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<b>14. ABSTRACT</b> During the normal aging process in the prostate a variety of histologic changes are seen including regions termed inflammatory atrophy where mononuclear infiltrating cells are seen adjacent to atrophic glands. Moreover, both clinical and subclinical prostatitis is a risk factor for the development of prostate cancer. This thesis describes a causative link between prostatic inflammatory processes and reductions in expression of the prostate suppressor protein NKX3.1. Reduction of the suppressor protein NKX3.1 by either loss of heterozygosity at 8p21, gene methylation, or both is an early event in prostate cancer. We have found that the inflammatory cytokines TNF-alpha and IL-1beta induce rapid ubiquitination and proteasomal degradation of NKX3.1 in vitro. Within 2 hours of exposing LNCaP cells to TNF-alpha we observed marked reductions of either endogenous or exogenously expressed NKX3.1. Inhibitors of proteasomal degradation blocked the loss of NKX3.1 and allowed accumulation of NKX3.1 protein complexed with polyubiquitin. The C-terminal domain of NKX3.1 representing the 51 amino acids distal to the homeodomain can undergo posttranslational modification to regulate ubiquitination and turnover of NKX3.1. Deletion of the C-terminal domain results in prolonged protein half life and absence of ubiquitination in response to TNF-alpha. The C-terminus directs ubiquitination, but is not the site for ubiquitin ligation since site-directed mutation of either or both of the two lysines (193 and 201) in the C-terminal domain affects neither ubiquitination nor protein half-life. The ubiquitination signal can be localized to amino acids (184-192) in the C-terminal domain and mutation of serine 185 prolonged NKX3.1 half-life, but did not affect TNF-alpha induced ubiquitination. Mutation of serine 196 inhibited TNF-alpha induced ubiquitination and degradation demonstrating differential regulation of NKX3.1 levels by inflammatory cytokines versus basal protein turnover.						
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## I. INTRODUCTION

## **A. Proliferative Inflammatory Atrophy – A Putative Precursor Lesion**

Cellular atrophy in the prostate was first identified as a putative precursor lesion of carcinoma over a half century ago.(1;2) This hypothesis was based largely on observational studies. Similar to carcinoma, atrophic lesions are commonly found in the peripheral zone of the prostate and are associated with increased age.(1;3;4) Despite these findings, the biological behavior and molecular fingerprint of atrophic lesions were unknown. Recently, the cellular kinetics and molecular properties of such lesions were identified and resembled certain characteristics of carcinoma suggesting an evolutionary relationship.

Focal atrophy in the prostate is classified in two forms, postatrophic hyperplasia (PAH) and simple atrophy (SA). Both forms of atrophy contain atrophic, anaplastic secretory cells lining the acinar lumen of the prostatic duct. However, PAH is characterized by crowded, smaller glands and can be differentiated from SA, which have larger, well-spaced glands.(5) Compared to normal prostate tissue, PAH and PA regions are characterized by high levels of proliferation versus benign tissue and low rates of apoptosis.(5) The term “atrophy” in this case refers only to the histological appearance and not biological behavior. In addition to the high rate of proliferation, chronic inflammation was found in 32% of all cases leading to the term proliferative inflammatory atrophy (PIA).(5;6) Analysis of the molecular characteristics of PIA is consistent with proliferation. PIA has increased levels of Bcl-2, an anti-apoptotic protein, increased expression of Ki-67, and reduced levels of the cell cycle inhibitor, p27, findings all consistent with regenerative tissue.(6)

Prostate carcinogenesis involves the progression of normal epithelium to carcinoma through the precursor lesion, prostatic intraepithelial neoplasia (PIN). PIN lesions are identified by proliferating epithelial cells with atypical cytology including increased nuclear size and prominent nucleoli.(7;8) Morphological transitions of PIA into PIN were found 42.5% of samples.(9) Moreover, PIA was found adjacent to or near high grade PIN in 46% of samples.(9) Therefore, almost 90% of high grade PIN lesions were found in close proximity to PIA. In addition to histological studies, molecular analysis also identified PIA as a potential precursor of PIN. *GSTπ* protein levels, which are undetectable in most PIN lesions and carcinomas due to methylation of the *GSTP1* promoter, are globally increased in PIA, possibly as a response to oxidative stress.(6) However, laser microdissected PIA revealed promoter methylation of the *GSTP1* gene in 6.3% of lesions further suggesting that PIA may be an evolutionary precursor of PIN and carcinoma.(10) *P53* mutations were also found in 5.3% of atrophic lesions comparable to the

mutation rate found in high grade PIN (4.2%).(11) Mutations of *P53* were not found in benign prostate tissue.(11) Taken together, this data provides evidence demonstrating the evolution of PIA into PIN, which then gives rise to carcinoma. Although most studies suggest a PIN intermediate, it is unclear whether carcinoma can develop directly from PIA.

## **B. Chronic Inflammation and Prostate Carcinogenesis**

### *Epidemiology*

Inflammation is a vascular and cellular process that serves to protect the host from foreign organisms. The inflammatory process is responsible for the removal of harmful stimuli and for cellular regeneration and healing. Acute inflammation is the initial host response which is characterized by the infiltration of neutrophils and other leukocytes. In the case of a persistent infection or prolonged exposure to a noxious agent, acute inflammation gives way to a chronic process consisting of mature macrophages and lymphocytes. Typically, the inflammatory process subsides as the cellular injury is resolved. However, when it does not subside, many changes seen in inflammation are reminiscent of tissue alterations associated with carcinogenesis, for example connective tissue remodeling and vascularization.(12;13)

Prostatitis may not be associated with an identifiable infectious pathogen and may occur without symptoms which makes diagnosis difficult.(14) Prior to 1999, epidemiological studies and clinical treatment strategies were problematic due to the uncertainties and vagueness of the diagnostic criteria.(15) A national survey of physician visits found that prostatitis was the most common urologic diagnosis in men under the age of 50.(16) Therefore, prostatitis was very common, but there was difficulty diagnosing and treating the condition.(17) In 1999, the National Institute of Health impaneled a group of researchers, advocates and physicians to revise the prostatitis classification system to provide clear criteria for clinical studies.(18) A well defined classification system has facilitated a comprehensive understanding of prostatitis and improved epidemiological studies.(19)

Using the NIH classification system, the relative odds of prostate cancer in men was 1.7 (1.1-2.6) with a history of any prostatitis, 2.5 (1.3-4.7) with acute (Type I) prostatitis, and 1.6 (0.8-3.1) with chronic bacterial (Type II) prostatitis.(20) This confirmed the results of a meta-analysis looking at all studies performed from 1966 through 2000, which calculated the odds ratio of prostate cancer to be 1.6 (1.0-2.4) among men with a history of any prostatitis.(21) These studies suggest a potential link between inflammation and prostate cancer, but a conclusive

association has been difficult to establish due to weaknesses in epidemiological studies such as recall bias, detection bias, or misclassification.(20;21)

Infection is one clear etiology of prostatitis. One study found that 32% of men with detectable levels of bacterial 16S rDNA in a prostate specimen had evidence of prostatic inflammation.(22) Moreover, Krieger et al showed that almost half of patients with Type III prostatitis had evidence of bacterial infection suggesting a causal relationship between infection and inflammation. In addition, it was determined that a history of sexually transmitted disease has an odds ratio of 1.8 for a history of prostatitis.(23) Since inflammation in the form of prostatitis has been linked to cancer, a logical hypothesis is that infection may be a risk factor for cancer. The association between sexually transmitted diseases/infections (STD) and prostate cancer has been speculated since the 1970's.(24) In recent years, two meta-analysis studies showed a history of any STD conferred an increase risk of prostate cancer although this risk was different for each organism.(25;26) Since the risk of prostate cancer was associated with different bacteria and viruses and not one specific organism, this suggested the resulting inflammation and not the infection itself was the true risk factor.(27;28)

Converse to the link between prostatitis and prostate cancer is the notion that anti-inflammatory agents may decrease prostate cancer risk. Clinical trials involving Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) have shown modest reductions in prostate cancer risk.(29) However, during longer-term follow-up, incidence curves come together and there are no statistically significant effects. Thus clinical trials of vitamins, anti-inflammatory drugs, or antioxidants to lower prostate cancer risk have been inconclusive.(30)

### *Genetic Predisposition*

History of prostate cancer in a first degree relative confers a substantial influence on risk for developing the disease. Interestingly, some genes that segregate with familial risk for prostate cancer appear to play a role in inflammation or in response to infection.(31)

RNaseL encoded by *RNASEL/HPCI* is an interferon regulated, 2'-5'linked oligoadenylate dependent endoribonuclease that mediates the antiviral 2-5A system, induces apoptosis, and inhibits proliferation.(32-35) In 1996, a genome-wide search of 66 prostate cancer families linked a region of chromosome 1 (1q24-25) to prostate cancer susceptibility which identified the locus.(36) Germline mutations in *RNASEL/HPCI* (1q25) were found to cosegregate with prostate cancer cases in high-risk families.(37) Carpten et al also showed diminished activity of

the resulting mutant RNase L transcripts that suggested *RNASEL/HPCI* is a potential cancer susceptibility gene. Further studies investigating the effect of RNaseL mutations on prostate cancer risk showed conflicting results. Although the Arg462Gln polymorphism was implicated in 13% of prostate cancers in one study, a recent follow-up study found no effect of the polymorphism on prostate cancer risk.(38;39) However, Li and Tai determined that *RNASEL/HPCI* sequence variants may manifest their effects on prostate cancer risk in the Caucasian population. Caucasian men with the Asp541Glu polymorphism had a significantly increased risk of prostate cancer (OR 1.29; 95% CI, 1.12-1.48). Although the entire effect of *RNASEL/HPCI* on prostate cancer incidence is unclear, a proposed mechanism for prostate carcinogenesis in the presence of a polymorphism or mutation may involve the evasion of tumor cells from a defective apoptotic process.(40-42)

*MSR1* (macrophage scavenger receptor 1) encodes a multidomain, integral membrane receptor capable of binding numerous polyanionic ligands.(43) In addition to binding and removing oxidized Low Density Lipoproteins (LDL), these receptors mediate the innate immune response against bacterial infection.(44) *MSR1* maps to chromosome 8p22 and is shown to undergo loss of heterozygosity (LOH) in 69% of prostate cancers.(45) Linkage analysis studying 159 hereditary prostate cancer families found evidence of a prostate cancer susceptibility locus at 8p22-23.(46) Although analysis of *MSR1* germline mutations found modest evidence linking six rare *MSR1* mutations to hereditary prostate cancer families, mutations occurred with greater frequency in European men with non-hereditary prostate cancer versus unaffected controls (4.4% v. 0.78%, P=0.009).(47) One missense mutation (Asp174Tyr) was found almost exclusively in African American men and occurred with twice the frequency in prostate cancer cases versus controls.(48) This suggests the effect of *MSR1* mutation on prostate cancer risk may be unevenly distributed among racial groups. A potential mechanism for carcinogenesis in the presence of a macrophage scavenger receptor defect may relate to chronic inflammation. Since oxidative stress can increase *MSR1* gene expression and many known ligands of the receptor bind in an oxidation dependent manner, one hypothesis is that a mutated receptor has reduced ability to remove reactive oxygen species.(47;49) Therefore, inability to mediate an inflammatory reaction may potentiate mutagenesis leading to oncogenic damage.

#### *Mediators of Inflammation-induced Genotoxicity*

Acute inflammation involves the initial recruitment of neutrophils followed by the infiltration of monocytes, which mature into macrophages. Activated macrophages then release reactive oxygen species (ROS) through a

process called the respiratory burst. The respiratory burst involves the receptor mediated activation of NADPH oxidase which converts oxygen ( $O_2$ ) to superoxide radical ( $O_2^{\cdot-}$ ) and hydrogen peroxide ( $H_2O_2$ ).<sup>(50)</sup> The free radicals function as antimicrobial agents in an immune response, but the unpaired electrons are also highly reactive with DNA and protein.<sup>(51)</sup> ROS can (1) modify DNA bases causing mutagenesis and (2) induce posttranslational modifications of proteins which affect signaling pathways regulating cell growth and proliferation.<sup>(52)</sup>

Oxidative DNA damage occurs at a rate of approximately 10,000 events/day per human cell.<sup>(53)</sup> Over time DNA repair enzymes are unable to resolve all incurred damage resulting in an age-dependent accumulation of oxidized DNA lesions.<sup>(51;53)</sup> Oxidized DNA bases, including 8-hydroxyguanosine, 2-hydroxyadenine, 5-hydroxycytosine, and 5-hydroxyuracil, have been shown to introduce mutations through replicative mismatches.<sup>(54)</sup> Since prostate cancer primarily affects older men, the effect of ROS on the aging process may interrelate with prostate carcinogenesis.<sup>(55)</sup> This hypothesis is partially supported by the study of two genes, *OGGI* and *GSTP1*.

