MECHANISMS OF PROSTATE TUMOR INITIATION:

INTERPLAY OF ANTIOXIDANTS AND GENETIC ALTERATIONS

By

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Ad majorem Dei gloriam

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

OVERVIEW

Prostate cancer is the most diagnosed non-skin cancer among American men. Its high prevalence and the lack of an ultimate cure for late-stage disease makes the disease a significant public health concern. Research in the Abdulkadir Laboratory has long focused on the molecular genetics of prostate cancer with the goal of gaining a deeper understanding of this complex disease to improve the health of the thousands affected by prostate cancer each year. As in other cancer types, prostate cancer development occurs with the accumulation of common genetic lesions or changes in gene expression that lead to transformation of cells. These changes include gain of expression or function of oncogenes and loss of expression of tumor suppressor genes. My dissertation work focuses on a tumor suppressor gene whose expression is lost during prostate cancer progression, *NKX3.1*. Serving as a useful mouse model of the very earliest changes which lead to prostate cancer, *Nkx3.1*-deficient mice display early, pre-cancerous lesions.

Our studies attempt to elucidate the mechanisms by which NKX3.1 loss is related to cancer development. One such proposed link is the increase in oxidative stress in Nkx3.1-deficient mouse prostates. Many studies, including clinical trials and mouse models of cancer, have suggested that increased oxidative stress promotes prostate tumorigenesis. As shown in Chapter III, I tested the hypothesis that Nkx3.1 loss-mediated reactive oxygen species (ROS) promote prostate tumorigenesis by quenching ROS in the *Nkx3.1*-deficient prostate. Surprisingly, I found that antioxidant supplementation

increased rather than decreased proliferation in the *Nkx3.1*-deficient prostate. These results resemble the findings of the recent Selenium and Vitamin E Cancer Prevention Trial (SELECT) which showed that the antioxidant vitamin E increased the risk of prostate cancer development in disease-free men. To determine if antioxidant supplementation has a similar effect in the human prostate in the setting of NKX3.1 loss, I tested the influence of polymorphisms in NKX3.1 on prostate cancer risk in the four randomization arms of the SELECT trial. Our findings in Chapter IV highlight the importance of gaining a more thorough understanding of oxidative stress in the development of prostate cancer.

As a transcription factor, another way that NKX3.1 may influence tumorigenesis is through regulation of its direct target genes. Chapter V of my dissertation focuses on the role of one of these target genes, peroxiredoxin 6 (*PRDX6*). PRDX6 is also a direct target gene of the important oncogene MYC. PRDX6 is a dual function enzyme with peroxidase and phospholipase A2 function. In a mouse model with focal high MYC expression, areas of MYC expression are tightly correlated with loss of Prdx6 expression. In order to investigate the role Prdx6 plays in prostate tumor progression, I rescued Prdx6 expression in mouse prostate cancer cell line Myc-CaP, showing that Prdx6 promotes *in vitro* and *in vivo* proliferation and anchorage-independent growth. I show that although high MYC levels are associated with decreased Prdx6 expression in early prostate cancer lesions of the mouse prostate, Prdx6 promotes tumor progression in advanced prostate cancer cells. Thus, the antioxidant protein PRDX6 may have diverse functions throughout tumor progression, highlighting the complexity of the role of antioxidants in prostate cancer. Further studies will be needed to elucidate the role of PRDX6 in human

prostate tumorigenesis and to determine the mechanism of MYC-associated decrease in PRDX6 expression.

My dissertation makes significant contributions to the understanding of antioxidant chemoprevention in prostate cancer and the role of the NKX3.1 and MYC target gene *PRDX6* in prostate tumorigenesis.

CHAPTER II

INTRODUCTION

Prostate anatomy and physiology

The prostate is an exocrine gland surrounding the urethra and the bladder neck in men (Figure 1). The function of the prostate is to produce a slightly alkaline secretion that makes up about one-sixth the volume of seminal fluid of humans (*1*) . These secretions contain metal ions, proteases, and highly charged organic molecules and are believed to promote the survival and motility of sperm in the female reproductive tract (*1*). The human prostate is made up of several major regions, including the central, transition, and peripheral zones (Figure 1). These zones have a differing propensity for cancer development.

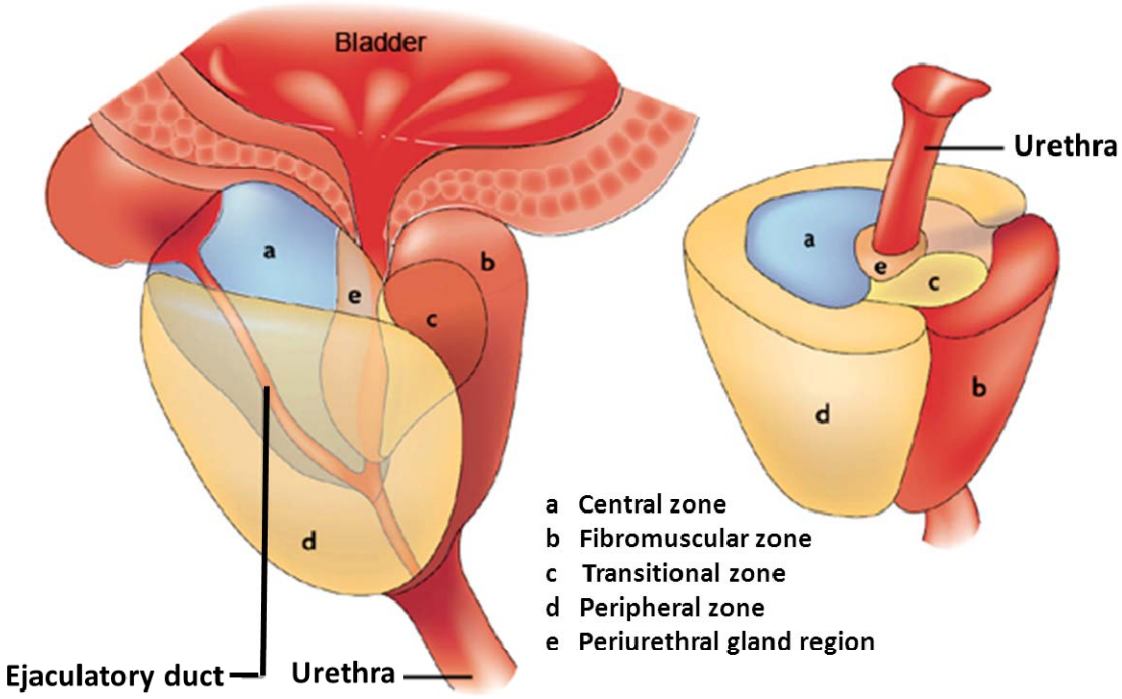

Figure 1. Human prostate anatomy and zones
The human prostate is located inferior to the bladder surrounding the urethra and ejaculatory ducts. Pictured are a sagittal view (left) and a coronal view (right) of the prostate including five prostate zones. The location of the bladder, ejaculatory duct, and urethra are also noted. Figure is adapted from (*2*).

The substructure of the prostate consists of exocrine gland tubules filled with prostatic secretions. The lumen of a prostate tubule is lined with the androgen-dependent, secretory luminal epithelial cells and basal epithelial cells (Figure 2). In the normal human prostate, basal epithelial cells reside beneath luminal epithelial cells in a continuous layer, with contacts to the surrounding basement membrane. The tubules are surrounded by a layer of smooth muscle cells in the stroma, which help to propel the prostatic secretions into the urethra during ejaculation. Also contained in the epithelial cell layer are rare neuroendocrine cells, which produce peptide hormones.

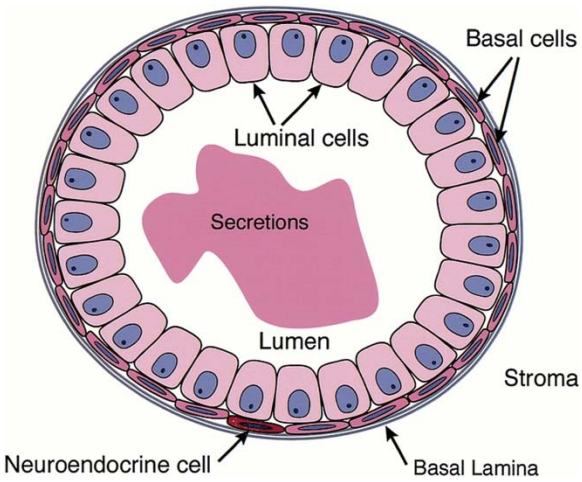

Figure 2. Normal human prostate histology
General cartoon of prostate histology including luminal, basal, neuroendocrine cells, smooth muscle cells, and surrounding basal lamina. The lumen of the normal prostate gland is usually filled with prostatic secretions.

Prostate cancer incidence and mortality

Prostate cancer is the second leading cause of cancer-related death among American men. As the most common non-skin cancer in men, an estimated 238,590 prostate cancer diagnoses will be made in 2013 (*3*). Fortunately, the majority of these cases will remain indolent and not progress to metastatic disease. However, in those cases that do progress, the disease is ultimately incurable. An estimated 29,720 men will die of prostate cancer in the U.S. in 2013 (*3*). There are over 2.5 million men in the U.S. with a history of prostate cancer who are alive, making prostate cancer a major public health concern (*3*). One out of every six American men will be diagnosed with prostate cancer at some point during their lifetime and one out of every 36 men will die from the disease.

Prostate cancer risk factors

At present, the etiology of prostate cancer is not completely understood. Those factors with proven links to the disease include age, race, family history/genetics, and obesity/diet.

Age

The risk of prostate cancer in those over age 65 is almost 14 times higher than those under 65 (*4*). In 2010 (the most recent year assessed) the prostate cancer rate per 100,000 individuals was 10.2 for men 20-49 years old, 310.4 for men 50-64 years old, 858.8 for men 65-74 years old, and 619.6 for men 75 years or older (*3*). Some propose that the correlation of advanced age with prostate tumorigenesis may be due to increased levels of oxidative stress as the body ages (*5–7*). This elevated oxidative stress during aging may be due to decreased antioxidant capacity of cells with age or due to chronic

inflammatory processes. The prostate is an organ with an especially high presence of age-associated inflammation, with the majority of men having subclinical inflammation of some degree in the prostate gland, and clinically evident prostatitis presenting in approximately 16% of the U.S. male population (*8*, *9*). Ultimately, the mechanistic link between age and prostate cancer development is not clear, and possible mechanisms relating elevated oxidative stress and inflammation to prostate cancer development are currently under intense current investigation.

Race

Race strongly influences the chance a man will develop and die from prostate cancer in the U.S. Age-adjusted prostate cancer incidence rates per 100,000 U.S. males tabulated by SEER showed a rate of 144.9 for whites, 228.5 for blacks, 81.8 for Asian/Pacific Islanders, 77.8 for American Indian/Alaskan Natives, and 125.8 for Hispanics (*3*). Mortality rates also greatly vary with age-adjusted prostate cancer mortality rates per 100,000 U.S. males of 21.2 for whites, 50.9 for blacks, 10.1 Asian/Pacific Islanders, 20.7 for American Indian/Alaskan Natives, and 19.2 for Hispanics. Alarmingly, African-American men have the highest rates of prostate cancer incidence in the world (*10*). The reasons for the discrepancies in incidence and mortality rates have been widely discussed, including genetic factors, environmental factors, and socio-economic factors, but no final consensus has been reached (*10*).

Family history/genetics

A positive family history, i.e. having at least one first degree relative with prostate cancer, increases risk 2.4-fold (*11*). Historically, genetics behind this link had not been well described and consistent susceptibility loci had not been identified for a wide

population (*12*, *13*). This may have been due to the fact that several incompletely penetrant prostate cancer susceptibility loci contribute to the prostate cancer phenotype and these combinations of loci are heterogeneous throughout diverse populations (*13*).

Single nucleotide polymorphisms and prostate cancer

However, recent findings from genome wide association studies (GWAS) have identified several single nucleotide polymorphisms (SNPs) which may significantly contribute to increased risk of prostate cancer. SNPs are DNA bases at specific loci that vary among individuals of a species. SNPs may confer alterations to gene function (if present regions of DNA which code for protein) or expression (if present in regulatory regions of DNA). SNPs can affect tendency to manifest a specific phenotype, i.e. prostate cancer development. Recent studies have found several SNPs on chromosome 8q24, housing the oncogene *MYC*, which may account for a large portion of hereditary prostate cancer (*14*, *15*). SNPs in other genes found to be linked to increased prostate cancer risk in GWAS studies include β-microseminoprotein (*MSMB*), *NKX3.1*, and G-protein coupled receptor family C group 6 member A *(GPRC6A*) (*16*).

Obesity/diet

Another set of risk factors that have been described in prostate tumorigenesis are obesity and dietary factors. Obesity is thought to alter hormone levels (such as testosterone and leptin), which may contribute to prostate tumor development (*17*). Obesity has been clearly shown to increase risk of recurrence and mortality from prostate cancer (*18*, *19*). However, only a weak, non-significant correlation has been observed between body mass index (BMI) and prostate cancer incidence (*20*, *21*). Intake of animal fat, red meat, and dairy fat have been shown to increase the risk of total or advanced

prostate cancer, while fish and seafood intake are negatively correlated with prostate cancer risk (*17*). The reasons for the association may include altered circulating levels of hormones due to increased fat mass (*22*), and the presence of mutagenic compounds formed during the cooking of meat such as heterocyclic amines and polycyclic aromatic hydrocarbons (*23*). The fact that the well-established prostate cancer risk factors are either non-modifiable or modifiable only with difficulty makes prostate cancer a truly challenging disease to prevent.

Cigarette smoking

There is no consistent effect of cigarette smoking on prostate cancer risk (*24*). Some studies have shown as much as a 30% increased risk in prostate cancer with cigarette smoking (*25*). Another study has shown different results, indicating duration of cigarette use and cumulative amount of cigarette use are not related to increased risk (*26*). There is a consensus, however, that current and former smokers have an increased risk of mortality due to prostate cancer (*24*). The most definitive prospective study to show this was the Health Professionals Follow-Up Study (*27*). This study found that current smokers had an increased risk of prostate cancer-specific mortality compared to never smokers (HR, 1.61; 95% confidence interval [CI], 1.11-2.32) and also had an increased risk of biochemical recurrence (*27*). Interestingly, those who had quit smoking for more than 10 years or had quit less than 10 years prior but had smoked less time overall did not have a significant increase in prostate cancer-specific death, indicating that current use of cigarettes has the most meaningful effect on prostate cancer-associated death (*27*). The link between current cigarette use and prostate cancer progression and death is not understood.

Prostate cancer diagnosis

Screening

Prostate cancer is diagnosed by several techniques. The first screening technique which has been used historically is the digital rectal exam (DRE). The American Urological Association (AUA) recommends that men over the age of 40 with a life expectancy of more than 10 years have a yearly DRE to examine if the prostate has nodules, hardening, gross asymmetry, or gland fixation. The second screening technique is serum prostate specific antigen (PSA) screening, which detects the PSA protein, a protein expressed and secreted only from prostate epithelial cells. PSA is present at higher levels in the blood when the prostate is growing abnormally. The test was made widely available in the late 1980s and early 1990s and has greatly increased the proportion of prostate tumors that are caught at a very early, treatable stage. The AUA also suggests that PSA screening be performed yearly on men over 40.

While PSA screening does increase the number of early stage tumors detected, it also increases the number of tumors found that would have remained indolent for the life of the individual, not invading or metastasizing, until the death of the man by other causes. Therefore, PSA screening can result in overtreatment. Side effects of prostate cancer treatment can include urinary incontinency, sexual impotency, bowel dysfunction, and loss of fertility which may have been avoided if a tumor was destined to remain indolent and did not require treatment. Thus, in 2012 the U.S. Preventive Services Task Force reported that the PSA screening in prostate cancer diagnosis of relatively healthy men was not recommended (*28*). Nevertheless, DRE and PSA remain as the major

screening modalities to detect prostate cancer at as early a stage as possible. The inability to reliably determine the clinical outcome of a tumor at diagnosis, and the necessity for treatment, is a major challenge in prostate cancer.

Biopsy

Upon a positive DRE or a repeated PSA above 4.0 ng/L, a prostate biopsy is performed to assess stage and grade of the tumor (*29*). Generally, 8-12 evenly spaced cores of prostate tissue are taken to survey the presence of cancer in the gland. Prostate cancer is commonly multifocal, with an average presence of five independent loci at diagnosis (*30*). A "Gleason Pattern" is given by a pathologist to represent the histological appearance and differentiation of the prostate (*31*). The scores range from 1 to 5, with 5 being the least differentiated and most aggressive cancer tissue. The "Gleason Score" is given by providing the sum of the most prevalent pathology type, with the next most prevalent type (e.g. 4 + 3 = 7) (*31*).

Prostate cancer treatment

Low-risk prostate cancer treatment - prostatectomy and radiation

Using the Gleason Score, DRE findings, and sometimes information from an imaging modality such as transrectal ultrasound (TRUS), doctors estimate the grade of the tumor and discuss treatment options for the patient following guidelines accepted by clinical practice (*32*). There are no definitive rules for treatment and much is decided as a discussion between patient and doctor comparing the mortality risk with the side effect risk, taking into consideration the patient's life expectancy and current health status.

Patients with low risk tumors, i.e. with a Gleason score of less than six, a low tumor stage, and a PSA ≤ 10 ug/L, may opt for "active surveillance." Active surveillance consists of no immediate treatment, but instead monitoring status by PSA and DRE every 6 to 12 months followed by a biopsy if changes are observed in either test (*29*).

Patients with a low-risk tumor may also opt for radical prostatectomy, removal of the entire prostate gland. This can be curative, but comes with possibility of side effects such as incontinency and impotency. However, recent minimally invasive nerve-sparing laparoscopic and robotic-assisted surgery has decreased side effect occurrence (*33*).

Another primary treatment option for low risk disease, with similar results as prostatectomy, is brachytherapy (*34*). This is performed by putting radioactive seeds into the prostate and irradiating the tissue to ablate the functional prostate gland. The vast majority of low-risk prostate cancer patients are cured by radical prostatectomy or brachytherapy, with 10-year biochemical free recurrence of about 80-90% each (*34*), (*35*).

Definitive grading of the prostate histology is possible after radical prostatectomy due to having access to the entire gland. This allows for the determination of whether additional therapy would be beneficial to the patient. If the tumor was found to have spread beyond the prostate gland, invading nearby tissue or regional lymph nodes, androgen deprivation therapy is often begun immediately after surgery (*29*). In addition, external beam radiation may be started 3-6 months post-surgery if the tumor was determined to be invasive from positive surgical margins (*29*).

Androgen-deprivation therapy

PSA testing is used to monitor for recurrence after surgery or radiotherapy. A rise in the PSA of 0.2 ng/L after surgery or 2 ng/L after radiation is considered biochemical recurrence. If androgen-deprivation therapy (ADT) was not already started immediately post-surgery due to tumor invasion outside the prostate, it will be started upon biochemical recurrence. ADT involves decreasing the action of androgens in the body by either inhibiting their circulating levels or their ability to carry out their normal biological effects (*36*). ADT causes inhibition of tumor growth and PSA to fall in almost all cases. However, in most cases there is recurrence of tumor growth, with an average survival of 5 years after PSA rise following initial ADT in patients with no evidence of metastatic disease (*37*). A prostate tumor which is no longer inhibited by lack of androgens is termed castrate-resistant prostate cancer (CRPC) and has often metastasized to distant sites. The most common site for prostate cancer metastasis is bone, followed distantly by liver and lung (*37*).

CRPC treatments

Treatment for CRPC is not curative but can only prolong survival for a short period of time. The overall survival time for men with CRPC is 2-3 years (*38*). In the last decade, docetaxel chemotherapy with prednisone has been the standard treatment for CRPC (*32*). Docetaxel prolongs survival for an average of 3 months (*39*). Many studies are investigating additional therapies for CRPC. In 2012, a new anti-androgen enzalutamide was approved for treatment of CRPC. Enzalutamide (also called MDV3100) is an androgen receptor antagonist which binds AR more strongly than previous antiandrogens and prevents translocation of AR to the nucleus (*40*).

Enzalutamide increased survival in CRPC patients after chemotherapy almost 5 months over placebo (*41*). A recently FDA-approved immunotherapy called Sipuleucil-T activates the body's own immune system against prostate cancer cells (*42*). Other treatments that have shown a survival benefit in Phase III clinical trials include novel taxanes, other androgen signaling inhibitors, and bone-directed agents (*43*). Unfortunately, all of these agents prolong survival for only 4 months or less.

Challenges in prostate cancer

There are several major challenges in the prostate cancer field. First, the challenge of distinguishing tumors which will remain indolent from those that will quickly progress to metastatic disease is a major clinical problem. While at least 70% of those diagnosed with prostate cancer will not progress to metastatic disease, the current grading and staging of tumors is not completely accurate in identifying high-risk patients. Many patients are over treated, while some may have benefited from more aggressive therapy. Secondly, effective therapies for each stage of the disease are needed to increase survival and ultimately to completely prevent progression to end stage disease. Ultimately, a prevention method is greatly desired, to decrease the widespread prevalence of the disease and avoid the high treatment-related morbidity and health care costs associated with prostate cancer. However, to determine an efficacious prevention method, a more thorough understanding of prostate tumor progression is needed.

Prostate cancer etiology

Prostate cancers are primarily adenocarcinomas, or cancers of the prostate glandular epithelial cells. Despite the prevalence of the disease, the mechanisms behind prostate carcinogenesis are not completely understood. The two major mechanisms behind prostate tumorigenesis that are highly investigated include androgen-driven mechanisms and oxidative stress-driven mechanisms.

Androgen-driven mechanisms

The prostate gland is an organ whose development and function is largely dependent on circulating androgens. The androgen receptor (AR) is a transcription factor that is expressed in the epithelial and stromal compartments of the prostate. In normal physiology, activation of the androgen receptor via hormone binding causes it to translocate to the nucleus to mediate its effects on gene transcription. The global set of androgen receptor target genes has been identified via ChIP-seq technology (*44*, *45*) and include genes involved in prostate growth and differentiation. Androgen treatment *in vitro* promotes cell proliferation, differentiation, and survival (*46*, *47*).

The vast majority of prostate tumors are androgen-dependent at diagnosis and when androgens are depleted, tumors strongly regress, supporting androgen mediation of prostate cancer (*36*). The regression usually lasts for 1-3 years (*48*), but inevitably, almost all tumors recur, with an average survival of 9-13 months post-recurrence (*49*). This suggests that the normal androgen signaling, mediated by circulating androgens binding normally to AR, has become aberrant and is fueling cancer growth. This can occur through mutations to the AR gene that allow the protein to respond to other ligands

or become ligand-independent (*50*). Therefore, while androgens drive prostate tumor growth, tumors often attain mutations which allow them to proliferate in the absence of androgens. Other factors must be identified to find an effective treatment of hormone-refractory tumors.

Oxidative stress-driven mechanisms

Elevated oxidative stress is present in many cancer types, and prostate cancer is no exception (*5*, *51*, *52*). Oxidative stress, damage which occurs in cells due to an excess of ROS, can result from an overproduction of ROS or an incomplete quenching of ROS. ROS, class of free radicals, are highly reactive chemicals containing oxygen with at least one unpaired electron in their outer shell. Some level of ROS are required for normal cellular functions, but an imbalance of ROS levels often lead to substantial cellular pathology.

Sources of ROS

ROS can be generated through several mechanisms in the cell. ROS are generated as normal byproducts of cellular respiration (*53*). In some cases, poor functioning of the electron transport chain allows further elevated levels of oxidative radicals to be formed (*53*). Several pro-oxidant enzymes, such as NADPH oxidase enzymes (NOX) in the cell membrane, produce ROS in response to cellular signals (*54*). In addition, inflammatory cells release ROS upon activation during an immune response causing oxidative stress in the tissue (*55*).

ROS types

There are many different types of ROS, each with diverse physiological functions and abilities to react with substrates causing damage to cellular components. Superoxide

($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO^{\bullet}$) are the most common ROS in cells (*56*). Superoxide is formed as a byproduct of cellular respiration and by NADPH oxidase enzymes and is converted by cellular antioxidants to H_2O_2 (*53*, *54*). These common ROS react with other compounds in the cell to form other reactive species such as lipid peroxides, which can also damage cellular components by oxidation. Other ROS include the hypochlorite ion (OCl^-) and singlet oxygen ($^1O_2$).

Cellular antioxidants

ROS are quenched by antioxidant molecules and enzymes in the cell. The most abundant and important antioxidant molecule in cells is glutathione (GSH). Glutathione is a tri-peptide (L-gamma-glutamyl -L-cysteinyl-glycine) which is oxidized itself to quench ROS and is then converted to its reduced from by antioxidant enzymes known as glutathione peroxidases (Gpx) (*57*). Other non-enzymatic antioxidants include essential nutrients such as alpha-tocopherol (α-T), gamma tocopherol (γ-T), and vitamin C. The unique chemical properties of antioxidant molecules dictate the specific types of reactive species they quench. Selenium, another important antioxidant compound, exerts its antioxidant effect by acting as a required component in a set selenium-containing proteins (selenoproteins) which act as cellular antioxidant enzymes. Table 1 lists several antioxidant molecules used as supplements in humans that have been studied in prostate tumorigenesis, highlighting the ROS they preferentially quench and other mechanisms of antioxidant action.

Table 1. Antioxidant supplements and their functions

Antioxidant	Solubility	ROS preferentially quenched	Other antioxidant functions	Citations
N-acetyl cysteine	water soluble	hydroxyl radical, nitrogen dioxide, carbonate radical	increases intracellular glutathione (GSH) levels by providing cysteine	(*58*, *59*)
Selenium	water soluble	N/A	required for synthesis of vital antioxidant selenoproteins	(*60*)
Vitamin C (ascorbic acid)	water soluble	aqueous peroxyl radicals; regenerates oxidized α-T to assist in quenching of lipid peroxides	increase antioxidant protein expression	(*61–64*)
Alpha-tocopherol	lipid soluble	lipid peroxyl radicals	induction of antioxidant enzymes via the Nrf2 transcription factor	(*65*, *66*)
Gamma-tocopherol	lipid soluble	lipid peroxyl radicals; quenches reactive nitrogen species better than α-T	with α-T, induces the Nrf2 transcription factor and antioxidant enzymes	(*65*, *67*, *68*)
Beta-carotene	lipid soluble	lipid peroxyl radicals	vitamin A precursor	(*69*, *70*)
Lycopene	lipid soluble	lipid peroxyl radicals	induction of antioxidant enzymes via the Nrf2 transcription factor	(*69*, *70*)
Soy isoflavanones	lipid soluble	lipid peroxyl radicals	induction of antioxidant enzymes via the Nrf2 transcription factor	(*71*, *72*)

Mammalian cells contain a wide variety of antioxidant enzymes, including superoxide dismutase, catalase, selenoproteins such as glutathione peroxidases, and thioreductases. Proper levels of cellular antioxidant molecules and enzymes are required to prevent damage to cells by ROS that are generated normally during cellular metabolism and those induced by exogenous sources. Antioxidant enzymes are normally upregulated upon oxidative stress to rid the cells of the damaging ROS. If antioxidant molecules and proteins cannot quench ROS adequately, high levels of ROS can cause significant damage to cellular components and greatly alter normal cell homeostasis.

ROS effects

Classically, oxidative stress has been known to cause oxidative damage to DNA, lipids, and proteins. DNA oxidation can lead to mutations which can either decrease cell viability or cause cellular transformation (*73*). Lipid and protein oxidation can significantly alter the function of proteins, leading to substantial pathology (*74*).

However, recent research has shown that the effect oxidative stress has on cells can vary greatly depending on the level of ROS, the type of ROS, and the type of cell (normal or cancerous). For example, low levels of ROS are necessary for intracellular signaling processes and can promote proliferation in many circumstances (*75–77*). However, high levels of ROS often lead to cell cycle arrest, senescence, or cell death (*78–82*). In addition, a certain level of ROS in a normal cell might cause the cell to die, but the same level in a cancer cell may allow the cell to live and even promote proliferation (Figure 3). Due to the various effects ROS can have, the precise role that they play in the development of tumors is not completely understood.

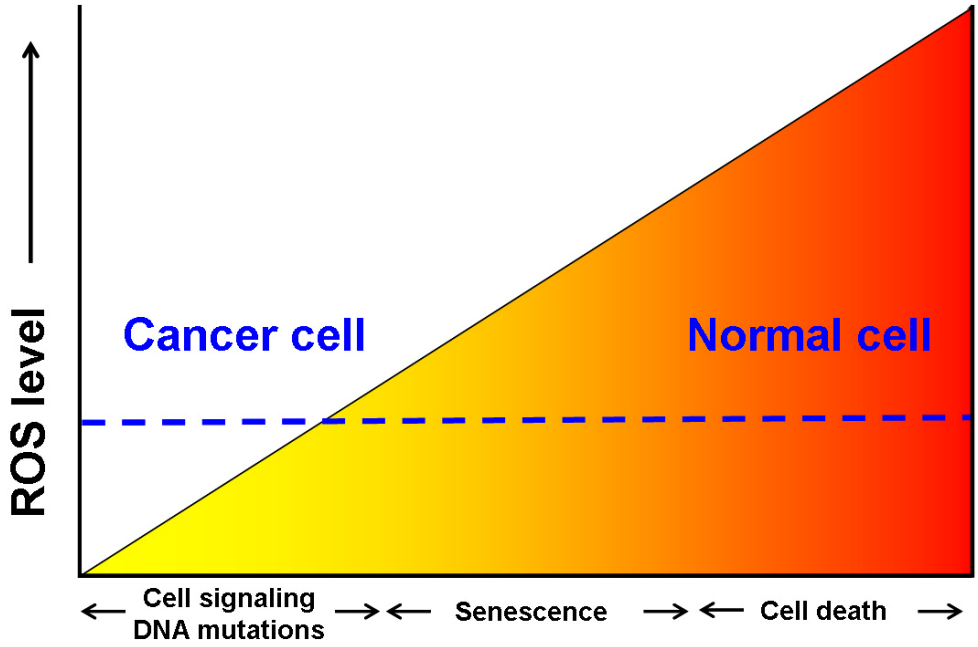

Figure 3. Differential effects of ROS depending on level and cell type
ROS can have very different effects on a cell depending on several factors. In addition to the actual type of reactive species, the amount of a certain reactive species and the cell type with which it is interacting play a major role in determining the effect. The image depicts how a higher level of ROS may cause cell death while lower levels may promote cell signaling. In addition, a certain level of ROS (indicated by the dashed blue line) may cause cell death in a normal cell, but may promote proliferative cell signaling in a cancer cell.

Oxidative stress has been implicated in the etiology of prostate cancer in several studies. Studies in human prostate cancer cell lines have suggested that ROS promote tumorigenicity (*51*, *83–86*). However, it has not been conclusively shown that ROS initiate prostate cancer, promote later stages of the disease, or are merely a side effect of the process. Studies such as these and along with pre-clinical studies (described below)

have prompted clinicians to determine the efficacy of antioxidant chemoprevention in human prostate cancer.

Prostate cancer chemoprevention

Because of the high prevalence of prostate cancer world-wide, the high treatment-related morbidity, and the ultimate lack of cures for advanced disease, prostate cancer is a key target for cancer prevention measures. Studies have investigated the ability of dietary factors to prevent disease, suggesting several foods that decrease prostate cancer risk such as fish/seafood (*87*), cruciferous vegetables (*88*), and tomato products (*89*). While some moderate links to modifiable lifestyle factors have been described, the desire for an easily usable supplement has spurred many years of research dedicated to identifying chemical compounds which can prevent prostate cancer. The two major classes of chemoprevention agents target the two major factors in prostate cancer epidemiology, androgens and oxidative stress. Chemopreventative agents have been studied in pre-clinical and clinical studies of prostate cancer development, and while they have shown a temporary promise for efficacy, ultimately have proven ineffective.

Pre-clinical prostate chemoprevention studies

Models

Chemoprevention has primarily been tested in genetically engineered and carcinogen-driven mouse models of prostate cancer. The most commonly used transgenic mouse model employed in these studies is the transgenic adenocarcinoma of mouse prostate (TRAMP) model, which is driven by prostate-specific expression of large and

small T antigens, inactivating the p53 and pRb tumor suppressor pathways (*90*). These mice develop neuroendrocrine prostate cancer by 10 weeks of age and distant metastasis by as early as 12 weeks of age (*90*, *91*). TRAMP mice provide a reliable source of tumors and metastases for use in chemoprevention trials. However, the model progresses very quickly, unlike most human prostate cancers, and employs changes in protein function (loss of p53 and pRb) which are not generally observed early in human prostate tumorigenesis (*92*, *93*). In addition, TRAMP mice display neuroendocrine tumors, tumors which express markers of neuroendocrine cell differentiation, while the vast majority of human prostate cancers are adenocarcinomas. Therefore, TRAMP is not the best model to use for prostate cancer prevention, as it may instead better reflect treatment of neuroendocrine prostate tumors. Other mouse models which have been used for chemoprevention studies include LADY mice (a somewhat slower progressing, large-T antigen driven model) (*94*) and *PTEN*-deficient mice (model with lack of the prostate tumor suppressor gene *PTEN*) (*95*). Arguably, the best models for chemoprevention would be those which exhibit the earliest changes in the initiation of prostate cancer and have a slow progression over the lifespan of the animal, as is seen in human prostate cancer. Many other models of early-stage tumorigenesis in mouse prostate have been developed, but few, if any, have been used in chemoprevention studies (*96*).

