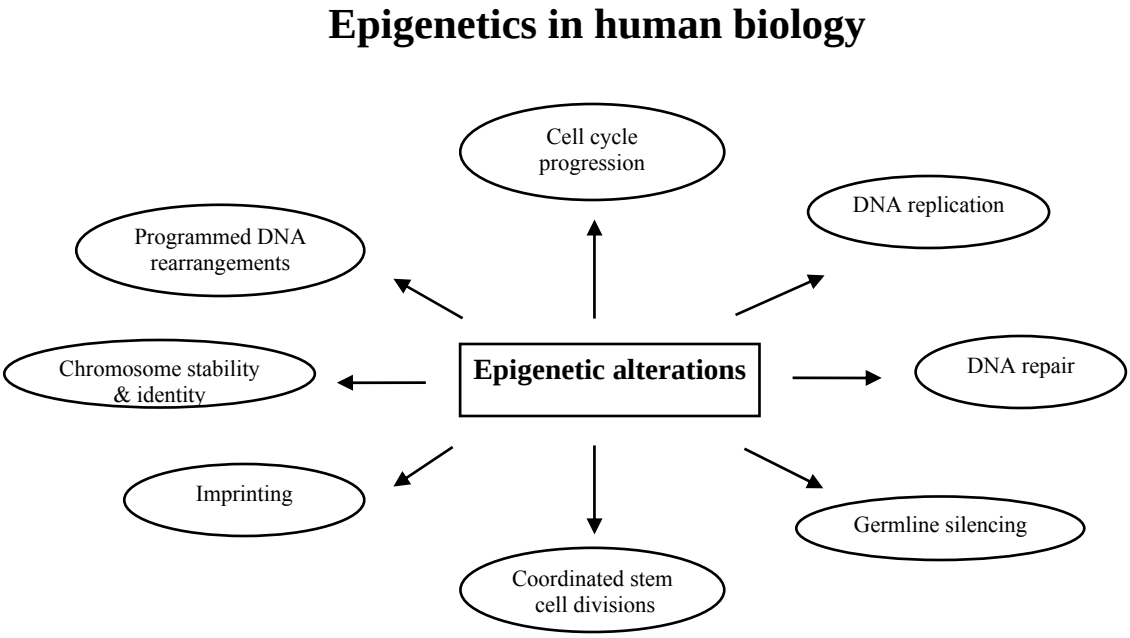


Figure 16. Epigenetics in human biology: Epigenetic alterations play a significant role in various key regulatory processes in cell biology such as cell cycle progression, DNA replication and repair, germline silencing, imprinting, genomic stability and stem cell divisions.

DNA methylation:

DNA methylation is one of the best-known epigenetic signals. DNA is methylated by DNA methyltransferases (DNMTs) at the 5-position (C5) of the cytosine ring, almost exclusively in the context of CpG dinucleotides. Low levels of DNA methylation in the promoter region is often correlated with active gene expression. Approximately 50% of genes are associated with CpG islands in their promoter region and these are usually hypo-methylated and hence, capable of transcriptional activation (Figure 17). By contrast, methylation near the transcriptional start site often inhibits gene expression which occurs through the recruitment of transcriptional repressor proteins such as methyl binding proteins (MBDs) and histone deacetylases (HDACs) (Fujita et al., 1999).

DNA methylation differentially regulates gene expression in a tissue-specific and developmental-stage-specific manner. The methylation pattern is established during development and is normally maintained throughout the life of an individual. However, during older age this pattern can become less stringent, leading to an increase in genomic instability (Yoo and Jones, 2006).

DNA methylation is important in the initiation and progression of cancer and this relationship has been extensively studied. Interestingly, promoter hypermethylation but genome wide hypomethylation have been observed in cancer leading to transcriptional inactivation of genes and genomic instability respectively. Furthermore, global DNA hypomethylation is linked with the chromatin remodeling and nuclear disorganization in cancer cells causing chromosomal instability (Hoffmann and Schulz, 2005). In terms of epigenetic cancer therapy, targeting the

DNMTs has been found to be the most effective way to inhibit DNA methylation and restore the normal methylation pattern. However, targeting DNMTs is not a specific approach and can lead to a genome wide hypomethylation and activation of certain deleterious oncogenes.

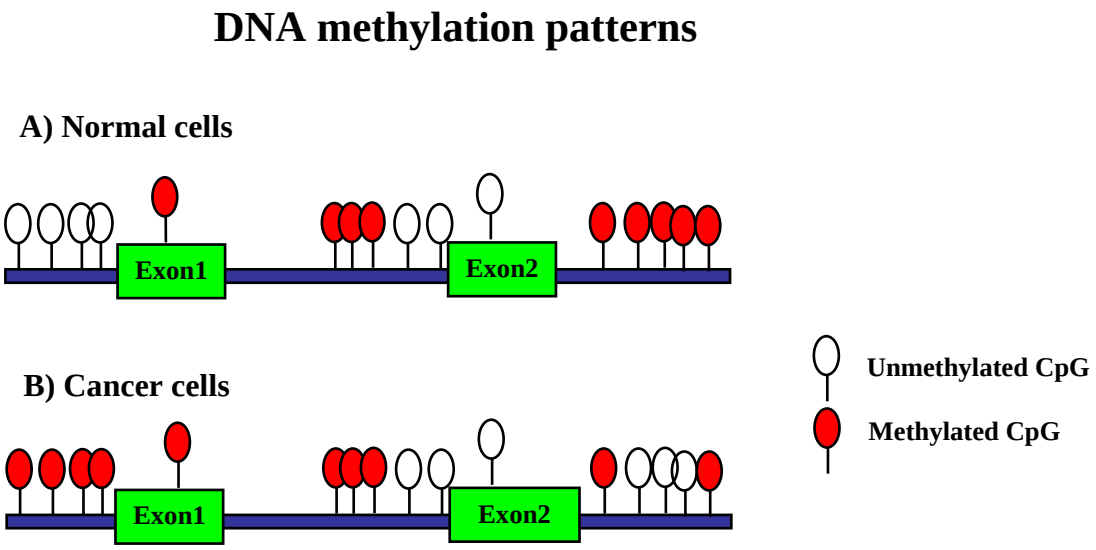


Figure 17. Schematic representation of DNA methylation: A) In normal cells, the CpG islands in the 5' regions of many genes are unmethylated and ready to be expressed B) In cancer cells, CpG islands in the 5' regions of many tumor-suppressor genes are methylated, and silenced. This is considered as one of the major mechanisms of tumor-suppressor gene inactivation.

Histone modifications:

Eukaryotic DNA is intimately associated with a family of small basic histone proteins, to form a highly ordered and condensed protein: DNA complex called 'chromatin'. In other words, chromatin is the physiological template of eukaryotic genetic information. The fundamental unit of the chromatin is called 'nucleosome' (Figure 18), which consists of approximately 147 base pairs of DNA wrapped around an octamer of histone core proteins. This octamer is composed of two copies of each H2A, H2B, H3 and H4 or sometimes natural variants of histone proteins (Hake et al., 2004). Generally, two different forms of chromatin have been described: 'heterochromatin' a tightly compacted form which leads to transcriptional repression and 'euchromatin' a more open conformation which leads to transcriptional activation (Wolffe and Kurumizaka, 1998).

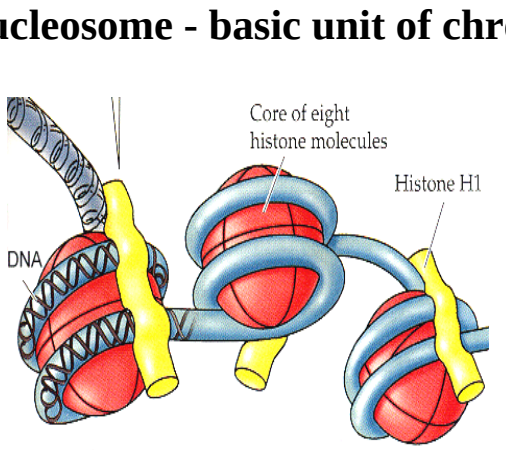


Figure 18. Structure of nucleosome: Fundamental unit of chromatin is called as a 'nucleosome' that consists of 147 bp DNA wrapped around an octamer of two copies of each histone H2A, H2B, H3 and H4

Remodeling of chromatin can be achieved by several different but interconnected mechanisms 1) covalent modifications of histones 2) exchange of 'generic' core histones with histone variants 3) disruption of the basic nucleosome structure and histone DNA contacts and 4) modification of DNA itself (Hake et al., 2004). Among these, covalent modifications of histones have been extensively investigated. Histone tail domains are subject to a diverse array of covalent modifications that include: acetylation, methylation, phosphorylation, ADP-ribosylation, ubiquitination, sumoylation, glycosylation, biotinylation and carbonylation.

Histone acetylation is most probably the best-studied modification and occurs on the lysine residues of tail domains of histones mainly histone H3 and H4. The overall level of acetylation of core histones results from the steady-state balance between the opposing activities of two groups of histone modifying enzymes: histone acetyl transferases (HATs) which cause acetylation while histone deacetylases (HDACs) which are responsible for deacetylation. HATs and HDACs are super-families of enzymes which are summarized in (Table 6 and 7).

In general, an increased level of histone acetylation (hyperacetylation) is associated with the euchromatin state (open chromatin), while lower level of histone acetylation (hypoacetylation) is often found in heterochromatin state (closed chromatin) (Fischle et al., 2003b; Strahl and Allis, 2000; Turner, 2000). HATs add an acetyl group (negative charge) on the lysine residues of histone tails, loosening the contacts between histones and a negatively charged DNA (open chromatin). On the other hand, removal of acetyl group leads to a tighter association between histones

and DNA (closed chromatin). Thus, histone acetylation plays significant role in transcriptional regulation of multiple genes by altering chromatin structure. Histone hyperacetylation leads to transcriptional activation by opening chromatin structure whereas histone hypoacetylation leads to transcriptional repression by closing chromatin structure (Figure 19). Histone deacetylase inhibitors (HDACi) inhibit the deacetylases and they are summarized in (Table 8). HDACi are commonly used to study histone acetylation as they can induce histone hyperacetylation and activate transcription of various genes. Above all, several of HDACi are currently in different phases of clinical trials which can prove as effective anticancer agents in future.

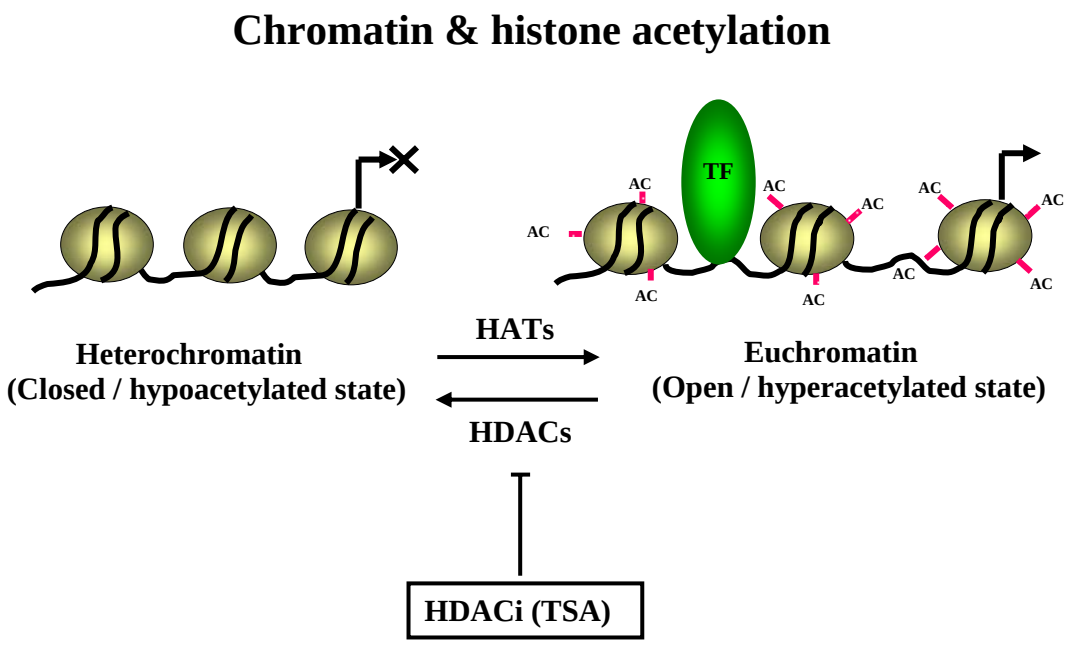


Figure 19. Histone acetylation and chromatin alterations: Histone acetylation is a dynamic modification. Histone acetylation (euchromatin) is caused by histone acetyl transferases (HATs) while deacetylation (heterochromatin) is caused by histone deacetylases (HDACs). HDACi can lead to hyperacetylation and in turn transcriptional activation of certain genes

Table 6. Known histone acetyltransferases (HATs)

HAT	Organism	Transcriptional/ other function	Histone specificity
GNAT family			
Gcn5	Yeast	Coactivator	**H3**/H4
GCN5	Human	Coactivator	**H3**/H4
PCAF	Human	Coactivator	**H3**/H4
Hat1	Yeast	Histone deposition	H4
Elp3	Yeast	Transcript elongation	-
Hpa2	Yeast	-	**H3**/H4
MYST family			
Esa1	Yeast	Cell cycle progression	**H4**/H3/H2A
Sas2	Yeast	Silencing	-
Sas3	Yeast	Silencing	**H3**/**H4**/H2A
Tip60	Human	Coactivator	**H4**/H3/H2A
HBOI	Human	Coactivator	-
MORF	Human	Coactivator	**H3**/**H4**/H2A
MOZ	Human	Coactivator	-
MOF	Human	Dosage compensation	**H4**/H3/H2A
p300/CBP	Human	Global Coactivator	**H2A**/**H2B**/**H3**/**H4**
Others			
SRC-1	Human	Coactivator	**H3**/H4
ACTR	Human	Coactivator	**H3**/**H4**
TIF2	Human	Coactivator	-
$TAF_{II}250$	Yeast/Human	RNA Pol-II transcription	**H3**/H4
TFIIIC	Human	Coactivator	**H2A**/**H3**/**H4**

Table 7. Known histone deacetylases (HDACs)

Class/HDACs	Organism	Transcriptional / other function	Complexes
Class I			
Hos1	Yeast	Repressor / activator	-
Hos2	Yeast	Repressor	Set3C
Rpd3	Yeast	Repressor/ activator	Rpd3S,L
HDAC1	Human	Repressor	mSin3-HDAC, NuRD,
HDAC2	Human	Repressor	Co-REST, N-CoR-2
HDAC3	Human	Repressor	N-CoR-1
HDAC8	Human	Repressor	
Class II			
Hda1	Yeast	Repressor	Hda1-associated complex
Hos3	Yeast	Repressor	Homo dimmer
HDAC4	Human	Repressor	
HDAC5	Human	Repressor	
HDAC6	Human	Repressor/ cell motility	
HDAC7	Human	Repressor/ activator	
HDAC9	Human	Repressor	
HDAC10	Human	Repressor	
Class III			
Hst1-4	Yeast	Silencing	Set3C
Sir2	Yeast	Silencing	Sir4, RENT
SIRT1	Human	Repressor	
SIRT2	Human	Mitotic exit	
SIRT3	Human	Metabolic regulation	
SIRT4	Human		
SIRT5	Human		
SIRT6	Human	DNA repair	
SIRT7	Human	Pol-I transcriptional activator	
Class IV			
HDACII	Human	-	

Table 8. Known histone deacetylases inhibitors (HDACi)

Class	HDACi	*In vitro* IC_{50} range	Clinical trial
Hydroxamic acids	Trichostatin A (TSA)	nM	
	Suberoyl anilide bihydroxamide (SAHA)	μM	Phase II
	CBHA	μM	
	Pyroxamide	μM	
	Oxamflatin	nM	
Short-chain fatty acids	Butyrate	mM	
	Phenylbutyrate	mM	Phase I
	Valproic acid	mM	Phase I & II
Cyclic tetrapetides / epoxides	Trapoxin	nM	
	HC-toxin	nM	
	Chlamydocin	nM	
	Depudesin	μM	
	Apicidine	nM- μM	
	Depsipeptide (FK228)	nM	Phase II
Benzamides	N-acetyldinaline (CI-994)	μM	
	MS-275	μM	Phase I

Histone methylation has been of a great interest in the last few years in chromatin biology. In contrast to histone acetylation which can occur only on lysine residues, methylation of histones can occur on both lysine and arginine residues. In addition, lysines can be mono-, di-, or tri-methylated whereas arginines can be mono- or di-methylated (symmetrically or asymmetrically), thus adding another layer of complexity to this modification dependent gene regulation (Hake et al., 2004). Lysine methylation is carried by a group of enzymes called as lysine methyltransferases which have enormous specificity. Histone methyl transferases (HMTs) are characterized by a conserved SET domain. The SET domain proteins are divided into subgroups (depending upon homology within the SET domain) as SUV3, SET1, SET2 and RIZ (Yoo and Jones, 2006). Lysine methylation was considered to be a stable or permanent modification for many years until the discovery of first demethylase, LSD1 (Shi et al., 2004). This study was one of the major breakthroughs in the field of histone methylation and provided the first piece of evidence that histone methylation is a dynamic process. As opposed to histone acetylation which often leads to gene activation, histone methylation can cause either gene activation or repression depending upon the lysine residue which gets methylated. Three methylation sites are so far implicated in transcriptional activation: H3K4, H3K36 and H3K79 and in repression: H3K9, H3K27 and H4K20 (Kouzarides, 2007).

There are two types of demethylase domains, with distinct catalytic reactions: the LSD domain and JmjC domain. As per Shi's initial observations, LSD1 can specifically demethylate H3K4 and repress transcription (Shi et al., 2004). Interestingly Metzger et al demonstrated that LSD1 can associate with androgen

receptor (AR), and demethylate H3K9 leading to transcriptional activation of AR-target genes (Metzger et al., 2005). Furthermore, JMJD2A, JMJD2B, JMJD2C and JMJD2D have been reported to demethylate H3K9 (Cloos et al., 2006; Fodor et al., 2006; Shin and Janknecht, 2007; Tsukada et al., 2006). Since 2004, discovery of the first histone demethylase LSD1, a total 7 histone demethylases have been documented. The precise functional significance of all of these enzymes is still not clear. Similarly, arginine methylation can also cause activation or repression of transcription and the enzymes responsible (protein arginine methyltransferase, PRMT) are recruited to the promoters by transcription factors (Lee et al., 2005).

Phosphorylation is another important and well understood histone modification. Histone phosphorylation is often associated with chromosome condensation that includes mitosis, meiosis, apoptosis and DNA damage. One of the earliest recognized responses to DNA damage is the phosphorylation of the histone variant γ-H2AX in mammalian cells (Fillingham et al., 2006). During DNA replication, condensation and decondensation of chromatin are two important processes. The phosphorylation at H3S10 by aurora B kinase and the phosphorylation at H3T3 by Has-pin kinase play significant roles during mitosis (Kouzarides, 2007). Little is known about histone phosphorylation and gene expression, however, a role for H3S10 has been demonstrated for activation of NFKB-regulated genes and immediate early genes such as c-fos and c-jun. Recently, global ChIP on CHIP analysis of many kinases in budding yeast has shown that they are present on the chromatin of specific genes and this might have implications in signal transduction (Pokholok et al., 2006).

Histone code hypothesis:

Strahl and Allis (2000) proposed the 'histone code' hypothesis to explain the complex nature of differential patterns of histone modification readouts (Strahl and Allis et al., 2000). According to this hypothesis, one histone modification or specific combinations of histone modifications can affect distinct downstream events either by altering chromatin structure and or by generating a binding platform for protein effectors molecules which can specifically recognize the modifications and initiate gene transcription or repression (Fischle et al., 2003a; Strahl and Allis, 2000). In one of the very first examples demonstrating the cooperativity of histone modifications in the regulation of gene expression, phosphorylation of H3-S10 together with H3-K14 acetylation was shown to prevent the methylation of H3-K9.

Histone modifications either promote or prevent the binding of proteins and protein complexes that drive particular regions of the genome into active transcription or repression. Proteins containing bromodomains and chromodomains have been shown to have affinity for acetylated and methylated lysine residues respectively (Figure 20). The histone code hypothesis states that the modification would recruit proteins or protein complexes which in turn would cause chromatin to take as a certain conformation and spread the pattern to the neighboring region. Heterochromatin protein-1 (HP1) associated heterochromatin formation is one of the best examples of this phenomenon. The chromodomain of HP1 interacts with a trimethylated H3-K9 and recruits SUV39H1 which is a histone H3-K9 methylase that subsequently methylates the H3 tail of the adjacent nucleosome at lysine 9 to mediate HP1 binding and further spreading of heterochromatic regions (Lachner et al., 2001).

Translation of histone code

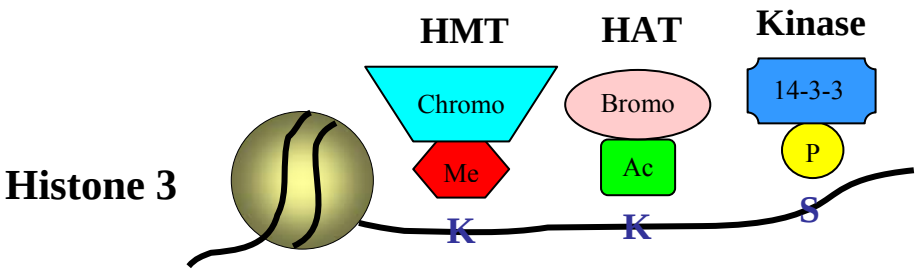


Figure 20. Translation of histone code: Protein modules of histone modifying enzymes that have been shown to interact with site-specific methylation (chromodomain), acetylation (bromodomain) and phosphorylation (domain in 14-3-3 proteins) marks in histone amino-tails. Ultimately, these modifications can act alone or in combination to alter chromatin structure and initiate specific gene transcription or repression.

Gaps in the knowledge:

The molecular events that occur during the initiation of prostate cancer remain enigmatic. However, it is clear that a first genetic hit (haploid loss of Nkx3.1) during tumor development confers a selective growth advantage to the affected cells, creating a reservoir of cells that are more susceptible for subsequent mutations. Magee and colleagues (Magee et al., 2003) performed gene expression profiling on the prostates of *Nkx3.1*-mutant mice and identified a class of genes that shows exquisite dosage sensitivity i.e. the expression of these genes in *Nkx3.1*$^{+/-}$ cells was similar to that in *Nkx3.1*$^{-/-}$ cells (Magee et al., 2003). In addition, this study suggested that Nkx3.1 dosage might regulate target gene activation in a stochastic manner. These observations are consistent with a model of haploinsufficient tumor suppression (Figure 13) in which reduction in Nkx3.1 dosage increases the probability of inactivation of select target genes important for tumorigenesis (Abdulkadir, 2005; Magee et al., 2003). Nevertheless, at the molecular level, the mechanisms involved in haploinsufficient tumor suppression and dosage-sensitive, stochastic gene regulation by Nkx3.1 have remained unclear. In addition, none of these dosage-sensitive target genes have been shown to functionally affect prostate cell growth or tumorigenicity. As my dissertation work, we have explored the molecular mechanism of haploinsufficient tumor suppression by Nkx3.1 and examined the dosage-sensitive and stochastic Nkx3.1 target gene regulation in prostate. Furthermore, we have functionally characterized one of the dosage-sensitive

targets, intelectin / omentin and demonstrated its role in suppressing prostate cell growth and tumorigenicity.

Hypothesis:

We hypothesize that dosage-sensitive, stochastic target gene regulation by Nkx3.1 is one of the mechanisms of haploinsufficiency in prostate tumor initiation and that phenomenon can be mediated by alterations in the active (on) versus inactive (off) states of chromatin.

Rationale behind hypothesis:

Previous studies in transgenic mice and Drosophila support that the dosage of transcription factors and stochastic on and off gene expression is related to changes in higher order chromatin (Ahmad and Henikoff, 2001; Lundgren et al., 2000). Lundgren and colleagues investigated the mechanisms of transcriptional activation in heterochromatin by utilizing FISH technique to directly visualize changes in chromatin organization during activation of the heterochromatic λ5 transgene in mice (Lundgren et al., 2000). They observed that activation of transcription was stochastic and dependent on the dosage of activating transcription factor early B cell factor (EBF). Further, reduction in dosage of EBF resulted in a reduced frequency of localization of the transgene to the outside of the heterochromatin complex and lower levels of transcription. These observations provided evidence that transcription factor dosage can initiate changes in higher order chromatin during the initial stages of gene expression (Lundgren et al., 2000).

Ahmad and Henikoff examined the behavior of a GFP reporter subject to position-effect variegation (PEV) in Drosophila. In their studies, the reporter gene displayed an expression pattern suggestive of repeated rounds of stochastic activation and inactivation of gene expression, resulting in patches of cells (mosaic pattern) expressing the reporter (Ahmad and Henikoff, 2001). In addition, their study established a correlation between fluctuations of chromatin states with the stochastic activation or inactivation of gene expression. Firstly, their results revealed that the fluctuations in the chromatin state of reporter genes can uncover transcription factor binding sites. Secondly, they observed that the recruitment of chromatin modulating activities after transcription factor binding can reduce the rate of heterochromatin reformation (Ahmad and Henikoff, 2001).

More recent studies in budding yeast indicate a role for chromatin remodeling and interconversion between inactive to active promoter states in the control of stochasticity in gene expression (Raser and O'Shea, 2004). Raser and O'Shea employed the two reporter system to measure the noise in gene expression in yeast cells. These studies revealed that the intrinsic noise in the transcription results from the slow interconversion between inactive to active promoter states due to stochastic chromatin remodeling events as shown in Figure 15 (Raser and O'Shea, 2004). Furthermore, this study revealed that mutations in the TATA box upstream of the repressible acid phosphatase PHO5 do not change the level of noise associated with PHO5 expression. However, mutations in genes (SNF6, ARP8, GCN5), which encode the components of chromatin remodeling complex increase the level of noise

supporting the role for chromatin remodeling in the control of noise in gene expression (Raser and O'Shea, 2004).