*OGGI* encodes a DNA glycosylase that excises 8-hydroxyguanosine (8-OH-G), a common lesion that results from exposure of guanosine to ROS.<sup>(56;57)</sup> In the absence of repair, replication of 8-OH-G results in a GC to TA transversion.<sup>(58)</sup> Therefore, mutation or loss of *OGGI* may confer an increase risk of cancer. In the prostate, three polymorphisms of *OGGI* (6803C/G, 7143 A/G, 11657A/G) confer an increased relative risk for hereditary or sporadic prostate cancer (2.72, 5.21, 9.8).<sup>(59)</sup>

One of the best studied genetic modifications of an inflammation related gene is the somatic inactivation of *GSTP1*, which encodes glutathione S-transferase  $\pi$ -class.<sup>(60)</sup> Glutathione S-transferases neutralize electrophilic carcinogens and other reactive oxygen species by conjugation to reduced glutathione.<sup>(61)</sup> Hypermethylation of regulatory sequences in the *GSTP1* promoter was found in 20 of 20 prostate cancer specimens and loss of protein expression occurred in 88 of 91 samples.<sup>(62)</sup> The remarkably high prevalence of *GSTP1* methylation in prostate cancer suggest irrefutably a role for this protein in maintaining the genomic integrity of prostate epithelial cells.<sup>(63)</sup> Inhibition of DNA methyltransferases *in vitro* reverses *GSTP1* promoter methylation and results in the restoration of GST $\pi$  protein expression.<sup>(64)</sup> However, restoring GST $\pi$  expression did not inhibit cell growth suggesting that *GSTP1* is not a tumor suppressor gene.<sup>(64)</sup> In histologic regions of proliferative inflammatory atrophy, levels of GST $\pi$  are elevated and thought to be induced in response to oxidative stress resulting from chronic inflammation.<sup>(6)</sup> In contrast, *GSTP1* hypermethylation occurs in approximately 70% of prostatic intraepithelial neoplasia (PIN) lesions.<sup>(65)</sup>

### *Inflammatory Cytokines*

Inflammatory cells in tumors and surrounding tissue include lymphocytes, macrophages and dendritic cells. Tumor associated macrophages have multiple actions that may contribute to carcinogenesis. Macrophages have been shown to generate nitric oxide, induce angiogenesis, and produce cytokines.(4;66) Among the cytokines commonly produced by macrophages are the proinflammatory cytokines, tumor necrosis factor-alpha (TNF- $\alpha$ ) and interleukin-1 beta (IL-1 $\beta$ ). (67)

TNF- $\alpha$  is a major mediator of inflammation involved in tissue destruction and remodeling.(68) TNF- $\alpha$  may play a role both as a tumor promoter and an apoptosis inducer, which may be dose dependent or cell type specific.(69) Although high doses of TNF- $\alpha$  have been shown to have anti-cancer actions by targeting tumor vasculature, low chronic levels of TNF- $\alpha$  may act as a tumor promoter.(69;70) As a proapoptotic ligand, TNF- $\alpha$  interacts with tumor necrosis factor receptor 1 (TNFR1) and activates a cascade of cysteine proteases.(71) Although TNF- $\alpha$  can induce cell death in prostate cancer cell lines and not benign tissue, severe cytotoxicity limits the efficacy of TNF- $\alpha$  as a therapeutic treatment.(72;73) In addition to the apoptotic pathway, TNF- $\alpha$  can also stimulate cellular proliferation and survival through the same receptor. In mice, homozygous deletion of the TNF- $\alpha$  gene attenuated chemically-induced skin carcinogenesis, but the rates of malignant progression for existing lesions were unaffected suggesting that TNF- $\alpha$  is necessary for cancer initiation in that model.(74) Recent studies suggest TNF- $\alpha$  may induce ROS production and identified TNF- $\alpha$  as an endogenous mutagen.(75;76) The importance of TNF- $\alpha$  in inflammation based cancers is due, in part, to the activation of NF- $\kappa$ B.(77) NF- $\kappa$ B mediates a number of oncogenic cellular activities including proliferation, invasion, and angiogenesis.(78) In hepatocellular cancer, suppression of NF- $\kappa$ B activation using an anti-TNF- $\alpha$  neutralizing antibody induced apoptosis in malignant cells.(79) With respect to the prostate, TNF- $\alpha$  levels have been correlated with loss of androgen sensitivity, a common even in prostate cancer progression.(80) However, the role of TNF- $\alpha$  in the initiation of prostate cancer is not elucidated.

IL-1 $\beta$  is a secreted 17 kDa proinflammatory cytokine formed from the cleavage of the IL-1 gene product by Interleukin-1 $\beta$  Converting Enzyme (ICE, caspase 1).(81) High levels of IL-1 $\beta$  are found in the tumor microenvironment and may be important in the procarcinogenic role of local inflammation.(82) In IL-1 $\beta$  gene

targeted mice, chemical induced tumor incidence was reduced compared to wild type and *Il-1 $\alpha$* <sup>(-/-)</sup> mice.(83) Moreover, IL-1 $\beta$  was shown to be required for tumor invasiveness and angiogenesis and may enhance its own oncogenic properties by inducing the expression of other tumorigenic cytokines.(84;85) IL-1 $\beta$  and TNF- $\alpha$  have related signaling pathways linked through the activation of TNF Receptor Associated Factor (TRAF) and NF- $\kappa$ B-inducing kinase (NIK) and mediating NF- $\kappa$ B activation.(86) The ability of okadaic acid and 12-O-tetradecanoylphorbol-13-acetate (TPA) to induce tumor formation in *Tnf- $\alpha$* <sup>(-/-)</sup> mice may be due to the up regulation of IL-1 $\beta$  suggesting an intrinsic signaling redundancy.(87) This suggests that inhibition of TNF- $\alpha$  signaling cannot completely prevent tumorigenesis due to a compensatory effect of IL-1 $\beta$ . Thus, the carcinogenic potential of each cytokine may be attributed to the activation of a common downstream signaling pathway.

In the prostate, chronic inflammation is characterized by stromal or epithelial lymphocyte and macrophage infiltration.(4;13) Lymphocytic prostatitis has been detected in foci of basal cell hyperplasia, benign prostatic hyperplasia, and prostatic intraepithelial neoplasia.(88;89) Interestingly, in prostatic adenocarcinoma, lymphocytes do not infiltrate malignant glands and remain in adjacent nonmalignant glands, stroma, and premalignant areas.(88) Although other studies have identified tumor-infiltrating lymphocytes in carcinomas, conclusive evidence exists identifying the presence of inflammatory cells, including neutrophils and macrophages, in putative precursor lesions.(13;90;91) In these lesions, cytokines are secreted by the immune cell infiltrate and mediate the inflammatory reaction in a paracrine manner. Therefore, predisposition to adenocarcinoma may result from pathologic exposure to secreted cytokines originating from nearby inflammatory cells.

### C. NKX3.1

#### *NK Family of Homeobox Genes*

Homeotic genes in *Drosophila melanogaster* control anatomical differentiation and mutation at these loci leads to the transformation of one body segment into another unrelated body segment.(92) The complex function of these genes are further underscored in their precise temporal and spatial expression patterns which are necessary for normal development.(93) Interestingly, homeotic genes are clustered on chromosome 3 in two complexes, *Antennapedia*-complex (ANT-C) and *bithorax* complex (BX-C), suggesting that these gene clusters evolved from a common origin.(94;95) The DNA sequences of *antp* of the ANT-C locus, *ubx* of the BX-C locus, and a segmentation gene, *ftz*, all contain conserved sequences. Cross-hybridization using repetitive DNA sequences from

each gene revealed a conserved 189 base pair open reading frame, known as the homeobox, that encoded a 63 amino acid region, the homeodomain.(96) Scott and Weiner also showed a 77% and 81% sequence identity between all three homeoboxes and homeodomains, respectively. With respect to function, a section of the homeodomain was found in a conserved, DNA binding domain of certain bacterial proteins suggesting that homeodomain proteins also bind DNA through a helix-turn-helix motif.(97) The DNA binding ability and function of homeodomain proteins as transcriptional regulators was later also shown in yeast.(98) Therefore, homeodomain proteins may effect development by regulating the transcription of other developmental genes. Although discovered in *Drosophila*, homeobox genes are evolutionarily conserved throughout higher organisms.(99)

In 1989, the NK family of four homeobox genes (NK1, NK2, NK3. and NK4) was discovered in *Drosophila*.(100) Each gene was later renamed to represent their mutational defect: NK1 (*slouch, slou*), NK2 (*ventral nervous system defective, vnd*), NK3 (*bagpipe, bag*), NK4 (*tinman, tin*).(101) NK3/*bagpipe*, which clusters with NK1 and NK4 on *Drosophila* chromosome 3, mediates the differentiation of the visceral mesoderm during the formation of gut musculature.(100;102) In mammals, two homologues of NK3/*bagpipe* have been discovered, *Nkx3.1* and *Nkx3.2*.(103;104) The function of each gene was identified through mutational analysis. Murine *Nkx3.2* (*BapX1*) mutation disrupts the formation of the axial skeleton and also affects the gut musculature, a function of *bagpipe* in *Drosophila*.(105) *Nkx3.1* encodes a homeodomain protein expressed progressively during somitogenesis in murine development and later found in the budding urethral epithelium in newborn mice with restricted expression in the adult prostate.(103;106) A null mutation of *Nkx3.1* resulted in altered ductal morphogenesis in the prostate, bulbourethral glands, and minor salivary glands.(107;108) Most interest in *Nkx3.1* stems from its restricted expression in the prostate potentially effecting development and epithelial proliferation.(107)

### *Structure*

Human *NKX3.1* full length mRNA is approximately 3.5 kb, largely comprised of a long 2575 bp 3' untranslated region.(109;110) The open reading frame of *NKX3.1* spans two exons and encodes a 234 amino acid protein.(109) The NKX3.1 homeodomain is 60 amino acids in length, distally situated near the C-terminus of the protein (AA124-183). The homology of the homeodomain most closely resembled that of NK3 (78% sequence identity) and lead to the name NKX3.1.(109) Four in-frame, alternative splice variants of *NKX3.1* have been detected in prostate tissue cDNA libraries.(110) Only one form of NKX3.1 protein has been found in cells.

### *Function*

The function of NKX3.1 is complex with a broad spectrum of gene regulation. Like NK3, NKX3.1 is a DNA-binding transcriptional regulator.(111) Further investigation identified NKX3.1 as a transcriptional repressor capable of inhibiting the transcription of androgen receptor (AR) in the mouse.(112) Interestingly, many of the known effects of NKX3.1 on transcription are mediated through interaction with another protein. For example, NKX3.1 interacts with prostate derived Ets factor (PDEF) and SP-family proteins to inhibit prostate specific antigen (PSA) transcription and subsequent expression.(113;114) NKX3.1 interacts with serum response factor (SRF) to activate the smooth muscle  $\gamma$ -actin (SMGA) promoter.(115) SRF effects smooth muscle differentiation by controlling gene expression patterns.(116) SRF had previously been shown to interact with Nkx2.5 and Nkx3.2 to affect expression of atrial natriuretic factor and smooth muscle specific gene expression, respectively.(117) Coactivation of the SMGA promoter by NKX3.1 and SRF was partially mediated by regions of NKX3.1 outside of the homeodomain. Residues 29-35 (tinman motif), residues 88-96 (acidic domain), and residues 99-105 (SRF-interacting motif) were observed to physically interact with SRF.(118) Therefore, the function of NKX3.1 is not entirely dependent on the homeodomain and may be modulated by the N-terminal and C-terminal domains. The physiological relevance of the interaction between NKX3.1 and SRF is unclear.

NKX3.1 interacts with the DNA-resolving enzyme, topoisomerase I (topo I), a novel function for a homeodomain protein.(119) Topo I is capable of relaxing positive and negative supercoiled DNA during transcription and replication.(120) Bowen et al showed NKX3.1 enhances the ability of topo I to cleave and relax double stranded DNA. Since topo I is also involved in repairing ultraviolet (UV) induced DNA damage, the ability of NKX3.1 to mediate the function of a DNA repair enzyme suggests that NKX3.1 may protect the genome from oncogenic mutations.(121)

Recent data identified additional functions of NKX3.1 such as stabilizing P53 via interaction with Histone Deacetylase 1 (HDAC1) and inhibiting AKT phosphorylation.(112) Lei et al propose that decreased levels of NKX3.1 lead to reduced P53 protein, activation of the PI3K/AKT pathway, and increased levels of androgen receptor which may promote prostate carcinogenesis in mouse model systems. Moreover, P53 has been show to inhibit androgen induced NKX3.1 induction potentially identifying a negative feedback mechanism regulating P53 and NKX3.1 protein levels.(122)

NKX3.1 interacts with multiple protein partners and regulates transcription of a number of genes. The complexity and depth of NKX3.1 function demonstrates how the loss or reduction of NKX3.1 may alter the molecular homeostasis of prostate epithelial cells.

#### *Candidate Tumor Suppressor Gene*

NKX3.1 is a prostate specific, androgen-responsive homeodomain protein that affects epithelial cell proliferation and differentiation in the prostate.(109;123) The *NKX3.1* gene maps to human chromosome 8p21, a region that frequently undergoes loss of heterozygosity in prostate cancer and leads to decreased NKX3.1 expression in prostate epithelial cells. (45;109;124) Reduced NKX3.1 levels occur at very early stages of malignant transformation and further loss of NKX3.1 expression correlates with tumor progression in human prostate cancer.(125;126) Although no somatic inactivating mutations have been identified, a mutation in the homeodomain of NKX3.1 has recently been discovered that segregates with early onset prostate cancer in a high-incidence family.(127;128) The mutant protein has decreased DNA binding affinity. Another genetic NKX3.1 variant is caused by a polymorphism, C154T, present in 11% of the population. The polymorphic gene codes for a variant protein with a cysteine for arginine substitution at residue 52 and is associated with an increased risk of developing prostate cancer with a higher Gleason score (>7).(129) The polymorphism also confers an increased risk of prostate enlargement.(130)

The growth suppressive properties of NKX3.1 have been validated using gene-targeted mice. Both heterozygous and homozygous deleted *Nkx3.1* mice develop prostatic dysplasia, a premalignant change in the prostate epithelium.(107;131;132) Moreover, *Nkx3.1* loss cooperates with *Pten* loss to enhance the formation of murine prostatic intraepithelial neoplasia (PIN) and development of invasive adenocarcinoma.(133;134) Loss of *Nkx3.1* expression also leads to deregulated levels of antioxidant enzymes and increased oxidative DNA damage, which suggests that *NKX3.1* may function as a caretaker gene in addition to its growth suppressing effects.(135) Since loss of *GSTP1* expression is also an early event in carcinogenesis, reduced levels of NKX3.1 protein may potentiate the development of inflammatory induced prostate cancer via the accumulation of oxidative damage.

#### *Prostatic Inflammation and NKX3.1 Loss*

In human tissue, NKX3.1 levels are reduced in areas of atrophy.(136) Recently, NKX3.1 levels were found to be controlled, in part, by the ubiquitin proteasome pathway in prostate cancer cell lines.(137) Thus, increased ubiquitination and degradation of NKX3.1 may account for reduced protein levels in inflammatory atrophy. Histologic analysis has suggested that cellular transformation occurs adjacent to foci of inflammatory atrophy and therefore the effects of the inflammatory cytokines in reducing NKX3.1 activity could explain how prostate cancer is initiated by some inflammatory reactions.(9) Furthermore, this implies that a pharmacological intervention that maintains NKX3.1 expression levels in prostate epithelium may have a role in prostate cancer prevention or treatment and potentially provides a theoretical underpinning for use of anti-inflammatory agents in prostate cancer prevention.