The most common rat model used is the Dunning rat model (*97*), in which a spontaneously arising rat prostate tumor was used to generate cell lines that are injected orthotopically to study inhibition of disease progression. Induced models of rat prostate cancer used include hormone and carcinogen-induced models (reviewed in (*98*)). Spontaneous prostate cancer can also be studied in canines, but the inefficiency of studies

with this species have prevented any significant progress in chemoprevention research (*96*).

Androgen-directed prevention measures

Androgen inhibition slows growth or causes apoptosis of currently existing prostate tumor cells in cell line models and transgenic mouse models. The ability to test the efficacy of androgen directed chemoprevention in transgenic mouse models of prostate cancer is difficult as the prostate-specific promoters used to drive transgene expression are androgen-responsive. For example, the anti-androgen flutamide was able to inhibit tumorigenesis in the TRAMP model (*99*); however, this was associated with a decrease in T-antigen expression from the probasin promoter, which could have mediated the observed effect. A similar effect was seen with the 5-α reductase inhibitor dutasteride in TRAMP mice (*100*). Despite the lack of good models for testing androgen-directed prevention measures in the pre-clinical testing, the centrality of androgens in prostate growth and proliferation makes androgen inhibition a good target for chemoprevention.

Oxidative stress-directed prevention measures

Numerous studies have been performed in mouse models of prostate cancer to analyze the efficacy of various antioxidant compounds for prostate cancer prevention (*101*), (*102*). Many compounds have been shown to slow tumor development in the TRAMP mouse including N-acetylcysteine (*103*), compounds from spinach leaves, green tea, cruciferous vegetables (*103–105*), and tomatoes (*106*, *107*), tocopherols and tocotrienols (*68*, *108*), and selenium-containing compounds (*109*). A combination of vitamin E (as α-tocopherol succinate), selenium, and lycopene in the diet were shown to strikingly inhibit cancer development in the LADY mouse (*110*), but the omission of

lycopene prohibited this effect (*111*) indicating it was the crucial component. Two compounds with purported antioxidant properties, curcumin and resveratrol, inhibited tumor development in the Pten-deficient mouse prostate (*112*). Selenium and vitamin E failed to prevent prostate cancer development in carcinogen and androgen driven model of rat prostate cancer, and vitamin E even showed a marginally significant increase in prostate cancer formation (*113*). While some studies showed "chemoprevention" of prostate cancer, the weakness of most of these studies is that a rapid progression to advanced cancer is seen in these models, due to inactivation of potent tumor suppressor genes. Therefore, they are not ideal models for chemoprevention. Some, but not all of these compounds have been tested in human studies, with mostly negative results, questioning the accuracy of commonly used pre-clinical rodent models of prostate cancer for chemoprevention.

Clinical prostate cancer chemoprevention studies

Androgen-directed prevention measures

Because androgens are crucial in the development of prostate cancer, and removal of them by physical or chemical means inhibits prostate tumor growth, inhibition of androgen levels in the normal prostate has been investigated as a chemopreventative measure. The major target for this measure has been the enzyme 5-alpha-reductase, the rate limiting enzyme responsible for the conversion of testosterone to the more biologically potent dihydrotestosterone (DHT). Two drugs, finasteride and dutasteride, have been used in human trials to inhibit 5-alpha-reductase (*114*, *115*).

The Prostate Cancer Prevention Trial (PCPT), one of the largest randomized controlled clinical chemoprevention studies performed in U.S. history, began in 1993

(*116*). The PCPT investigated the ability of the 5-alpha-reducatse inhibitor finasteride (5 mg per day), to prevent prostate cancer development in a healthy male population. Finasteride had received FDA approval for use in BPH in 1992, where it effectively decreased prostate volume and improved urinary symptoms (*117*, *118*). As finasteride decreases PSA levels, participants received a prostate biopsy, regardless of clinical signs, at study completion (year 7). The final results of PCPT published in 2003 showed an almost 25% reduction in total prostate cancer risk with finasteride treatment (*119*). Results of the trial were not completely well received, however, due to the fact that they observed an increased risk of advanced cancer (Gleason 7-10) in the finasteride-supplemented group (*119*). Subsequent analysis by the investigators suggested that this finding may be explained by an increased chance of detecting advanced cancer due to a similar number biopsies taken from the significantly smaller prostate volume in the finasteride group (*114*). Tumor extent was lower and detected earlier in the finasteride group (*114*).

A subsequent trial, Reduction by Dutasteride of Prostate Cancer Events (REDUCE), was performed with a more potent 5-alpha-reducatse inhibitor and on men at an increased risk of prostate cancer due to slightly elevated PSA. The results of REDUCE showed a similar reduction in overall risk as PCPT and an increased risk of advanced disease in years 3 and 4 of the study (*115*). The unexpected increase in advanced tumor development with 5-alpha-reductase inhibitors has spurred much controversy (*120*, *121*) with scientists and clinicians questioning the study design and the implications for clinical practice. This problem has prevented these agents from receiving FDA approval for prostate cancer chemoprevention (*122*).

Oxidative stress-directed prevention measures

Positive results from antioxidant chemoprevention in mouse models of prostate cancer and antioxidant effects on human prostate cancer cells suggested that oxidative stress may be a causative mechanism for human prostate cancer. Several epidemiological studies and clinical trials supported this idea. In a U.S. male population, high plasma levels of γ-tocopherol were significantly associated with a decreased risk of prostate cancer, and higher selenium levels had a trend toward a decreased risk of prostate cancer (*123*). Higher plasma levels of lycopene were associated with a decreased risk of prostate cancer (*124*, *125*). Intake of cruciferous vegetables moderately decreases risk (*88*, *126*), and components of green tea seem to decrease risk of prostate cancer (*127*, *128*).

Two intervention studies assessing antioxidant chemoprevention for other cancer types suggested that supplementation with the antioxidants vitamin E and selenium may substantially decrease prostate cancer risk. The first of these studies, the Nutritional Prevention of Cancer (NPC) trial, assessed the ability of selenium to prevent recurrent nonmelanoma skin cancer in the Eastern U.S (*129*). While the authors did not observe a decrease in skin cancer recurrence as hypothesized, in secondary analysis of the results, a decrease in incidence of prostate cancer (63% decrease), lung cancer, colorectal cancer, total cancer incidence, and total cancer-associated mortality was noted (*130*). The second study, the Alpha-tocopherol Beta-Carotene Cancer Prevention Study (ATBC), tested the ability of α-tocopherol and beta-carotene to decrease the incidence of lung cancers and other cancers (*131*). α-T did not decrease the risk of lung cancer, and beta-carotene

increased the risk of lung cancer by 16% (*132*). Secondary analyses showed that α-tocopherol supplementation decreased prostate cancer risk by 34% (*133*).

However, the evidence for an overall role of antioxidant compounds in preventing prostate cancer development was not completely clear. For example, while increased plasma levels of γ-T were associated with a lower risk of prostate cancer, high α-T levels alone were not (*123*). Significant decreases in risk with higher α-T and selenium levels were only seen with high γ-T levels (*123*). In another important investigation, supplementation with the important antioxidant beta-carotene did not decrease prostate cancer risk (*134*).

Nevertheless, using the results from NPC and ATBC as rationale, and in order to determine the efficacy of vitamin E and selenium for prostate cancer chemoprevention, the Selenium and Vitamin E Cancer Prevention Trial (SELECT) was launched in 2001. SELECT was the largest randomized controlled prostate cancer chemoprevention trial to date (*135*, *136*). It tested the ability of α-tocopherol (400 IU/day) and selenium (200 μg/d from L-selenomethionine) to prevent prostate cancer in over 32,000 men in the U.S., Canada, and Puerto Rico with no history of prostate cancer, low PSA, and negative DRE.

SELECT began recruitment in August 2001 and continued through June 2004. 35,533 men were recruited from 427 sites and randomized into four intervention groups: vitamin E, selenium, vitamin E + selenium, or placebo (*137*). Unlike the PCPT, prostate cancer diagnoses in SELECT were made by community standard of care. There was widespread hope for the confirmation of an effective chemopreventative measure for prostate cancer. Unfortunately, however, when an interim analysis was performed on data gathered until August 1, 2008, discontinuation of study supplement was recommended

since there was convincing evidence that neither trial supplement reduced the risk of prostate cancer (*137*). An initial publication of study results in January 2009, reported no statistically significant change of prostate cancer risk in any of the intervention groups (*137*). However, a non-statistically significant increase in prostate cancer was seen in the vitamin E arm ($p = 0.06$) in the analysis.

A second analysis of results published in 2011 including, 54,464 additional person-years of follow-up, again showed that there were no significant decreases in prostate cancer risk in any randomization arm (*138*). Alarmingly, there was a 17% increased risk of prostate cancer in the vitamin E arm (hazard ratio [HR], 1.17; 99% CI, 1.004-1.36, $p = 0.008$). Interestingly, the vitamin E + selenium arm did not show a significantly elevated risk, suggesting that selenium is somehow protective in the setting of vitamin E supplementation (*138*).

These disappointing results have led to controversy over the failure of vitamin E and selenium to prevent prostate cancer (*139*). Some have critiqued the form and dosage of selenium used as major reasons for the failure of the trial (*140*, *141*). Other authors note that the form of vitamin E used in the trial, α-T, decreases the levels of γ-tocopherol in the body (*142*). γ-T is the form which has stronger epidemiological evidence for association with decreased prostate cancer risk; and thus, decreasing γ-T levels could be to blame for an increased risk. Others have suggested that while vitamin E and selenium may prevent cancer in a subpopulation, they are not protective in the general population (*143*).

Due to the large amount of money and effort spent on the SELECT trial, and the anticipation for finding a simple, broadly applicable prevention measure, current research

is focused on determining the mechanisms by which vitamin E may increase prostate cancer risk and on identifying subgroups of participants who may have been at significantly increased or decreased risk. Determining these factors will deepen understanding of prostate tumorigenesis, influence future attempts at prostate cancer chemoprevention, and direct current recommendations for prostate cancer prevention and treatment.

NKX3.1 in prostate tumorigenesis

Molecular genetics of prostate cancer

While much remains unknown about prostate cancer initiation, the steps of tumor progression have been studied in more detail. A widely accepted general progression of gene expression changes occurring during the progression towards malignant and metastatic disease has been described (*144*) (Figure 4). First, the proliferation of the prostate epithelial cells increases and the gland becomes hyperplastic. This hyperplasia can continue uncontrolled and eventually lead to the cells becoming dysplastic, altering their normal size and shape. Such changes result in the appearance of prostatic intraepithelial neoplasia (PIN) lesions, an accepted precursor to prostate cancer. These PIN lesions can progress with additional changes to become localized adenocarcinoma. With additional alterations, the adenocarcinoma can become locally invasive and then metastasize to distant sites.

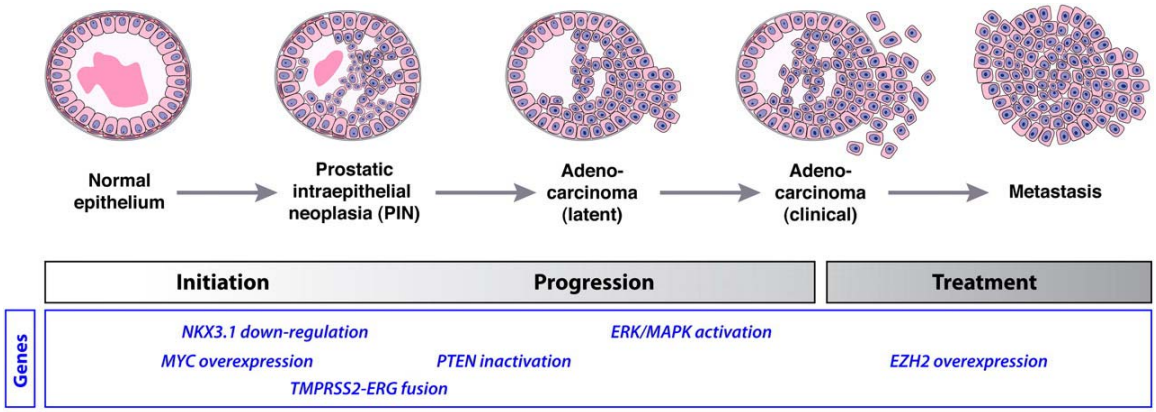

Figure 4. Model of prostate cancer progression and associated genetic alterations
Cartoon depicting the initiation and progression of human prostate tumorigenesis with the common genetic lesions or gene expression changes that occur during each step of progression. Adapted from Abate-Shen and Shen (*144*).

Each of these steps in prostate tumorigenesis is accompanied by changes in gene expression, either through loss or gain or DNA encoding genes, or through modulation of gene expression at the RNA or protein level. One of the earliest gene expression changes in prostate cancer, thought to occur prior to PIN development, is loss of expression of the tumor suppressor gene *NKX3.1* (*145*).

While these lesions can and do occur in the manner described above, the disease is heterogeneous. Different patients can have widely different gene expression changes (*146*, *147*). This makes finding a widespread treatment for recurrent prostate cancer difficult.

NKX3.1 homeobox transcription factor

Nkx3.1 was first described in mice by Charles Bieberich and colleagues in 1996 as a member of the NK family of homeobox genes (*148*). Homeobox proteins were classically described in body segment determination in *Drosophila* development and contain a consensus homeodomain which binds DNA and directs gene transcription

(*149*). While the homeodomain is similar among homeobox proteins, additional specificity to binding sites is conferred by interaction with other transcription factors (*149*). Thus, *Nkx3.1* is a homeobox transcription factor which regulates gene transcription of a distinct set of target genes.

NKX3.1 expression and *Nkx3.1*-null phenotype

NKX3.1 expression is almost completely confined to the prostate epithelial cells and its expression is highly androgen-dependent, as castration greatly diminishes *Nkx3.1* expression in mice (*148*). Shortly after its description in mice, the human *NKX3.1* gene was isolated and shown to be at a gene locus which is commonly deleted in prostate cancer (*150*). This discovery prompted investigators to hypothesize that *NKX3.1* plays an important role in maintaining the differentiation state of the prostate epithelium, preventing cancer development as a tumor suppressor.

Subsequent studies in *Nkx3.1*-deficient mice have shown that Nkx3.1 is crucial for the proper development and maintenance of the prostate gland (*151*, *152*). *Nkx3.1*-deficient prostates display increased epithelial proliferation, leading to hyperplasia, with the epithelial cells growing into the lumen of the glands (*151*, *152*). Additional studies showed that indeed, Nkx3.1 expression is completely lost in 5% of benign prostatic hyperplasias, 20% of high-grade prostatic intraepithelial neoplasias, 34% of hormone-refractory prostate cancers, and 78% of metastases (*153*), suggesting that Nkx3.1 acts as a tumor suppressor gene in the prostate.

NKX3.1 functions

In order to determine the functional role played by Nkx3.1 in the prostate, microarray studies were performed in *Nkx3.1*$^{+/+}$ and *Nkx3.1*$^{-/-}$ mouse prostate (*154*),

(*155*). Gene expression profiles can be analyzed on the level of groups of genes using Gene Set Enrichment Analysis (GSEA), highlighting the major gene sets regulated by a gene of interest (*156*, *157*). GSEA analysis of the *Nkx3.1*$^{+/+}$ and *Nkx3.1*$^{-/-}$ mouse prostate microarrays showed many significantly changed gene sets, including gene sets involved in oxidative stress and cell cycle regulation (*158*).

Oxidative stress regulation

Nkx3.1$^{-/-}$ mice have elevated prostatic oxidative stress, as shown by increased ROS levels in prostate tissue (*159*). In addition, they show an increased presence of oxidative damage to DNA and protein (*155*, *159*). Loss of Nkx3.1 in the prostate epithelium may induce ROS in an indirect or direct manner. ROS may be elevated in *Nkx3.1*-null epithelium partially due to the elevated level of proliferation, indicating elevated metabolic activity in cells, which can lead to increased oxidative stress (*160*). However, Nkx3.1 also appears to have a direct role in regulation of oxidative stress through direct regulation of anti- and pro-oxidant target genes. Chromatin immunoprecipitation with massively parallel DNA sequencing (ChIP-seq) analysis coupled with analysis of gene expression changes in *Nkx3.1*$^{-/-}$ mice showed that the antioxidant genes glutathione peroxidase 2 *(Gpx2*) and peroxiredoxin 6 (*Prdx6*), and the pro-oxidant gene quiescin Q6 sulfhydryl oxidase 1 (*Qsox1*) are direct target genes of Nkx3.1 (*158*). Expression of the antioxidants *Gpx2* and *Prdx6* is decreased, while expression of the pro-oxidant *Qsox1* is increased in *Nkx3.1*$^{-/-}$ mice suggesting an environment which promotes increased oxidative stress. It has been proposed that this increased oxidative stress is a mechanism through which Nkx3.1-loss promotes tumorigenesis (*155*, *161*, *162*).

Cell cycle regulation

The *Nkx3.1*$^{-/-}$ mouse displays prostatic hyperplasia and increased levels of proliferation markers Ki67 and PCNA relative to the *Nkx3.1*$^{+/+}$ mouse (*151*, *163*). NKX3.1 expression has also been shown to decrease proliferation of human prostate cell lines (*164–168*). Studies by Magee *et al.* showed that *Nkx3.1*-deficiency extended the proliferative phase of prostate regeneration after castration and testosterone replacement in mice (*154*). These studies suggest that Nkx3.1 plays a role inhibiting progression of the cell cycle.

Response to DNA damage

NKX3.1 has also recently been shown to play a role in repairing DNA damage, thus promoting cell survival after genetic insult. NKX3.1 localizes to sites of DNA damage, recruiting and activating ataxia telangiectasia, mutated protein (ATM), a protein essential for the DNA repair process (*169*). NKX3.1 was also shown to bind the DNA unwinding enzyme topoisomerase I, enhancing its DNA cleavage activity and promoting survival in the presence of DNA damage (*170*, *171*).

NKX3.1 target gene regulation

For many years, the direct target genes of Nkx3.1 were not known. Initial microarray studies performed in wild type and *Nkx3.1*-deficient mice identified genes dysregulated upon Nkx3.1-loss (*154*, *155*, *158*). Significant gene networks dysregulated upon Nkx3.1 loss were those involved in aminoacyl-tRNA synthesis, oxidative stress control, and cell cycle control (*158*). Recent ChIP-seq experiments performed by the Abdulkadir laboratory and others have identified the genome-wide set of genes bound by Nkx3.1 (*158*, *172*). Integration of these data with gene expression profiling data yielded

a set of 282 “direct” Nkx3.1 target genes, genes either activated (153 genes) or repressed (129 genes) by Nkx3.1 binding (*158*). The major groups of genes in the Nkx3.1 direct target genes were the aminoacyl-tRNA synthesis pathway and MAPK signaling (*158*). This suggests that Nkx3.1 plays a role in regulation of protein biosynthesis and signaling related to cell cycle progression.

Nkx3.1 and Myc co-regulate a set of target genes

Changes in gene expression, classically increases in oncogenes and decreases in tumor suppressor genes, accompany and drive carcinogenesis. However, in most cancer systems, the ability of tumor suppressors and oncogenes to regulate expression of the same genes has not been described. In a recent study published by the Abdulkadir laboratory, 65 target genes were found to be directly regulated by Nkx3.1 and Myc via GeneGO analysis of ChIP-seq data for both transcription factors (*158*). The shared direct target genes were enriched in pathways involved in tumorigenesis, and many were regulated in the mouse prostate (*158*).

Peroxiredoxin 6 (PRDX6)

One of the direct targets co-regulated by Nkx3.1 and Myc is the dual-function enzyme peroxiredoxin 6 (PRDX6). PRDX6 is a member of the peroxiredoxin superfamily, which is a group of cysteine-dependent peroxidases (*173*), which reduce hydrogen peroxide and other peroxides, functioning as antioxidant enzymes. PRDX6 is of interest to prostate tumorigenesis as it may regulate prostatic oxidative stress. PRDX6 is unique among the 6 member family in that it also has a second catalytic function, phospholipase A2 (PLA2) activity (*174*). This is also interesting as some of the main

products of PLA2 activity are prostaglandins, a class of compounds that have been suggested to play an important role in prostate tumorigenesis (*175–177*).

PRDX6 is expressed widely, but has only been extensively studied in the lung where it functions to protect against oxidative stress (*178–180*) and to promote proper lung surfactant metabolism (*181*, *182*). The role of PRDX6 in cancer has not been widely investigated. In general it has been shown to be upregulated in cancer cell lines and tumor tissue relative to normal cells and tissue (*183–186*) and to increase proliferation, migration, and tumorigenicity in cancer cell lines (*187–189*). It is not known how PRDX6 may function in the normal or transformed prostate; therefore, significant investigation is needed to determine the role of PRDX6 and other NKX3.1-MYC co-regulated genes in prostate tumorigenesis.

Summary and Rationale of Studies

One of the most important challenges in prostate cancer today is determining efficacious prevention measures for the disease. Recent studies have shown that success in this endeavor will require having a more thorough knowledge of the molecular mechanisms behind prostate cancer. After large scale clinical trials, it is still not conclusively known if antioxidant chemoprevention will prevent development of the disease. In fact, data from the SELECT trial suggest that in some cases these compounds may even promote prostate cancer (*138*).

To begin to address these challenges, I have conducted studies in mouse models of prostate tumorigenesis and in human clinical samples. To help determine if oxidative stress is a causative mechanism early in prostate tumorigenesis, and if antioxidant

chemoprevention may prevent disease, I tested antioxidant chemoprevention in mice which develop an early prostate cancer precursor lesion. I measured aspects of tumorigenesis to determine if ROS play a role in this very early stage of disease.

Next, to attempt to explain some of the mechanisms of early prostate tumorigenesis in humans, and to determine if there are certain populations who perform better or worse with antioxidant chemoprevention, I used samples from the SELECT trial to genotype genetic variants. I set out to investigate if variants in the tumor suppressor gene *NKX3.1* modified prostate cancer risk associated with antioxidant supplementation. During this analysis, I serendipitously found that a SNP in the Bcl-2 family member gene *BNIP3L* also modified risk with antioxidant supplementation in SELECT. Findings from this study will help further our understanding of the molecules and processes involved in early prostate tumorigenesis.

Lastly, while the transcription factor NKX3.1 is known to play a role in prostate tumorigenesis, all of its mechanisms of action are not understood. Due to its possible role in regulation of oxidative stress in the prostate, I decided to investigate one of the direct target genes of NKX3.1, the antioxidant gene *PRDX6*. My studies began to analyze the effect that *PRDX6* regulation may have in the initiation and progression of prostate cancer.

Through my use of preclinical and clinical samples, I have completed a study of antioxidant chemoprevention and NKX3.1 target gene regulation in early prostate tumorigenesis.

CHAPTER III

ANTIOXIDANT SUPPLEMENTATION PROMOTES PROSTATE EPITHELIAL CELL PROLIFERATION IN *Nkx3.1* MUTANT MICE

Introduction

Due to the high prevalence and significant treatment-related morbidity associated with human prostate cancer, there is a strong interest in preventive approaches. In order to accomplish this, a more thorough understanding of the relationship between oxidative stress and the steps of prostate tumor progression is needed. In recent years, extensive research has been devoted to the relationship between oxidative stress and the etiology of prostate cancer (*5*, *7*, *52*, *101*). In addition, the prostate gland has been associated with chronic inflammation (*8*), a condition linked to elevated oxidative stress. Many studies have proposed a positive correlation between elevated oxidative stress and prostate cancer progression and have argued the value of antioxidants in preventing prostate cancer (reviewed in (*102*)). However, it is notable that the majority, if not all, of these studies have employed models of late stage, aggressive disease, focusing on later steps in carcinogenesis rather than prevention of prostate cancer initiation (*68*, *103*, *104*, *83*, *110*, *112*, *190*).

The Selenium and Vitamin E Cancer Prevention Trial (SELECT) was initiated in 2001 to conduct a large, randomized controlled clinical trial on the efficacy of the antioxidants selenium and vitamin E in the prevention of prostate cancer (*135*). Results

from two previously published clinical trials (*130*, *191*) suggested that these two antioxidants could prevent prostate cancer development. However, initial results published in 2009 (*137*) showed that neither selenium, vitamin E, nor their combination significantly prevented prostate cancer in the study population. Follow-up results published in late 2011 (*138*) showed that vitamin E supplementation increased rather than decreased the risk of development of prostate cancer. This troubling finding highlights the importance of understanding the role of ROS in prostate tumorigenesis. In fact, one of the lead authors of the SELECT trial has suggested that any success in future chemoprevention may reside in the identification of specific risk factors in individuals that will help determine the effect any agent may have on their tumor development (*192*).

NKX3.1 is a homeodomain transcription factor whose loss of expression correlates with human prostate cancer progression (*150*, *153*, *193*). NKX3.1 expression is lost early in tumorigenesis, suggesting that it is an early step in the progression to malignant disease. While several studies have investigated the role Nkx3.1 loss plays in prostate cancer (*145*, *154*, *167*, *169*, *170*, *172*, *194–198*), much remains unknown. *Nkx3.1*$^{-/-}$ mice are a model of the early stages of prostate tumorigenesis, exhibiting hyperplasia and dysplasia at 8 weeks of age and progressing to prostatic intraepithelial neoplasia (PIN), a precursor lesion to prostate cancer, later in life (*151*, *152*, *199*). With additional genetic lesions, such as the loss of one allele of the Pten tumor suppressor gene, these mice develop prostate cancer (*151*, *152*, *200*). Ouyang *et al*. showed that prostates of *Nkx3.1*$^{-/-}$ mice show dysregulation of several antioxidant and pro-oxidant control enzymes, accompanied by elevated oxidative stress (*155*). They and others have suggested that increased oxidative stress may be an important way in which Nkx3.1 loss

promotes prostate tumor initiation (*161*), . However, the ability of oxidative stress to mediate the hyperplasia of the *Nkx3.1*$^{-/-}$ mouse prostate has not been examined.

In this study, I tested the ability of antioxidant supplementation to prevent the prostate pathology of *Nkx3.1*$^{-/-}$ mice. Interestingly, Ifound that antioxidant supplementation did not inhibit, but instead promoted, the hyperplastic phenotype of the *Nkx3.1*$^{-/-}$ prostate. NAC supplementation of *Nkx3.1*$^{-/-}$ prostate also induced expression of a pro-proliferative gene signature, as demonstrated by Genome Set Enrichment Analysis (GSEA). This suggests that ROS restrain the proliferative potential of the prostate epithelium in the setting of Nkx3.1-loss. Our studies give new insight into the failure of antioxidants to prevent prostate cancer in healthy men.

Methods

Animals

Nkx3.1$^{-/-}$ mice have been described (*152*). Mice were maintained at Vanderbilt University Medical Center in compliance with national and institutional animal welfare standards. For NAC supplementation, *Nkx3.1*$^{+/+}$ and *Nkx3.1*$^{-/-}$ pups were weaned at 3 weeks of age and littermates were divided between NAC treatment cages or vehicle cages. Mice received vehicle or 5mM NAC (Sigma) in drinking water *ad lib* beginning at weaning for 13 weeks. The pH of NAC solution was adjusted to that of regular drinking water. Analysis of water intake and weight data after the conclusion of the experiment showed that the NAC dosage achieved was 158.5 mg/kg/day in *Nkx3.1*$^{+/+}$ mice and 140.7 mg/kg/day in *Nkx3.1*$^{-/-}$ mice. At the end of 13 weeks of supplementation, the mice were euthanized following BrdU intraperitoneal injection (50mg/kg) for prostate

histological analysis. Animal protocol M/08/047 was approved by Vanderbilt's Institutional Animal Care and Use Committee.

Quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was extracted from snap-frozen mouse anterior prostate tissue according to the Trizol® manufacturer's protocol. RNA was treated with RQ1 Rnase-free DNAse (Promega) according to manufacturer's protocol and incubated at 37°C for 20 minutes, followed by purification using the RNA Clean Up protocol from the RNeasy Mini Kit (Qiagen). 1 μg RNA was subjected to reverse transcription using M-MLV Reverse Transcriptase (Invitrogen). Quantitative real time PCR was performed using SYBR® Green and the Applied Biosystems 7300 Real Time PCR system with gene-specific primers designed using Applied Biosystems Primer Express® software. The following primers were used: *18s* forward (5'-CGCCGCTAGAGGTGAAATTCT-3'), *18s* reverse (5'-CGAACCTCCGACTTTCGTTCT-3'), *Gpx2* forward (5'-TGACCCGTTCTCCCTCATG-3'), *Gpx2* reverse (5'-GCGCACGGGACTCCATAT-3'), *Prdx6* forward (5'-TCTGGCAAAAAATACCTCCGTTA-3'), *Prdx6* reverse (5'-GCCCCAATTTCCGCAAAG-3'), *Qsox1* forward (5'-GGCTGGGAGGGTGACAGTT-3'), and *Qsox1* reverse (5'-std 18 GCCCCTACCACCAAGCAA-3'). The expression of each mRNA was normalized to *18s* rRNA expression.

ChIP-qPCR of Nkx3.1 binding sites in LNCaP cells

Chromatin immunoprecipitation (ChIP) was performed using the ChIP Assay kit (Millipore) as described by the manufacturer with the following modifications. LNCaP cells (ATCC) were grown in RPMI medium supplemented with 10% fetal bovine serum (FBS) and 1 nM dihydrotestosterone (DHT) for 48 hours. Cells were fixed in 1%

formaldehyde at 37°C for 10 minutes to crosslink protein-DNA complexes. Next, cells were thoroughly washed with ice-cold PBS, pelleted, and resuspended in SDS lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris at pH 8.1]. Chromatin was sheared to a size of ~300-500 base pairs and diluted 1:10 with ChIP dilution buffer. An aliquot of the diluted sample (1%) was saved as input. Samples were precleared and precipitated overnight at 4°C with anti-NKX3.1 (Santa Cruz Biotechnology) or normal goat IgG (Santa Cruz Biotechnology) antibodies. Antibody complexes were collected with Protein A Agarose/Salmon Sperm DNA (Millipore) for 2 h and washed extensively per manufacturer's instructions. Samples were reverse cross-linked at 65°C overnight with 0.3 M NaCl and 30 μg of RNase A (Qiagen). Input and bound DNA were purified with a PCR Purification kit (Qiagen) and analyzed by qPCR (Applied Biosystems 7300) using SYBR Green. The following primers were used for qPCR: *QSOX1* forward (5'-CCTTCATTGCTATTCACTGGCTAA-3'), *QSOX1* reverse (5'-TCCCCAACTGCAATGCAAA-3'), *PRDX6* forward (5'-GGTGGCCGAAAGACTTTTTG-3'), *PRDX6* reverse (5'-TGGCTCTTCCTAAAGCTGTTATCA-3'), *GPX2* forward (5'-GAATCAGTCTAGCAAAGGATCAAACA-3'), and *GPX2* reverse (5'-GCATAGAGGGTGTAGTTACTGAGAACA-3'). Immunoprecipitated DNA was normalized to 1% input. Results are presented as mean ± SD.

Dihydroethidium staining

DHE staining was performed on anterior prostate tissue frozen in Tissue Tek® OCT embedding medium. 10 μm sections were cut and stained with 10 μM dihydroethidium (Molecular Probes) for 30 minutes in a 5% CO_2 incubator and

visualized on a Zeiss fluroescent microscope. Fluorescence intensity of each image was scanned and scored using Bio Rad GS-700 Imaging Densitometer and BioRad Quantity One ® software.

Histology and immunohistochemistry

Tissue was fixed overnight in 10% formalin solution and washed in 70% ethanol. Tissue processing and hematoxalin and eosin (H&E) staining was performed by the Vanderbilt Translational Pathology Shared Resource. For immunohistochemistry, paraffin embedded sections were deparaffinized, rehydrated, and steam/pressure antigen retrieval was performed. The following antibodies were used: anti-BrdU (mouse, 1:200, Santa Cruz Biotechnology), anti-phospho histone H3 (rabbit, 1:500, Millipore), anti-cleaved caspase-3 (rabbit, 1:200, Cell Signaling), anti-smooth muscle actin (mouse, 1:2000, Sigma), anti-p63 (PIN cocktail, Biocare Medical), anti-AR (rabbit, 1:600 Santa Cruz Biotechnology), anti-p16 (rabbit, 1:1000, Santa Cruz Biotechnology), anti-8-Hydroxydeoxyguanosine (8-OHdG) (mouse, 1:1000, QED Bioscience), anti-p27 (mouse, 1:2000, BD Transduction Laboratories), and anti-p21 (mouse, 1:50, Santa Cruz Biotechnology). Horseradish peroxidase (HRP) conjugated secondary antibodies (BioRad) were used to detect primary antibodies and 3, 3'-diaminobenzidine (Sigma) or Nova Red (Vector Laboratories) were used as the chromogenic substrates. Counterstain was performed with hematoxylin.

Immunohistochemistry quantification

Three independent fields of anterior prostate using a box objective at 60x were observed for 8-OHdG immunohistochemical staining in one year old *Nkx3.1*$^{+/+}$ and *Nkx3.1*$^{-/-}$ mice and for BrdU, pHH3, and/or activated caspase 3 staining in the *Nkx3.1* $^{+/+}$

and *Nkx3.1*$^{-/-}$ vehicle and NAC-supplemented mice. Number of total cells and cells staining positive for each of the markers were recorded and data was reported as percent cells positive for the marker. In all cases, at least 500 total cells were counted per mouse.

Statistical Analysis

Statistical analysis for immunohistochemistry, qRT-PCR, and fluorescence intensity image data was performed using two tailed Student's t-Test, with two samples of unequal variance. All results are presented as mean ± Standard Deviation. P values ≤ 0.05 are considered significant.