Specific aims:

We tested our hypothesis in *Nkx3.1*-mutant mouse prostates with the following specific aims:

Aim I: We first determined the effects and mechanism of alterations in active (open) versus inactive (close) chromatin states on the dosage-sensitive, stochastic Nkx3.1 target gene regulation (Chapter IV).

Aim II: We established the functional significance of dosage-sensitive Nkx3.1 target genes in prostate cancer (Chapter V).

CHAPTER II

MATERIALS AND METHODS

ChIP assays, two-step ChIP assays:

ChIP assays were performed using the ChIP assay kit from Upstate Biotechnology (Lake Placid, NY) with the following modifications. Anterior prostates from *Nkx3.1*-mutant mice (Abdulkadir et al., 2002) were excised, snap-frozen and ground into a powder under liquid nitrogen. The tissue was fixed in 1% formaldehyde/PBS supplemented with the Complete protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland), 1 mM Na_3VO_4 and 1 μg ml^{-1} pepstatin for 15 min at room temperature. Fixation was stopped by the addition of glycine to a final concentration of 125 mM. After several washes in ice-cold PBS, the tissue pellet was resuspended in NEBA buffer (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA) supplemented with Complete protease inhibitor cocktail, 1 mM Na_3VO_4 and 1 μg ml^{-1} pepstatin, dounce homogenized and incubated for 15 min on ice. NP40 (0.5%) was added and samples were vortexed for 30 s before collecting the nuclei. Subsequently, the nuclear fraction was lysed in nuclear lysis buffer (50 mM Tris-HCl pH 8.1, 10 mM EDTA, 1% SDS, Complete protease inhibitor cocktail, 1 mM vanadate and 1 μg ml^{-1} pepstatin). Chromatin was sheared to a size of approximately 500-700 base pairs (Figure 21) and diluted 1:10 with ChIP dilution buffer. Samples were pre-cleared and precipitated overnight at 4 °C with the following antibodies: pan-acetylated H3 (Upstate, 06-599), pan-acetylated H4 (Upstate, 06-598), Nkx3.1

(Santa Cruz, T-19X), PCAF (Santa Cruz, H-369X) and rabbit or mouse IgG (Santa Cruz). Immune complexes were collected with salmon-sperm DNA−saturated protein-A/G Sepharose for 3 h and washed extensively following the manufacturer's protocol. Samples were reverse cross-linked at 65 °C overnight with 0.3 M NaCl and 30 µg RNAse. Input and bound DNA was extracted with PCR purification kit (Qiagen) and analyzed by quantitative PCR (Applied Biosystems 7300) using SYBR-Green. ChIP values are presented as fold enrichment and calculated as [IP Ab/ Input Ab] / [IP IgG/ Input IgG] as described earlier (Magee et al., 2006; Polo et al., 2007).

Two-step ChIP assays were performed as described earlier (Kim et al., 2005). In brief, components were eluted from the first immunoprecipitation reaction by incubation with 10 mM dithiothreitol (DTT) at 37 °C for 30 min and diluted 1:50 in ChIP dilution buffer followed by reimmunoprecipitation with the second antibodies. Two-step ChIP was performed in essentially the same way as the first immunoprecipitations.

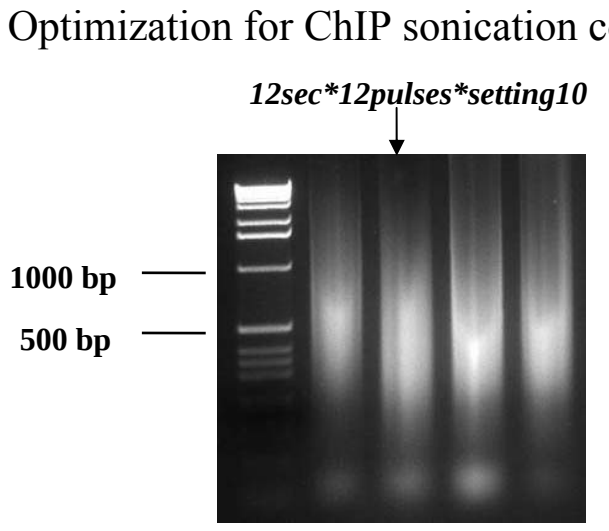


Figure 21. Optimization of sonication conditions for ChIP assays: Note the optimal chromatin shearing (500-700 bp) by Fisher scientific sonicator (12s*12 pulses*setting 10)

Trichostatin A Preparation:

Trichostatin A was obtained (Sigma biochemical) and dissolved into ethanol. Several aliquots of stock solutions of 500 μg/ml, 200 μg/ml and 100 μg/ml were prepared and stored at -20^{0}C. Fresh aliquots were used for separate experiments.

Mouse prostate explants and TSA treatment:

Mouse prostate explants were performed as described previously (Mogal et al., 2006). Briefly, anterior prostates were excised, minced and treated with either vehicle (ethanol), Trichostatin A (Sigma) at a concentration of 500 ng/ml for 8 h or 5-azacytidine 1 μM for 24 h. Prostate tissue was harvested in a complete DMEM/F12 50/50 media. RNA was extracted with Trizol reagent, cDNA was synthesized and analyzed by quantitative RT-PCR (Applied Biosystems 7300) using the SYBR-Green method as described (Mogal and Abdulkadir, 2006). The explant experiments were independently repeated under the same conditions.

To our knowledge, conditions for TSA treatment of mouse prostate explants have not been determined. We therefore, first determined the effects of TSA treatment on histone acetylation in mouse prostate tissue explants using different concentrations of TSA (250 ng/ml, 500 ng/ml and 1000 ng/ml) and at different time points (8 hr, 16 hr and 24 hr) and found 500 ng/ml TSA for 8 hrs to be the optimal condition for inducing histone H3 acetylation (Figure 22).

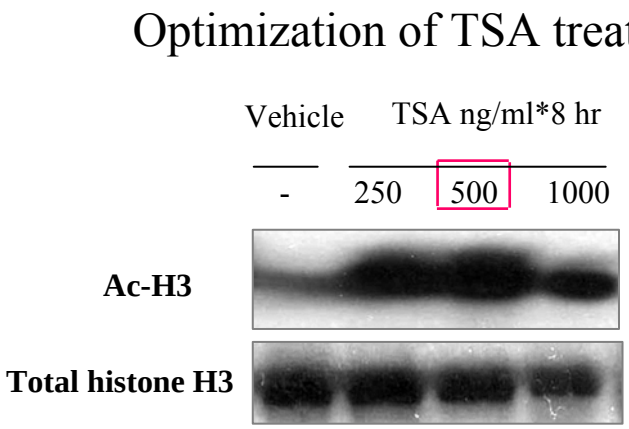


Figure 22. Optimization of TSA treatment for prostate explants: Mouse prostates were treated with either vehicle or TSA (250 ng/ml, 500 ng/ml and 1000 ng/ml for 8h) and efficiency of TSA treatment was confirmed by western blot analysis for acetylated histone H3. Note that maximum acetylation was achieved at 500 ng/ml concentration of TSA.

RNA isolation and Quantitative RT-PCR Analysis:

RNA was extracted with Trizol reagent (Invitrogen) after 8 and 24 h treatments from mouse prostate tissue and human prostate and breast cancer cells respectively. Samples were dissolved in RNase-free 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. 1 µg of total RNA was reverse transcribed 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. RNase free water was used to make final volume of 250 µl. cDNA samples

were then boiled for 5 min and stored at −20 ^{0}C. PCR was performed by SYBR® green PCR Master Mix (Applied Biosystems).The increase in fluorescence of the SYBR green dye was monitored using a GeneAmp 5700 sequence detection system (Applied Biosystems). All of the PCR reactions were performed in triplicate and independently repeated at least two to three times.

Western Blot Analysis:

Mouse prostate explants were treated with TSA as described above and lysates were prepared using extraction buffer (50 mM Tris-HCl-buffered saline, pH 7.4, 1% Triton X-100, 1% Nonidet P-40, 5 mM $CaCl_2$, 2 mM phenylmethylsulfonyl fluoride, and 3 mM hydrogen peroxide). Nuclear protein was extracted by the method of Dignam et al (Dignam et al., 1983). Protein concentrations were measured using the Bio-Rad D_C protein assay reagent. Extracts containing 20–30 μg of protein were electrophoresed on a 12% SDS-polyacrylamide gel and blotted onto an Immobilon™ membrane (Millipore). The blotted membrane was blocked with 5% fat-free dry milk for 1 h at room temperature and incubated with the rabbit polyclonal acetylated histone 3 antibody (1:1000, Upstate) at 4°C overnight. The membrane was then incubated for 1 h at room temperature with a peroxidase-labeled goat anti-rabbit 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 at room temperature for 30 sec to 1 min. Membranes were stripped and then incubated with rabbit polyclonal total histone 3 antibody (1:1000, Upstate) and Nkx3.1 antibodies (Santa Cruz T19). To confirm knockdown efficiency for stable LNCaP cell lines, lysates

from LNCaP cells were prepared and probed with Nkx3.1, intelectin (Phoenix pharmaceuticals Inc) and actin (Santa Cruz) antibodies.

Immunohistochemistry assays:

Immunohistochemistry for paraffin-embedded section was performed as described (Abdulkadir et al., 2001). In brief, paraffin-embedded sections (6 μm thick) were deparaffinized, rehydrated, and subjected to antigen retrieval in 10 mmol/L citrate buffer, pH 6.0. The following antibodies were used: acetylated H3 (1:100) and acetylated H4 (1:100) antibodies. Immunoreactivity was detected using 3,3‘-diaminobenzidine substrate, and slides were counterstained with hematoxylin before they were mounted and viewed.

Co-immunoprecipitation:

For immunoprecipitation experiments, LNCaP cells were grown in RPMI 1640 supplemented with 5% FBS at 37^0C with 5% CO_2. The cells were lysed in 0.5 ml of extraction buffer. The lysates were incubated on ice for 30 min and subsequently cleared by centrifugation at 12,000 rpm for 15 min at 4 °C. The protein concentration of the lysates was determined by Bio-Rad DC protein assay reagent. 50 μl of protein A/G Plus-agarose beads (Santa Cruz) were added to 1.5-ml microtubes containing 500 μl of cellular lysate (500 μg protein) and rotated at 4 °C for 1 h. The samples were centrifuged for 1 min at 2000 rpm. The samples were incubated with primary antibodies [2 μg each of rabbit IgG, goat IgG, Nkx3.1 or PCAF antibodies (Santa Cruz)] and the mixture was rotated overnight at 4 °C. Immune-complexes were

collected with 50 µl of protein A/G Plus-agarose beads for 2 hr at 4 °C. The beads were pelleted by gentle centrifugation and washed five times with 1 ml of ice-cold extraction buffer. After the final wash, the precipitated protein complexes were resuspended in SDS sample loading buffer and boiled for 5 min. The samples were vortexed, centrifuged and the supernatants were analyzed by Western blotting.

RNA Interference:

Lentiviral NKX3.1 shRNA construct (RHS4186) and retroviral intelectin shRNA construct (RHS1764) were purchased from Open Biosystems. Intelectin shRNA was cloned into the same lentiviral vector for a valid comparison with shNKX3.1. The constructs were transfected into LNCaP cells with superfect reagent (Qiagen). Stable clones were selected in a complete medium supplemented with 500 µg/ml of G418 for two weeks. The NKX3.1 and intelectin protein knockdown efficiencies were confirmed by western blotting.

Cell growth analysis:

For cell growth analysis, 1×10^5 cells were seeded in 10 cm plates in duplicates supplemented with a complete RPMI medium. Two different Nkx3.1 and intelectin knockdown cell clones were used. Cells were counted at different time points: day 1, day 3, day 6 and day 8. At least two independent experiments were performed.

MTT assays:

The antiproliferative effects of intelectin were determined by MTT (ATCC) proliferation assays. Briefly, DU145 cells or shNKX3.1 LNCaP cells were seeded at a density of 10^3 cells in quadruplicate in a 96-well flat-bottomed tissue culture plate in 100 µl of complete medium. Intelectin (generous gift from Dr. Gong (Yang et al., 2006)) or control vectors (stratagene) were transiently transfected for 24 h and the transfection efficiency was confirmed by western blot analysis. At variable indicated times 10 µL of 3-[4,5-dimethylthiazolyl-2]-2,5-diphenyl-tetrazolium bromide (MTT reagent) was added to each well and incubated for 2-4 h at 37°C until purple precipitates were visible. The reaction was terminated by addition of 100 µL of detergent reagent (ATCC) in the dark for 2 h and the absorbance was recorded at 570 nm. At least two independent experiments were performed. Results are presented as Mean ± SD.

Soft agar colony formation assays:

For soft agar colony formation assay, 0.8×10^5 cells were suspended in a complete RPMI medium containing 0.3% of agarose and overlaid onto a bottom layer of solidified 0.5% agar in a complete RPMI medium. The colonies were allowed to grow for 2 weeks. The colonies (greater than 0.5 mm size) were counted under light microscope. At least two independent experiments were performed.

***In Vivo* Xenograft studies:**

Approximately, 2×10^6 cells suspended in Matrigel (BD Biosciences) at 50:50 volumes for a total volume of 400 µl/ injection site were injected subcutaneously in right and left flanks of male nude mice (8 weeks old, Jackson Lab). All the mice were monitored daily and once the tumor xenograft started growing, the tumor sizes were measured twice weekly in two dimensions (Width and Length) with calipers. Average tumor volume was calculated for each tumor site by formula $V = 0.5 \times L \times W^2$. At the termination of the experiment, mice were sacrificed; tumors were excised and weighed to record mean tumor mass. All animal protocols are followed as per approved institutional guidelines of Vanderbilt University.

Statistical analysis:

Data were analyzed by t-test and results were considered significant at $P \leq 0.05$. Results are presented as Mean ± SD.

Mathematical Model:

In collaboration with Dr. Philip Crooke from the Department of Mathematics, Vanderbilt University, we developed a mathematical model based on our previous and current observations. We primarily developed a model for two reasons; firstly to recapitulate our experimental findings and secondly, to make some predictions that can be tested experimentally in future.

Let $G_a(t)$ denote the level of active genes at time t, $G_{in}(t)$ the level of inactive genes, $Nkx(t)$ the level of Nkx3.1, $HAT(t)$ the level of histone acetyltransferase,

NkxHAT(t) the level of the Nkx3.1-HAT complex and *mRNA(t)* the level of target gene mRNA (either *intelectin* or *probasin*). The mRNA pathway is characterized by three constants: *K1*, *K2* and *K3*. We assume that the total number of genes, G_0, is conserved so that $G_a(t)+G_{in}(t) = G_0$. Using compartment analysis, we can write down individual differential equations for a compartment, e.g. the active gene compartment. Individual components in the pathway do not necessarily mean creation or consumption of the substance in the compartment. For example, mRNA dynamics are governed by $G_a(t)$ and *Nkx(t)*, but these components are not consumed in the creation of *mRNA(t)*. Using the above scheme and the diagram in Fig. 33A, we construct a system of ordinary differential equations:

$$\frac{d(NkxHAT)}{dt} = \lambda \cdot Nkx \cdot HAT \quad (1)$$

$$\frac{d(G_a)}{dt} = -K2 \cdot G_a + K1 \cdot NkxHAT \cdot G_{in} \quad (2)$$

$$\frac{d(G_{in})}{dt} = K2 \cdot G_a - K1 \cdot NkxHAT \cdot G_{in} \quad (3)$$

$$\frac{d(mRNA)}{dt} = K3 \cdot Nkx \cdot G_a \quad (4)$$

Notice that if we add the second and third differential equations, we find:

$$\frac{d(G_a)}{dt} + \frac{d(G_{in})}{dt} = 0 \Rightarrow G_a(t) + G_{in}(t) = G_0 = \text{constant}$$

(5)

This permits us to replace $G_{in}(t)$ by $G_0 - G_a(t)$. Using this expression in the differential equation for $G_a(t)$, we find

$$\frac{d(G_a)}{dt} = -K2 \cdot G_a + K1 \cdot NkxHAT \cdot (G_0 - G_a).$$

(6)

Hence, the model simplifies to the coupled system:

$$\frac{d(G_a)}{dt} = -K2 \cdot G_a + K1 \cdot NkxHAT \cdot (G_0 - G_a) \quad (7)$$

$$\frac{d(NkxHAT)}{dt} = \lambda \cdot Nkx \cdot HAT \Rightarrow NkxHAT(t) = NkxHAT(0) + \lambda \int_0^t Nkx(\tau) \cdot HAT(\tau) d\tau \quad (8)$$

where λ is a constant that determines the rate of formation of the Nkx3.1-HAT complex, *HAT(t)* is a prescribed function and *Nkx(t)* an experimentally determined function (Magee et al., 2003), assuming that Nkx3.1 mRNA levels reflect protein levels. The outcome variable for the model is *mRNA(t)*. This can be computed by integrating the differential equation for *mRNA(t)*. In particular,

$$mRNA(t) = mRNA(0) + K3 \int_0^t Nkx(\tau) \cdot G_a(\tau) d\tau.$$

(9)

We will take *mRNA*(0) = 0. The *mRNA*(*t*) represents target gene levels (intelectin or probasin). The dynamics of the model are dependent on the ratio of initial conditions, $\frac{Ga(0)}{Gin(0)}$, which reflects target gene chromatin accessibility and introduces a stochastic component into the model, and the functions *Nkx(t)* and *HAT(t)*. For the simulations in this paper, we have assumed that $HAT(t) \equiv \text{constant}, HAT(0) \text{and } NkxHAT(0) = 0.$

For modeling the effects of TSA, the kinetic parameter and the chromatin accessibility represented by the initial active/inactive gene ratio $R_0 = \frac{G_a(0)}{Gi_n(0)}$ are

assumed to depend on the TSA level, which we assume is constant over the course of the experiment. Our mathematical formulation for the effect of TSA (suitably normalized to be dimensionless) on *K2* is given by the relationship:

$$K2 = \alpha(1 - \mu(TSA)\sigma(R_0)) \tag{10}$$

where α is a constant, $\mu(\mathrm{TSA}) = \dfrac{\mathrm{TSA}}{\mathrm{K}_\mu + \mathrm{TSA}}$ and $\sigma(\mathrm{R}) = 1 - \dfrac{\mathrm{R}}{\mathrm{K}_\sigma + \mathrm{R}}$.

In the simulations, we have assumed that TSA and R_0 are fixed in time. The constants K_μ and K_σ were chosen to fit the experimental data. In our model, the rate constants for intelectin and probasin were kept the same, although in theory they could be different. Remarkably, differentiating between probasin and intelectin by the initial active/inactive gene ratio (i.e. chromatin accessibility) alone, keeping all other parameters constant, was sufficient to simulate experimental findings. All simulations were performed with *Mathematica* (Wolfram Research, Inc., Champaign, IL).

CHAPTER III

DETERMINATION OF SUITABLE HOUSEKEEPING GENE FOR QRT-PCR ANALYSIS FOLLOWING TRICHOSTATIN A TREATMENT

Introduction:

Assessment of gene expression by RT-PCR is based on comparison with internal standards, so called housekeeping genes. The use of such genes as internal control relies on the fact that ideally they should exhibit a constant basal level of expression which is consistent, non-regulated and independent of the cell cycle (Selvey et al., 2001). 18S ribosomal RNA (rRNA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA (mRNA) and β actin mRNA are commonly used as such internal standards. However, it has now become clear that the expression of these genes can be affected by various factors like developmental and differentiation status, cell-cycle phase, pathological conditions and drug treatments (Matyas et al., 1999; Wu and Rees, 2000; Yamada et al., 1997). GAPDH is a glycolytic enzyme, encoded by a single gene and it has the advantage of being highly conserved in different species (Bhatia et al., 1994; Fort et al., 1985). β actin mRNA and 18S rRNA are constitutively expressed in eukaryotic cells and their expression is thought to remain constant even during cell growth and during different phases of the cell cycle (Edwards and Denhardt, 1985). However, some studies have indicated increased expression of GAPDH and β actin mRNAs in carcinomas, in regenerating tissues after resection and during postnatal development (Calvo et al., 1991; Cohen et al., 1991). Therefore, 18S rRNA is widely used as a housekeeping gene. However, no one single housekeeping gene is perfect under all experimental conditions and

therefore, it is necessary to characterize the suitability of various housekeeping genes to serve as internal controls under particular experimental conditions (Zhong and Simons, 1999).

An emerging area of research in cancer involves the study of epigenetic aberrations and their contribution to malignant transformation and progression of cancer. DNA methylation and histone modifications (such as acetylation, methylation, phosphorylation and ubiquitination) by specific chromatin-modifying enzymes play essential roles in both tumor initiation and progression (Li et al., 2005). Among all of these histone modifications acetylation is arguably the best-studied modification. The actual levels of acetylation of the core histones result from steady state balance between the opposing activities of histone acetyl-transferases (HATs) and histone deacetylases (HDACs) (Hake et al., 2004). Over the years many different types of HDAC inhibitors (HDACi) have been developed, ranging from complicated structures of bacterial or fungal origin [trichostatin A (TSA), trapoxin] to the very simple butyrate. HDACi are capable of inhibiting HDACs with varying efficiencies (at nanomolar to millimolar concentrations) leading to hyperacetylation of histones followed by transcriptional activation of certain genes (de Ruijter et al., 2003). TSA (at nanomolar concentrations) is the most commonly used HDACi. Furthermore, modulation of histone acetylation is currently being explored as a therapeutic strategy in treatment of cancer. Specifically, inhibition of histone deacetylases by trichostatin A (TSA) has been shown to prevent tumorigenesis and metastasis (Ailenberg and Silverman, 2002). We have been interested in studying the role of chromatin state and histone acetylation on stochastic, dosage-sensitive gene regulation by Nkx3.1 in

prostate cancer. Nkx3.1 is a homeodomain-containing transcription factor and candidate tumor suppressor gene whose deletion in mice leads to the formation of prostatic intraepithelial neoplasia (PIN) (Abdulkadir et al., 2002). Loss of Nkx3.1 protein expression is common in human prostate carcinomas and prostatic intraepithelial neoplasia (PIN) lesions and correlates with tumor progression. Further, in the prostate, tumor initiation is often linked to loss of heterozygosity at the Nkx3.1 locus and microarray analysis has identified Nkx3.1 target genes, some of which show exquisite dosage sensitivity (Magee et al., 2003). The number of Nkx3.1 alleles determines the relative probabilities of stochastic activation or inactivation of a given target gene (Magee et al., 2003). In order to study the stochastic and dosage-sensitive expression of these genes we needed an accurate internal standard following TSA treatment. Here we report the use of ribosomal highly basic 23-kDa protein (rb 23-kDa, RPL13A) as an adequate internal standard following TSA treatment.

Results:

Effects of TSA on histone acetylation in mouse prostate explants

Mouse prostate explants were treated with TSA (500 ng/ml) or vehicle for 8 hours and tissue extracts were used for western blot analysis. We observed increased levels of acetylated histone H3 in TSA treated samples as compared to vehicle (ethanol) treated samples (Figure 23). Our western blot findings confirmed that trichostatin A (TSA) treatment was effective in our experimental system.

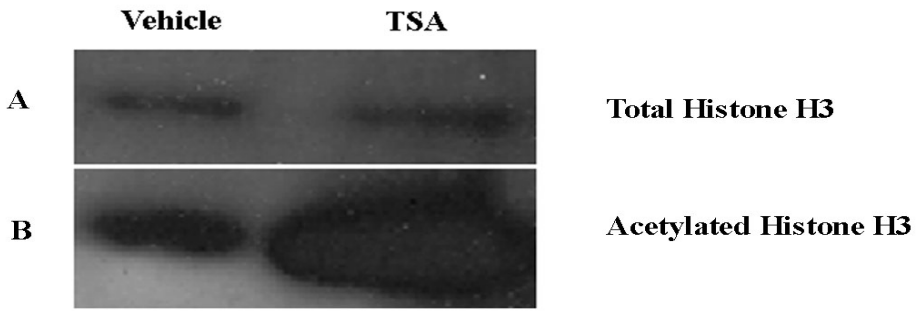


Figure 23. Western blot analysis for acetylated and total histone H3 using mouse prostate explants: Mouse prostate explants were treated with either a vehicle (ethanol) or trichostatin A (TSA) at a concentration of 500 ng/ml for 8 hours. Proteins were extracted, electrophoresed and blotted for acetylated H3 and total histone H3 levels. A) Effect of trichostatin A (TSA) on the levels of total histone H3 B) Effect of trichostatin A (TSA) on the levels of acetylated histone H3. *[Mogal et al, 2006, Mole Cell Probes]*

RT-PCR quantitation of housekeeping genes following trichostatin A (TSA) treatment in mouse prostate tissue and human prostate cancer (LNCaP) cell line

In mouse prostate tissue explants, 18S rRNA ($P < 0.002$) and β actin mRNA ($p < 0.01$) levels were significantly reduced in TSA treated tissue samples as compared to the vehicle treated samples (Figure 24). However, RPL13A and GAPDH mRNA levels remained unaffected and showed constant expression in both the vehicle as well as TSA treated samples.