In contrast to other prostate cancer cell lines, LNCaP cells express NKX3.1 protein which allows for examination of the degradation pathway of the endogenous protein. We will exploit this NKX3.1-expressing cell line to understand the physiologic regulation of NKX3.1 expression in the presence and absence of inflammatory cytokines. Moreover, LNCaP cells are an appropriate cellular milieu to identify the region of NKX3.1 regulating *in vitro* protein turnover. We will selectively use another prostate cancer cell line, PC-3, which does not express NKX3.1, in order to determine whether NKX3.1 levels are differentially regulated between distinct cell lines. The relevance of our biochemical findings to pathogenic events in human prostatic tissue may then be determined by analyzing NKX3.1 expression *in situ* in regions of inflammation compared with nonneoplastic and neoplastic regions of the same gland. In addition, the effect of degradation resistant NKX3.1 mutants on inflammation induced prostate carcinogenesis may be analyzed in transgenic mouse models.

#### *NKX3.1 C-terminus*

Although homeodomain proteins have been well studied, very few have been shown to undergo ubiquitin mediated proteasomal degradation. Vsx-1 is a *paired*-like homeodomain protein which may regulate retinogenesis in zebrafish.(138) Regulation of Vsx-1 levels is mediated by C-terminal dependent ubiquitination.(139) In Kurtzman et al, truncation of the entire C-terminus showed the greatest effect on protein stabilization. Cdx2 is another homeodomain protein that requires an intact C-terminus for ubiquitination. Cdx2, a mammalian orthologue

of Caudal, is involved in intestinal development and differentiation.(140) Unlike Vsx-1, a C-terminal phosphorylation site is the trigger for ubiquitination.(141) Cyclin-dependent kinase 2 phosphorylates Cdx2 on a C-terminal serine residue within a  $\beta$ -catenin degradation motif (four evenly spaced serines).(141) Mutation of these serines abrogated ubiquitination and increased half-life.(141)

NKX3.1 is a 234 amino acid protein consisting of an N-terminus (1-119), homeodomain (120-183), and a C-terminus (184-234). The C-terminus of NKX3.1 represses NKX3.1 regulated transcriptional activity of the smooth muscle  $\gamma$ -actin promoter in the presence and absence of the transcriptional coactivator, Serum Response Factor.(115) Although the C-terminus does not mediate the physical interaction with SRF, a C-terminal truncated form of NKX3.1 had greater transcriptional activity versus wild type suggesting that the C-terminus may inhibit NKX3.1 function.(118) In addition, Carson et al showed the CMV driven expression of exogenous Nkx3.1(1-183) was approximately 10 fold higher than exogenous wild type protein. Since the N-terminus of NKX3.1 contains a phosphorylation site that stabilizes the protein and truncation of the N-terminus lowers exogenous protein expression (unpublished observations), we hypothesized that the removal of the C-terminus may extend protein half-life.(115;137)

#### **D. Phosphorylation-dependent Ubiquitination**

In the late 1970's an ATP dependent proteolysis system called ubiquitination was discovered in rabbit reticulocytes.(142) Ubiquitination is an ATP-dependent, multistep process involving the conjugation of a ubiquitin polypeptide to a lysine residue of a target protein resulting in proteolysis.(143) Ubiquitin is a 76 amino acid protein that is highly conserved from yeast to humans.(144;145) The catalytic transfer of ubiquitin to substrate involves three types of ubiquitin ligases: E1, E2 and E3. E1 or ubiquitin activating enzyme uses ATP to facilitate the covalent bond formation of ubiquitin to a cysteine residue on E1 which puts the ubiquitin molecule in an "activated" state.(146) Ubiquitin is then transferred to an E2 or ubiquitin conjugating enzyme, which can then interact with an E3 ubiquitin ligase.(147) The E3 is primarily responsible for substrate recognition and serves as a docking protein for E2.(148) Ubiquitin is then transferred directly from E2 to the substrate protein completing the ubiquitination process.(143;148)

Ubiquitin can modify proteins as individual units called monoubiquitination or can form polyubiquitin chains. Monoubiquitination regulates protein location and activity whereas the consequence of polyubiquitination is dependent on the manner of intrachain linkage between ubiquitin moieties.(149) The classical mechanism of polyubiquitination involves a polyubiquitin chain linked through lysine 48 which targets the protein for degradation at the 26S proteasome.(150) The 26S proteasome is composed of a 20S catalytic core and a 19S component on each end which recognize the polyubiquitinated proteins.(150;151)

Phosphorylation is a known signal that marks a protein for ubiquitination. Phosphorylated substrates are recognized by receptor proteins such as F-box proteins and recruited to the ubiquitin ligase complex.(152) Many ubiquitination targets are phosphorylated prior to ubiquitination including  $\beta$ -catenin and P53.(153) However, the quintessential example is the degradation of I $\kappa$ B which leads to the activation of NF- $\kappa$ B.(154) Extracellular stimuli, including IL-1 $\beta$  and TNF- $\alpha$ , induce the activation of IKK $\alpha$  and IKK $\beta$ .(155) IKK subsequently phosphorylates I $\kappa$ B leading to the recruitment of a Skp1-Cullin-F-box-type E3 ubiquitin-protein ligase.(156;157) I $\kappa$ B, which is bound to NF- $\kappa$ B in the cytoplasm, is ubiquitinated and degraded. Free NF- $\kappa$ B can then be imported to the nucleus and regulate transcription.(155) Thus, phosphorylation mediates cell signaling pathways through the potentiation of kinase activation and induction of protein degradation via ubiquitination.

NKX3.1 is already known to undergo phosphorylation at serine 48 by protein kinase C and threonine 89 and 93 by CK2, which suggests that NKX3.1 is a target of cellular kinases.(129;137) Since TNF- $\alpha$  activates multiple kinases and induces phosphorylation and degradation of I $\kappa$ B, we investigated the ability of TNF- $\alpha$  to induce NKX3.1 phosphorylation.. Understanding the role of phosphorylation in the ubiquitin mediated degradation of NKX3.1 could foster the development of a pharmacologic intervention designed to inhibit the responsible kinase and maintain NKX3.1 expression.

## **II. MATERIALS AND METHODS**

## **A. Cell culture and reagents**

LNCaP and PC-3 cells were routinely cultured in Improved Minimal Essential Medium (IMEM) supplemented with 5% Fetal Bovine Serum (FBS) (Life Technologies, Gaithersburg, MD) at 37°C in 5% CO<sub>2</sub>. LNCaP cells were treated with 40ng/ml TNF- $\alpha$  (Roche Diagnostics, Indianapolis, IN), 40ng/ml IL-1 $\beta$  (Leinco Technologies, St. Louis, MO), and 40ng/ml IL-6 (Sigma Chemical Co., St. Louis, MO) for 6 hours except when time course experiments were performed. All cytokines were recombinant, human proteins purified from *E.Coli*. For inhibitor experiments, cells were pretreated for 1 hour prior to treatment with TNF- $\alpha$ . MG132, actinomycin D, and TLCK were purchased from Sigma Chemical Co.. Lactacystin was purchased from A.G. Scientific (San Diego, CA) and zVAD was purchased from MP Biomedicals (Aurora, OH). Bortezomib was a generous gift of Millennium Pharmaceuticals (Cambridge, MA). To determine relative NKX3.1 stability, cells were treated with either 10 or 50  $\mu$ M cycloheximide (Sigma Chemical Co.) for 1 hour except where indicated. When testing the effect of TNF- $\alpha$  on exogenous NKX3.1 levels, LNCaP cells were treated with 100 $\mu$ M cycloheximide for 1 hour prior to the addition of TNF- $\alpha$ .

## **B. Plasmid construction and expression**

Full length NKX3.1 and NKX3.1(1-183) cDNA were cloned into the mammalian expression vector, pcDNA3.1 (Invitrogen, Carlsbad, CA), as previously described.(111) A plasmid expressing a full length NKX3.1 protein with a NH<sub>2</sub>-terminal FLAG epitope was constructed.(129) Mammalian expression vectors were engineered to express full length NKX3.1 and COOH-terminal deletion mutants with an NH<sub>2</sub>-terminal MYC epitope. Sequential NKX3.1 deletion mutant cDNA was amplified by PCR, excised on a 1% agarose gel, and purified using a MinElute Gel Extraction Kit (Qiagen, Valencia, CA). Isolated NKX3.1 cDNA was ligated into a cloning vector using a TOPO-TA cloning kit (Invitrogen). NKX3.1 cDNA was digested with *EcoRI* and *XhoI* for directional cloning into pCMV-MYC (Clontech, Mountain View, CA). All point mutated NKX3.1 sequences were generated using a Quickchange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. All constructs were fully sequenced to confirm the presence of the desired mutations and ensure that no additional mutations were generated. Twenty four hours prior to transfection, 750,000 LNCaP and PC-3 cells were plated in FBS-IMEM. Cells were transfected overnight with 500 ng MYC-NKX3.1 expression vector or MYC empty vector, 500 ng GFP expression vector, or 1.5  $\mu$ g of NKX3.1 expression vector or pcDNA empty vector using

lipofectAMINE Reagent (Invitrogen). The transfection media was replaced with FBS-IMEM for 24 hours prior to treatment.

### **C. Western blotting/immunoprecipitation**

Cells were harvested in cold PBS, centrifuged, and resuspended in RIPA buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, .1% (w/v) Sodium dodecyl sulfate (SDS)] with protease inhibitors (Complete Mini, Roche Diagnostics). Fifty  $\mu\text{g}$  of total cellular protein was resolved on 4-20% or 10-20% SDS-polyacrylamide gels (Invitrogen) and transferred to nitrocellulose membranes (Trans-Blot transfer medium; Bio-Rad Laboratories, Hercules, CA). After blocking in 5% milk, membranes were probed with rabbit anti-human NKX3.1 antiserum (1:1000), (126) mouse monoclonal anti-MYC antibody (1:1000) (Santa Cruz Biotechnologies, Santa Cruz, CA), rabbit anti-GFP antibody (1:1000) (BD Biosciences, San Jose, CA), and mouse monoclonal anti- $\beta$ -actin (Sigma Chemicals Co) (1:5000). Membranes were revealed with goat anti-mouse or anti-rabbit HRP-linked secondary antibody (1:5000) (Pierce Biotechnology, Rockford, IL). For immunoprecipitation experiments,  $1.5 \times 10^6$  LNCaP cells were plated in FBS-IMEM. Cells were transfected with 3  $\mu\text{g}$  HA tagged ubiquitin expression vector (gift of Dirk Bohmann) or pcDNA3.1 empty vector. After 24 hours in FBS-IMEM, 10  $\mu\text{M}$  MG132 or vehicle was added to cells for 1 hour followed by treatment with TNF- $\alpha$  for 6 hours. 400  $\mu\text{g}$  total cellular lysate was combined with 3  $\mu\text{g}$  goat polyclonal anti-human NKX3.1 antibody (Santa Cruz Biotechnologies) or goat IgG (Santa Cruz Biotechnologies) in PBS overnight at 4°C. 50  $\mu\text{l}$  of protein A/G slurry was added to the protein-antibody mixture for 2 hours at 4°C with rotation. Each sample was washed three times in cold RIPA buffer and immunoprecipitated protein was eluted in 2X SDS sample buffer with boiling. Ubiquitinated NKX3.1 was detected by mouse monoclonal anti-ubiquitin antibody (1:1000) (Santa Cruz Biotechnologies) or mouse monoclonal anti-HA antibody (1:1000) (Santa Cruz Biotechnologies). Detection of NKX3.1 protein using mouse monoclonal anti-human NKX3.1 antibody (Zymed, San Francisco, CA) (1:750) was used as a loading control.

### **D. NKX3.1 ubiquitination assay**

To assess the ubiquitination of exogenous NKX3.1, a His Bind Purification Kit was used (Novagen, Madison, WI).  $2 \times 10^6$  LNCaP cells were plated in T75 flasks (Corning, Corning, NY) and transfected overnight with 2  $\mu\text{g}$  MYC-NKX3.1 or MYC-empty expression vector and 3  $\mu\text{g}$  of histidine tagged ubiquitin (His-Ub) expression

vector (gift of Dirk Bohmann). Cells were pretreated for 1 hour with 100 nM bortezomib followed by a 6 hour treatment of 40 ng/ml TNF- $\alpha$ . Cells were harvested in cold PBS, pelleted by centrifugation, and resuspended in 1X Binding Buffer (diluted to volume with 6M Urea). Following 10 minute incubation on ice, samples were sonicated and centrifuged for 30 minutes at 14,000 x g at 4°C. The supernatant was incubated with 50  $\mu$ l precharged Ni<sup>2+</sup> agarose bead slurry for 3 hours at room temperature with rotation. Samples were washed four times with 1X Binding Buffer (6M Urea) followed by three wash steps with 1X Wash Buffer (6M Urea). Ubiquitinated proteins were eluted from the Ni<sup>2+</sup> agarose beads with 40  $\mu$ l 4X SDS sample buffer with boiling and resolved on a 4-20% SDS-polyacrylamide gel. Ubiquitinated NKX3.1 was detected using a mouse monoclonal anti-MYC antibody (1:1000) (Santa Cruz Biotechnologies).

#### **E. Reverse transcriptase PCR analysis**

LNCaP cells were plated in IMEM with 5% FBS and grown to 80% confluency. Cells were treated with 40 ng/ml TNF- $\alpha$  for 6 hours and total RNA was extracted using a RNeasy mini kit (Qiagen). NKX3.1 and  $\beta$ -actin primers were added at a final concentration of 0.6 $\mu$ M to 1.5  $\mu$ g template RNA. Qiagen OneStep RT-PCR enzyme mix was used for each reaction. RT-PCR reactions were carried out for the indicated number of cycles. Primer sequences: NKX3.1 Forward – GCCGCACGAGCAGCCA

GAGACA, NKX3.1 Reverse – TTCAGGGCCGGCAAAGAGGAGTG,  $\beta$ -actin Forward – GGCCACGGCTGCTTC,  $\beta$ -actin Reverse – GTTGGCGTACAGGTCTTTGC

#### **F. *In vivo* phosphorylation and immunoprecipitation**

For labeling of endogenous NKX3.1, LNCaP cells were incubated in phosphate-free Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 1 mCi/ml [<sup>32</sup>P]P<sub>i</sub> in carrier-free HCl (Amersham Biosciences, Piscataway, NJ) for 4 hours. Cells were then treated with 40 ng/ml TNF- $\alpha$  for 6 hours and labeled NKX3.1 was immunoprecipitated as described above. Immunoprecipitates were resolved on a 10-20% SDS polyacrylamide gel followed by gel drying. NKX3.1 phosphorylation was measured by autoradiography. Immunoprecipitated NKX3.1 protein levels were detected by western blot analysis and used to determine relative phosphorylation.