Microarray and Genome Set Enrichment Analysis (GSEA)

Total prostate RNA from four vehicle and four NAC-supplemented *Nkx3.1*$^{-/-}$ mice was extracted from snap-frozen mouse anterior prostate tissue according to the Trizol® manufacturer's protocol. RNA was treated with RQ1 Rnase-free DNAse (Promega) according to manufacturer's protocol, followed by purification using the RNA Clean Up protocol from the RNeasy Mini Kit (Qiagen). RNA was processed and microarray analysis was performed by the Vanderbilt Genome Sciences Resource Core. Briefly, RNA was quantified using the Qubit RNA assay and RNA quality was assessed with the Agilent Bioanalyzer. cDNA was generated using the Ambion® WT Expression Kit. After fragmentation, the cDNA was labeled and hybridized to Affymetrix Mouse Gene 1.0 ST arrays. Arrays were scanned with Affymetrix Gene Chip Scanner [version 3.2.2]. CEL files were imported to R [version 2.15.1] for quality control and pre-processing. Arrays for three vehicle and four NAC-supplemented mice passed quality control. Using the Affy package [version 1.34.0] (*201*), raw intensity scores for probes were normalized by quantiles, background corrected with RMA (*202*), and summarized by median polish

using PM-only probes. The C2 (curated) gene sets of MSigDB [version 3.0] were queried using GSEA [version 2.07] (*156*) to test for differences between vehicle and NAC-supplemented prostates. Relationships between functional terms were visualized in Cytoscape [version 2.8.3] (*203*) with the Enrichment Map package [version 1.2] (*204*). All microarray and GSEA analysis was performed on a node running Debian Linux [version 6.0.5].

Results

Nkx3.1 directly regulates antioxidant and pro-oxidant genes in the prostate

Previous gene expression analyses have revealed mis-expression of antioxidant and pro-oxidant genes in the *Nkx3.1* null mouse prostate, including Glutathione peroxidase (*Gpx2*), Peroxiredoxin 6 (*Prdx6*), and quiescin Q6 sulfhydryl oxidase 1 (*Qsox1* or *Qscn6*) (*154*, *155*, *158*). I performed qRT-PCR analysis on anterior prostates to confirm these gene expression changes. Expression of the antioxidant genes Gpx2 and Prdx6 was decreased in 10-11-week-old and 16-17-week-old *Nkx3.1*$^{-/-}$ mice, while expression of the pro-oxidant gene Qsox1 was elevated in these mice (Figure 5A). Examination of chromatin immunoprecipitation coupled to massively parallel sequencing (ChIP-seq) analysis for Nkx3.1 in mouse prostate (*158*) and the human prostate cancer cell line LNCaP (PDA, ML and SAA, manuscript in preparation) performed by our laboratory revealed binding sites for Nkx3.1 in both human and mouse tissue at all three genes (Figure 5B, 5C). Binding in LNCaP was confirmed via ChiP-qPCR (Figure 5D) Therefore, Gpx2, Prdx6 and Qsox1 are direct target genes of the Nkx3.1 transcription factor.

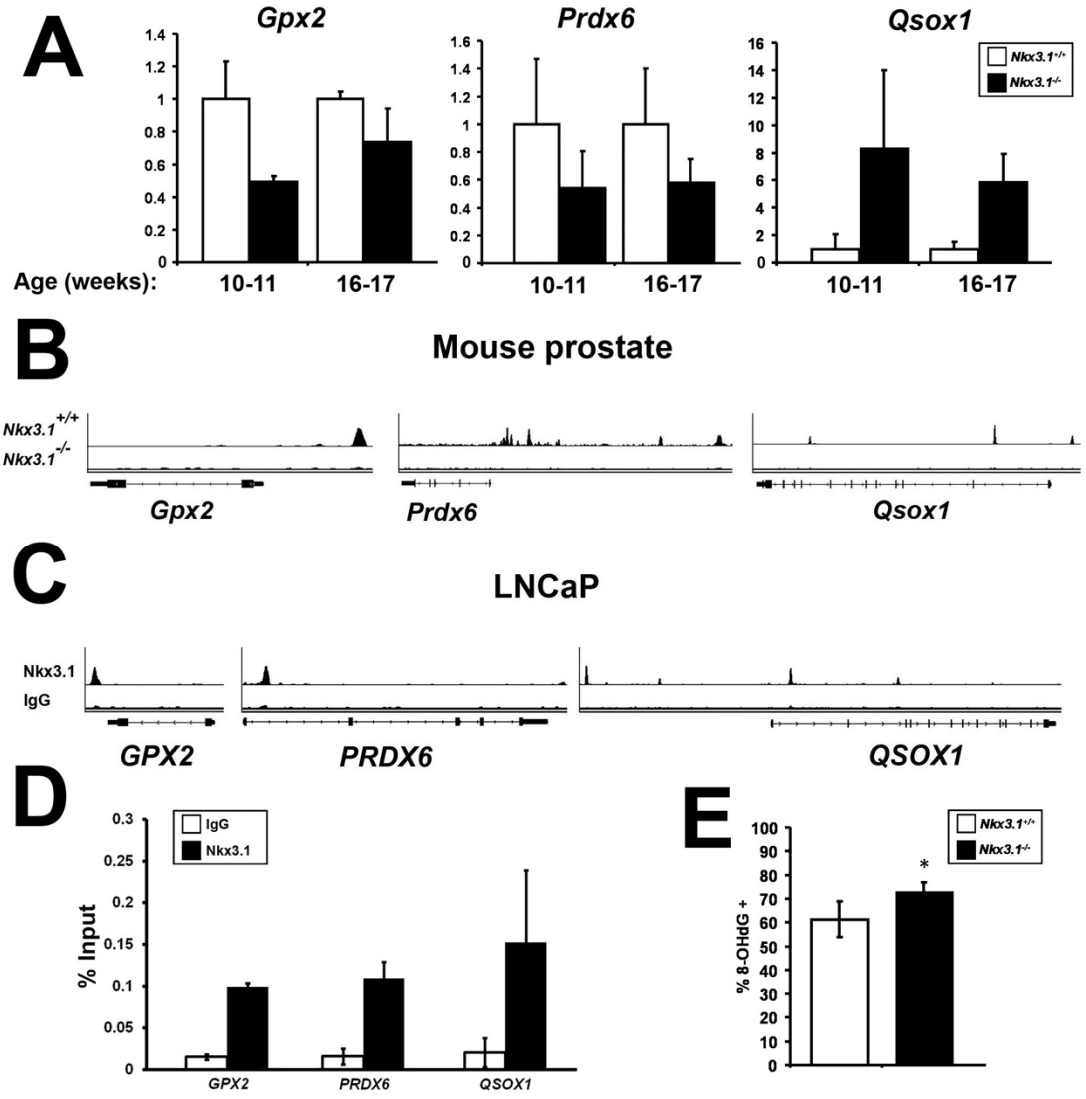

Figure 5. ***Nkx3.1*$^{-/-}$ mouse prostate shows dysregulation of oxidative stress genes and increased oxidative stress levels**

(A) Quantitative reverse transcriptase-PCR analysis of RNA from 10-11-week and 16-17-week-old *Nkx3.1*$^{+/+}$ and *Nkx3.1*$^{-/-}$ mouse anterior prostate for the expression of *Gpx2*, *Prdx6*, and *Qsox1*. Expression levels are relative to 18s rRNA. (10-11 weeks: n = 4 *Nkx3.1*$^{+/+}$, n = 2 *Nkx3.1*$^{-/-}$; 16-17 weeks: n = 3 *Nkx3.1*$^{+/+}$, n = 5 *Nkx3.1*$^{-/-}$) (B) ChIP-seq screen shots from Integrative Genomics Viewer (IGV) displays direct binding of Nkx3.1 to the gene loci of *Gpx2*, *Prdx6* and *Qsox1* in mouse prostate, (C) and to *GPX2*, *PRDX6* and *QSOX1* in the human prostate cancer cell line LNCaP. (D) ChIP-qPCR analysis for Nkx3.1 binding sites in *GPX2*, *PRDX6*, and *QSOX1*. Results are presented for each binding site primer set with anti-NKX3.1 antibody and IgG control. Immunoprecipitated DNA was normalized to 1% input. (E) Percent positive stained anterior prostate epithelial cells from immunohistochemical staining for 8-OHdG in one-year-old *Nkx3.1*$^{+/+}$ and *Nkx3.1*$^{-/-}$ anterior prostate. (n = 5 in each group) Student's t-Test * = $p \leq 0.05$.

Nkx3.1$^{-/-}$ mouse prostate displays increased oxidative stress

The most common oxidative DNA base lesion, 8-OHdG, is commonly used as a marker of persistent oxidative stress (*205*). Immunohistochemical staining of one-year-old mouse anterior prostate showed significantly increased staining in *Nkx3.1*$^{-/-}$ mice (Figure 5E). These results confirm earlier findings of increased oxidative DNA damage in the prostates of independently generated *Nkx3.1*$^{-/-}$ mice (*155*)

NAC supplementation of *Nkx3.1*$^{-/-}$ mice does not inhibit hyperplastic prostate phenotype

To determine if increased oxidative stress plays a causative role in the hyperplasia and dysplasia observed in the *Nkx3.1*$^{-/-}$ mouse prostate, I supplemented *Nkx3.1*$^{-/-}$ mice with 5mM NAC in their drinking water from 3 weeks of age until mice were sacrificed at 16 weeks of age (Figure 6A). The 5mM NAC concentration was chosen to achieve a dosage of approximately 125 mg/kg/day for 13 weeks, a dosage and treatment duration shown to inhibit plasma ROS, decrease oxidative DNA and protein lesions in the prostate, and decrease the incidence of prostate anterior lobe hyperplasia in the Transgenic Adenocarcinoma Mouse Prostate (TRAMP) model (*103*, *190*). Examination of water intake and weight data revealed that the achieved dosage for the *Nkx3.1*$^{-/-}$ mice was approximately 140 mg/kg/day. The 13 week NAC supplementation decreased ROS levels in the anterior prostate as shown by decreased staining for superoxide using the fluorescent dye dihydroethidium (DHE) (Figure 6B, 6C).

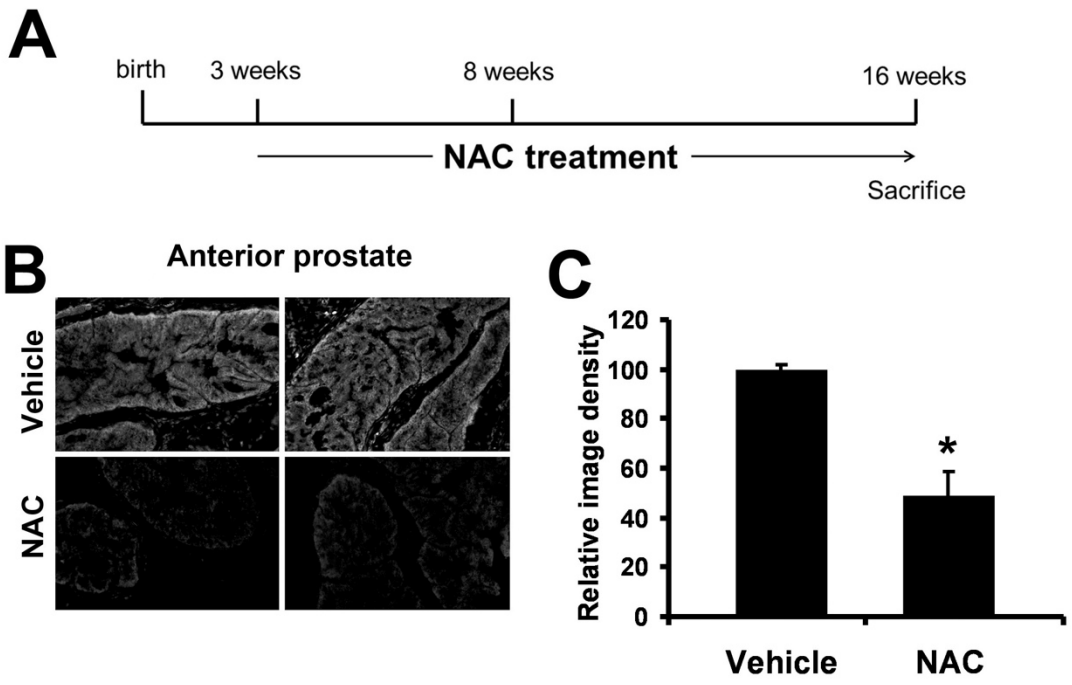

Figure 6. Antioxidant supplementation of *Nkx3.1*$^{-/-}$ mice decreases prostatic ROS
(A) *Nkx3.1*$^{-/-}$ mice were supplemented with 5mM N-acetylcysteine (NAC) *ad lib* in their drinking water postweaning for 13 weeks. Mice were sacrificed for analysis at the end of supplementation (16 weeks of age). (B) Dihydroethidium (DHE) staining of frozen anterior prostate from *Nkx3.1*$^{-/-}$ vehicle or NAC-supplemented mice. (C) Quantification of DHE staining density. (n = 3 in each group) Student's t-Test * = $p \leq 0.05$.

Histological analysis of *Nkx3.1*$^{-/-}$ anterior prostate, the prostatic lobe which displays the severest *Nkx3.1*$^{-/-}$ phenotype, showed that the NAC supplementation did not reverse the *Nkx3.1*$^{-/-}$ phenotype. Observation of 23 control and 24 NAC-supplemented *Nkx3.1*$^{-/-}$ prostates revealed that the NAC-supplemented prostates did not have less hyperplasia or dysplasia than the control prostates (Figure 7A). Immunohistochemical staining for smooth muscle actin was unchanged between supplemented and control mice, suggesting the prostate epithelial cells did not alter gland structure or invade the stromal compartment (Figure 7B). Immunostaining for p63 (basal cell marker) and androgen receptor (AR) remained unchanged with supplementation, showing no major histological alterations of the prostate epithelium after NAC supplementation (Figure 7B).

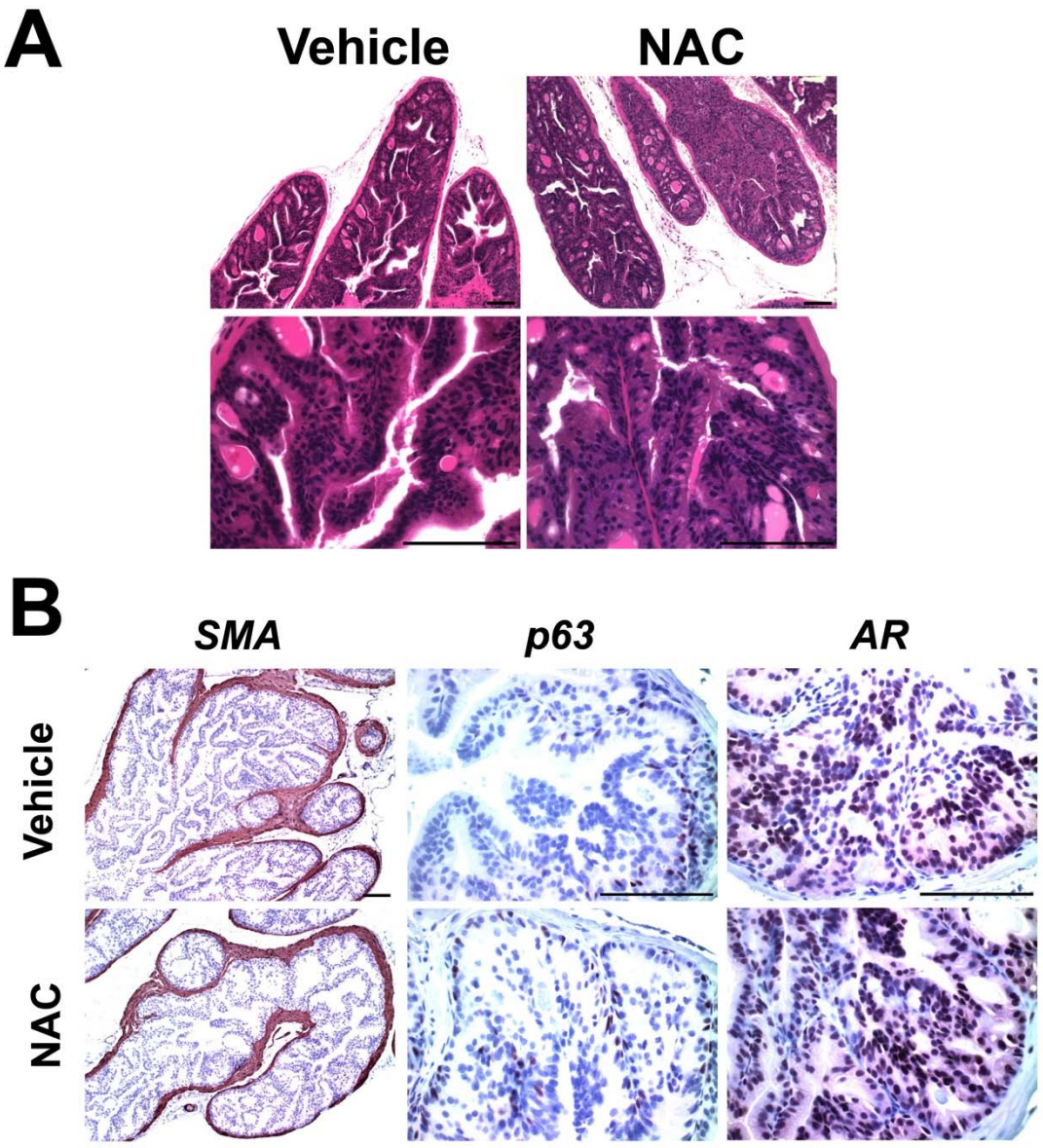

Figure 7. NAC supplementation does not alter prostate histology in *Nkx3.1*$^{-/-}$ mice
(A) Hematoxalin and eosin stained sections of *Nkx3.1*$^{-/-}$ anterior prostate do not display significant histological changes with NAC supplementation. (B) Immunohistochemical staining of anterior prostate for smooth muscle actin (SMA), p63, and androgen receptor (AR) do not have significant changes in staining pattern. Scale bar = 0.1mm.

NAC supplementation of *Nkx3.1*$^{-/-}$ mouse prostate promotes increased proliferation

To assess cell proliferation in the prostate after NAC supplementation, mice were injected with BrdU three hours prior to sacrifice to label cells undergoing DNA synthesis, indicating the proportion of cells progressing though the cell cycle. Surprisingly, the percentage of anterior prostate epithelial cells staining positive for BrdU was increased by 60% in the NAC-supplemented *Nkx3.1*$^{-/-}$ mice ($p = 0.02$, $n = 10$ in each group, Figures 8A, 8B). Staining for the mitotic cell marker pHH3 was also increased by 30% in the NAC-supplemented animals ($p = 0.05$, $n = 15$ vehicle, $n = 16$ NAC, Figures 8C, 8D). However, activated caspase-3 staining revealed that apoptosis was unchanged with NAC supplementation ($p = 0.59$, $n = 10$ in each group, Figures 8E, 8F). The observed increase in proliferation without a concurrent decrease in apoptosis suggests NAC supplementation increases prostate epithelial cell numbers in the *Nkx3.1*$^{-/-}$ prostate.

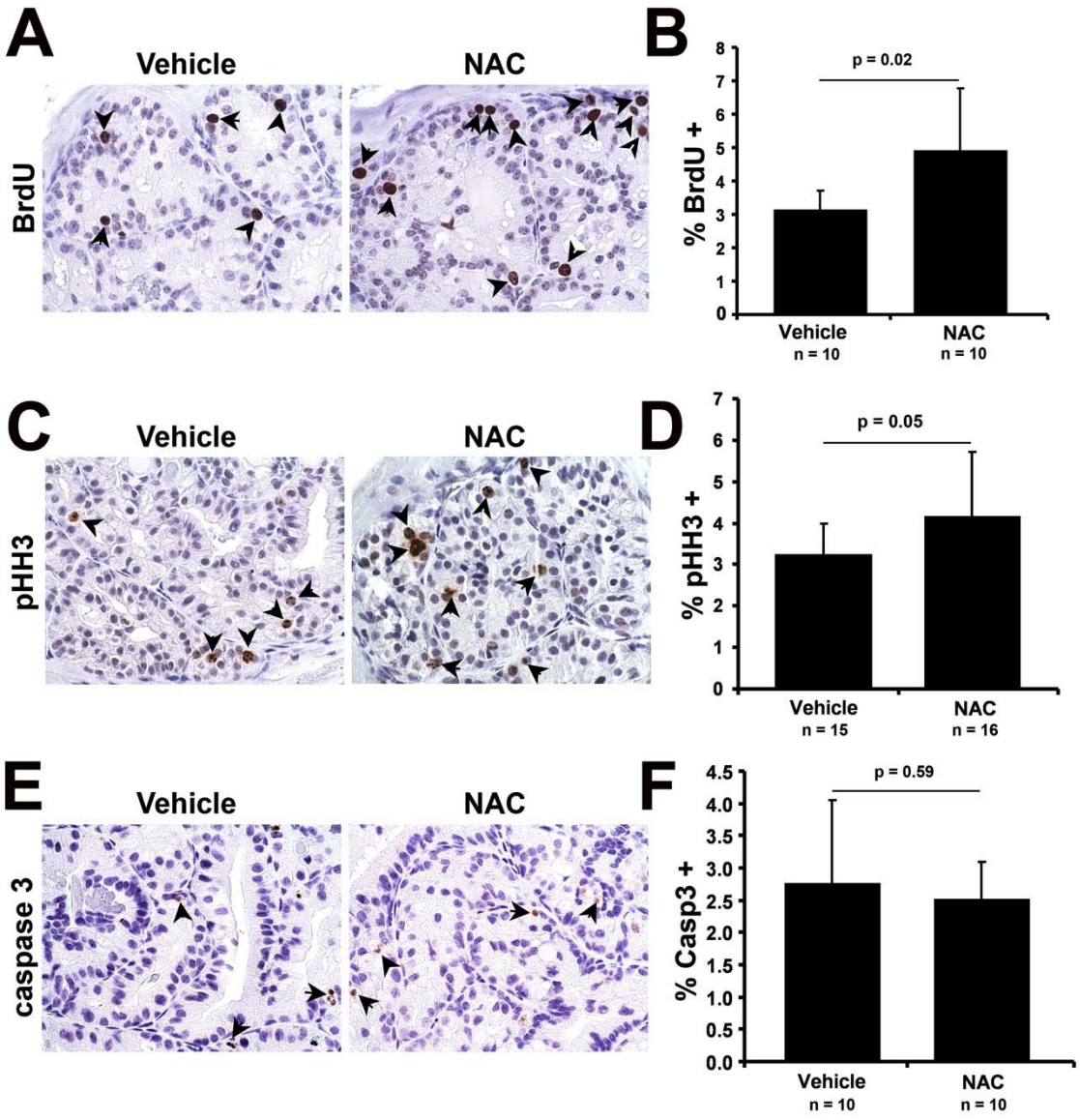

Figure 8. NAC supplementation promotes epithelial proliferation in the *Nkx3.1*$^{-/-}$ prostate
(A), (C), (E) Representative images from immunohistochemical staining of *Nkx3.1*$^{-/-}$ vehicle and NAC-supplemented anterior prostate with antibodies specific to BrdU (A), pHH3 (C), and activated caspase-3 (E). (B), (D), (F) Quantification of immunohistochemical stains. p values for a Student's t-Test are shown.

NAC supplementation of *Nkx3.1*$^{+/+}$ mouse prostate does not affect proliferation

To determine if NAC supplementation affects prostate epithelial cell proliferation in the absence of Nkx3.1-loss and elevated oxidative stress, I supplemented *Nkx3.1*$^{+/+}$ mice with NAC in the same manner as was used for the *Nkx3.1*$^{-/-}$ mice. The dosage achieved in the *Nkx3.1*$^{+/+}$ mice was comparable to the *Nkx3.1*$^{-/-}$ mice at approximately 160 mg/kg/day. The NAC supplementation did not alter overall prostate histology in the *Nkx3.1*$^{+/+}$ mice (Figure 9A). BrdU and pHH3 immunohistochemical analyses showed that NAC supplementation did not alter the proliferation index of the *Nkx3.1*$^{+/+}$ anterior prostate (Figure 9B, 9C).

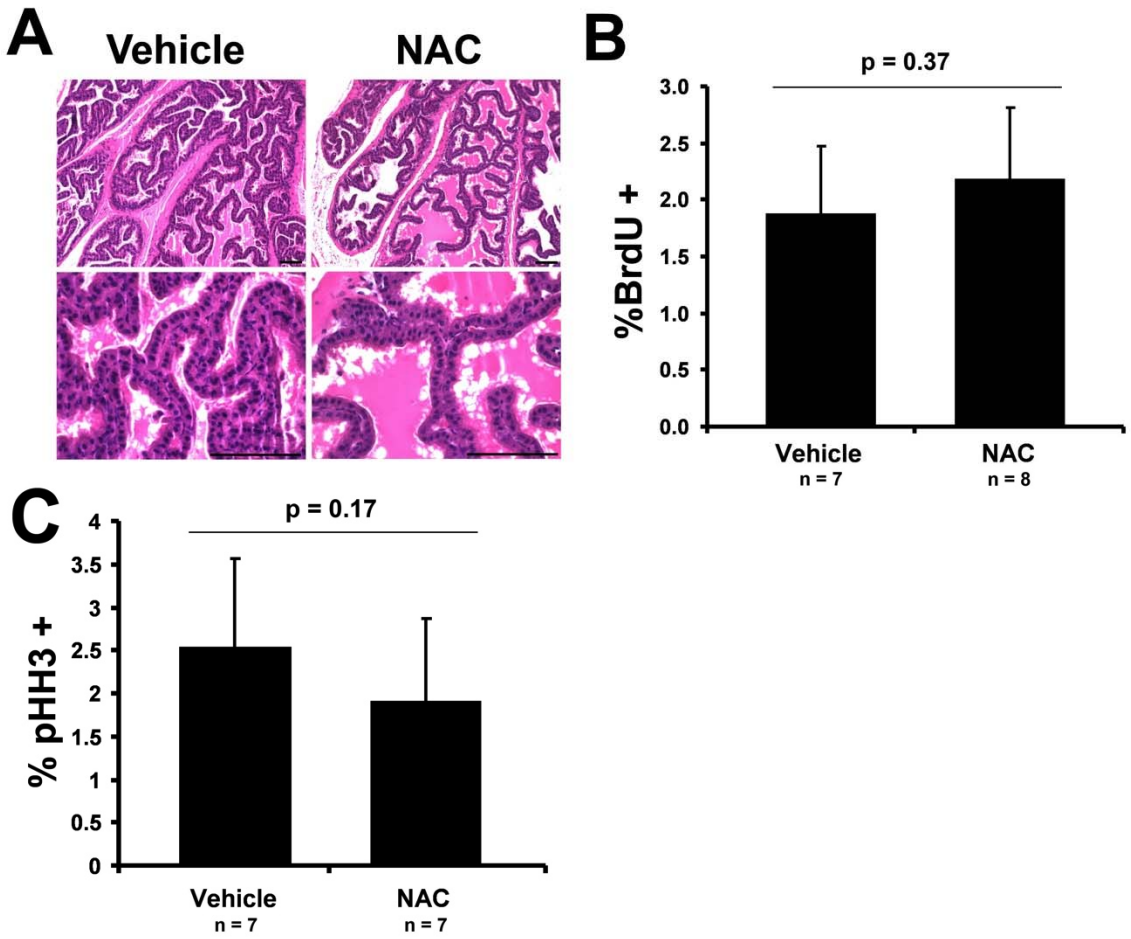

Figure 9. NAC supplementation does not alter epithelial proliferation in the *Nkx3.1*$^{+/+}$ prostate
(A) H&E sections of *Nkx3.1*$^{+/+}$ vehicle and NAC-supplemented anterior prostate show no change in histology. Scale bar = 0.1mm. (B) Quantification of BrdU immunohistochemical staining in *Nkx3.1*$^{+/+}$ vehicle and NAC-supplemented anterior prostate. (C) Quantification of pHH3 immunohistochemical staining in *Nkx3.1*$^{+/+}$ vehicle and NAC-supplemented anterior prostate. p value for a Student's t-Test is shown.

NAC supplementation of the *Nkx3.1*$^{-/-}$ mouse prostate promotes expression of a pro-proliferative gene signature

ROS have been shown to induce senescence and quiescence in human and mouse models of disease (*206*). Because quenching of prostatic ROS with NAC increased epithelial cell proliferation, I hypothesized that oxidative stress in the *Nkx3.1*-null prostate induces cell cycle arrest. I performed immunohistochemical staining for well-

defined markers of senescence (p16, p21) and quiescence (p27) in *Nkx3.1*$^{-/-}$ vehicle and NAC-supplemented prostates. Expression of these markers remains unchanged with NAC supplementation (Figure 10).

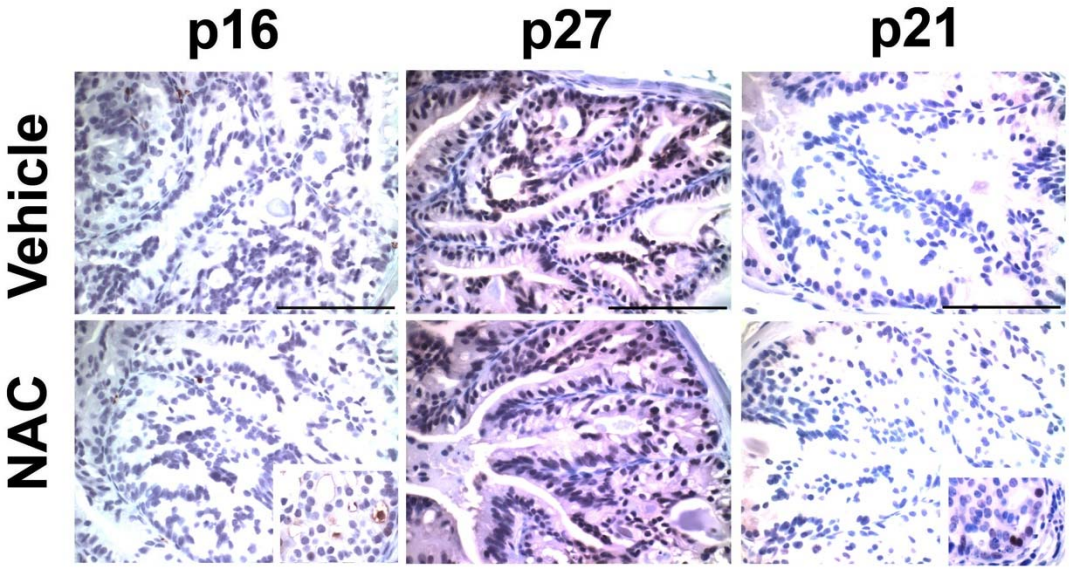

Figure 10. NAC supplementation of the *Nkx3.1*$^{-/-}$ prostate does not alter expression of well-established senescence and quiescence markers
Immunohistochemical staining of *Nkx3.1*$^{-/-}$ vehicle and NAC-supplemented anterior prostate for p16, p27, and p21. *p16 inset*: positive control for p16 staining from *PbCre4; Pten*$^{f/f}$ prostate (*207*). *p21 inset*: positive control for p21 staining from *PbCre4; Pten*$^{f/f}$*; p53*$^{f/+}$ prostate(*208*). Scale bar = 0.1mm.

In order to analyze global gene expression changes associated with NAC supplementation of the *Nkx3.1*$^{-/-}$ prostate, we performed Affymetrix microarray analysis on total RNA extracted from three *Nkx3.1*$^{-/-}$ vehicle and four *Nkx3.1*$^{-/-}$ NAC-supplemented anterior prostates. Genome Set Enrichment Analysis (GSEA) (*156*, *157*) is used to determine if the expression of *a priori* defined gene sets, relating to biological pathways or experimental conditions, is significantly altered in the experimental tissue of interest. GSEA allows for detection of modest gene expression changes of many genes in

one pathway that as a group may have a functional biological effect. The GSEA Molcular Signatures Database (MSigDB) collections consist of sets of human genes. We compared our mouse gene expression data to the human gene sets using the human genes orthologous to the mouse genes. Using the C2 (curated) gene sets collection, we identified many gene sets that were significantly enriched or depleted in NAC-supplemented *Nkx3.1*$^{-/-}$ prostates, including several that are associated with proliferation control and quiescence (Appendix A and Appendix B).

To obtain a broader picture of the relationships between the significantly altered gene sets in NAC-supplemented *Nkx3.1*$^{-/-}$ prostates, we performed Enrichment Map analysis (*204*). This is a method for GSEA interpretation and visualization which constructs networks from gene sets (nodes) containing overlapping genes. Analysis of identified networks using Enrichment Map can yield important information about the broad biological processes altered in a treatment group. Enrichment Map results for all networks containing ≥5 nodes are presented in Figure 11A. The first network I term "proliferation control" and consists of 7 nodes. One of these upregulated "proliferation control" gene sets (GRAHAM_NORMAL_QUIESCENT_VS_NORMAL_DIVIDING_DN) is a gene set consisting of transcripts that are downregulated during quiescence of hemopoetic stem cells (HSCs) and another is a set upregulated in dividing leukemia stem cells compared to quiescent HSCs (GRAHAM_CML_DIVIDING_VS_NORMAL_QUIESCENT_UP) (Figure 7B, (*209*)). Another upregulated "proliferation control" gene set is ROSTY_CERVICAL_CANCER_PROLIFERATION_CLUSTER, consisting of genes controlling cell division and proliferation and associated with an increased severity and

early relapse in cervical cancer (Figure 11B, (*210*)). Enrichment of this network in the NAC-supplemented prostate serves as further quantitative evidence of increased proliferation in *Nkx3.1*$^{-/-}$ prostate upon NAC supplementation. Another network upregulated in the NAC-supplemented *Nkx3.1*$^{-/-}$ prostates contains gene sets comprised in a large part by chemokine/growth factor genes such as REACTOME_G_ALPHA_I_SIGNALLING_EVENTS (Figure 11A, 11B). A network consisting of sets involved in immune regulation was depleted in NAC-supplemented *Nkx3.1*$^{-/-}$ prostates (Figure 11A).