Next to examine the effect of TSA on the expression of housekeeping genes in a cell line we used human prostate cancer cell line (LNCaP). In LNCaP cells, at a concentration of 100 ng/ml TSA, 18S rRNA ($P < 0.002$) and β actin mRNA ($p < 0.01$) levels were significantly up-regulated in TSA treated samples (Figure 25A). RPL13A and GAPDH mRNA levels remained unaffected and showed constant expression in both the vehicle as well as TSA treated samples. Further in LNCaP cells, at a concentration of 200 ng/ml TSA, GAPDH mRNA levels were most significantly reduced ($P <0.001$) followed by 18S rRNA levels ($P <0.01$) and β actin mRNA levels (Figure 25B). However, RPL13A mRNA levels remained unaffected at higher concentrations of TSA treatment as well and showed constant mRNA level expression.

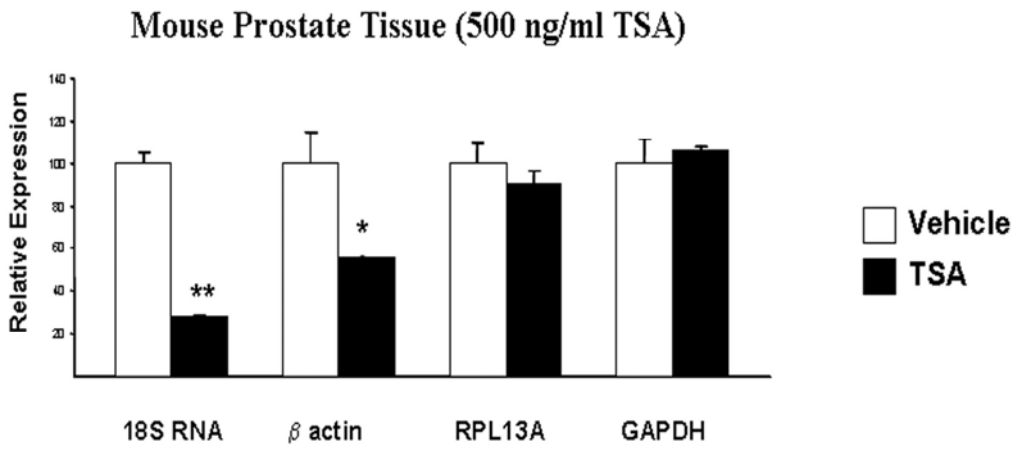


Figure 24. Quantitative RT-PCR analysis of housekeeping genes following trichostatin A (TSA) treatment in mouse prostate tissue: 18S rRNA ($P < 0.002$) and β actin mRNA ($P < 0.01$) levels were significantly down-regulated in TSA treated tissue samples as compared to the vehicle treated samples. However, RPL13A and GAPDH mRNA levels remained unaffected. The data here shown represents the average of three different experiments performed in triplicate using same conditions. (**$P < 0.005$, *$P < 0.05$) *[Mogal et al, 2006, Mole Cell Probes]*

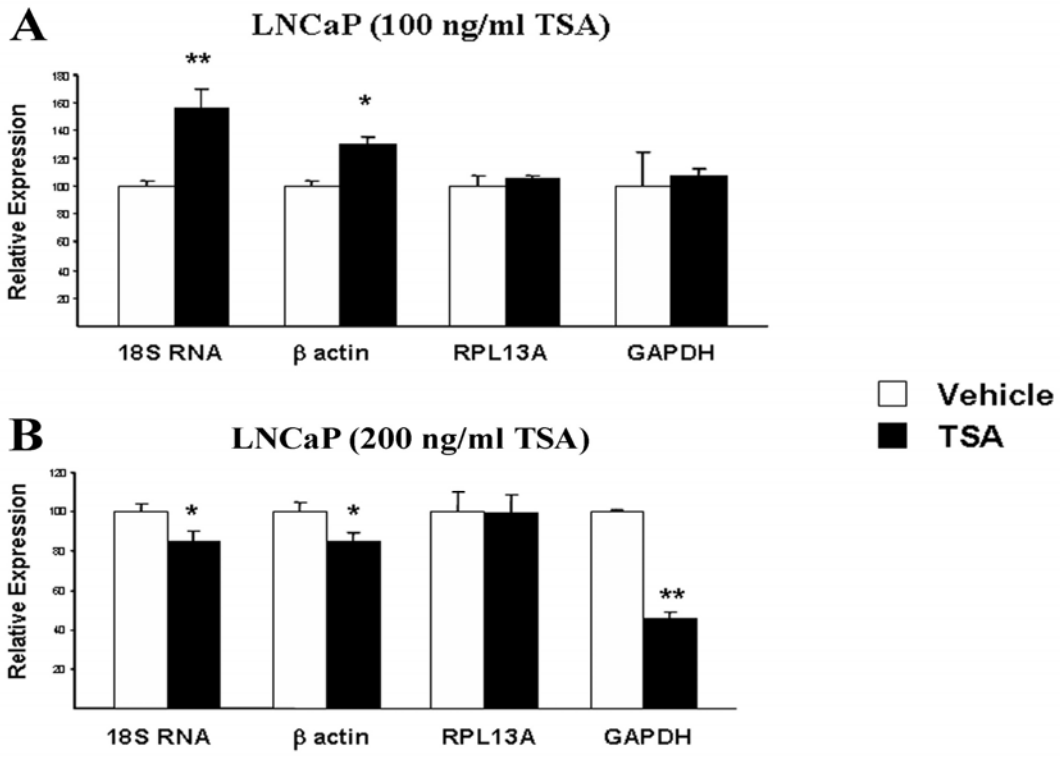


Figure 25. Quantitative RT-PCR analysis of housekeeping genes following trichostatin A (TSA) treatment in human prostate cancer (LNCaP) cell line. *A)* LNCaP (100 ng/ml TSA): 18S rRNA ($P < 0.002$) and β actin mRNA ($P < 0.01$) levels were significantly up-regulated in TSA treated samples as compared to the vehicle treated samples. However, RPL13A and GAPDH mRNA levels remained unaffected. ***B)* LNCaP (200 ng/ml TSA):** GAPDH mRNA ($P < 0.001$) and 18S rRNA ($P < 0.01$) levels were significantly down-regulated in TSA treated samples as compared to the vehicle treated samples. However, RPL13A and mRNA levels remained unaffected. The data here shown represents the average of two different experiments performed in triplicate using same conditions. (**$P < 0.005$, *$P < 0.05$) *[Mogal et al, 2006, Mole Cell Probes]*

RT-PCR quantitation of housekeeping genes following trichostatin A (TSA) treatment in human breast cancer (T-47D and ZR-75-1) cell lines

Further in order to extend our investigation in non-prostate cells we used two different human breast cancer cell lines (T-47D and ZR-75-1). In T-47D cells, at a concentration of 100 ng/ml TSA, 18S rRNA ($P < 0.005$), β actin mRNA ($p < 0.003$) and GAPDH mRNA ($P < 0.01$) levels were significantly reduced in TSA treated samples (Figure 26A). RPL13A mRNA levels remained unaffected. Further at a concentration of 200 ng/ml TSA, β actin mRNA ($p < 0.005$) and GAPDH mRNA ($P < 0.003$) levels were significantly reduced, however 18S rRNA and RPL13A mRNA levels remained unaffected (Figure 26B).

In ZR-75-1 cells, at 100ng/ml TSA, 18S rRNA ($P < 0.001$), β actin mRNA ($p < 0.001$) and GAPDH mRNA ($P < 0.01$) levels were significantly up-regulated in TSA treated samples, again RPL13A showed constant expression (Figure 26C). Similarly, at a concentration of 200 ng/ml TSA, 18S rRNA ($P < 0.05$), and GAPDH mRNA ($P < 0.04$) levels were significantly altered in TSA treated samples, however RPL13A mRNA and β actin mRNA showed constant expression (Figure 26D).

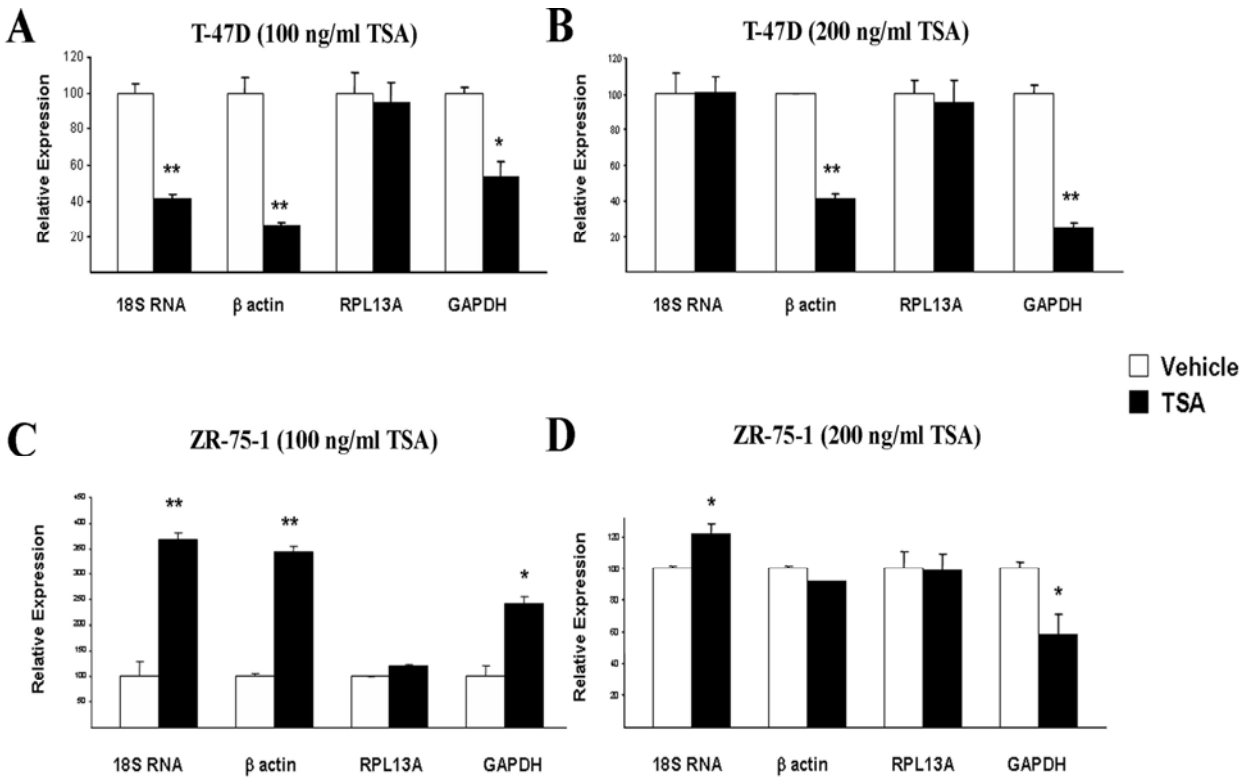


Figure 26. Quantitative RT-PCR analysis of housekeeping genes following trichostatin A (TSA) treatment in human breast cancer (T-47D and ZR-75-1) cell lines. ***A)*** **T-47D (100 ng/ml TSA)**: 18S rRNA ($P < 0.005$), β actin mRNA ($p < 0.003$) and GAPDH mRNA ($P < 0.01$) levels were significantly reduced in TSA treated samples as compared to the vehicle treated samples. However, RPL13A mRNA levels remained unaffected. ***B)*** **T-47D (200 ng/ml TSA):** β actin mRNA ($p < 0.005$) and GAPDH mRNA ($P < 0.003$) levels were significantly reduced in TSA treated samples as compared to the vehicle treated samples. However 18S rRNA and RPL13A mRNA levels remained unaffected. ***C)*** **ZR-75-1 (100 ng/ml TSA):** 18S rRNA ($P < 0.002$), β actin mRNA ($p < 0.001$) and GAPDH mRNA ($P < 0.01$) levels were significantly up-regulated in TSA treated samples, on the other hand RPL13A mRNA remained unaffected and showed constant expression. ***D)*** **ZR-75-1 (200 ng/ml TSA):** 18S rRNA ($P < 0.05$), and GAPDH mRNA ($P < 0.04$) levels were significantly altered in TSA treated samples, however RPL13A mRNA and β actin mRNA showed constant expression. The data here shown represents the average of two different experiments performed in triplicate using same conditions. (**$P < 0.005$, *$P < 0.05$) *[Mogal et al, 2006, Mole Cell Probes]*

Table 9. Quantitative RT-PCR Primer Sequences

Gene	Species	Primer Sequences
18S rRNA	Mouse / Human	5'-CGCCGCTAGAGGTGAAATTCT-3' 5'-CGAACCTCCGACTTTCGTTCT-3'
β actin	Mouse Human	5'-ACCAGTTCGCCATGGATGAC-3' 5'-TGCCGGAGCCGTTGTC-3' 5'-CCAGCTCACCATGGATGATG-3' 5'-ATGCCGGAGCCGTTGTC-3'
RPL13A	Mouse Human	5'-GAGGTCGGGTGGAAGTACCA-3' 5'-TGCATCTTGGCCTTTTCCTT-3' 5'-CATCGTGGCTAAACAGGTACTG-3' 5'-GCACGACCTTGAGGGCAGCC-3'
GAPDH	Mouse Human	5'-CATGGCCTTCCGTGTTCCTA-3' 5'-GCGGCACGTCAGATCCA-3' 5'-CCCATGTTCGTCATGGGTGT-3' 5'-TGGTCATGAGTCCTTCCACGATA-3'

Discussion and conclusions:

The expression of housekeeping genes such as 18S rRNA, β actin mRNA and GAPDH mRNA ideally should remain constant under all experimental conditions, in all normal as well as pathological states and during cell growth and various phases of the cell cycle (Edwards and Denhardt, 1985). However, some reports suggested that these genes may either be up-regulated or down-regulated depending on the circumstances (Matyas et al., 1999; Wu and Rees, 2000; Yamada et al., 1997). To explore whether expression of these housekeeping genes is modulated under the effect of the commonly used histone deacetylase inhibitor (HDACi) trichostatin A (TSA), we compared the expression level of these housekeeping genes (18S rRNA, β actin and GAPDH) along with the novel ribosomal highly basic 23-kDa protein (rb 23-kDa, RPL13A). We examined the expression pattern of all four housekeeping genes in mouse prostate tissue, human prostate cancer (LNCaP) cell line and human breast cancer (T-47D and ZR-75-1) cell lines following vehicle (Ethanol) or trichostatin A (TSA) treatment. To our knowledge, this is the first report of testing housekeeping genes as RNA internal standards under the effect of trichostatin A (TSA).

Our results showed that the commonly used housekeeping genes such as 18S rRNA, β actin mRNA, GAPDH mRNA levels were significantly altered in the TSA treated samples as compared to the vehicle treated samples. It is interesting that TSA can either up-regulate (e.g. LNCaP and ZR-75-1 at 100 ng/ml TSA) or down-regulate (e.g. prostate explants, T-47D, LNCaP and ZR-75-1 at 200 ng/ml TSA) expression of

these genes. This could be due to an 'optimal dose response effect' of TSA (100 ng/ml Vs 200 ng/ml) in the particular tissue or cell line. Regardless of the underlying reason, these findings underscore the importance of testing the suitability of housekeeping genes in different experimental systems.

The levels of ribosomal RNA, which make up ~ 80% of total RNA, are thought to be less likely to vary under conditions that affect the expression of mRNAs, since they are transcribed by a distinct RNA polymerase. 18S rRNA has been described as a preferable internal control and most widely used as a housekeeping gene (Thellin et al., 1999). It is expressed at constant levels in normal liver versus liver metastasis (Blanquicett et al., 2002). It is also stably expressed in various cancer tissues which may result from its lack of involvement in cellular metabolism (Finnegan et al., 1993). However, other studies have shown that 18S rRNA is not a suitable control as it can be regulated (Solanas et al., 2001) and its synthesis is independent from synthesis of mRNA (Radonic et al., 2004). Our study also showed that 18S rRNA can be affected by trichostatin A (TSA) at least in prostate and breast tissue and probably in other tissues as well, hence we would not recommend 18S rRNA as a suitable housekeeping gene under the effects of histone deacetylase inhibitors (HDACi) such as trichostatin A (TSA).

β actin mRNA remains a widely used housekeeping gene internal control in molecular biology (Bernard et al., 1999), despite the fact that many studies have reported its cell cycle dependent expression pattern and regulation in specific circumstances. Some studies have also questioned the use of β actin mRNA as

suitable internal control in RT-PCR since it does not satisfy certain basic requirements for application as a housekeeping gene (Serazin-Leroy et al., 1998; Yamada et al., 1997). Further, some studies have specifically shown that using β actin mRNA as an internal control can detrimentally affect the accuracy of RT-PCR results (Selvey et al., 2001). Similar to 18S rRNA, our study showed that β actin mRNA can also be regulated by trichostatin A (TSA) at least in prostate and breast tissue and probably in other tissues as well. Thus, we would not recommend β actin mRNA as suitable housekeeping gene with the use of trichostatin A (TSA).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a key enzyme in glycolysis, which makes it an abundant RNA species for use as a potential internal RNA standard. This housekeeping gene is constitutively expressed in many tissues. However, wide variation in GAPDH expression levels have been observed in tissues at different developmental stages (Oikarinen et al., 1991), in cells treated with insulin (Nasrin et al., 1990), dexamethasone (Oikarinen et al., 1991), mitogens (Rao et al., 1990) as well as virally transformed or oncogene-transfected fibroblasts (Bhatia et al., 1994). Different tissue types exhibited marked differences in the expression of GAPDH gene. Furthermore, within the same tissue, GAPDH expression was up-regulated in the presence of inflammation or malignant transformation (Jesnowski et al., 2002). The up-regulated expression levels of GAPDH have been previously reported in human pancreatic or colon adenocarcinoma (Schek et al., 1988). In tumor cells this could be due to an increase in glycolysis and glucose turnover. Further, GAPDH mRNA levels were up-regulated in the presence of hypoxia and by hypoxia inducible factor 1 (HIF-1) (Zhong and Simons, 1999). Moreover, GAPDH is

pathologically implicated in neurodegeneration and apoptosis (Tatton et al., 2000). Our study showed that GAPDH mRNA can also be regulated by trichostatin A (TSA) at least in prostate and breast tissue and probably in other tissues as well. Thus, we would not recommend GAPDH mRNA as a suitable housekeeping gene with the use of trichostatin A (TSA).

Lastly we studied expression of the ribosomal highly basic 23-kDa protein (rb 23-kDa, RPL13A) following trichostatin A (TSA) treatment. Previous studies have shown that the expression of the gene for the ribosomal highly basic 23-kDa protein (RPL13A) was remarkably constant between different tissue types (Jesnowski et al., 2002). Furthermore, its expression was not affected by malignant transformation or inflammation in the same tissue in contrast to GAPDH (Jesnowski et al., 2002). One research group recommended RPL13A as a standard for normalization for at least the pancreas and prostate (Jesnowski et al., 2002). In our study, the expression of this gene was very stable following TSA treatment.

The stable and non-regulated expression of housekeeping genes is critical for accurate interpretation of RT-PCR results. Thus, it is essential to choose proper housekeeping genes when normalizing RNA concentrations (Rubie et al., 2005). Our study supports the notion that no one housekeeping gene is perfect under all experimental conditions, and that it is necessary to characterize the suitability of various housekeeping genes to serve as internal controls under particular experimental conditions. In conclusion, we recommend the use of the ribosomal

highly basic 23-kDa protein (RPL13A) as a suitable standard for normalization with the use of histone deacetylase inhibitors (HDACi) such as trichostatin A (TSA).

CHAPTER IV

MECHANISM OF HAPLOINSUFFICIENT PROSTATE TUMOR SUPPRESSION AND ROLE FOR CHROMATIN ACCESSIBILITY

Introduction:

As discussed earlier, transcription factor haploinsufficiency is a predisposing factor to a wide variety of disorders including cancer (Seidman and Seidman, 2002), but the precise mechanisms involved have remained elusive. We have established a mouse model of prostate tumor initiation due to haploinsufficiency of the homeodomain transcription factor Nkx3.1 (Abdulkadir et al., 2002; Magee et al., 2003), which provided us with a unique opportunity for deciphering the mechanisms of transcription factor haploinsufficiency. In *Nkx3.1*-mutant mice, loss of even a single allele of the tumor suppressor Nkx3.1 stochastically inactivates the expression of a class of dosage-sensitive target genes (Magee et al., 2003). Interestingly, some Nkx3.1 target genes, such as intelectin/omentin, were exquisitely sensitive to a reduction in Nkx3.1 dose, while others such as probasin were only slightly affected (Magee et al., 2003). These results are consistent with a model of haploinsufficient tumor suppression in which reduction in Nkx3.1 dosage increases the probability of complete loss of expression of select target genes in prostate epithelial cells.

Here, we show that dosage-sensitivity of Nkx3.1 target genes is largely dictated by the differential histone H3/H4 acetylation states and Nkx3.1 occupancy at the target gene loci. In *Nkx3.1*$^{+/-}$ prostates, the dosage-insensitive gene *probasin* was

hyperacetylated in its regulatory region, bound by Nkx3.1 and expressed. In contrast, the dosage-sensitive target *intelectin* was hypoacetylated, not bound by Nkx3.1 and silenced. Importantly, inducing histone acetylation with the histone deacetylase inhibitor, Trichostatin-A, allowed binding of Nkx3.1 and reactivation of dosage-sensitive target gene expression in *Nkx3.1*$^{+/-}$ cells. We incorporated our findings into a mathematical model of Nkx3.1 gene regulation that predicts the involvement of Nkx3.1 in chromatin remodeling. We subsequently showed that Nkx3.1 is indeed involved in setting target gene histone acetylation levels through interactions with the histone acetyltransferase P/CAF (p300/CBP-associated factor).

In conclusion, our results reveal how the interplay between transcription factor dosage and chromatin affects target gene expression in tumor initiation. We demonstrate the importance of chromatin accessibility in dosage-sensitive and stochastic gene regulation and provide a mechanistic basis for haploinsufficient tumor suppression by Nkx3.1.

Results and Discussion:

Differential histone H3/H4 acetylation at the dosage-sensitive and dosage-insensitive target gene loci

We reasoned that Nkx3.1 dosage-sensitive and stochastic gene regulation may be mediated by the differential chromatin accessibilities of target gene loci. To investigate this, we examined the histone H3/H4 acetylation states of the promoter regions of two representative target genes in mouse prostate: intelectin and probasin (Magee et al., 2003). Probasin is a prostate specific differentiation marker (Kasper and Matusik, 2000) that is insensitive to Nkx3.1 dosage, while intelectin is a D-galactosyl specific lectin that is also known as omentin, an adipocytokine (Tsuji et al., 2001) (Schaffler et al., 2005). Intelectin shows exquisite sensitivity to Nkx3.1 dosage, with its expression in *Nkx3.1*$^{+/-}$ cells approximating the levels seen in *Nkx3.1*$^{-/-}$ cells (Magee et al., 2003). We used chromatin immunoprecipitation assays (ChIP) to profile the histone H3 and H4 acetylation patterns in the 2-kb regions of the *intelectin* and *probasin* promoters in *Nkx3.1*-mutant mouse prostate tissues (Figure 27A,B). The *intelectin* promoter showed higher levels of histone H3 and H4 acetylation in *Nkx3.1*$^{+/+}$ mice compared to *Nkx3.1*$^{+/-}$ or *Nkx3.1*$^{-/-}$ animals. In particular, we observed the maximum level of acetylation in a region (Int5) that was subsequently found to contain a consensus Nkx3.1 binding site (TAAGTG) (Steadman et al., 2000) by sequence analysis (Figure 27A). By contrast, on the *probasin* promoter, H3 and H4 acetylation levels were high in *Nkx3.1*$^{+/+}$ and *Nkx3.1*$^{+/-}$ mice and low in *Nkx3.1*$^{-/-}$ mice (Figure 27B). Thus expression of intelectin and probasin correlated with the

overall histone H3/H4 acetylation states of their promoter regions in mice of various genotypes.

Differential Nkx3.1 occupancy at the dosage-sensitive and dosage-insensitive target gene loci

We examined *in vivo* binding of Nkx3.1 at the *intelectin* and *probasin* promoters in *Nkx3.1*-mutant mice by ChIP. Nkx3.1 was bound at the Int5 region of the *intelectin* promoter in *Nkx3.1*$^{+/+}$ but not in *Nkx3.1*$^{+/-}$ mice, while Nkx3.1 binding to the Pbn1 region of the *probasin* promoter (containing a consensus Nkx3.1 site) was observed in both *Nkx3.1*$^{+/+}$ and *Nkx3.1*$^{+/-}$ mice (Figure 28). We used a mouse androgen receptor promoter region containing a consensus Nkx3.1 binding site (AR) as a positive control (Lei et al., 2006) for Nkx3.1 ChIP while Int2 and Pbn4 regions (which do not contain Nkx3.1 consensus) served as negative controls. Thus expression of intelectin and probasin correlated with the Nkx3.1 occupancy in mice of various genotypes.