### **III. RESULTS**

## **A. TNF- $\alpha$ induced NKX3.1 ubiquitination and degradation**

### **1. Effect of TNF- $\alpha$ on NKX3.1 expression in LNCaP cells.**

In the course of studying the apoptotic effects of TNF- $\alpha$  on LNCaP cells, we observed that TNF- $\alpha$  induced NKX3.1 loss by 24 hours, which is prior to the onset of apoptosis. To better understand the time of TNF- $\alpha$  induced NKX3.1 loss, LNCaP cells were treated with a final concentration of 40 ng/ml over a 24 hour time course (Figure 1). Two hours after exposure to TNF- $\alpha$ , NKX3.1 levels were modestly effected.

The rate of change in NKX3.1 levels relative to  $\beta$ -actin was greatest during the 2 to 6 hour time period and began to plateau at longer exposures.

### **2. Dose dependence of TNF- $\alpha$ induced NKX3.1 loss.**

To rule out a nonspecific mechanism of NKX3.1 downregulation due to cytotoxic levels of TNF- $\alpha$ , we assessed the effect of different TNF- $\alpha$  concentrations. TNF- $\alpha$  reduced NKX3.1 levels at an initial concentration of 5 ng/ml (Figure 2). Reductions in the level of NKX3.1 were enhanced at increasing concentrations of 10, 20, and 40 ng/ml TNF- $\alpha$ . The greatest effect on NKX3.1 protein expression was observed at 40 ng/ml and did not increase at higher concentrations.

### **3. Effect of different proinflammatory cytokines on NKX3.1 expression.**

We sought to determine whether another cytokine induced a similar effect. LNCaP cells were treated with 40 ng/ml IL-1 $\beta$  and NKX3.1 levels were assessed over a 24 hour time course (Figure 3). Although the effect of TNF- $\alpha$  on NKX3.1 was evident 2 hours after exposure, the onset of IL-1 $\beta$  induced NKX3.1 loss was delayed. A reduction in NKX3.1 levels was not observed until 12 hours after IL-1 $\beta$  exposure and a maximum effect was evident at the 24 hour time point. To determine whether the loss of NKX3.1 expression was specific to the TNF- $\alpha$  and IL-1 $\beta$  signaling pathways, we examined the effect of 40 ng/ml interleukin-6 (IL-6). IL-6 did not effect NKX3.1 levels.

### **4. Effect of TNF- $\alpha$ on NKX3.1 mRNA levels.**

LNCaP cells were treated for 6 hours with TNF- $\alpha$  and mRNA levels were assessed by semiquantitative RT-PCR analysis (Figure 4a). TNF- $\alpha$  caused a modest reduction in NKX3.1 mRNA observed after 28 cycles. To determine whether TNF- $\alpha$  was inhibiting transcription or affecting mRNA stability, we assessed the effect of a transcriptional inhibitor on TNF- $\alpha$  induced NKX3.1 loss. LNCaP cells were treated with 4  $\mu$ g/ml actinomycin D

for 1 hour to ensure complete transcriptional inhibition and treated with 40 ng/ml TNF- $\alpha$  over a 5 hour time course (Figure 4b). In comparison to vehicle treatment, TNF- $\alpha$  enhanced NKX3.1 loss. NKX3.1 was not detected after 3 hours of TNF- $\alpha$  treatment versus 5 hours of vehicle treatment. Since TNF- $\alpha$  may be regulating NKX3.1 at multiple levels, we wanted to determine if TNF- $\alpha$  had a posttranslational effect on NKX3.1. Following pretreatment with cycloheximide, a translational inhibitor, LNCaP cells were treated with TNF- $\alpha$  for 6 hours (Figure 4c). In the presence of cycloheximide, TNF- $\alpha$  induced NKX3.1 loss in proportion to the effect seen without translational inhibition suggesting a posttranslational effect.

**Figure 4 Effect of TNF- $\alpha$  on NKX3.1 mRNA levels.**

(A) LNCaP cells were counted and plated at 750,000 cells per T25 flask. Cells were grown to 80% confluency and the media was refreshed prior to treatment. 40 ng/ml TNF- $\alpha$  was added to the cells for 6 hours and total cellular RNA was harvested using a Qiagen RNeasy kit. 1.5  $\mu$ g of RNA per sample was used for semi-quantitative RT-PCR analysis. Samples were made in duplicate for amplification of NKX3.1 and  $\beta$ -actin mRNA. For each set of treatments (untreated and TNF- $\alpha$  treated), the PCR reaction was terminated at different cycle numbers, as indicated, to prevent signal saturation. Results are representative of three separate experiments. (B) 750,000 LNCaP cells were plated in T25 flasks and remained in full serum for 24 hours. Cells were pretreated for 1 hour with 4  $\mu$ g/ml actinomycin D except in the untreated control sample. Following pretreatment, the media was refreshed with IMEM supplemented with 5% FBS. Cells were treated with 40 ng/ml TNF- $\alpha$  or vehicle (PBS) for 1, 2, 3, 4, and 5 hrs and whole cell lysates were subject to western blot analysis. (C) 750,000 LNCaP cells in T25 flasks were pretreated with 50  $\mu$ M cycloheximide or vehicle (dH<sub>2</sub>O) for 1 hour. After pretreatment, cells were stimulated with 40 ng/ml TNF- $\alpha$  for 6 hours and whole cell lysate was harvested. NKX3.1 levels were determined by western blot. Result is representative of two experiments.

**5. Effect of zVAD, TLCK, and MG132 on TNF- $\alpha$ -induced NKX3.1 loss.**

The mechanism of NKX3.1 loss was studied using chemical inhibitors. We determined whether caspase (zVAD), serine protease (TLCK), and proteasome (MG132) inhibitors stabilized NKX3.1 levels in TNF- $\alpha$  treated LNCaP cells. Following pretreatment with zVAD, TLCK, and MG132 for 1 hour, LNCaP cells were exposed to 40 ng/ml TNF- $\alpha$  for 6 hours (Figure 5). 40  $\mu$ M zVAD and 25  $\mu$ M TLCK did not abrogate the effect of TNF- $\alpha$  on

NKX3.1. However, pretreatment with MG132 increased basal levels of NKX3.1 and prevented TNF- $\alpha$  induced NKX3.1 loss suggesting regulation of NKX3.1 protein levels at the proteasome.

#### **6. Effect of different proteasome inhibitors on TNF- $\alpha$ induced NKX3.1 degradation.**

To confirm that TNF- $\alpha$  induced NKX3.1 loss is mediated by the 26S proteasome, we assessed the effect of different proteasome inhibitors on NKX3.1 levels in the presence of TNF- $\alpha$ . LNCaP cells were pretreated with different concentrations of MG132, lactacystin, and bortezomib for 1 hour, followed by the addition of 40 ng/ml TNF- $\alpha$  for 6 hours (figure 6). All three proteasome inhibitors successfully blocked the effect of TNF- $\alpha$  on NKX3.1 loss.

#### **7. Effect of TNF- $\alpha$ on NKX3.1 ubiquitination.**

Since the ubiquitination of target proteins is required prior to proteasomal degradation, we examined the extent of TNF- $\alpha$  induced NKX3.1 ubiquitination. LNCaP cells were pretreated with 10 $\mu$ M MG132 for 1 hour to prevent the degradation of NKX3.1, but allow the ubiquitination of NKX3.1 to occur. Subsequent to pretreatment, LNCaP cells were exposed to 40 ng/ml TNF- $\alpha$  for 6 hours. NKX3.1 was immunoprecipitated and the extent of TNF- $\alpha$  induced ubiquitination of NKX3.1 was determined by western blot analysis (Figure 7a). A control immunoprecipitation with an IgG antibody was performed to identify ubiquitinated, nonspecific proteins. NKX3.1 antibody detected bands at molecular weights much higher than 30 kDa, which may represent polyubiquitinated forms of NKX3.1 (Figure 7a, bottom panel). Moreover, the detection of these high molecular weight forms of NKX3.1 increased in the presence of TNF- $\alpha$ . NKX3.1 ubiquitination was determined by western blot detection of ubiquitin residues. Polyubiquitinated NKX3.1 was detected only in the samples pretreated with MG132 and immunoprecipitated with anti-NKX3.1 antibody (Figure 7a, top panel). Furthermore, the extent of NKX3.1 ubiquitination increased with TNF- $\alpha$  treatment.

We sought to confirm the high molecular weight forms of NKX3.1 in Figure 7a were the result of ubiquitination and not due to the cross-reactivity of the anti-ubiquitin antibody. A mammalian expression vector was engineered to express a fusion protein of ubiquitin with a hemagglutinin (HA) peptide tag on the N-terminus. The HA peptide tag is 9 amino acids (YPYDVDPYA) specifically recognized by an anti-HA antibody. Immunodetection of the HA tag with an anti-HA antibody was used as a surrogate for measuring the ubiquitination of NKX3.1. LNCaP cells were transiently transfected with the HA-ubiquitin expression vector and incubated in full

serum to ensure protein expression. Following proteasomal inhibition with MG132, LNCaP cells were treated with 40 ng/ml TNF- $\alpha$  for 6 hours (Figure 7b). Immunoprecipitated NKX3.1 was ubiquitinated in the presence of MG132 alone and increased with TNF- $\alpha$  treatment (Figure 7b, top panel). In the presence of TNF- $\alpha$  and MG132, NKX3.1 ubiquitination was substantially increased compared to treatment with MG132 alone. Taken together, these results suggest the effect of TNF- $\alpha$  on NKX3.1 degradation is mediated by ubiquitination.

#### **8. Effect of bortezomib on NKX3.1(1-183) in LNCaP cells.**

To determine the effect of the C-terminus on NKX3.1 stability, we investigated the effect of 100 nM bortezomib on exogenous NKX3.1 levels in LNCaP cells. The purpose of this experiment was to measure the increase in NKX3.1 levels after treatment with bortezomib, indicating that NKX3.1 is being targeted for proteasomal degradation. LNCaP cells were transfected with a C-terminal truncated NKX3.1, NKX3.1(1-183), expression construct. Bortezomib had no effect on NKX3.1(1-183) levels suggesting NKX3.1(1-183) is not degraded at the proteasome (Figure 8). Endogenous NKX3.1 levels increased in the presence of bortezomib indicating successful proteasomal inhibition in these cells.

#### **9. Effect of bortezomib on exogenous NKX3.1 and NKX3.1(1-183) in PC-3 cells.**

To demonstrate that bortezomib effects exogenous NKX3.1 protein turnover and C-terminal truncation inhibits protein turnover, we transfected the NKX3.1 negative prostate carcinoma cells line, PC-3, with NKX3.1 and NKX3.1(1-183) expression constructs. After treatment with 100 nM bortezomib for 6 hours, NKX3.1 levels were assessed by western blot (Figure 9). Consistent with effect of bortezomib on endogenous NKX3.1, bortezomib increased exogenous NKX3.1 protein levels suggesting exogenous NKX3.1 is degraded at the proteasome (Figure 9, top panel). As demonstrated previously, NKX3.1(1-183) levels were not effected by bortezomib. Moreover, a longer exposure of the western blot revealed higher molecular weight forms of NKX3.1, which is consistent with ubiquitination (Figure 9, bottom panel). No high molecular weight forms of NKX3.1(1-183) were observed.

#### **10. Relative stability of NKX3.1 and NKX3.1(1-183) in PC-3 cells.**

We sought to determine the relative half-life of NKX3.1(1-183). PC-3 cells were transfected with NKX3.1 and NKX3.1(1-183) expression vectors and treated with cycloheximide over a 2 hour time course. NKX3.1 levels were steadily reduced throughout the time course, but cycloheximide had little effect on NKX3.1(1-183) (Figure 10). NKX3.1(1-183) levels were maintained at the 2 hour time point suggesting a greater relative stability compared to wild type protein.

### **11. Effect of TNF- $\alpha$ on the ubiquitination of NKX3.1 and NKX3.1(1-183).**

Since degradation at the proteasome requires modification with ubiquitin, we measured the extent of NKX3.1(1-183) ubiquitination in the presence and absence of TNF- $\alpha$ . LNCaP cells were transfected with a MYC-tagged NKX3.1 or a MYC-tagged NKX3.1(1-183) expression vector. In addition to the MYC expression constructs, LNCaP cells were cotransfected with a polyhistidine-tagged ubiquitin mammalian expression construct. In this experiment, following pretreatment with 100 nM bortezomib for 1 hour and treatment with 40ng/ml TNF- $\alpha$  for 6 hours, whole cell lysate was incubated with Ni<sup>2+</sup> charged agarose beads. The Ni<sup>2+</sup> charged agarose beads were able to bind the electron rich polyhistidine tag and isolate ubiquitination protein. Following the pull down reaction, ubiquitinated NKX3.1 was detected by western blot using an anti-MYC antibody (Figure 11). MYC-NKX3.1 was ubiquitinated following treatment with bortezomib and increased upon TNF- $\alpha$  stimulation. In contrast, ubiquitinated NKX3.1(1-183) was not detected after treatment with bortezomib alone or in the presence of TNF- $\alpha$ . This result suggests that NKX3.1 ubiquitination may be dependent on the presence of the C-terminus.

### **12. Effect of TNF- $\alpha$ on NKX3.1 and NKX3.1(1-183) levels in LNCaP cells.**

To determine the effect of TNF- $\alpha$  on NKX3.1(1-183) stability, LNCaP cells were transfected with MYC-NKX3.1 and MYC-NKX3.1(1-183) expression constructs. LNCaP cells were pretreated with 100  $\mu$ M cycloheximide for 1 hour to inhibit all CMV-driven protein production and treated with 40 ng/ml TNF- $\alpha$ . At the 60 minute time point, TNF- $\alpha$  enhanced the loss of MYC-NKX3.1 in comparison to vehicle treatment (Figure 12). In contrast, MYC-NKX3.1(1-183) levels were unchanged in the presence of TNF- $\alpha$  versus vehicle throughout the time course. The resistance of NKX3.1(1-183) to TNF- $\alpha$  induced degradation suggests the C-terminus may mediate NKX3.1 protein degradation.