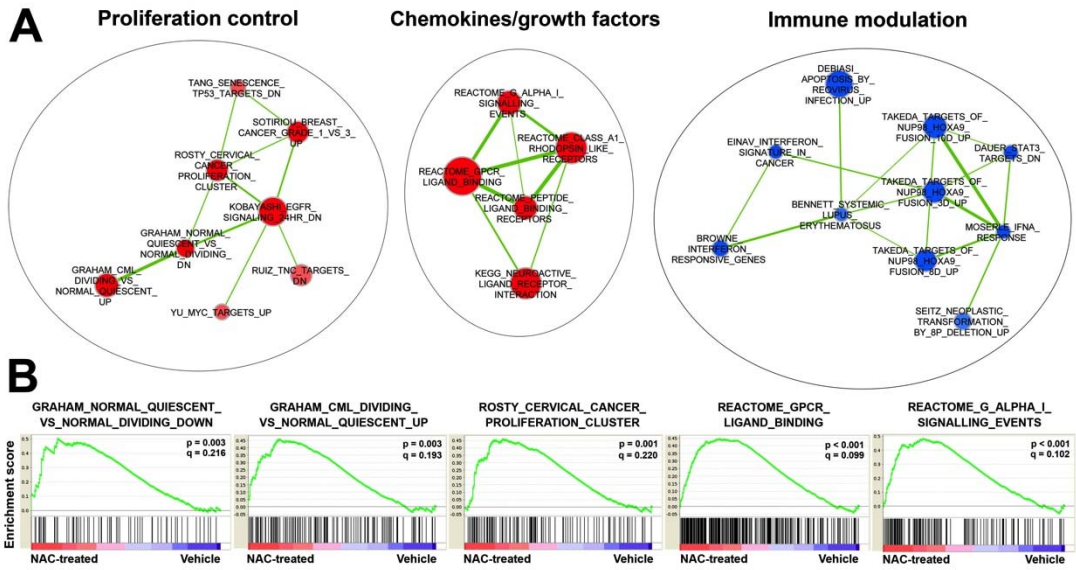

Figure 11. NAC supplementation promotes proliferation of a pro-proliferative gene expression signature in *Nkx3.1*$^{-/-}$ prostate

(A) Enrichment Map (*204*) analysis for Genome Set Enrichment Analysis (GSEA) C2 (curated) gene set data obtained from vehicle and NAC-supplemented *Nkx3.1*$^{-/-}$ anterior prostate. Map displays the related gene networks containing ≥5 gene sets with a false discovery rate (FDR) q value <0.25. Node size corresponds to gene set size. Hue designates which manner in which the gene sets are altered (red = enriched in NAC-supplementation, blue = depleted in NAC-supplementation). Color intensity represents significance by enrichment p value. Line thickness connecting the gene set nodes represents the degree of gene overlap between the two sets. (B) GSEA Enrichment plots (*156*, *157*) for selected gene sets from the "proliferation control" network and the "chemokines/growth factors" network. Nominal p value (statistical significance of the enrichment) and the FDR are presented.

The ‘leading edge’ is the subset of genes within a specific MSigDB gene set which drives the observed association in GSEA. Analysis of the leading edge genes may help to determine which changes in gene expression are responsible for a given phenotype. Leading edge genes from the “proliferation control” network (Table 2) include many classic pro-proliferative genes such *Ccna2* (*CCNA2* in human), *Cdc6, Tk1*, and *Gmnn.* Leading edge genes in the “chemokines/growth factors” network (Table 3) include many involved in pathways that have proven links to prostate cancer, including chemokines/chemokine receptors (*Ccl2, Cxcl5, Cxcr1, Cxcr2*) (*211*),(*212*), the endothelin axis (*Ednrb, Ednra*) (*213*), and neuropeptides (*Npy, Npy1r, Npy5r, Pyy*) (*214*).

Table 2. Leading edge genes from a sample of "proliferation control" gene sets with significant enrichment

Gene set name	Leading edge genes
GRAHAM_NORMAL_QUIESCENT_ VS_NORMAL_DIVIDING_DN	CD36, TK1, CPA3, RACGAP1, DLGAP5, CDC6, PRC1, COTL1, DTL, BUB1, MCM10, CDC20, CCNB2, RRM2, MCM6, MELK, NDC80, CCNA2, CENPM, GMNN, RAD51AP1
GRAHAM_CML_DIVIDING_ VS_NORMAL_QUIESCENT_UP	CD36, TUBB6, CCL2, SERPINB2, XIST, PF4, TK1, CPA3, HGF, RACGAP1, FAM38B, DLGAP5, CDC6, MPO, PRC1, COTL1, BUB1, MCM10, CDC20, CCNB2, PBK, RRM2, PPBP, UBE2S, CDC7, TPX2, CLEC11A, NEK2, MICAL2, MELK, NDC80, ASPM, KPNA2, HMMR, CCNA2, CENPM, GMNN, RAD51AP1, BRCA1, ECT2, PMP22, AURKA, CSTA, ESPL1, ACOT7, ELOVL6
ROSTY_CERVICAL_CANCER_ PROLIFERATION CLUSTER	TK1, SHCBP1, NETO2, RACGAP1, DLGAP5, HN1, PLK1, CDC6, MKI67, PRC1, CDCA3, DTL, BUB1, ASF1B, E2F1, MCM10, CDC20, CCNB2, PBK, RRM2, CDCA8, UBE2S, DBF4, TPX2, NEK2, MELK, NDC80, ASPM, KPNA2, CELSR3, HMMR, CCNA2, CENPM, GMNN, RAD51AP1, BRCA1, ECT2, AURKA, ESPL1, HMGA1, AURKB, NCAPH, TACC3, TTK, E2F8, LRP8, LMNB1

Table 3. Leading edge genes from a sample of "chemokines/growth factors" gene sets with significant enrichment

REACTOME_GPCR_ LIGAND_BINDING	EDNRB, CXCR2, CCL7, CCL2, CXCL13, FFAR1, PF4, NPY, NPY1R, OPN4, C3, HTR5A, ADORA2B, GRM3, HEBP1, PROK2, CCL3, S1PR3, CCL11, NPS, C5AR1, CNR1, AVPR1B, VIP, SSTR1, FPR1, ANXA1, CALCRL, OPRM1, P2RY13, WNT2B, PDYN, UTS2, F2, TSHR, UTS2R, S1PR2, CCL4, GNG3, TAC1, CXCL11, APLN, GNB3, HRH3, DARC, HTR1A, AVPR1A, ADORA1, ADORA3, DRD5, TAS1R2, TACR3, FSHB, NPY5R, CCR3, CCL22, PPBP, RHO, HTR1D, HTR4, HCRT, BDKRB2, C3AR1, MC4R, ADM2, APLNR, CXCR3, TAS1R1, SSTR2, WNT6, OPRL1, GRM5, PROKR2, ADRA1D, LPAR4, OPRK1, FZD4, CHRM5, NPSR1, TAAR1, GPBAR1, MC2R, FFAR2, WNT4, WNT8A, HTR6, CCL17, CXCR5, SCT, ADCYAP1, ADRB3, LPAR1, TSHB, SSTR3, SSTR4, OPRD1, GHRHR, TRH, HRH4, PYY, CCL25, CCR10, OPN5, GALR2, QRFPR, HCRTR2, ADRA2C, CXCR1, GPR17, AGT, PPYR1, FZD10, CALCB, KISS1R, CASR, CCR7, EDNRA, HTR1B, CRHR2, MTNR1B, P2RY2, BDKRB1, HRH1, PRLH, CCR1, TRHR, OXT, P2RY4, GIPR, CXCL5
REACTOME_G_ALPHA_ I_SIGNALLING_EVENTS	CXCR2, CXCL13, PF4, NPY, NPY1R, C3, HTR5A, HEBP1, ADCY2, S1PR3, C5AR1, CNR1, ADCY4, SSTR1, FPR1, ANXA1, OPRM1, P2RY13, PDYN, S1PR2, GNG3, CXCL11, APLN, GNB3, HRH3, HTR1A, ADORA1, ADORA3, ADCY10, NPY5R, CCR3, PPBP, RHO, HTR1D, BDKRB2, C3AR1, APLNR, CXCR3, SSTR2, OPRL1, OPRK1, CXCR5, LPAR1, SSTR3, SSTR4, OPRD1, HRH4, PYY, CCL25, CCR10, OPN5, GALR2, ADRA2C, CXCR1, GPR17, AGT, PPYR1, CASR, CCR7, HTR1B, MTNR1B, BDKRB1, ADCY8, CCR1, GNAT1, P2RY4, CXCL5

Discussion

Our study has provided novel evidence of prostate tumor promotion by antioxidant supplementation. Using *Nkx3.1*-null mice, I have modeled antioxidant chemoprevention in the early stages of prostate tumorigenesis and shown an increase in prostate epithelial proliferation upon NAC supplementation. These results suggest that ROS can be anti-tumorigenic in the early stages of prostate cancer and that antioxidant chemoprevention may be ineffective or harmful in many circumstances.

In this report I have confirmed that *Nkx3.1*$^{-/-}$ mice display increased prostatic oxidative stress. The hyperproliferative state of the *Nkx3.1*$^{-/-}$ prostate may promote increased oxidative stress through one of many indirect mechanisms. However, I have shown that the oxidative stress regulatory genes *Gpx2*, *Prdx6*, and *Qsox1* are dysregulated in the mutant mice and are shown to be direct targets of the Nkx3.1 transcription factor in both the mouse and human prostate. Therefore, I propose that loss of Nkx3.1 expression may directly affect oxidative stress maintenance through dysregulation of these target genes.

To determine if elevated oxidative stress is a causative mechanism for the hyperplasia observed in the *Nkx3.1*$^{-/-}$ prostate, I supplemented *Nkx3.1*$^{-/-}$ mice with the antioxidant NAC. NAC is a precursor for the most prevalent antioxidant molecule in cells, glutathione (GSH). NAC has been safely used for many years in mice and humans and has been shown in previous studies to increase GSH concentration, decrease oxidative stress, and have beneficial clinical effects (*58*, *215*). While NAC supplementation did decrease ROS levels in the *Nkx3.1*$^{-/-}$ prostate, it did not alter the

hyperplastic phenotype. Upon immunohistochemical staining with BrdU and pHH3, I observed that NAC supplementation promoted proliferation in the *Nkx3.1*$^{-/-}$ prostate. Surprisingly, rather than inhibit the hyperplastic phenotype, NAC supplementation promotes hyperplasia in the *Nkx3.1*$^{-/-}$ prostate. In the *Nkx3.1*$^{+/+}$ prostate, NAC supplementation did not increase proliferation, suggesting that the mechanism by which NAC increases proliferation in the *Nkx3.1*$^{-/-}$ prostate is related to elevated oxidative stress.

I propose that in the setting of Nkx3.1-loss, ROS are preventing further increases in proliferation of the prostate epithelium or inducing cell cycle arrest, a phenomenon which has been observed in other systems (reviewed in (*216*)). I hypothesized that elevated ROS in the *Nkx3.1*$^{-/-}$ prostate is activating an anti-proliferative pathway or inhibiting a pro-proliferative pathway, reducing the proliferative potential of the prostate epithelial cells. By decreasing these ROS by antioxidant supplementation, the epithelial cells are free to proliferate at a higher level. To first test this hypothesis, I performed immunohistochemical analysis of well-established senescence and quiescence markers. This did not reveal any changes with NAC supplementation of the *Nkx3.1*$^{-/-}$ prostate. To further investigate the possible mechanism behind the increased proliferation upon NAC supplementation, we performed global gene expression analysis on vehicle and NAC-supplemented *Nkx3.1*$^{-/-}$ prostate. Analysis of the gene expression data with GSEA and Enrichment Map revealed a significant enrichment in expression of gene sets involved in proliferation control and chemokine/growth factor function and depletion of an immune modulation cluster in NAC-supplemented prostates. Some of the gene expression changes may be a result of elevated proliferation, whereas others may be the factors

directly modulated by antioxidant supplementation and causing the change in proliferation. Pro-proliferative gene sets enriched in *Nkx3.1*$^{-/-}$ prostate upon NAC supplementation included gene sets such as GRAHAM_NORMAL_QUIESCENT_VS_NORMAL_DIVIDING_DN, which is a set of genes which are downregulated in normal quiescent cells as compared to normal dividing cells. These genes were upregulated in NAC-supplemented prostates, indicating that a pro-cell division phenotype was present. Genes in these pro-proliferative gene sets included many from classic pro-proliferative gene families such as cyclin genes, cell division cycle (cdc) genes, and aurora kinase genes. In addition other genes in these sets classically implicated in proliferation and cell cycle progression include thymidine kinase 1 (*TK1*), polo-like kinase 1 (*PLK1*), and *E2F1*.

NAC-enriched chemokine/growth factors gene sets included genes involved in pathways that been implicated in prostate tumorigenesis, including chemokines/chemokine receptors (*Ccl2, Cxcl5, Cxcr1, Cxcr2*) the endothelin axis (*Ednrb, Ednra*) and neuropeptides (*Npy, Npy1r, Npy5r, Pyy*). Upregulation of genes in these pathways could play a role in the increased proliferation with NAC supplementation. While direct ROS-mediated inhibition of chemokines has not described, this is a possible mechanism by which NAC quenching of ROS could allow for increased pathology of the prostate.

The third major cluster of gene sets which was altered in the NAC-supplemented prostates was an immune modulation cluster. This cluster of gene sets was depleted in the NAC-supplemented group. One of the most common genes present in these gene sets is STAT1, a transcription factor that is known to inhibit proliferation, induce cell cycle

arrest, and induce apoptosis in cancer cells (*217*). STAT1 is generally considered to promote anti-cancer immune responses (*218*). Therefore, depletion of STAT1 and related genes could help promote increased cell division upon NAC supplementation.

In addition to gene expression changes at the RNA level, ROS may modulate activity of proteins to affect proliferation, as has been thoroughly described in the literature (*216*). For example, the important cell cycle regulator CDK1 has been shown to be inhibited by oxidative stress by changing its phosphorylation status (*219*), causing cell cycle arrest and apoptosis. Protein phosphatases are a class of molecules that have been shown to be modulated by oxidative stress (*220*). Many protein phosphatases have been shown to be inhibited by oxidative stress, allowing for phosphorylation and activation of proteins involved in pro-proliferative signaling such as MAPK and Akt (*221*). However, elegant studies have also shown in non-transformed cells that oxidative stress can activate protein phosphatase 2A, which dephosphorylates pRb, prohibiting cell cycle progression (*222*, *223*). Thus, antioxidants could inhibit these changes, allowing for greater proliferation.

Based upon our findings, I propose a potential model for Nkx3.1-loss associated ROS and NAC supplementation in prostate tumor initiation (Figure 12). Loss of Nkx3.1 expression in the prostate causes dysregulation of antioxidant and pro-oxidant direct target genes, resulting in elevated ROS in the hyperplastic *Nkx3.1*$^{-/-}$ prostate. These ROS may actually limit proliferation in the *Nkx3.1*$^{-/-}$ prostate by inhibiting expression of pro-proliferative genes. ROS have been shown to induce cell cycle arrest or decrease proliferation in several models of non-cancerous and cancerous cells (*78–80*, *224*) and, in some of these cases, antioxidant supplementation has been explicitly shown to reverse

these ROS-induced effects. Thus, NAC may be increasing proliferation of the *Nkx3.1*-null prostate by decreasing ROS-mediated inhibition of pro-proliferative genes. An alternative hypothesis would be that NAC works through a ROS-independent mechanism to ellicit its effect. NAC has been shown to modulate gene expression, cellular signaling pathways, intracellular trafficking, secretion, immune function, and mitochondrial function (*59*).

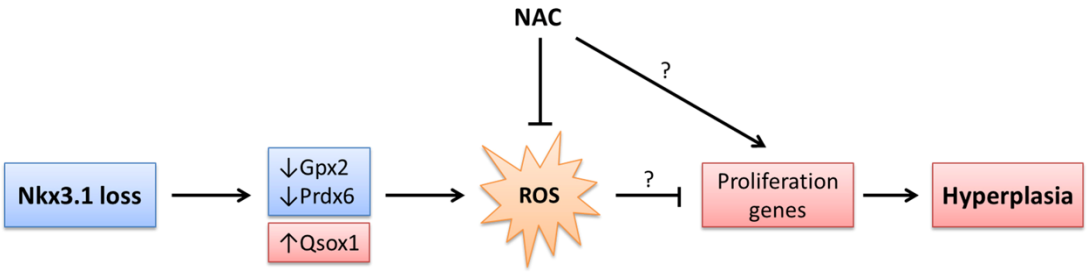

Figure 12. Potential model for Nkx3.1-loss associated ROS and NAC supplementation in prostate tumor initiation.

Results from this study emphasize the need for a deeper understanding of the role ROS play in prostate tumor progression. The effect of ROS on cells is not always pro-tumorigenic. The level of ROS present in a tissue can influence the effect seen, with high levels of ROS promoting senescence or cell death, but lower levels promoting DNA mutations or activating pro-proliferative signaling. The cell type with which ROS interacts also determines its effect. In a normal cell, a certain level of ROS may kill the cell or cause a cell to undergo cell cycle arrest, while in a cancer cell the same level of ROS may promote proliferation and invasion.

Despite the ability of antioxidants to inhibit cancer in several mouse models, I have shown that the antioxidant NAC promotes proliferation in the *Nkx3.1*$^{-/-}$ prostate. I propose that the *Nkx3.1*$^{-/-}$ mouse is a good model for antioxidant chemoprevention, exhibiting early lesions similar to those of cancer-naïve men in whom clinicians desire to prevent malignant disease. Indeed, antioxidants may function to inhibit tumor progression at later stages, effectively treating cancer in some settings. However, at early stages, ROS may actually slow or prevent tumor progression from occurring (*225*, *226*). In addition, different antioxidant compounds may affect the prostate in unique ways. Alternatively, ROS may have different effects on prostate tumor progression based on the genetic lesions or gene expression changes present.

The recent alarming results from the SELECT trial, in which "antioxidant chemoprevention" increased prostate cancer risk, can be informed by our study. While the proliferation upon NAC supplementation is not increased to an extremely large degree, it is nevertheless a significant increase and could become more pronounced with long term supplementation. NAC was not the specific antioxidant used in the SELECT trial; however, the results can yield important information due to the fact that NAC should decrease the overall oxidative state and possibly reflect possible results seen by other antioxidants. In fact, a recently published study using selenium and vitamin E in a rat model of prostate tumorigenesis showed a similar finding, that vitamin E supplementation showed a marginally significant increase in prostate tumor formation (*113*).

In the setting of certain genetic lesions or expression changes, such as Nkx3.1-loss, depleting ROS may actually allow cells to escape a ROS-mediated inhibition of

proliferation, increasing the chance of transformation of the prostate epithelium. The increased prostate cancer risk in the SELECT study population may indeed be driven by a subset of participants with an inherited polymorphism in Nkx3.1 (rs11781886) that is associated with increased prostate cancer risk (*227*). Depletion of ROS by vitamin E may have modified the risk from the levels normally associated with the polymorphism, producing the surprising detrimental effect with vitamin E chemoprevention. Oxidative stress and antioxidant levels have been shown in previous studies to modify cancer risk associated with inherited polymorphisms (*228–232*). Studies are ongoing using the SELECT biorepository to determine if antioxidant supplementation increased the prostate cancer risk associated with the functional *NKX3.1* variant (rs11781886) (*233*).

Our report provides valuable insight into the inconsistent results among preclinical and clinical studies on the efficacy of prostate cancer antioxidant chemoprevention (*102*). I suggest that investigation of prostate cancer chemoprevention specifically in physiologically relevant models, with analysis of the complexities of specific gene expression changes, is critically needed if clinically applicable results are desired. Caution should be taken when using antioxidants for prostate cancer prevention, because the effect which they have, beneficial or harmful, may lie in the makeup of the prostate gland of each unique individual.

CHAPTER IV

GENETIC VARIANTS AND PROSTATE CANCER RISK IN THE SELECT TRIAL

Introduction

Secondary results from two previous clinical trials (the Nutritional Prevention of Cancer Trial [NPCT] (*191*), and the Alpha-Tocopherol Beta-Carotene Cancer Prevention Study [ATBC] (*130*)) indicated that selenium or vitamin E supplementation was able to reduce prostate cancer incidence. Therefore, to test the ability of these agents to effectively prevent prostate cancer development, the multi-center Selenium and Vitamin E Cancer Prevention Trial (SELECT) was initiated in 2001 (*135*, *136*).

SELECT had over 32,000 prostate cancer naïve participants in the United States, Puerto Rico, and Canada. Upon initiation of the study, there was great hope and confidence in the field that a new prevention strategy for prostate cancer prevention would emerge that could decrease disease burden in a widespread population. Unfortunately, neither selenium nor vitamin E alone or in combination was able to prevent prostate cancer development, and vitamin E supplementation actually increased the risk of prostate cancer (*138*).

Recent studies from our laboratory have identified the prostate cancer tumor suppressor *NKX3.1* as possible genetic risk factor for prostate cancer development upon antioxidant supplementation. I reported that supplementation with the antioxidant N-

acetyl-cysteine promotes prostate epithelial cell proliferation in the *Nkx3.1*$^{-/-}$ mouse, a mouse model of the earliest stages of transformation of the prostate gland (*159*). These mice are deficient in the homeobox transcription factor *Nkx3.1,* and at advanced age display a hyperplastic/dysplastic prostate phonotype similar to the prostate cancer precursor lesion seen in humans, prostatic intraepithelial neoplasia (PIN) (*151*, *152*, *199*). NKX3.1 expression is significantly decreased during human prostate tumorigenesis (*150*, *153*, *193*), suggesting that NKX3.1 acts as a tumor suppressor in the prostate. *Nkx3.1*-null mice exhibit dysregulation of oxidative stress control genes and display increased oxidative stress (*155*, *159*) which has been proposed as a mechanism of tumor promotion upon NKX3.1 loss. However, antioxidant supplementation of these mice increased rather than decreased prostate epithelial proliferation in the *Nkx3.1-null* mice (*159*). These results are reminiscent of the SELECT trial, where vitamin E supplementation in cancer naïve individuals increased prostate cancer risk in a moderate, but significant manner (*138*). Therefore, I propose that loss of NKX3.1 expression may play an important role in elevated prostate cancer risk upon antioxidant supplementation.

In order to test this hypothesis in a human population, I set out to investigate if individuals with altered NKX3.1 expression or activity due to two prostate cancer-related genetic polymorphisms (rs11781886 and rs2228013) have an increased cancer risk upon antioxidant supplementation in the SELECT trial. Using the SWOG-defined case-cohort from the SELECT trial, we genotyped rs2228013 and rs11781886 to investigate the interaction of *NKX3.1* genotype and prostate cancer risk in all intervention arms of the study. I hypothesized that individuals with low NKX3.1 expression or activity (possessing risk alleles for rs11781886 or rs2228013) and supplemented with the

antioxidants vitamin E or selenium, would have an elevated prostate cancer risk compared to those with the polymorphisms in the placebo group. Our results show that prostate cancer risk associated with rs11781886 genotype is significantly increased by supplementation with selenium and vitamin E. These findings suggest that supplementation with antioxidants can alter risk associated with established genetic risk factors for prostate cancer and highlight specific populations with especially elevated risk.

In our investigation of genetic variants that may modify the increased prostate cancer risk with antioxidant supplementation in SELECT, I made the chance finding that the polymorphism rs11781866 on 8p21 in the region of the Bcl-2 and adenovirus E1B 19 kDa interacting protein 3 like (*BNIP3L*) gene modified risk with vitamin E supplementation. BNIP3L is a member of the BH3-only type of Bcl-2 family members and has been implicated in cancer as having both pro-survival and pro-apoptotic functions. Our results on rs11781866 are the first data showing a genetic polymorphism not previously described in prostate tumorigenesis that modifies the risk of prostate cancer in the vitamin E arm of SELECT. These findings shed important light on the mechanisms of prostate tumor initiation and may help explain the reasons behind the increased risk of prostate cancer upon vitamin E supplementation.

Methods

Study Populations

Nashville Men's Health Study (NMHS)

Study samples were composed of 790 men over 40 years of age who were scheduled for a diagnostic prostate biopsy from 2002-2008 at Vanderbilt University Medical Center, the Tennessee Valley Veteran's Administration Hospital, or Urology Associates, in Nashville, TN. Patient data, such as Gleason scores, clinical outcome, and PSA levels, were recorded as described (*234*).

SELECT

Study samples were composed of the SELECT trial case-cohort study defined by the Southwestern Oncology Group (SWOG). A subcohort representative of SELECT participants was created *a priori* as the comparison group for biomarker studies using the following approach. Men randomized into the study were stratified into 9 age/race cohorts: <55 (African American only), and 55-59, 60-64, 65-69, ≥70 years for both African Americans and others. Beginning in 2005 and annually until 2009, men with new diagnoses of prostate cancer had matching men randomly selected for the subcohort from the set of men with blood samples available within the same age-race stratum. A ratio of 1:3 was used for African Americans and 1:1.5 for others. Cases used in this analysis are as of July 31, 2009. The SELECT trial study population characteristics have been described (*137*, *138*). The subjects from the SWOG defined case-cohort genotyped for this analysis included 1,866 cases and 3,135 non-cases for a total of 5,001 samples. Study case-cohort characteristics are presented in Table 6.

SNP selection and genotyping

Nashville Men's Health Study

SNP analysis was performed using the ABI Taqman Allelic Discrimination Assay for rs11781886. Single SNP allelic discrimination was carried out using the ABI 7900HT machine. The genotyping was performed at the DNA Resources Core at the Vanderbilt Center for Human Genetics Research.

SELECT

SNP Analysis was performed using the ABI Prism Taqman Allelic Discrimination Assays for rs11781866, rs11781886, and rs2228013. Single SNP allelic discrimination was carried out using the ABI 7900HT, which allows single-plex SNP interrogation over a large volume of samples. The genotyping was performed at the Dana Farber/Harvard Cancer Center High Throughput Genotyping Core Facility.

Genotype statistical analysis for SELECT genotyping

SNP association with total, low grade (Gleason ≤6), and high grade (Gleason 7-10) prostate cancer risk was assessed over all four intervention arms of SELECT. Hazard ratios and associated p-values were calculated using a Cox proportional hazards model. Cases outside the subcohort enter the proportional hazards model just prior to diagnosis and remain in until diagnosis. Noncases in the subcohort enter the model at randomization and continue until they are censored. Cases in the subcohort appear in the model twice: once treated as noncases in the subcohort (entering at randomization, censored just prior to diagnosis), and once treated as cases outside the subcohort (*235*). We chose the weighting method of Prentice because it produced less biased estimates in a simulation study (*236*).

Because the sampling scheme used in creating the subcohort was stratified, all analyses were stratified based on the nine age/race groups. The different strata were weighted based on their subcohort selection probability.

Noncases were censored as of the earlier of July 31, 2009 or the date they were last known to be alive/date of death.

While genotype at rs2228013 was not correlated with baseline PSA level category ($p = 0.3090$ and $p = 0.7852$), rs11781886 genotype was significantly correlated with elevated baseline PSA category ($p < 0.0001$). However, the difference in PSA between genotypes was too small to have relevance for prostate cancer detection and was consistent among intervention arms (Table 4). Therefore, multivariate analysis for prostate cancer risk associated with all SNPs does not include baseline PSA level.

Table 4. Mean baseline PSA levels with genotype at rs11781886 in SELECT case-control cohort

Genotype	Intervention arm											
	Placebo			Vitamin E			Selenium			Vitamin E + Selenium		
rs11781886	Mean PSA (ng/mL)	SD	N	Mean PSA (ng/mL)	SD	N	Mean PSA (ng/mL)	SD	N	Mean PSA (ng/mL)	SD	N
TT	1.68	1.06	607	1.67	1.08	718	1.69	1.04	704	1.66	1.04	653
CT	1.73	1.08	496	1.78	1.05	478	1.86	1.10	445	1.74	1.01	481
CC	1.78	1.04	93	1.92	1.10	105	1.88	1.08	78	1.79	1.05	68

Target SNPs rs11781886 and rs11781866 were modeled in a joint effects model relative to the TT genotype in the placebo arm. Individual hazard ratios were calculated for each of the three possible genotypes and four intervention arms. An additional analysis was done to test for linear trend, where the genotypes were modeled 0, 1, and 2 for TT, CT, and CC respectively. Also, a joint effects model and linear trend analysis were performed using a 2 level model for these SNPs, with TT compared to CT and CC genotypes combined for rs11781886 and TT and CT genotypes combined compared to CC for rs11781866.

The target SNP rs2228013 was modeled in a joint effects model relative to the GG genotype in the placebo arm. Individual hazard ratios were calculated for the GG compared to AG and AA genotypes combined, due to the small number of samples with the AA genotype, and the four intervention arms. An additional analysis was done to test for linear trend, where the genotypes were modeled 0 and 1 for GG and AG/AA respectively.

Additional baseline covariates included in the models were family history of prostate cancer (yes or no), smoking status (nonsmoker, current smoker, former smoker), and body-mass index (linear).

All statistical analyses were performed using SAS version 9.2 software (SAS Institute, Cary, NC, USA). All statistical tests are two-sided, and $P < 0.05$ was considered statistically significant. Genotype effects for rs11781886 and rs11781866 were calculated using a 3 level model (TT, CT, CC), unless otherwise noted. Genotype effects for rs2228013 were calculated using a 2 level model (GG, AG/AA).

Microarray analysis of SELECT trial supplement clinical study

The microarray experiment by Tsavachidou and colleagues (*237*) was downloaded from Array Express (http://www.ebi.ac.uk/arrayexpress/experiments/E-MEXP-1327/). Affymetrix HG-U133A Arrays and matching patient data were analyzed for quality control in Bioconductor using packages affy (version 1.38.1), affyPLM (version 1.36.0), affyIO (version 1.28.0), and simpleaffy (version 2.36.0) in R version 3.0.0. Four of the 85 arrays did not pass quality control (DI02, DI04, DI45 and DI81). The remaining 81 arrays were normalized by quantiles, background corrected by RMA, and probesets were summarized by median polish. A Welch T-test was used to assess expression differences between individual genes.

Transcription Factor Motif Analysis

The sequence containing the rs11781866 SNP was extracted from Ensembl human genome version 71 and analyzed for vertebrate transcription factor binding sites at Jaspar (found at: http://jaspar.genereg.net/cgibin/jaspar_db.pl?rm=browse&db=core&tax_group=vertebrates). The score threshold was set at 80% [a measure of degeneracy] and the top transcription factors were recorded.

Results

Nashville Men's Health Study (NMHS) pilot genotyping study

To determine the ability to successfully genotype rs11781886, 790 DNA samples from the Nashville Men's Health Study were genotyped. The minor allele frequencies in

the white and black individuals for rs11781886 do differ from the MAF in the worldwide population of 0.301 (*238*, *239*) (Chi-Squared goodness of fit test p = 0.001565 and p = 0.028643 for white and black respectively) (Table 5). I then compared the observed MAFs for whites and blacks in the NMHS cohort to the subpopulations most similar to the race of the subgroups (CEU - Utah Residents (CEPH) with Northern and Western European ancestry- with a MAF of 0.265 for whites, and ASW - Americans of African Ancestry in SW USA - with a MAF of 0.311 for blacks) (*238*, *239*). The MAFs observed were not significantly different to race-specific MAFs (Chi-Squared goodness of fit test p = 0.811 for whites and p = 0.061 for blacks). It should be noted, however, that the MAFs calculated from general populations while the NMHS consists of men who are at a higher risk for prostate cancer diagnosis as they were selected due to the fact they were scheduled for a diagnostic biopsy of the prostate. Therefore, results from the NMHS genotyping may not be representative of the overall population. For this reason, I also did not observe an increase in prostate cancer diagnosis related to rs11781886 (*227*) (Table 5).

Table 5. Nashville Men's Health study rs11781866 genotyping results

	Overall	**White**	**Black**
Total genotypes (N)	790	698	92
Minor allele frequency	0.275	0.262	0.375
Proportion of cancer outcome with CC genotype	0.368	0.395	0.286
Proportion of cancer outcome with TT genotype	0.369	0.373	0.324

SELECT biorepository for case-control cohort genotyping analysis

To determine if *NKX3.1* levels/activity could modulate prostate cancer risk upon antioxidant supplementation, we genotyped a cohort of SELECT participants for the rs1781886 and rs2228013 variants in a cohort of SELECT participants (n = 5,001). SNP rs11781886 is found in the 5' untranslated region (UTR) of *NKX3.1* and leads to lower *NKX3.1* expression (*227*). Located in the second exon of *NKX3.1*, rs2228013 alters NKX3.1 phosphorylation and activity (*240*). The Southwestern Oncology Group (SWOG)-defined case-cohort characteristics are described in Table 6. In general, the case-cohort participants reflected the characteristics of the overall SELECT population. One exception to this is race distribution, as SWOG over selected from the proportion of African American cases and controls to increase the power to detect significant findings in this important group.