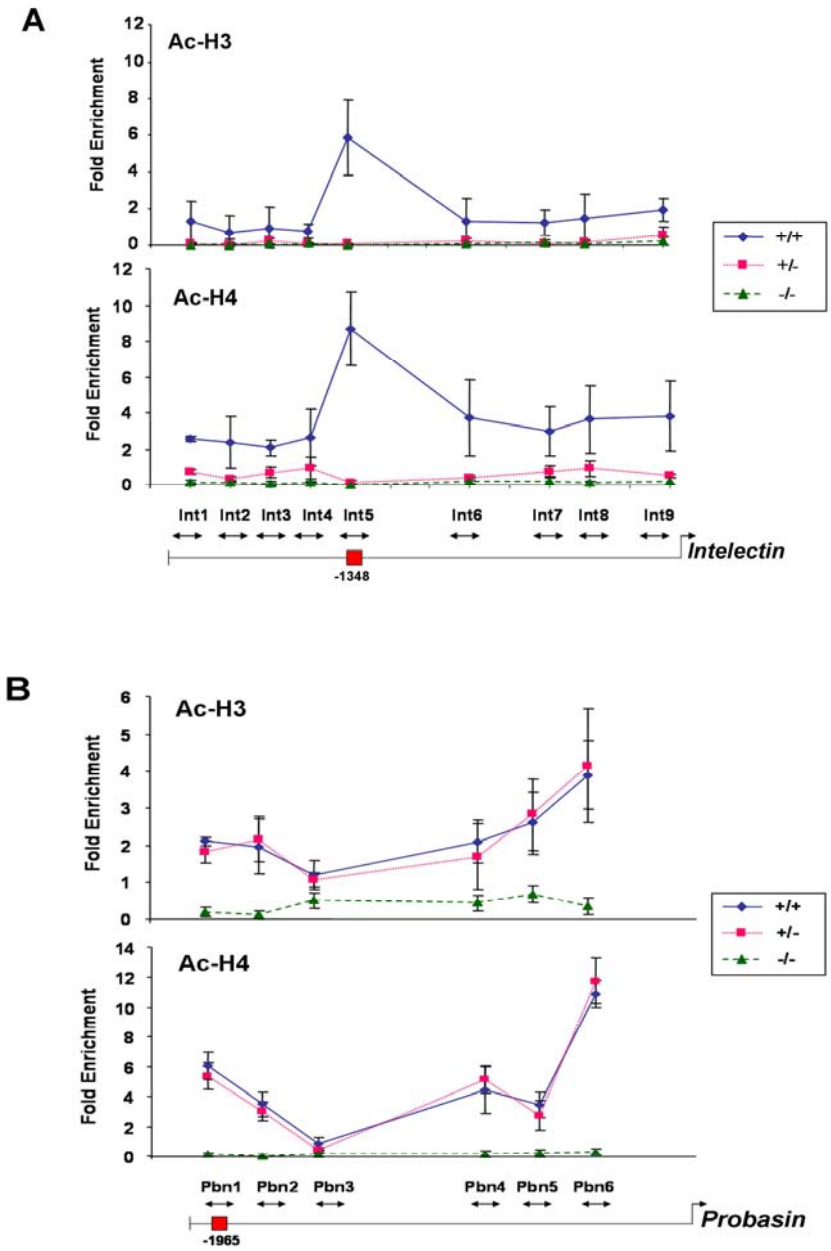


Figure 27. Dosage-sensitive and dosage-insensitive Nkx3.1 target gene loci show differential histone H3/H4 acetylation states: (A, B) ChIP analysis on prostate tissues from *Nkx3.1*-mutant mice show enrichment of acetylated histone H3 (Ac-H3) and acetylated histone H4 (Ac-H4) in the 2-Kb region of the *intelectin* and *probasin* promoters respectively. Schematics of the promoters with locations of quantitative PCR primers and putative Nkx3.1 binding sites (TAAGTG- red boxes) are shown below. ChIP values are represented as fold enrichments and calculated as [IP Ab/ Input Ab] / [IP IgG/ Input IgG]. Results are presented as Mean ± SD (N=6 per genotype).

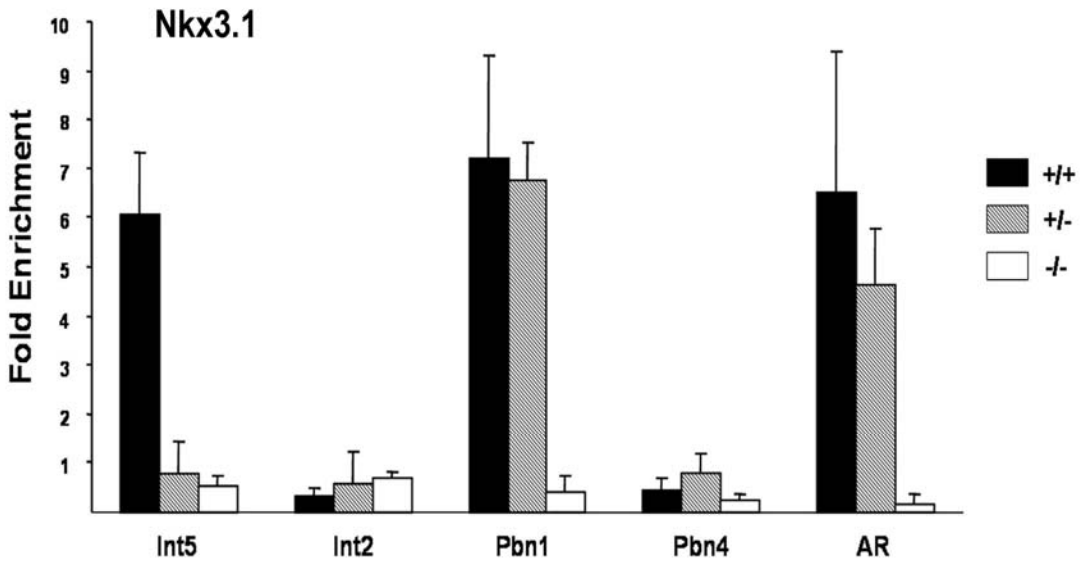


Figure 28. Dosage-sensitive and dosage-insensitive Nkx3.1 target gene loci show differential Nkx3.1 occupancy *in vivo*: *In vivo* binding of Nkx3.1 to consensus sites on the *intelectin* (Int5) and *probasin* (Pbn1) promoters in *Nkx3.1*-mutant mouse prostates by ChIP analysis. A mouse androgen receptor promoter region containing a consensus Nkx3.1 binding site (AR) was used as a positive control while Int2 and Pbn4 regions served as negative controls. Results are presented as Mean ± SD (N=6 per genotype).

Induction of histone hyperacetylation with a HDAC inhibitor, TSA in *Nkx3.1*$^{+/-}$ mouse prostates

Our results thus far indicate that in *Nkx3.1*$^{+/-}$ prostate cells, the *probasin* promoter is hyperacetylated at histones H3/H4, resulting in an "open" chromatin configuration that allows Nkx3.1 binding and activation of gene expression. The *intelectin* promoter on the other hand is in a hypoacetylated, "closed" chromatin state which precludes Nkx3.1 binding and gene activation. If so, then increasing chromatin accessibility by inducing histone H3/H4 hyperacetylation might allow Nkx3.1 binding to the *intelectin* promoter and gene activation in *Nkx3.1* heterozygous cells. To ascertain this, we treated mouse prostate tissue explants with the histone deacetylase inhibitor Trichostatin-A (TSA) (Yoshida et al., 1995). The efficacy of our treatment regimen was evident from results of western blot analyses for acetylated histone H3 (Figure 29 upper panel). TSA treatment did not have a discernible effect on Nkx3.1 protein expression (Figure 29 lower panel).

ChIP analysis of the *intelectin* promoter indicates that TSA treatment led to a significant increase in H3/H4 acetylation in *Nkx3.1*$^{+/-}$ mouse prostates, particularly in the Int5 region (Figure 30B and 30D). Notably, the levels of histone H3 acetylation observed at the Int5 region in *Nkx3.1*$^{+/-}$ mice after TSA treatment approximate the baseline levels seen in *Nkx3.1*$^{+/+}$ mice (Figure 30B). By contrast, TSA treatment had modest or minimal effects on histone H3/H4 acetylation at the *intelectin* promoter in *Nkx3.1*$^{+/+}$ and *Nkx3.1*$^{-/-}$ mice or the *probasin* promoter in all genotypes (Figure 30B, 30C and Figure 30E). These results support the notion that acetylation is a dynamic process and there may be a limit to the maximum acetylation achievable at any

promoter. They also suggest that Nkx3.1 may be involved in setting the histone H3/H4 acetylation states of its target genes.

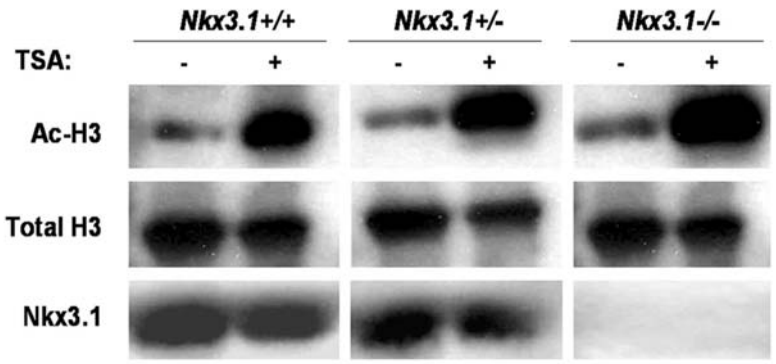


Figure 29. The histone deacetylase inhibitor, trichostatin A (TSA) induces histone acetylation in prostate explants: Western blot analyses with *Nkx3.1*-mutant mice prostate tissue explants following TSA treatment show significant increase in the Ac-H3 protein levels (upper panel). Total histone H3 was used as a loading control (middle panel). Notably, TSA treatment did not affect Nkx3.1 protein expression (lower panel).

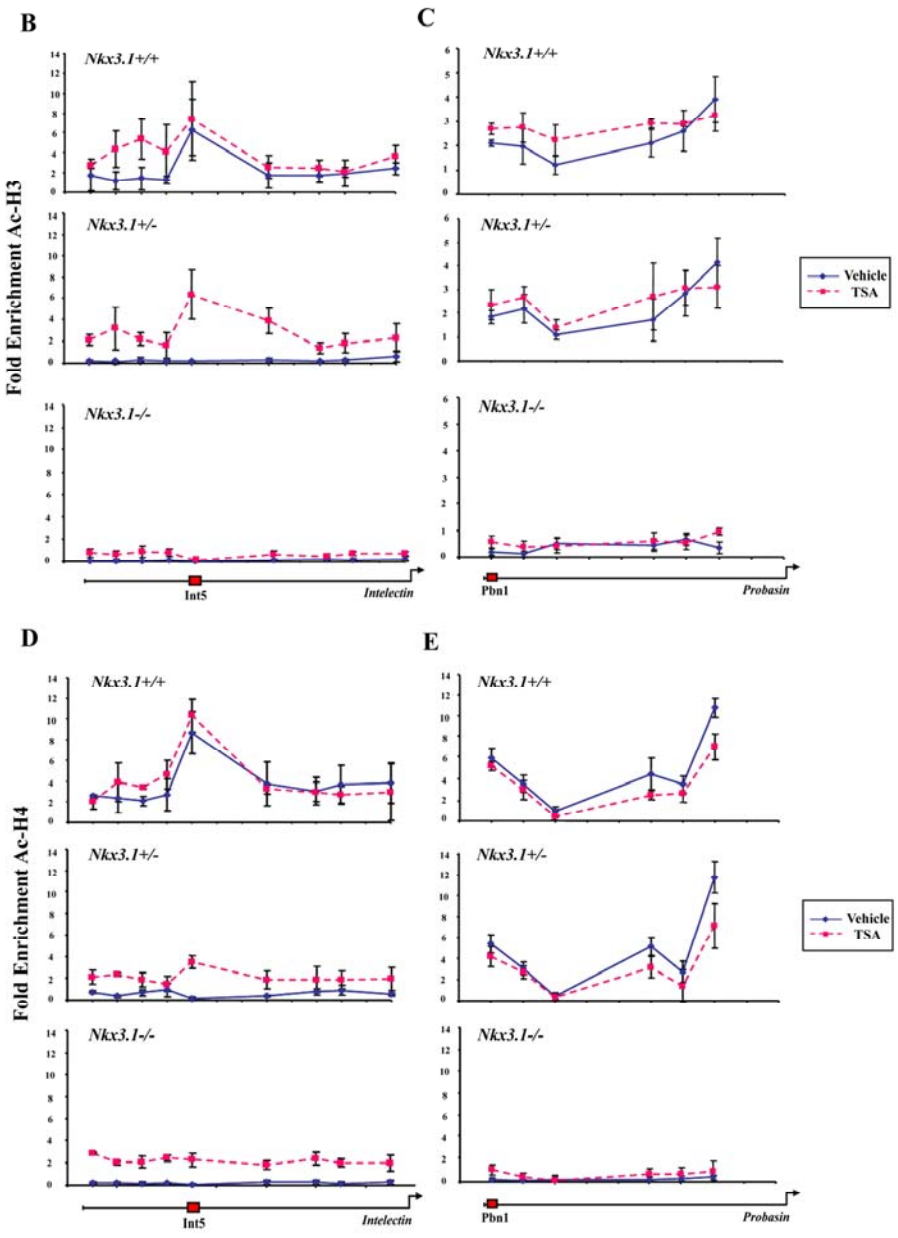


Figure 30. The histone deacetylase inhibitor, trichostatin A (TSA) induces histone acetylation at the dosage-sensitive gene loci in *Nkx3.1*$^{+/-}$ mouse prostate explants: (B, C) Prostate tissue ChIP assays for Ac-H3 on the *intelectin* and *probasin* promoters after TSA treatment. Note the significant increase in Ac-H3 enrichment on the *intelectin* promoter particularly in the region of the Nkx3.1 consensus site (Int5) in *Nkx3.1*$^{+/-}$ mice. (D, E) Prostate tissue ChIP assays for Ac-H4 on the *intelectin* and *probasin* promoters after TSA treatment demonstrate the significant increase in Ac-H4 enrichment on the *intelectin* promoter particularly in the region Int5 in *Nkx3.1*$^{+/-}$ mice. Results are presented as Mean ± SD (N=6 per genotype).

Induction of histone hyperacetylation with TSA allows Nkx3.1 binding and reactivation of dosage-sensitive target gene expression in *Nkx3.1*$^{+/-}$ mouse prostates

Using ChIP, we next showed that TSA treatment promotes the binding of Nkx3.1 to the Int5 region but not a control region in *Nkx3.1*$^{+/-}$ mice (Figure 31). Importantly, TSA treatment also significantly induced intelectin mRNA expression in *Nkx3.1*$^{+/-}$ mice as assessed by quantitative RT-PCR (Figure 32 upper panel). The expression of another dosage-sensitive Nkx3.1 target gene, Pdzk1, was also induced by TSA treatment in *Nkx3.1*$^{+/-}$ mice (Figure 32 upper panel), while the expression of the dosage-insensitive genes probasin and Sel1L (Magee et al., 2003) remained largely unaffected (Figure 32 lower panel). Notably, none of these genes was reactivated by TSA treatment in *Nkx3.1*$^{-/-}$ mice, emphasizing the requirement for Nkx3.1 in gene induction. We note that both intelectin and Pdzk1 were induced by TSA in *Nkx3.1*$^{+/-}$ mice to levels 3 to 4 fold above their respective expression levels in wild type mice, and that TSA treatment did not lead to a further increase in the expression of any of the genes tested in *Nkx3.1*$^{+/+}$ prostates.

In addition to histone modifications, DNA methylation is another common epigenetic event important for regulating gene expression (Jones and Baylin, 2002). Hence, we investigated the possible involvement of DNA methylation in Nkx3.1 target gene regulation by using the DNA methyltransferase inhibitor, 5-azacytidine (Groudine et al., 1981). Unlike TSA, treatment with 5-azacytidine did not have consistent effects on the expression of dosage-sensitive versus dosage-insensitive target genes (Figure 32). In our prostate explant experiments, longer period of

treatment of 5-azacytidine could not be tested due to toxicity effects. Reactivation of dosage-sensitive target genes by TSA emphasizes the role of histone acetylation in the regulation of these Nkx3.1 target genes. These results point to the chromatin accessibility of target gene loci as a major determining factor in the response of target genes to reduced dosages of activating transcription factors. The ability to restore the expression of dosage-sensitive genes with HDAC inhibitors may have important therapeutic implications for disorders due to haploinsufficiency of transcription factors.

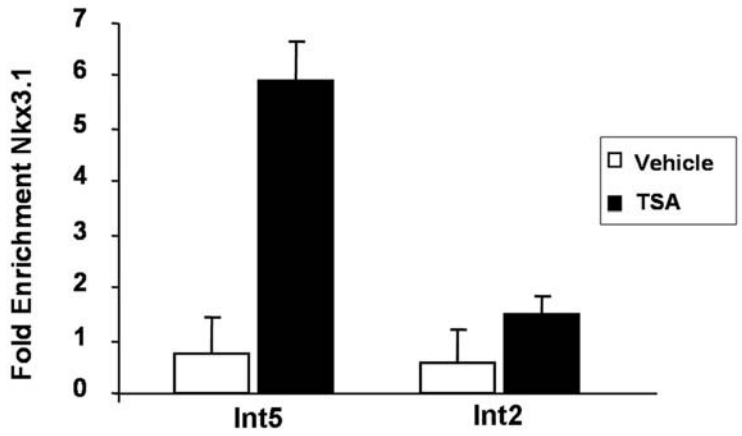


Figure 31. TSA treatment promotes Nkx3.1 binding *in vivo* in *Nkx3.1*$^{+/-}$ mouse prostate explants: ChIP assays on the *intelectin* promoter show Nkx3.1 binding to Int5 site but not Int2 control region following TSA treatment in *Nkx3.1*$^{+/-}$ mice. Results are presented as Mean ± SD from N ≥ 4.

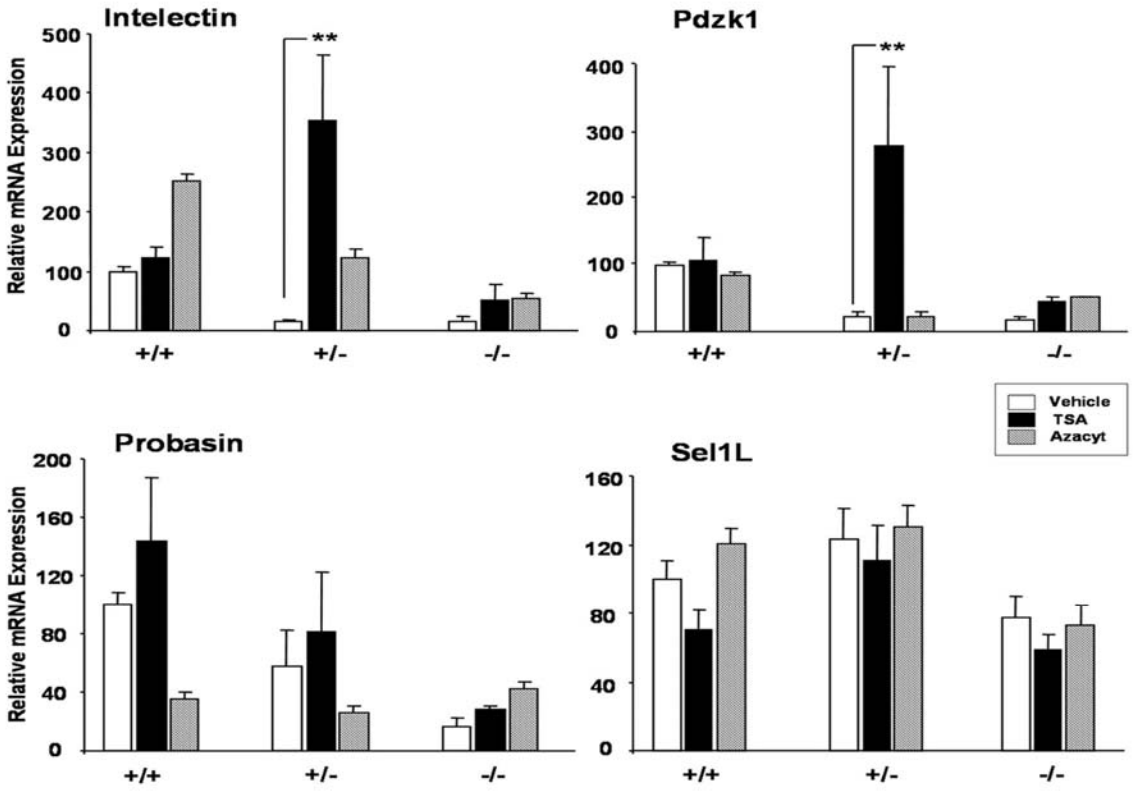


Figure 32. The HDAC inhibitor, TSA, reactivates dosage-sensitive target gene expression in *Nkx3.1*$^{+/-}$ mouse prostate explants: Quantitative RT-PCR analysis for Nkx3.1 target gene expression following TSA treatment. Dosage-sensitive mRNAs like intelectin and Pdzk1 (upper panel) were significantly up-regulated in TSA-treated *Nkx3.1*$^{+/-}$ mice prostate explants compared to dosage-insensitive targets like probasin and Sel1L (lower panel). 5-Azacytidine (Azacyt), a DNA hypo-methylating agent was used as additional control (N=3 per genotype). Results are presented as Mean ± SD. **, $p < 0.05$.

Table 10. Primers used for ChIP assays and qRT-PCR

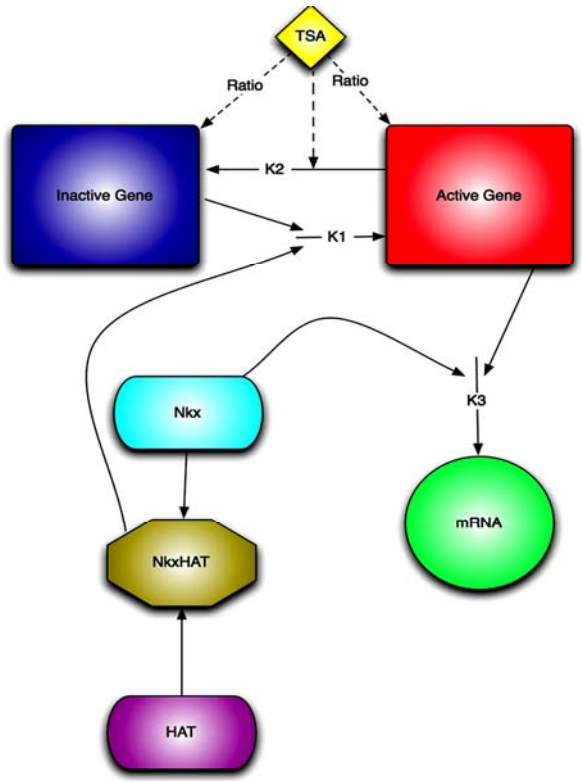
ChIP Assay Primer	Sequence
Int1	F: 5'- GGACACTATGCCCCTCCTTAG-3' R: 5'-CAGCGTTTGGATGCTGATTA-3'
Int2	F: 5'- CGAAAGGACCCAGATGTAGC-3' R: 5'-GCCCTGTGATCCATCCATTA-3'
Int3	F: 5'- CCAAGGAGCTAAAGGGATCTG-3' R: 5'-CCAATGGACCTCTCTTTCCA-3'
Int4	F: 5'-GAATTCCAGGGCCAAAAAG-3' R: 5'-TTAGCTCATTTACATTTCCAATGC-3'
Int5	F: 5'-GATTGAAGAAGATAGCCCATGC-3' R: 5'-TTGGGTTTTGAAAGTTTCTTATTTG-3'
Int6	F: 5'-AAAGGATGTCATTTCCTTCTGC-3' R: 5'-TTTCTTCCTTATCAATCCCTAAGC-3'
Int7	F: 5'-TGCAGTGCATGCCTTTAATC-3' R: 5'-CACGGAGGACTACTTTTCCAA-3'
Int8	F: 5'-CACACCCATTGTGATTGTGC-3' R: 5'-TGGTGGTGGGTTTTGAGATT-3'
Int9	F: 5'-ATTTGTCTTTTAGGGCCCACT-3' R: 5'-GGTCCTTTTATGGGGAAAGC-3'
Pbn1	F: 5'-TGGTATTTCCTACTGCCAGACA-3' R: 5'-TTATGTGCAGTCATGAGATTGTC-3'
Pbn2	F: 5'-ATGCCACTTTGAGGAATTGG-3' R: 5'-AAGGTCACTGTTTCGGAGGT-3'
Pbn3	F: 5'-TTCCTTCTGTATGTTGCATTTTC-3' R: 5'-CGCACATCTCAGGAACACAG-3'
Pbn4	F: 5'-TCAGCACAGGTTCCTGAAAA-3' R: 5'-TTGAGTTCATGGGTGTGTGG-3'
Pbn5	F: 5'-TTGTCGTGATTGACAAGCTG-3' R: 5'-TGGAACTGGAGGTTCTAGCC-3'
Pbn6	F: 5'-GCCATGTTTTTGGTCATGTG-3' R: 5'-TGGAAGAATATCAAGTCTGACCA-3'
RT-PCR Primer	**Sequence**
mIntelectin1	F: 5'-AGCGGCCAGTGCCTTGT-3' R: 5'-ACCGATGCAGTGATGTTCAGTATT-3'
mPdzk1	F: 5'-TCTGCGGAGTCCGAGCAT-3' R: 5'-GAGTGTGAGGCTGTGCTGAGAGT-3'
mSel1L	F: 5'-AGAAAACAGCCTTAACCAACTTGAG-3' R: 5'-TCCGGGAAGCAACGAATCTA-3'
mProbasin	F: 5'-CTCCTGCTCACACTGCATGTG-3' R: 5'-CCAAGGCCCGTCAATCTTC-3'
hINTELECTIN1	F: 5'-GGAGAAGGAAAGTGTTGGACTGA-3' R: 5'-GGCGTCGCCAAAATCATAGA-3'
hPDZK1	F: 5'-CCACTGGAAACCATTCACCAT-3' R: 5'-TCTGCAATAGCCGCCTGTAA-3'
hSEL1L	F: 5'-GCGTGATTCACTTTAGCCTGATTAT-3' R: 5'-TAGTCTAAATGTTACTGTGTGGTACG-3'
hPSA	F: 5'-TGAGAAACCTGAGATTAGGA-3' R: 5'-ATCTCTCTCAGATCCAGGCT-3'
hANGIOPOIETIN 2	F: 5'-GACTTCCAGAGGACGTGGAAAG-3' R: 5'-CTCATTGCCCAGCCAGTACTC-3'
hNKX3.1	F: 5'-GCAGAGACCGAGCCAGAAA-3' R: 5'-GAACTTCCTCTCCAACTCGATCA-3'

A mathematical model of gene activation by Nkx3.1

To gain further insights into the interactions between Nkx3.1 dosage and chromatin remodeling in target gene regulation, we incorporated our current results and earlier findings (Magee et al., 2003) into a mathematical model of gene activation by Nkx3.1 (Figure 33A). According to this model, a target gene can exist in an inactive/closed chromatin (hypoacetylated state) or an active/open chromatin (hyperacetylated state) competent for transcription. Due to the fact that we observed reduced histone H3/H4 acetylation in Nkx3.1 null cells and focal hyperacetylation in a region containing the Nkx3.1 binding site on the *intelectin* promoter, we assumed in the model that Nkx3.1 interacts with a histone acetyltransferase (8) and influences the active to inactive gene ratio. TSA also affects this ratio by inhibiting the transition from the active to the inactive state due to its known ability to inhibit HDACs. In addition, we assume that Nkx3.1 directly affects the rate of transcription from the active gene. Input values for levels of Nkx3.1 in *Nkx3.1*$^{+/+}$ and *Nkx3.1*$^{+/-}$ mice were obtained from experimental measurements (Magee et al., 2003). Further details of the model and its representation by a set of ordinary differential equations can be found in the materials and methods section. We find that simulations from this model for the expression of intelectin and probasin in the presence of one or two *Nkx3.1* alleles (Figure 33B and 33C) closely paralleled experimental findings for probasin and intelectin mRNA expression in a castration/testosterone replacement paradigm in *Nkx3.1*$^{+/+}$ and *Nkx3.1*$^{+/-}$ mice (Magee et al., 2003). We also modeled the effects of TSA. Simulations for the effects of TSA showed the most significant induction over baseline in the case of intelectin in the presence of one *Nkx3.1* allele (Figure 33D-G),

similar to the experimental observations for *Nkx3.1*$^{+/-}$ mice (see Figure 32), although the fold induction observed in the simulation is not as high as that observed experimentally. The mathematical relationship between gene inactivation rate constant, K2, TSA, and the initial ratio of active to inactive genes is shown in Figure 34. Overall, the modeling results closely simulated experimental observations regarding dosage-sensitive target gene regulation. We have not modeled stochastic variations in Nkx3.1 itself as done by others (Karmakar and Bose, 2006); rather, in our model the initial ratio of active to inactive genes which reflects chromatin accessibility/acetylation introduces a stochastic component into the model and is a major determinant of the dynamics of the model.