### **13. Role of C-terminal lysines in NKX3.1 degradation.**

We examined the role of NKX3.1 C-terminal lysine residues, 193 and 201, in the turnover of the protein by mutation of each lysine to arginine. PC-3 cells were transfected with mammalian constructs expressing NKX3.1 containing the C-terminal lysine mutations. Relative protein stability was assessed following a 2 hour treatment with 50  $\mu$ M cycloheximide. NKX3.1(1-183) was resistant to degradation and used as a negative control (Figure 13a, inset). Mutation of either lysine residue individually or both in combination did not effect to stability of NKX3.1 (Figure 13a). Degradation of each mutant was comparable to NKX3.1 wild type suggesting that the C-terminus is

not the primary site of ubiquitination for normal cellular turnover of NKX3.1. We tested the effect of TNF- $\alpha$  on the ubiquitination of NKX3.1(K193R,K201R) using the Ni<sup>2+</sup> charged agarose bead pulldown assay in LNCaP cells. TNF- $\alpha$  induced the ubiquitination of NKX3.1 and NKX3.1(1-183) and were used as a positive and negative control, respectively (Figure 13b). Mutation of both C-terminal lysine residues did not attenuate TNF- $\alpha$  induced ubiquitination suggesting the C-terminus does not contain the ubiquitination site involved in TNF- $\alpha$  induced NKX3.1 degradation.

#### **14. Deletion analysis of the NKX3.1 C-terminal domain.**

We sought to identify the region of the C-terminus that governs NKX3.1 ubiquitination and subsequent degradation in the presence and absence of TNF- $\alpha$ . Mammalian constructs were created to express MYC-tagged NKX3.1 with serial deletions of the C-terminus. (Figure 14a) LNCaP cells were transfected with each mammalian expression construct and treated with cycloheximide for 1 hour. The stability of each mutant was assessed by comparing NKX3.1 levels in treated versus untreated samples via western blot analysis. Each deletion mutant had the same relative stability compared with NKX3.1 wild type except NKX3.1(1-192) and NKX3.1(1-183) (Figure 14b). NKX3.1(1-192) levels were partially stabilized and modestly effected by cycloheximide whereas NKX3.1(1-183) was unaffected. NKX3.1(1-183) levels were not reduced following treatment with cycloheximide for 1 hour. To confirm this result, we expressed each deletion construct in LNCaP cells and treated with 100 nM bortezomib for 6 hours. NKX3.1 levels were examined by western blot to determine the degree of proteasomal degradation (Figure 14c). Consistent with the cycloheximide experiment, a modest effect of bortezomib on increased NKX3.1 protein levels was seen on NKX3.1(1-192) and NKX3.1(1-183) levels were unchanged. Taken together these results suggest amino acids 184-192 are the region of the C-terminus mediating protein turnover.

#### **15. Effect of S185A, S195A, and S196A mutation on NKX3.1 stability**

Located within AA184-199 are a number of potential phosphorylation sites (Figure 15a). Using an internet based program, S185, S195, and S196 were identified as being candidate phosphorylation targets (<http://www.cbs.dtu.dk/services/NetPhos/>). S186 scored very low as a potential phosphorylation site and was not studied further. To determine the effect of these sites on NKX3.1 stability, each serine was mutated to an alanine. LNCaP cells were transfected with a MYC-tagged NKX3.1 expression construct containing the desired serine to alanine mutation. After treatment with cycloheximide for 1 hour, relative NKX3.1 levels were assessed by western blot (Figure 15b). NKX3.1(S185A) levels were not effected by cycloheximide after 1 hour suggesting that

disruption of a candidate phosphorylation site may reduce NKX3.1 degradation. NKX3.1(S195A) and NKX3.1(S196A) behaved analogous to wild type NKX3.1. To confirm the effect of the S185A mutation on NKX3.1 stability, transfected LNCaP cells were treated with bortezomib for 6 hours. Bortezomib had little effect on NKX3.1(S185A) levels suggesting that NKX3.1(S185A) degradation at the proteasome is reduced (Figure 15c). Mutation of serines 195 and 196 did not attenuate the effect of bortezomib on NKX3.1 levels.

#### **16. Determination of NKX3.1, NKX3.1(1-183), NKX3.1(S185A), and NKX3.1(S196A) half-life.**

We sought to determine the effect of the S185A mutation on relative stability. The half-life of NKX3.1 sequence variants and point mutants were determined by evaluating protein levels over a 2 hour time course following cycloheximide treatment. NKX3.1 wild type levels steadily decreased with cycloheximide treatment and the protein half-life was determined to be approximately 1 hour (Figure 16). Mutation of serine 185 increased the relative stability of the protein versus NKX3.1 wild type. However, truncation of the entire C-terminus was the most stable of all NKX3.1 variants tested. Although the half-life of NKX3.1(1-183) was 4 hours, the increased stability of NKX3.1(S185A) suggests that serine residue 185 may partially regulate protein turnover. NKX3.1(S196A) had a comparable half-life (59 minutes) to that of NKX3.1 wild type and is not involved in mediating basal protein levels.

#### **17. Effect of S196A mutation on TNF- $\alpha$ induced NKX3.1 ubiquitination and degradation.**

Since mutation of a potential phosphorylation site stabilizes the protein, we investigated the effect of S185A, S195A, and S196A on TNF- $\alpha$  induced ubiquitination. Using the Ni<sup>2+</sup> bead ubiquitination assay, TNF- $\alpha$  increased the ubiquitination of NKX3.1 and NKX3.1(S185A) suggesting that cytokine induced NKX3.1 ubiquitination is regulated on a site other than serine 185 (Figure 17a). Although a serine to alanine mutation at serine 185 increased the relative stability of the protein, the level of ubiquitination in the presence of bortezomib alone was similar to wild type NKX3.1. Mutation of serine 195 reduced the ability of TNF- $\alpha$  to induce ubiquitination and was completely abrogated by a S196A mutation. Since the ubiquitination of NKX3.1(S196A) is not increased in the presence of TNF- $\alpha$ , we determined the effect of S196A mutation on TNF- $\alpha$  induced degradation. In the absence of protein synthesis, mutation of serine 196 attenuated the effect of TNF- $\alpha$  on NKX3.1 loss (Figure 17b). Taken together, these results suggest that serine 196 may be the key site regulating TNF- $\alpha$  induced ubiquitination and degradation.

### **B. Role of phosphorylation in NKX3.1 ubiquitination**

#### **18. Effect of TNF- $\alpha$ on the phosphorylation of NKX3.1.**

We performed an initial *in vivo* phosphorylation experiment to determine the extent of NKX3.1 phosphorylation in the presence of TNF- $\alpha$ . LNCaP cells were metabolically labeled in phosphate free DMEM supplemented with 1 mC/ml [ $^{32}$ P]PO $_4$ . Following the labeling incubation, 40 ng/ml TNF- $\alpha$  was added to the media for 6 hours. Labeled NKX3.1 was immunoprecipitated and the degree of phosphorylation was determined by autoradiography. NKX3.1 levels were measured by western blot and used to determine relative phosphorylation. NKX3.1 was successfully immunoprecipitated and TNF- $\alpha$  induced NKX3.1 protein degradation (Figure 18). Treatment with TNF- $\alpha$  increased endogenous NKX3.1 phosphorylation in the presence of a lowered protein level.

## **V. DISCUSSION**

### **A. Effect of TNF- $\alpha$ on NKX3.1 levels**

The effect of the inflammatory cytokine, TNF- $\alpha$ , on NKX3.1 loss was an incidental finding during apoptotic studies. To determine whether TNF- $\alpha$  induced NKX3.1 loss was part of the apoptotic process, we examined the temporal effect, dose dependence, and mechanism of this finding. Although TNF- $\alpha$  induced apoptosis in LNCaP initially at 48 hours, NKX3.1 levels were substantially reduced by 24 hours.(158) A time course experiment showed an initial effect of TNF- $\alpha$  on NKX3.1 levels at 2 hours, well before the onset of apoptosis. In addition to the temporal effect, we investigated the dose response of TNF- $\alpha$  on NKX3.1 loss. The effect of TNF- $\alpha$  on cell death or survival is dose dependent with chronic, low levels linked to tumor promotion. (159) We found that TNF- $\alpha$  reduced NKX3.1 levels at low concentrations (5 ng/ml) which in prior publications did not induce apoptosis in LNCaP cells.(158) Furthermore, the maximum effect of TNF- $\alpha$  on NKX3.1 levels was observed at 40 ng/ml, a concentration previously shown to induce NF- $\kappa$ B nuclear localization.(158) Taken together, the dose response of NKX3.1 loss is consistent with an antiapoptotic process. With respect to mechanism, TNF- $\alpha$  activates caspase and serine protease activity in LNCaP cells as part of the apoptotic process.(158;160) We found that inhibition of these pathways did not attenuate the effect of TNF- $\alpha$  on NKX3.1 loss. Moreover, this result was consistent with our previous data that TNF- $\alpha$  induced caspase activity beginning at 48 hours and serine protease activity at 24 hours.(158) Evaluation of the timing, dosage, and mechanism suggest that loss of NKX3.1 expression in response to a proinflammatory cytokine is not a consequence of apoptosis.

Inflammatory reactions produce a number of cytokines, including TNF- $\alpha$  and IL-1 $\beta$ , which are implicated in carcinogenesis.(67) In addition to TNF- $\alpha$ , we found that IL-1 $\beta$ , but not IL-6 induced NKX3.1 loss in LNCaP cells. IL-1 $\beta$  and TNF- $\alpha$  utilize TRAF intermediates and mutual downstream mediators which are not shared with IL-6.(161;162) Although IL-6 has been shown to be an autocrine growth factor in prostate cancer cells, its inability to affect NKX3.1 levels suggest that cytokine induced NKX3.1 loss is specific to signaling pathways likely involving IL-1 $\beta$  and TNF- $\alpha$  activated intermediates.(163) While the cytokine profile in prostatic inflammation is heterogeneous and complex, the study of TNF- $\alpha$  as a representative cytokine may elucidate the pattern of early

molecular changes in prostate cells exposed to chronic inflammation.(164) Therefore, we used TNF- $\alpha$  exclusively to investigate the mechanism of NKX3.1 loss.

## **B. Mechanism of TNF- $\alpha$ induced NKX3.1 loss**

Although the major effect of TNF- $\alpha$  on NKX3.1 was posttranslational, we also observed decreased mRNA levels. The ability of TNF- $\alpha$  to degrade NKX3.1 in the presence of both a transcriptional and translational inhibitor suggests that diminished mRNA levels have a minor effect on protein loss in this instance. It is unclear whether the decrease in mRNA levels is the direct result of TNF- $\alpha$  affecting NKX3.1 transcription or if reduced NKX3.1 protein has a secondary effect on its own promoter. Furthermore, in regions of prostate carcinoma with diminished NKX3.1 expression, mRNA was observed to be reduced, unchanged, or increased suggesting mRNA levels are not good predictors of protein expression.(136;165;166) Regardless, the effect of TNF- $\alpha$  at the transcriptional and posttranslational level highlights the complexity of NKX3.1 regulation and is consistent with Bethel et al. In foci of PIA, Bethel et al found NKX3.1 down regulation in the presence of reduced and normal levels of mRNA suggesting different levels of regulation.

At the posttranslational level, we confirmed the results of Li et al demonstrating basal regulation of NKX3.1 via ubiquitin mediated degradation. Moreover, we determined the half-life of NKX3.1 to be approximately 1 hour which differed somewhat from the estimated 80 minute half-life previously shown.(137) TNF- $\alpha$  treatment increased NKX3.1 ubiquitination resulting in protein degradation. Since NKX3.1 is a putative tumor suppressor protein, maintaining protein levels in the presence and absence of TNF- $\alpha$  may have therapeutic value. We found that proteasome inhibition by different proteasome inhibitors increased basal NKX3.1 levels and prevented degradation in the presence of TNF- $\alpha$ . Lactacystin inhibits the 20S and 26S proteasomes by targeting the  $\beta$ -catalytic subunits.(167) In addition, lactacystin is cell permeable, but is not suitable for clinical use due to its irreversibility.(168) Bortezomib is a selective and potent proteasome inhibitor currently used in the treatment of multiple myeloma.(169) Unlike lactacystin, bortezomib is a reversible inhibitor.(170) Bortezomib has been shown *in vitro* to induce cell death and inhibit cell growth in LNCaP cells.(171) In humans, bortezomib alone showed limited therapeutic value in a Phase I trial as a treatment for androgen independent prostate cancer.(172) However, in conjunction with an additional chemotherapeutic agent, bortezomib demonstrated greater antitumor activity.(173)

Use of bortezomib as a chemopreventative agent against prostate carcinogenesis has not been investigated. Although bortezomib increased NKX3.1 levels and prevented TNF- $\alpha$  induced NKX3.1 degradation, the side effects of treatment may prevent the prophylactic administration of bortezomib to protect against prostate cancer development.(172) Therefore, we sought to find a region that directs the ubiquitination of protein and subject this site to mutation analysis.

### **C. Role of the C-terminus in NKX3.1 protein stability**

Using an internet based screening program, we discovered a putative PEST sequence within the N-terminus of NKX3.1 at AA81-97 (<https://emb1.bcc.univie.ac.at/toolbox/pestfind/pestfind-analysis-webtool.htm>). A PEST sequence is a polypeptide region comprised predominantly of proline, glutamic acid, serine, and threonine which targets proteins for rapid degradation primarily at the proteasome.(174) Within the PEST sequence of NKX3.1, CKII has been shown to phosphorylate threonine 89 and 91 and protect the protein from ubiquitin dependent degradation.(137) The stabilization effect of phosphorylation in NKX3.1 is opposite of the phosphorylation dependent degradation induced by CKII in other proteins.(175;176) Phosphorylation of the PEST domain may induce degradation or stabilization of target proteins depending on the conformational changes in structure rather than affecting substrate recognition.(177) In the case of NKX3.1, phosphorylation may increase protein stability by altering the N-terminal secondary structure which may effect the proteasomal degradation initiation site or hide the targeted lysine residues.(178)

The ubiquitination of proteins containing PEST sequences can be regulated by other domains. I $\kappa$ B $\alpha$  contains a C-terminal PEST sequence that regulates intrinsic stability mediated by CKII phosphorylation.(179) However, TNF- $\alpha$  induces phosphorylation and degradation of I $\kappa$ B $\alpha$  at N-terminal residues, serine-32 and -36 which is unaffected by a phosphorylation deficient PEST sequence.(179;180) Thus, certain proteins contain multiple sites directing proteasomal degradation. In NKX3.1, C-terminal truncation can rescue basal protein turnover and TNF- $\alpha$  induced degradation. More specifically, AA184-192 is the major region regulating NKX3.1 levels. A serine to alanine mutation at residue 185 approximately doubled protein half-life compared to wild type. Interestingly, ubiquitination of NKX3.1(S185A) was similar to NKX3.1. This may be attributed to the sensitivity of the Ni<sup>2+</sup> bead ubiquitination assay or the result of monoubiquitination on multiple lysine residues. Deletion of the entire C-terminus extended the half life almost 4 fold suggesting phosphorylation may only partially mediate ubiquitination.