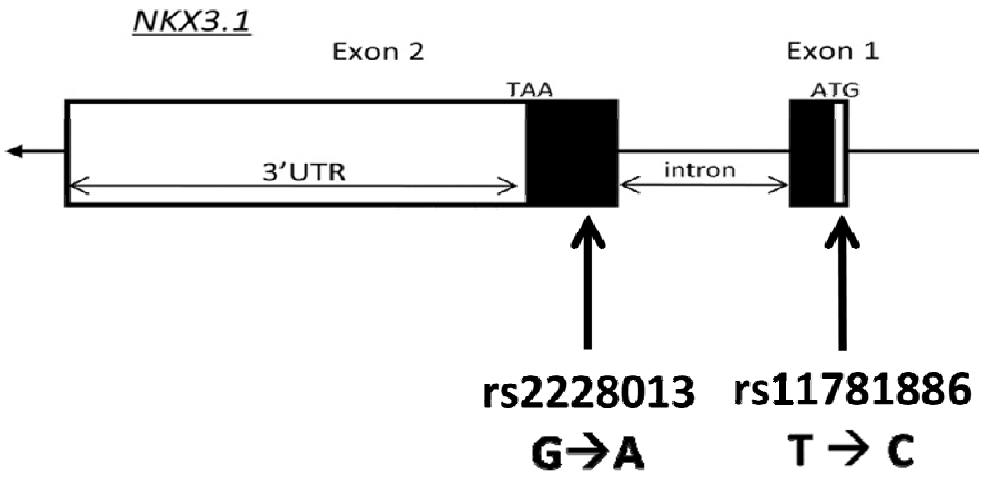

Figure 13. Location of *NKX3.1* SNPs genotyped in this study. Modified from (*227*), this image depicts the genetic location of SNP rs11781886 in the 5' UTR of the *NKX3.1* gene (*227*), and rs2228013 in the 2nd exon of the *NKX3.1* gene (*240*).

Table 6. Baseline characteristics of SELECT case-control cohort (n = 5,001)

Characteristic	Noncases N = 3,135 N (%)	Cases N = 1,866 N (%)	High-Grade Cases N = 540 N (%)	Low-Grade Cases N = 1,081 N (%)
Age, y				
<55	126 (4.0%)	42 (2.3%)	11 (2.0%)	22 (2.0%)
55-59	832 (26.5%)	503 (27.0%)	123 (22.8%)	311 (28.8%)
60-64	926 (29.5%)	565 (30.3%)	150 (27.8%)	339 (31.4%)
65-69	724 (23.1%)	437 (23.4%)	138 (25.6%)	242 (22.4%)
≥70	527 (16.8%)	319 (17.1%)	118 (21.9%)	167 (15.4%)
Race				
White (non-Hispanic)	2,175 (69.4%)	1,521 (81.5%)	439 (81.3%)	899 (83.2%)
African American	756 (24.1%)	253 (13.6%)	79 (14.6%)	130 (12.0%)
Other	204 (6.5%)	92 (4.9%)	22 (4.1%)	52 (4.8%)
Body mass index (kg/m ^{2})				
<25	606 (19.3%)	357 (19.1%)	98 (18.1%)	207 (19.1%)
25-<30	1,466 (46.8%)	950 (50.9%)	244 (45.2%)	583 (53.9%)
≥30	1,052 (33.6%)	556 (29.8%)	197 (36.5%)	289 (26.7%)
Unknown	11 (0.4%)	3 (0.2%)	1 (0.2%)	2 (0.2%)
Smoking Status				
Never	1,292 (41.2%)	894 (47.9%)	263 (48.7%)	516 (47.7%)
Former	1,553 (49.5%)	868 (46.5%)	246 (45.6%)	500 (46.3%)
Current	267 (8.5%)	99 (5.3%)	28 (5.2%)	63 (5.8%)
Unknown	23 (0.7%)	5 (0.3%)	3 (0.6%)	2 (0.2%)
Baseline PSA				
0.00-0.99	1,391 (44.4%)	134 (7.2%)	33 (6.1%)	73 (6.8%)
1.00-1.99	1,070 (34.1%)	486 (26.0%)	140 (25.9%)	269 (24.9%)
2.00-2.99	444 (14.2%)	627 (33.6%)	196 (36.3%)	360 (33.3%)
≥3	230 (7.3%)	618 (33.1%)	171 (31.7%)	378 (35.0%)
Unknown	0 (0.0%)	1 (0.1%)	0 (0.0%)	1 (0.1%)
History of Diabetes				
No	2,737 (87.3%)	1,733 (92.9%)	491 (90.9%)	1,018 (94.2%)
Yes	398 (12.7%)	133 (7.1%)	49 (9.1%)	63 (5.8%)
First-degree relative with prostate cancer				
None	2,626 (83.8%)	1,284 (68.8%)	384 (71.1%)	733 (67.8%)
≥1	507 (16.2%)	582 (31.2%)	156 (28.9%)	348 (32.2%)
Unknown	2 (0.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)

Table 6, continued

Characteristic	Noncases N = 3,135 N (%)	Cases N = 1,866 N (%)	High-Grade Cases N = 540 N (%)	Low-Grade Cases N = 1,081 N (%)
SELECT intervention assignment				
Vitamin E + Selenium	772 (24.6%)	448 (24.0%)	138 (25.6%)	257 (23.8%)
Vitamin E alone	800 (25.5%)	518 (27.8%)	148 (27.4%)	291 (26.9%)
Selenium alone	782 (24.9%)	465 (24.9%)	138 (25.6%)	265 (24.5%)
Placebo	781 (24.9%)	435 (23.3%)	116 (21.5%)	268 (24.8%)
SNP: RS11781886				
TT	1,717 (54.8%)	984 (52.7%)	273 (50.6%)	579 (53.6%)
CT	1,184 (37.8%)	737 (39.5%)	227 (42.0%)	411 (38.0%)
CC	211 (6.7%)	136 (7.3%)	37 (6.9%)	86 (8.0%)
Unknown	23 (0.7%)	9 (0.5%)	3 (0.6%)	5 (0.5%)
SNP: RS2228013				
GG	2,864 (91.4%)	1,693 (90.7%)	488 (90.4%)	978 (90.5%)
AG	233 (7.4%)	150 (8.0%)	45 (8.3%)	90 (8.3%)
AA	5 (0.2%)	2 (0.1%)	0 (0.0%)	2 (0.2%)
Unknown	33 (1.1%)	21 (1.1%)	7 (1.3%)	11 (1.0%)
SNP: RS11781866				
TT	1,424 (45.4%)	842 (45.1%)	238 (44.1%)	488 (45.1%)
CT	1,374 (43.8%)	809 (43.4%)	246 (45.6%)	463 (42.8%)
CC	318 (10.1%)	201 (10.8%)	52 (9.6%)	120 (11.1%)
Unknown	19 (0.6%)	14 (0.8%)	4 (0.7%)	10 (0.9%)

The increased total prostate cancer risk for the vitamin E arm in the case-cohort (HR 1.178, 95% CI 0.987-1.405, p = 0.0688) (Table 8) was similar to that of the entire SELECT study (HR 1.17, 95% CI 1.004-1.36, p = 0.008) (*138*). The increase in risk with vitamin E supplementation did not reach statistical significance for low grade prostate cancer (HR 1.070, 95% CI 0.867-1.319, p = 0.5294) (Table 9) or high grade prostate cancer (HR 1.250, 95% CI 0.942-1.658, p = 0.1224) (Table 10) in the case-cohort.

Effect of *NKX3.1* polymorphisms rs11781886 and rs2228013 on prostate cancer risk in SELECT

The minor allele frequency in the case-cohort for rs11781886 was 0.2631, which is similar to the frequency in a worldwide population of 0.301 (*238*, *239*). The observed minor allele frequency of 0.0386 for rs2228013 was similar to the published frequency of 0.022 (*238*, *239*).

rs11781886

Genotype at rs11781886 was not associated with a significant change in overall, low or high grade prostate cancer risk in the case-cohort as a whole, including all intervention arms (Table 7). I analyzed the interaction of risk associated with rs11781886 with intervention arm to determine if antioxidant supplementation modifies prostate cancer risk associated with genotype. None of the intervention arms significantly altered the overall trend in total, low grade, or high grade prostate cancer risk associated with rs11781886 (Tables 8-10).

Table 7. Effect of polymorphisms rs11781886 and rs2228013 total, low grade, and high grade prostate cancer risk in all participants of SELECT case-control cohort

Polymorphism	Hazard Ratio	95% HR Confidence Limits	p value
Total			
rs11781886	1.072	0.967-1.188	0.1852
rs2228013	0.953	0.759-1.196	0.6773
Low grade			
rs11781886	1.076	0.951-1.218	0.2463
rs2228013	1.008	0.771-1.318	0.9529
High grade			
rs11781886	1.099	0.939-1.286	0.2390
rs2228013	0.933	0.654-1.329	0.6994

Table 8. Effect of genotype at rs11781886 and rs2228013 on total prostate cancer risk in each intervention arm of the SELECT case-control cohort

Genotype	Intervention arm											
	Placebo N = 1220			Vitamin E N = 1318			Selenium N = 1247			Vitamin E + Selenium N = 1216		
rs11781886	**HR**	**95% CI**	**p value**	**HR**	**95% CI**	**p value**	**HR**	**95% CI**	**P value**	**HR**	**95% CI**	**P value**
All genotypes	1.000	ref	Ref	1.178	0.987-1.405	0.0688	1.091	0.913-1.304	0.3367	1.021	0.853-1.222	0.8171
TT	1.000	ref	Ref	1.142	0.891-1.463	0.2951	1.074	0.836-1.379	0.5753	1.218	0.947-1.567	0.1238
CT	1.175	0.895-1.542	0.2448	1.500	1.124-1.971	0.0036	1.218	0.918-1.617	0.1710	0.966	0.733-1.272	0.8048
CC	1.144	0.690-1.898	0.6015	1.233	0.744-2.042	0.4162	1.676	1.011-2.777	0.0450	0.987	0.541-1.803	0.9671
p trend	0.2897			0.8548			0.6457			0.0634		
rs2228013												
All genotypes												
GG	1.000	ref	Ref	1.165	0.968-1.403	0.1066	1.064	0.882-1.283	0.5191	1.037	0.859-1.252	0.7074
AG or AA	0.912	0.574-1.447	0.6952	1.137	0.742-1.740	0.5557	1.365	0.876-2.128	0.1685	0.740	0.456-1.203	0.2247
p trend	0.6952			0.8330			0.2934			0.4722		

Table 9. Effect of genotype at rs11781866 and rs2228013 on low grade prostate cancer risk in each arm of the SELECT case-control cohort

Genotype	**Intervention arm**											
	Placebo			**Vitamin E**			**Selenium**			**Vitamin E + Selenium**		
Low grade cases												
	HR	**95% CI**	**p value**	**HR**	**95% CI**	**p value**	**HR**	**95% CI**	**p value**	**HR**	**95% CI**	**p value**
All genotypes	1.00	ref	Ref	1.070	0.867-1.319	0.5294	1.004	0.812-1.241	0.9728	0.932	0.752-1.154	0.5166
rs11781886												
TT	1.000	ref	Ref	1.159	0.864-1.554	0.3251	1.018	0.754-1.375	0.9054	1.182	0.874-1.599	0.2764
CT	1.261	0.917-1.734	0.1537	1.267	0.910-1.765	0.1608	1.172	0.836-1.643	0.3560	0.915	0.658-1.273	0.5977
CC	1.160	0.649-2.074	0.6156	1.341	0.742-2.424	0.3312	1.811	1.016-3.228	0.0441	1.010	0.487-2.095	0.9795
p trend	0.2212			0.7016			0.6488			0.0802		
rs2228013												
All genotypes												
GG	1.000	ref	Ref	1.079	0.865-1.346	0.4997	0.987	0.789-1.235	0.9082	0.956	0.764-1.197	0.6968
AG or AA	0.974	0.568-1.670	0.9232	1.036	0.621-1.728	0.8915	1.386	0.833-2.308	0.2091	0.761	0.431-1.343	0.3461
p trend	0.9232			0.9705			0.3317			0.6115		

Table 10. Effect of genotype at rs11781866 and rs2228013 on high grade prostate cancer risk in each arm of the SELECT case-control cohort

Genotype	**Intervention arm**											
	Placebo			**Vitamin E**			**Selenium**			**Vitamin E + Selenium**		
High grade cases												
	HR	95% CI	p value	HR	95% CI	p value	HR	95% CI	p value	HR	95% CI	p value
All genotypes	1.00	ref	ref	1.250	0.942-1.658	0.1224	1.201	0.902-1.598	0.2099	1.212	0.911-1.612	0.1868
rs11781886												
TT	1.000	ref	Ref	1.015	0.672-1.533	0.9447	1.059	0.710-1.579	0.7796	1.297	0.871-1.931	0.2002
CT	1.027	0.656-1.609	0.9058	1.753	1.146-2.680	0.0096	1.308	0.839-2.039	0.2355	1.078	0.692-1.678	0.7401
CC	1.164	0.519-2.611	0.7123	1.131	0.515-2.484	0.7600	1.325	0.559-3.140	0.5224	1.079	0.435-2.676	0.8695
p trend	0.7586			0.3596			0.6433			0.4247		
rs2228013												
GG	1.000	ref	Ref	1.209	0.896-1.630	0.2138	1.173	0.869-1.584	0.2969	1.197	0.887-1.616	0.2407
AG or AA	0.909	0.444-1.862	0.7938	1.223	0.635-2.354	0.5472	1.240	0.593-2.589	0.5677	0.928	0.448-1.922	0.8413
p trend	0.7938			0.8278			0.7724			0.7592		

However, allelic variation at rs11781886 in the different intervention arms was associated with significantly altered prostate cancer risk compared to the homozygous major allele genotype (TT) in the placebo group (Tables 8-10). The CT genotype at rs11781886 in the vitamin E arm was strongly associated with an increased risk of total (HR 1.500, 95% CI 1.124-1.971, p = 0.0036) and high grade (HR 1.753, 95% CI 1.146-2.680, p = 0.0096) prostate cancer (Table 8 and Table 10). There is a significant 45% increased overall prostate cancer risk (HR 1.450, 95% CI 1.117-1.882, p = 0.0052) and a significant 64% increase in high grade prostate cancer risk (HR 1.638, 95% CI 1.089-2.463, p = 0.0178) associated with having at least one C allele (genotype CT or CC) at rs11781886 with vitamin E supplementation (Table 8 and Table 10).

The CC genotype at rs11781886 in the selenium arm was associated with an increased risk of total (HR 1.676, 95% CI 1.011-2.777, p = 0.045) and low grade (HR 1.811, 95% CI 1.016-3.228, p = 0.0441) prostate cancer risk relative to the TT genotype in the placebo group (Table 8, Table 9). There is a marginally significant increased overall prostate cancer risk associated with having at least one C allele at rs11781886 with selenium supplementation (HR 1.277, 95% CI 0.976-1.669, p = 0.0744) (Table 8). Presence of the C allele in the vitamin E + selenium arm, however, was not associated with an increased risk (HR 0.968, 95% CI 0.742-1.264, p = 0.8134) (Table 8).

rs2228013

Contrary to a previously published report (*240*), rs2228013 was not associated with an increased risk of high grade prostate cancer (Table 7), nor was it associated with an increased overall risk of total prostate cancer in the study-wide population. Randomization arm did not significantly modify prostate cancer risk with rs2228013

genotype (Table 8). There were no significant associations with any genotype at rs2228013 in any randomization arm with prostate cancer risk for total prostate cancer (Table 8) or for low (Table 9) or high grade cancers (Table 10).

Effect of *BNIP3L* polymorphism rs11781866 on prostate cancer risk in SELECT

The rs11781866 minor allele frequency (MAF) in the case-cohort was 0.324, which is similar to the published frequency in a worldwide dataset of 0.284 (*238*, *239*). The dbSNP MAF ranges from 0.184 in a population of Mexican ancestry in California to 0.350 in a population of Northern and Western European ancestry in Utah (*241*).

rs11781866 modulates prostate cancer risk in the vitamin E arm of SELECT

I first analyzed the effect of genotype at rs11781866 on overall prostate cancer risk in all case-cohort subjects Genotype at rs11781866 did not effect overall risk in the case-control cohort as a whole (HR 0.994, 95% CI 0.904-1.093, p = 0.9045) (Table 11). Risk of high grade and low grade disease was also not altered by rs11781866 in the total case-cohort population (Table 11).

Table 11. Effect of polymorphism rs11781866 on total, low grade, and high grade prostate cancer risk in all participants of SELECT case-control cohort

	Hazard Ratio	95% HR Confidence Limits	p value
Total	0.994	0.904-1.093	0.9045
Low grade	0.984	0.877-1.104	0.7823
High grade	1.009	0.874-1.164	0.9060

However, when subjects were examined by intervention arm, I observed a significant interaction between rs11781866 genotype on total prostate cancer risk (p = 0.0372) only in the vitamin E arm (Table 12). When the risks for genotype with vitamin

E supplementation were compared to placebo with TT genotype, I observed 26% higher risk with the presence of at least one T allele in the vitamin E arm (HR 1.262, 95% CI 1.046-1.521, p = 0.0149), but no increased risk for those homozygous for the minor allele C in the vitamin E arm. Interestingly, individuals with the TT genotype in the vitamin E arm had a substantially higher risk (+42%) than those in the placebo group (HR 1.423, 95% CI 1.092-1.855, p = 0.0089) (Table 12). This risk decreased with each addition of a C allele to the genotype (Table 12). Notably, this significant genotype-specific modification of risk was not observed in the other intervention arms. The risk of high grade disease was not modulated by rs11781866 genotype in the vitamin E arm or in any other arm of the trial; instead, the risk modulation was specific to low-grade cases (Table 13).

Table 12. Effect of genotype at rs11781866 on total prostate cancer risk in each intervention arm of the SELECT case-control cohort

Genotype	Intervention arm											
	Placebo N = 1220			Vitamin E N = 1318			Selenium N = 1247			Vitamin E + Selenium N = 1216		
	HR	95% CI	p value	HR	95% CI	p value	HR	95% CI	p value	HR	95% CI	p value
All genotypes	1.000	ref	ref	1.178	0.987-1.405	0.0688	1.091	0.913-1.304	0.3367	1.021	0.853-1.222	0.8171
TT	1.000	ref	ref	1.423	1.092-1.855	0.0089	1.259	0.962-1.648	0.0929	1.127	0.861-1.475	0.3839
CT	1.125	0.858-1.476	0.3947	1.256	0.958-1.646	0.0995	1.171	0.893-1.536	0.2537	1.164	0.883-1.533	0.2807
CC	1.47	0.930-2.324	0.0989	1.115	0.736-1.689	0.6062	1.216	0.801-1.846	0.3576	1.104	0.717-1.701	0.6531
p trend	0.1069			0.0372			0.1504			0.2472		

Table 13. Effect of genotype at rs11781866 on low and high grade prostate cancer risk in each arm of the SELECT case-control cohort

Genotype	**Intervention arm**											
	Placebo			**Vitamin E**			**Selenium**			**Vitamin E + Selenium**		
Low grade cases												
	HR	**95% CI**	**p value**	**HR**	**95% CI**	**p value**	**HR**	**95% CI**	**p value**	**HR**	**95% CI**	**p value**
All genotypes	1.00	Ref	ref	1.070	0.867-1.319	0.5294	1.004	0.812-1.241	0.9728	0.932	0.752-1.154	0.5166
TT	1.000	Ref	ref	1.450	1.059-1.985	0.0206	1.263	0.915-1.743	0.1549	1.058	0.766-1.462	0.7308
CT	1.226	0.891-1.687	0.2111	1.115	0.803-1.549	0.5145	1.079	0.778-1.496	0.6496	1.088	0.781-1.517	0.6177
CC	1.624	0.957-2.753	0.0721	0.996	0.575-1.620	0.8953	1.141	0.690-1.886	0.6074	1.167	0.699-1.947	0.5543
p trend	0.0559			0.0063			0.0583			0.2629		
High grade cases												
	HR	**95% CI**	**P value**	**HR**	**95% CI**	**P value**	**HR**	**95% CI**	**P value**	**HR**	**95% CI**	**P value**
All genotypes	1.00	Ref	ref	1.250	0.942-1.658	0.1224	1.201	0.902-1.598	0.2099	1.212	0.911-1.612	0.1868
TT	1.000	Ref	ref	1.303	0.847-2.005	0.2281	1.256	0.815-1.936	0.3011	1.282	0.835-1.969	0.2562
CT	1.081	0.691-1.693	0.7326	1.420	0.924-2.181	0.1097	1.395	0.908-2.144	0.1287	1.461	0.949-2.251	0.0852
CC	1.085	0.496-2.371	0.8386	1.328	0.717-2.462	0.3672	1.105	0.547-2.233	0.7814	0.993	0.490-2.012	0.9845
p trend	0.7405			0.9101			0.7804			0.6828		

rs11781866 may regulate BH3-only family member gene BNIP3L

To begin to investigate a possible mechanism behind the modification of prostate risk in the vitamin E group with rs11781866 genotype, I examined the gene locus around the SNP. rs11781866 is found at position 26,298,209 on chromosome 8p21, in a large intron of *BNIP3L* (Figure 14A). Other genes in this region include *PPP2R2A*, *PNMA2*, and *DPYSL2*. To investigate change in expression of genes around rs11781866 with SELECT trial supplements, we analyzed a randomized, placebo-controlled phase IIA study of prostate cancer patients before prostatectomy that was conducted using the identical supplements and dosage as used in SELECT (*237*). Participants scheduled for a prostatectomy indicated for prostate cancer treatment were randomized to take 200 μg of L-selenomethionine (selenium), 400 IU of all-rac-alpha-tocopheryl acetate (vitamin E), a combination of 200 μg of L-selenomethionine and 400 IU of vitamin E, or placebo for 3-6 weeks prior to prostatectomy. Using laser-capture microdissection, tumor tissue, normal epithelial tissue, and normal stromal tissue were isolated and RNA was extracted for microarray analysis (*237*). Analysis of these data showed that *BNIP3L* was overexpressed in vitamin E-supplemented tumor tissue relative to placebo-supplemented tumor tissue (Figure 14B). Expression of other genes in the region of rs11781866 was not affected by randomization arm (Figure 14B). This suggests Bcl-2 family member protein BNIP3L may play a role in the increased prostate cancer risk upon vitamin E supplementation in SELECT.

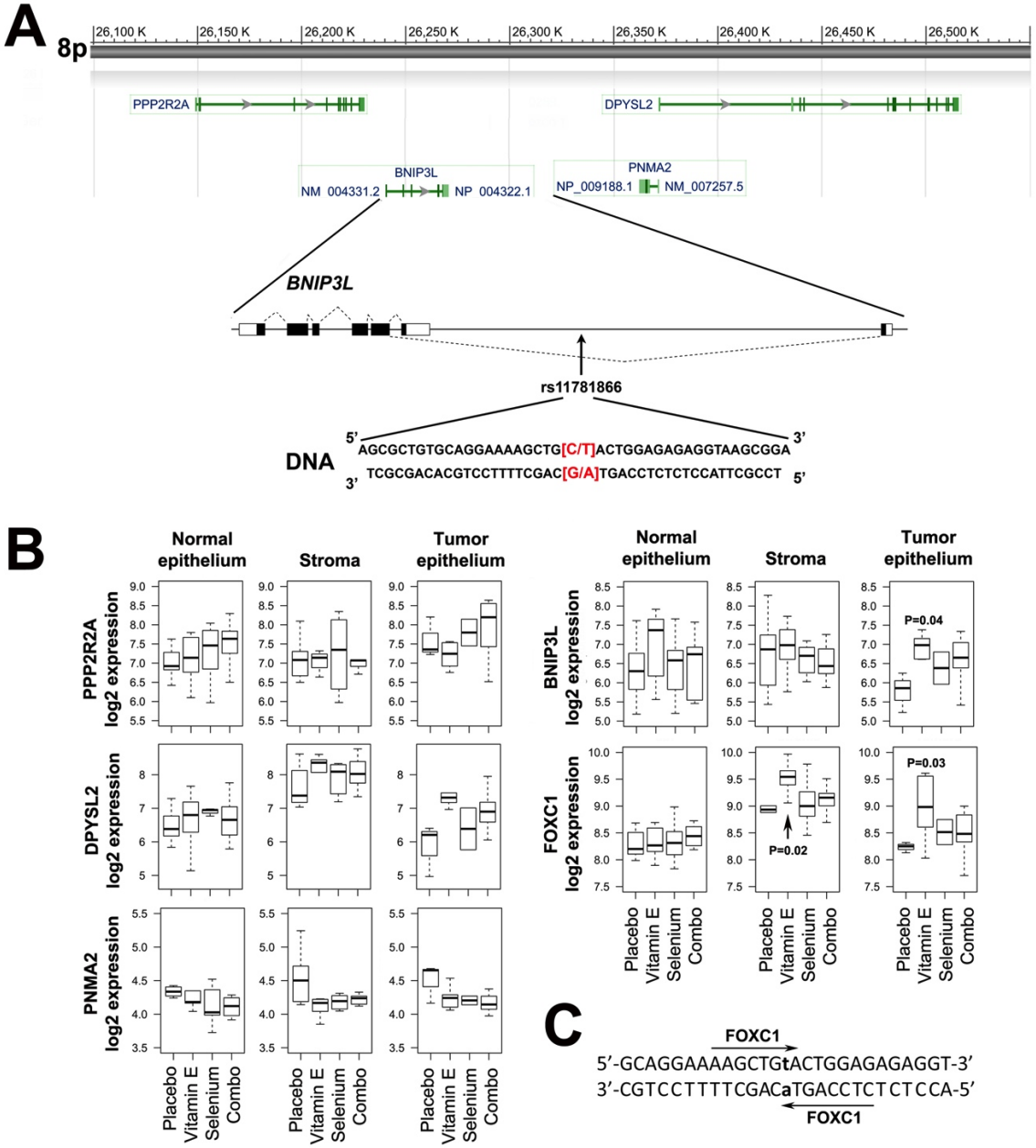

Figure 14. rs11781866 locus and nearby gene regulation with SELECT trial supplements
A. Genome view of the rs11781866 locus on chromosome 8p21. Image modified from NBI sequence viewer. Gene positions and symbols in their actual genomic positions are shown at the top of the figure in green. A schematic cartoon (not drawn to scale) of the *BNIP3L* gene with the position of rs11781866 is shown below in as an enlarged image. B. Relative expression of genes in prostate tissue from Tsavachidou *et al.* (*237*) supplemented with SELECT trial supplements (200 µg of L-selenomethionine (Selenium), 400 IU of all-rac-alpha-tocopheryl acetate (Vitamin E), a combination of 200 µg of L-selenomethionine and 400 IU of vitamin E (Combo), or placebo for 3-6 weeks prior to prostatectomy in laser-capture microdissected tumor cells and adjacent normal epithelium and stromal cells. C. Diagram of the sequence around rs11781866 including the putative FOXC1 binding site.

To explore the mechanism by which BNIP3L may be regulated by rs11781866 genotype, we determined the consensus transcription factor binding motifs at the rs11781866 locus. Table 14 lists the transcription factor motifs present with the major allele (T) and minor allele (C) at rs11781866. Notably, with the major allele, two FOXC1 binding motifs are present, while the minor allele genotype eliminates both of these putative binding site motifs (Table 14 and Figure 14C). Interestingly, FOXC1 expression was also elevated by 400 IU of all-rac-alpha-tocopheryl acetate (vitamin E) in the Phase IIA clinical trial in normal stroma and tumor cells (Figure 14B).

Table 14. Transcription factor consensus motifs at rs11781866

rs11781866 Major allele GCAGGAAAAGCTG**t**ACTGGAGAGAGGT								
Model ID	**TF name**	**Score**	**Relative score**	**Start**	**End**	**Strand**	**predicted motif sequence**	**predicted site sequence**
MA0032.1	FOXC1	4.238	0.81912061	8	15	1	GTA	AAGCTGtA
MA0032.1	FOXC1	5.653	0.89827216	14	21	-1	GTA	CTCCAGTa
MA0158.1	HOXA5	4.334	0.80557451	11	18	1	CNNNAAT	CTGtACTG
MA0113.1	NR3C1	8.522	0.80019789	1	18	-1	ANNNNGTNC	CAGTaCAGCTTTTCCTGC
rs11781866 Minor allele GCAGGAAAAGCTG**c**ACTGGAGAGAGGT								
Model ID	**TF name**	**Score**	**Relative score**	**Start**	**End**	**Strand**	**predicted motif sequence**	**predicted site sequence**
MA0122.1	Nkx3-2	5.659	0.80999296	12	20	1	NTAAGT(G/A)NN	TGcACTGGA

Discussion

SELECT was initiated as a large phase III clinical trial to test the efficacy of the antioxidants α-T and selenium to prevent prostate cancer development in a widespread population. What was believed to be strong rationale for the study was provided by data from NPCT (*191*), which showed a 49% decreased prostate cancer risk with selenium supplementation, and ATBC, which showed a 34% decreased prostate cancer risk with vitamin E supplementation (*130*). However, these were secondary results from trials designed to study prevention of other cancer types.

Men enrolled in the study had no evidence of previous prostate cancer and had low serum PSA (≤ 4 ng/mL) and a digital rectal exam not suspicious for prostate cancer. The primary clinical endpoint was incident prostate cancer cases as diagnosed by the standard of care at each study site. At the start of the trial, the authors predicted a 25% decrease in prostate cancer risk from supplementation with either vitamin E or selenium in the SELECT trial.

SELECT began enrollment in August 22, 2001 and continued blinded to the trial results until October 23, 2008. Surprisingly, when the 7 year planned interim results of the trial were compiled in late 2008, neither selenium nor vitamin E alone or in combination decreased the risk of prostate cancer (*137*). Study coordinators ended the supplementation after this analysis, as the study supplements had shown no beneficial effects. Follow-up continued and another analysis in late 2011 showed that participants in the vitamin E-alone arm had a 17% increased risk of prostate cancer (*138*). The

observation that vitamin E, a widely-used, "natural" agent, increases prostate cancer risk in a cancer-naïve population has caused significant controversy in the field (*242*, *243*).

The unexpected increase in prostate cancer risk with high dose (400 IU/day) vitamin E, an important molecule and nutrient in humans, is a significant public health concern. In recent times, a significant body of research has been dedicated to explaining the role of oxidative stress in prostate tumorigenesis (*5*, *7*, *9*, *52*, *101*). Numerous studies propose that reactive oxygen species promote cancer development through induction of DNA damage or promotion of pro-proliferative signaling, showing that antioxidants prevent prostate cancer progression (reviewed in (*102*)). However, most preclinical studies have been performed in models of advanced disease, and therefore, are not ideal models for prostate cancer prevention. Thus, while decreasing oxidative stress with antioxidants may inhibit progression to advanced prostate cancer, additional studies must be performed to determine their efficacy in prostate tumor prevention. At later stages in cancer progression, ROS may promote proliferation and tumor progression by DNA mutations and signaling mechanisms. However, at early stages, elevated ROS may inhibit cancer progression, as has been seen in other recent studies (*225*, *226*). Further, recent clinical studies have called into question the prostate cancer antioxidant chemoprevention hypothesis (*134*, *137*, *138*, *244*, *245*). Thus, it is not surprising that antioxidant supplementation may not always be efficacious in preventing prostate cancer, as was observed in SELECT.

There are many possible reasons for the failure of SELECT to show the ability of selenium or vitamin E to prevent prostate cancer development. Some propose that the formulations or doses of the study supplements were incorrect (*140*, *142*, *246*, *247*).

Others suggest that the previous beneficial effects were only efficacious in a certain population (e.g. low plasma selenium status in NPC or smokers in ATBC) (*243*, *248*). These concerns are warranted and have been widely debated.

Selenium and prostate cancer prevention

SELECT showed that selenium as a daily dose of 200 μg L-selenomethione was not able to prevent prostate cancer (*137*, *138*). Many have argued that the form of selenium used in SELECT may have been incorrect; however, two recently published studies dispute this argument. A recently published study using selenized yeast (the same type that was used in the NPC trial (*130*)) did not show a protective effect in men at high risk for prostate cancer (*245*). A recent investigation comparing selenized yeast to selenomethionine in aged canines (the only other species besides humans known to consistently develop sporadic prostate cancer with age) showed no differences in the levels of prostatic selenium, DHT, or testosterone and did not have alterations in DNA damage, proliferation or apoptosis in the prostate gland (*249*).

Thus, instead of reconciling the null effect of selenomethionine in SELECT due to its difference in selenium form from NPC, I believe it is most likely that only those with low baseline selenium levels will derive benefit from selenium supplementation, as was seen in NPC. The average baseline plasma selenium level in SELECT was about 136 μg/L (*137*). The NPC trial was conducted with participants from low selenium areas in the Eastern U.S. and only showed a preventative effect of selenium supplementation in the two lowest quartiles of baseline selenium, at or below 123.2 μg/L (*130*). In those in the highest quartile of baseline selenium, there was no protective effect (*130*).

Indeed, as many others have suggested, I propose there may be a "U-shaped curve" for the benefit of nutritional components: low or high a concentrations of these compounds both have the possibility to be detrimental to human health (*250*) (Figure 15). Identification of plasma antioxidant levels may be required for efficacious cancer prevention and for avoiding detrimental effects such as those seen in SELECT and other studies (*251*, *252*).

