# THE ROLE OF INHIBITOR OF APOPTOSIS (IAP) FAMILY MEMBER SURVIVIN IN PROSTATIC DISEASE

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## Dedication

This dissertation is dedicated to my family; parents Gordon and Sheila McIlwain, sisters Amy McIlwain and Becca Rankin (and Ted), niece Kairi, fiancé Alexis Glore and dogs Siena and Strawberry. All of you have in some capacity helped me through this process called graduate school. It is amazing how much a little support goes such a long way.

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David W. McIlwain

THE ROLE OF INHIBITOR OF APOPTOSIS (IAP) FAMILY MEMBER SURVIVIN IN PROSTATIC DISEASE

Continual and recalcitrant inflammation is an extremely common condition in the human prostate and has been found to be associated with a number of prostatic diseases including prostate cancer and benign prostatic hyperplasia (BPH). While much has been described regarding prostate disease resulting from oxygen and nitrogen radicals during inflammation, proliferative mechanisms associated with repair and regeneration are less understood. The Inhibitor of Apoptosis (IAP) family member survivin has been found to be overexpressed during inflammation and associated with prostate cancer progression. Apurinic/apyrimidinic endonuclease 1/redox effector factor 1 (APE1/Ref-1) is a multifunctional protein that is essential in activating inflammatory transcription factors. Because APE1/Ref-1 is expressed and elevated in prostate cancer, we sought to characterize APE1/Ref-1 expression and activity in human prostate cancer cell lines and determine the effect of selective reduction-oxidation (redox) function inhibition on prostate cancer cells in vitro and in vivo. Due to the role of inflammatory transcriptional activators NFκB and STAT3 in survivin protein expression, and APE1/Ref-1 redox activity regulating their transcriptional activity, we assessed selective inhibition of APE1/Ref-1's redox function as a novel

method to halt prostate cancer cell growth and survival. Our study demonstrates that survivin and APE1/Ref-1 are significantly higher in human prostate cancer specimens compared to noncancerous controls and that APE1/Ref-1 redox-specific inhibition with small molecule inhibitors APX3330 and APX2009 decreases prostate cancer cell proliferation and induces cell cycle arrest. Inhibition of APE1/Ref-1 redox function significantly reduced NFκB transcriptional activity, survivin mRNA and survivin protein levels. These data indicate that APE1/Ref-1 is a key regulator of survivin and a potentially viable target in prostate cancer.

Tao Lu, Ph.D., Chair

## Table of Contents

## List of Tables

## List of Figures

## List of Abbreviations

| Abbreviation | Full name |
|---|---|
| **ABC** | ATP-binding cassette |
| **ADT** | Androgen deprivation therapy |
| **AKT** | Serine/threonine-specific protein kinase |
| **ANOVA** | Analysis of variance |
| **AP** | Apurinic/apyrimidinic |
| **AP** | Anterior prostate |
| **AP-1** | Activator protein 1 |
| **APE1/Ref-1** | Apurinic/apyrimidinic endonuclease 1/redox factor-1 |
| **AR** | Androgen Receptor |
| **BCA** | Bicinchoninic acid |
| **Bcl-2** | B-cell lymphoma 2 |
| **BER** | Base excision repair |
| **BIRC1** | Baculoviral IAP repeat-containing protein 1 |

| | |
|---|---|
| **BIRC6** | Baculoviral IAP Repeat Containing protein 6 |
| **BPH** | Benign prostatic hyperplasia |
| **BrdU** | 5-bromo-2'-deoxyuridine |
| **CD** | Cluster of differentiation |
| **Cdc2** | Cyclin-dependent kinase 1 |
| **c-IAP1** | Baculoviral IAP repeat-containing protein 2 |
| **c-IAP2** | Baculoviral IAP repeat-containing protein 3 |
| **CK** | Cytokeratin |
| **c-MET** | Tyrosine protein kinase Met |
| **Co-IP** | Co-Immunoprecipitation |
| **CPPS** | Chronic pelvic pain syndrome |
| **CYP17** | Cytochrome P450 17A1 |
| **Cys** | Cysteine |
| **DI** | Distilled |
| **DLP** | Dorsolateral Prostate |

| | |
|---|---|
| **DRE** | Digital rectal examination |
| **E.coli** | Escherichia coli |
| **ECM** | Extracellular matrix |
| **EGF** | Epidermal growth factor |
| **ELISA** | Enzyme-linked immunosorbent assay |
| **ER** | Estrogen Receptor |
| **ERKs** | Extracellular-signal-regulated kinases |
| **ET-1** | Endothelin-1 |
| **ETS** | E26 transformation-specific transcription factor |
| **FasL** | Fas ligand |
| **FGF** | Fibroblast growth factor |
| **FOXO** | Forkhead box |
| **GPCRs** | G-protein-coupled receptors |
| **GST-π** | Glutathione S-transferase P |
| **H&E** | Hematoxylin and eosin |

| | |
|---|---|
| **HCl** | Hydrochloric acid |
| **HER-2** | Receptor tyrosine-protein kinase erbB-2 |
| **HGF** | Hepatocyte growth factor |
| **HIF-1α** | Hypoxia-inducible factor 1-alpha |
| **HSP90** | Heat shock protein 90 |
| **IAP** | Inhibitors of Apoptosis |
| **IF** | Immunofluorescence |
| **IGF-1** | Insulin-like growth factor 1 |
| **IKK** | IκB kinase complex |
| **IL** | Interleukin |
| **ILP2** | IAP-like protein 2 |
| **INCENP** | Inner centromere protein |
| **IP** | Intraperitoneal |
| **JAK** | Janus Kinase |
| **JNKs** | c-Jun N-terminal kinases |

| | |
|---|---|
| **KGF** | Keratinocyte growth factor |
| **LB** | Lysogeny Broth |
| **LC3** | Microtubule-associated protein 1A/1B-light chain 3 |
| **LNRH** | Luteinizing hormone-releasing hormone |
| **LUTS** | Lower urinary tract symptoms |
| **Lys** | Lysine |
| **MAPKs** | Mitogen activated protein kinases |
| **MKK** | Mitogen-Activated Protein Kinase Kinase |
| **ML-IAP** | Melanoma inhibitor of apoptosis |
| **mTOR** | Mechanistic target of rapamycin |
| **NE** | Neuroendocrine |
| **NEM** | N-Ethylmalimide |
| **NF-κB** | Nuclear factor kappa B |
| **NIH** | National Institutes of Health |
| **NKX3.1** | Homeobox protein Nkx-3.1 |

| | |
|---|---|
| **NSAIDs** | Nonsteroidal anti-inflammatory drugs |
| **PARP** | Poly (ADP-ribose) polymerase |
| **PBS** | Phosphate buffered saline |
| **PBST** | PBS-Tween |
| **P-gp** | P-glycoprotein |
| **PI** | Propidium Iodide |
| **PI3K** | Phosphoinositide 3-kinase |
| **PIP2** | Phosphatidylinositol (4,5) bisphosphate |
| **PIP3** | Phosphatidylinositol (3,4,5) trisphosphate |
| **PSA** | Prostate-specific antigen |
| **PSMA** | Prostate surface membrane antigen |
| **PTEN** | Phosphatase and tensin homolog |
| **PTHrP** | Calcitonin and parathyroid hormone-related proteins |
| **ROS** | Reactive oxygen species |
| **RT** | Room temperature |

| | |
|---|---|
| **RTK** | Receptor tyrosine kinases |
| **RT-PCR** | Reverse transcription-polymerase chain reaction |
| **Shh** | Sonic hedgehog |
| **siRNA** | Small (or short) interfering RNA |
| **SLC** | Solute Carrier |
| **SMA** | Smooth muscle actin |
| **SMAC/DIABLO** | Second mitochondria-derived activator of caspase |
| **Sp1** | Specificity protein 1 |
| **STAT** | Signal transducer and activator of transcription |
| **TGF** | Transforming growth factor |
| **TNFR1** | TNF-α Receptor 1 |
| **TNFR2** | TNF-α Receptor 2 |
| **TNF-α** | Tumor necrosis factor α |
| **TRE** | TPA DNA response elements |

| | |
|---|---|
| **TUNEL** | Terminal deoxynucleotidyl transferase dUTP nick end labeling) |
| **TURP** | Transurethral resection of the prostate |
| **UGS** | Urogenital sinus |
| **VEGF** | Vascular endothelial growth factor |
| **VIP** | Vasoactive intestinal peptide |
| **VP** | Ventral Prostate |
| **WT** | Wild Type |
| **XIAP** | X-Linked Inhibitor of Apoptosis |

# Chapter 1 Introduction

## Prostate Anatomy

The prostate is a male-specific small, walnut-sized gland that sits underneath the bladder and in front of the rectum. The prostate also surrounds the urethra, which is a narrow tube connected to the bladder that runs through the length of penis and carries both semen and urine out of the body. On top of the prostate resides the seminal vesicles which contain and secrete a significant portion of the ejaculate. The neurovascular bundle, a bundle of nerves and vessels, run alongside the prostate and are responsible for controlling erectile function. The prostate gland itself is surrounded by a layer of connective tissue called the prostatic capsule.

The prostate is composed of three different zones: the peripheral, the transitional and the central **(Figure 1)**. The peripheral zone is the area that is nearest to the rectum. It is the zone felt by doctors during a digital rectal examination (DRE) and where approximately 75% of prostate cancers are found [1]. By volume, the peripheral zone is the largest zone of the prostate. The transitional zone is in the middle of the prostate and resides between the other two zones. The urethra passes through the transitional zone and for most men under the age of 40 the transitional zone accounts for 20% of the prostate

volume. As men age, this region of the prostate can grow and cause urinary tract symptoms in a process called benign prostatic hyperplasia (BPH). [2] The central zone is in front of the transitional zone and is the farthest zone from the rectum.

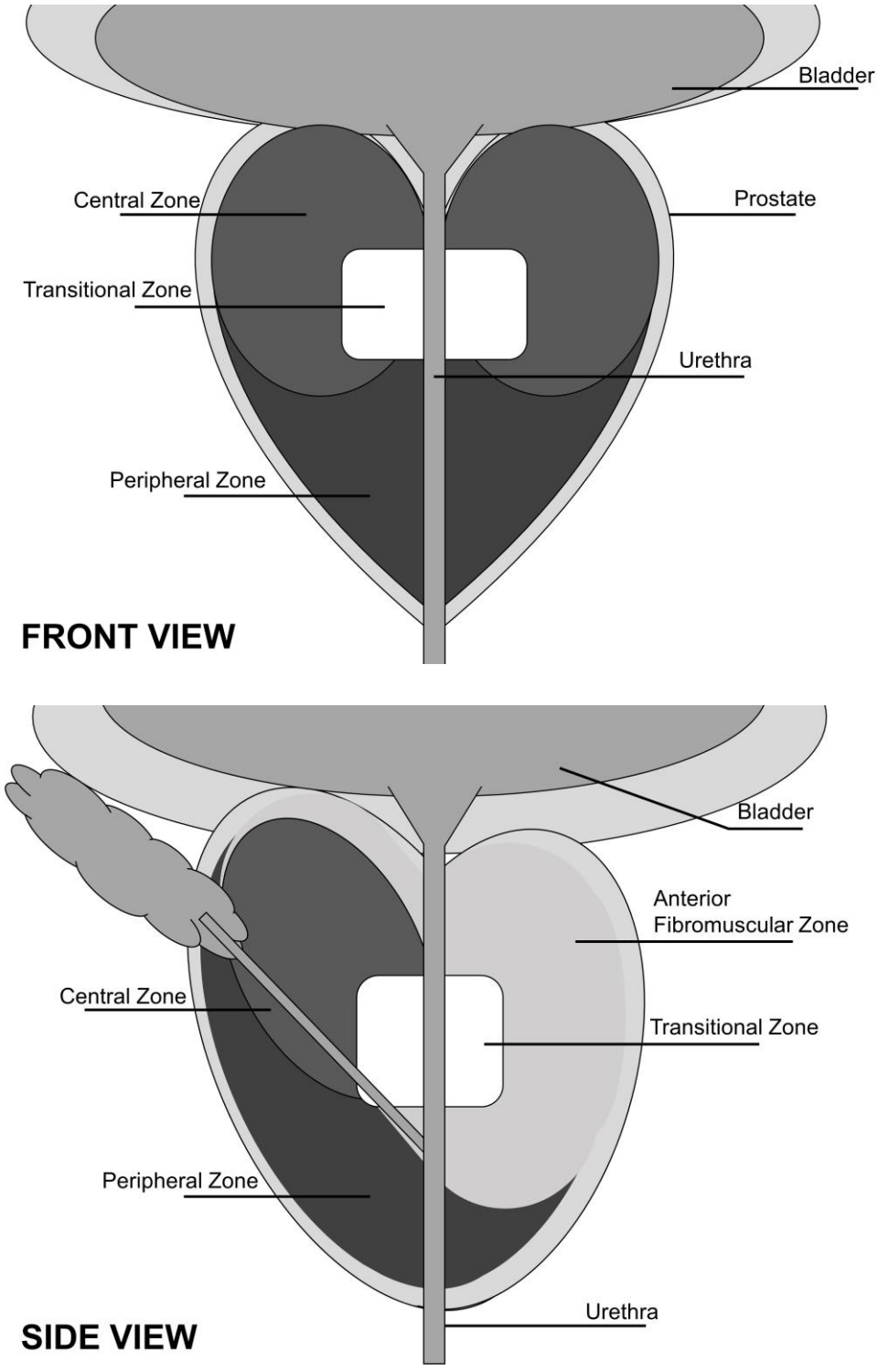



**Figure 1.** Schematic of human prostate anatomy

The prostate is composed of three different zones: the peripheral, the transitional and the central. The peripheral zone is the largest zone by volume and is where the majority of prostate cancers occur. The central zone is in front of the transitional zone and is the farthest zone from the rectum.

## Prostate Biology

The prostate is important for reproduction supplying proteins and minerals necessary for sperm survival, transit and fertilization. [3] It is not however, essential for life. Zinc, citrate and fructose are made from the seminal vesicles and provide the sperm energy for the migration up the uterine tract. During ejaculation, smooth muscle surrounding the prostate contract and propel fluid and semen into the urethra while simultaneously closing off the bladder. The prostate also releases enzymes, like Prostate-specific antigen (PSA), which are necessary for liquefying the semen to allow the sperm to reach the egg. PSA is used as a biomarker for prostate cancer. [4]

## Prostate Histology

The prostate is a set of tubulo-alveolar glands with luminal lining by epithelium and surrounded by a connective tissue stromal compartment. There are two histologically distinct layers in the epithelium with a secretory luminal layer made of up of tall columnar cells responsible for production of seminal fluid components and a basal layer composed of cuboidal epithelial cells which play a role in tissue replenishment **(Figure 2)**. [5-7] Both layers are surrounded by a basement membrane consisting of extracellular matrix (ECM) which forms a divide between the epithelia and stroma. The stromal compartment is comprised

of a number of different cell types with the smooth muscle cell being the most abundant. These smooth muscle cells are derived from the mesenchyme of the embryonic urogenital sinus (UGS). [8] Fibroblasts, nerves, endothelial cells and vascular smooth muscle cells are also very abundant in the stroma of the adult prostate. [9]

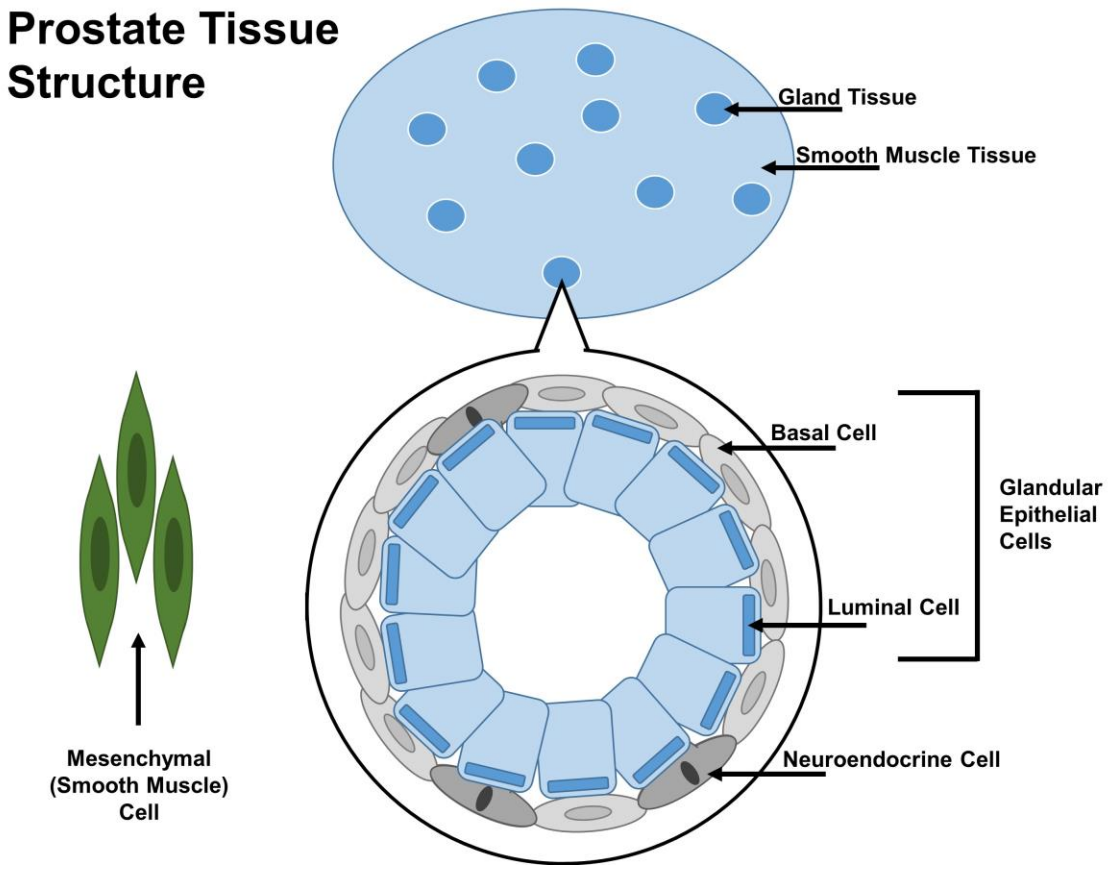



**Figure 2.** Schematic of the human prostatic gland

The prostate is a set of tubulo-alveolar glands with luminal lining by epithelium and surrounded by a connective tissue stromal compartment. The epithelium compartment is traditionally composed of three distinct cell types; the basal cell, the neuroendocrine cell and luminal cell type. The stromal compartment is comprised of a number of different cell types with the smooth muscle cell being the most abundant.