A



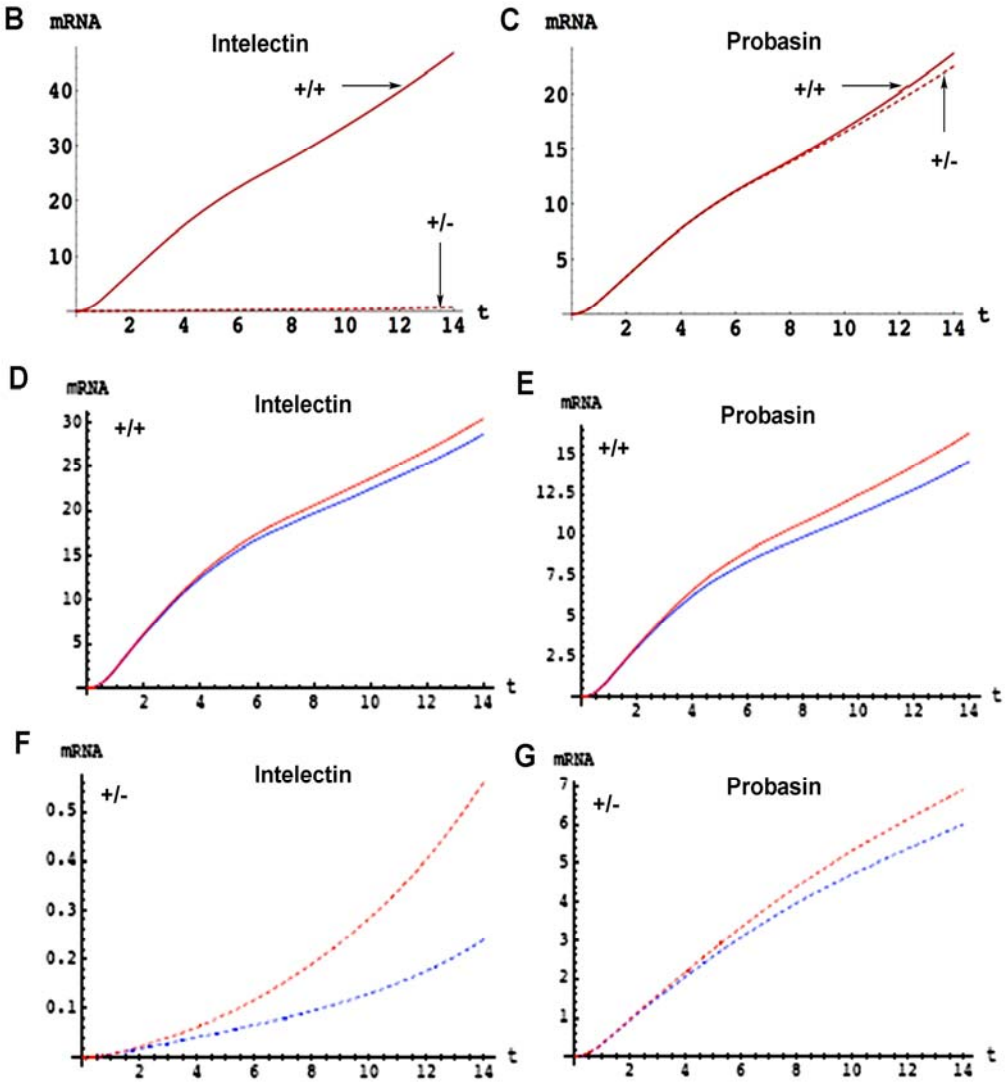


Figure 33. A mathematical model of gene activation by Nkx3.1: (A) Network depiction of gene activation by Nkx3.1. TSA is shown as inhibiting the transition from active gene (hyperacetylated, open chromatin) to inactive gene (hypoacetylated, closed chromatin). Nkx3.1 levels are derived from experimental observations (Magee at el., 2003). A histone acetyltransferase, HAT complexes with Nkx3.1 and is assumed to be constitutively synthesized. (B, C) Results of simulations showing expression levels of intelectin and probasin in *Nkx3.1*$^{+/+}$ and *Nkx3.1*$^{+/-}$ mice. (D-G) Results of simulations showing the effects of TSA on target gene expression. Red lines, +TSA; Blue lines, -TSA

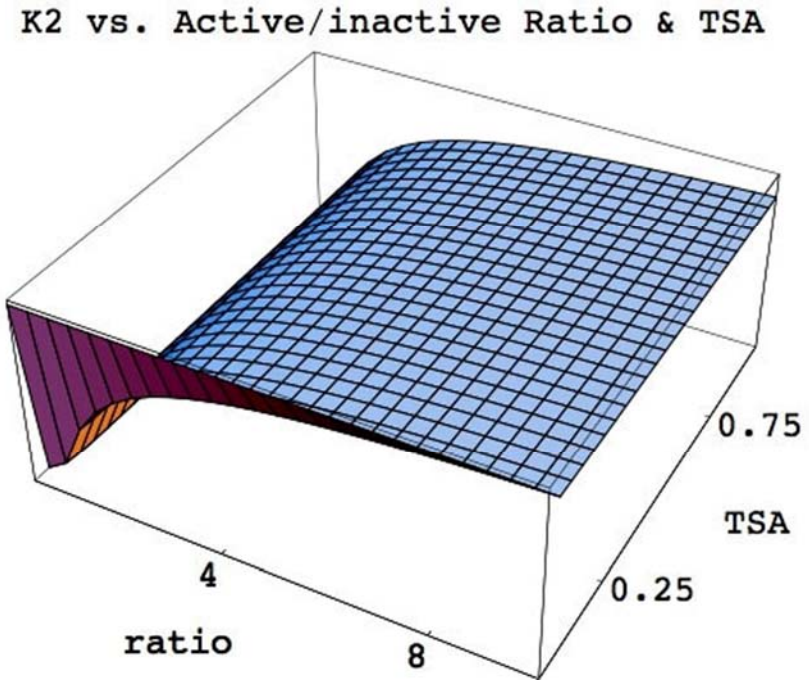


Figure 34. Graph showing effect of TSA on gene activation: The mathematical relationship between the rate constant K2 which controls the rate at which an active gene is inactivated, the level of TSA and the initial ratio of active to inactive genes, R_0. This is a plot of Equation 10 in the Methods section.

Nkx3.1 associates with, and recruits the histone acetyltransferase PCAF to chromatin

The modeling results coupled with our results showing reduced histone acetylation at the *probasin* and *intelectin* promoters in *Nkx3.1*-deficient mice prompted us to examine the possibility that Nkx3.1 is associated with histone acetyltransferase activity. Using co-immunoprecipitation analyses, we found evidence of interaction between Nkx3.1 and PCAF in human prostate cancer cells (LNCaP) and mouse prostate tissue (Figure 35).

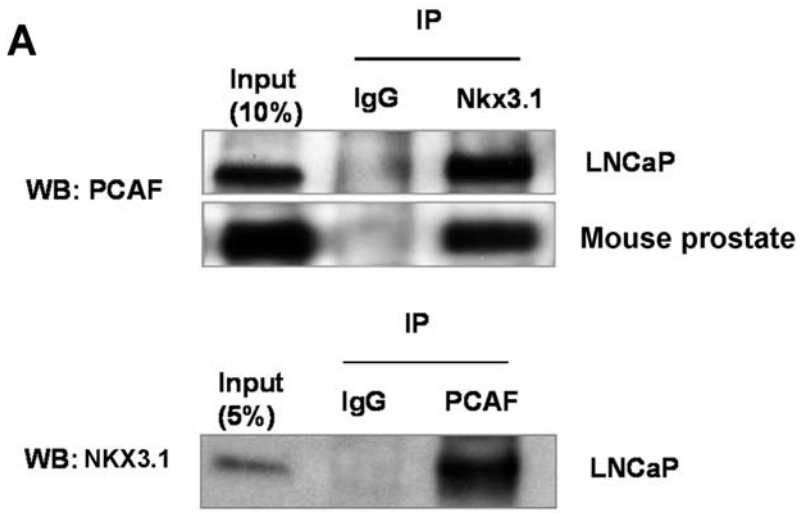


Figure 35. Nkx3.1 interacts with histone acetyl transferase, PCAF *in vivo*: Co-immunoprecipitation assays demonstrate association of Nkx3.1 and PCAF in LNCaP cells and mouse prostate tissue. Lysates were immunoprecipitated with anti-Nkx3.1 antibody and then blotted for anti-PCAF antibody (upper panel) or vice versa (lower panel).

Next we assessed whether Nkx3.1 recruits PCAF to target gene promoters. ChIP assays with mouse prostate tissues showed PCAF binding to the Nkx3.1 consensus sites of both the *intelectin* (Int5 in *Nkx3.1*$^{+/+}$) and *probasin* promoters (Pbn1 in *Nkx3.1*$^{+/+}$ and *Nkx3.1*$^{+/-}$) (Figure 36). These results are consistent with the 'gene-specific targeting model' of histone acetylation (Struhl, 1998) which implies recruitment of HATs to particular promoter regions by sequence-specific DNA binding proteins. We also observed PCAF binding at the Pbn4 control region that does not contain an Nkx3.1 consensus in *Nkx3.1*$^{+/+}$ and *Nkx3.1*$^{+/-}$ mice, presumably due to the ability of PCAF to bind to acetylated histones through its bromodomain (Dhalluin et al., 1999).

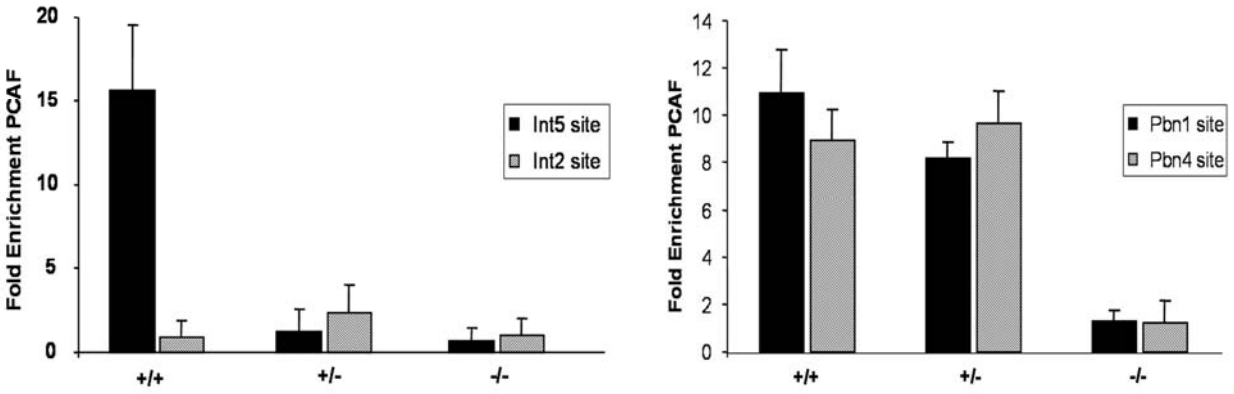


Figure 36. Nkx3.1 recruits PCAF at the chromatin: ChIP assays on the *intelectin* (left) and *probasin* (right) promoters demonstrate the recruitment of PCAF to the Nkx3.1 DNA binding site. Results are presented as Mean ± SD from N ≥ 4.

As we observed Nkx3.1 binding to the *intelectin* promoter in *Nkx3.1*$^{+/-}$ mice following TSA treatment (see Figure 31), we sought to determine if Nkx3.1 can recruit PCAF to the *intelectin* promoter under these conditions as well. ChIP assays established that PCAF was recruited to the Int5 region in *Nkx3.1*$^{+/-}$ mice after TSA treatment (Figure 37). Hyperacetylation in the Int5 region after TSA treatment could have also contributed to PCAF recruitment to this site, in addition to the interaction with Nkx3.1. Nevertheless, failure to observe recruitment of PCAF at a control region (Int2) after TSA treatment suggests that Nkx3.1 is involved in the targeted recruitment of PCAF to the Nkx3.1 binding site (Int5) on the intelectin promoter.

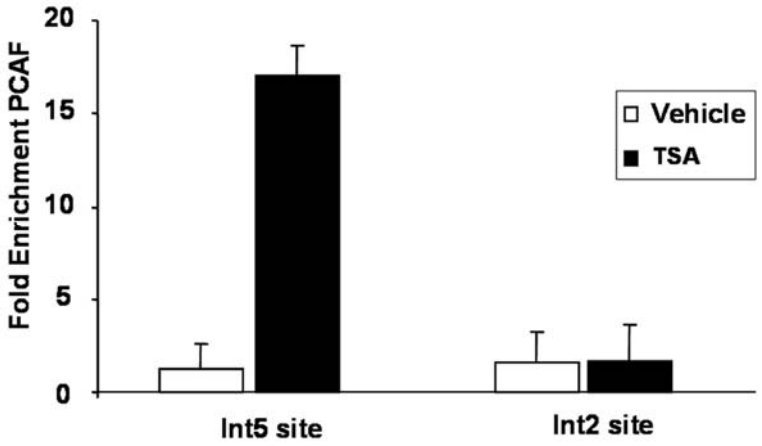


Figure 37. TSA treatment leads to recruitment of PCAF at the intelectin promoter in *Nkx3.1*$^{+/-}$ mice: Recruitment of PCAF to the Nkx3.1 site (Int5) on the *intelectin* promoter in *Nkx3.1*$^{+/-}$ mice following TSA treatment by ChIP analysis. Results are presented as Mean ± SD from N=4.

To establish whether Nkx3.1 and PCAF can form a complex at the same region of DNA on the *intelectin* promoter, we performed two-step ChIP assays. The results indicate that Nkx3.1 and PCAF were bound at the same Int5 region (Figure 38). Thus Nkx3.1 can recruit PCAF to DNA in a dosage-sensitive and promoter-specific manner, likely to set histone acetylation states of target genes. Nkx3.1 can both repress and activate transcription, and has been reported to interact with HDAC1 (Lei et al., 2006), raising the possibility that differential complex formation with HATs or HDACs may determine whether Nkx3.1 activates or represses a target gene.

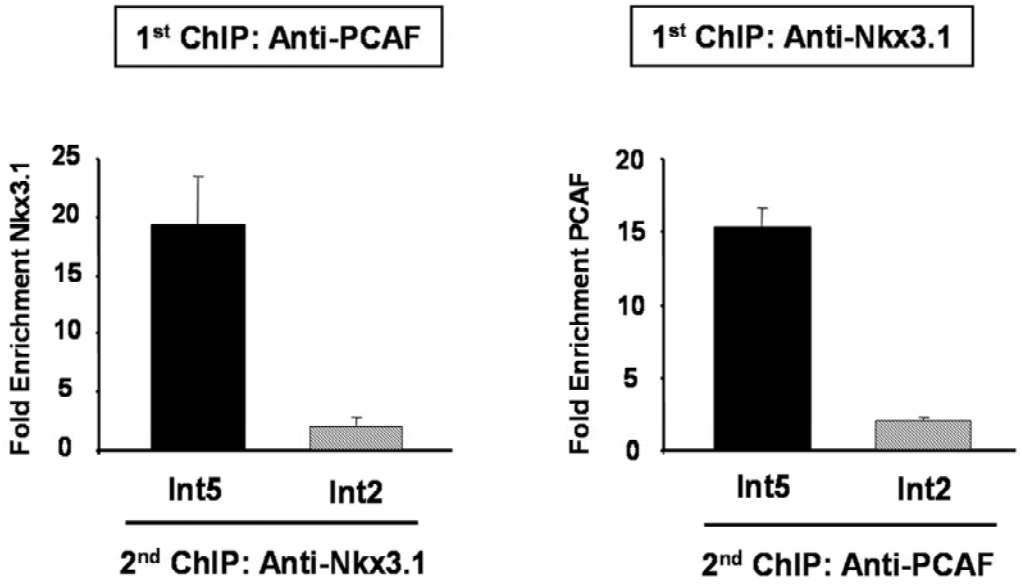


Figure 38. Nkx3.1 and PCAF form complex at the chromatin: Two-step ChIP assays demonstrate that Nkx3.1 and PCAF can interact at the Nkx3.1 site (Int5) on the *intelectin* promoter in *Nkx3.1*$^{+/+}$ mice. Results are presented as Mean ± SD from N=4.

Conclusions:

In summary, based on our results we propose a chromatin mechanism for Nkx3.1 haploinsufficiency in prostate tumor initiation. We propose a model (Figure 39) whereby dosage-sensitive and dosage-insensitive Nkx3.1 target gene loci show differential chromatin accessibilities, due to differential Nkx3.1/PCAF occupancy. Nkx3.1/PCAF are involved in setting differential histone acetylation states and target gene expression patterns. Our results provide an example of how a genetic lesion (haploid loss of the Nkx3.1 tumor suppressor) can engender epigenetic changes (alterations in histone H3/H4 acetylation) that selectively inactivate a dosage-sensitive target gene important for suppressing tumorigenicity. This mechanism may be applicable to other disorders caused by transcription factor haploinsufficiency. The reduced chromatin accessibility of some target genes can lead to their selective inactivation in heterozygous cells, with attendant pathology.

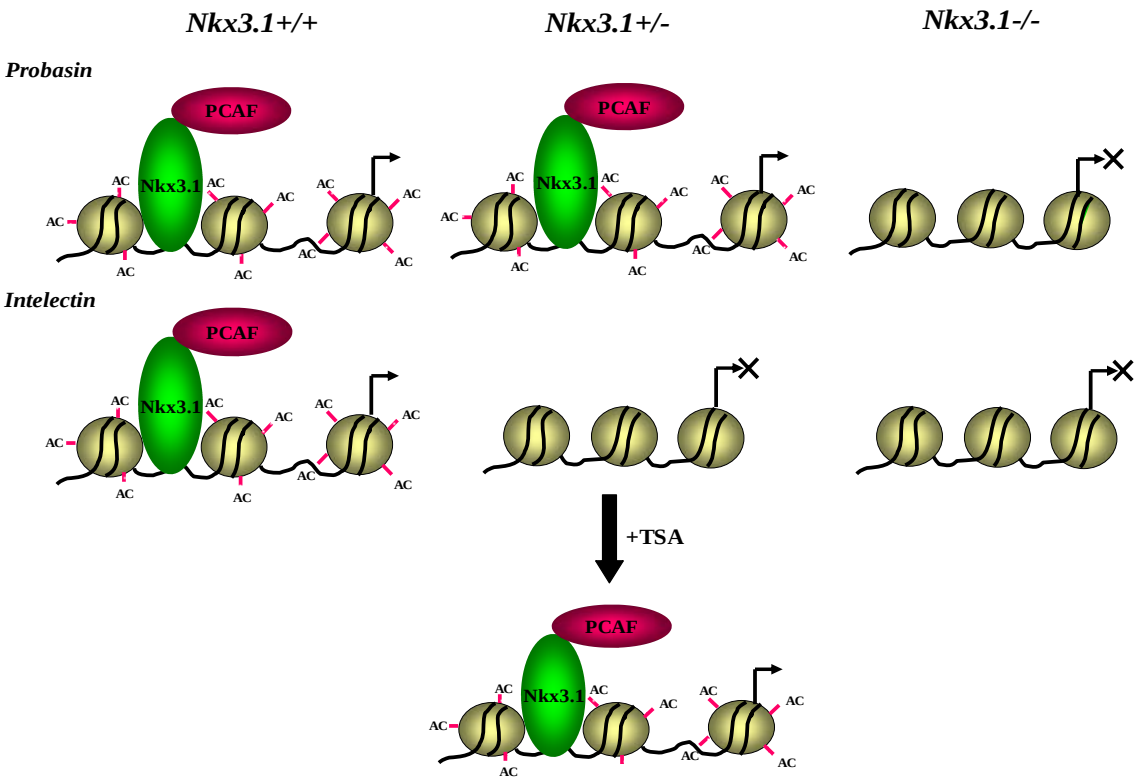


Figure 39. Dosage-sensitive, stochastic Nkx3.1 target gene activation model: A schematic representation of the dosage-sensitive, stochastic Nkx3.1 target gene activation model. The differential chromatin states, Nkx3.1 occupancy and PCAF recruitment at the *probasin* (dosage-insensitive) and *intelectin* (dosage-sensitive) promoters in *Nkx3.1*-mutant mice are illustrated. Note the effects of inducing histone acetylation by Trichostatin A (TSA), at the *intelectin* promoter in *Nkx3.1*$^{+/-}$ animals.

CHAPTER V

FUNCTIONAL ANALYSIS OF NKX3.1 TARGET GENE INTELECTIN/ OMENTIN IN PROSTATE CANCER

Introduction:

The Nkx3.1 model of haploinsufficiency in prostate tumor initiation (Abdulkadir, 2005; Magee et al., 2003) posits that in *Nkx3.1* heterozygous cells, selective inactivation of dosage-sensitive target genes could confer a growth advantage. This would lead to a clonal expansion of the heterozygous cells, providing a larger target cell population for additional genetic mutations. A key unproven assumption of this model is that some dosage-sensitive Nkx3.1 targets have prostate tumor suppressive properties. In my dissertation work, we functionally characterized the dosage-sensitive target intelectin / omentin to explore its role in the differentiation and immortalization pathways involved in prostate tumorigenesis.

Human intelectin (hIntL) is a soluble lectin that recognizes galactofuranose in carbohydrate chains of bacterial cell wall and plays a role in the recognition of bacteria-specific components in the host (Tsuji et al., 2001). The mature hIntL is a secretory glycoprotein consisting of 295 amino acids and N-linked oligosaccharides. The basic structural unit of hIntL consists of a 120-kDa homotrimer in which 40-kDa polypeptides are bridged by disulfide bonds. The hIntL gene consists of 8 exons located on chromosome 1q21.3 and shows high levels of homology with mouse intelectin. Previous mRNA study has identified that intelectin is expressed in the heart, small intestine, colon, and thymus (Tsuji et al., 2001).

Very recently, sequence analysis studies have revealed 100 % identity between human intelectin and omentin (Schaffler et al., 2005), an adipocytokine which profoundly affects insulin sensitivity and plays significant role in obesity, insulin resistance and cardiovascular disease (Kralisch et al., 2005). Intelectin is a mammalian Ca^{2+} -dependent D-galactosyl-specific lectin which is structurally related to the intestinal lactoferrin receptor. It is expressed in paneth and goblet cells of the small intestine where it plays protective role in the innate immune response against various parasite infection (Wrackmeyer et al., 2006).

The role of intelectin in cancer however, is not very well studied. Wali and colleagues reported that the intelectin gene is over-expressed in malignant pleural mesothelioma tumor samples compared to autologous normal epithelium by serial analysis of gene expression (SAGE) (Wali et al., 2005). Apart from this study the functional relevance of the intelectin gene in other cancers has not yet been explored. We have examined the role of intelectin in prostate tissue and we present strong evidence suggesting that intelectin has a tumor suppressor-like activity in prostate cancer.

Results and Discussion:

Establishment of shRNA mediated stable knockdown cell lines of NKX3.1 and Intelectin

We examined the role of intelectin using LNCaP prostate cells which express appreciable amounts of NKX3.1 and intelectin. We used a lentiviral mediated shRNA

approach to stably knockdown NKX3.1 and intelectin in LNCaP cells and confirmed shRNA mediated knockdown efficiency by western blot analysis. Our results showed that both NKX3.1 and intelectin proteins were significantly knocked down in LNCaP cells (Figure 40A and 40B). Consistent with it being a dosage-sensitive NKX3.1 target, the expression of intelectin was dramatically reduced in NKX3.1 knockdown clones (Figure 40A). In contrast, NKX3.1 protein levels remained unaffected in intelectin knockdown clones (Figure 40B).