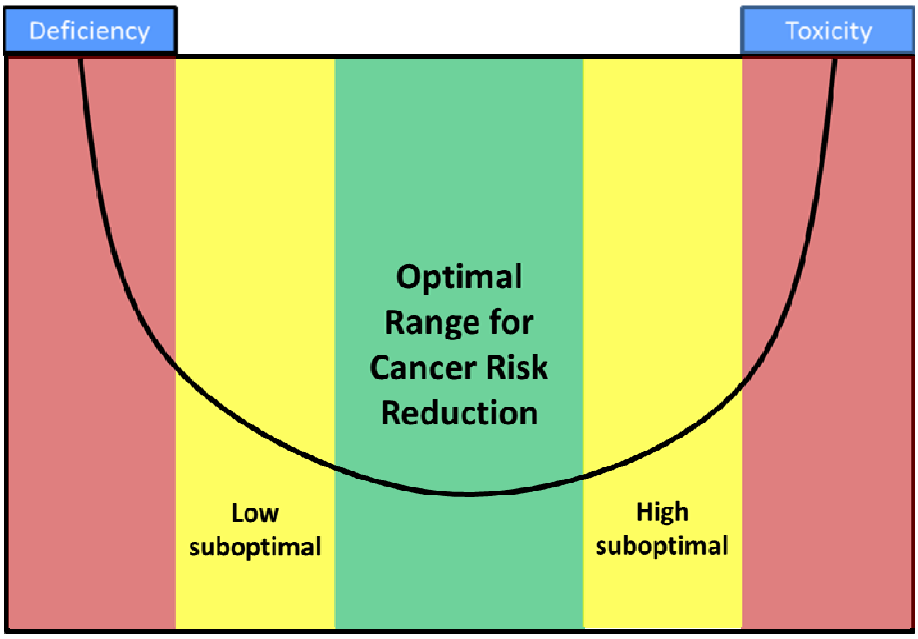

Figure 15. A "U-shaped" curve may describe the relationship of many essential nutrients with cancer risk reduction. This image redrawn from Walters *et al.* (*250*) shows the relative cancer risk associated different concentrations of essential nutrients in the body. Lower than optimal concentrations of a certain nutrient can increase cancer risk and therefore, individuals specifically deficient in this nutient may benefit from supplementation. However, higher than optimal concentrations of the same nutrient may also increase cancer risk. Individuals with optimal or already elevated concentrations of the nutrient, would therefore have an increased cancer risk with supplementation.

Vitamin E and prostate cancer prevention

SELECT and other studies have also shown that α-T did not prevent and, in some cases, promoted prostate cancer development (*123*, *138*, *253*). Indeed, it has been argued that the vitamin E form used was incorrect, that the dosage was too high, or that vitamin E may only be efficacious in a defined subpopulation (*113*, *139*, *141–143*).

α-T and γ-T differ only in the presence of one methyl group on the chromanol ring of the common tocopherol structure (*254*). The additional methyl group in α-tocopherol may influence its ability to quench certain radical species. For example, γ-tocopherol has a greater ability than α-tocopherol to trap reactive nitrogen species and has been proposed to have greater anti-inflammatory properties than α-tocopherol (*67*, *255*). These differences in the two forms of vitamin E could have important biological effects.

SELECT investigators chose 400 IU α-T primarily because of the striking decrease in prostate cancer incidence in the large ATBC trial seen with this form of vitamin E (*191*). This supplementation decreased plasma γ-T levels by 50%. Some pre-clinical and clinical studies suggest that γ-T may be a more potent chemopreventative agent than α-tocopherol. The NIH-AARP Diet and Health Study, a large prospective questionnaire study about supplemental and dietary α- and γ-T intake, showed no protective effect of α-T supplementation, but did show that dietary γ-tocopherol intake was associated with a decreased risk of prostate cancer (*253*). Another large prospective study showed that prostate cancer cases were associated with a lower γ-T level, but not a significantly lower α-T level (*256*). Therefore, the inability of α-T supplementation to prevent prostate cancer in SELECT and other studies may be due to the fact that the important γ-T levels are severely decreased.

The high dosage (400 IU) of α-T used in SELECT, more than 13 times the daily recommended value of 30 IU, may have played a role in the promotion of prostate cancer. Not only does high dose α-T decrease plasma γ-T (the possible effects of which are discussed above), but high dosages of vitamins and minerals may also have detrimental effects in other ways. Supra-nutritional dosages of other vitamins and minerals have been linked to increased mortality and cancer risk in several recent studies (*251*, *252*, *257*). While the mechanism of increased prostate cancer risk in SELECT by high dose α-T remains unknown, my studies and the studies of many other investigators (*233*) will attempt to help explain this concerning finding.

***NKX3.1* SNPs and prostate cancer risk in SELECT**

In the wake of the debate over the SELECT results, Dr. Eric Klein, a lead researcher of SELECT, has suggested that identification of efficacious chemopreventative agents may rely upon finding unique risk factors in individual patients (*192*). Towards this goal, the specimens from the SELECT biorepository (consisting of plasma, serum, WBC-derived DNA, and toenails) have been made available to the scientific community to investigate the possible reasons for the inability of selenium to prevent prostate cancer and the promotion of prostate cancer by the vitamin E in the study-wide population.

In order to help determine the mechanism between increased prostate cancer risk in SELECT with antioxidant supplementation and to identify subpopulations with modified risk levels, I investigated the relationship between risk associated with two functional variants in the prostate tumor suppressor gene *NKX3.1* and SELECT intervention arm using the SELECT biorepository. Our analysis was prompted by our

observation that *Nkx3.1*-deficient mice showed *increased* rather than decreased proliferation with antioxidant supplementation (*159*). *Nkx3.1*$^{-/-}$ mice exhibit dysregulation of anti- and pro-oxidant genes and increased ROS (*159*). Inhibition of ROS caused a pro-tumorigenic phenotype, perhaps due to lifting a ROS-mediated inhibition of proliferation in these early lesions. Therefore, I hypothesized that the significantly increased risk of prostate cancer with vitamin E supplementation in SELECT, and the lack of prostate cancer prevention with selenium or the combination of selenium and viatmin E, was partially driven by individuals deficient in *NKX3.1* expression.

I found that antioxidant supplementation did affect the prostate cancer risk associated with allelic variation at rs11781886. In both the vitamin E and selenium arms, presence of the minor allele at rs11781886 was associated with an increased risk of prostate cancer (Tables 8-10). The strong increase in prostate cancer risk even with only one minor allele with vitamin E supplementation may be explained by the fact that *NKX3.1* has been shown to be haploinsufficient in many settings (*152*, *154*, *258*). Vitamin E supplementation more strongly increased high grade risk while selenium more strongly increased low grade prostate cancer risk (Tables 8-10). Thus, the increased risk of prostate cancer with antioxidant supplementation in SELECT may be partially due to a significant increase in risk upon antioxidant supplementation in those with low *NKX3.1* expression. At present, the biological mechanism behind this increased risk is unknown; however, antioxidant supplementation witn NAC in *Nkx3.1*-deficient mice promoted increased expression of gene sets involved in positive regulation of cell proliferation and chemokine/growth factor signaling (*159*). Interestingly, however, combination vitamin E and selenium supplementation did not significantly modulate risk (Tables 8-10).

These data are not the first example of a genetic polymorphism affecting the prostate cancer risk associated with antioxidant capacity. Mikhak *et al.* showed that the polymorphism which causes a Ala16Val substitution in the manganese superoxide dismutase (*SOD2*) gene affects high grade prostate cancer risk in the presence of low plasma lycopene status (*228*), with the Ala/Ala genotype having higher risk than the Ala/Val or Val/Val genotype. One hypothesis to explain this is that the Ala/Ala variant, which has higher SOD2 activity, leads to more hydrogen peroxide from the superoxide reduced by SOD2. This elevated hydrogen peroxide, if it cannot be quenched by sufficient levels of plasma antioxidants such as lycopene, can go to cause oxidative damage, leading to increased advanced prostate cancer risk (*259*). In another a series of independent studies analyzing prostate cancer risk with plasma tocopherol, selenium, and lycopene levels, SNPs in *SOD1* and *SOD2* were analyzed (*230*, *232*, *260*). While there was not an increased risk of total or aggressive prostate cancer with the *SOD1* and *SOD2* SNPs, SNP genotype modified the risk of prostate cancer associated with plasma antioxidant levels. Also, the effect of polymorphisms in DNA repair enzymes hOGG1 and XRCC1 on prostate cancer risk is modified by plasma levels of carotenoids and α-T (*231*). Description of these and other variants that modify risk associated with antioxidant status may help to highlight molecular pathways involved in the mechanisms behind the unexpected increased prostate cancer risk with vitamin E supplementation in SELECT.

Unlike Gelmann *et al.*(*240*), I found no elevation in high-grade prostate cancer risk due to rs2228013 in the SELECT case-cohort, nor did rs2228013 affect total or low grade risk in the case-cohort overall or in any intervention arm. rs2228013 has been

shown to modulate NKX3.1 function *in vitro* (*240*); however, unlike rs11781886 (*227*), *in vivo* and human tissue studies to analyze the SNP's effect on NKX3.1 expression or activity in the human prostate have not been reported. Therefore, the effect of rs2228013 may not be merely a decrease in NKX3.1 function as would be seen with rs11781886 due to overall decreased expression of NKX3.1. Instead, genotype at rs228013 may cause different, more complex alteration of NKX3.1 function which has alternate physiological effects, resulting in differential influence of antioxidant supplementation. In addition, the case-cohort has a relatively small number of high grade cases, due to the fact that the participants were pre-screened for prostate cancer at the initiation of the study. Therefore, the population may not be the best representative of high grade cases in the general US population.

Our studies have identified a genetic risk factor (rs11781886) for prostate cancer which is modulated by supplementation with the antioxidants selenium and α-T. Indeed, presence of the minor allele at rs11781886 may have partially contributed to the increased risk of prostate cancer upon vitamin E supplementation, and the lack of prostate cancer prevention with selenium supplementation. These results suggest that an individual's prostate cancer risk associated with antioxidant supplementation is dependent upon genotype at rs11781886. Future studies in mouse models of prostate cancer and in human clinical samples will be required to determine the molecular events associated with NKX3.1 polymorphisms and prostate cancer development in the presence of antioxidant supplementation.

Putative *BNIP3L* SNP rs11781866 and prostate cancer risk in SELECT

Our work has suggested a possible novel role for BNIP3L in prostate tumorigenesis in the setting of vitamin E supplementation. *BNIP3L* is a member of the BH3-only class of the Bcl-2 gene family on chromosome 8p21 (*261*). Bcl-2 proteins are master controllers of apoptosis and share one or many of the four conserved BH domains (BH1, BH2, BH3, and BH4). The BH domains allow for interactions between the family members, some of which are pro-apoptotic and others of which are anti-apoptotic (*261*). The BH3-only class contains only the BH3 domain, and expression of these proteins tightly regulated by cellular stress. They antagonize the interaction of the anti-apoptotic family members with the pro-apoptotic family members, allowing for the pro-apoptotic members to promote apoptosis through permeabilization of the mitochondrial membrane (*261*). BH3-only proteins serve as master regulators of the cellular response to acute stressors.

BNIP3L shows ~65% sequence homology with *BNIP3*, with the areas of greatest similarity in its BH3 domain and transmembrane domain (*262–264*). BNIP3L localizes primarily to the mitochondria and its RNA is expressed ubiquitously in most human tissues(*263*, *264*). Its transmembrane domain is required for promotion of apoptosis (*264*).

Multiple reports support the role of BNIP3L as a tumor suppressor. BNIP3L has been shown to promote apoptosis in cells, especially in hypoxic conditions (*264–266*). BNIP3 and BNIP3L are upregulated by hypoxia, mediated by HIF-1α (*265*, *267*). Interestingly, *BNIP3L* has been shown to be a direct target of p53 in hypoxia, promoting apoptosis (*265*), and therefore may be a key dysregulation of gene expression in cancer

upon p53 loss. Further support for a tumor suppressor role for BNIP3L comes from a report that showed *BNIP3L* undergoes homozygous deletions in prostate cancer tissue (*268*), and that it is found in a chromosomal region (8p21) which is well-established to show loss of heterozygosity in human prostate cancer (*150*, *269*).

However, other lines of evidence suggest a role for BNIP3L in promoting cell survival, perhaps acting as an oncogene. BNIP3 and BNIP3L have been implicated in autophagy (*270–272*), a process by which cells can break down and recycle organelles (*273*). The process is critical for normal cellular homeostasis and survival in times of cellular stress. Autophagy can be triggered due to damage to organelles or stressful conditions which require the production of energy from cellular components.

Autophagy has been shown to play both pro-tumorigenic or anti-tumorgenic roles depending on the cellular context (*274*). Breast, prostate, and ovarian cancers have a high chance of deletion of the essential autophagy gene *BECLIN1* (*BECN1*) (*275–278*), *BECN1*$^{+/-}$ mice develop many types of tumors with age (*279*), and expression of BECN1 decreases tumorigenicity in breast cancer cells (*280*), highlighting the role of autophagy in cancer inhibition. Also, BNIP3L has been shown to be deleted in a small portion of prostate tumors and deletion is associated with increasing tumor grade (*281*). On the other hand, autophagy also acts as cellular survival mechanism in times of stress and can assist cancer cells undergoing metabolic stress to survive (*274*). mTORC1 is a master sensor of cellular metabolic flux which inhibits autophagy in times of high nutrient conditions. Elevation of ROS, increased hypoxia, stress, and low nutrient conditions promote autophagy (*274*).

The precise mechanism by which BNIP3L promotes autophagy is not conclusively known; however, several hypotheses have been suggested. The first idea is that BNIP3L causes mitochondrial dysfunction and depolarization, leading to increased ROS which induce autophagy(*282*, *283*). The second possibility is that BNIP3L induces autophagy by disrupting Bcl-2-Beclin 1 complexes, freeing Beclin-1 from repression by Bcl-2 and allowing it to induce autophagy (*271*). *BNIP3L* is a target gene of the transcription factor FOXO3, which is inhibited by Akt signaling, and when activated, triggers autophagy (*284*). These and other mechanisms have been proposed (*270*) and will require extensive investigation before a thorough understanding of BNIP3L induction of autophagy is reached.

While support for the role of BNIP3L as a tumor suppressor is abundant in the literature, several studies have also suggested that BNIP3L can promote cancer (*272*, *273*). Expression of BNIP3L protects breast and prostate cancer cells from hypoxia-induced cell death (*272*). Another very recent study showed that BNIP3L expression was associated with high NF-κB expression in glioma (*285*), which is known to correlate with increased tumor grade (*286*). There was increased expression of *BNIP3L* RNA with increasing grade (*286*). Interestingly, higher BNIP3L levels were correlated with different phenotypes depending on the stage of the tumor. In astrocytoma tumors, high BNIP3L was correlated with significantly lower proliferation, a trend towards higher apoptosis, and better tumor free survival. However, high BNIP3L in high grade tumors (grade III anaplastic astrocytoma, and grade IV glioblastoma) was correlated with no change in proliferation, with lower apoptosis, and no change in tumor free survival (*286*). While this study was done on a relatively small number of human samples, and more definitive

results would be beneficial, this work highlights a possible diversity of effects BNIP3L may have depending on the cellular context.

Another way that BNIP3L could play a pro-tumorigenic role is though modification of the tumor microenvironment. Several have suggested that autophagy plays an important part in this interplay between tumor and surrounding stroma cells. In what has been termed the "Autophagic Tumor Stroma Model of Cancer Metabolism," oxidative stress in the microenviroment created by tumor cells promotes aerobic glycolysis and autophagy in stroma cells, which causes the release of nutrients needed for tumor cell survival (*274*). Evidence for this has been shown in experiments that suggest tumor associated fibroblasts undergo autophagy due to oxidative stress induced by MCF7 breast cancer cells (*287*). Data suggest that these fibroblasts protect breast tumor cells from cell death through apoptosis (*287*). HIF-1 alpha expression in stromal cells (which induced BNIP3L expression) promoted autophagy and promoted tumor growth, while HIF-1 alpha expression in tumor cells suppressed tumor growth (*288*). Thus, BNIP3L expression and autophagy in the tumor stroma has been correlated with increased tumor growth, and may be relevant pro-tumorgenic role for BNIP3L. Our microarray analysis of SELECT trial supplement exposed prostates (Chapter IV) did not show elevation of *BNIP3L* specifically in the normal stroma; however, data were not available for tumor stroma, so determining the relevance of this mechanism in human prostate tumorigenesis would require additional experiments.

Autophagy induction by BNIP3 or BNIP3L may occur through three possible mechanisms (*270*). The proteins could mediate mitochondrial membrane depolarization inducing intracellular ROS, which can lead to autophagy. BNIP3 or BNIP3L can

displace autophagy promoter Beclin-1 from Bcl-2, promoting autophagy. Lastly, the proteins may influence autophagy by regulation of mTORC1 activity, which inhibits autophagy. BNIP3L has been clearly shown to play a role in mitochondrial clearance in reticulocytes, a process which requires functioning autophagy (*289*). Thus, alterations in expression of BNIP3L could logically either inhibit or promote prostate tumorigenesis.

In our study, the TT (homozygous major allele) genotype at rs11781866 was associated with a significant increase in prostate cancer risk with vitamin E supplementation, which was diminished with each addition of a C allele (Table 12 and Table 13). This effect was not seen, however, in the selenium and selenium and vitamin E combination arms, suggesting a vitamin E-specific effect rather than an overall antioxidant effect may be responsible for the findings. In order to determine which if any genes in the vicinity of rs11781866 might be regulated in a way to affect prostate tumorigenesis, we queried published data using the SELECT trial supplements in humans prior to prostatectomy (*237*). The only gene in the vicinity of rs11781866 that was modulated by SELECT supplementation was *BNIP3L*, whose expression was increased in tumor tissue from the vitamin E group. If one assumes the major allele of rs11781866 is associated with normal expression of BNIP3L, and the minor allele with less expression of rs11781866, then vitamin E could be promoting BNIP3L expression to promote prostate tumorigenesis.

Because *BNIP3L* levels are elevated with vitamin E supplementation, I hypothesize that *BNIP3L* is acting as an oncogene in the setting of elevated α-T levels. Using the JAPSAR transcription factor binding profile database, we found that the rs11781866 locus contains two putative binding sites for the transcription factor FOXC1,

which are eliminated by substitution with the minor allele. FOXC1 levels are also significantly increased in vitamin E- supplemented prostate (*237*), which suggests that FOXC1 is positively regulating *BNIP3L*.

I propose the following model summarizing our current results (depicted in Figure 16). Vitamin E increases FOXC1 levels, then, through positive regulation of BNIP3L by FOXC1 binding at the rs11781866 locus, BNIP3L levels increase. I propose that increased FOXC1 expression with vitamin E supplementation more strongly upregulates BNIP3L expression in the presence of the TT allele since there are two binding sites for FOXC1. The BNIP3L induction decreases with each addition of a C allele due to decreased binding of FOXC1. In the placebo group, the FOXC1 levels are not as high, and therefore BNIP3L is not induced to a high level. Therefore, differences in FOXC1 binding to the rs11781866 locus in the placebo group do not alter BNIP3L levels and prostate cancer risk to a significant degree. Further studies are required to confirm the changes proposed at each step of this model.

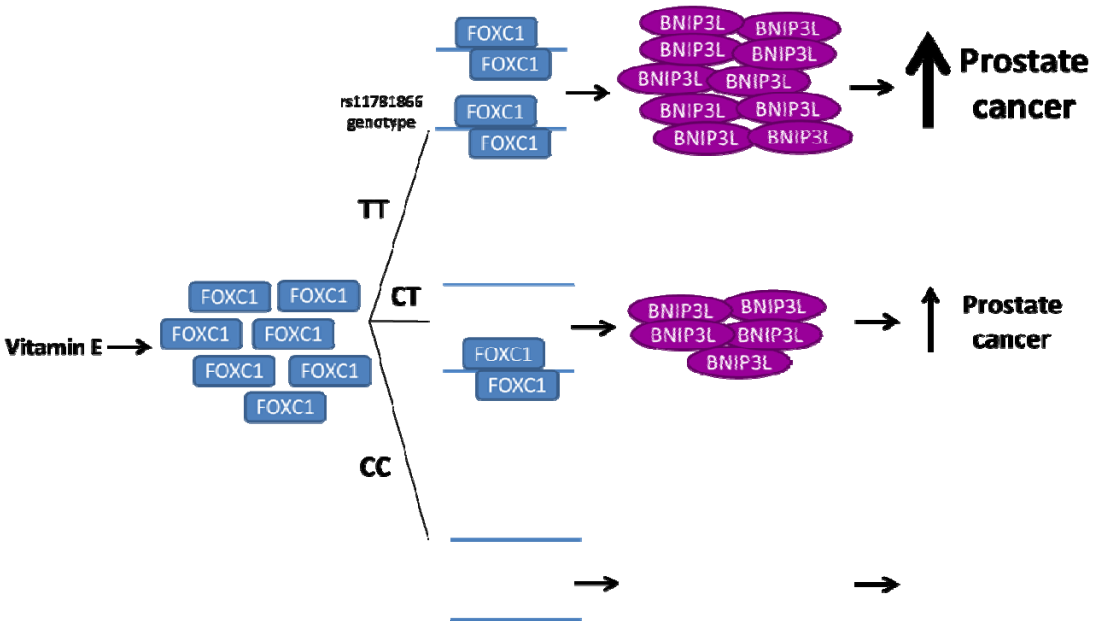

Figure 16. **Model of rs11781866 influenced and BNIP3L-mediated promotion of prostate cancer with vitamin E supplementation in SELECT.** At the start of SELECT, all participants are screened and designated as 'cancer naïve,' thus the model starts at left with a normal prostate gland. When the participants were supplemented with vitamin E, this caused upregulation of the transcription factor FOXC1 (as shown in Figure 14, indicated here by the presence of more FOXC1 molecules with vitamin E supplementation). I propose that FOXC1 positively regulates *BNIP3L* expression by binding to a putative regulatory region at rs11781866 with the major allele (T), with one binding site on each DNA strand for a total of 2 binding sites per chromosome at rs117818866. This putative FOXC1 binding site is lost in presence of the minor allele (C). Therefore, in the presence of larger quantities of FOXC1, FOXC1 occupies its binding sites maximally and upregulates *BNIP3L* and promoting cancer development to the greatest degree via *BNIP3L* upregulation. There is less upregulation of *BNIP3L* in the presence of one or two minor alleles at rs11781866 and correspondingly less promotion of cancer. Without vitamin E supplementation, there is no induction of FOXC1, and thus, no promotion of cancer even with the major allele at rs11781866. Therefore, genotype at rs11781866 does not alter prostate cancer risk in the placebo group.

CHAPTER V

ROLE OF NKX3.1 AND MYC TARGET GENE *PRDX6* IN PROSTATE TUMORIGENESIS

Introduction

While many correlative gene expression changes have been described in the development of prostate cancer, mechanistic information about the role these changes play in cancer development is lacking. Gain of expression of the oncoprotein MYC is a common early event in prostate cancer development (*290*), (*291*) with 76% of PIN, 81.6% of adenocarcinoma, and 68% of metastatic lesions overexpressing MYC (*292*). MYC expression drives cell cycle progression, helps cells maintain stem cell characteristics, increases cellular metabolic pathways, and increases protein synthesis (*293*). Another common gene expression change in prostate cancer is loss of expression of the tumor suppressor NKX3.1. NKX3.1 expression is completely lost in 5% of benign prostatic hyperplasias, 20% of high-grade prostatic intraepithelial neoplasias, 34% of hormone-refractory prostate cancers, and 78% of metastases (*153*). In addition, *NKX3.1* has been shown to be haploinsufficient (*258*); thus, a complete loss of NKX3.1 protein expression may not be necessary for important biological effects. Concurrent gain of MYC expression with loss of NKX3.1 expression is a common event in prostate tumorigenesis.

Our laboratory has shown that the transcription factors Myc and Nkx3.1 cooperate in a mouse model of MYC-driven prostate tumorigenesis, sharing 65 common direct target genes (*158*). One of the genes bound by NKX3.1 and MYC is *peroxiredoxin 6* (*Prdx6*). *Prdx6* mRNA expression is decreased in *Nkx3.1*$^{-/-}$ mice (*155*, *159*) and in MYC+, *Nkx3.1*$^{-/-}$ lesions, Prdx6 protein expression is greatly depleted (*158*), suggesting that Nkx3.1 positively regulates Prdx6, while MYC negatively regulates Prdx6.

PRDX6 is a member of the peroxiredoxin superfamily of selenium-independent peroxidases. As a peroxidase, PRDX6 can reduce short-chain, fatty acid, and phospholipid peroxides (*294*). It is singular among the peroxiredoxins in its ability to reduce phospholipid peroxides, as none of the other family members have this ability. Uniquely, PRDX6 is also a dual function enzyme, containing phospholipase A2 activity in addition to its peroxidase activity. Phosholipase A2 activity cleaves phospholipids at the second carbon of glycerol, releasing lysophospholipids and free fatty acids (such as arachidonic acid). Prdx6 is expressed in all major organs of the body and has the highest expression in lung (*295*). Prdx6 has been extensively studied in the lung, but its role in prostate physiology has not been investigated.

Here I show that high MYC expression is specifically associated with depleted Prdx6 expression in mouse prostate. Our findings suggest that MYC may directly repress Prdx6 by binding to the Prdx6 promoter. Interestingly, although MYC is correlated with decreased Prdx6 expression in early prostate lesions, Prdx6 appears to have a pro-tumorigenic function in aggressive prostate cancer cells, promoting proliferation *in vitro* and *in vivo* and by promoting anchorage-independent growth. Our studies describe a novel functional role for PRDX6 in prostate tumorigenesis.

Methods

Cell lines and mice

The Myc-CaP mouse prostate cancer cell line (*296*) was derived from a prostate cancer from the Hi-Myc mouse (*297*) and was a gift from Charles Sawyers, Memorial Sloan Kettering Cancer Center. Myc-Cap cells were cultured in a 5% CO_2 incubator at 37°C in DMEM with 10% fetal bovine serum (FBS). LNCaP, PC3, and DU145 cells were purchased from ATCC. LNCaP and PC3 cells were maintained in RPMI with 10% FBS and DU145 cells were maintained in DMEM/F12 with 10% FBS. *Nkx3.1*$^{f/f}$, and *PBcre4;Z-MYC, PBcre4;Z-MYC;Nkx3.1*$^{f/f}$, and *PBcre4;Z-MYC;Pten*$^{f/+}$*;p53*$^{f/+}$ mice have been described (*152*, *158*, *207*, *208*).

Lentiviral constructs

FM-1 control lentiviral transfer vector was a kind gift from Dr. Jeffrey Milbrandt, Washington University. Wild type Prdx6, peroxidase mutant C47S, and phopholipase mutant H26A constructs (kind gift from Aron Fisher, University of Pennsylvania) were cloned into the FM-1 lentiviral constructs. Lentiviruses generated specifically for this study were packaged in the African green monkey kidney fibroblast-like cell line COS-1 (*298*) to avoid ethical concerns over use of other lentiviral packaging cell lines. Lentivirus was made by cotransfecting the transfer vector containing the gene of interest with the VSVG envelope glycoprotein plasmid and the HIV-1 packaging vector Δ8.9 (gift from Dr. David Baltimore) into COS-1 cells (ATCC) using polyethylenimine (Polysciences Inc.). Virus was concentrated by filtration in Centricon Plus-70 filter units (Millipore). Viral titer was determined by infecting HT1080 cells with serial dilutions of

virus and followed by quantification of GFP-positive cells by flow cytometry after two days of infection.

***In vitro* proliferation assay**

Myc-CaP FM-1-control, FM-1-Prdx6, FM-1-C47S, and FM-1-H26A cell lines were plated in 96-well plates at a density of 200 or 100 cells per well. Cells were incubated for 3 or 4 days at which time 20 μl CellTiter 96® AQ_{ueous} One Solution (Promega) was added to each well. Cells were incubated for one to three hours in a 5% CO_2 incubator at 37°C. Absorbance at 490 nm was read on a Bio Tek Elx800 Plate Reader (Fisher Scientific).

Soft agar colony formation assay

Each well of a 6 well plate was coated with 1 mL DMEM containing 0.5% agar (Fisher). Cells were then resuspended in DMEM containing 0.35% agarose (DNA grade) at a concentration of 10,000 cells per mL and 1 mL was plated in each well. Cells were incubated in a 5% CO_2 incubator overnight. 2 mL DMEM was added to each well the next day. Media was changed every 2-4 days, adding replacing 2 or 3 mL per well. Cells were incubated in a 5% CO_2 incubator for 14 days. Number of colonies >30 μm per 10x field was quantified by selecting three random fields per well and counting four wells.

Myc-CaP allograft experiments

Myc-CaP cells were infected with FM-1-control, FM-1-Prdx6, FM-1-C47S, or FM-1-H26A plates with 8 μl/mL polybrene in DMEM for ~24 hrs. Cells were sorted for YFP positivity via fluorescence activated cell sorting (FACS) to establish stable cell lines with lentiviral-mediated gene expression. 250,000 non-infected parental Myc-CaP cells were combined with 250,000 stable FM-1-control, FM-1-Prdx6, FM-1-C47S, or FM-1-

H26A lines for each graft. 0.2 mL matrigel (BD Matrigel™ Matrix Phenol Red-Free) was combined with the cells for each graft. Cells with matrigel were injected subcutaneously in male athymic Nude (*Foxn1 nu/nu*) mice. Grafts were allowed to grow for 15 days before harvest.

Histology and immunostaining

Tissue was fixed overnight in 10% formalin solution and washed in 70% ethanol. Tissue processing and hematoxalin and eosin (H&E) staining were performed by the Vanderbilt Translational Pathology Shared Resource. For immunohistochemistry, paraffin embedded sections were deparaffinized, rehydrated, and steam/pressure antigen retrieval was performed. The following antibodies were used: anti-phospho histone H3 (rabbit, 1:500, Millipore), anti-cleaved caspase-3 (rabbit, 1:200, Cell Signaling), anti-Prdx6 (1:200, gift from Aron Fisher laboratory), c-Myc (rabbit, 1:300, with ABC amplification, or 1:30,000 with TSA amplification, Abcam), and anti-green fluorescent protein (chicken, 1:200, Abcam). For immunofluorescence, fluorescent-tagged secondary antibodies, including Alexa 488 (anti-rabbit, Invitrogen), and Alexa 546 (anti-chicken, Invitrogen) were used. Slides were mounted with Vectashield Mounting media with DAPI (Vector Laboratories). For non-fluorescent stains, DAB substrate (Sigma) was used and slides were counterstained with hematoxalin.

Immunofluoresent image quantification for percent YFP stained area

For percent YFP+ area analysis in Myc-CaP allograft tissue, random images of the YFP immunofluoresent stain were taken with a 20x objective of all samples in a blinded fashion. Percent stained area and total area was obtained using the Fovea Pro

plugin for Adobe Photoshop. Percent YFP+ area was determined by dividing the %YFP+ value by the % of field filled by cells (total area).

Quantification of pHH3 and caspase 3 staining index

In the Myc-CaP allograft experiment, random fields including YFP+ tissue were chosen at 20x. At 40x, the total number of YFP+ cells and the number of pHH3 or caspase 3 positive cells were counted. The Fisher's Exact Test was used to test the differences in percent of cells staining positive between all four groups.

Results

Prdx6 expression in mouse prostate

Prdx6 is significantly expressed in wild type mouse prostate, with high expression in the distal region and almost a complete loss of expression in the extreme proximal region (Figure 17 A,B).

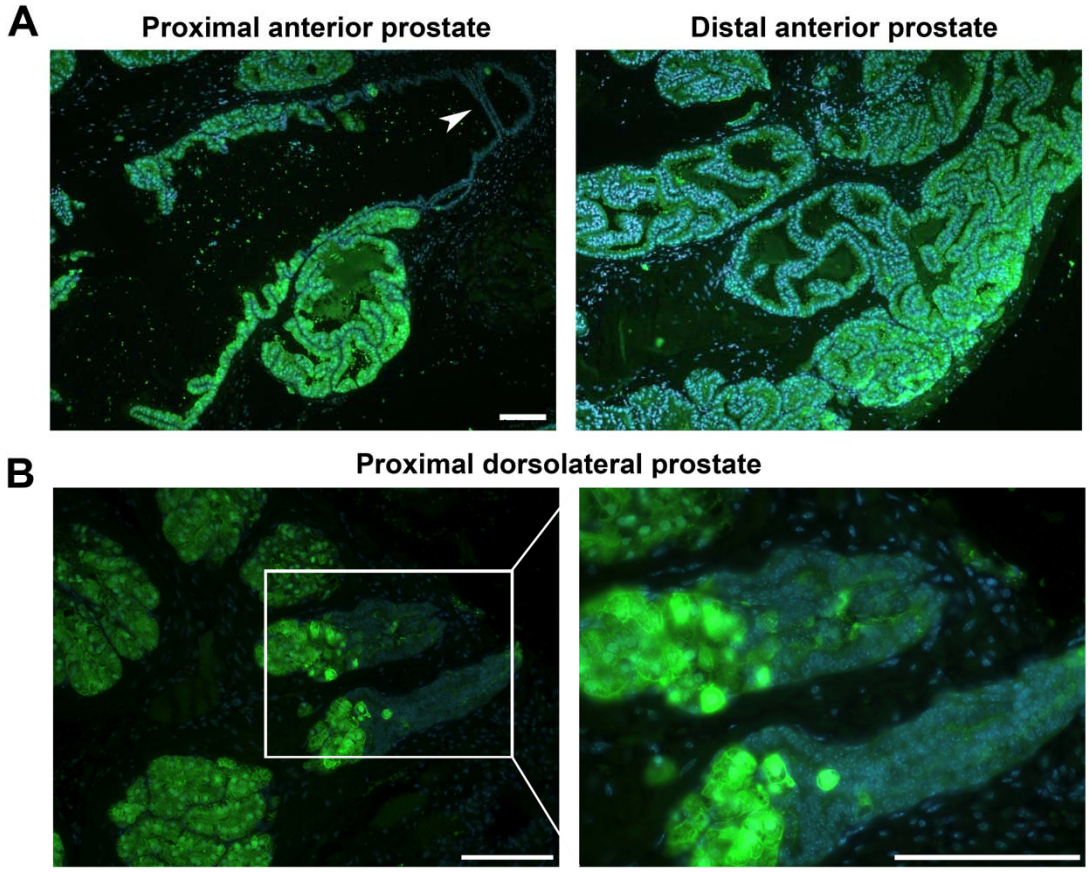

Figure 17. Spatial distribution of Prdx6 expression in mouse prostate. Immunofluorescent stain for Prdx6 (indicated in green) in anterior (A) and dosolateral (B) prostate. Nuclei are stained with DAPI (blue). Scale bar = 0.1 mm.