Traditionally, there are considered three different epithelial cell types in the prostate; luminal, basal and neuroendocrine (NE) cells. However, it has recently been shown that there is an additional heterogeneous subpopulation of cells called transit amplifying cells that migrate from the basal to the luminal layer. [10] These transit amplifying cells are of intermediate phenotype sharing characteristics of the early progenitor basal cells and the terminally differentiated secretory luminal cells. [11]

NE cells are considered an androgen-independent and non-proliferating cell type. [12] The NE cells are scattered across the luminal and basal layers and the exact role of this cell type is not known. [13] NE cells are most commonly characterized by the expression of chromogranin A, serotonin, bombasin, vasoactive intestinal peptide (VIP), calcitonin and parathyroid hormone-related proteins (PTHrP). [14-24] Even though NE cell function has yet to be elucidated, it has been implicated in prostatic carcinogenesis. [25-26]

Basal cells are believed to be the proliferative cell type of the epithelial compartment. [27-28] These cells are commonly characterized by the expression of cytokeratin (CK) 5 and 14 and the cell-surface marker CD44. Some other possible markers include CK10, 11, 15 and 17, p63, integrin α2, B-cell lymphoma 2 (Bcl-2), P-cadherin, pp32, Glutathione S-transferase P (GST-π), tyrosine-

protein kinase Met (c-MET), HGF (hepatocyte growth factor) Receptor and Receptor tyrosine-protein kinase erbB-2 (HER-2). [29-42]

Luminal cells are considered androgen-dependent, requires androgen to live, and are most commonly characterized by the expression of CK8/18 and cluster of differentiation (CD) 57. [43-44] As the result of differentiation, luminal cells do not express Bcl-2 like the basal cell type. Also, the androgen receptor is expressed and mediates the production and secretion of androgen-dependent proteins like PSA. [45] Low levels of prostate surface membrane antigen (PSMA) and endothelin-1 (ET-1) have been detected in luminal cells. [46-47]

The prostate stroma is comprised of a multitude of different cells including endothelial cells, immune cells, nerve cells and fibroblasts, also called myofibroblasts. These cells play an important role in the homeostasis of the prostate providing the right environment for cell proliferation, movement and differentiation. [48] The prostate stromal compartment also plays a critical role in signaling with the epithelial compartment and has been found to contribute to epithelial tumor progression. [49]

Myofibroblasts, which make up smooth muscle, are the most abundant stromal cell type and play a critical role in tissue remodeling. These cells synthesize and express different extracellular matrix proteins like collagen,

tenascin, versican, laminin, smooth muscle actin (SMA), CD34 and fibronectin while simultaneously producing different proteases and growth factors needed for angiogenesis. [50] These smooth muscle cells are maintained by unipotent progenitor populations in the adult prostate. [51] Both of the prostatic tissue compartments expand in the presence of inflammation.

**Prostate Inflammation**

Prostate inflammation, also called prostatitis, is a common occurrence in the adult male population of the United States. [52] Dietary influences, environmental exposures, metabolic disorders and infectious agents [52-53] have all been tied to prostatic inflammation but the cause is most likely multi-factorial. Prostate inflammation has been extensively tied with the development and progression of both prostate cancer and BPH. [55-56] Inflammation causes imbalances of pro-inflammatory and anti-inflammatory cytokines, increased production of angiogenic and lymphangiogenic growth factors, and generation of reactive oxygen species (ROS) from infiltrating inflammatory cells. [57] All of which are considered potential initiators of prostatic neoplastic growth. Prostatitis is classified into four different groups; acute bacterial prostatitis, chronic bacterial prostatitis, inflammatory/non-inflammatory chronic pelvic pains syndrome

(previously known as chronic nonbacterial prostatitis) and asymptomatic inflammatory prostatitis **(Table 1)**.

| NIH CLASSIFICATION | DEFINITION |
|---|---|
| **Category I**<br>Acute Bacterial Prostatitis | Acute infection of the prostate |
| **Category II**<br>Chronic Bacterial Prostatitis | Chronic infection of the prostate |
| **Category IIIa & b**<br>Inflammatory/ Non-Inflammatory CPPS | Chronic Pelvic Pain Syndrome – accounts for majority of prostatitis cases. Nonbacterial in nature |
| **Category IV**<br>Asymptomatic Inflammatory Prostatitis | No history of genitourinary pain, but leukocytosis is noted during evaluation for other condition |

**Table 1.** National Institutes of Health (NIH) defined classification system for prostatitis.

Prostatitis is classified into four different categories; acute bacterial prostatitis, chronic bacterial prostatitis, inflammatory/ non-inflammatory chronic pelvic pain syndrome and asymptomatic inflammatory prostatitis.

Acute bacterial prostatitis is caused by bacteria that travel up the urethra and into the prostate. [58] It is the least common form of prostatitis and its symptoms consist of high fever, chills, joint/muscle aches and fatigue. In addition to those symptoms, pain around the base of the penis and behind the scrotum, pain in the lower back, and the feeling of a full rectum may also occur. Fluoroquinolone antibiotics are the standard of care with ciprofloxacin, levofloxacin, and ofloxacin most often prescribed. Trimethoprim/sulfamethoxazole is also sometimes used for the treatment of acute bacterial prostatitis. [59]

Chronic bacterial prostatitis is also caused by bacteria and is most commonly found in older men with other urological disorders. [60] Unlike acute bacterial prostatitis, its symptoms are mild and is considered a low grade infection that lasts for an extended amount of time. Symptoms include intermittent urges to urinate, frequent urination, painful urination, or nocturia. Low back pain, rectum pain, scrotum pain, and painful ejaculations can also occur. Antibiotics are also used for this form of prostatitis with treatment lengths ranging from 1 to 3 months. [61]

Chronic pelvic pain syndrome (CPPS), also known as chronic nonbacterial prostatitis, is the most common form of prostatitis and its symptoms reflect those

of chronic bacterial prostatitis. [62] No bacteria is discovered making treatment hard. Antibiotics, nonsteroidal anti-inflammatory drugs (NSAIDs) and alpha-adrenergic antagonists are used to ease the symptoms of CPPS. [63] It is hypothesized that this form of prostatitis is actually an overactive pain syndrome that connects the pelvic floor, bladder, prostate and rectum in an overactive neural network. [64]

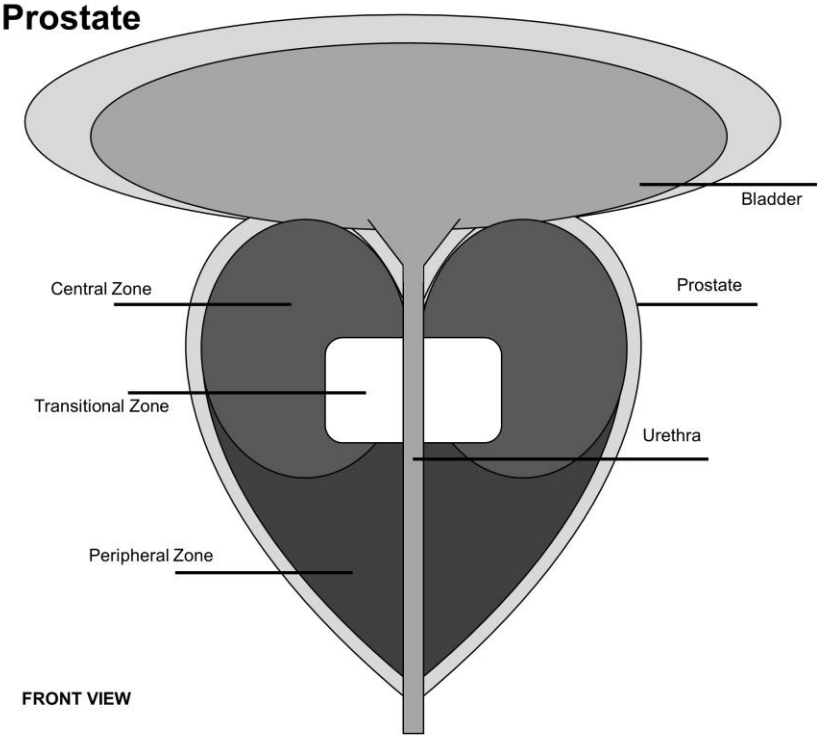
Asymptomatic inflammatory prostatitis has no symptoms and is usually diagnosed during tests for other medical problems. [65] Since there are no symptoms treatment is usually not needed. Prostatic inflammation is associated with the development of prostatic diseases including Benign Prostatic Hyperplasia and prostate cancer.

**Benign Prostatic Hyperplasia**

Benign prostatic hyperplasia (BPH) is a non-malignant condition where the epithelial and stromal compartments of the transitional zone, also the periurethral areas, enlarge to the point of causing unwanted urinary symptoms and occurs primarily in older men **(Figure 3)**. The prostate gland continues to grow throughout life but it is not entirely known what causes this specific type of prostatic enlargement. Untreated, BPH can cause the blockage of urine flow out of the bladder ultimately leading to urinary tract and/or kidney problems. [66] In

the United States, BPH has a prevalence of 40% amongst men by 60 years of age and 90% of men by 80 years of age. [67] BPH symptoms, also known as lower urinary tract symptoms (LUTS), include increased urinary urgency, urinary frequency, weak urine stream and inability to empty the bladder. Urinary tract infection and hematuria are also possible symptoms. [68] Treatment of BPH usually involves alpha-adrenergic antagonists to relax bladder neck and prostate muscles and/or 5-alpha reductase inhibiters to shrink the prostate by preventing testosterone to dihydrotestosterone conversion which is known to cause prostatic growth. [69] Surgery, transurethral resection of the prostate (TURP), might also be recommended in severe cases. [70]

## Normal Prostate




## Benign Prostatic Hyperplasia

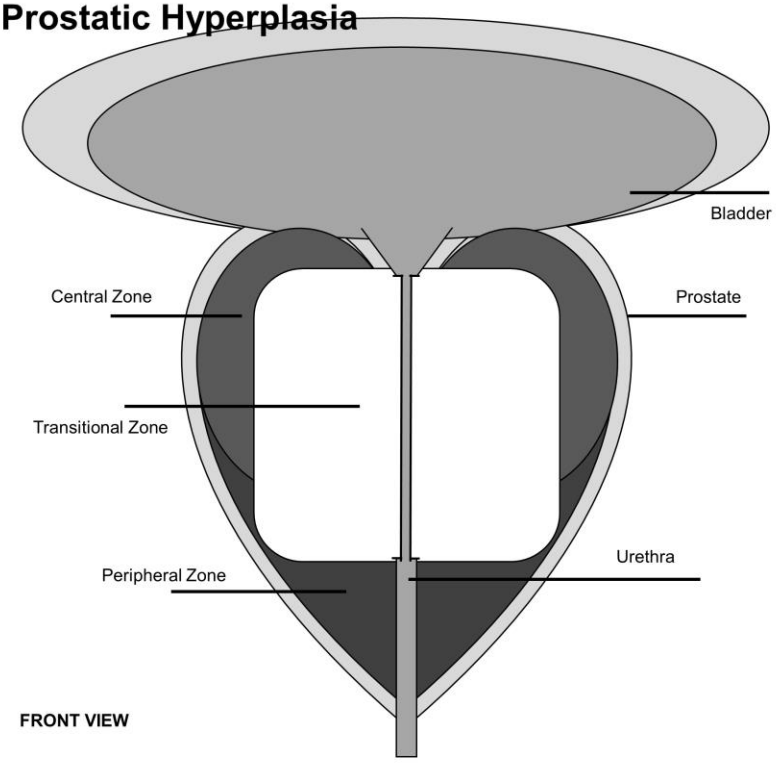


**Figure 3.** Benign Prostatic Hyperplasia

Prostate with Benign Prostatic Hyperplasia (BPH) showing enlarged transitional zone narrowing the urethra. BPH is a non-malignant condition where the epithelial and stromal compartments of the transitional zone, also periurethral areas, enlarge to the point of causing unwanted urinary symptoms. This primarily occurs in older men.

There are no clear triggering events causing BPH; one plausible hypothesis is that both acute and chronic inflammation may lead to increased proliferation within the prostate through a variety of mechanisms. Oxidative stress is known to cause tissue damage and may lead to compensatory proliferation resulting in hyperplastic growth. [71] Unregulated pro-inflammatory chemokines and cytokines by chronic inflammatory infiltrates have also been proposed to contribute to uncontrolled cellular proliferation, specifically stromal cell proliferation. Chronically active T-cells and macrophages are associated with nodules found in BPH and are known to secrete interleukin (IL) -6, IL-8 and IL-15. [72-74] Based on the available literature, BPH prevalence is most likely multifaceted with an unregulated immune system and systemic hormonal changes playing crucial roles. BPH is not a risk factor for prostate cancer even though inflammation is a risk factor for both BPH and prostate cancer.

**Prostate Cancer**

Prostate cancer is the uncontrolled malignant growth of cells within in the prostate and the most commonly cancer diagnosed in American men. Most prostate cancers are adenocarcinomas, meaning the cancer originates from the luminal gland cells of epithelial origin. Other classifications of prostate cancer are rare and include sarcomas, small cell carcinomas, neuroendocrine tumors and

transitional cell carcinomas. Majority of prostate cancers are indolent and slow growing but there are subclasses that can grow and spread quickly. [75] Behind lung and colorectal cancer, prostate cancer is the third leading cause of cancer-related death in American men with 1 man in 39 dying from prostate cancer. [76]

Prostate cancer is usually diagnosed using PSA blood tests, DRE and prostate biopsy. [77] Several newer tests are being implemented to determine prostate cancer risk and treatment including: the phi (combines results of total PSA, free PSA and its precursor proPSA), the 4Kscore test (combines results of total PSA, free PSA, intact PSA and human kallikrein 2), the progensa (investigates prostate cancer antigen 3 (PCA3) levels in urine after DRE), the ConfirmMDx (genetic test from prostate biopsy) and genetic tests that look for the abnormal gene fusion called TMPRSS2:ERG in urine prostate cells after DRE. [78] Determining the stage of prostate cancer plays a key role in establishing treatment options.

The Gleason grading system is used by pathologists to determine the stage of prostate cancer. This system is based exclusively on the architectural pattern of prostatic glands within the tumor after biopsy and it evaluates the differentiation, normal gland architecture, of the tumor with the belief that a more differentiated tumor is less aggressive than an undifferentiated tumor. [79] A

Gleason grade 1 is considered well differentiated with a Gleason grad 5 considered poorly differentiated. The Gleason score is the combined sum of the two most common architectural patterns (Gleason grades). A high Gleason score means the cancer is poorly differentiated and more likely to metastasize **(Figure 4)**.