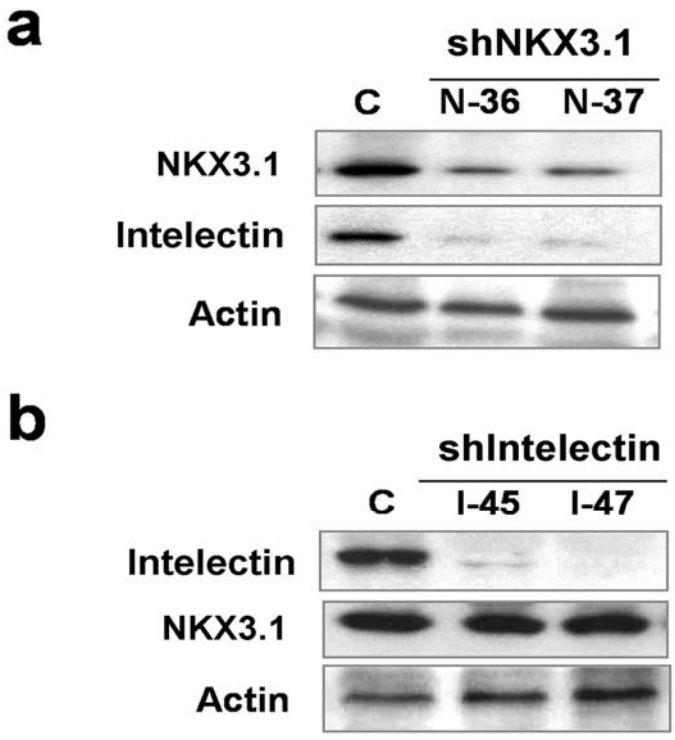


Figure 40. Establishment of stable knockdown cells of NKX3.1 and intelectin: (A) Western blot analyses to confirm the stable knockdown of NKX3.1 protein levels in LNCaP prostate cells (upper panel). Note the reduced level of intelectin protein in NKX3.1 knockdown clones (middle panel). Actin was used as a loading control (lower panel). (B) Western blot analyses to confirm the stable knockdown of intelectin protein levels in LNCaP prostate cells (upper panel). NKX3.1 protein levels were unaffected in intelectin knockdown clones (middle panel). Actin was used as a loading control (lower panel).

Altered NKX3.1 target gene expression in stable NKX3.1 knockdown cells

Magee and colleagues examined Nkx3.1 target gene expression in *Nkx3.1*-mutant mice and identified a spectrum of positively and negatively regulated target genes (Magee et al., 2003). We examined mRNA expression of NKX3.1 target genes in NKX3.1 knockdown LNCaP cells by qRT-PCR analysis (Figure 41). Our results showed that positively regulated target genes such as *INTELECTIN*, *PDZK1*, *and SEL1L* mRNA levels were significantly down-regulated (Figure 41B-D) in NKX3.1 knockdown cells compared to control cells.

Moreover, we examined the mRNA expression of negatively regulated NKX3.1 target genes such as *PSA* and *ANGIOPOIETIN2.* Interestingly, both *PSA* and *ANGIOPOIETIN2* were significantly up-regulated in NKX3.1 knockdown cells compared to control cells (Figure 41E and 41F). These results are consistent with the gene profiling microarray data from *Nkx3.1*-mutant mice (Magee et al., 2003).

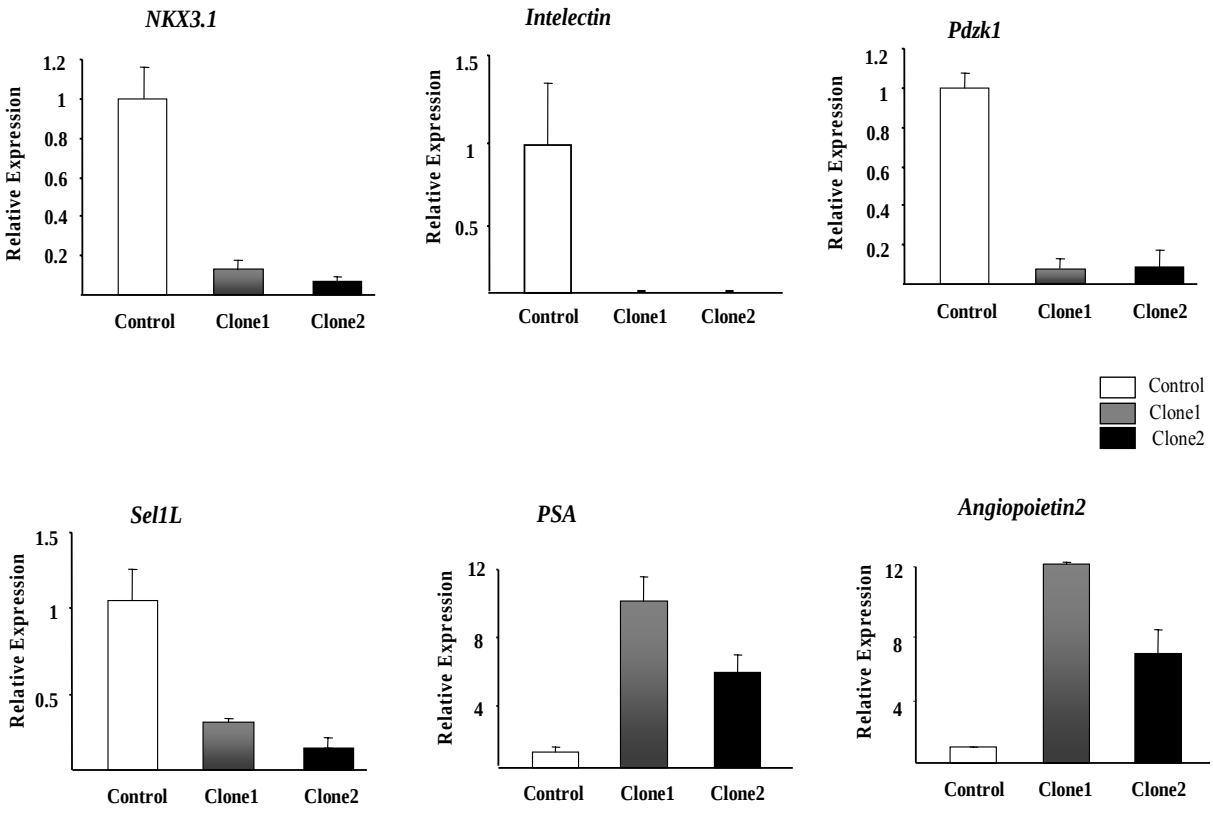


Figure 41. Altered NKX3.1 target gene expression in stable NKX3.1 knockdown cells: Quantitative RT-PCR analysis for NKX3.1 target genes in stable NKX3.1 knockdown LNCaP cells. (A) *NKX3.1* mRNA level was significantly reduced in NKX3.1 knockdown cells compared to control cells. (B-F) Significant reduction in the mRNA levels of positively regulated genes such as *INTELECTIN (B), PDZK1 (C)* and *SEL1L (D)* was observed in NKX3.1 knockdown clones. *PSA (E)* and *ANGIOPOIETIN 2 (F)* are negatively regulated by NKX3.1 and their mRNA levels were significantly up-regulated in knockdown clones compared to control clones.

The dosage-sensitive Nkx3.1 target gene intelectin suppresses prostate cell growth

To determine the effects of intelectin on prostate cell growth, we performed a series of *in vitro* proliferation assays. First we performed cell growth analysis and found that the proliferation rates of both the intelectin and NKX3.1 stable knockdown clones were significantly increased compared to control cells (Figure 42).

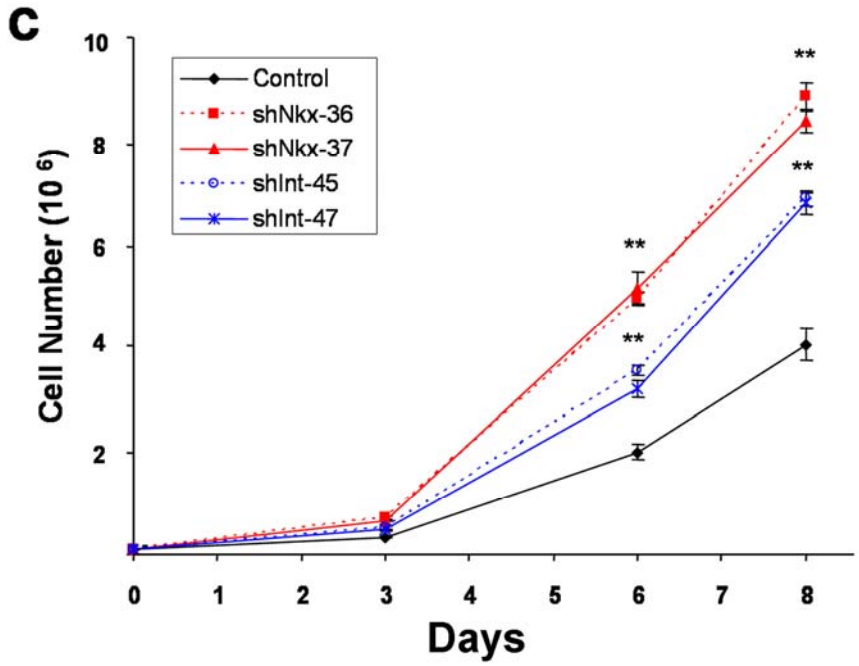


Figure 42. Intelectin suppresses prostate cell growth *in vitro*: Cell growth analyses show the significant increase (**, $p < 0.05$) in growth of NKX3.1 and intelectin stable knockdown LNCaP cells compared to empty vector control. Two independent experiments in duplicates were performed and results are presented as Mean ± SD.

Additionally, we examined the antiproliferative effects of intelectin by MTT assays in DU145 prostate cells which express very low intelectin endogenously (Figure 43: inset). Interestingly, our analysis revealed that cell viability was significantly decreased in cells transfected with intelectin/omentin compared to cells transfected with control vector (Figure 43). Furthermore, we rescued the growth phenotype in Nkx3.1 knockdown cells with low intelectin expression by exogenous intelectin (Figure 43). Thus, cell growth analysis and cell viability studies suggest that intelectin/omentin may have tumor suppressing ability in prostate cells.

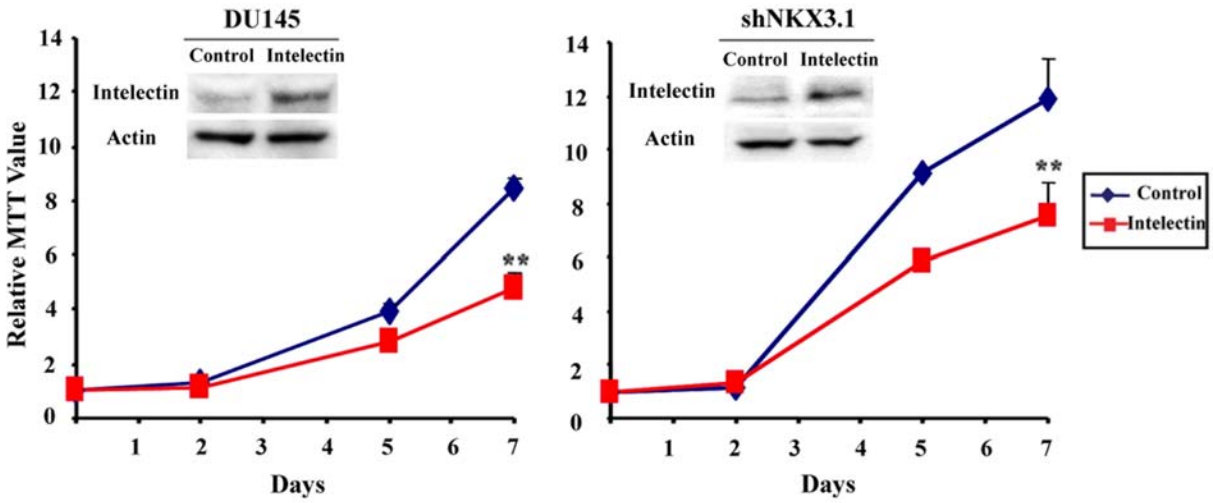


Figure 43. Intelectin inhibits prostate cell viability *in vitro*: MTT cell viability assays demonstrate the significant decrease (**, $p < 0.05$) in cell viability of DU145 and shNKX3.1-LNCaP cells with overexpression of intelectin /omentin compared to the empty control vector. Insets: Western blot analysis to demonstrate the transfection efficiency. Two independent experiments in quadruplicates were performed and results are presented as Mean ± SD.

Intelectin / omentin is a bona-fide prostate tumor suppressor *in vivo*

We next examined the tumorigenicity of the intelectin knockdown cells by performing soft-agar colony formation assays. Consistent with the *in vitro* proliferation assays, increased colony formation was observed for both the intelectin and NKX3.1 knockdown clones compared to control cells (Figure 44) suggesting that intelectin has tumor suppressor-like activity in prostate cells.

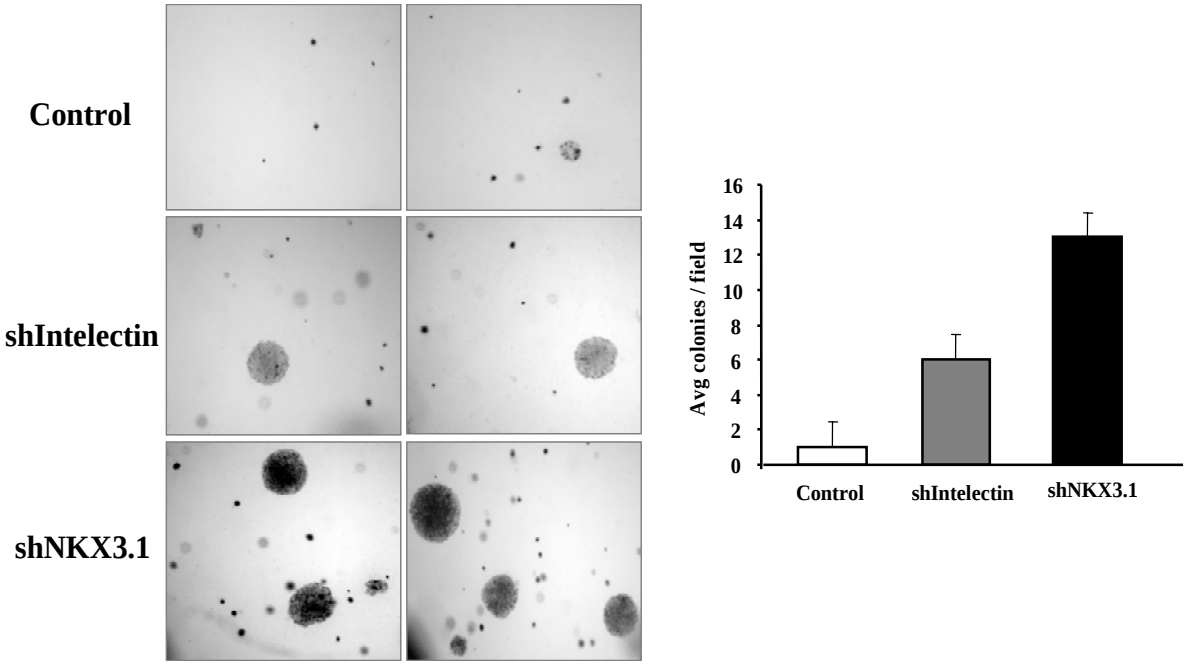


Figure 44. Anchorage-independent growth in intelectin knockdown cells: Soft agar colony formation assays reveal increased anchorage-independent growth for NKX3.1 and intelectin knockdown LNCaP cells (*, $p < 0.05$). Representative fields are shown (2.5 x Magnification). The results are presented as Mean ± SD.

To establish the tumor suppressor function of intelectin *in vivo*, we performed xenograft studies in immuno-compromised mice. We found that the tumors in mice injected with intelectin and NKX3.1 knockdown cells grew faster and to greater volumes than the tumors in mice injected with control cells (Figure 45). Mean tumor weight was also significantly greater in the intelectin and NKX3.1 knockdown group than the control group (Figure 46). These studies strongly support a tumor suppressor role for intelectin in prostate cells.

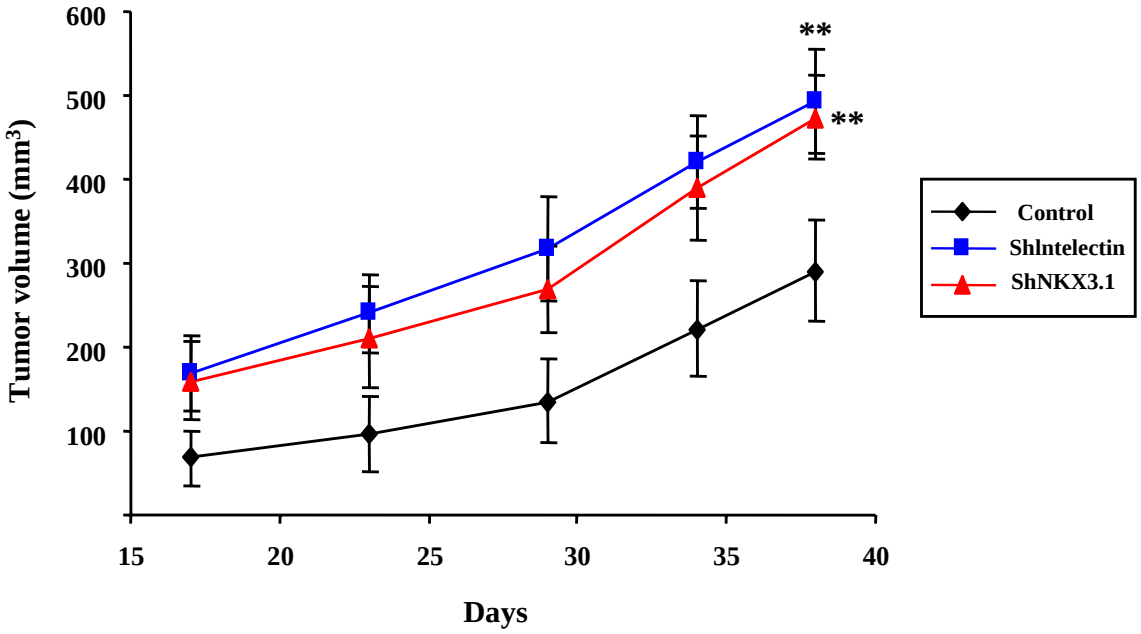


Figure 45. Intelectin shows a prostate tumor suppressor function *in vivo*: Xenograft studies demonstrate the *in vivo* tumor suppressor-like activity for intelectin in prostate cancer. Tumors from intelectin/shRNA (N=8) and NKX3.1/shRNA (N=6) LNCaP cells grew faster than the control group (N=6).

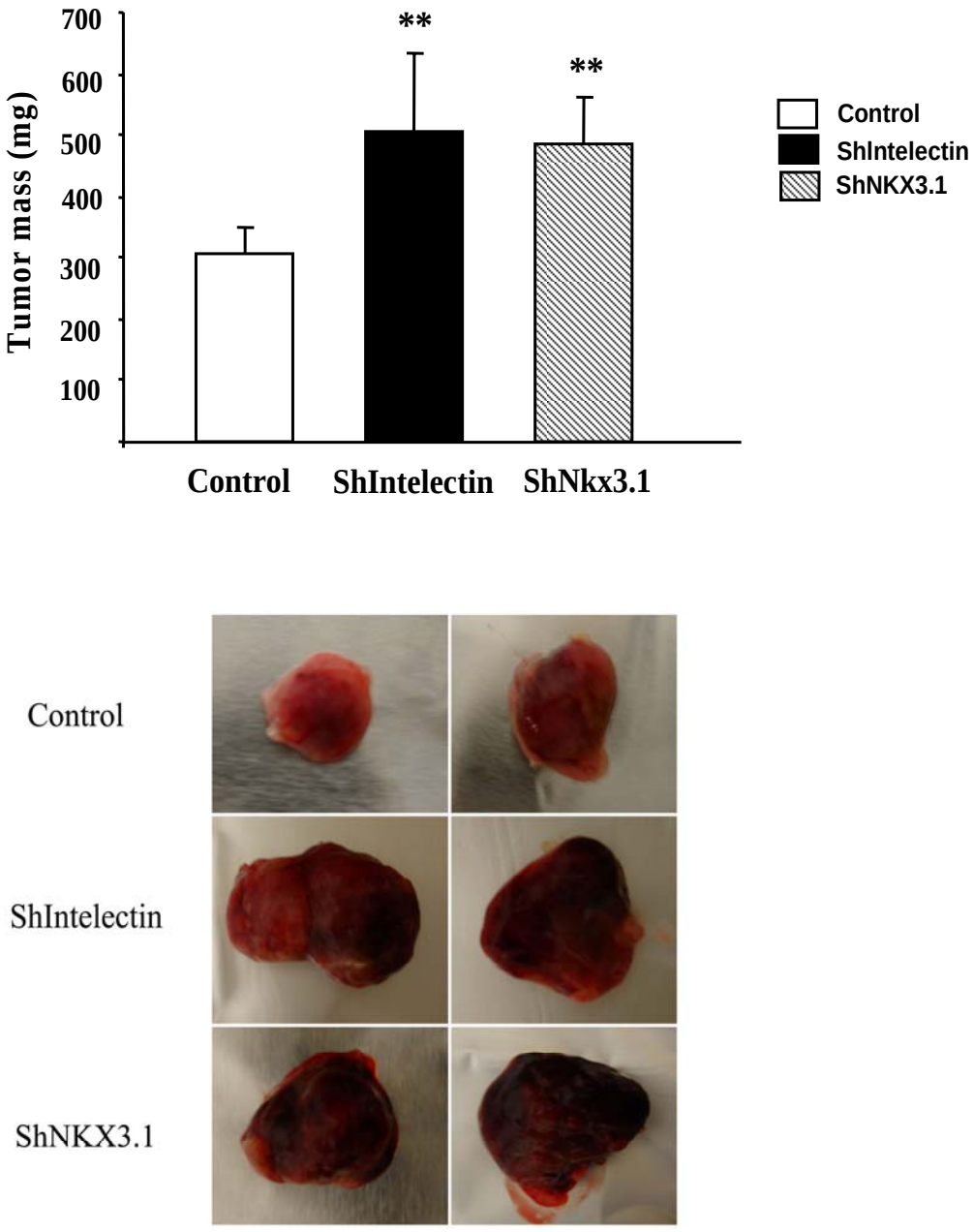


Figure 46. Intelectin is a bona-fide prostate tumor suppressor: Xenograft studies demonstrate the *in vivo* tumor suppressor-like activity for intelectin in prostate cancer. Note the significant increase in the mean tumor mass in intelectin and NKX3.1 knockdown group compared to control group (top panel). Representative tumors from each group are shown in (bottom panel). The results are presented as Mean ± SD.

Conclusions:

In conclusion, we provide evidence that Nkx3.1 target genes may have a functional significance in prostate tumorigenesis. Here, we performed a series of *in vitro* and *in vivo* experiments which indicate that the Nkx3.1 dosage-sensitive target, intelectin / omentin may have tumor suppressor-like activity in prostate. We observed that prostate cells deficient in intelectin / omentin demonstrate rapid proliferation rates and increased anchorage-independent cell growth. Furthermore, transfection of exogenous intelectin / omentin in prostate cells with low endogenous intelectin expression results in significantly decreased in cell viability. Consistent with our *in vitro* findings, Xenograft studies demonstrate that athymic mice injected with intelectin knockdown cells develop tumors at faster rates and at greater volumes compared to the control cells. Overall, our study strongly suggests a tumor suppressor like function for intelectin / omentin in prostate cancer *in vivo*. Furthermore, as this is the first piece of evidence for functional significance of Nkx3.1 targets, it opens new avenues to explore the functional relevance of other Nkx3.1 targets that may help to characterize the molecular pathways involved in prostate tumorigenesis.

CHAPTER VI

CELL CYCLE REGULATION BY NKX3.1

Introduction:

Delayed cell cycle exit in *Nkx3.1*-deficient mice

Nkx3.1-deficient mice develop prostatic hyperplasia that can progress to prostatic intraepithelial neoplasia (PIN) lesions over time (Abdulkadir et al., 2002); (Bhatia-Gaur et al., 1999). Further, conditional knockout *Nkx3.1* mice also develop hyperplasia and PIN lesions which resemble to the human PIN lesions (Abdulkadir et al., 2002) suggesting that Nkx3.1 somehow establishes or maintains luminal epithelia in a growth arrested state. Magee and colleagues utilized the castration testosterone replacement (see Figure 9) and demonstrated that Nkx3.1 regulates the cell cycle exit of luminal epithelia during prostate regeneration. *Nkx3.1*-deficient luminal cells fail to appropriately exit the transient proliferation state (Magee et al., 2003).