Prdx6 expression is depleted in MYC-driven mouse prostate cancer

Our previous study (*158*) identified *Prdx6* as a co-direct target gene of Myc and the prostate tumor suppressor Nkx3.1. Myc and Nkx3.1 were shown to bind to the *Prdx6* locus via ChIP-seq, and in MYC+; Nkx3.1-null lesions, Prdx6 was greatly depleted, suggesting that Myc represses Prdx6 expression. To determine if Myc represses Prdx6 in other models of mouse of cancer, I analyzed Prdx6 expression in other published

microarray studies. Importantly, Prdx6 expression was decreased over 9-fold in Myc-driven tumors of the hi-MYC mouse prostate (*297*) and was decreased over 2-fold in MYC tumor allografts (*158*, *299*). Via qRT-PCR analysis, *Prdx6* expression was also decreased by about 48% in the *Nkx3.1*$^{-/-}$ mouse prostate (*159*). Interestingly, *Prdx6* expression was also decreased more than 5-fold in *PBCre4; Pten*$^{loxp/loxp}$ tumors (*95*), a model which is driven by loss of the tumor suppressor Pten instead of high MYC expression, suggesting loss of Prdx6 may play a role in additional mechanisms of prostate tumor initiation. At the protein level, Prdx6 expression was not notably decreased in *Nkx3.1*$^{-/-}$ mice compared to wild type (*Nkx3.1*$^{+/+}$) mice (Figure 18). Prdx6 expression was lower in *PBCre;Pten*$^{f/f}$ mouse tumors than in normal wild type mouse prostate (Figure 16).

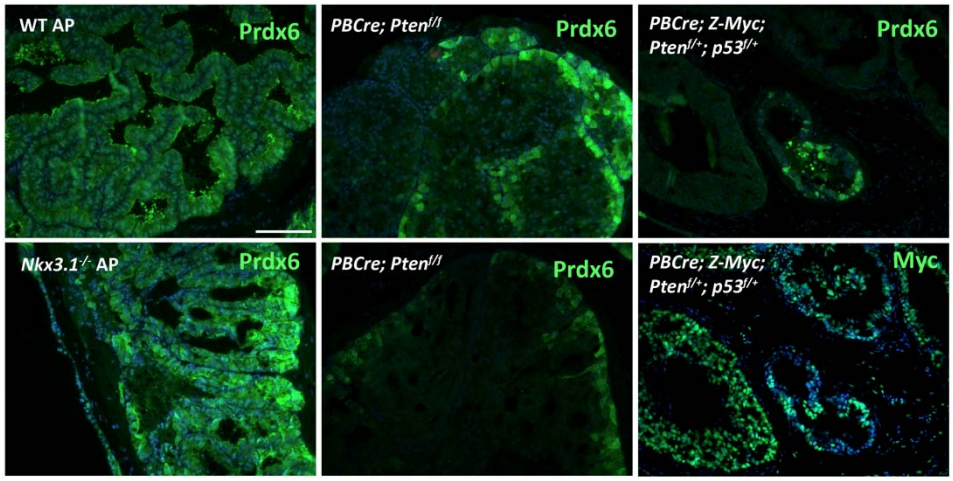

Figure 18. **Prdx6 expression is decreased in mouse models of prostate cancer.** Immunofluoresent stain for Prdx6 (indicated in green) wild type anterior prostate, *Nkx3.1*$^{-/-}$ anterior prostate, *PBCre;Pten*$^{f/f}$, and *PBCre;Z-MYC;Pten*$^{f/f}$*;p53*$^{f/+}$. Immnohistochemical staining for MYC (indicated in green) is also presented for *PBCre;Z-MYC;Pten*$^{f/f}$*;p53*$^{f/+}$. Nuclei are stained with DAPI (blue). Scale bar = 0.1 mm.

I analyzed Prdx6 expression at the protein level in mouse prostates with focal MYC expression (the *PBCre;Z-MYC* mouse (*207*) and *PBCre; Z-MYC; Nkx3.1*$^{f/f}$ mouse (*158*) and saw that MYC expression tightly correlated with areas of Prdx6 depletion (Figure 19 A,B). Prdx6 expression was also strongly decreased in MYC-expressing areas of the *PBcre4;Z-MYC;Pten*$^{f/+}$*; p53*$^{f/+}$ tumor model (Figure 18). These data suggest that Myc represses Prdx6 expression in the prostate.

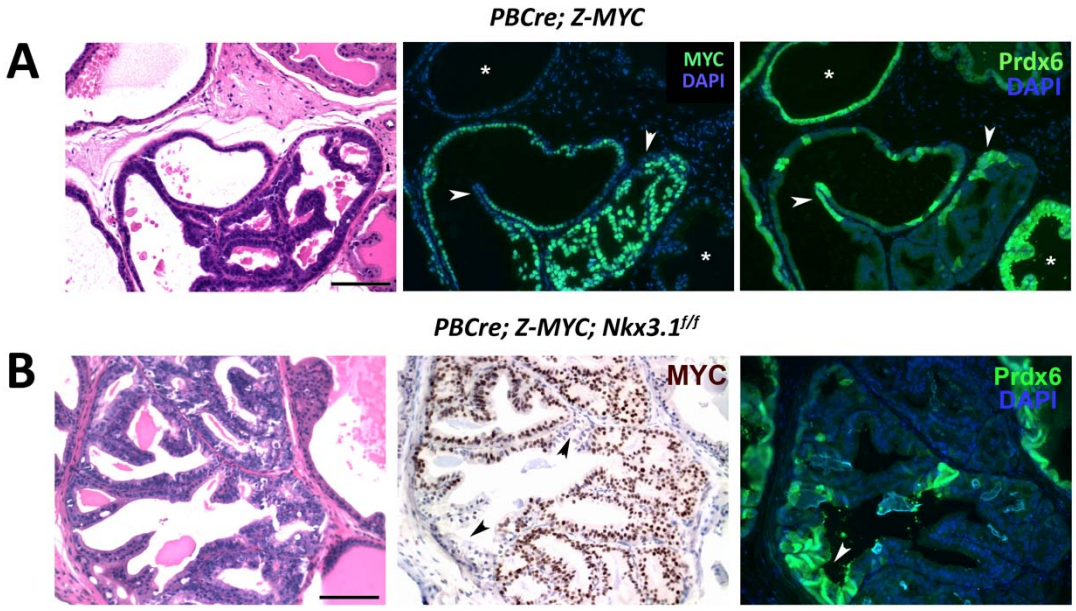

Figure 19. Prdx6 is specifically depleted in MYC+ regions of mouse prostate. (A) H&E (left), immunofluorescent stain for MYC (center, in green), and immunofluorescent stain for Prdx6 (right, in green) in *PBCre; Z-MYC* prostate. (B) H&E (left), immunohistochemical stain for MYC (center, in brown), and immunofluorescent stain for Prdx6 (right, in green) in *PBCre; Z-MYC; Nkx3.1*$^{f/f}$ prostate tissue recombination graft. Arrowheads indicate areas of low MYC expression on MYC stained sections and the corresponding areas in the Prdx6 stain on the adjacent section where Prdx6 expression is retained due to lack of MYC expression. Asterisk in the MYC immunofluorescence image for the PBCre;Z-MYC mouse indicates an entire gland which is MYC negative; the entire gland on the adjacent Prdx6 immunofluorescence image is positive for Prdx6 expression.

Prdx6 promotes prostate cancer cell proliferation and tumorigenicity

To determine functional effect of Prdx6 on prostate cancer cells, Prdx6 expression was driven in the Myc-CaP mouse prostate cancer cell line, a cell line derived from the hi-Myc mouse (*296*, *297*). The Myc-CaP line has high Myc expression and no Nkx3.1 expression. Lentivirus-mediated expression of wild type Prdx6, peroxidase mutant Prdx6 (C47S), and phosholipase A2 mutant Prdx6 (H26A) was achieved in Myc-CaP cells (Figure 20 A, B).

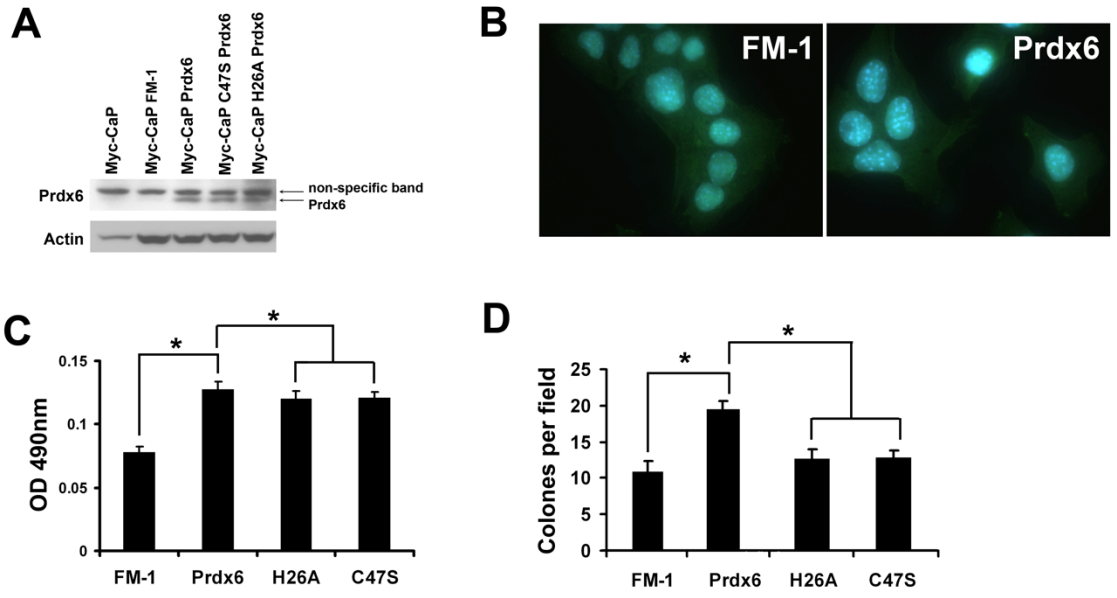

Figure 20. Prdx6 drives *in vitro* proliferation and anchorage-independent growth in Myc-CaP mouse prostate cancer cells. (A) Immunoblot for Prdx6 in lysates from Myc-CaP cell lines with lentiviral-mediated expression of Prdx6. Actin immunoblot is presented as a loading control. (B) Immunocytochemical stain in Myc-CaP FM-1 cells (FM-1) and Myc-CaP Prdx6 cells (Prdx6) shows cytoplasmic expression of Prdx6. (C) Optical density measurements at 490 nm (OD 490nm) measurement for the *in vitro* proliferation assay with Myc-CaP-Prdx6 expressing cell lines. N = 5 wells, experiment performed once with these conditions. (D). Soft agar colony formation assay results presented as colonies per field for Prdx6-expressing Myc-CaP cell lines. Number of colonies >30 μm per 10x field was quantified by selecting three random fields per well and counting four wells. * $p < 0.05$ for Student's T-test.

Prdx6 promoted *in vitro* proliferation of the Myc-CaP cells in a manner that appeared to be dependent on both the peroxidase and phospholipase A2 activities of the Prdx6 enzyme (Figure 20C). Prdx6 increased anchorage-independent growth of Myc-CaP in the soft agar colony formation assay, in a manner that was dependent on both the peroxidase and phospholipase A2 functions of Prdx6 (Figure 20D). These functional assays suggest that Prdx6 promotes tumorigenicity of aggressive prostate cancer cells.

To determine the ability of Prdx6 to modulate tumorigenicity *in vivo*, I performed allografts of the Prdx6-Myc-CaP cell lines mixed with equal numbers of uninfected, parental Myc-CaP cells (Figure 21A). Myc-CaP Prdx6 lines and the empty vector line (Myc-CaP-FM-1, Myc-CaP-Prdx6, Myc-CaP-C47S, and Myc-CaP-H26A) express YFP while the parental Myc-CaP line does not, resulting in a mosaic YFP expression in the grafts, designating the lentiviral-construct-expressing cells (Figure 21B).

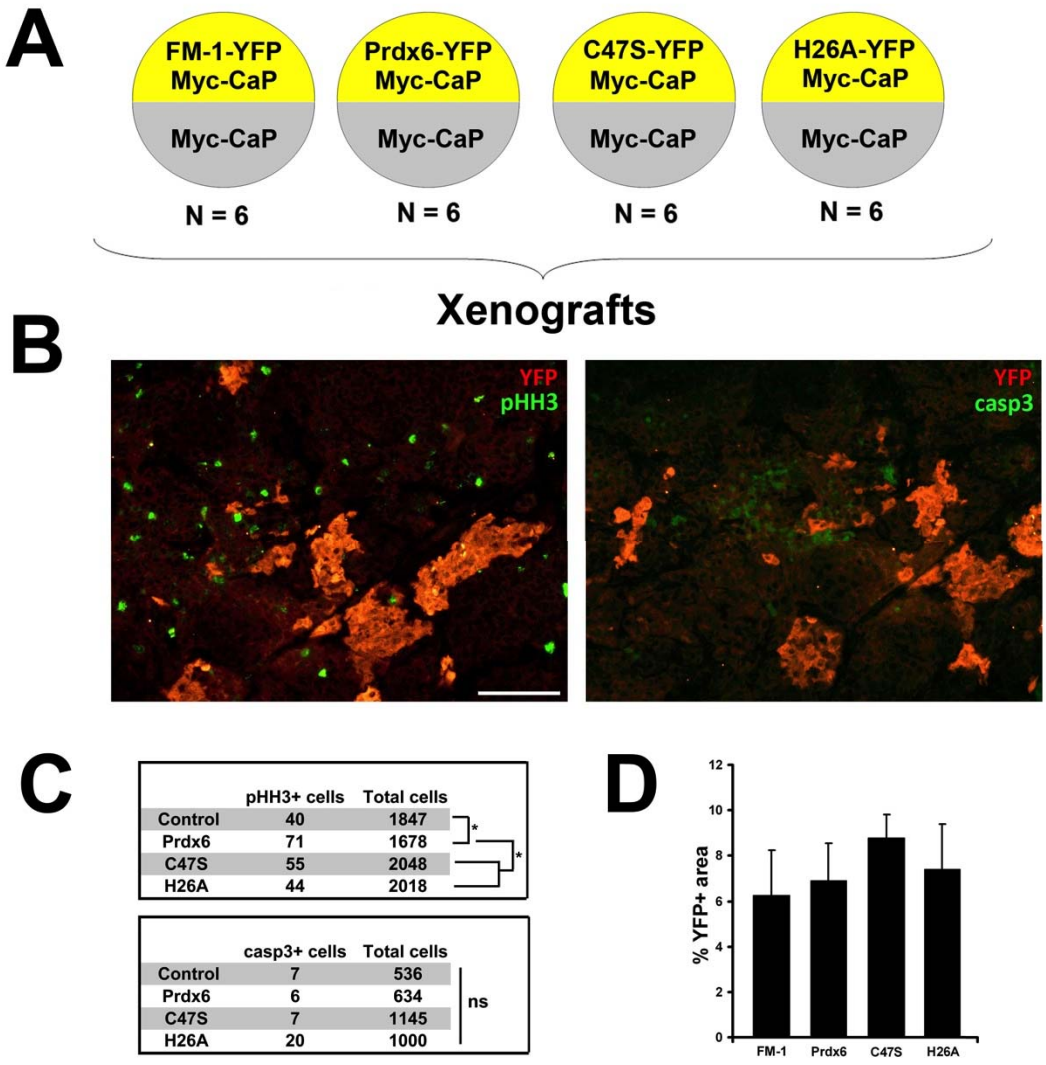

Figure 21. Prdx6 drives proliferation in Myc-CaP subcutaneous allografts. (A) Model of allograft scheme: 500,000 total cells, including 250,000 non-YFP expressing parental Myc-CaP cells and 250,000 YFP-expressing Prdx6-expressing or control Myc-CaP cells, were injected subcutaneously in nude mice. Allografts were allowed to grow 15 days before harvest. (B) Represensative immunohistofluorescent images from double staining for YFP (in red) and phosphorylated histone H3 (pHH3, in green) (left) and YFP (in red) and activated caspase 3 (casp3, in green) (right). Scale bar = 0.1mm. (C) Quantification of pHH3+ and casp3+ cells in YFP positive areas immunofluorescent stains of Prdx6-expressing and control Myc-CaP allografts. The total cells indicates the total YFP positive cells counted in each group and the pHH3+ or casp3+ cell totals are the total number of cells which stained positive for these markers in the YFP positive area. Fisher's Exact test was used to test if the distribution of positive cells differed in each group. * $p < 0.05$ by Fisher's Exact test. (D) Immunofluoresent image quantification for percent YFP stained area in Prdx6-expressing and control Myc-CaP allograft tissue as obtained using the Fovea Pro plugin for Adobe Photoshop.

As Prdx6 promoted proliferation of Myc-CaP cells *in vitro*, I hypothesized that Prdx6 expression would increase proliferation in the allograft and, therefore, increase the percent of YFP-positive cells in the grafts. While none of cell lines significantly modulated the percent of YFP positive cells (Figure 21D), the number of pHH3-positive cells in the wild-type Prdx6 expressing cells was significantly higher than the control or Prdx6 mutant cell lines (Figure 21C). Apoptosis, as indicated by activated capspase 3 staining, was not significantly altered (Figure 21C).

Discussion

Extensive research has been performed to analyze the role of the oncoprotein MYC in prostate tumorigenesis (*158*, *207*, *293*, *300*). MYC is overexpressed at early stages of human prostate cancer and is elevated in a high percentage of advanced disease (*292*). MYC's important role in normal cell physiology and in carcinogenesis cannot be overstated. Classically, MYC has been thought of as transcription factor with distinct target genes, which when regulated, have crucial effects on the cell. MYC target gene regulation stimulates proliferation, cell growth, cell metabolism, and apoptosis. To carry out these changes, MYC promotes expression of some genes and represses expression of others. However, the recent publications that have suggested MYC functions as a general amplifier, rather than a specifier, of gene expression (*301*, *302*), have led to some controversy over the function of Myc. Supporting the role of MYC as a specifier of gene expression, our studies have characterized a unique MYC target gene which is distinctly repressed by MYC expression in the prostate, Prdx6. I have shown a sharp correlation of

high MYC expression with depleted Prdx6 expression in mouse prostate, suggesting that MYC directly represses Prdx6 expression; however, additional studies will be required to confirm direct transcriptional repression or to identify other mechanisms by which MYC inhibits Prdx6 protein levels.

Our study has also described a functional role for the dual-function enzyme Prdx6 in prostate tumorigenesis. Prdx6 has been only recently studied in the setting of carcinogenesis. Li *et al.* found that Prdx6 was upregulated in a highly metastatic variant of the MBA-MD-435 breast cancer cell line (*303*). They went on to show that exogenously driven Prdx6 expression in MBA-MD-435 and MBA-MD-231 cells increased *in vitro* proliferation and invasion, and increased tumor growth and metastasis in a allograft model (*189*). Knockdown of endogenous Prdx6 inhibited *in vitro* breast cancer cell invasion and tumor growth and metastasis in the allograft model (*189*). Prdx6 knockdown in A549 lung cancer cells decreases invasiveness (*188*). Further studies suggested that the peroxidase activity of Prdx6 is responsible for its growth-promoting function and the PLA2 activity is responsible for its invasion-promoting function (*187*). Prdx6 is upregulated in pancreatic cancer (*184*), squamous cell carcinoma of the tongue (*183*), ovarian cancer (serum) (*186*), and endometrial cancer (*185*). Investigators have shown that Prdx6 is upregulated in cells that gain resistance to chemotherapy (*304*). Therefore, the majority of studies in cancer cell lines and human cancer tissues indicate that Prdx6 is associated with aggressive cancer.

The role of Prdx6 specifically in prostate tumorigenesis is completely unknown. Expression of Prdx1-6 were analyzed in normal and cancerous human prostate tissue by Basu *et al.* (*305*). While Prdx3 and Prdx4 appear to be consistently upregulated in cancer

compared to normal tissue, Prdx6 was not shown to be differentially expressed in this study. To our knowledge, our studies are the first investigation into the role of Prdx6 prostate tumorigenesis.

Our studies suggest that Prdx6 promotes a tumorigenic cell phenotype when expressed in cancer cells. Prdx6 expression in Myc-CaP, an aggressive, Myc-driven mouse prostate cancer cell line, promoted proliferation *in vitro* and *in vivo* and promoted anchorage-independent growth. The growth-promoting activity of Prdx6 appeared to be dependent on both the peroxidase and phospholipase A2 activities of Prdx6. Peroxidase activity may help to maintain lower oxidative stress levels in cells, allowing for increased cell viability and proliferation. Phospholipase A2 activity can generate arachidonic acid, a precursor of prostaglandins, many of which have been implicated in promotion of prostate tumorigenesis (*176*, *177*).

MYC expression is often associated with elevated aggressiveness and poor outcome in cancer (*306*). Then, how does MYC inhibition of Prdx6, a pro-tumorgenic protein, make sense? Our models of focal MYC expression in mouse prostate (*PB-Cre;Z-MYC* and *PB-Cre;Z-MYC;Nkx3.1* $^{f/f}$) display early lesions, PIN and early microinvasion, rather than extremely aggressive lesions with complete loss of glandular structure and massive invasion. The cell line model used, Myc-CaP, was derived from a very aggressive tumor from the hi-Myc mouse and therefore may not be representative of the early lesions in our focal MYC models. Indeed, a recent publication by Rolfs ***et al.*** (*307*) has described a dual role of Prdx6 in mouse model of skin carcinogenesis: at early stages, Prdx6 inhibits tumor formation, but at late stages, Prdx6 promotes tumor progression of existing tumors. In addition, human tissue studies which have suggested

an upregulation of PRDX6 in cancer may also be representative of more advanced lesions. Therefore, I propose that MYC normally represses PRDX6 in untransformed prostate epithelial cells, resulting in a decrease in the possible tumor-preventative activity of Prdx6. However, advanced lesions may attain genetic mutations or other cellular changes that allow for escape from MYC repression of PRDX6, thereby allowing PRDX6 expression to promote tumor progression in aggressive prostate cancer cells.

CHAPTER VI

DISCUSSION AND FUTURE DIRECTIONS

In the preceding studies, I set out to investigate the relationship between oxidative stress and genetic alterations in prostate tumor initiation. I investigated the role of ROS in a mouse model of early prostate tumorigenesis with the lack of expression of a crucial tumor suppressor gene (Chapter III), explored the influence of genetic risk factors for prostate cancer development upon antioxidant supplementation (Chapter IV), and studied the function of an antioxidant protein in prostate tumorigenesis (Chapter V). These analyses have provided new insight into the interplay of oxidative stress with prostate tumor initiation, antioxidant chemoprevention for prostate cancer, and helped to elucidate the roles of established prostate tumor suppressor *NKX3.1* and novel prostate cancer associated genes *BNIP3L* and *PRDX6* in prostate tumorigenesis.

NKX3.1 and antioxidant chemoprevention in prostate tumorigenesis

Due to the failure of SELECT and other antioxidant cancer chemoprevention trials, the need is apparent for a deeper understanding of the role ROS plays in tumor initiation depending on cellular context. Clinical trials are almost always performed in populations of diverse genetic background and diverse environmental contexts to determine measures which can be applicable to a widespread population. However, as has been proposed for many essential nutrients a (*250*), I propose that a personalized approach to antioxidant chemoprevention, with genetics and environmental factors taken

into account, will be required for true success at improving prostate cancer and other health outcomes.

In order to begin to address the complexities of antioxidant chemoprevention for prostate cancer in the pre-clinical setting, I supplemented *Nkx3.1*-deficient mice, a mouse model which accurately represents the very earliest stages of prostate tumorigenesis, with the antioxidant NAC. After finding that this antioxidant promoted prostate epithelial cell proliferation in the mouse, I then assessed the rate of prostate cancer development in *NKX3.1*-deficient men upon antioxidant supplementation in SELECT. While rs2228013 did not significantly modify prostate cancer risk overall or in any intervention arm, presence of the minor allele at rs11781886 genotype was associated with an increased risk of prostate cancer in the vitamin E and selenium arms. This suggests that antioxidant supplementation in the presence of low *NKX3.1* expression promotes development of prostate cancer, a finding consistent with our mouse study.

In individuals with normal prostatic *NKX3.1* expression, who have the major allele (T) at rs11781886, the tumor suppressor NXK3.1 can prevent proliferation and maintain low oxidative stress via regulation of oxidative control genes. However, men with the minor allele (C) at rs11781886, associated with decreased *NKX3.1* expression (*227*), have heightened prostate cancer risk with vitamin E and selenium supplementation. Figure 22 summarizes the proposed mechanism behind this observation. In subjects who have the minor allele (C) at rs1781886, a binding site for the transcription factor Sp1 is created (*227*) causing repression of *NKX3.1* expression. In these individuals, we propose there is an increase in proliferation in the prostate epithelium, as seen in the *Nkx3.1*$^{-/-}$ mouse, but this proliferation is held in check by

elevated ROS in the cell. Thus, individuals with the minor allele at rs11781886 who are not supplemented with antioxidants do not have a very large increase in prostate cancer risk. However, those with the rs11781886 minor allele individuals who are supplemented with antioxidants will have further elevated prostate epithelial proliferation and increased prostate cancer risk due to quenching of the NKX3.1-loss associated ROS that normally inhibit proliferation to some degree (Figure 22). Thus, these data suggest that individuals with an elevated prostate cancer risk due to low *NKX3.1* levels likely contributed to the overall 17% increased risk of prostate cancer with vitamin E supplementation in SELECT.

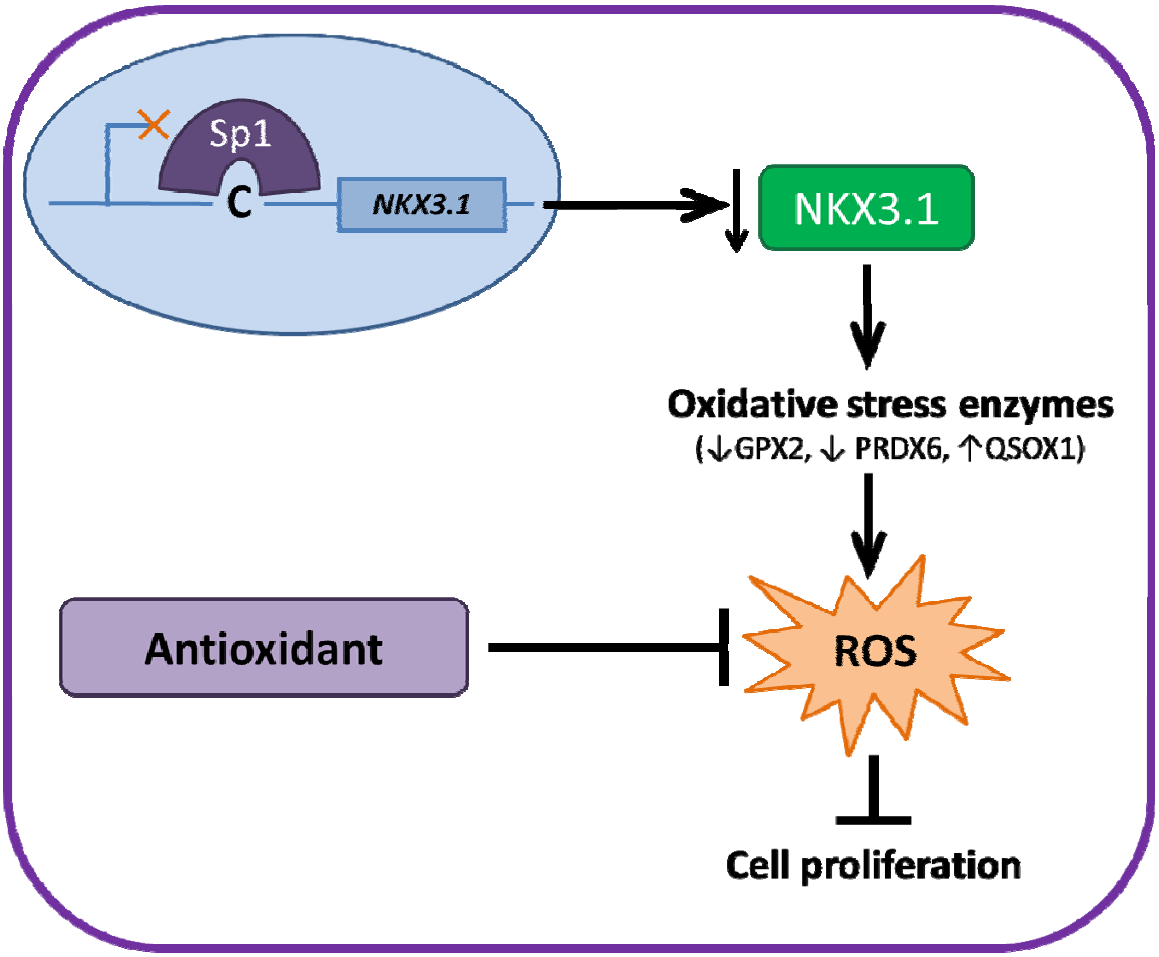

Figure 22. Effects of antioxidant supplementation in prostate epithelial cells with the rs11781886 minor allele (C). The C genotype at rs11781886 allows for the transcription factor Sp1 to bind and inhibit *NKX3.1* expression. This results in increased oxidative stress due to direct dysregulation of oxidative stress enzymes, which would usually partially inhibit the increased proliferation associated with loss of *NKX3.1* expression. Antioxidant supplementation helps to quench the elevated oxidative stress, releasing the cells from ROS-mediated inhibition of proliferation, increasing the risk for transformation of the cell and cancer development.

Future directions

Specificity of antioxidant promotion of prostate epithelial proliferation

Several investigations could expand our understanding of the effects of ROS modulation in early prostate tumorigenesis. In my mouse study, I observed increased proliferation upon NAC supplementation in *Nkx3.1*-deficient prostates, and in my human genotyping study, I observed an increased prostate cancer risk with a genotype reported to be associated with decreased *NKX3.1* expression. Although I propose that a similar mechanism is at play in the mouse and human prostate, and among the different antioxidants as they could all result in an overall decrease in oxidative stress in the prostate epithelium, the possibility exists for species-specific and supplement specific effects.

In order to determine if the pro-proliferative effect seen with NAC supplementation in *Nkx3.1*$^{-/-}$ mice is due to an overall antioxidant effect or due to an effect specific to NAC, *Nkx3.1*$^{-/-}$ mice could be supplemented with other antioxidants such as vitamin E, vitamin C, selenium, soy, lycopene, and beta-carotene. Also, as some investigators have claimed that the decrease in serum γ-T was responsible for the increased risk of vitamin E supplementation in SELECT (*142*), it would be especially interesting to supplement with vitamin E as either α-T as was done in SELECT or γ-T to see if there is a different effect. Duration and dosage of supplementation could also be altered to determine specific effects on tumor initiation.

Similarly, in human studies, influence of rs11781866 genotype on prostate cancer risk with plasma levels or supplementation with of various antioxidants could be investigated. Large studies such as the Health Professionals Follow-Up Study (HPFS)

(*308*) and the Physicians Health Study (PHS) (*309*), with data on plasma vitamin E levels, selenium levels, lycopene levels, beta carotene levels, or other antioxidants, could be analyzed to confirm the effect and determine whether a similar effect is seen with other antioxidants.

Effect of antioxidant supplementation at different stages of prostate tumor progression

In addition to supplementing early in life to study chemoprevention, mice which develop tumors slowly (such as *Nkx3.1*$^{+/-}$*; Pten*$^{+/-}$ mice) could be supplemented with antioxidants after tumor formation in order to determine the effect of quenching ROS on prostate tumor progression. Mice which develop advanced tumors, such as *PB-Cre; Z-MYC; Pten*$^{f/+}$*; p53*$^{f/+}$ (*208*), will also be supplemented after advanced tumor formation to determine the effect of antioxidants in late-stage tumors. These studies would help inform the discordant results from pre-clinical and clinical trials of prostate cancer chemoprevention and treatment.

Molecular mechanism responsible for antioxidant-mediated promotion of proliferation

Using accurate prostate cancer mouse models, global gene expression could be performed to determine changes in expression due to each antioxidant chemoprevention or cancer treatment. Differences in expression with supplementation of each antioxidant compound and at each stage of tumor formation will help elucidate the complexity of findings observed in human clinical trials and possibly inform molecular mechanisms of early prostate tumorigenesis. Findings may help determine if any antioxidant compounds may be efficacious for chemoprevention or in later stages of tumor development. As these models use similar genetic changes to those observed during human prostate

tumorigenesis, they may better represent effects of antioxidant compounds in chemoprevention than in historically used models.

If particular antioxidants show a protective or detrimental effect in a specific genetic context, this could then be investigated in human studies. For example, if one agent promotes cancer development in mice with *PTEN*-deficiency, samples from clinical trials using that agent could be stratified based on PTEN genetic or expression status to confirm that the agent affects human prostate tumorigenesis in a similar manner to the mouse model.