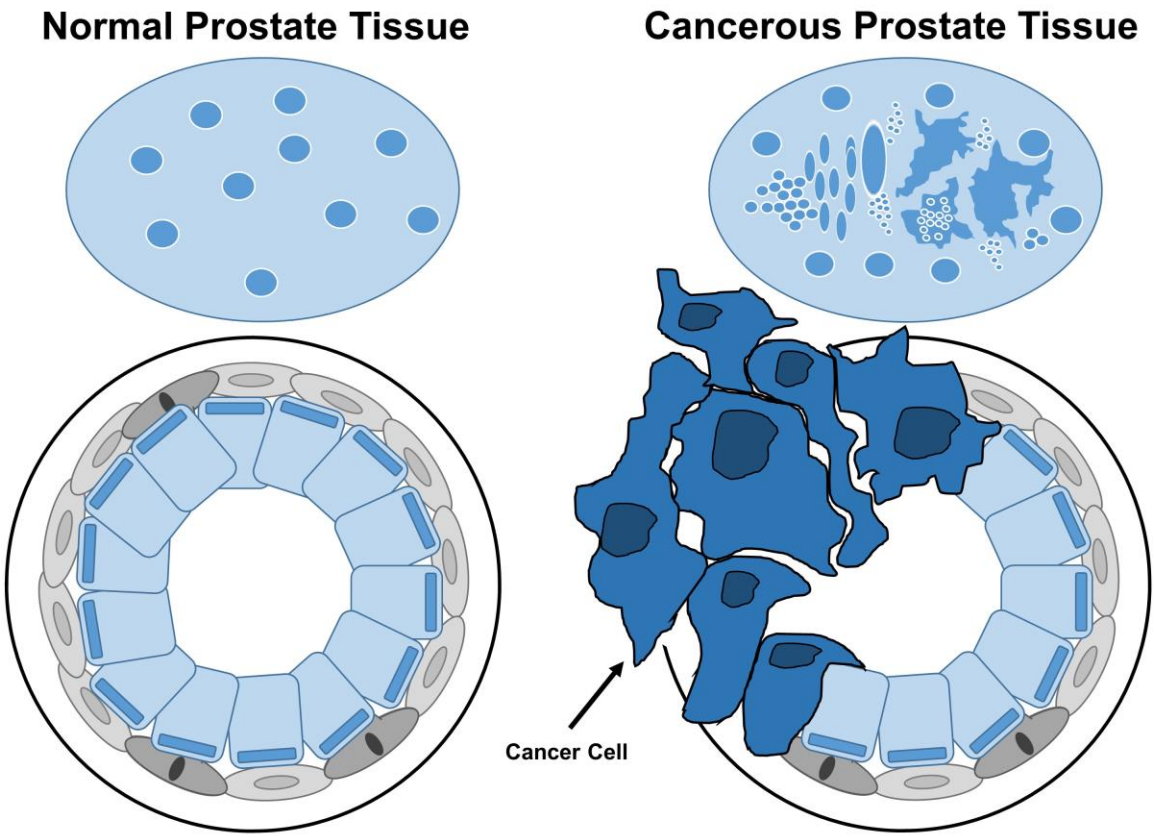



**Figure 4.** Prostate cancer glandular structure

Prostate cancer is the uncontrolled malignant growth of cells within the prostate (**Right Panel**). It is characterized by the increased ratio of epithelial cells to stromal cells compared to normal prostate tissue (**Left Panel**). Unorganized glandular structures are a hallmark of prostate cancer and these cancer cells invade through the gland basement membrane where they metastasize to lymph node and bone.

Treatment of prostate cancer depends on the stage of tumor. Those tumors that have a low Gleason score, monitoring the cancer through active surveillance or watchful waiting, PSA tests and biopsies, might be preferable to the possible side effects of treatment. [80] For those localized tumors with moderate risk of progression, radiation therapy is often used. Conformal radiation therapy, intensity modulated radiation therapy, proton bean radiation and brachytherapy are used to minimize damage to surrounding normal tissues. [81] Destroying the tumor using high-intensity focused ultrasound, highly focused ultrasonic beams, is also gaining popularity for these localized tumors. [82] For those moderate and high risk tumors that are still localized to the prostate, prostate surgery/removal is used to cure the cancer. [83]

Metastatic prostate cancer or high risk prostate cancers in patients who cannot have surgery, hormone deprivation therapy and chemotherapy may be treatments of choice. Hormone deprivation therapy is used to reduce the levels of androgens and/or activity of the androgen receptor. There are a number of treatments aimed at lowering androgen levels or androgen receptor activity: orchiectomy (surgical castration), luteinizing hormone-releasing hormone (LNRH) agonists and antagonists (plays on hormonal feedback systems), Cytochrome P450 17A1 (CYP17) inhibitors (blocks production of androgens) and androgen

receptor inhibitors (blocks androgen receptor activity). [84-89] Side effects of these drugs include reduced sexual desire, erectile dysfunction, osteoporosis, decreased mental sharpness, loss of muscle mass, weight gain, fatigue and depression. [90] Once the cancer becomes resistant to androgen deprivation therapy, chemotherapy is used. Docetaxel and cabazitaxel have been shown to help men live longer with metastatic prostate cancer and now combinations with androgen deprivation therapy are being investigated. [91] Resistance to both of these treatments are unfortunately inevitable **(Figure 5)**.

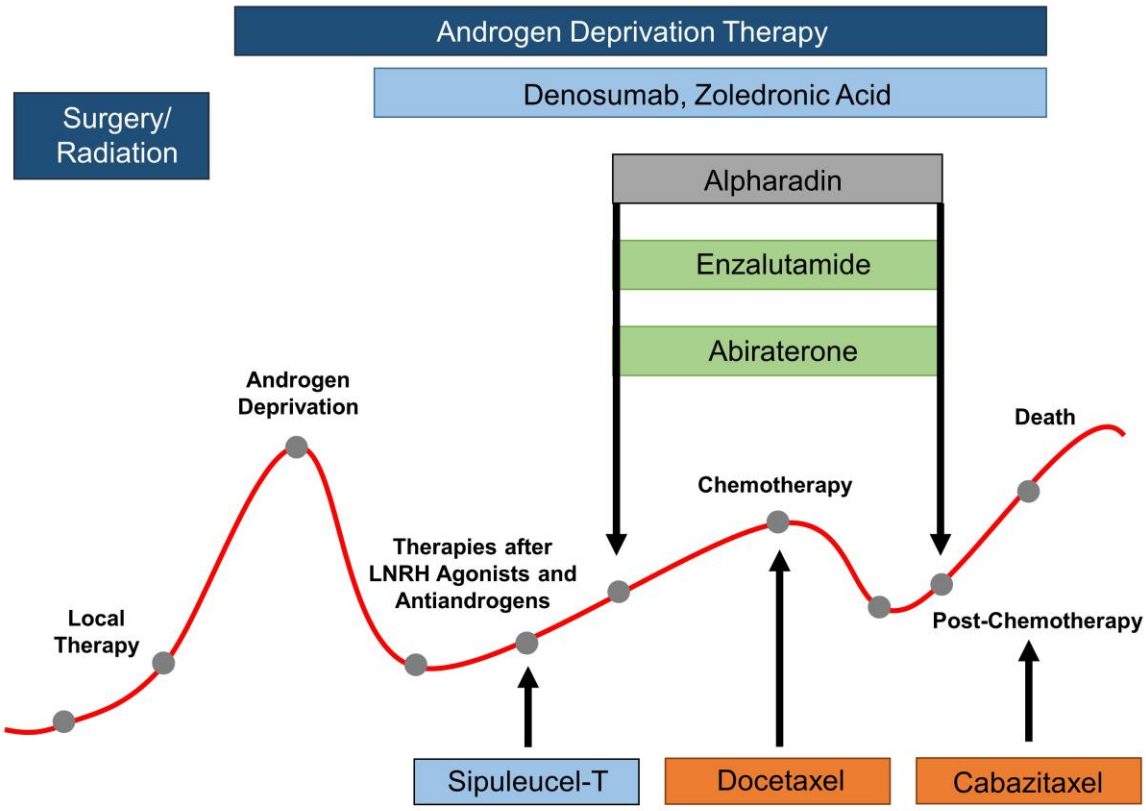



**Figure 5.** Current treatment landscape for prostate cancer

(**Red line**) signifies PSA levels overtime and **(Grey Dot)** signifies treatment. When the cancer is localized to the prostate, surgery and radiation is most often used to cure the cancer. If surgery/radiation is not an option or if PSA levels continue to increase after treatment, androgen deprivation therapy (ADT) is then administered. This would include LNRH agonists and anti-androgens (Enzalutamide and Abiraterone). Once ADT fails to stop the growth/metastasis of the cancer immunotherapy (Sipuleucel-T) and chemotherapy (Docetaxel and Cabazitaxel) are used in conjunction with ADT. Denosumab and Zoledronic Acid are also used to treat bone problems in patients who have cancer.

The most-accepted risk factors for prostate cancer development are age, family history (genetics) and African American ancestry but there is an increased incidence of prostate cancer in those men who adopt a westernized lifestyle. [92] This suggests that environmental factors along with hereditary factors are playing a role in the development of prostate cancer. One such potential environmental factor is the induction of chronic inflammation through infections, dietary factors, hormonal changes and/or environmental chemical exposures. This inflammatory microenvironment created by the immunobiology of the prostate leads to the influx of chemokines and cytokines that potentially aid to unregulated cell growth.

**Prostate Cancer Signaling**

Interestingly, there is a relatively low rate of mutations in prostate cancer compared to other tumors of different origins and a high prevalence of non-random copy number variations involving well-known oncogenes and tumor suppressors. [93-94] The most common genomic alterations in prostate cancer signaling pathways include the androgen receptor pathway, phosphoinositide 3-kinase (PI3K) pathway, the loss of tumor suppressor Homeobox protein Nkx-3.1(NKX3.1) and the pro-growth chromosomal rearrangement TMPRSS2:ERG. [95-98] This chromosomal rearrangement involving E26 transformation-specific transcription (ETS) factors under androgen receptor control is found in 60-70% of

prostate cancers. [99] Aside from these genomic alterations, multiple inflammatory signaling pathways have been identified as possible mediators between inflammation and carcinogenesis.

The nuclear factor kappa B (NF-κB) signaling pathway has been associated with inflammation, autoimmune disorders and cancer. [100] The family consists of 5 members: p65 (also known as RelA), RelB, c-Rel, p100/p53 and p105/p52 which form homo or heterodimers that function as transcriptional activators through binding consensus sites along DNA. The canonical NF-κB pathway is activated by a broad spectrum of stimuli like tumor necrosis factor α (TNF-α) and IL-1 and involves the phosphorylation of the inhibitory IκB proteins by the IκB kinase complex (IKK). This phosphorylation results in the ubiquitination and degradation of IκB by the proteasome, releasing NF-κB dimers to translocate to the nucleus and activate responsive target genes. Some of these target genes are involved in proliferation and apoptosis and have been shown to contribute to the development and progression of certain cancers including prostate cancer. [101-102]

NF-κB inhibition has been shown to suppress prostate cancer *in vivo* and *in vitro*. [103] In prostate cancer cell lines PC-3 and DU145, NF-κB was found to be constitutively active and inhibition of NF-κB was found to inhibit cell growth

and induce cell death. [104-106] NF-κB activator TNF-α is highly expressed in prostate cancer along with its receptors TNFR1 and TNFR2 compared to noncancerous prostatic epithelium and the expression of TNF- α has been correlated with prostate cancer cell proliferation, metastasis and drug resistance. [107] High levels of TNF-α levels in human serum have also been associated with poor prognosis of prostate cancer patients. [108]

The Janus Kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway is an important cascade required for normal development and cell homeostasis. [109] It is recognized as an important membrane-to-nucleus cascade that can be activated by reactive oxygen species, cytokines and growth factors. The JAK/STAT pathway has been implicated in hematopoiesis, gland development, immune response and sexually dimorphic growth as well as in the development of both hematological and solid tumor malignancies. [110] Once signaling activation occurs and the specific inducer (like IL-6) binds to its respective receptor, the receptors undergo oligomerization leading to the phosphorylation of the JAK family of kinases, JAK 1-3. These activated JAK proteins act as docking sites for STAT monomers. The phosphorylation of the STATs allow for dimerization and the effective translocation to the nucleus and activation of STAT-dependent target genes.

STAT3, along with NF-κB, has been shown to be constitutively active in a number of cancers including prostate **(Figure 6)**. [111] STAT3 gene targets are associated with cell cycle regulation and apoptosis and control various cellular events such as proliferation, differentiation and cell survival. [112] Upregulation of antiapoptotic proteins Bcl-2, Bcl-XL, survivin and Mcl-1 by STAT3 have been shown in prostate cancer and many other tumors. [113-114] The proangiogenic factor vascular endothelial growth factor, VEGF, has also been tied to STAT3 and been shown to be involved in in tumor invasion and migration. [115] Additionally, inhibition of the JAK/STAT pathway has been found to suppress prostate cancer growth and survival. [116]

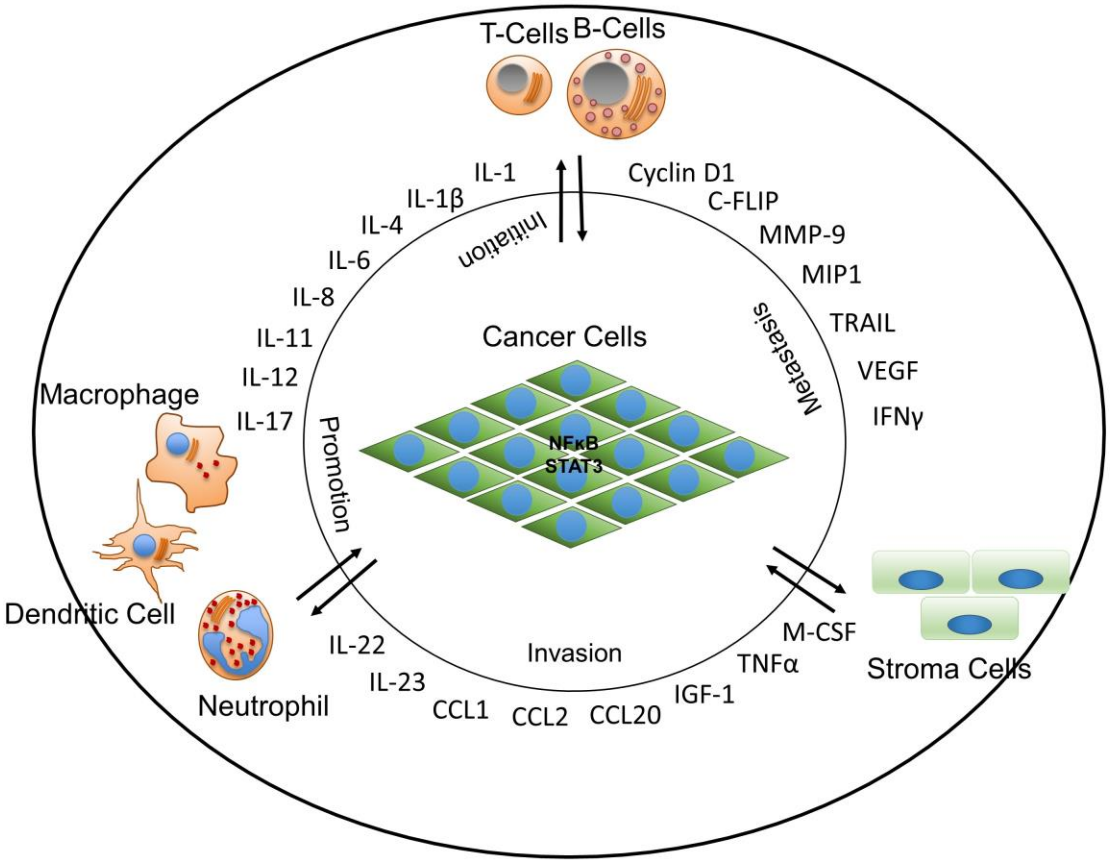



**Figure 6.** NF-κB and STAT3 in cancer

NF-κB and STAT3 are found to be activated in a number of cancers including prostate cancer but also play major roles in other cell types. These two signaling pathways work collaboratively to continue positive feed-forward signaling, including the signaling molecules shown in the diagram above, creating an inflammatory tumor microenvironment. Inflammatory chemokines and cytokines are secreted by local immune cells, cancer cells and stromal cells creating the perfect stimuli for cancer initiation, promotion, invasion and metastasis.

The PI3K/ serine/threonine-specific protein kinase (AKT) pathway is a signal transduction pathway that connects a multitude of membrane receptors to essential cellular functions such as cell survival and proliferation. [117-119] PI3K is divided into three major classes: class I (IA and IB) molecules that contain one catalytic and regulatory subunit and have the ability to bind to receptor tyrosine kinases (RTKs), G-protein coupled receptors and oncogenic proteins to transduce their signals, and class II and III molecules which contain a single catalytic subunit and bind to several receptors, such as RTKs or cytokine receptors. After PI3K activation, recruitment and activation of AKT occurs through phosphatidylinositol (4, 5) bisphosphate (PIP2) conversion into phosphatidylinositol (3, 4, 5) trisphosphate (PIP3). Activated AKT can phosphorylate and activate several other proteins like mechanistic target of rapamycin (mTOR), glycogen synthase kinase 3, and Forkhead box (FOXO) members. Ultimately, AKT's actions regulates a large array of cellular processes related to cell survival and proliferation.