To determine the effects of Nkx3.1 on growth arrest in the regenerating prostate, Magee and colleagues analyzed Ki-67 mRNA expression in $Nkx3.1^{+/+}$, $Nkx3.1^{+/-}$ and $Nkx3.1^{-/-}$ prostates by RT-PCR. Ki-67 mRNA profiles showed that $Nkx3.1^{+/-}$ and $Nkx3.1^{-/-}$ mice exhibit an extended proliferative phase (Magee et al., 2003). Next they counted the number of cells that incorporated Brdu at 3 and 7 days post-TR and found no difference in fraction of Brdu positive cells in $Nkx3.1^{+/+}$, $Nkx3.1^{+/-}$ and $Nkx3.1^{-/-}$ at day 3. However, by day 7, the fraction of Brdu positive cells

in *Nkx3.1*$^{+/+}$ was dramatically reduced compared to the day 3, while a much larger population of cells continued to divide in *Nkx3.1*$^{+/-}$ and *Nkx3.1*$^{-/-}$ mice (Figure 47A-F). These results support the notion that Nkx3.1 regulates luminal cell exit from cell cycle, in other words, loss of one or both alleles of Nkx3.1 precludes timely withdrawal of luminal epithelia from cell cycle. Nevertheless, the underlying molecular mechanism behind this phenomenon has not been explored. We present some data which suggest that this may be mediated through potential Nkx3.1-HDAC1-cyclin D/E pathway.

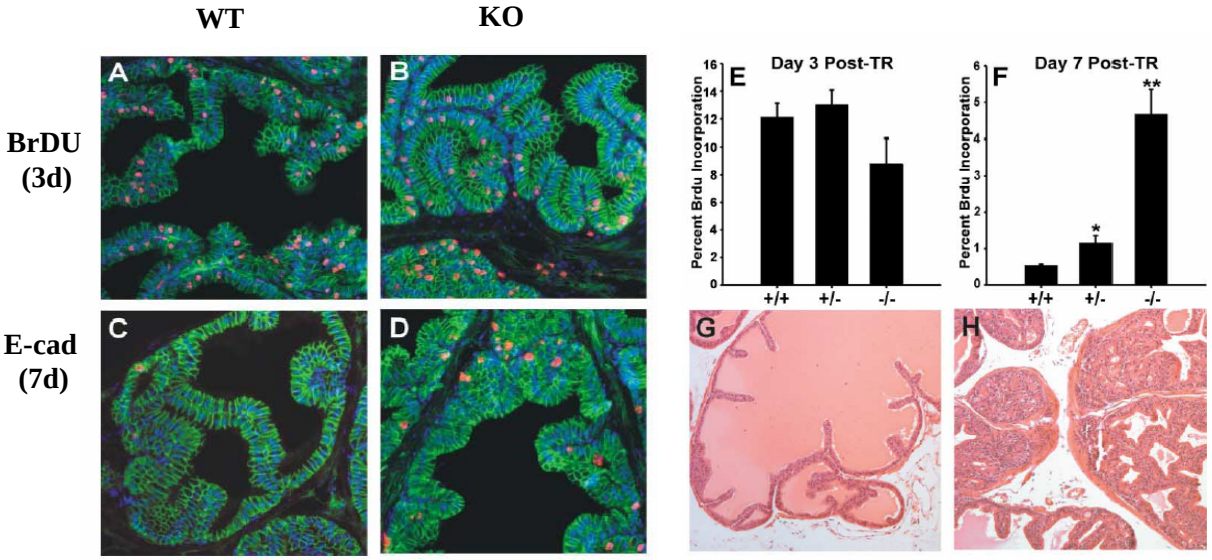


Figure 47: Loss of Nkx3.1 results in extended periods of proliferation and luminal hyperplasia in prostate: [A-D] Brdu incorporation assays for cell proliferation at 3 (A,B) and 7 (C,D) days post-TR. Brdu: Red; E-cad: Green (luminal epithelial cell marker). [E-F] Note the percentages of Brdu incorporating cells at 3 (E) and 7 (F) days in different genotypes. At day 7 cells from *Nkx3.1*$^{+/-}$ and *Nkx3.1*$^{-/-}$ prostates continue to divide and fail to exit cell cycle. [G-H] H&E staining showed dramatic hyperplasia in *Nkx3.1*$^{-/-}$ prostates (H) at 14 days post-TR relative to the *Nkx3.1*$^{+/+}$ (G) *(Adapted and modified from Magee et al., 2003 Cancer Cell).*

Role of HDAC1 in cell cycle regulation:

The histone deacetylase 1 (HDAC1) gene has been implicated in controlling the transcription of core cell cycle regulators, but the *in vivo* role of HDACs in cell cycle regulation is still not well understood. HDAC1 has been shown to be recruited by the Retinoblastoma (Rb) protein and related proteins to the promoters of several cell cycle genes (Zhang and Dean, 2001). Rb represses the transcription of cell cycle genes that contain E2F binding sites in their promoters and this repression is required for Rb-mediated growth suppression (Zhang et al., 1999). The recruitment of HDAC1 by Rb leads to deacetylation of histones at target gene promoters (Luo et al., 1998) and inhibition of HDAC activity blocks Rb-mediated repression of genes activated at G1/S transition, including cyclin E (Zhang et al., 2000).

In contrast, some *in vivo* studies have indicated that HDACs can promote proliferation. Mouse embryos mutant for HDAC1 show an overall reduction of proliferation and decrease in cyclin-dependent kinase (CDK) activity and die before the stages of organogenesis (Lagger et al., 2002). Additionally studies in zebrafish found that hdac1 mutant can survive longer but display hindbrain underproliferation (Cunliffe, 2004). Lastly, HDAC inhibitors (HDACi) cause growth arrest in several tumors and are therefore targeted as a potential anticancer therapeutics.

Recently, Stadler and colleagues demonstrated that HDAC1 is cell-autonomously required for cell cycle exit and differentiation in the retina of zebrafish (Stadler et al., 2005). To determine the role of hdac1 in the differentiation of zebrafish retina, the authors utilized BrdU incorporation and surprisingly found more proliferation in retina and optic stalk in the eyes of hdac1 mutants compared to wild

type (Figure 48 A,B). The fraction of mitotic cells was assessed by anti-phospho-histone H3 staining which was also elevated in hdac1 mutants (Figure 48 C,D).

Cyclin D and E are known to drive cell cycle progression in various organisms; hence Stadler and colleagues examined cyclin D1 and cyclin E2 RNA expression by in situ hybridization in hdac1 mutant retina (Stadler et al., 2005). Interestingly, cyclin D1 and E2 were expressed in wild type retina, but were down-regulated in differentiating cells (Figure 48K,M). In contrast, expression of both cyclin D1 and E2 was failed to down-regulate and were strongly expressed in hdac1 mutant retina (Figure 48L,N). These studies support the idea that HDAC1 is required for transcriptional repression of cyclin D1 and E2 and for cell cycle exit in zebrafish retina (Stadler et al., 2005).

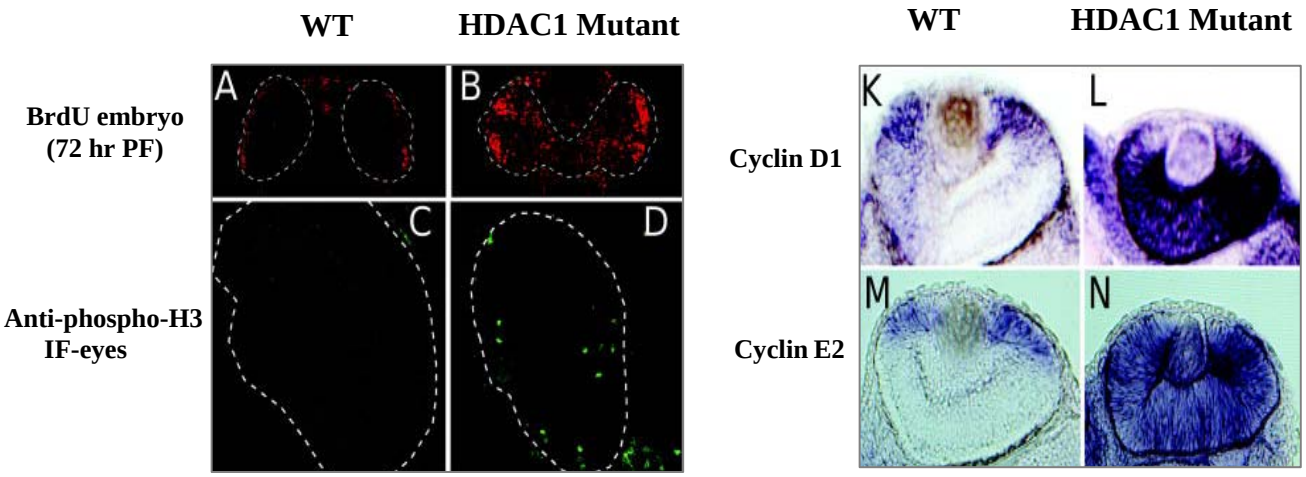


Figure 48: Loss of hdac1 activity results in failure of retinal cells to exit the cell cycle: (A-B) Brdu incorporation in 72 h postfertilization (hpf) in wild type (A) or hdac1 mutants (B); (C-D) Anti-H3 immunofluoresence on cryosections of 72 hpf wild type (C) and hdac1 mutants (D). (K-N) Whole mount in situ hybridization of cyclin D1 (K,L) and cyclin E2 (M,N) RNA *(Adapted and modified from Stadler et al., 2005 Develpomental Dynamics).*

Preliminary results and Discussion:

Altered expression of cyclins in *Nkx3.1*-deficient mice

To investigate the molecular mechanism of cell cycle regulation by Nkx3.1, we first examined the expression of different cyclins including cyclin D1, cyclin E2, cyclin A2 and cyclin B1 in *Nkx3.1*-mutant mouse prostates by qRT-PCR analysis. Intriguingly, we found that cyclin D1 and cyclin E2 mRNA levels were significantly up-regulated in *Nkx3.1*$^{+/-}$ and *Nkx3.1*$^{-/-}$ prostates while cyclin A2 and cyclin B1 mRNA levels remained unaffected (Figure 49).

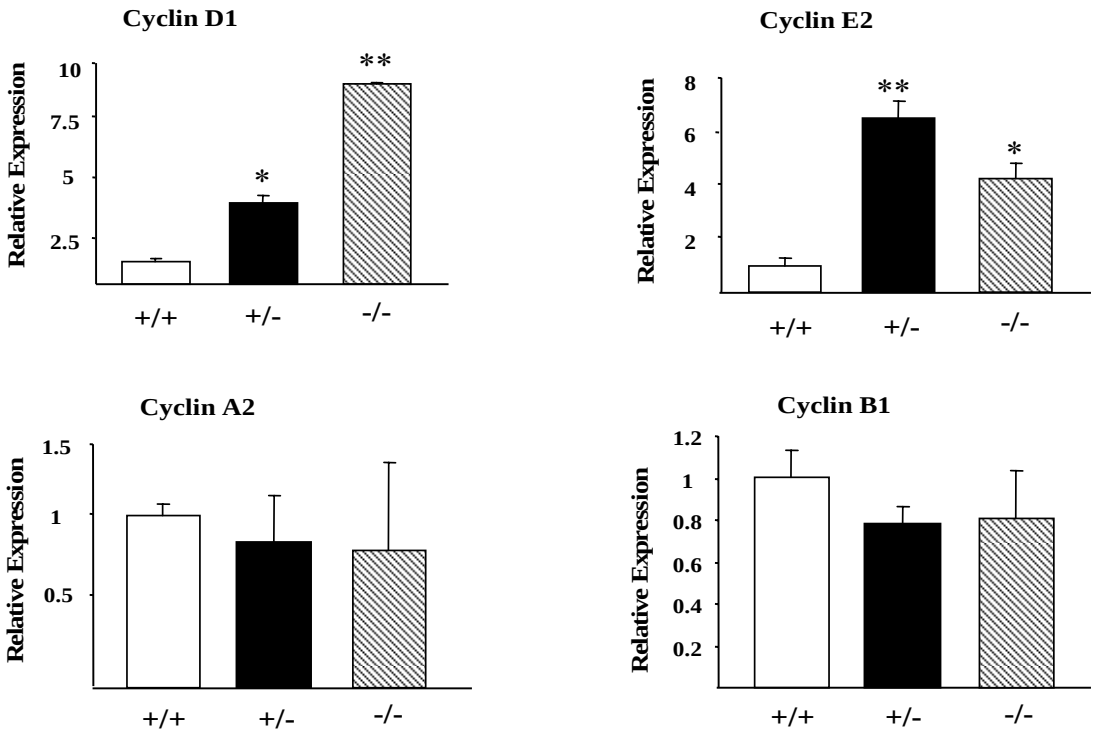


Figure 49. Altered expression of cyclins in *Nkx3.1*-deficient mice: QRT-PCR analysis on *Nkx3.1*-mutant mice prostates indicate that cyclins D1 (A) and cyclin E2 (B) mRNA are dramatically up-regulated ($^{*}P<0.05$, $^{**}P<0.001$) in *Nkx3.1*-deficient mice compared to wild type mice while cyclin A2 (C) and cyclin B1 (D) mRNA remain largely unaffected.

We further examined the expression of cyclins in LNCaP cells in which NKX3.1 was transiently knocked down. We found that cyclin E2 and cyclin A2 were significantly up-regulated while cyclin D1 and cyclin B1 remained unaffected (Figure 50).

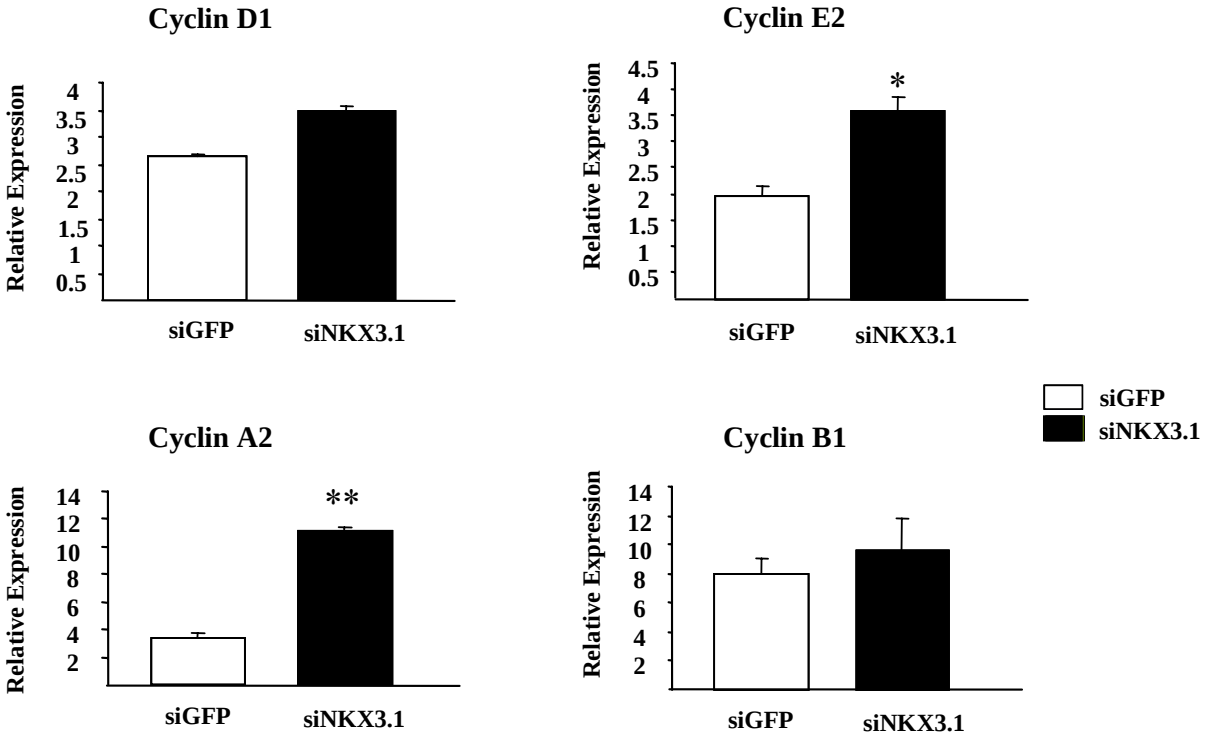


Figure 50. Altered expression of cyclins in response to siNKX3.1 in LNCaP cells: QRT-PCR analysis in either siGFP or siNKX3.1 LNCaP cells indicate that cyclins E2 and cyclin A2 mRNA are dramatically up-regulated ($^{*}P<0.05$, $^{**}P<0.001$) upon NKX3.1 downregulation. Note that cyclin D1 and cyclin B1 mRNA remain unaffected.

Differential HDAC expression in *Nkx3.1*-mutant mice

Histone deacetylases (HDACs) have been consistently linked to the cell cycle regulation. To our knowledge, HDAC expression in mouse prostate has not been yet studied, so we first examined the expression of different HDACs by quantitative RT-PCR in *Nkx3.1*-mutant prostates. Our analysis revealed that HDAC1 and HDAC2 were expressed abundantly in the wild type mouse prostate tissue. We found that HDAC3, HDAC5, HDAC6, HDAC7 and HDAC8 were expressed at low levels and HDAC9 was not expressed in wild type mouse prostate tissue (Figure 51). Interestingly, we observed that HDAC1 and HDAC2 expression was dramatically reduced in *Nkx3.1*$^{+/-}$ and *Nkx3.1*$^{-/-}$ prostates compared to their wild type levels. Furthermore, none of the other HDACs tested showed significant differences in their expression among different genotypes of *Nkx3.1*-mutant prostates (Figure 51).

Among different HDACs, HDAC1 has been shown to play significant role in the cell cycle exit in zebrafish visual system (Stadler et al., 2005). Magee and colleagues (Magee et al., 2003) provided evidence that prostate epithelia in *Nkx3.1*-deficient mice exhibit delayed cell cycle exit. Furthermore, we observed dramatic reduction in mRNA levels of HDAC1 in *Nkx3.1*-deficient prostates (Figure 51). These observations suggest that HDAC1 may play a critical role in delayed cell cycle exit of prostate epithelia in *Nkx3.1*-deficient mice. We therefore examined the HDAC1 protein expression in *Nkx3.1*-mutant mice prostate tissue by western blot analysis. Consistent with our mRNA data, western blot studies demonstrate that HDAC1 protein expression was significantly reduced in *Nkx3.1*$^{+/-}$ and *Nkx3.1*$^{-/-}$ prostates compared to the wild type expression (Figure 52).

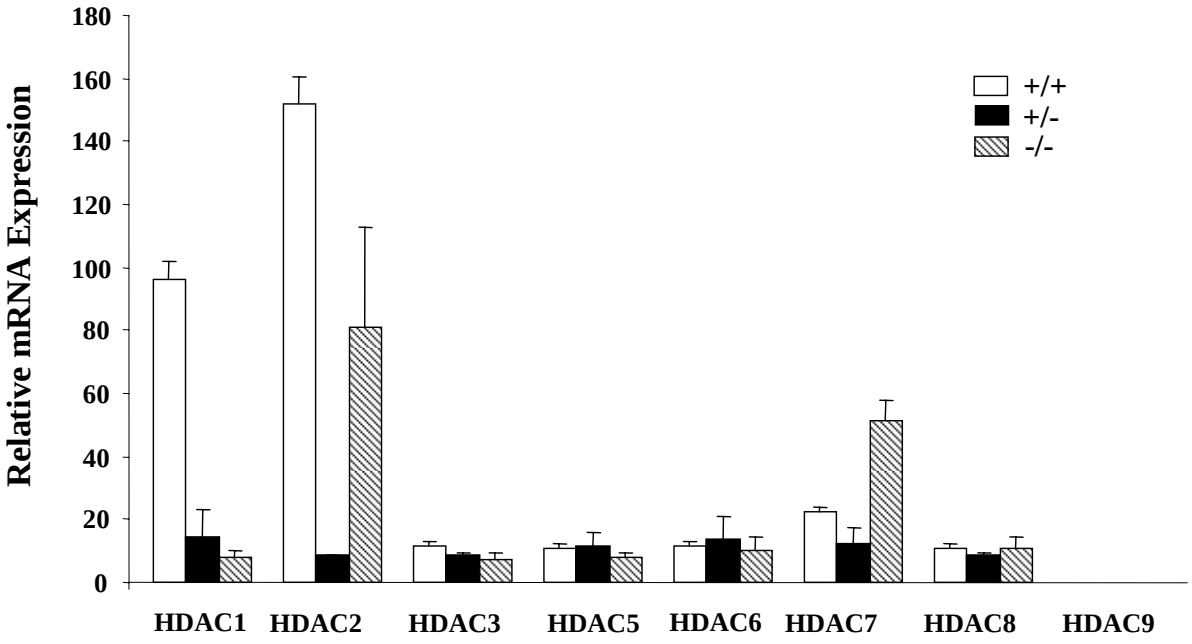


Figure 51. Differential expression of HDACs in *Nkx3.1*-mutant prostate tissue: QRT-PCR analysis demonstrates that HDAC1 and HDAC2 were abundantly expressed in wild type mouse prostate tissue. Note that HDAC1 and HDAC2 mRNA levels were dramatically reduced in *Nkx3.1*$^{+/-}$ and *Nkx3.1*$^{-/-}$ prostates compared to their wild type expression levels

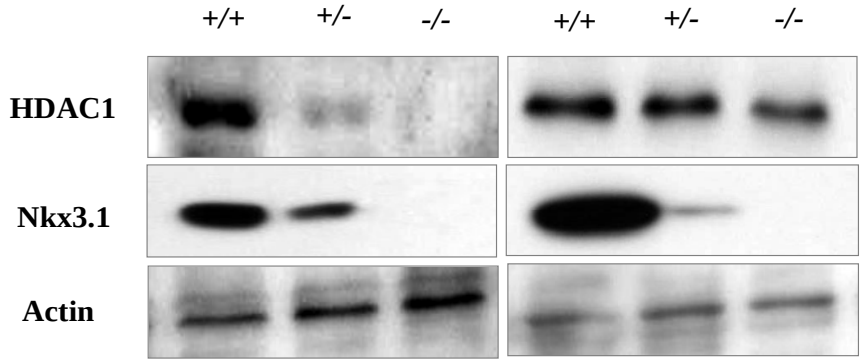


Figure 52. HDAC1 protein expression in *Nkx3.1*-mutant prostate tissue: Western blot analysis to show HDAC1 (upper panel) and Nkx3.1 (middle panel) protein expression in *Nkx3.1*$^{+/+}$, *Nkx3.1*$^{+/-}$ and *Nkx3.1*$^{-/-}$ prostates. Note that HDAC1 protein expression was markedly reduced in *Nkx3.1*$^{+/-}$ and *Nkx3.1*$^{-/-}$ prostates compared to the wild type expression. Actin (lower panel) was used as a loading control.

Altered HDAC enzyme activity in *Nkx3.1* -deficient prostates

QRT-PCR analysis coupled with western blot analysis indicates that HDAC1 mRNA and protein expression is reduced in *Nkx3.1*-deficient prostates. To further explore the role of HDACs in cell cycle regulation in *Nkx3.1*-mutant mice, we examined the HDAC enzymatic activity using a HDAC colorimetric assay. Again consistent to the mRNA and protein studies, HDAC activity assays revealed that HDAC enzymatic activity was also significantly reduced in *Nkx3.1*$^{+/-}$ and *Nkx3.1*$^{-/-}$ mice prostates than the wild type activity level (Figure 53).

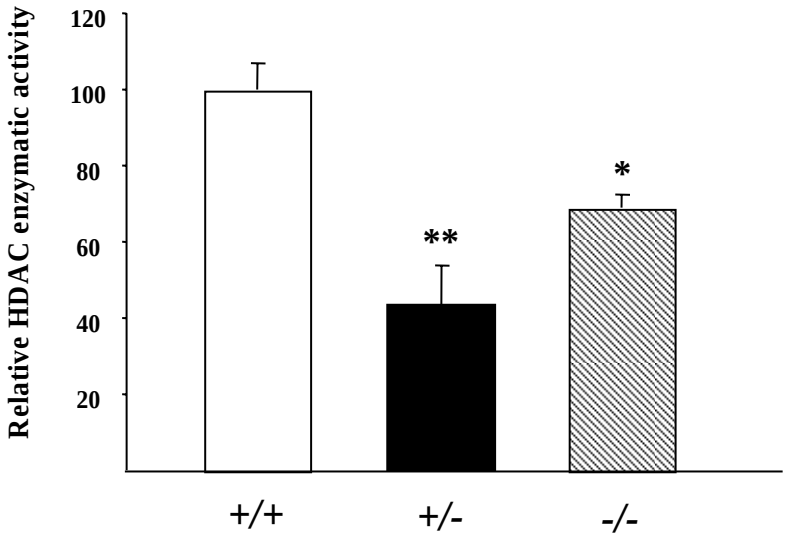


Figure 53. Altered HDAC enzyme activity in *Nkx3.1*-deficient prostates: HDAC activity assay demonstrate that HDAC enzymatic activity is significantly reduced in *Nkx3.1*$^{+/-}$ and *Nkx3.1*$^{-/-}$ prostates ($^{*}P<0.05$, $^{**}P<0.001$).

Increased global histone acetylation in *Nkx3.1*-deficient prostates

Altered expression and enzymatic activity of HDACs in *Nkx3.1*-mutant mice prostates suggest that histone acetylation may be involved in the cell cycle regulation by Nkx3.1. To further determine the role of histone acetylation, we performed immunohistochemistry assays and examined the global histone acetylation pattern (Ac-H3 and Ac-H4) in *Nkx3.1*-deficient mouse prostate tissues. Our immunohistochemical analysis identified global histone hyperacetylation in *Nkx3.1*$^{-/-}$ prostates compared to wild type prostates (Figure 54).