Importantly, the gene expression changes seen in mice after short term supplementation with α-tocopherol acetate and selenomethionine as used in SELECT should be compared to gene expression changes seen in the Phase II clinical study which performed microarray in men after supplementation with the SELECT antioxidants (*237*). This important comparison will help to determine the accuracy of our preclinical models and their ability to predict the efficacy of chemoprevention compounds in prostate cancer.

To determine the mechanism of increased risk with *NKX3.1* downregulation due to rs11781886 in the human prostate, global gene expression analysis could be performed on tissue from individuals with different genotypes at rs11781886 who were supplemented with SELECT trial or other antioxidants. These data could be compared to the antioxidant- supplemented prostate from accurate models of mouse prostate cancer to determine if similar pro-proliferative gene signatures are enriched with antioxidant supplementation. This analysis may help to uncover genes which could be implicated in

the pro-tumorigenic phenotype, providing possible targets for cancer treatment or prevention.

Alternative mechanisms

While the effects of antioxidant compounds are often described as working through alterations in prostatic oxidative stress, there remains the possibility that these compounds act through mechanisms distinct from ROS regulation. To help determine if modulation of oxidative stress is actually occurring, detailed studies of ROS levels or oxidative damage in the prostate in observational and intervention studies are needed in mouse models and human studies. Even if ROS are altered, it may be the case that the preventative effects or promotion seen by compounds are actually due to other effects.

Personalized chemoprevention

"Personalized medicine" is a recent phenomenon in cancer treatment which has come about due to our increased knowledge of the disease pathology. It allows for determination of the type of treatment has the greatest probability of helping at patient due to patient-specific information. A similar process may be extended in the future to determine the correct supplementation to prevent cancer development depending on patient-specific risk factors, serving as "personalized chemoprevention." This can be used is to approximate the risk or benefit associated with a single individual taking a specific chemopreventative agent. For example, although the average of vitamin E supplementation across participants of all genotypes in SELECT was to increase prostate cancer risk by 17%, the information gained from genetic modifications of this risk (such as genotype at rs11781886 and rs11781866) could be used to accurately estimate an individual's probable risk or benefit. This information could be clinically useful, for

example, as some may derive benefit from vitamin E supplementation for another disease. If these individuals knew how their specific genetic profile affected the prostate cancer risk associated with taking vitamin E, they could make an informed decision about whether taking vitamin E for benefit for another disease was worth the risk.

Therefore, through integrating new information gained on genetic context, point in tumor progression, relationship between animal models and human disease processes, and specific antioxidant compounds employed proposed by these studies, a deeper understanding of the complex molecular events involved in prostate cancer chemoprevention will be obtained.

BNIP3L SNP rs11781866 and prostate cancer risk in SELECT

In another such investigation, I described another genetic polymorphism (rs11781866) that modifies the risk of prostate cancer associated with SELECT randomization arm. While the SNP did not influence prostate cancer risk in the entire study population, I made the surprising finding that presence of the minor allele at rs11781866 in the region of *BNIP3L* decreases the risk of prostate cancer associated with vitamin E supplementation.

At present, the effect of rs11781866 genotype on *BNIP3L* expression or expression of other genes is unknown. Because *BNIP3L* levels are elevated with vitamin E supplementation, I hypothesize that *BNIP3L* is acting as an oncogene in the setting of elevated α-T levels. Thus, if having the minor allele negates this effect, I hypothesize that rs11781866 decreases BNIP3L expression or function to cause this change.

Figure 23 summarizes the proposed mechanism behind increased prostate cancer risk with major allele (T) at rs11781866. Vitamin E supplementation elevates *FOXC1* expression as was observed in our analysis of microarray data from individuals supplemented with SELECT antioxidants (Chapter IV, (*237*)). Interestingly, whereas FOXC1 upregulation has been shown to be important for the response to oxidative stress (*310*, *311*), we are the first to propose upregulation of *FOXC1* in response the antioxidant α-T. Therefore, the mechanism of elevated FOXC1 upon α-T supplementation is unknown and its discovery will require significant investigation. I propose that FOXC1 binding to the rs11781866 locus positively regulates BNIP3L expression. Thus, with high FOXC1 levels in the setting of α-T supplementation, BNIP3L levels are also high. As we observed an increased risk of prostate cancer development with the major allele at rs11781866, I hypothesize that elevated BNIP3L promotes prostate cancer development, possilbe through induction of autophagy and survival of the prostate epithelial cells.

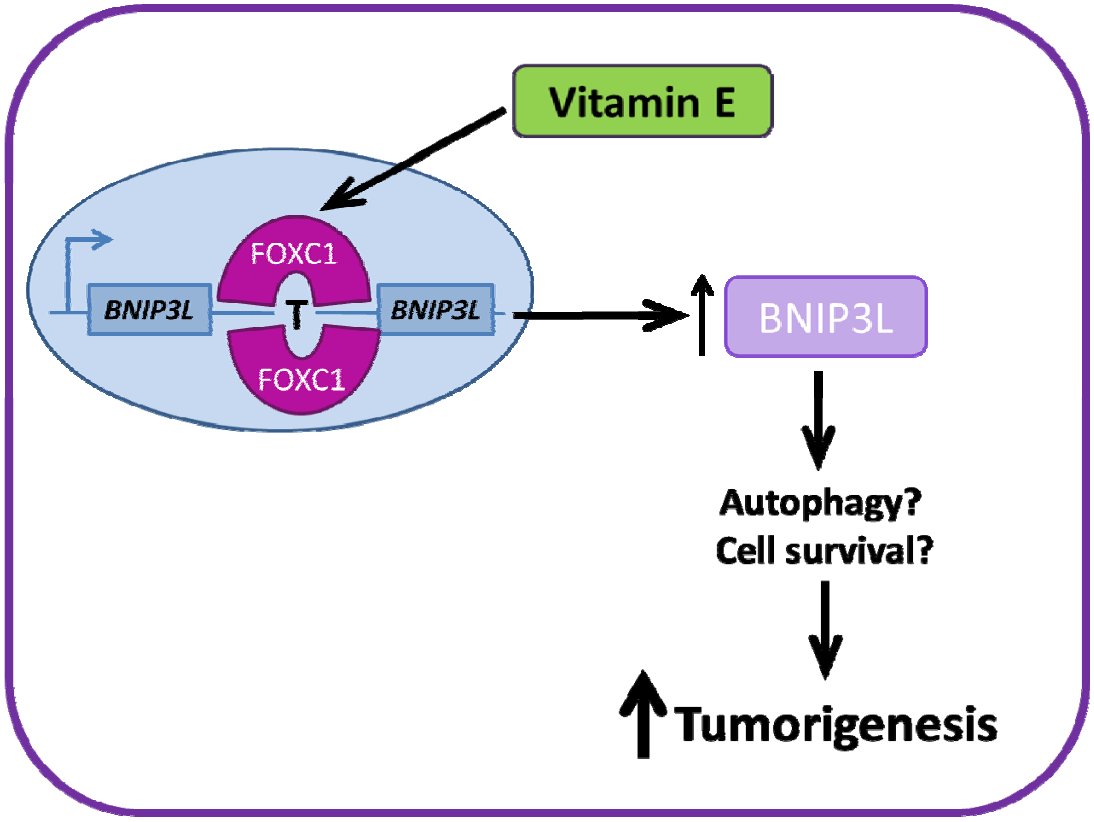

Figure 23. Model displaying vitamin E promotion of tumorigenesis via regulation of BNIP3L by FOXC1. Vitamin E supplementation increases FOXC1 levels in the prostate epithelial cells. The major allele at rs11781866 creates two FOXC1 binding sites. As I propose FOXC1 binding to rs11781866 positively regulates BNIP3L, vitamin E supplementation upregulates BNIP3L. BNIP3L then promotes transformation of the prostate epithelial cells, possibly through induction of autophagy and cell survival, increasing the risk of prostate tumorigenesis.

Future directions

Direct modulation of BNIP3L by FOXC1 at rs11781866

As my results in the rs11781866 study suggest a possible novel link of BNIP3L to prostate tumorigenesis, many studies could be performed to investigate the role of this gene in the disease. The direct modulation of BNIP3L expression by the rs11781866 SNP could be studied using molecular genetics approaches. The Cancer Genome Atlas (TCGA) is a database which includes genetic and gene expression data from many tissue and tumor types which can be queried to determine the relationship between rs11781866 and expression of BNIP3L. I hypothesize that the minor allele (C) at rs11781866 will be associated with decreased *BNIP3L* expression.

As I hypothesize that differential binding of FOXC1 to rs11781866 largely determines the degree of upregulation of BNIP3L, experiments could be performed to confirm differential binding of FOXC1 to the rs11781866 *in vitro* and *in vivo.* To begin to investigate functional changes in gene regulation due to the rs118781866 allele, a luciferase assay with the rs11781866 putative enhancer region controlling transcription at a minimal promoter region could be carried out. I hypothesize that the presence of the minor allele at rs11781866 will decrease activity of the enhancer region.

It is possible that rs11781866 is not the functional SNP, but merely a SNP in linkage with the actual polymorphism causing the alteration in phenotype. Thus, it may not be through differential regulation and binding of FOXC1 that *BNIP3L* levels are altered. If this is the case, additional SNPs in the vicinity of rs11781866 must be genotyped in SELECT to determine the quantitative trait locus (QTL) associated with the enhanced risk of prostate cancer with antioxidant supplementation. Additional functional

analysis of SNPs in the rs11781866 QTL will be required to then determine the functional SNP responsible for the phenotype.

Functional role of BNIP3L in prostate tumorigenesis

As the role of BNIP3L in prostate tumorigenesis has not been established, basic analyses of its effect on tumor cell proliferation, apoptosis, migration, invasion, and tumorigenicity could be performed using established prostate cancer cell lines. However, the functional significance of BNIP3L in prostate tumor initiation would be best studied using a tissue recombination approach with modulation of BNIP3L expression in nonmalignant prostate stromal or epithelial cells. This could be done using primary cells from mouse models of prostate cancer or human prostate epithelial and stromal cell lines. Tissue recombination would uniquely allow for a direct test of the "Autophagic Tumor Stroma Model of Cancer Metabolism" in the prostate, where tumor and stromal cell interactions are highly studied and extremely important to glandular pathology.

To further assess the importance of BNIP3L regulation in human prostate cancer patients even in the absence of high dose antioxidant supplementation, clinical data could be queried for expression of BNIP3L upon differing levels of plasma α-T or γ-T to determine if these compounds in the normal physiological range can influence BNIP3L levels and therefore change prostate cancer risk. Also, patient tissue samples from the SELECT biorepository, or other studies where antioxidant supplementation has been performed with subsequent biopsy, should be analyzed for expression via BNIP3L immunohistochemistry to determine if changes in *BNIP3L* RNA truly correlate with altered BNIP3L protein levels.

While extensive future experiments are needed to elucidate the role of increased BNIP3L in prostate tumorigenesis and the effect of rs11781866 on prostate cancer risk with antioxidant supplementation, this investigation has uncovered a possible new molecule related to prostate tumorigenesis. As with the NKX3.1 SNP rs11781886, *BNIP3L* SNP genotype may contribute to important "personalized chemoprevention" in the future, especially due to the greater than 40% increased risk of prostate cancer development with vitamin E supplementation in those with the TT genotype. If BNIP3L is found to play a functional role in promotion of prostate tumor progression, it may also possible serve as a new molecular chemoprevention or therapeutic target.

Role of NKX3.1 and MYC target gene Prdx6 in prostate tumorigenesis

The preceding discussion has highlighted that the roles of oxidative stress and antioxidants in prostate cancer are complex and much remains to be understood. I began studying the role of PRDX6 in prostate tumorigenesis not only because it was a co-direct target of NKX3.1 and MYC, but because it is an antioxidant protein which may play a significant role in the development of prostate cancer. Based on previous work in our laboratory which showed that Myc expression and loss of Nkx3.1 decreased Prdx6 in early lesions in the mouse prostate (*158*, *159*), I hypothesized that Prdx6 expression inhibits prostate tumor initiation. Significant future investigation will be required to test this hypothesis. In my analysis of the functional role of Prdx6 in the aggressive mouse prostate cancer cell line Myc-CaP, however, I found that Prdx6 promoted proliferation and tumorigenesis. Therefore, I hypothesize that Prdx6 plays a dual role in prostate

tumorigenesis, preventing tumor formation at an early stage, but promoting aggressiveness in established tumors.

I propose two ways in which Prdx6 may both inhibit and promote prostate tumorigenesis depending on the cellular context. ROS can have diverse roles depending on their level, the exact species, and the cell with which it is interacting. At very early stages in prostate tumorigenesis, Prdx6 may be important in quenching ROS which could cause damaging mutations to cells, thus preventing tumor initiation. However, at advanced stages, Prdx6 quenching of ROS may inhibit oxidative-stress induced apoptosis or cell cycle arrest. On the other hand, the dual function of Prdx6 may explain its dual role during tumorigenesis. Again, early in tumor initiation, Prdx6 peroxidase activity may prevent initiating mutations, but late in tumorigenesis, the phospholipase A2 activity of Prdx6 may promote elevated levels of prostaglandins which promote tumor growth.

Future directions

Regulation of PRDX6 by MYC

The regulation of PRDX6 by Myc should be investigated in more detail. First, siRNA-mediated depletion of MYC in normal and malignant human prostate cell lines could be performed to determine if decreased MYC expression correlates with increased PRDX6 expression, suggesting that MYC could repress PRDX6. Because the genome-wide MYC binding sites in PRDX6 have been described in non-prostate cell types, a reporter assay using the PRDX6 promoter containing the MYC binding sites could be carried out. Thus, I expect that MYC transfection will repress the basal level of transcription of the PRDX6 promoter. ChIP could be performed for MYC human prostate cell lines and human tissue lysates to confirm MYC binding to the *in vivo*

PRDX6 locus. Presence of well-known MYC cofactors involved in transcriptional repression, such as DNMT3a (*312*), could be assessed to support the direct transcriptional repression of PRDX6 by MYC.

Functional role of PRDX6 in early and late prostate tumorigenesis

The proposed dual role of PRDX6 in prostate tumorigenesis could be tested by several investigations. PRDX6 expression could be driven or depleted via shRNA in non-malignant and malignant human prostate epithelial cells and tested in a tissue recombination model for tumorigenicity. Overexpression experiments could be performed with wild type and mutant PRDX6 constructs to determine the role of the distinct Prdx6 functions in the phenotype observed. In addition, animal hosts of the tissue recombination grafts could be treated with specific inhibitors to Prdx6 peroxidase (mercaptosuccinate) or phospholipase A2 (MJ33) (*313*) activity to determine the Prdx6 function responsible for the phenotype and possibly define a therapeutic target for Prdx6-overexpressing tumors.

To investigate the relevance of PRDX6 in clinical prostate tumorigenesis, human tissue microarrays containing tissue from normal prostate, hyperplastic prostate, PIN lesions, early, low grade carcinoma, and advanced, high grade carcinoma could be stained for MYC, PRDX6, and NKX3.1. I hypothesize that in early, pre-malignant lesions, high MYC and low NKX3.1 expression will be correlated with low PRDX6 expression, as seen in our mouse model. However, in advanced lesions, I expect to see high PRDX6 in many cases, even in the presence of high MYC expression as PRDX6 may be able to escape normal repression by MYC in advanced lesions to exert its tumor promoting effects.

Explanation of the role of PRDX6 in human prostate tumorigenesis may help in understanding the molecular mechanisms of the disease, allowing for efficacious prevention and treatment strategies to be developed.

Conclusions

Prostate cancer is a major worldwide public health concern and will most likely remain so for many years to come. Due the widespread prevalence of the disease, success in prostate cancer prevention is highly desired, yet has been largely unsuccessful to date. The lack of success is most likely because of the complex and diverse mechanisms of prostate cancer development, which are highlighted by the intricate roles of oxidative stress in prostate tumorigenesis.

My dissertation work has provided valuable insight, showing that antioxidants do not always prevent cancer, and in prostate cancer, may even promote malignancy in certain populations. My observations would suggest that one cannot assume that taking high dose antioxidants can always be done without harm. Although many do not view antioxidants and other supplements as "drugs" because prescriptions are not required, they can and do alter normal physiological processes, sometimes resulting in various forms of pathology. By defining subpopulations with differential response to antioxidant treatment, I have supported the future of possible "personalized chemoprevention" which could allow for individualized determination of risk or benefit from antioxidant supplementation.

My data confirm the crucial role of NKX3.1 in prostate tumorigenesis, as loss of NKX3.1 expression in both the mouse and human prostate increases cancer risk with antioxidant supplementation. In addition, I have identified another possible gene, *BNIP3L*, which may promote prostate tumorigenesis with vitamin E supplementation.

Lastly, I have proposed a complex role for the antioxidant enzyme PRDX6 in prostate tumorigenesis, a protein which may have a significant function in MYC-driven tumor initiation. Gaining an understanding of how PRDX6 relates to prostate cancer development with its antioxidant and phospholipase A2 functions at different stages of tumorigenesis may allow for future development of preventative and treatment interventions. Further investigation will help to delineate the complex interplay of oxidative stress and genetic alterations in prostate tumor initiation.

Appenidix A. TOP GENE SETS ENRICHED IN NAC-SUPPLEMENTED *Nkx3.1*$^{-/-}$ ANTERIOR PROSTATE

* Gene sets with a FDR q-value < 0.25 are listed*

Gene Set Name	Gene Set Size	Enrichment Score	Normalized Enrichment Score	NOM p-value	FDR q-value
LEE_LIVER_CANCER_MYC_E2F1_DN	54	0.6687	2.0786	0.0000	0.0296
LIAN_LIPA_TARGETS_3M	63	0.6321	2.0697	0.0000	0.0165
LEE_LIVER_CANCER_E2F1_DN	54	0.6587	2.0367	0.0000	0.0150
LEE_LIVER_CANCER_MYC_DN	51	0.6449	2.0146	0.0000	0.0159
LIAN_LIPA_TARGETS_6M	78	0.6114	2.0052	0.0000	0.0152
HESS_TARGETS_OF_HOXA9_AND_MEIS1_DN	80	0.5729	1.9349	0.0000	0.0459
NAKAYAMA_SOFT_TISSUE_TUMORS_PCA2_DN	63	0.6068	1.9301	0.0000	0.0424
REACTOME_PEPTIDE_LIGAND_BINDING_RECEPTORS	147	0.5361	1.9250	0.0000	0.0408
GAL_LEUKEMIC_STEM_CELL_DN	185	0.5161	1.9233	0.0000	0.0379
CADWELL_ATG16L1_TARGETS_UP	95	0.5614	1.9072	0.0000	0.0433
KEGG_PPAR_SIGNALING_PATHWAY	57	0.6099	1.9063	0.0000	0.0398
REACTOME_CLASS_A1_RHODOPSIN_LIKE_RECEPTORS	250	0.4960	1.9009	0.0000	0.0393
PAL_PRMT5_TARGETS_DN	28	0.6665	1.8969	0.0000	0.0387
REACTOME_STRIATED_MUSCLE_CONTRACTION	28	0.6805	1.8848	0.0000	0.0434
LEE_LIVER_CANCER_DENA_DN	62	0.5935	1.8843	0.0000	0.0409
TAKEDA_TARGETS_OF_NUP98_HOXA9_FUSION_3D_DN	25	0.7011	1.8740	0.0000	0.0457
LEE_LIVER_CANCER_MYC_TGFA_DN	55	0.5976	1.8576	0.0000	0.0532
KEGG_ADIPOCYTOKINE_SIGNALING_PATHWAY	65	0.5750	1.8510	0.0000	0.0548
CHIARADONNA_NEOPLASTIC_TRANSFORMATION_KRAS_CDC25_DN	50	0.5925	1.8478	0.0000	0.0546
NAKAYAMA_SOFT_TISSUE_TUMORS_PCA1_DN	69	0.5638	1.8430	0.0000	0.0560
GERY_CEBP_TARGETS	112	0.5311	1.8426	0.0000	0.0535
KEGG_HEMATOPOIETIC_CELL_LINEAGE	65	0.5724	1.8285	0.0000	0.0619
POOLA_INVASIVE_BREAST_CANCER_UP	225	0.4793	1.8273	0.0000	0.0601

VERRECCHIA_EARLY_RESPONSE_TO_TGFB1	48	0.5884	1.8126	0.0016	0.0696
NAKAYAMA_SOFT_TISSUE_TUMORS_PCA2_UP	75	0.5459	1.7943	0.0000	0.0852
SHEPARD_BMYB_MORPHOLINO_DN	146	0.4956	1.7939	0.0000	0.0821
ICHIBA_GRAFT_VERSUS_HOST_DISEASE_35D_UP	139	0.5060	1.7934	0.0000	0.0795
CROONQUIST_NRAS_VS_STROMAL_STIMULATION_DN	72	0.5433	1.7903	0.0030	0.0793
FURUKAWA_DUSP6_TARGETS_PCI35_DN	57	0.5595	1.7769	0.0000	0.0909
NADERI_BREAST_CANCER_PROGNOSIS_UP	32	0.6239	1.7765	0.0032	0.0884
RICKMAN_HEAD_AND_NECK_CANCER_F	50	0.5676	1.7704	0.0030	0.0920
KEGG_COMPLEMENT_AND_COAGULATION_CASCADES	58	0.5550	1.7651	0.0015	0.0953
REACTOME_PLATELET_DEGRANULATION	80	0.5312	1.7592	0.0000	0.0992
URS_ADIPOCYTE_DIFFERENTIATION_DN	28	0.6437	1.7525	0.0078	0.1039
REACTOME_G_ALPHA_I_SIGNALLING_EVENTS	151	0.4838	1.7517	0.0000	0.1020
MCLACHLAN_DENTAL_CARIES_UP	161	0.4818	1.7502	0.0000	0.1010
REACTOME_GPCR_LIGAND_BINDING	346	0.4463	1.7500	0.0000	0.0986
LEE_LIVER_CANCER_TOP50	32	0.6084	1.7461	0.0015	0.1007
SETLUR_PROSTATE_CANCER_TMPRSS2_ERG_FUSION_DN	17	0.7022	1.7350	0.0137	0.1108
LE_EGR2_TARGETS_UP	97	0.5114	1.7336	0.0000	0.1097
MCLACHLAN_DENTAL_CARIES_DN	184	0.4631	1.7209	0.0000	0.1236
LEE_LIVER_CANCER	32	0.6084	1.7202	0.0049	0.1217
BERTUCCI_INVASIVE_CARCINOMA_DUCTAL_VS_LOBULAR_DN	39	0.5845	1.7065	0.0077	0.1387
SABATES_COLORECTAL_ADENOMA_DN	227	0.4517	1.7058	0.0000	0.1368
STEARMAN_TUMOR_FIELD_EFFECT_UP	40	0.5818	1.7028	0.0047	0.1382
YU_MYC_TARGETS_UP	37	0.5795	1.6982	0.0082	0.1420
RICKMAN_HEAD_AND_NECK_CANCER_D	20	0.6706	1.6958	0.0016	0.1423
KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION	215	0.4493	1.6925	0.0000	0.1436
WIELAND_UP_BY_HBV_INFECTION	70	0.5189	1.6798	0.0014	0.1600
CAIRO_HEPATOBLASTOMA_POOR_SURVIVAL	15	0.6968	1.6752	0.0190	0.1646

KEGG_LINOLEIC_ACID_METABOLISM	17	0.6617	1.6745	0.0193	0.1623
MISHRA_CARCINOMA_ASSOCIATED_FIBROBLAST_UP	18	0.6576	1.6639	0.0163	0.1779
GRAHAM_CML_DIVIDING_VS_NORMAL_QUIESCENT_UP	148	0.4584	1.6540	0.0026	0.1926
YAO_HOXA10_TARGETS_VIA_PROGESTERONE_UP	74	0.5057	1.6509	0.0057	0.1951
NAKAYAMA_SOFT_TISSUE_TUMORS_PCA1_UP	55	0.5309	1.6487	0.0030	0.1962
SMID_BREAST_CANCER_RELAPSE_IN_LUNG_DN	31	0.5850	1.6456	0.0065	0.1985
LIU_VAV3_PROSTATE_CARCINOGENESIS_UP	76	0.5012	1.6444	0.0030	0.1972
VALK_AML_WITH_CEBPA	29	0.6001	1.6438	0.0092	0.1948
VERRECCHIA_RESPONSE_TO_TGFB1_C1	18	0.6466	1.6433	0.0178	0.1922
JEON_SMAD6_TARGETS_UP	20	0.6340	1.6364	0.0201	0.2011
SHEPARD_BMYB_TARGETS	55	0.5091	1.6326	0.0079	0.2041
SMID_BREAST_CANCER_LUMINAL_A_UP	74	0.4925	1.6280	0.0029	0.2095
VARELA_ZMPSTE24_TARGETS_DN	43	0.5380	1.6241	0.0115	0.2138
LE_EGR2_TARGETS_DN	107	0.4678	1.6240	0.0014	0.2108
GRAHAM_NORMAL_QUIESCENT_VS_NORMAL_DIVIDING_DN	69	0.5041	1.6193	0.0031	0.2161
KEGG_ECM_RECEPTOR_INTERACTION	79	0.4958	1.6108	0.0029	0.2296
TONKS_TARGETS_OF_RUNX1_RUNX1T1_FUSION_GRANULOCYTE_DN	15	0.6550	1.6105	0.0256	0.2266
NAKAJIMA_EOSINOPHIL	18	0.6406	1.6102	0.0272	0.2239
REACTOME_IMMUNOREGULATORY_INTERACTIONS_BETWEEN_A_LYMPHOID_AND_A_NON_LYMPHOID_CELL	45	0.5225	1.6097	0.0122	0.2216
REACTOME_MUSCLE_CONTRACTION	48	0.5235	1.6089	0.0164	0.2201
REACTOME_REGULATION_OF_LIPID_METABOLISM_BY_PEROXISOME_PROLIFERATOR_ACTIVATED_RECEPTOR_ALPHA	51	0.5165	1.6074	0.0138	0.2199
ROSTY_CERVICAL_CANCER_PROLIFERATION_CLUSTER	119	0.4605	1.6061	0.0014	0.2196
TONKS_TARGETS_OF_RUNX1_RUNX1T1_FUSION_ERYTHROCYTE_UP	134	0.4508	1.6017	0.0053	0.2251
KOBAYASHI_EGFR_SIGNALING_24HR_DN	215	0.4235	1.6010	0.0000	0.2234
SOTIRIOU_BREAST_CANCER_GRADE_1_VS_3_UP	118	0.4513	1.6008	0.0029	0.2208

CROMER_TUMORIGENESIS_DN	38	0.5523	1.5996	0.0106	0.2201
KEGG_NEUROACTIVE_LIGAND_RECEPTOR_INTERACTION	248	0.4199	1.5982	0.0000	0.2199
FLECHNER_BIOPSY_KIDNEY_TRANSPLANT_REJECTED_VS_OK_UP	76	0.4839	1.5968	0.0045	0.2201
COLIN_PILOCYTIC_ASTROCYTOMA_VS_GLIOBLASTOMA_DN	28	0.5756	1.5968	0.0201	0.2173
CLASPER_LYMPHATIC_VESSELS_DURING_METASTASIS_DN	32	0.5735	1.5945	0.0224	0.2193
REACTOME_NA_CL_DEPENDENT_NEUROTRANSMITTER_TRANSPORTERS	16	0.6504	1.5935	0.0162	0.2184
KEGG_CELL_ADHESION_MOLECULES_CAMS	102	0.4578	1.5864	0.0057	0.2301
EBAUER_MYOGENIC_TARGETS_OF_PAX3_FOXO1_FUSION	45	0.5218	1.5834	0.0168	0.2331
VERRECCHIA_RESPONSE_TO_TGFB1_C2	18	0.6282	1.5831	0.0232	0.2310
SABATES_COLORECTAL_ADENOMA_UP	105	0.4568	1.5818	0.0041	0.2307
MOROSETTI_FACIOSCAPULOHUMERAL_MUSCULAR_DISTROPHY_UP	17	0.6469	1.5818	0.0362	0.2280
LEE_LIVER_CANCER_CIPROFIBRATE_DN	55	0.5065	1.5812	0.0107	0.2265
MARKEY_RB1_CHRONIC_LOF_DN	115	0.4538	1.5806	0.0069	0.2251
SHEN_SMARCA2_TARGETS_DN	247	0.4185	1.5802	0.0000	0.2234
COULOUARN_TEMPORAL_TGFB1_SIGNATURE_DN	97	0.4599	1.5797	0.0028	0.2216
AMIT_SERUM_RESPONSE_240_MCF10A	53	0.5031	1.5745	0.0163	0.2294
REACTOME_HEMOSTASIS	245	0.4095	1.5714	0.0013	0.2332
KONDO_PROSTATE_CANCER_WITH_H3K27ME3	139	0.4389	1.5674	0.0027	0.2386
TANG_SENESCENCE_TP53_TARGETS_DN	33	0.5529	1.5669	0.0175	0.2373
DELYS_THYROID_CANCER_UP	364	0.3931	1.5637	0.0000	0.2411
RUIZ_TNC_TARGETS_DN	112	0.4460	1.5626	0.0085	0.2407
WANG_CISPLATIN_RESPONSE_AND_XPC_UP	118	0.4399	1.5614	0.0028	0.2411
YAO_TEMPORAL_RESPONSE_TO_PROGESTERONE_CLUSTER_6	70	0.4786	1.5604	0.0075	0.2406
KUNINGER_IGF1_VS_PDGFB_TARGETS_DN	19	0.6155	1.5566	0.0287	0.2458

Appendix B. TOP GENE SETS DEPLETED IN NAC-SUPPLEMENTED *Nkx3.1*$^{-/-}$ ANTERIOR PROSTATE

* Gene sets with a FDR q-value < 0.25 are listed*

Gene Set Name	Gene Set Size	Enrichment Score	Normalized Enrichment score	NOM p-val	FDR q-val
MOSERLE_IFNA_RESPONSE	20	-0.8749	-2.4193	0.0000	0.0000
TAKEDA_TARGETS_OF_NUP98_HOXA9_FUSION_3D_UP	132	-0.5704	-2.2829	0.0000	0.0033
DAUER_STAT3_TARGETS_DN	28	-0.7439	-2.2618	0.0000	0.0026
DER_IFN_BETA_RESPONSE_UP	68	-0.5917	-2.1501	0.0000	0.0181
BENNETT_SYSTEMIC_LUPUS_ERYTHEMATOSUS	18	-0.7845	-2.1353	0.0026	0.0216
EINAV_INTERFERON_SIGNATURE_IN_CANCER	22	-0.7500	-2.1197	0.0000	0.0226
SHEN_SMARCA2_TARGETS_UP	353	-0.4734	-2.1077	0.0000	0.0220
MILI_PSEUDOPODIA_HAPTOTAXIS_UP	464	-0.4643	-2.1023	0.0000	0.0203
BROWNE_INTERFERON_RESPONSIVE_GENES	54	-0.6101	-2.0896	0.0000	0.0212
ZHAN_MULTIPLE_MYELOMA_LB_DN	34	-0.6481	-2.0617	0.0000	0.0296
TAKEDA_TARGETS_OF_NUP98_HOXA9_FUSION_8D_UP	125	-0.5218	-2.0523	0.0000	0.0301
KIM_LRRC3B_TARGETS	18	-0.7318	-1.9726	0.0000	0.0778
LIANG_SILENCED_BY_METHYLATION_2	28	-0.6323	-1.9469	0.0000	0.0943
DER_IFN_ALPHA_RESPONSE_UP	47	-0.5755	-1.9350	0.0031	0.1000
FARMER_BREAST_CANCER_CLUSTER_1	32	-0.6243	-1.8871	0.0000	0.1458
DEBIASI_APOPTOSIS_BY_REOVIRUS_INFECTION_UP	197	-0.4416	-1.8764	0.0000	0.1525
SEITZ_NEOPLASTIC_TRANSFORMATION_BY_8P_DELETION_UP	61	-0.5236	-1.8509	0.0031	0.1826
MAHADEVAN_RESPONSE_TO_MP470_UP	15	-0.6978	-1.8291	0.0074	0.2095
TAKEDA_TARGETS_OF_NUP98_HOXA9_FUSION_10D_UP	148	-0.4444	-1.8280	0.0000	0.1997
DACOSTA_UV_RESPONSE_VIA_ERCC3_COMMON_DN	375	-0.4060	-1.8250	0.0000	0.1945
KANG_CISPLATIN_RESISTANCE_UP	15	-0.6962	-1.8135	0.0000	0.2057
DACOSTA_UV_RESPONSE_VIA_ERCC3_XPCS_DN	69	-0.5121	-1.8112	0.0000	0.2005
YANG_BREAST_CANCER_ESR1_BULK_UP	16	-0.6918	-1.8026	0.0095	0.2072

POMEROY_MEDULLOBLASTOMA_PROGNOSIS_DN	31	-0.5722	-1.7809	0.0185	0.2369
ZHANG_BREAST_CANCER_PROGENITORS_UP	384	-0.3911	-1.7775	0.0000	0.2334
REACTOME_RNA_POLYMERASE_I_III_AND_MITOCHONDRIAL_TRANSCRIPTION	80	-0.4892	-1.7769	0.0069	0.2258
SCHLOSSER_MYC_TARGETS_AND_SERUM_RESPONSE_DN	39	-0.5457	-1.7614	0.0053	0.2469
REACTOME_TRANSCRIPTION	150	-0.4293	-1.7588	0.0000	0.2429
ZHU_CMV_8_HR_UP	27	-0.5904	-1.7581	0.0051	0.2359

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