PI3K/AKT pathway is hyper activated in the majority of solid tumors. PI3K/AKT/mTOR signaling is up-regulated in 30%–50% of prostate cancers and is most often due to the loss of Phosphatase and tensin homolog (PTEN) function and AKT hyper activation. [120] PTEN dephosphorylates PIP3 to PIP2

and negatively controls the PI3K/AKT pathway. PTEN has been found to be haploinsufficient in some prostate cancers and its genetic loss has been associated with prostate cancer progression and increased metastatic potential. [121] Therapeutics targeting the deregulation of the PI3K/AKT pathway have been developed and are currently being tested in prostate cancer.

Mitogen activated protein kinases (MAPKs) comprise a family of kinases tied to the regulation of intracellular metabolism, gene expression, cell growth and differentiation and stress response mechanisms. [122-123] MAPKs are divided into three families: the extracellular-signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs), and p38 MAPKs. A multitude of signals initiate MAPK signaling by the binding and activation RTKs and/or G-protein-coupled receptors (GPCRs). The activated RTKs interact with Ras, or other members of its superfamily, and through Mitogen-Activated Protein Kinase Kinase (MKK) 4 and MKK7 kinases activate JNKs. Downstream targets of the MAPKs include activator protein 1 (AP-1) and p53 and these transcriptional activators mediate expression of target genes containing a TPA DNA response elements (TRE). The MAPK/ERK pathway has been shown to be activated in the later stages of prostate cancer often due to deregulated PI3K/AKT signaling or aberrant growth factor signaling. [124] Although members of the Ras family are rarely mutated in

prostate cancer, Ras is activated by epidermal growth factor (EGF), Insulin-like growth factor 1 (IGF-1), keratinocyte growth factor (KGF), and fibroblast growth factors (FGFs), which are often deregulated in prostate cancer. [125-126]

These inflammatory signaling pathways have been shown to upregulate a family of proteins called the Inhibitors of Apoptosis.

**Inhibitors of Apoptosis (IAP) Protein Family**

Inhibitors of Apoptosis (IAP) deregulation is found in many types of human tumors and are associated with chemotherapy-resistance, disease progression and poor prognosis. [127] Consistent with the idea that different types of cancer cells are addicted to IAPs for cell survival, the inactivation of IAPs under tissue culture conditions results in the induction of apoptosis in most tumor cells. Furthermore, IAP inhibition does not seem to be harmful to normal differentiated cells. [128-130] IAPs were thought to function primarily by inhibiting cysteine proteases called caspases, which are involved in apoptosis. However, recently it has been that IAPs also influence a multitude of other cellular processes important for inflammation, immunity, cell migration and cell survival. [131]

There are 8 different IAP family members in humans; Baculoviral IAP repeat-containing protein 1 (BIRC1), Baculoviral IAP repeat-containing protein 2

(c-IAP1), Baculoviral IAP repeat-containing protein 3 (c-IAP2), X-Linked Inhibitor of Apoptosis (XIAP), survivin (Birc5), Baculoviral IAP Repeat Containing protein 6 (BIRC6), Melanoma inhibitor of apoptosis (ML-IAP) and IAP-like protein 2 (ILP2). All IAP family members contain 1-3 70 amino acid long Baculoviral IAP repeat (BIR) domains with a few members (c-IAP1, c-IAP2, XIAP and ML-IAP) containing a carboxy-terminal RING domain. [132] In addition to these two domains, IAP family members also contain several other domains that are most likely involved in protein-protein interactions. The BIR domain is thought to allow these proteins to bind caspases while the RING domain allows these proteins to act as an E3 ubiquitin ligase. The antiapoptotic function of IAP proteins appears to be highly dependent on these two domains. [133-135]

There are classically considered two apoptosis (programmed cell death) signaling pathways: the extrinsic pathway of apoptosis and the intrinsic pathway of apoptosis. [136] The extrinsic pathway is usually initiated through the binding of a death ligand to its respective receptor on the cell surface causing receptor oligomerization. [137] Some of these death ligands include Fas ligand (FasL), TNFα, APO-3L/TWEAK, and APO-2L/TRAIL. The binding of these factors ultimately leads to the recruitment of specialized adaptor proteins and the activation of the caspase cascades. The extrinsic pathway primarily works

through activated caspase 8 that can directly cleave and activate caspase 3 or indirectly lead to mitochondrial perturbations via the cleavage of BID. BID stimulates mitochondrial outer membrane permeabilzation. Activated caspase 3 is responsible for the proteolytic cleavage of the nuclear enzyme poly (ADP-ribose) polymerase (PARP) among other key proteins. PARP cleavage facilitates cellular disassembly. The intrinsic pathway is usually mediated by the release of pro-apoptotic proteins cytochrome c and second mitochondria-derived activator of caspase (SMAC/DIABLO) from the mitochondrial intermembrane space. [138] Once released these proteins activate caspase 9 which subsequently directly cleaves and activates caspase 3. SMAC/DIABLO is known to bind several IAP family members including XIAP, c-IAP1 and c-IAP2 promoting caspase cleavage and apoptosis. [139]

It has recently been found that XIAP is the only IAP family member that directly binds and inhibits caspases; specifically caspase 3, caspase 7 and caspase 9. [141] The first and second BIR repeats are involved in the inhibition of caspase 3 and caspase 7 while the third repeat is responsible for caspase 9 inhibition via the interaction of XIAP and the N termini of the processed caspase subunits. The other IAP family members are not considered potent caspase inhibitors but have an active E3 ubiquitin ligase activity. This ligase activity allows

IAP family members to regulate cell death modulator's and effector's protein stability contributing to cell survival. [142] Survivin is unique as an IAP family member in the fact it does not contain E3 ligase activity and is not a potent caspase inhibitor. Survivin is also associated with prostate cancer cell survival and proliferation.

**Survivin**

Survivin is encoded by the BIRC5 gene on chromosome 17q25 and is the smallest member of the IAP family. The BIRC5 gene encodes wild type survivin (4 exons, 142 amino acids) and five splice variants; ΔEX3 (deletion of exon 3, 137 amino acids), 2B (additional exon, 165 amino acids), 3B (5 exons, 120 amino acids), 2α (2 exons, 74 amino acids) and 3α (two exons, 78 amino acids). [143-144] All survivin isoforms contain a complete sequence identity with the N-terminus region but vary sequence on the carboxyl end. Isoform localization and expression patterns vary widely compared to wild type survivin and have been shown to associate with prostate cancer activity and progression. [145] Overall, there is a consensus that ΔEx3 is anti-apoptotic and 2B is pro-apoptotic and that these isoforms may perform divergent functions in tumor progression and therapy response though much more research is need for the additional variants.

[146] Wild-type survivin is a dual functioning protein, playing both a role in cell death regulation (anti-apoptotic) and cell division (pro-mitotic) **(Figure 7)**.

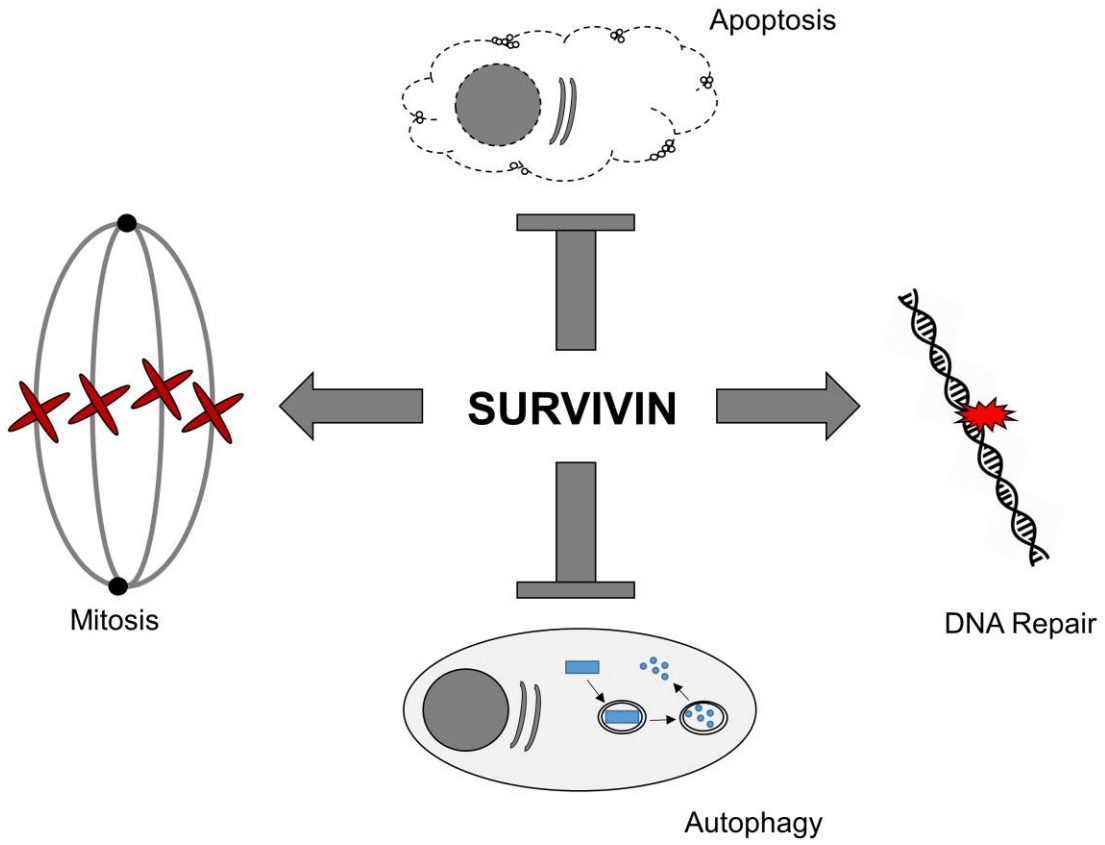



**Figure 7.** Survivin cellular roles in cancer

Survivin has anti-apoptotic and pro-mitotic roles within in cancer cells. Survivin is instrumental in cell division controlling microtubule stability/chromosomal arrangement and has been shown to play a role in DNA repair, specifically non-homologous recombination promoting cell growth and survival. Survivin also, in conjunction with other IAP members, prevents apoptosis and autophagy through caspase cleavage and autophagosome inhibition.

Survivin regulation at the transcriptional level has been found to be mediated by a number of factors associated with inflammatory and growth signaling. PI3K/AKT, MAPK, JNK and JAK/Stat3 have been shown to regulate survival and proliferation of normal and of tumor cells through IAP protein regulation. Transcriptional regulation of survivin has been found to be mediated by Stat3 in some cancers. [147] Stat3 inhibitor, STATTIC, down-regulates survivin protein levels in human breast and ovarian cancer cells. [148] However, it has been reported that the specific protein 1 (Sp1) transcription factor, rather than Stat3, primarily regulates survivin promoter activity in esophageal cancer and gastric cancer; [149] thus suggesting the regulation mode of survivin appears to be highly cell-type specific. Survivin's promoter contains two critical Sp1 sites and some reports suggest that p53-mediated transcriptional repression is another possible manner of regulation. [150] NF-κB has been shown to regulate survivin transcription in liver cancer and squamous cell carcinoma and the androgen receptor has been found to regulate survivin protein levels in prostate cancer. [151-152]

Survivin has a relative short protein half-life at around 30 minutes, regulated by proteasomal degradation. [153] Phosphorylation of Threonine 34 on survivin by Cyclin-dependent kinase 1 (cdc2) is known to stabilize survivin

protein levels by slowing down the clearance of survivin through the proteasomal degradation. [154] In cervical carcinoma cell lines and ovarian cell lines, the combined treatment of Cyclin-dependent kinase inhibitor and paclitaxel down-regulated survivin protein levels through the inhibition of Threonine 34 phosphorylation and enhanced caspase-dependent apoptosis. [155-156] Heat shock protein 90, Hsp90, a chaperone protein, has also been reported to regulate survivin protein levels. [157] The disruption of survivin–Hsp90 interaction results in the proteasomal degradation of survivin, leading to mitotic defects and apoptosis. [158-159]

The bifunctionality of survivin can be sorted by the individual subcellular compartments that survivin occupies and its secondary protein modifications. Survivin is localized in the cytoplasm, the mitochondria, the cytoskeleton and the nucleus with the distribution being cell type dependent. [160-162] Survivin has been shown to inhibit apoptosis induction with other IAP family members by preventing caspase activation in the cytoplasm and in mitochondria. In the nucleus, survivin is a part of a complex called the chromosomal passenger proteins. The chromosomal passenger protein complex is composed of the proteins Aurora-B kinase, inner centromere protein (INCENP), and Borealin. This

complex is necessary for regulating mitosis and chromosomal arrangement. [163]

The subcellular location and specific protein functional properties are regulated by signal-dependent secondary modifications of the protein. Survivin is phosphorylated at threonine 117 by the Aurora-B kinase and this localizes survivin to chromosome arms and inner centromeres from prophase through metaphase. [164] It also localizes survivin to kinetochores in metaphase, distributes survivin to the midzone microtubules in anaphase and at telophase, localizes survivin exclusively to the midbody. [166] Phosphorylation at threonine 34 is critical for survivin protein stability and its anti-apoptotic function. [165]

Survivin is expressed during development, but is variable and sometimes undetectable in adult fully-differentiated tissues. The overexpression of survivin protein has been reported in almost all solid tumor human malignancies including prostate cancer, lung cancer, breast cancer, stomach cancer, esophageal cancer, liver cancer, and ovary cancer. Similar to its protein expression, survivin promoter (transcriptional) activity is largely null in normal cell types, but is increased in malignant cell lines. The specific up-regulation of survivin in cancer cells is further supported by the immunological responses detected against it. Cancer cells seem to utilize the protective character of survivin during cancer progression.

During colon cancer progression, the level of survivin protein was found to increase from adenoma with low-grade dysplasia (2.3%) to high-grade dysplasia (52.4%) to carcinoma in adenoma (63.3%). [166] Similar results has been found in the tumorigenesis of pancreatic ductal adenocarcinoma where no survivin protein level has been found in normal pancreatic ducts but only in low-grade pancreatic intraepithelial neoplasia to high-grade lesions and to the highest in pancreatic ductal adenocarcinoma tissues. [167] Survivin expression has been shown to induce a global transcriptional change in the tumor microenvironment that may promote tumor progression and has been implicated in angiogenesis. Survivin showed a cytoprotection effect, possibly through the PI3K pathways, in endothelial cells thought to be mediated by VEGF. Survivin inhibition led to not only decreased tumor cell growth, but also a reduction in tumor-derived blood vessels. [169]

Targeting survivin with small molecules has proven difficult and none have made it to clinic. YM155, a survivin inhibitor, showed promise but failed in clinical trials due to toxicity. Because of this, finding alternative ways of inhibiting survivin is of great appeal. The signaling pathways involved in survivin mRNA and protein regulation are known targets of a protein called Apurinic/apyrimidinic

endonuclease 1/redox factor-1 (APE1/Ref-1) which has roles in DNA repair and transcription factor activation.

**APE1/Ref-1**

A number of human diseases, including cancer, result from oxidative damage caused by endogenous and exogenous drivers. The base excision repair (BER) pathway is the repair pathway that fixes the majority of DNA base damage in both the nucleus and mitochondria. [169] APE1/Ref-1, a multifunctional enzyme that is part of this BER pathway, has both a DNA repair activity and a role in the activation of many transcription factors. These two activities are encoded by the two termini regions of the APE1/Ref-1 protein: the N-terminal region redox function and the C-terminal DNA repair function **(Figure 8)**. [170-172] The DNA repair activity includes AP endonuclease activity, 3′ phosphodiesterase activity, 3′ phosphatase activity, and 3′–5′ exonuclease activity and is responsible for protein–protein interactions within the BER pathway. [173]

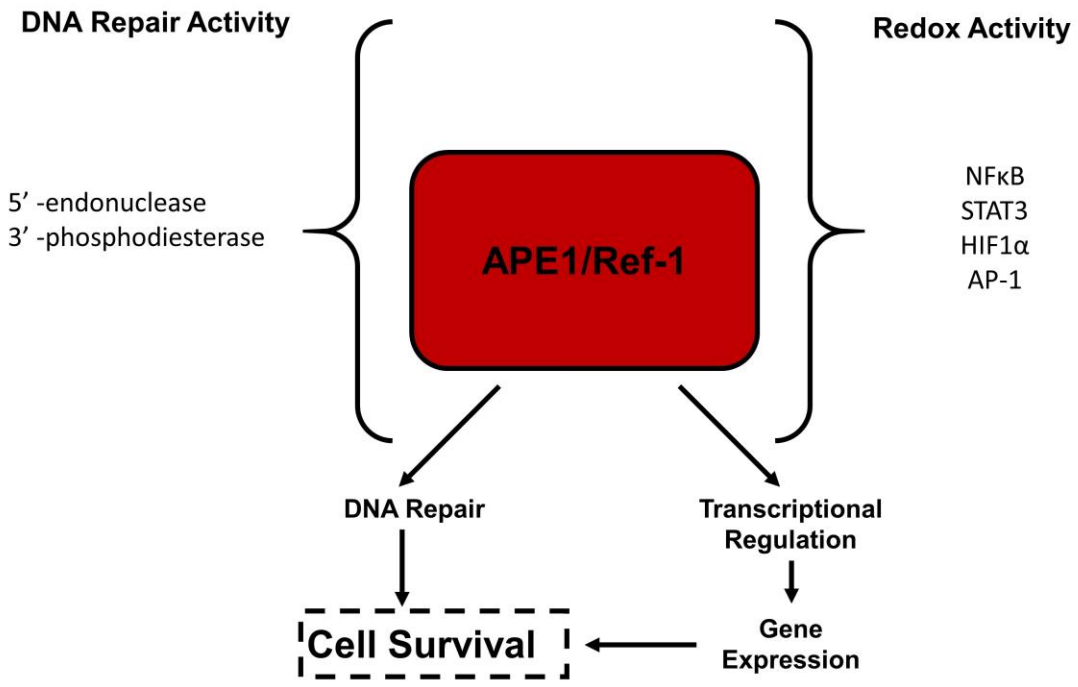



**Figure 8.** APE1/Ref-1 cellular roles in cancer

APE1/Ref-1 is a bifunctional protein with roles in DNA repair and redox regulation. APE1/Ref-1 is part of the base excision repair pathway (BER) and is responsible for repairing Apurinic/apyrimidinic (AP) sites with its 5’ –endonuclease and 3’ –phosphodiesterase activity. It is also responsible for regulating the activity of a number of transcription factors including NFκB, STAT3, Hypoxia-inducible factor 1-alpha (HIF-1α) and AP-1. Together these two functions promote cell survival.