Therefore, despite the presence of the CKII phosphorylation site in the PEST domain, NKX3.1 levels can be mediated through its C-terminal domain. Thus, the existence of multiple phosphorylation sites regulating NKX3.1 turnover may allow for recognition by different kinases or provide a potential hierarchy of regulation. Further analysis is necessary to clarify the differential ability of the C-terminus and PEST sequence to regulate NKX3.1 levels.

The increase in TNF- $\alpha$  induced NKX3.1 ubiquitination was inhibited by a C-terminal serine to alanine mutation at serine 196. Although serine 196 mutation did not affect the half-life of the protein, TNF- $\alpha$  was unable to degrade the S196A mutant. The region of NKX3.1 that mediates TNF- $\alpha$  induced degradation differs from the region controlling basal turnover. Thus, the mechanism of NKX3.1 loss during chronic inflammation is independent from the normal cellular regulation of the protein. Targeting the region affecting TNF- $\alpha$  induced NKX3.1 degradation may stabilize NKX3.1 protein levels in the presence of inflammation without altering the physiologic half-life of the protein.

#### **D. NKX3.1 degradation motif and transcription factor, AP-2 $\alpha$**

The C-terminus of homeodomain proteins is not conserved. However, we have identified a region of NKX3.1 that may function as a degradation signal in other unrelated proteins. Although deletion of 193-199 had only a modest effect on protein stabilization, we included this region in our search. A BLAST analysis of the AA184-199 region of NKX3.1 identified AP-2 $\alpha$  as having a homologous sequence. NKX3.1(184-191) had an 87.5% sequence identity to AP-2 $\alpha$ (134-141) (Figure 19). AP-2 $\alpha$  is a transcription factor implicated in a number of diseases including breast and ovarian cancer.(181;182) In immortalized breast epithelial cells, AP-2 $\alpha$  undergoes ubiquitin mediated degradation resulting in a 1 hour half-life.(183) The half-life of the protein in breast cancer cell lines is increased to approximately 30 hours due to diminished ubiquitination.(183) However, Li et al did not isolate the domain directing the ubiquitination of AP-2 $\alpha$ . Mutation of the NKX3.1 ubiquitination directing domain in AP-2 $\alpha$  would shed important light on the significance of this region on protein turnover.

#### **E. Potential role of NKX3.1 phosphorylation in ubiquitin mediated proteasomal degradation**

Phosphorylation may be a process through which the function and stability of NKX3.1 are regulated. NKX3.1 phosphorylation by protein kinase C on serine 48 reduced DNA binding ability, but did not effect coactivation of SRF.(129) Interestingly, a cysteine for arginine polymorphic substitution at amino acid 52 reduced phosphorylation at serine 48 and may confer an increase in prostate cancer risk.(129) Thus, the potential deleterious effect of the R52C polymorphism may be related to the altered phosphorylation status of the protein. Mutation of another candidate phosphorylation site at threonine 179 in the homeodomain completely abrogated the DNA binding ability of NKX3.1 suggesting that the effect of phosphorylation may be dependent on the domain of the protein.(129)

TNF- $\alpha$  induced phosphorylation is a known trigger for ubiquitination of I $\kappa$ B and served as the basis for investigating NKX3.1 phosphorylation.(155) We showed that NKX3.1 is phosphorylated in response to TNF- $\alpha$  treatment. In addition, NKX3.1 is phosphorylated in the absence of TNF- $\alpha$  which suggests that phosphorylation may also regulate basal NKX3.1 turnover. However, the high level of phosphorylation in the absence of treatment indicates that NKX3.1 has numerous phosphorylation sites which may confound future site-directed mutagenesis experiments attempting to confirm the specific TNF- $\alpha$  phosphorylation site at serine 196 or basal regulation at serine 185.

NKX3.1 contains 23 serines, 12 threonines, and 9 tyrosines of which 31 have been identified as possible phosphorylation sites (Figure 20). Along with candidate phosphorylation sites, several proteins have been identified as potential kinases for each site. No common pathway is evident within the list of putative kinases, but this diversity may reflect the broad range of NKX3.1 function and multiple levels of regulation. Moreover, in addition to TNF- $\alpha$  induced NKX3.1 degradation being inhibited by a serine to alanine mutation at residue 196, mutation of serine 185 extended protein half life suggesting that different kinases may regulate NKX3.1 expression. Therefore, multiple kinases phosphorylating different sites, including CKII induced phosphorylation of threonine 89 and 91, may affect the expression of NKX3.1.

#### **F. Effect of TNF- $\alpha$ induced NKX3.1 loss on carcinogenesis**

TNF- $\alpha$  may contribute to malignancy through a number of mechanisms including the induction of COX-2 expression, production of a network of growth factors, or generation of ROS.(184-186) In particular, TNF- $\alpha$  induced production of ROS caused *in vivo* oxidative stress that was relieved with antioxidant treatment.(76) GSTP1,

a key enzyme responsible for detoxification of free oxygen radicals, was shown to inhibit TNF- $\alpha$  signaling via interaction with TRAF2 suggesting the protective effect of GSTP1 may go beyond free radical removal.(187) However, TNF- $\alpha$  increased GSTP1 expression which may act as part of a negative feedback loop attenuating the effect of TNF- $\alpha$  in an immune response.(188) Since NKX3.1 has been shown to regulate the expression of pro- and antioxidant enzymes, the role of NKX3.1 in maintaining genomic stability may be interrelated with GSTP1 expression.(135) Silencing of GSTP1 expression *in vitro* increased TNF- $\alpha$  induced ASK1 and JNK activation which suggests that increased TNF- $\alpha$  signaling may result from hypermethylation of the *GST $\pi$*  gene *in vivo*.(187) A more active TNF- $\alpha$  signaling axis may increase NKX3.1 degradation. Reduction in NKX3.1 levels may affect the expression of enzymes regulating the removal of free oxygen species which, in the absence of GSTP1 expression, could enhance the formation of oxidative DNA damage. Thus, TNF- $\alpha$  induced NKX3.1 loss may have a profound effects not directly related to its growth suppressive properties.

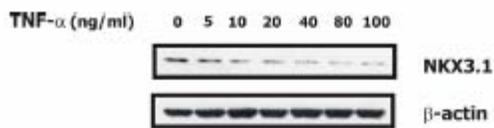
Polymorphisms in the TNF- $\alpha$  promoter have been shown to increase the relative risk of prostate cancer.(189) A GA polymorphism at -308 and 488 increased the relative risk of prostate cancer 14 and 17 fold, respectively.(189) Furthermore, polymorphisms at -308 increased TNF- $\alpha$  production.(190) Our finding of TNF- $\alpha$  induced NKX3.1 loss may provide mechanistic evidence linking the -308 polymorphism in the TNF- $\alpha$  promoter and the resulting increased TNF- $\alpha$  expression with the increase in prostate cancer risk.

Our findings directly link inflammation with simultaneous loss of a suppressor protein that increases the susceptibility of prostate epithelial cells to transformation. Identifying the molecular mechanism of NKX3.1 loss sheds important light on the ability of inflammatory cytokines to regulate NKX3.1 expression and provides an opportunity to design a pharmacological intervention to maintain NKX3.1 expression in the presence of chronic inflammation. The ubiquitin proteasome pathway regulates NKX3.1 expression and is utilized by inflammatory cytokines to enhance degradation which, in the context of proper environmental stimuli, may provide an opportunity for carcinogenesis.



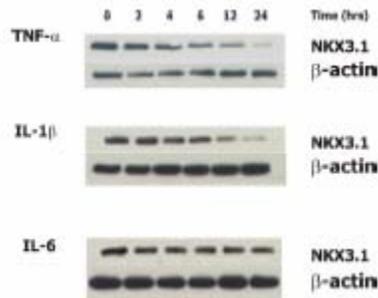
**Figure 1** Effect of TNF- $\alpha$  on NKX3.1 expression in LNCaP cells.

LNCaP cells were plated at 750,000 cells per T25 flask in DMEM supplemented with 5% FBS for 24 hours. Cells were treated with a final concentration of 40 ng/ml TNF- $\alpha$  for 2, 4, 6, 12 and 24 hours. NKX3.1 levels were assessed by western blot and normalized to  $\beta$ -actin levels to determine relative expression. Result shown is representative of two experiments.

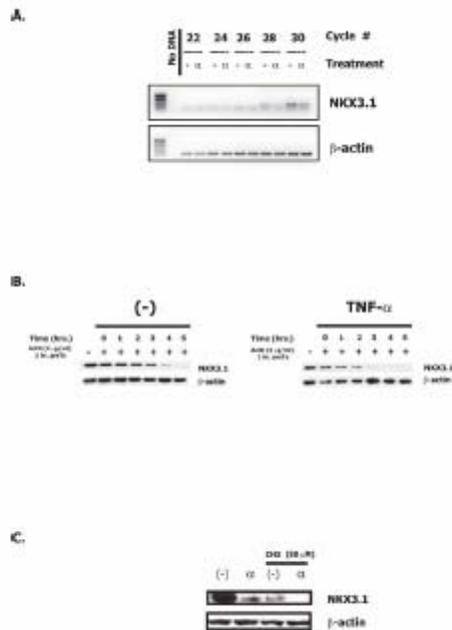


**Figure 2** Dose dependence of TNF- $\alpha$  induced NKX3.1 loss.

LNCaP cells were plated at 750,000 cells per T25 flask in full serum for 24 hours. Different final concentrations of TNF- $\alpha$  were added to each sample for 6 hours. Whole cell extracts were analyzed for NKX3.1 expression by western blot.  $\beta$ -actin levels were used as a loading control.

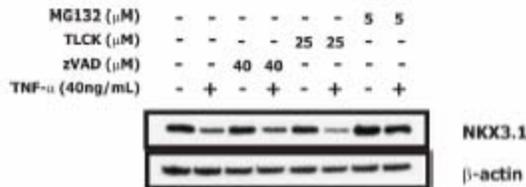


**Figure 3** Effect of three proinflammatory cytokines on NKX3.1 expression. 750,000 LNCaP cells were plated per T25 flask and incubated in full serum for 24 hours. A final concentration of 40 ng/ml IL-1 $\beta$  and IL-6 was added to the cells for 2, 4, 6, 12, and 24 hours. NKX3.1 levels were determined by western blot and normalized to  $\beta$ -actin levels. The effect of TNF- $\alpha$  on NKX3.1 levels, as previously determined by western blot, was included for comparison. Experiments were repeated and data shown is representative of the result.



**Figure 4** Effect of TNF- $\alpha$  on NKX3.1 mRNA levels.

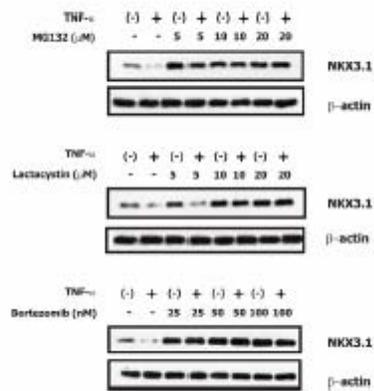
(A) LNCaP cells were counted and plated at 750,000 cells per T25 flask. Cells were grown to 80% confluency and the media was refreshed prior to treatment. 40 ng/ml TNF- $\alpha$  was added to the cells for 6 hours and total cellular RNA was harvested using a Qiagen RNeasy kit. 1.5  $\mu$ g of RNA per sample was used for semi-quantitative RT-PCR analysis. Samples were made in duplicate for amplification of NKX3.1 and  $\beta$ -actin mRNA. For each set of treatments (untreated and TNF- $\alpha$  treated), the PCR reaction was terminated at different cycle numbers, as indicated, to prevent signal saturation. Results are representative of three separate experiments. (B) 750,000 LNCaP cells were plated in T25 flasks and remained in full serum for 24 hours. Cells were pretreated for 1 hour with 4  $\mu$ g/ml actinomycin D except in the untreated control sample. Following pretreatment, the media was refreshed with IMEM supplemented with 5% FBS. Cells were treated with 40 ng/ml TNF- $\alpha$  or vehicle (PBS) for 1, 2, 3, 4, and 5 hrs and whole cell lysates were subject to western blot analysis. (C) 750,000 LNCaP cells in T25 flasks were pretreated with 50  $\mu$ M cycloheximide or vehicle (dH<sub>2</sub>O) for 1 hour. After pretreatment, cells were stimulated with 40 ng/ml TNF- $\alpha$  for 6 hours and whole cell lysate was harvested. NKX3.1 levels were determined by western blot. Result is representative of two experiments.



**Figure 5** Effect of zVAD, TLCK, and MG132 on TNF- $\alpha$ -induced NKX3.1

loss.

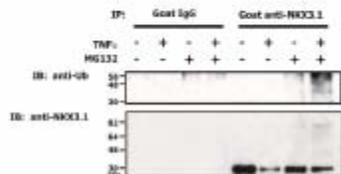
Chemical inhibitors were used to identify the degradation pathway involved in TNF- $\alpha$  induced NKX3.1 loss. 750,000 LNCaP cells were plated and incubated in full serum for 24 hour prior to treatment. Cells were pretreated with vehicle (DMSO), 40 $\mu$ M zVAD, 25  $\mu$ M TLCK, and 5  $\mu$ M MG132 for 1 hour. 40 ng/ml TNF- $\alpha$  was then added for 6 hours and reduction in NKX3.1 levels was determined by western blot. Result is representative of three experiments.



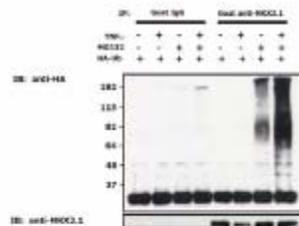
**Figure 6** Effect of different proteasome inhibitors on TNF- $\alpha$  induced NKX3.1 degradation.

The effect of three proteasome inhibitors on TNF- $\alpha$  induced NKX3.1 degradation was measured in LNCaP cells plated at 750,000 cells per T25 flask. Cells were pretreated for 1 hour with different concentrations of MG132, lactacystin, and bortezomib. After proteasomal inhibition, 40 ng/ml TNF- $\alpha$  was added to the indicated samples for 6 hours. NKX3.1 levels were detected by western blot.