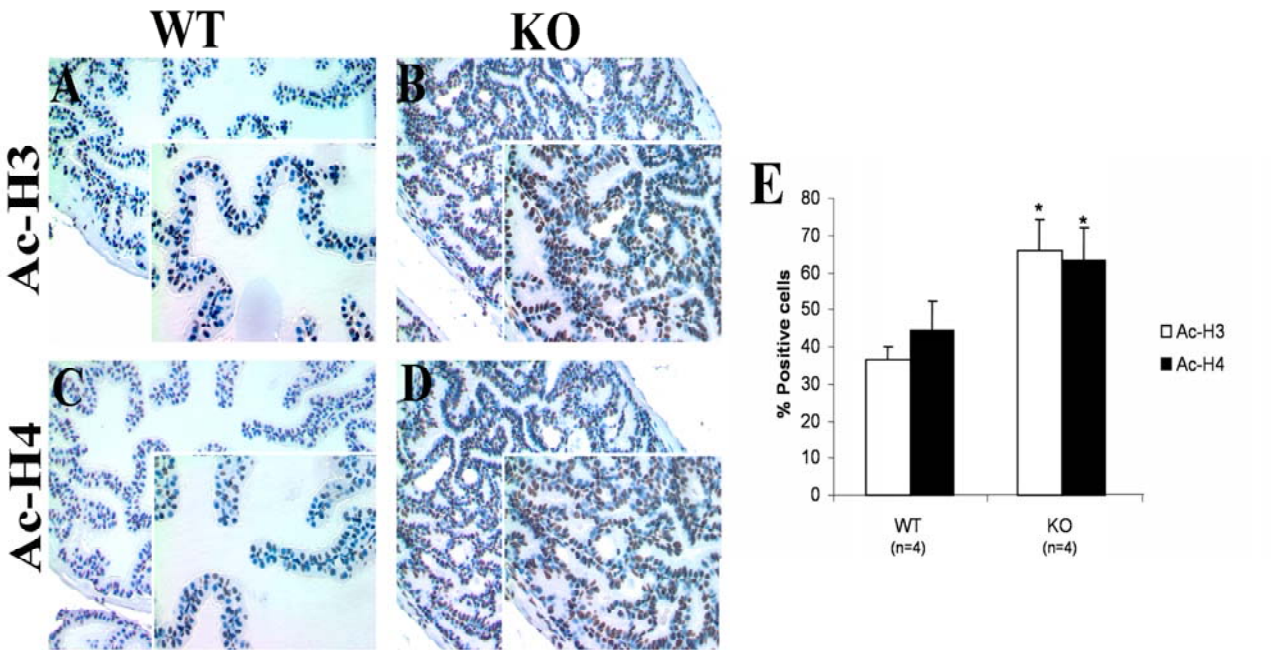


Figure 54. Increased global histone acetylation in *Nkx3.1*-deficient prostates: Immunohistochemistry analyses indicate significantly increased expression of Ac-H3 and Ac-H4 in *Nkx3.1*$^{-/-}$ prostates (B and D) compared to *Nkx3.1*$^{+/+}$ prostates (A and C). Quantitation of percentage positive cells (Ac-H3/H4) is shown in panel (E). (*P<0.05)

Up-regulation of cyclins in response to HDAC inhibition

Our results so far suggest that high expression of HDACs in *Nkx3.1*$^{+/+}$ prostates may lead to the lower expression of cyclin D1 and E2. Thus, we hypothesize that treatment of wild type prostate tissue with HDAC inhibitor, TSA can induce expression of cyclin D1 and cyclin E2. Intriguingly, we observed that both cyclin D1 and cyclin E2 were significantly up-regulated upon HDAC inhibition in *Nkx3.1*$^{+/+}$ prostate explants (Figure 55).

We next examined the effects of HDAC inhibition on cyclins expression in LNCaP cells. Our results showed that cyclin E2 mRNA expression was significantly up-regulated in LNCaP cells following TSA treatment. On the other hand, cyclin D1, cyclin A2 and cyclin B1 mRNA remained unaffected and showed constant expression in vehicle treated or TSA treated samples (Figure 56).

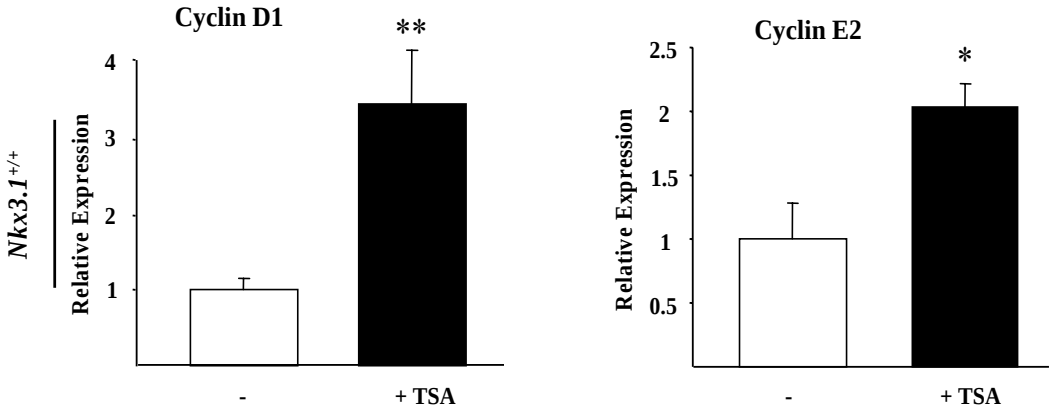


Figure 55. Up-regulation of cyclins in response to HDAC inhibition in *Nkx3.1 wild type* prostates: Prostate explants were treated with or without HDACi, TSA and subsequent QRT-PCR demonstrate that cyclin D1 and E2 were significantly up-regulated in response to TSA treatment ($^{*}P<0.05$, $^{**}P<0.001$).

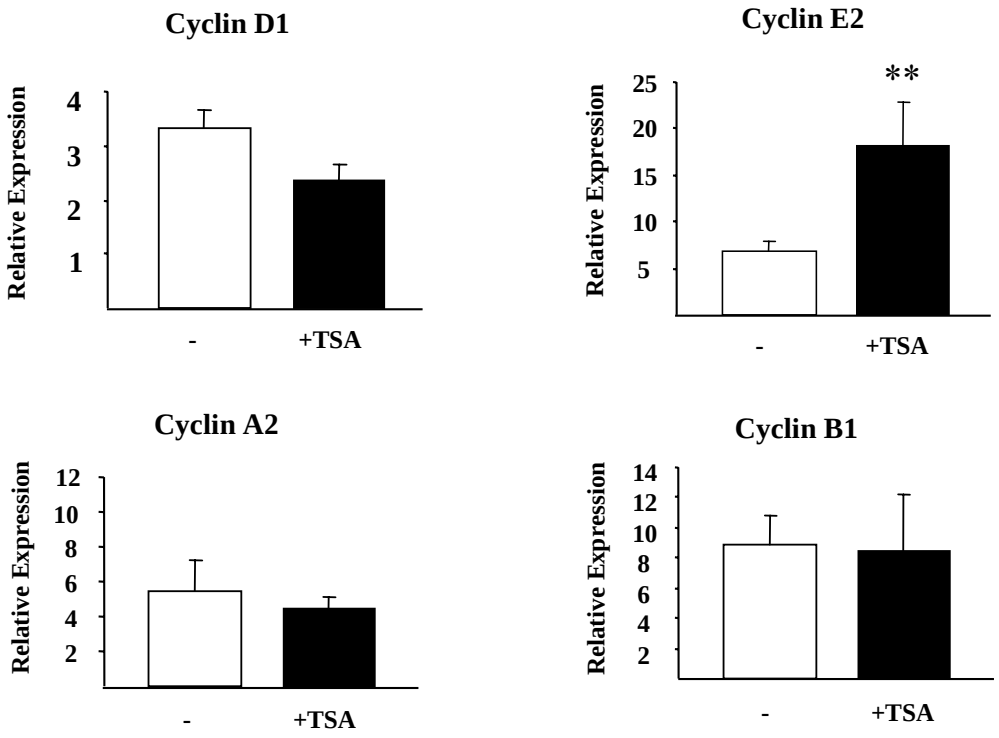


Figure 56. Up-regulation of cyclin E2 in response to HDAC inhibition in LNCaP cells: Quantitative RT-PCR analysis in LNCaP cells following TSA treatment indicate that cyclin E2 expression was significantly up-regulated ($^{**}P<0.001$) while cyclin D1, cyclin A2 and cyclin B1 expression remained unaffected by TSA.

Conclusions and Future Directions:

Based on our preliminary observations, we propose a working model (Figure 57) in which Nkx3.1 regulates cyclin D1 and cyclin E2 expression and cell cycle exit of prostate epithelia, mediated through HDAC1. Our data strongly suggest the reduced HDAC1 mRNA expression, protein expression and enzymatic activity in *Nkx3.1*-deficient prostates compared to the wild type prostates. Our study also indicates that cyclin D1 and cyclin E2 mRNA levels are up-regulated in *Nkx3.1*-deficient mice and NKX3.1 deficient LNCaP cells. As a part of future work, first we will confirm our previous findings. Secondly, we will explore the link between Nkx3.1 and HDAC1. We will investigate the molecular mechanism of how Nkx3.1 may be regulating HDAC1 in cell cycle regulation. Recently, Lei and colleagues have provided evidence that Nkx3.1 engages in cell cycle and cell death machinery via association with HDAC1, leading to increased p53 acetylation and half-life through MDM2-dependent mechanisms (Lei et al., 2005). However, neither the precise transcriptional regulation nor the exact role of cyclins in cell cycle regulation by Nkx3.1 has been explored. It will be interesting to determine if Nkx3.1 directly regulates transcriptional activity of cyclin D1 and cyclin E2. We will perform chromatin immunoprecipitation (ChIP) assays to determine the direct *in vivo* binding of Nkx3.1 at the regulatory regions of cyclin D1 and cyclin E2.

Working model

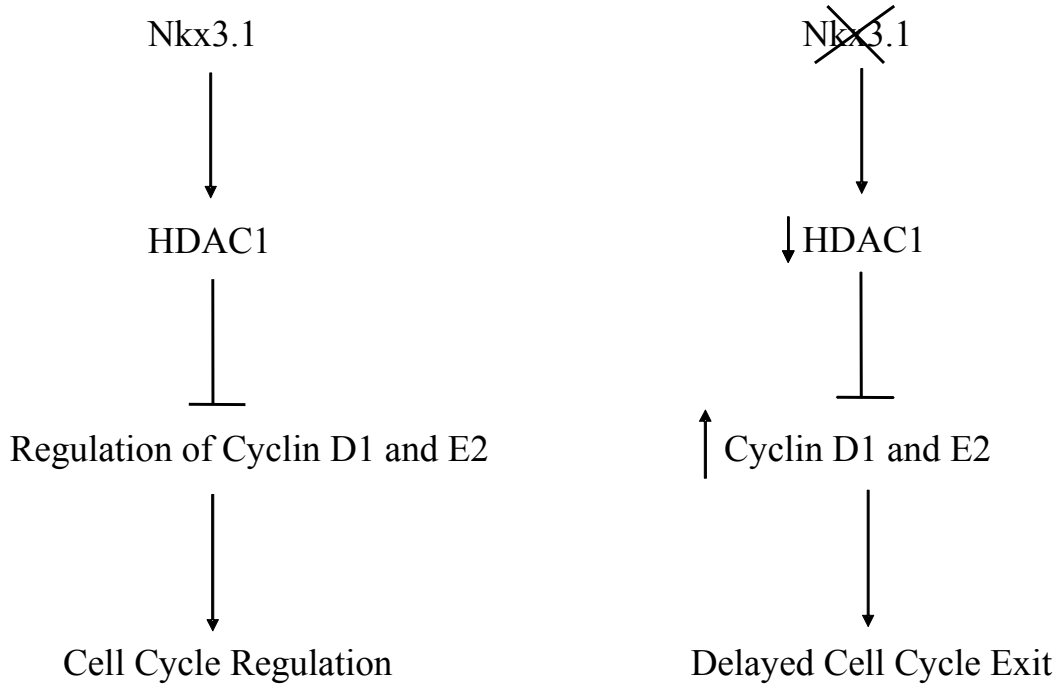


Figure 57. Model for role of Nkx3.1 in cell cycle regulation: We propose a model in which Nkx3.1 regulates cyclin D1 and cyclin E2 expression via HDAC1. Nkx3.1 deficiency leads to HDAC1 deficiency, which can alter cyclin expression resulting into delayed cell cycle exit.

CHAPTER VII

FUTURE DIRECTIONS AND OVERALL CONCLUSIONS

Future Directions:

As my dissertation work, we have investigated a molecular mechanism behind dosage-sensitive and dosage-insensitive Nkx3.1 target gene regulation. Nevertheless, an interesting question still remains unanswered, *why is the intelectin gene sensitive to the loss of Nkx3.1 dosage while the probasin gene is insensitive?* To answer this question, we propose two different hypotheses: 1) The number of functional Nkx3.1 binding sites at these target gene loci is different. I.e. Intelectin gene locus has more Nkx3.1 binding sites than the probasin gene locus or vice versa. 2) Nkx3.1 binds at these target gene loci with differential binding affinity. I.e. At the probasin gene locus Nkx3.1 binds with a stronger affinity than that of the intelectin locus.

To investigate these possibilities, we first calculated the binding affinities (which are presented as a percentage total input of ChIP) of Nkx3.1 at the intelectin (Int5 binding site) and probasin (Pbn1 binding site) promoters. No significant difference was observed in the affinities of Nkx3.1 at the intelectin and probasin promoter at least at the Int5 site versus Pbn1 site (Figure 58). However, this does not rule out binding affinities differences at other Nkx3.1 binding sites at the intelectin and probasin gene loci which we have not yet determined.

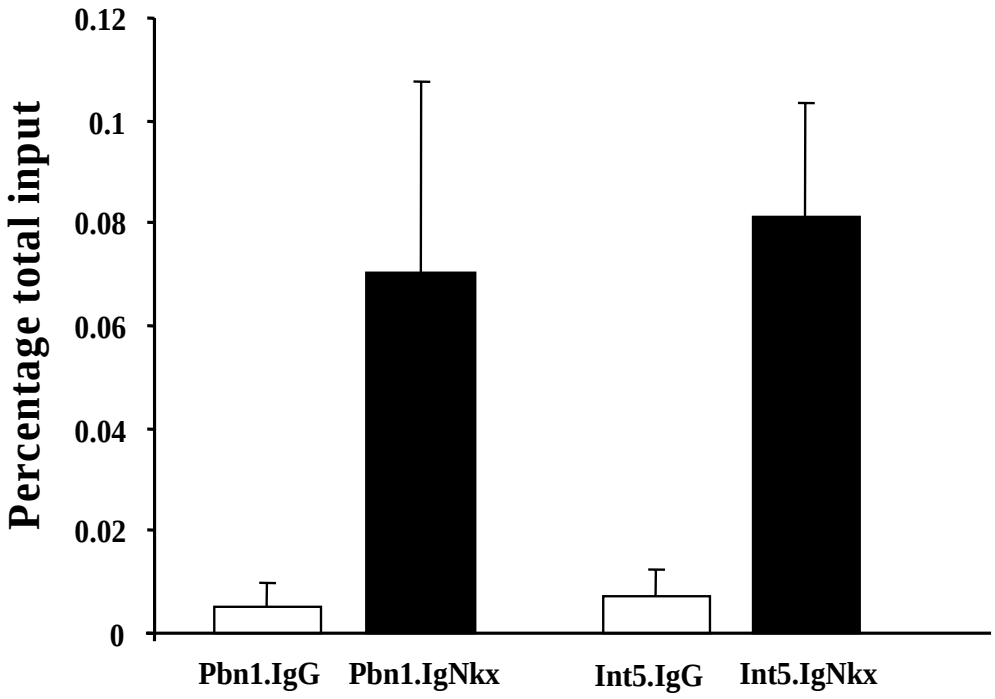
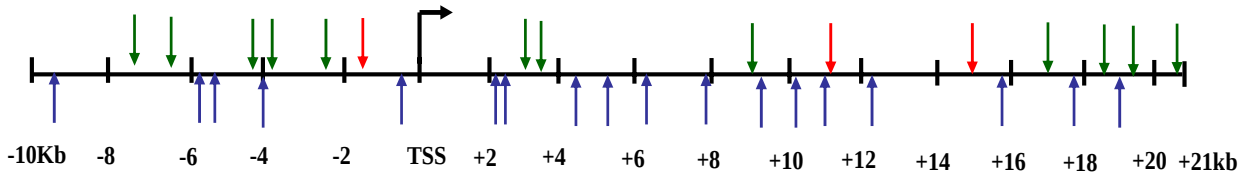


Figure 58. Binding affinities of Nkx3.1 at dosage-sensitive versus dosage-insensitive gene loci: Binding affinities were calculated as percentages of total input from ChIP assays. No difference was observed in the binding affinities of Nkx3.1 at the Int5 site versus Pbn1 site.

After this initial assessment regarding binding site affinities, we next searched for Nkx3.1 putative binding sequences (TAAGTA/G or C/TACTTA) on the 10 Kb upstream and 5 Kb downstream regions of the intelectin and probasin gene loci. We found (15+18 =33) putative sites at the intelectin locus and (10+16 =26) putative sites at the probasin locus. The schematic of the intelectin and probasin gene loci with approximate binding sequence location is shown in (Figure 59).

Intelectin



Probasin

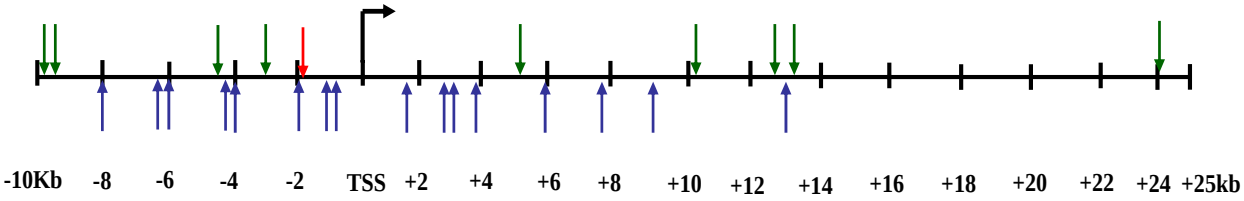


Figure 59. Schematic of the intelectin and probasin gene loci with approximate binding sequence location: We examined the sequences of the intelectin and probasin gene loci and mapped the putative Nkx3.1 binding sites which are represented as arrows. Green arrow: TAAGTG/A; Blue arrow: C/TACTTA; Red arrow: Nkx3.1 bound sites by ChIP analyses (see below); Black arrow: Transcription start site (TSS).

Next we performed ChIP assays in Nkx3.1 mutant prostates to examine *in vivo* binding of Nkx3.1 at these putative sites. Interestingly, by ChIP analysis, we found that 3 out of 33 sites were bound by Nkx3.1 at the intelectin locus. By contrast, only 1 out of 26 sites was bound by Nkx3.1 at the probasin locus (Figure 60). These preliminary results suggest that difference in number of binding sites may underlie the dosage-sensitive or –insensitive nature of Nkx3.1 target genes. However, we need to confirm these binding sites with more number of mice and ChIP assays.

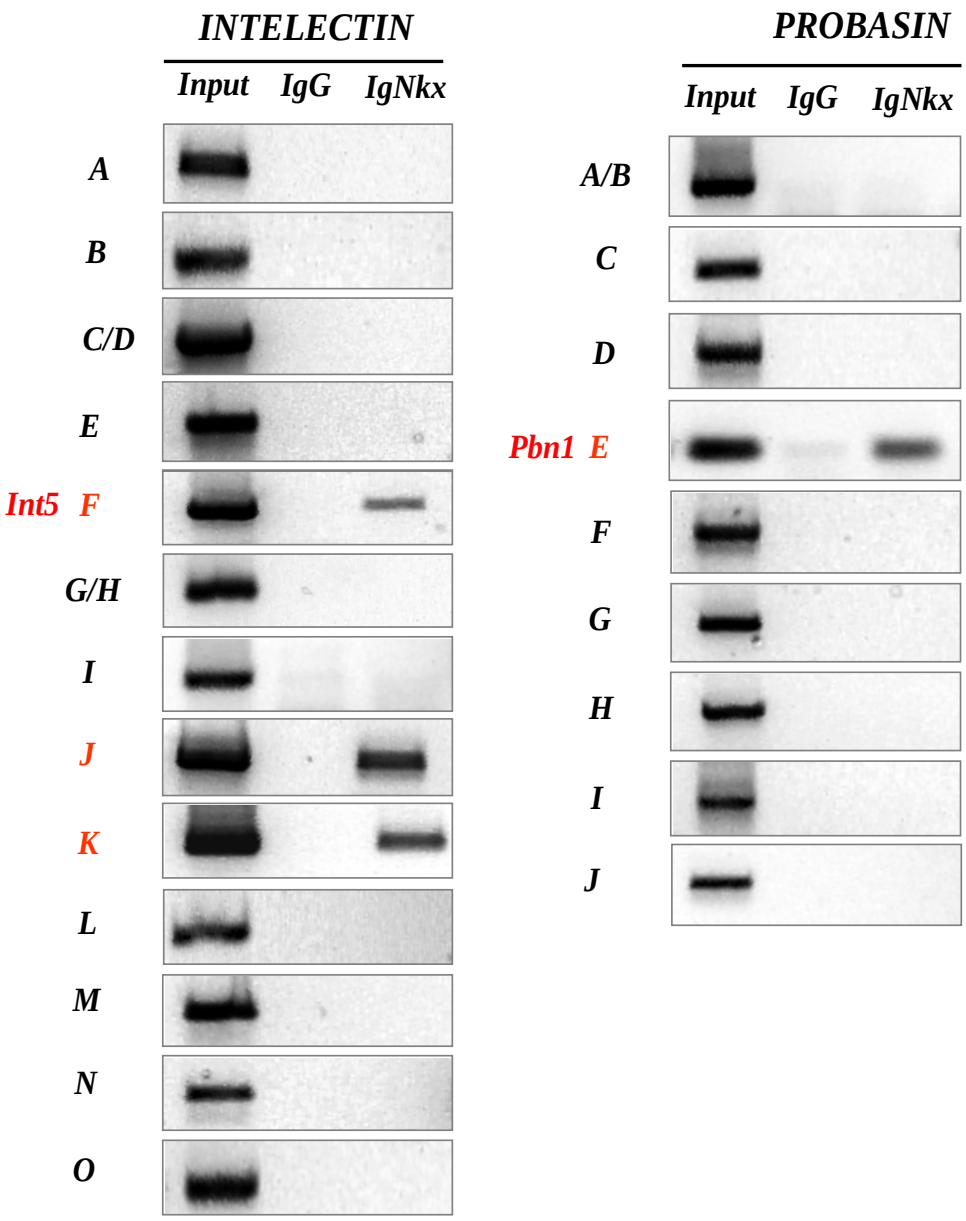


Figure 60. ChIP assays to demonstrate direct *in vivo* binding of Nkx3.1: ChIP assays identify 3 binding sites at the intelectin gene locus and 1 binding site at the probasin gene locus. Note the Nkx3.1 binding at the Int5 and Pbn1 sites which we have demonstrated earlier.

Overall Conclusions:

Magee and colleagues originally proposed a concept of dosage-sensitive, stochastic Nkx3.1 target gene regulation in prostate cancer haploinsufficiency (Magee et al., 2003) however; neither the underlying molecular mechanism nor the functional relevance of Nkx3.1 target genes in prostate cancer has been investigated. Here, we have explored the molecular basis of the dosage-sensitive and stochastic Nkx3.1 target genes and demonstrated the functional significance of one of the Nkx3.1 dosage-sensitive target, intelectin / omentin in prostate tumorigenesis.

We examined the dosage-sensitive and dosage-insensitive Nkx3.1 target gene regulation in *Nkx3.1*-mutant mice which is a model for prostate tumor initiation (Abdulkadir et al., 2002). Based on our results we propose a model in which dosage-sensitive (intelectin) and dosage-insensitive (probasin) target gene loci show differential histone acetylation pattern and differential Nkx3.1/PCAF binding *in vivo* which correlates with their expression. Our results strongly suggest the role for chromatin accessibility in the dosage-sensitive and -insensitive Nkx3.1 target gene regulation. Although our data strongly support a chromatin accessibility model, other possibilities could account for the differential dosage-sensitivity of Nkx3.1 target genes. Formation of differential protein complexes with different interactions at the dosage-sensitive (intelectin) versus dosage-insensitive (probasin) target genes promoters or acetylation of Nkx3.1 itself as a key concentration dependent event can not be ruled out at this point. Nevertheless, our results emphasize the role for epigenetic modifications (histone acetylation) in selective inactivation of dosage-sensitive target genes important for tumorigenesis and other developmental disorders

caused by transcription factor haploinsufficiency. Moreover, we demonstrate direct *in vivo* binding of Nkx3.1 at the target gene promoters by ChIP assays suggesting direct regulation of these genes by Nkx3.1. Lastly, we report a very novel finding that Nkx3.1 can associate with histone acetyl transferase (HAT), PCAF and to our knowledge neither PCAF nor any other HAT has been shown to be associated with Nkx3.1. The finding supports the notion that Nkx3.1 can act as a transcriptional activator *in vivo.*

In addition to the mechanism of dosage-sensitive Nkx3.1 target gene regulation, we explore the functional significance of one of the dosage-sensitive target intelectin / omentin in prostate cancer. Here, we provide evidence that intelectin / omentin can suppress prostate tumorigenicity *in vivo* in prostate cancer. Our series of *in vitro* studies including cell growth analyses, MTT assays and soft agar colony formation assays suggest that intelectin /omentin may have a tumor suppressor-like activity in prostate cells. Consistent with *in vitro* studies, our nude mice xenograft studies indicate that intelectin / omentin is a bona-fide tumor suppressor in prostate cancer *in vivo*. These findings provide the first piece of functional evidence for Nkx3.1 target genes in prostate tumorigenesis. Furthermore, our study underscores the importance of functional characterization of dosage-sensitive and -insensitive Nkx3.1 target genes that could illuminate the molecular pathways involved in prostate tumorigenesis.

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