APE1/Ref-1 reductively activates transcription factors including c-Jun, AP-1, NF-κB, p53 and Hypoxia-inducible factor 1-alpha HIF-1α, thereby stimulating their DNA-binding activity. [174] All of these transcription factors are involved in cellular processes such as survival, growth and inflammatory pathways. The mechanism by which APE1/Ref-1 reduces the cysteine residues within these transcription factors involves the exchange of a proton from one or two of the redox active cysteine residues in its N-terminus (Cys65, Cys93 or Cys99). [175] The subsequent oxidized form of APE1/Ref-1 is then reduced and returned to active state by thioredoxin, and oxidized thioredoxin is reduced by thioredoxin reductase. [176]

In thiol-mediated redox reactions, one cysteine residue of the redox factor (in this case APE1/Ref-1) serves as the nucleophilic cysteine, which is responsible for attacking the disulfide bond in another protein and forming a mixed disulfide bond. This mixed disulfide bond is then resolved by the attack of the “resolving” cysteine residue within APE1/Ref-1 and results in the formation of a disulfide bond in the redox factor; the transcription factor is thereby reduced and APE1/Ref-1 is oxidized in this reaction. The reduction of a cysteine within the transcription factor DNA binding domain allows for easier covalent bond formation with DNA. It is hypothesized that the disulfide bond formation involving

Cysteines 65 and 93 occurs while APE1/Ref-1 is in an unfolded state as both cysteine residues are normally buried and not appropriately positioned to form a disulfide bond.

APE1/Ref-1 is associated with the progression of various human diseases including cancer as observed in ovarian, gastro-esophageal, pancreatico-biliary, lung, prostate, cervical, colorectal, breast, hepatocellular, bladder, head and neck, gastric, and glial cancers. [177] A positive correlation has found between the redox activity of APE1/Ref-1 and tumor grade. [178] Various studies using APE1/Ref-1- siRNA in cancer cell lines have demonstrated that APE1/Ref-1 has a role in cancer development and progression. [179] In osteosarcoma, one study has found a significant association between APE1/Ref-1 overexpression and disease risk. Increased chemosensitivity after the use of APE1/Ref-1-siRNA was also shown in this study and many others. [180]

The expression pattern and subcellular localization of APE1/Ref-1 differs among normal and diseased cell types. In non-diseased cells APE1/Ref-1 is primarily expressed in the nucleus rather than in the cytoplasm. [181] In response to oxidative stress, cytoplasmic expression is often observed and associated with cells exhibiting active metabolism. [182] Altered APE1/Ref-1 cellular localization (mixed nuclear or cytoplasmic localization) has been

observed in many cancers. Nuclear APE1/Ref-1 expression has been observed in head and neck cancer, rhabdomyosarcomas, bladder, ovarian, gastro-esophageal and pancreatico-biliary cancers. Cytoplasmic APE1/Ref-1 expression has been found in thyroid, prostate and hepatocellular cancers. A positive correlation between aggressive tumor grade and nuclear APE1/Ref-1 has been found in ovarian, gastro-esophageal and pancreatico-biliary. [183] Nuclear localization and expression was also found to be correlated with cancer differentiation pattern and lymph node status. [184]

APE1/Ref-1 plays a role in apoptosis, angiogenesis and cell growth making it an attractive anti-cancer therapeutic target. It has been demonstrated that APX3330, also known as E3330, and APX3330 blocks only the redox function of APE1/Ref-1 and interferes with its ability to convert transcription factors from an oxidized to a reduced state, which affects their ability to bind to their target consensus sequences. [185] It is thought to do this through covalently modifying core cysteine residues within the redox domain of APE1/Ref-1 preventing its interaction with other proteins. Interestingly, APX3330 has no effect on APE1/Ref-1 DNA-repair endonuclease activity making it specific to only the redox function.

In previous studies it has been shown that only two of the seven cysteine residues in the redox domain are solvent accessible suggesting APE/Ref-1 may become unfolded in the presence of APX3330. [186] Cysteine 65, which is typically buried and critical for the redox function of APE1/Ref-1, becomes exposed and reacts with N-Ethylmaleimide (NEM) in the presence of APX3330 confirming the unfolding hypothesis. Furthermore, based on studies using liquid chromatography and tandem mass spectrometry, APX3330-mediated increase in disulfide bond formation of the critical APE1/Ref-1 cysteine residues Cysteine 65 and Cysteine 93 has been suggested as the cause of the redox activity inhibition **(Figure 9)**.

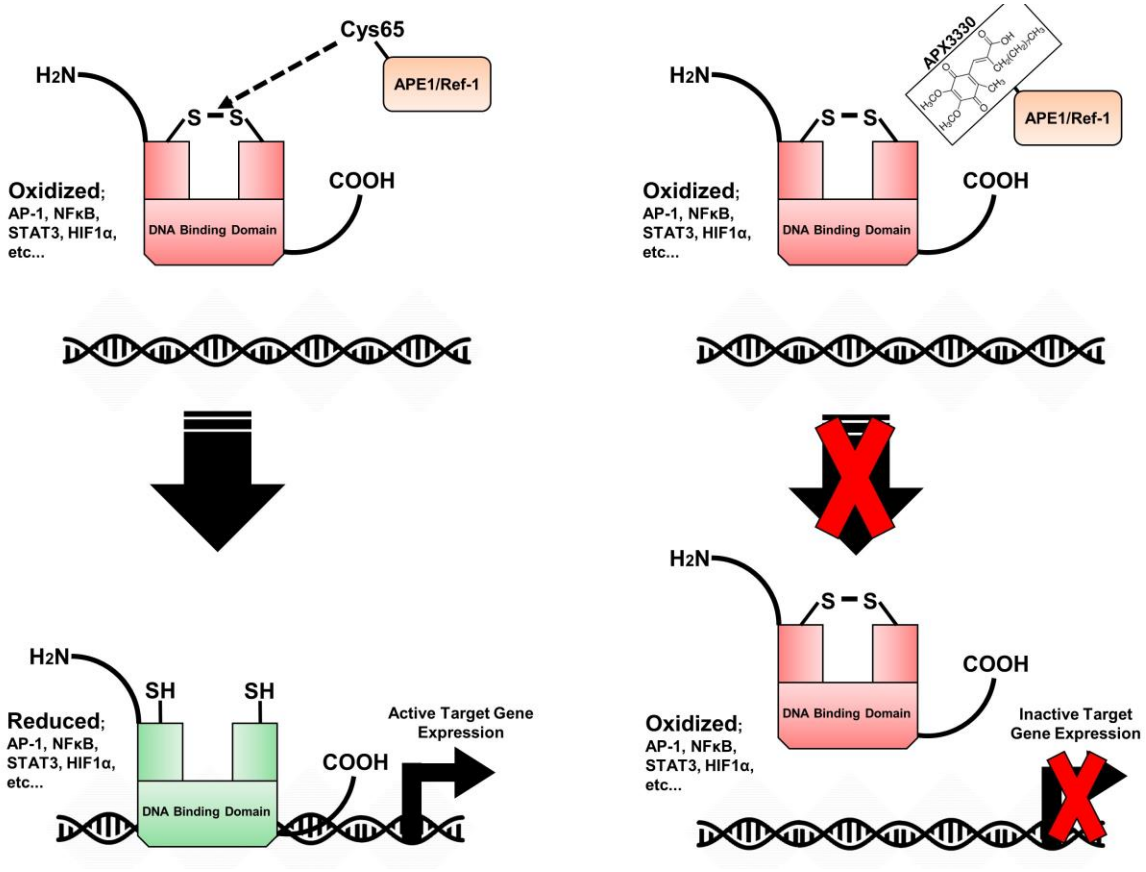



**Figure 9.** Mechanism of APE1/Ref-1 redox inhibition through APX3330

Cysteine 65 of APE1/Ref-1 is necessary for the reduction of transcription factors. APE1/Ref-1 interacts with downstream transcription factors such as NF-κB, HIF-1α and AP-1 converting them from oxidized to reduced forms, allowing them to bind to their target promoter sequences and switch on the transcription of genes. APX3330 binds and prevents Cysteine 65 from creating a disulfide bond with another thiol group in the accepting transcription factor thereby blocking the APE1/Ref-1's ability to convert the oxidized transcription factor to a reduced transcription factor keeping the target gene transcription turned off.

## Summary

Chronic inflammation is a common factor in a number of prostatic diseases including prostate cancer and BPH. However, it is not known how specialized cells in the prostatic epithelium are programmed to avoid cell death in noxious inflammatory microenvironments and survive in order to repopulate the tissue to the point of disease. It is also not known if APE1/Ref-1 is a key regulator of prostate cell growth and cancer progression or upstream of pathways that regulate survival proteins. In this dissertation I investigated these questions.

# Chapter 2 Material and Methods

## Methods

### Mouse prostate *E. coli* inflammation model

*E.coli* strain 1677 was cultured in 25 ml Lysogeny Broth (LB) medium at 37 ℃ overnight. The infected medium was then collected and centrifuged at 1,500 G's for 10 minutes. The bacteria pellet was then washed with sterile 1x Phosphate Buffered Saline (PBS) and was re-suspended in 10 ml sterile PBS. The absorbance, OD600, was measured to determine the concentration of *E.coli* and the *E.coli* suspension is lastly diluted to 1 X $10^6$/ml (OD600=0.118) in sterile PBS.

C57BL/6J wild type mice at 8-12 weeks were deprived from water for 2 hours and then were anesthetized using isoflurane. *E.coli* strain 1677 solution (1 X $10^6$/ml in sterile PBS) is instilled through a sterile catheter into the prostate (100 µl/mice) at day 0, with PBS being used as the vehicle. Mice were sacrificed after instillation based on the experiment design. 5-bromo-2'-deoxyuridine (BrdU) is intraperitoneal (IP) injected into animal (10 mM, 200 µl/mouse) 2 hours before sacrifice to label all proliferating cells. Prostates were collected within 30 minutes after animal sacrifice for following experiments.

### Tissue Microdissection

Mouse prostate was collected within 30 minutes after the animal is sacrificed. The fat tissue around prostate was removed and the prostate was dissected to separate different prostatic lobes in cold sterile PBS with antibiotics. All lobes (including Anterior Prostate (AP), Dorsolateral Prostate (DLP), and Ventral Prostate (VP)) were collected separately for following experiments.

### Tissue fixation and embedding

The DLP tissue for immunofluorescence was fixed in 10% formalin in PBS for overnight and then the tissue was washed with distilled (DI) water twice to remove excessive formalin. Then the tissue was dehydrated by incubating it in 50% ethanol, 70% ethanol, 2 X 95% ethanol, and 2 X 100% ethanol, at 30 minutes/step. The tissue was then treated with 100% xylene twice at 30 minutes/step followed by two 50% xylene + 50% paraffin treatments also at 30 minutes/step. The 50% xylene + 50% paraffin treatment was replaced with 100% paraffin and the tissue was incubated at 59°C in oven for 1 hour. Then the paraffin was refreshed and the tissue was incubating at 59°C overnight. The tissue was embedded in paraffin in the following day and was serially sectioned into 5µm slice for immunofluorescence (IF) or hematoxylin and eosin stain (H&E) staining.

**Immunofluorescence staining**

Paraffin embedded slides were heated in oven at 59°C for 2 hours to melt and remove paraffin. The slides were washed with xylene 3 times at 5 minutes each followed by 2 times methanol wash at 5 minutes each. The slides were then washed with distilled water for 5 minutes. Tissues were then subjected to heat-induced antigen retrieval in 10 mM citrate buffer (citrate buffer stock solution of monohydrate-free acid citric acid, sodium citrate dehydrate, pH 6.0) for 10 minutes followed by a 10 minute cool down period. The tissue was circled with hydrophobic pen and washed with 1x PBS-Tween (PBST) for 3 times at 5 minutes/step. Sections were blocked at room temperature with a bovine serum albumin (BSA)-serum mixture for 2 hours and incubated with primary antibody overnight at 4°C. The sections were then washed the following day with PBST for 15 minutes to remove excessive primary antibody. The tissue was then treated with a fluorophore labelled secondary antibody at room temperature (RT) for 1 hour. For nuclear staining, the tissue was treated with Hoechst 33342 in PBST at RT for 10 minutes followed by 2 PBS washes and 3 DI water washes, 5 minutes each. An aqua mounting medium was used on the slides and subsequently were kept in 4°C for short term and in -20°C in for long term storage.

**ELISA (Enzyme-linked immunosorbent assay)**

Prostate tissues were harvested and homogenized from 8 week-old C57BL/6J WT mice 0–14 days after *E. coli* strain 1677 instillation. For the release experiments, tissues were equilibrated for 1 hour in aerated Krebs physiological salt solution, with buffer changes of 15 minutes and then 30 minutes. Krebs was collected after the experiment and frozen in -80°C as the “released fraction.” Additional tissues were harvested for the total tissue content, and these tissues were snap frozen in liquid nitrogen, placed in sterile PBS, and homogenized using sonification. Tissue slurries were centrifuged at 14,000 G for 10 minutes and the supernatant was collected as the total tissue content fraction. All collections were analyzed by ELISA. Absorbance readings for each concentration were normalized as a ratio to control non-inflamed prostates (PBS vehicle treated).

**TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling)**

Prostate lobes (DLP) were fixed in 10% formalin overnight, processed and embedded in paraffin and serially sectioned at 5 μm with a microtome and rehydrated as previously mentioned. Tissue sections were incubated with Proteinase K working solution (10 μg/ml in 10 nM Tris/HCl, pH 7.4–8) at RT for 15 minutes and then rinsed twice with PBS. 50 microliters of TUNEL reaction

mixture were added to each section and slides were placed in a humidified atmosphere for 60 minutes at 37°C in the dark. The slides were then rinsed three times with PBS and stained with Hoechst 33258 as previously described. Tissues were then washed and covered with an aqueous medium and glass coverslips. Samples were directly analyzed under a fluorescence microscope using an excitation wavelength in the range of 450–500 nm and detection in the range of 515–565 nm (green).

**Immunoblotting (Protein quantification)**

After treatment, prostatic cells were washed with cold PBS and were lysed by adding 250-500 µl/well lysis buffer. The plate was kept at RT for 10-15 minutes and the cells were then broken apart with a cell scraper. The cell lysate was placed into an Eppendorf tube and kept on ice for 30 minutes. The cell lysate was then placed in -80°C for storage or used for bicinchoninic acid assay (BCA assay).

Serial dilutions of BSA standard solution (concentration: 0.125 mg/ml, 0.25 mg/ml, 0.5 mg/ml, 1 mg/ml and 2 mg/ml) were prepared using BSA standard and lysis buffer for BCA assay. In 96 wells plate, 35 µl of standard solution or protein samples was added into each well, 2 replicates per sample. The BCA reagent A and B were mixed in a 1:50 ratio to make BCA reaction buffer. 70 µl BCA reaction

buffer was added into each well (combined with samples) in the 96 wells plate. The plate was incubated at 37°C for 30 minutes and absorbance at 540 nM was read on plate reader. The protein concentration was calculated using standard curve in excel.

The protein samples were prepared using 2X laemmli loading buffer and lysis buffer. The protein concentration was adjusted to make the loading amount of protein the same between the samples. The samples were loaded on a precast 4%-20% gradient gel. The gel was ran under 115V for 1 hour. Bio-rad dual color precision ladder was used as protein marker on at least one side of the gel.

The gel was washed with DI water for 5 minutes. The gel and the thick blotting paper was soaked in transfer buffer for 15 minutes to acclimate. The PVDF membrane was activated in methanol for 1-2 minutes and was placed in transfer buffer for 10-15 minutes. The gel was transferred at 10V, 400mA for 20-30 minutes. The PVDF membrane was collected and blocked in milk/BSA blocking buffer for 2 hours at RT or overnight at -4°C.