**A.**

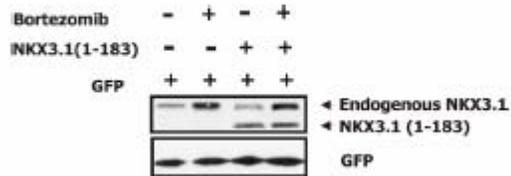


**B.**



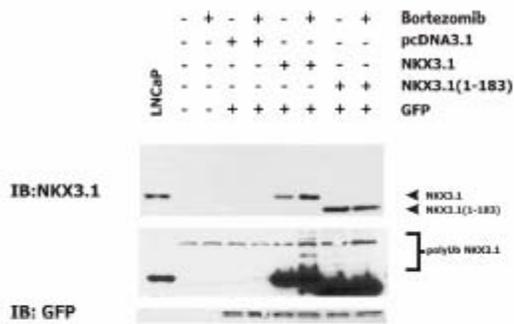
**Figure 7** Effect of TNF- $\alpha$  on NKX3.1 ubiquitination.

(A) LNCaP cells were plated at  $1.5 \times 10^6$  cells per T75 flask in IMEM containing 5% FBS. Following treatment with 10 $\mu$ M MGI32 for 1 hour, 40 ng/ml TNF- $\alpha$  was added for 6 hours. 400  $\mu$ g of total cellular lysate was incubated with 3  $\mu$ g anti-NKX3.1 antibody (or IgG) to isolate NKX3.1. *Top panel:* anti-ubiquitin western blot. *Bottom panel:* anti-NKX3.1 western blot. (B)  $1.5 \times 10^6$  LNCaP cells were plated in T75 flasks in full serum and incubated for 24 hours. Cells were transfected overnight with 3  $\mu$ g HA-ubiquitin expression vector (or pcDNA empty vector). Following transfection, the cells were pretreated with 10  $\mu$ M MGI32 for 1 hour prior to the addition of 40 ng/ml TNF- $\alpha$  for 6 hours. NKX3.1 was immunoprecipitated from 400  $\mu$ g total cellular lysate using 3  $\mu$ g anti-NKX3.1 antibody. A control immunoprecipitation was performed to identify nonspecific signal. *Top panel:* anti-HA western blot. *Bottom panel:* anti-NKX3.1 western blot.



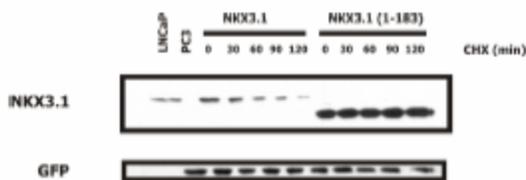
**Figure 8** Effect of bortezomib on NKX3.1(1-183) in LNCaP cells.

750,000 LNCaP cells were plated in T25 flasks and transfected with pcDNA3.1-NKX3.1(1-183) or pcDNA3.1-empty vector and a GFP expression vector. Twenty-four hours posttransfection, cells were treated with 100 nM bortezomib for 6 hours. NKX3.1 levels were determined through western blot analysis. Result is representative of two experiments. GFP levels were used as a loading and transfection control.



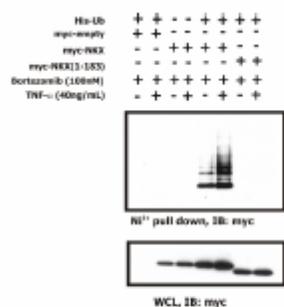
**Figure 9** Effect of bortezomib on exogenous NKX3.1 and NKX3.1(1-183) in PC-3 cells.

750,000 PC-3 cells were transfected with pcDNA3.1-empty vector, pcDNA-NKX3.1, or pcDNA3.1-NKX3.1(1-183) and cotransfected with a GFP expression vector. Cells were treated with bortezomib for 6 hours and total cellular lysates were isolated. NKX3.1 expression was measured by western blot. *Top panel:* short exposure (anti-NKX3.1). *Bottom panel:* long exposure (anti-NKX3.1). GFP levels were used as a transfection and loading control. Result is representative of two experiments.



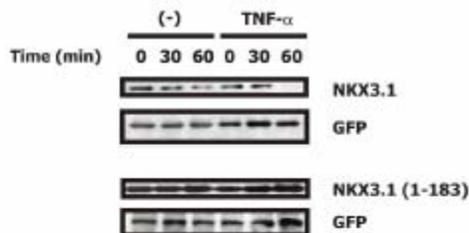
**Figure 10** Relative stability of NKX3.1 and NKX3.1(1-183) in PC-3 cells.

PC-3 cells were plated at 750,000 cells per T25 flask. Cells were transfected with pcDNA3.1-NKX3.1 or pcDNA3.1-NKX3.1(1-183). To determine the relative stability of NKX3.1 and NKX3.1(1-183), cells were treated with 50 $\mu$ M cycloheximide over a two hour time course. NKX3.1 levels were determined by western blot analysis. GFP levels were used as a transfection and loading control. Result is representative of three experiments.



**Figure 11** Effect of TNF- $\alpha$  on the ubiquitination of NKX3.1 and NKX3.1(1-183).

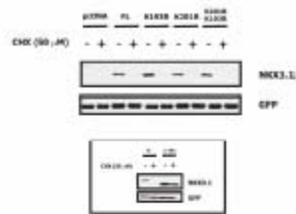
LNCaP cells were cotransfected with MYC tagged NKX3.1 or NKX3.1(1-183) expression constructs and a polyhistidine tagged ubiquitination construct. Cells were pretreated as indicated with 100 nM bortezomib for 1 hour prior to a 6 hour 40 ng/ml TNF- $\alpha$  treatment. Whole cell lysates were incubated with Ni<sup>2+</sup> charged agarose beads to isolate proteins ubiquitinated with exogenous polyhistidine tagged ubiquitin. Following Ni<sup>2+</sup> bead pull-down, ubiquitinated NKX3.1 was identified by western blot using an anti-MYC antibody. Input levels of NKX3.1 were determined by western blot analysis of each total cellular lysate prior to the addition of Ni<sup>2+</sup> beads. Result is representative of two experiments.



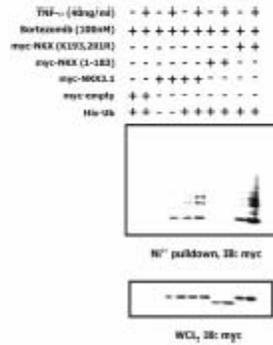
**Figure 12** Effect of TNF- $\alpha$  on NKX3.1 and NKX3.1(1-183) levels in LNCaP cells.

LNCaP cells were transfected with MYC tagged NKX3.1 or MYC tagged NKX3.1(1-183) expression constructs. To inhibit exogenous protein expression, cells were pretreated with 100  $\mu$ M cycloheximide for 1 hour. 40 ng/ml TNF- $\alpha$  was added to the cells for 30 or 60 minutes. NKX3.1 levels were assessed by western blot using an anti-MYC antibody. GFP levels were used as a loading and transfection control. Result is representative of two experiments.

A.



B.



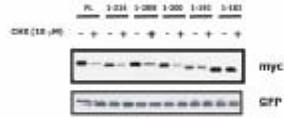
**Figure 13 Role of C-terminal lysines in NKX3.1 degradation.**

(A) 750,000 PC-3 cells were transfected with pcDNA3.1-NKX3.1, -NKX3.1(K193R), -NKX3.1(K201R), -NKX3.1(K193,201R), or -NKX3.1(1-183). Cells were treated with 50  $\mu$ M cycloheximide for 2 hours to ensure complete translational inhibition. NKX3.1 degradation was determined by western blot. *Just*: the effect of cycloheximide on NKX3.1(1-183) levels was used as a negative control. Result is representative of two experiments. (B) The ubiquitination of NKX3.1(K193,201R) was determined in LNCaP cells. Cells were cotransfected with MYC-NKX3.1, MYC-NKX3.1(1-183), or MYC-NKX3.1(K193,201R) expression constructs and a polyhistidine tagged ubiquitin expression construct. 100nM bortezomib was added to the cells to inhibit the proteasomal degradation of polyubiquitinated proteins. Ubiquitinated NKX3.1 was isolated by incubation of the total cellular lysate with precharged  $\text{Ni}^{2+}$  agarose beads for 3 hours. Western blot analysis of the  $\text{Ni}^{2+}$  pull down reaction with an anti-myc antibody revealed the extent of ubiquitination in the presence and absence of TNF- $\alpha$ . Prior to pull-down, NKX3.1 levels in the total cellular lysate were determined by western blot to ensure equal loading.

**A.**



**B.**



**C.**



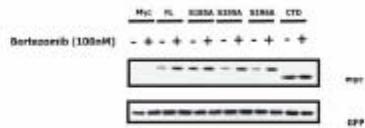
**A.**



**B.**



**C.**

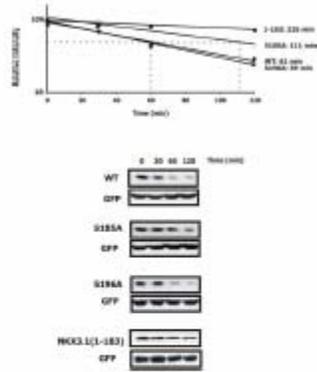


**Figure 14 Deletion analysis of the NKX3.1 C-terminal domain.**

(A) C-terminal amino acid sequence of NKX3.1 and sequential deletions. (B) 750,000 LNCaP cells were transfected overnight with the indicated MYC tagged NKX3.1 deletion construct and a GFP expression construct. To assess the effect of each mutant on NKX3.1 stability, cells were treated with 10  $\mu$ M cycloheximide for 1 hour. Clarified whole cell lysates were subject to western blot analysis to determine relative NKX3.1 levels. GFP levels were used as a loading and transfection control. Result is representative of three experiments (C) LNCaP cells were transfected overnight with the indicated MYC tagged NKX3.1 expression construct and a GFP expression construct. Following transfection, cells were treated with 100 nM bortezomib for 6 hours. The effect of bortezomib on NKX3.1 levels was determined by western blot. GFP levels were used as a loading and transfection control. Result is representative of two experiments

**Figure 15 Effect of S185A, S195A, and S196A mutation on NKX3.1 stability.**

(A) Primary sequence of the NKX3.1 C-terminus. Mutation of serines 185,195, and 196 to alanines are shown. (B) 750,000 LNCaP cells were transfected with different MYC-tagged NKX3.1 constructs and a GFP expression construct. Cells were treated with 10  $\mu$ M cycloheximide for 1 hour and exogenous NKX3.1 protein levels were assessed by western blot. GFP levels were used a loading and transfection control. Result is representative of two experiments. (C) LNCaP cells were transfected with the indicated MYC-NKX3.1 expression vector and treated with 100 nM bortezomib for 6 hours. NKX3.1 levels were assessed by western blot. Cotransfection of a GFP expression construct was employed as a loading and transfection control. Result is representative of two experiments.



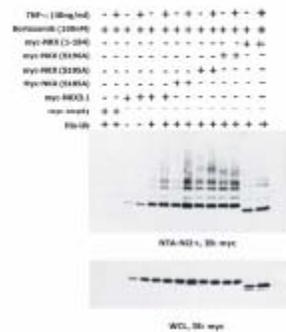
**Figure 16** Determination of NKX3.1, NKX3.1(1-183), NKX3.1(S185A), and NKX3.1(S196A) half-life.

750,000 LNCaP cells were cotransfected with MYC-NKX3.1, MYC-NKX3.1(1-183), MYC-NKX3.1(S185A), or MYC-NKX3.1(S196A) expression constructs and a GFP expression construct. Cells were treated with 10 $\mu$ M cycloheximide over a two hour time course and samples were collected at the indicated times. NKX3.1 levels were determined by western blot using an anti-MYC antibody. NKX3.1 levels were quantified by densitometry and normalized to GFP levels to determine relative expression.

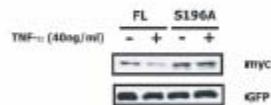
**Figure 17** Effect of S196A mutation on TNF- $\alpha$  induced NKX3.1 ubiquitination and degradation.

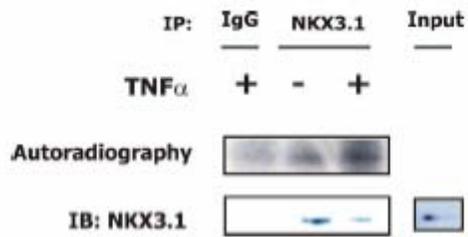
(A) LNCaP cells were cotransfected with MYC tagged NKX3.1, NKX3.1(S185A), NKX3.1(S196A), or NKX3.1(1-183) expression constructs and a polyhistidine tagged ubiquitin construct. Cells were pretreated as indicated with 100 nM bortezomib for 1 hour prior to a 6 hour 40 ng/ml TNF- $\alpha$  treatment. Whole cell lysates were incubated with Ni<sup>2+</sup> charged agarose beads to isolate proteins ubiquitinated with exogenous polyhistidine tagged ubiquitin. Following Ni<sup>2+</sup> bead pulldown, ubiquitinated NKX3.1 was identified by western blot using an anti-MYC antibody. Input levels of NKX3.1 were determined by western blot analysis of each total cellular lysate prior to the addition of Ni<sup>2+</sup> beads. Result is representative of two experiments. (B) 750,000 LNCaP cells were transfected with MYC-NKX3.1 and MYC-NKX3.1(S196A) expression constructs. Cells were pretreated with 100  $\mu$ M cycloheximide for 1 hour followed by a 1 hour treatment with 40ng/ml TNF- $\alpha$ . NKX3.1 levels were determined by western blot with an anti-MYC antibody. GFP levels were used as a transfection and loading control.

**A.**



**B.**





**Figure 18** Effect of TNF- $\alpha$  on the phosphorylation of NKX3.1.

LNCaP cells were plated at 750,000 cells per T25 flask in DMEM supplemented with 5% FBS for 24 hours. For the labeling of endogenous NKX3.1, cells were incubated in phosphate free medium containing 1 mCi/ml  $^{32}$ P-orthophosphate for 4 hours. 40 ng/ml TNF- $\alpha$  was added for 6 hours and cells were harvested and lysed in RIPA buffer. NKX3.1 was immunoprecipitated and visualized by western blot. Phosphorylation of NKX3.1 was determined by autoradiography.