The primary antibody was diluted in milk/BSA blocking buffer or just BSA blocking buffer depending on the antibodies instructions. The membrane was incubated with primary antibody at -4°C overnight. The membrane the following

day was washed with PBST for 6 times 5 minutes each to wash off unbound primary antibody. The secondary antibodies were diluted in the standard milk/BSA blocking buffer and the membrane was incubated with the secondary antibody at RT for 2 hours. The membrane was then washed with PBST for 4 times at 5 minutes each followed by 2 PBS washes at 10 minutes each. The membrane was then developed using Thermo fisher Femto or Pico developing buffer and was exposed on X-ray film in dark room. The film was scanned onto a joint lab computer and analyzed using Adobe Photoshop software for quantification.

After developing, the membrane was washed with PBST for 3 times to get rid of excess developing solution. The membrane was stripped using stripping buffer for 5-15 minutes and then washed with PBS 3 times at 5 minutes each. Then the membrane was blocked in blocking buffer again and placed in -4°C for storage.

**Cell culture**

Prostate cancer cell lines PC-3, LNCaP, C4-2 and noncancerous prostatic epithelial cell line E7 were cultured in complete growth medium at 37℃, 5% $CO_2$ condition. 0.05% trypsin was used to digest the cells and was neutralized with

new medium. Cells were passaged between 3-5 days depending on seeding and confluency.

**Cell proliferation assay**

Prostatic cell lines were seeded at concentrations previously determined in a 96-wells plate. Cells were treated with APE1/Ref-1 redox inhibitors APX3330 and APX2009 for various time points and the treatment medium was refreshed every day with new drug. The cells were then fixed with methanol for 5 minutes in RT and were dried overnight for methylene blue assay.

**Methylene blue assay**

Prostate cells were seeded 1,000-5000 per well (cell line/experiment-dependent) and treated with either APX3330, APX2009 or RN7-58 for 5 days. Media was then removed and cells were fixed with methanol for 10 minutes and stained with 100 μL of 0.05% of methylene blue (LC16920-1 diluted in 1X PBS) for 1 hour. The cells were then washed 3X with water and allowed to air dry overnight. 100 μL's of 0.5N HCl was added to each well to dissolve the methylene blue stain and absorbance (@630nm) was measured via spectrophotometry (**Figure 10**). The percent viabilities, normalized to DMSO

control, were graphed and $IC_{50}$ concentrations determined. DMSO control was not significantly different from untreated cells.

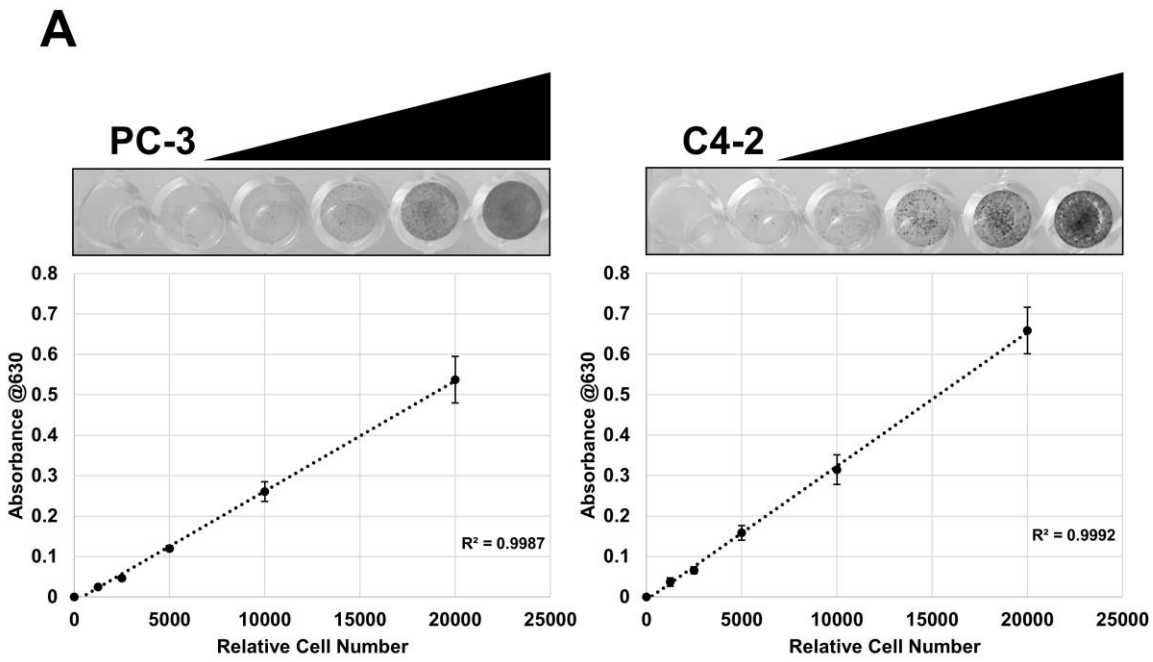



**Figure 10.** Methylene blue assay

Standard curve showing absorbance with relative cell number. PC-3 and C4-2 cells were seeded in increasing amounts in a 96 well plate, methanol fixed and stained with methylene blue. The stain was then dissolved with hydrochloric acid (HCl) and absorbance measured via spectrometry.

**RT-PCR (Reverse transcription-polymerase chain reaction)**

RNA isolation was performed using RNeasy Mini Kit. 10 nanograms of total RNA was reverse transcribed using Superscript III One-Step RT-PCR System. Real-time PCR was performed using the TaqMan Gene Expression Assay BIRC5 and HPRT1 and Applied Biosystems 7500 Fast Real-Time PCR System.

**CoIP (Co-Immunoprecipitation)**

Samples were co-immunoprecipitated using the Pierce Co-IP kit. Additionally, the cells were washed twice with 1X PBS and the proteins were cross-linked using DTBP (5 mm, for 30 min on ice). DTBP was quenched by washing with cold inactivation buffer (100 mm Tris-HCl, pH 8, 150 mm NaCl) and 1XPBS. Cells were then lysed and the lysates added to columns and after extensive washing, the bound proteins were eluted and prepared for immunoblot analysis.

**Luciferase assay**

C4-2 cells were co-transfected with constructs containing luciferase driven by NFκB and a Renilla luciferase control reporter vector pRL-TK at a 20:1 ratio by using Effectene Transfection Reagent. After 16 hours, cells were treated with

increasing concentrations of APX2009 in serum free media for 24 hours. Firefly and Renilla luciferase activities were assessed by using the Dual Luciferase Reporter Assay System. Renilla luciferase activity was used for normalization and all transfection experiments were performed in triplicate and repeated 3 times in independent experiments.

**Propidium Iodide (PI) cell cycle analysis**

PC-3 and C4-2 cells were treated with APX2009 (9 and 14 μM, respectively) for 48 hours. 500,000 cells were then aliquoted for cell cycle analysis and 0.1 mg/ml Propidium Iodide and 0.6% NP-40 PBS stain wash was added to the tubes. The cells were then centrifuged at 1900 rpms for 10 minutes with the brake on low and then decanted and blotted. RNAase and stain wash were added and cells incubated on ice for 30 minutes. Propidium Iodide intensity was measured via flow cytometry.

**siRNA transfection**

PC-3 and C4-2 cells were seeded into a 6 wells plate at a concentration of $2 \times 10^5$ cells/well. 50 nM control siRNA or 50 nM APE1/Ref-1 siRNA were transfected using HiPerfect transfection reagent following the instruction manual.

Cells were cultured overnight and then were subjected to various experiments. Transfection efficiency was confirmed via immunoblot.

### *In vivo* subcutaneous tumor

$2 \times 10^6$ C4-2 cells were subcutaneously implanted in the hind flank of male athymic nude mice using a 100µl volume of 50:50 solution of Matrigel: RPMI medium. When tumor volumes reached 150 -200 $mm^3$, the animals were treated with 25 mg/kg IP APX2009 or vehicle every 12 hours for 5 days. 5-bromo-2'-deoxyuridine (BrdU) was injected into the animals 2 hours prior to sacrifice and tumor tissues were analyzed for survivin levels (IF and immunoblotting) and BrdU incorporation (IF).

## Materials

### Animal models

C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME) and the athymic nude mice were purchased from Charles River's Laboratories (Wilmington, MA); Matrigel was form BD Biosciences (San Jose, CA); BrdU was purchased from Fisher Scientific (Hampton, NH).

**ELISA (Enzyme-linked immunosorbent assay)**

The ELISA kits for the death ligands, IL-1's and lL-6 were purchased from BioSource (Camarillo, CA). ELISA kits for sonic hedgehog (Shh), IGF's, and transforming growth factor betas (TGFβ's) were purchased from R&D Systems (Minneapolis, MN).

**TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling)**

In situ Cell Death Detection Kit, Fluorescein was purchased from Roche (Branford, CT). Proteinase K was purchased from Sigma Aldrich (St. Louis, MO).

**Cell culture**

PC-3, C4-2, LNCaP and E7 cells were maintained in Travis Jerde's lab. Cell culture medium, antibiotics, HEPES buffer, trypsin, and additives were from HyClone (Logan, Utah); flasks, culture dishes, and plates were from Corning (Tewksbury, MA) or BD Biosciences (San Jose, CA); Fetal Bovine serum was from Atlanta Biologics (Flowery Branch, GA).

**Immunoblotting**

4-20% gradient SDS-page precast gels and western apparatus obtained from Bio-rad (Hercules, CA). Peirce BCA buffer, chemiluminescent substrates

Femto and Pico, phosphatase and protease inhibitor, and Bovine Serum Albumin (BSA) were obtained from Fisher Scientific (Hampton, NH).

**Immunofluorescence**

The Normal Donkey Serum for blocking, the Hoechst 33342 for nuclear staining, and mounting medium were purchased from Fisher Scientific (Hampton, NH).

Anti-survivin, Anti-Bcl-2, Anti-Bcl-XL, Anti-Mcl-1, Anti-GAPDH, Anti-Cyclin B1, Anti-Cdc2, Anti-Cleaved Caspase 3, Anti-Total Caspase 3, Anti-APE1 antibodies were purchased from Cell Signaling Technology (Danvers, MA), Anti-BrdU and Anti-p65 antibodies were from Novus Biologicals (Littleton, CO); Anti-β-actin antibody was from Sigma Aldrich (St. Louis, MO); all the fluorescent labelled secondary antibodies were from Life Technologies (Carlsbad, CA); all the HRP-conjugated secondary antibodies were from Fisher Scientific (Hampton, NH).

**RT-PCR (Reverse transcription-polymerase chain reaction)**

RNeasy Mini Kit was purchased from Qiagen (Germantown, MD). Superscript III One-Step RT-PCR System was purchased from Thermo Fisher Scientific (Waltham, MA). The TaqMan Gene Expression Assays (BIRC5

(Hs04194392_s1) and HPRT1 (Hs02800695_m1) were purchase from Thermo Fisher Scientific (Waltham, MA).

**CoIP (Co-Immunoprecipitation)**

Pierce Co-IP kit and DTBP were purchased from Thermo Fisher Scientific (Waltham, MA).

**Luciferase assay**

pLuc-MCS with NFκB responsive promoter were purchased from PathDetect cis-Reporting Systems Stratagene (La Jolla, Ca) and a Renilla luciferase control reporter vector pRL-TK were purchased from Promega Corporation (Madison, WI). Effectene Transfection Reagent was purchased Qiagen (Germantown, MD). Firefly and Renilla luciferase activities were assessed by using the Dual Luciferase Reporter Assay System which was purchased from Promega Corporation (Madison, WI).

**Propidium Iodide (PI) cell cycle analysis**

Propidium Iodide (PI) was purchased from Sigma Aldrich (St. Louis, MO). NP-40 was purchased from Thermo Fisher Scientific (Waltham, MA).

### siRNA vectors and transfection reagent

Survivin #1 and #2 siRNA were purchased from Cell Signaling Technology (Danvers, MA); Allstar Negative Scramble siRNA was purchased from Qiagen (Germantown, MD); APE1 #1 siRNA was given to us form Dr. Melissa Fishel and Prevalidated APE1/Ref-1 siRNA (siAPE1 #2) was purchased from Life Technologies (Carlsbad, CA) (#s1446). HiPerFect Transfection Reagent was purchased from Qiagen (Germantown, MD).

### Facilities

Flow-Cytometry Sorter: BD Facs Aria; Fluorescent Microscope: Leica DMI6000B. RT-PCR: Applied Biosystems 7500 Fast Real-Time PCR System.

### Other materials

All the chemical reagents and lab supplies were purchase from Fisher Scientific (Hampton, NH) or Sigma Aldrich (St. Louis, MO).

## Statistical analysis

All statistical works were done using Microsoft Excel. The data were analyzed using student t-test, One-way ANOVA or Two-way ANOVA as appropriate. For each independent test, the result was calculated by the average of 3-6 replicates.

# Chapter 3: Coordinated Induction of Cell Survival Signaling in the Inflamed Microenvironment of the Prostate

## Introduction

Continual and recalcitrant inflammation is an extremely common condition in the human prostate and has been found to be associated with a number of prostatic diseases including prostate cancer and benign prostatic hyperplasia (BPH) [187-190]. Prostatic inflammation is characterized by the presence of inflammatory cells in the stroma, epithelium, and lumen of prostatic glands where the infiltrate is primarily lymphocytic with secondary accompanying macrophages juxtaposed to loci of reactive hyperplasia [187]. The origins of inflammation in the prostate remain a subject of debate and are most likely multi-factorial [191]. A role for a bacterial component in prostatic inflammation is controversial but is certainly plausible [192-195], and colonization by non-culturable organisms has been suggested by PCR assays of bacterial 16S ribosomal RNA in prostate biopsies as this has been associated with histological evidence of inflammation [196]. Numerous nonbacterial causes of inflammation have been investigated including viruses, environmental components, systemic hormones, and urinary reflux. Whatever the cause, inflammation in the prostate is of considerable

importance to urological research due to the prevalence and impacts of BPH and prostate cancer.

While much has been described regarding prostate disease resulting from oxygen and nitrogen radicals during inflammation, proliferative mechanisms associated with repair and regeneration are less understood. Repair and regrowth are co-regulated processes characteristic of many cellular responses to trauma and in order for tissue recovery to proceed, inflammation must orchestrate precise series of events directing damaged cells to die, inducing proliferation of protected tissue progenitors to repopulate damaged tissue, and promoting differentiation of those expanded cells into proper cell subtypes [197]. Errors in these processes allow for expansion of damaged cells in an environment saturated in growth promoting factors leading to hyperplasia and desmoplasia [198]. While there is an extensive literature in prostate cancer cells regarding survival and cell death escape, mechanisms of how inflammation directs benign cells to avoid death and proliferate are poorly understood.

In addition to the classically understood mechanisms by which cells survive noxious tissue conditions—inhibition of pro-apoptotic proteins, induction of pro-survival proteins, and the subsequent inactivation of caspases—autophagy represents a process that can be associated with both the promotion

of apoptosis and the promotion of cell survival and proliferation. Autophagy consists of autophagosome formation and effective macromolecule degradation [199]. The mechanisms of autophagy are diverse and depend on the origin of the stimulus. Autophagy is implicated in a number of diseases including cancer [200]. The removal of damaged organelles or proteins can be advantageous for a cell and may act as an escape mechanism from cell death [201].

It is not known how specialized cells in the prostatic epithelium are programmed to avoid cell death mechanisms in the noxious condition of inflammation and survive in order to repopulate the tissue as part of the innate repair and recovery process. In this study, we characterize the immediate induction of cell death mechanisms in our mouse model of prostatic inflammation that has been shown to transition from acute to chronic phases of inflammation similar to human prostates. Cell death signaling induction is followed by a coordinated induction of survival mechanisms that begin in basal epithelial cells and expand to include all layers of the epithelium. We also found that autophagy is induced during the recovery phases of inflammation. Finally, we found that the most consistently induced of the survival proteins, survivin, is associated with inflammation in human prostate specimens, and that survivin expression is more tightly correlated with inflammation than with disease state of the prostate.

**Inflammation Causes Tissue Damage and Hyperplasia in a Model of Prostatic Inflammation**

Mice instilled with uropathogenic E. coli 1677 exhibited widespread inflammation with varying degrees of hyperplasia and dysplasia consistent with previous papers on this model, and as has been previously published, the dorsolateral lobe of the prostate produced the most consistent and dynamic response to inflammation [192, 202]. Three days after instillation, WT animal epithelium develops distinct multilayers and display extensive inflammatory infiltrate **(Figure 11)**. Previous reports from this model indicate that the inflammatory infiltrate in this model is primarily neutrophilic 1–2 days post induction, and lymphocytic 3–5 days after inflammation with accompanying macrophages [192, 202]. This phase mimics what is observed in human prostates with chronic inflammation [203-204]. Intense loci of inflammation are juxtaposed to epithelial hyperplasia and the prostatic glands juxtaposed to intense inflammation show tremendously increased epithelial cell proliferation and hyperplasia. Mice exhibiting 1–3 days of inflammation exhibit numerous damaged and apoptotic cells as evidenced by pyknotic nuclei and retracted cytoplasm **(Figure 11)**. These data demonstrate that our model of prostatic

inflammation is associated with the damaging effects of inflammation and cellular damage.