#### IV. DISCUSSION

### **A. Effect of TNF- $\alpha$ on NKX3.1 levels**

The effect of the inflammatory cytokine, TNF- $\alpha$ , on NKX3.1 loss was an incidental finding during apoptotic studies. To determine whether TNF- $\alpha$  induced NKX3.1 loss was part of the apoptotic process, we examined the temporal effect, dose dependence, and mechanism of this finding. Although TNF- $\alpha$  induced apoptosis in LNCaP initially at 48 hours, NKX3.1 levels were substantially reduced by 24 hours.(158) A time course experiment showed an initial effect of TNF- $\alpha$  on NKX3.1 levels at 2 hours, well before the onset of apoptosis. In addition to the temporal effect, we investigated the dose response of TNF- $\alpha$  on NKX3.1 loss. The effect of TNF- $\alpha$  on cell death or survival is dose dependent with chronic, low levels linked to tumor promotion. (159) We found that TNF- $\alpha$  reduced NKX3.1 levels at low concentrations (5 ng/ml) which in prior publications did not induce apoptosis in LNCaP cells.(158) Furthermore, the maximum effect of TNF- $\alpha$  on NKX3.1 levels was observed at 40 ng/ml, a concentration previously shown to induce NF- $\kappa$ B nuclear localization.(158) Taken together, the dose response of NKX3.1 loss is consistent with an antiapoptotic process. With respect to mechanism, TNF- $\alpha$  activates caspase and serine protease activity in LNCaP cells as part of the apoptotic process.(158;160) We found that inhibition of these pathways did not attenuate the effect of TNF- $\alpha$  on NKX3.1 loss. Moreover, this result was consistent with our previous data that TNF- $\alpha$  induced caspase activity beginning at 48 hours and serine protease activity at 24 hours.(158) Evaluation of the timing, dosage, and mechanism suggest that loss of NKX3.1 expression in response to a proinflammatory cytokine is not a consequence of apoptosis.

Inflammatory reactions produce a number of cytokines, including TNF- $\alpha$  and IL-1 $\beta$ , which are implicated in carcinogenesis.(67) In addition to TNF- $\alpha$ , we found that IL-1 $\beta$ , but not IL-6 induced NKX3.1 loss in LNCaP cells. IL-1 $\beta$  and TNF- $\alpha$  utilize TRAF intermediates and mutual downstream mediators which are not shared with IL-6.(161;162) Although IL-6 has been shown to be an autocrine growth factor in prostate cancer cells, its inability to affect NKX3.1 levels suggest that cytokine induced NKX3.1 loss is specific to signaling pathways likely involving IL-1 $\beta$  and TNF- $\alpha$  activated intermediates.(163) While the cytokine profile in prostatic inflammation is heterogeneous and complex, the study of TNF- $\alpha$  as a representative cytokine may elucidate the pattern of early

molecular changes in prostate cells exposed to chronic inflammation.(164) Therefore, we used TNF- $\alpha$  exclusively to investigate the mechanism of NKX3.1 loss.

## **B. Mechanism of TNF- $\alpha$ induced NKX3.1 loss**

Although the major effect of TNF- $\alpha$  on NKX3.1 was posttranslational, we also observed decreased mRNA levels. The ability of TNF- $\alpha$  to degrade NKX3.1 in the presence of both a transcriptional and translational inhibitor suggests that diminished mRNA levels have a minor effect on protein loss in this instance. It is unclear whether the decrease in mRNA levels is the direct result of TNF- $\alpha$  affecting NKX3.1 transcription or if reduced NKX3.1 protein has a secondary effect on its own promoter. Furthermore, in regions of prostate carcinoma with diminished NKX3.1 expression, mRNA was observed to be reduced, unchanged, or increased suggesting mRNA levels are not good predictors of protein expression.(136;165;166) Regardless, the effect of TNF- $\alpha$  at the transcriptional and posttranslational level highlights the complexity of NKX3.1 regulation and is consistent with Bethel et al. In foci of PIA, Bethel et al found NKX3.1 down regulation in the presence of reduced and normal levels of mRNA suggesting different levels of regulation.

At the posttranslational level, we confirmed the results of Li et al demonstrating basal regulation of NKX3.1 via ubiquitin mediated degradation. Moreover, we determined the half-life of NKX3.1 to be approximately 1 hour which differed somewhat from the estimated 80 minute half-life previously shown.(137) TNF- $\alpha$  treatment increased NKX3.1 ubiquitination resulting in protein degradation. Since NKX3.1 is a putative tumor suppressor protein, maintaining protein levels in the presence and absence of TNF- $\alpha$  may have therapeutic value. We found that proteasome inhibition by different proteasome inhibitors increased basal NKX3.1 levels and prevented degradation in the presence of TNF- $\alpha$ . Lactacystin inhibits the 20S and 26S proteasomes by targeting the  $\beta$ -catalytic subunits.(167) In addition, lactacystin is cell permeable, but is not suitable for clinical use due to its irreversibility.(168) Bortezomib is a selective and potent proteasome inhibitor currently used in the treatment of multiple myeloma.(169) Unlike lactacystin, bortezomib is a reversible inhibitor.(170) Bortezomib has been shown *in vitro* to induce cell death and inhibit cell growth in LNCaP cells.(171) In humans, bortezomib alone showed limited therapeutic value in a Phase I trial as a treatment for androgen independent prostate cancer.(172) However, in conjunction with an additional chemotherapeutic agent, bortezomib demonstrated greater antitumor activity.(173)

Use of bortezomib as a chemopreventative agent against prostate carcinogenesis has not been investigated. Although bortezomib increased NKX3.1 levels and prevented TNF- $\alpha$  induced NKX3.1 degradation, the side effects of treatment may prevent the prophylactic administration of bortezomib to protect against prostate cancer development.(172) Therefore, we sought to find a region that directs the ubiquitination of protein and subject this site to mutation analysis.

### **C. Role of the C-terminus in NKX3.1 protein stability**

Using an internet based screening program, we discovered a putative PEST sequence within the N-terminus of NKX3.1 at AA81-97 (<https://emb1.bcc.univie.ac.at/toolbox/pestfind/pestfind-analysis-webtool.htm>). A PEST sequence is a polypeptide region comprised predominantly of proline, glutamic acid, serine, and threonine which targets proteins for rapid degradation primarily at the proteasome.(174) Within the PEST sequence of NKX3.1, CKII has been shown to phosphorylate threonine 89 and 91 and protect the protein from ubiquitin dependent degradation.(137) The stabilization effect of phosphorylation in NKX3.1 is opposite of the phosphorylation dependent degradation induced by CKII in other proteins.(175;176) Phosphorylation of the PEST domain may induce degradation or stabilization of target proteins depending on the conformational changes in structure rather than affecting substrate recognition.(177) In the case of NKX3.1, phosphorylation may increase protein stability by altering the N-terminal secondary structure which may effect the proteasomal degradation initiation site or hide the targeted lysine residues.(178)

The ubiquitination of proteins containing PEST sequences can be regulated by other domains. I $\kappa$ B $\alpha$  contains a C-terminal PEST sequence that regulates intrinsic stability mediated by CKII phosphorylation.(179) However, TNF- $\alpha$  induces phosphorylation and degradation of I $\kappa$ B $\alpha$  at N-terminal residues, serine-32 and -36 which is unaffected by a phosphorylation deficient PEST sequence.(179;180) Thus, certain proteins contain multiple sites directing proteasomal degradation. In NKX3.1, C-terminal truncation can rescue basal protein turnover and TNF- $\alpha$  induced degradation. More specifically, AA184-192 is the major region regulating NKX3.1 levels. A serine to alanine mutation at residue 185 approximately doubled protein half-life compared to wild type. Interestingly, ubiquitination of NKX3.1(S185A) was similar to NKX3.1. This may be attributed to the sensitivity of the Ni<sup>2+</sup> bead ubiquitination assay or the result of monoubiquitination on multiple lysine residues. Deletion of the entire C-terminus extended the half life almost 4 fold suggesting phosphorylation may only partially mediate ubiquitination.

Therefore, despite the presence of the CKII phosphorylation site in the PEST domain, NKX3.1 levels can be mediated through its C-terminal domain. Thus, the existence of multiple phosphorylation sites regulating NKX3.1 turnover may allow for recognition by different kinases or provide a potential hierarchy of regulation. Further analysis is necessary to clarify the differential ability of the C-terminus and PEST sequence to regulate NKX3.1 levels.

The increase in TNF- $\alpha$  induced NKX3.1 ubiquitination was inhibited by a C-terminal serine to alanine mutation at serine 196. Although serine 196 mutation did not affect the half-life of the protein, TNF- $\alpha$  was unable to degrade the S196A mutant. The region of NKX3.1 that mediates TNF- $\alpha$  induced degradation differs from the region controlling basal turnover. Thus, the mechanism of NKX3.1 loss during chronic inflammation is independent from the normal cellular regulation of the protein. Targeting the region affecting TNF- $\alpha$  induced NKX3.1 degradation may stabilize NKX3.1 protein levels in the presence of inflammation without altering the physiologic half-life of the protein.

#### **D. NKX3.1 degradation motif and transcription factor, AP-2 $\alpha$**

The C-terminus of homeodomain proteins is not conserved. However, we have identified a region of NKX3.1 that may function as a degradation signal in other unrelated proteins. Although deletion of 193-199 had only a modest effect on protein stabilization, we included this region in our search. A BLAST analysis of the AA184-199 region of NKX3.1 identified AP-2 $\alpha$  as having a homologous sequence. NKX3.1(184-191) had an 87.5% sequence identity to AP-2 $\alpha$ (134-141) (Figure 19). AP-2 $\alpha$  is a transcription factor implicated in a number of diseases including breast and ovarian cancer.(181;182) In immortalized breast epithelial cells, AP-2 $\alpha$  undergoes ubiquitin mediated degradation resulting in a 1 hour half-life.(183) The half-life of the protein in breast cancer cell lines is increased to approximately 30 hours due to diminished ubiquitination.(183) However, Li et al did not isolate the domain directing the ubiquitination of AP-2 $\alpha$ . Mutation of the NKX3.1 ubiquitination directing domain in AP-2 $\alpha$  would shed important light on the significance of this region on protein turnover.

#### **E. Potential role of NKX3.1 phosphorylation in ubiquitin mediated proteasomal degradation**

Phosphorylation may be a process through which the function and stability of NKX3.1 are regulated. NKX3.1 phosphorylation by protein kinase C on serine 48 reduced DNA binding ability, but did not effect coactivation of SRF.(129) Interestingly, a cysteine for arginine polymorphic substitution at amino acid 52 reduced phosphorylation at serine 48 and may confer an increase in prostate cancer risk.(129) Thus, the potential deleterious effect of the R52C polymorphism may be related to the altered phosphorylation status of the protein. Mutation of another candidate phosphorylation site at threonine 179 in the homeodomain completely abrogated the DNA binding ability of NKX3.1 suggesting that the effect of phosphorylation may be dependent on the domain of the protein.(129)

TNF- $\alpha$  induced phosphorylation is a known trigger for ubiquitination of I $\kappa$ B and served as the basis for investigating NKX3.1 phosphorylation.(155) We showed that NKX3.1 is phosphorylated in response to TNF- $\alpha$  treatment. In addition, NKX3.1 is phosphorylated in the absence of TNF- $\alpha$  which suggests that phosphorylation may also regulate basal NKX3.1 turnover. However, the high level of phosphorylation in the absence of treatment indicates that NKX3.1 has numerous phosphorylation sites which may confound future site-directed mutagenesis experiments attempting to confirm the specific TNF- $\alpha$  phosphorylation site at serine 196 or basal regulation at serine 185.

NKX3.1 contains 23 serines, 12 threonines, and 9 tyrosines of which 31 have been identified as possible phosphorylation sites (Figure 20). Along with candidate phosphorylation sites, several proteins have been identified as potential kinases for each site. No common pathway is evident within the list of putative kinases, but this diversity may reflect the broad range of NKX3.1 function and multiple levels of regulation. Moreover, in addition to TNF- $\alpha$  induced NKX3.1 degradation being inhibited by a serine to alanine mutation at residue 196, mutation of serine 185 extended protein half life suggesting that different kinases may regulate NKX3.1 expression. Therefore, multiple kinases phosphorylating different sites, including CKII induced phosphorylation of threonine 89 and 91, may affect the expression of NKX3.1.

#### **F. Effect of TNF- $\alpha$ induced NKX3.1 loss on carcinogenesis**

TNF- $\alpha$  may contribute to malignancy through a number of mechanisms including the induction of COX-2 expression, production of a network of growth factors, or generation of ROS.(184-186) In particular, TNF- $\alpha$  induced production of ROS caused *in vivo* oxidative stress that was relieved with antioxidant treatment.(76) GSTP1,

a key enzyme responsible for detoxification of free oxygen radicals, was shown to inhibit TNF- $\alpha$  signaling via interaction with TRAF2 suggesting the protective effect of GSTP1 may go beyond free radical removal.(187) However, TNF- $\alpha$  increased GSTP1 expression which may act as part of a negative feedback loop attenuating the effect of TNF- $\alpha$  in an immune response.(188) Since NKX3.1 has been shown to regulate the expression of pro- and antioxidant enzymes, the role of NKX3.1 in maintaining genomic stability may be interrelated with GSTP1 expression.(135) Silencing of GSTP1 expression *in vitro* increased TNF- $\alpha$  induced ASK1 and JNK activation which suggests that increased TNF- $\alpha$  signaling may result from hypermethylation of the *GST $\pi$*  gene *in vivo*.(187) A more active TNF- $\alpha$  signaling axis may increase NKX3.1 degradation. Reduction in NKX3.1 levels may affect the expression of enzymes regulating the removal of free oxygen species which, in the absence of GSTP1 expression, could enhance the formation of oxidative DNA damage. Thus, TNF- $\alpha$  induced NKX3.1 loss may have a profound effects not directly related to its growth suppressive properties.

Polymorphisms in the TNF- $\alpha$  promoter have been shown to increase the relative risk of prostate cancer.(189) A GA polymorphism at -308 and 488 increased the relative risk of prostate cancer 14 and 17 fold, respectively.(189) Furthermore, polymorphisms at -308 increased TNF- $\alpha$  production.(190) Our finding of TNF- $\alpha$  induced NKX3.1 loss may provide mechanistic evidence linking the -308 polymorphism in the TNF- $\alpha$  promoter and the resulting increased TNF- $\alpha$  expression with the increase in prostate cancer risk.

Our findings directly link inflammation with simultaneous loss of a suppressor protein that increases the susceptibility of prostate epithelial cells to transformation. Identifying the molecular mechanism of NKX3.1 loss sheds important light on the ability of inflammatory cytokines to regulate NKX3.1 expression and provides an opportunity to design a pharmacological intervention to maintain NKX3.1 expression in the presence of chronic inflammation. The ubiquitin proteasome pathway regulates NKX3.1 expression and is utilized by inflammatory cytokines to enhance degradation which, in the context of proper environmental stimuli, may provide an opportunity for carcinogenesis.



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