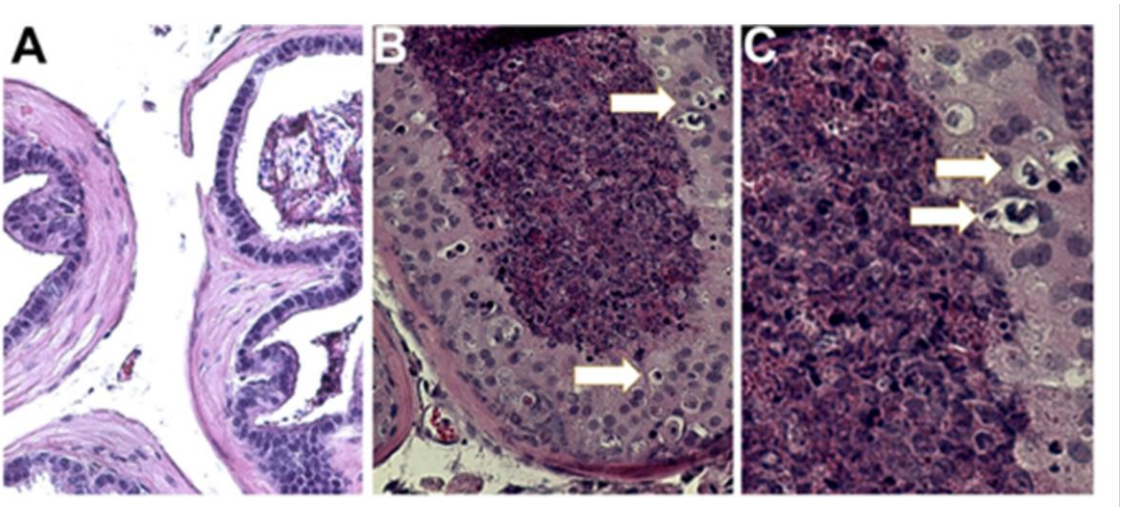



**Figure 11.** Hematoxylin and Eosin staining of 3 day inflamed dorsolateral prostates in the mouse prostatic inflammation model

**A:** (200×) Non-inflamed control prostate shows pseudostratified epithelium and very few apoptotic or autophagic cells. **B:** 200× Inflamed prostatic duct shows layering of epithelium characteristic of reactive hyperplasia during inflammation, but also shows numerous damaged and apoptotic cells as evidenced by pyknotic nuclei and retracted cytoplasm (arrows). **C:** 400× Image of damaged epithelial cells in hyperplastic epithelium in inflamed prostate.

**Inflammation Causes Rapid Apoptotic Response**

To characterize inflammation-induced apoptosis, we assessed control and inflamed mouse prostates for expression of executioner caspase 3, 7, and 6 cleavage via immunohistochemistry **(Figure 12A and 12B)**. We found an increase in cells exhibiting activation (cleavage) of all three executioner caspases with caspase 3 being the most dramatic, peaking at day 2 with 1.3% of cells expressing cleaved caspase 3 (n = 6 mice). Cleaved caspase 3 positive cells were primarily found in select basal and luminal epithelial cells of prostatic glands and absent from the fibromuscular stroma. In addition, we assessed these tissues for later stage apoptosis by staining for nick-end labeling of fragmented DNA. To determine this, a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) was performed to measure percent of epithelial cells in later stage apoptosis as described in histologic sections of prostate tissue and positive cells were detected by fluorescent microscopy **(Figure 12C)**. Few fluorescein-positive cells were found in days 0 and 1 of infection but a significant increase in apoptotic cells was found in day 2 with subsequent decrease by day 4. The fluorescein positive cells, like the caspase 3 positive cells, were primarily found in the basal and luminal compartments of the prostatic glands.

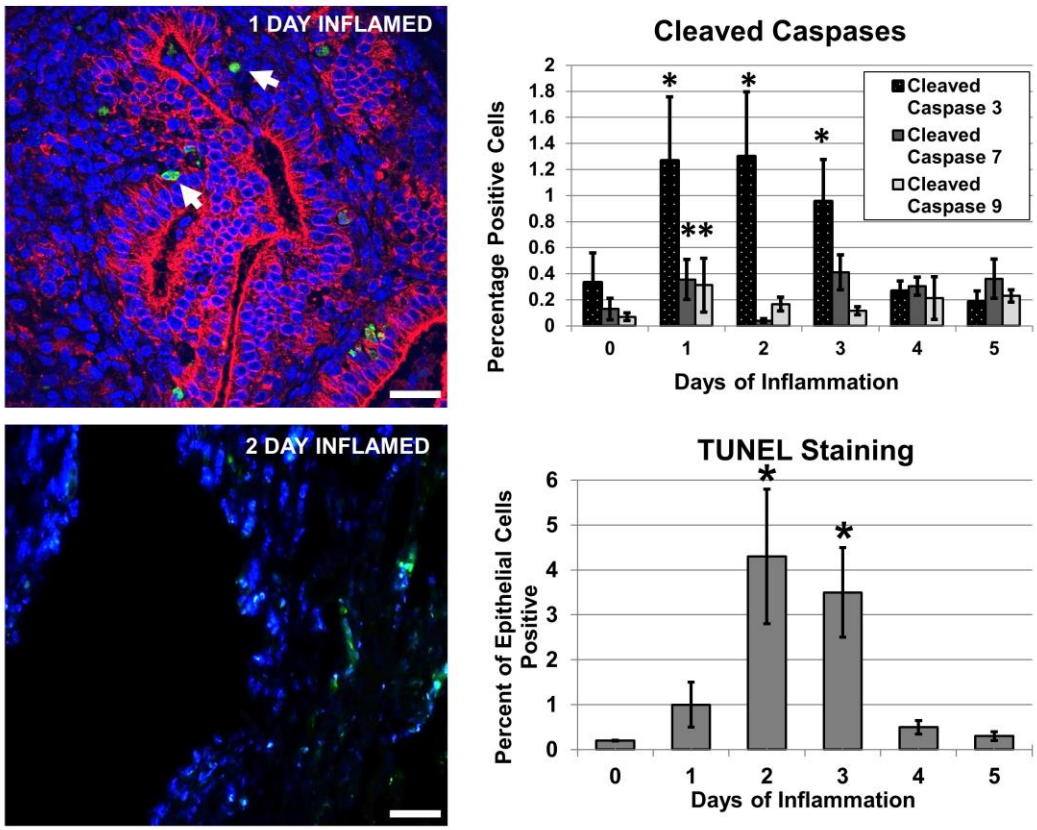


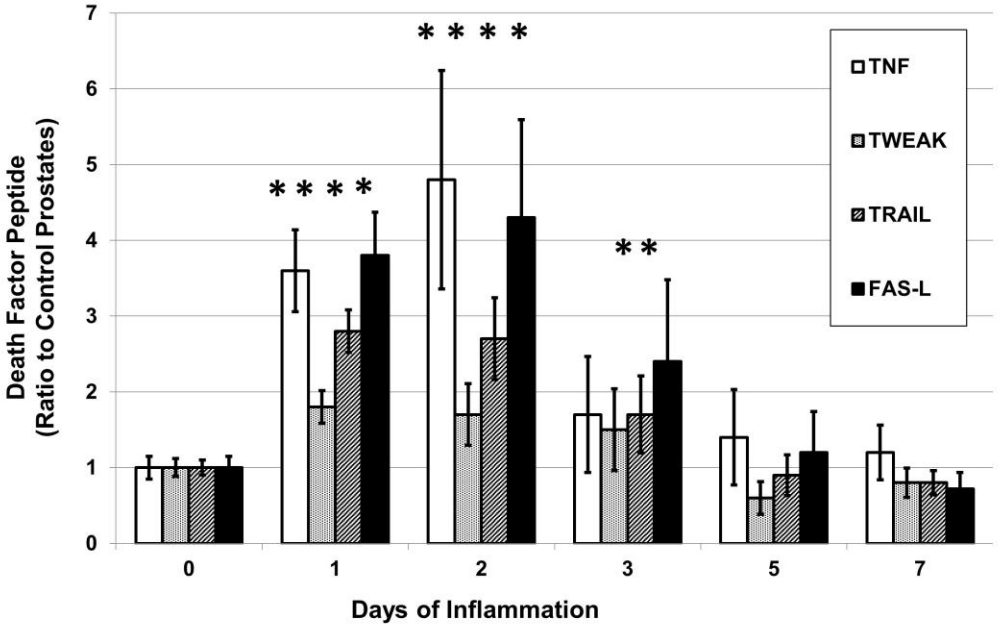

**Figure 12.** Inflammation of the mouse prostate results in induced apoptotic signaling

**A:** Fluorescent image (200×) of cleaved caspase 3 (green) in the epithelium (PanCK-red) of mouse prostates inflamed 3 days. **B:** Calculated data of epithelial cells for cleaved caspases 3, 7, and 6 from 6 mouse dorsal-lateral prostate lobes inflamed to the time points shown, expressed as percentage of cleaved caspase-positive cells within the epithelial compartment. **C:** Fluorescent image (200×) of TUNEL-positive cells. **D:** Calculated data of epithelial cells for TUNEL from 6 mouse dorsal-lateral prostate lobes inflamed to the time points shown, expressed as percentage of TUNEL-positive cells within the epithelial compartment. All data are expressed as mean ± s.e.m. *$P < 0.05$ versus PBS-instilled prostate; comparisons using analysis of variance (ANOVA), $n = 6$.

**Inflammation Induces Death and Survival Factor Production and Release**

Inflammation induces coordinated and temporal expression and release of known cell death factors, subsequently followed by an induction of a panel of known cell survival-inducing factors. As measured by ELISA, bona fide cell death-inducing mediators TNFα, TWEAK, TRAIL, and FAS ligand are all induced rapidly and transiently upon induction of acute inflammation in the dorsolateral prostate, maximizing at 1–2 days after induction **(Figure 13A)**. Subsequent to induction of death ligands, the production of known cell survival factors Shh, IGF-1, IL-1α, IL-6, TGFβ1, and TGFβ2 are substantially induced in the second day of inflammation, and maximizes at day 3 (**(Figure 13B)**, n = 6 mice)). IL-1α, IL-6, and TGFβ1 remained significantly induced for 5 days after inflammation induction. These data demonstrate that death factors coincide with the acute and neutrophilic phase of inflammation, and are coordinately followed by the induction of survival factors corresponding to the lymphocytic phase of inflammation.

**A**


Death Factor Peptide
(Ratio to Control Prostates)
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6
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TNF
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3
5
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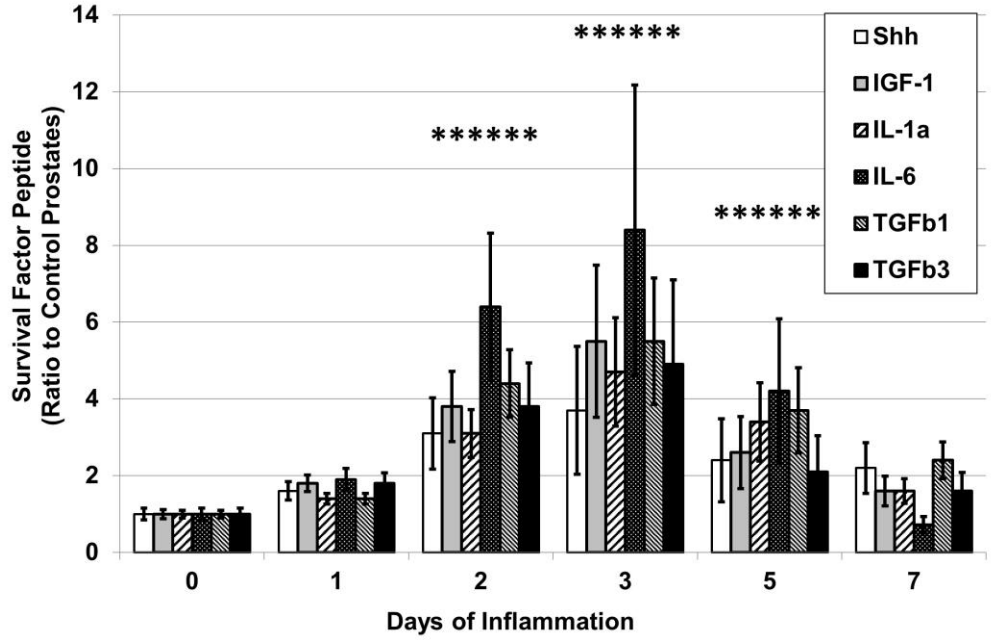
Days of Inflammation

**B**


Survival Factor Peptide
(Ratio to Control Prostates)
14
12
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Shh
IGF-1
IL-1a
IL-6
TGFb1
TGFb3
0
1
2
3
5
7
Days of Inflammation

**Figure 13.** Inflammation induces the expression and release of known cell death factors, followed by an induction of a panel of known cell survival-inducing factors

**A:** Total tissue content of TNFα, TWEAK, TRAIL, and FAS ligand are all induced rapidly upon induction of inflammation in the dorsolateral prostate, maximizing at 1–2 days after induction. **B:** Subsequently, the induction of known cell survival factors Shh, IGF-1, IL-1α, IL-6, TGFβ1, and TGFβ2 begins in the second day of inflammation, and maximizes at day 3. IL-1α, IL-6, and TGFβ1 remained induced for 5 days after inflammation induction. All peptides were assessed by ELISA of whole dorsolateral prostate lobes and calculated as picogram of peptide per gram of tissue. Data were then normalized as a ratio to control non-inflamed prostates at the given time point of induction, for presentation. All data are expressed as mean ± s.e.m. *$P < 0.05$ versus PBS-instilled prostate; comparisons using analysis of variance (ANOVA), $n = 6$. Asterisk-labeled time points are those that show significant inducibility of all death or survival factors.

**Inflammation Induces Cell Survival Signaling Pathways in Response to Apoptotic Signals**

We assessed the induction of four previously identified survival signaling molecules, previously known to regulate cell survival in prostate cells: survivin, Bcl-2, Bcl-XL, and Mcl-1 **(Figure 14)**. Our data indicate that inflammation induces the expression of survivin, Bcl-2 and Mcl-1, but had no effect on Bcl-XL expression (Not shown). The most prominently induced survival protein by inflammation was survivin, exhibiting an eight-fold induction at days 3 and 4 after inflammation relative to uninflamed control prostates (**(Figure 14A and 14B)**; n = 6 mice)). Since survivin was the most dynamic and consistently induced survival factor in inflamed prostates, we sought to further characterize its induction. Survivin is rarely expressed in control prostates, being expressed in 1% of epithelial cells, and primarily in select basal cells **(Figure 14C-top)**. Immunofluorescence of survivin expression increased linearly by day throughout the first 5 days of inflammation **(Figure 14D)**, and by 5 days 50% of the epithelial cells of inflamed prostates were positive for survivin **(Figure 14D)**, image depicted in Figure 4C-bottom. The percentage of survivin-positive cells remained increased for 7 days of infection. These data indicate that survival proteins are induced during prostatic inflammation, corresponding to induced survival factor

signals and the lymphocytic phase of inflammation, and temporally following the neutrophilic death factor/apoptotic phase.

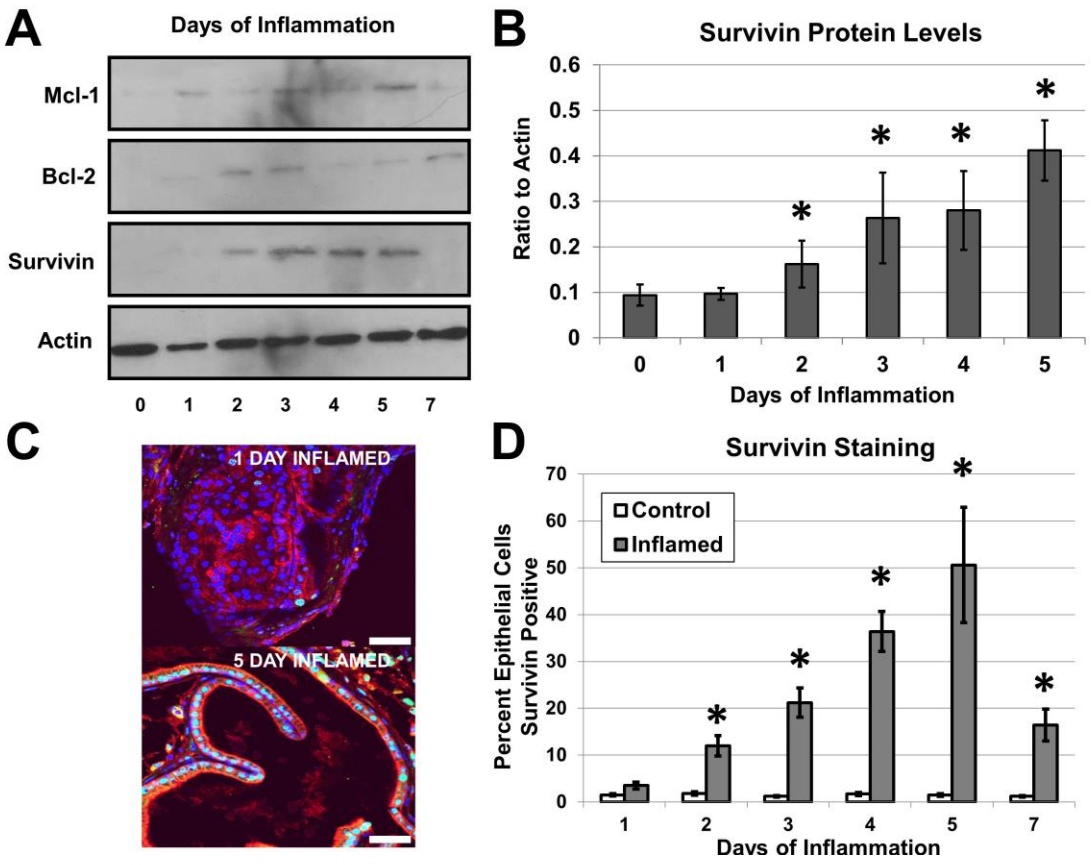



**Figure 14.** Inflammation induces three primary cell survival signaling pathways in mouse dorsolateral prostates, most prominently, survivin

**A:** Immunoblot example of induction of survivin, Bcl-2, and Mcl-1 during the time course of inflammation in mouse prostates. **B:** Quantified data of relative expression of survivin protein levels during prostatic inflammation; data were calculated as ratio of pixel intensity of the given survival protein, relative to β-actin, and expressed as ratio of expression to control prostates at the corresponding time of induction. Data are expressed as mean ± s.e.m. *$P < 0.05$ versus PBS-instilled (control) prostate; comparisons using analysis of variance (ANOVA), $n = 6$. **C:** Immunofluorescence of survivin expression (green) in 5 day instilled control (top) and inflamed (bottom) dorsolateral prostates demonstrating epithelial cell (PanCK, red) expression in the nucleus during inflammation. **D:** Quantified cell counting of epithelial cells positive for survivin expression; data expressed are the percentage of epithelial cells expressing survivin in control and inflamed prostates at each time point of inflammation. All data are expressed as

mean ± s.e.m. *P < 0.05 versus PBS-instilled prostate; comparisons using analysis of variance (ANOVA), n = 6.

**Inflammation Induces Autophagy: LC-3 Association with the Autophagosome**

As autophagy is a cell mechanism that can lead to either cell survival or cell death, we sought to characterize its induction in prostatic inflammation. Inflammation increases both the expression and vesicle association of the autophagy LC3 **(Figure 15)**. LC3 is expressed in the cytosol of cells and, upon initiation of autophagy, LC3 associates with vesicular membranes to form the autophagosome. This causes LC3 to run faster on gels, so a lower running band in immunoblotting is indicative of autophagosome-associated LC3, and therefore autophagy induction. Immunoblotting of proteins from inflamed prostates demonstrates that autophagy is induced by inflammation in the prostate, maximizing at 3 days of inflammation (**(Figure 15A and 15B)**; n = 6 mice)). Additionally, LC3 expression itself is also induced after three days of inflammation. To further characterize LC3 during prostatic inflammation, we assessed tissue localization by in immunofluorescence. Autophagosome-associated LC3 is associated with a punctate appearance as the protein is concentrated around the vesicle **(Figure 15C)**. We found that the number of prostatic epithelial cells exhibiting punctate LC3 increased from 4% of epithelial cells to 25% by the third day of inflammation **(Figure 15D)**. Autophagic cells were

found throughout the epithelium, in both luminal and basal layers. These data demonstrate that autophagy is induced by acute prostatic inflammation, and maximizes during the lymphocytic phase.

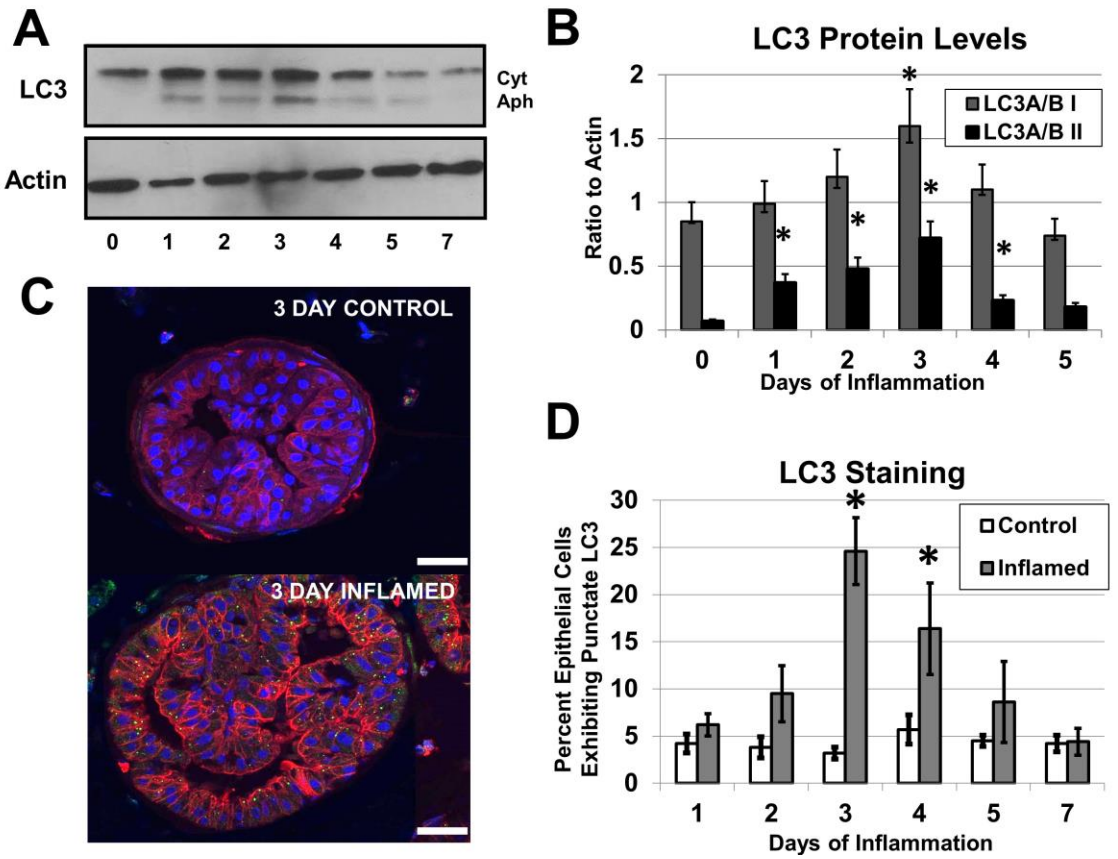



**Figure 15.** Inflammation autophagy marker expression in mouse dorsolateral prostates

**A:** Immunoblot example of induction of the autophagy marker LC3 at inflammation time points indicated; the higher running band (cyt) represents cytosolic LC3 while the lower running band (Aph) indicates LC3 associated in the autophagasome, and is indicative of autophagy induction. **B:** Quantified data of relative expression of all cytosolic and autophagasome-associated LC3; data were calculated as ratio of pixel intensity of the LC3 form, relative to β-actin, and expressed as ratio of expression to control prostates at the corresponding time of induction. Data are expressed as mean ± s.e.m. *$P < 0.05$ versus PBS-instilled (control) prostate; comparisons using analysis of variance (ANOVA), $n = 6$. **C:** Immunofluorescence of LC3 (green) localization in 3 day instilled control (top) and inflamed (bottom) dorsolateral prostates demonstrating the characteristic punctate LC3 Localization with the autophagasome of autophagic epithelial cells, a further indicator of autophagic cells (PanCK, red). **D:** Quantified cell counting of epithelial cells positive for punctate LC3 Localization; data expressed are the

percentage of epithelial cells expressing punctate LC3 in control and inflamed prostates at each time point of inflammation. All data are expressed as mean ± s.e.m. *P < 0.05 versus PBS-instilled prostate; comparisons using analysis of variance (ANOVA), n = 6.

**Survivin Is Induced Juxtaposed to Inflammation in Human Prostate Specimens**

In our in vivo mouse model of prostatic inflammation, survivin is the most consistently induced survival factor in the prostate in response to inflammatory signals. Because of this, we sought to determine if it is induced by inflammation in human prostate specimens. To address inflammation and survival protein expression in human tissue, we co-stained for CD45+ (immune cells) and survivin in non-diseased (prostates removed from cystectomies), BPH specimens (via TURP), and prostate cancer specimens (removed via prostatectomy), 12 tissues (from 12 separate patients) per group. As previously reported, survivin is induced in the majority of prostate cancer specimens, and is not expressed in the majority of non-diseased control prostates. However, our staining also demonstrated that regions of inflammation associated with survivin induction regardless of whether the region was found in non-diseased or diseased prostates **(Figure 16A)**. We defined both inflamed and non-inflamed regions in all three prostatic conditions (non-diseased, BPH, and cancer) by the number of CD45-evident 20× fields. Based on our previously established mouse model inflammatory scoring, we set the criteria for inflammation to be greater than or equal to 30 CD45+ cells per 20× field, and non-inflamed regions were defined as

less than 10 CD45+ cells per field. Using this criteria, we quantified the number of survivin-positive cells in sections from inflamed and non-inflamed regions in non-diseased, BPH, and cancerous prostate specimens. We found that regardless of condition, inflamed fields were associated with 60–70% of epithelial cells positive for survivin, while non-inflamed non-diseased and BPH fields exhibited less than 10% of epithelial cells positive for survivin, and non-inflamed cancerous fields were associated with 24% positive cells **(Figure 16B)**. While there was a threefold increase in survivin-positivity among prostate cancer specimens independent of inflammation, the primary difference between diseased and non-diseased prostates was the prevalence of inflammation **(Figure 16C)**. In non-diseased prostates, severe inflammation represented on average 11% of sections, while in BPH and prostate cancer sections severe inflammation constituted 82% and 71% of the section, respectively. There was no difference in the severity of inflammation in the 20× views quantified in this study once they were determined to be in the “severe” (>30 CD45-positive leukocytes per field) category. Additionally, we observed no significant difference in survivin-positivity between the transition zone and peripheral zone of non-diseased prostates.

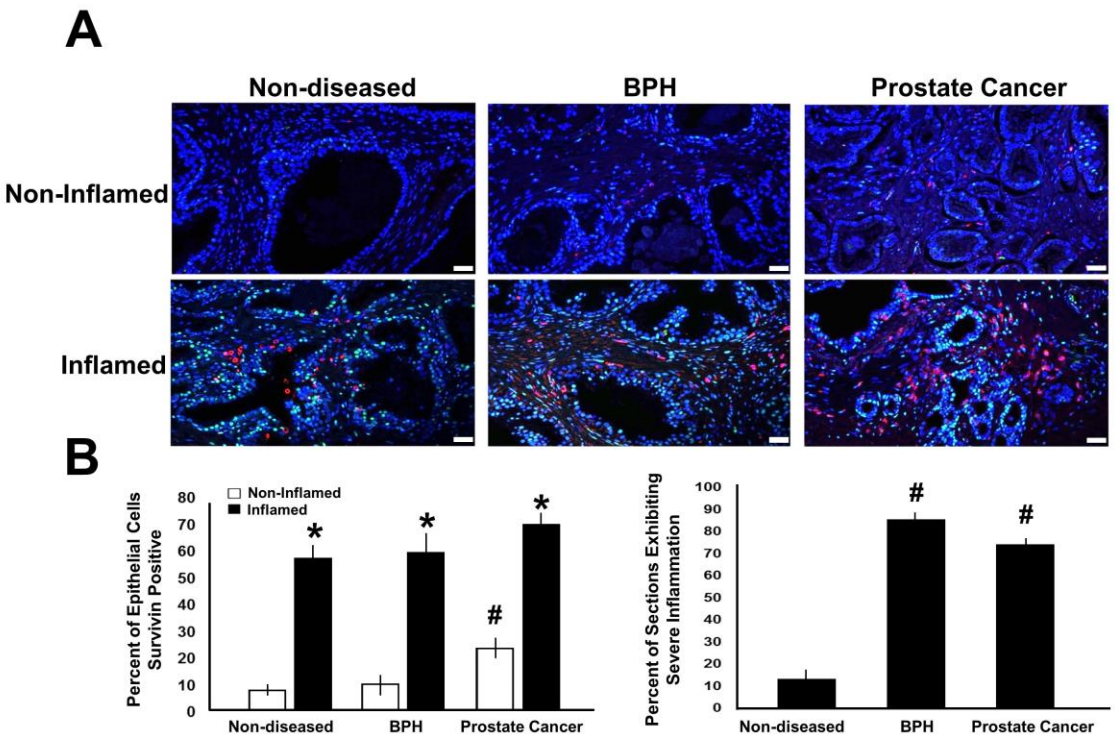



**Figure 16.** Human prostate specimens demonstrate intense survivin staining juxtaposed to regions of inflammation

**A:** Immunofluorescent images of non-inflamed or inflamed human prostates representing non-diseased controls (peripheral zone) taken from cystoprostatectomy cancer and BPH-free prostate specimens, BPH specimens (transition zone from TURP), or prostate cancer specimens, as indicated. Sections were stained for survivin (green) and CD45, a pan leukocyte marker (red) to identify regions of inflammation. Sections were deemed non-inflamed if they exhibited less than 10 Leukocytes per 20× field, and inflamed if they exhibited greater than 30 Leukocytes per field. **B:** Quantified cell counting of epithelial cells positive for survivin expression in human prostates; data expressed are the percentage of epithelial cells expressing survivin in non-inflamed and inflamed prostates at each time point of inflammation—three 20× fields per prostate section were averaged for each data point, and all data are expressed as mean ± s.e.m. *$P < 0.05$ inflamed versus non-inflamed prostate; #$P < 0.05$ disease condition versus non-diseased control. Analysis of variance

(ANOVA), n = 12 human prostates. **C:** percent of sections from each human prostate group (non-diseased, BPH, and cancer) that exhibited 30 Leukocytes per 20× section. Three 20× fields per prostate section were averaged for each data point, and all data are expressed as mean ± s.e.m. #P < 0.05, disease condition versus non-diseased control. Analysis of variance (ANOVA), n = 12 human prostates.

## Discussion

Inflammation is a common feature of prostate biology and is believed to be associated with the disease progression involved in both BPH and prostate cancer. Yet, inflammation is also a destructive process that involves a repair and recovery stage in which protected cells must survive the initial insults of inflammation, followed by their rapid proliferation as a means to repopulate the damaged tissue. The cell signaling mechanisms involved in coordinating these events is not understood, and little is known as to how the epithelium of tubular structures such as the prostate protects the specialized cells that are the keystones of epithelial repair and recovery.

The data in the present study indicate that inflammation induces a profile of cell death and cell survival-inducing factors, coordinated such that death factors and induction of cell death cellular mechanisms occurs within the first 48 hours after induction of inflammation, followed by a maximized expression of survival factors and signaling pathways. Inflammation causes visible death of the prostatic epithelium in the first 48 hours of inflammation as evidenced by H&E staining and confirmed by activation of caspases and nick-end labeling. Coordinate with this, there is a significant induction of cell death factors including TNFα, TWEAK, TRAIL, and FasL. Secondary to cell death, the acute

inflammation time course exhibits induction of known survival factors including growth factors, cytokines, and developmental morphogens that correspond to activation of survival pathways that include survivin, Mcl-1, and Bcl-2. We conclude from this that a population of epithelial cells resides in the prostatic epithelium that responds to inflammatory signals by inducing survival factors, and functions to repopulate the tissue during repair secondary to the damaging effects of inflammatory triggers.

Additionally, autophagy mechanisms maximize during this survival phase and may represent an additional cell survival mechanism in prostatic epithelial cells. Autophagy is indicated by the formation of autophagosomes for cell protein digestion during stress. Cells in autophagy can either use the digested material for survival, or they can be entered into the apoptotic cascade if the stress time period endures. During the induction of autophagy, the normally cytosolic protein Light Chain 3 (LC3) associates and participates in the formation of the autophagosome. As such, the association of LC3 in a lipid fraction—that of a membrane—indicates the induction of autophagy. In our study, autophagosome-associated LC3 was evident by two methods: the faster-running band at 14 KD in immunoblotting that indicates autophagosome association; secondly, the specific exhibition of LC3 into punctate formations indicating the presence of

autophagosomes. Our data demonstrate a substantial increase in the faster running band by immunoblotting, and in the number of epithelial cells exhibiting punctate LC3 containing autophagosomes. From this we conclude that inflammation induces autophagy in a subset of prostate epithelial cells in experimental models of prostatic inflammation.

The mouse model used in this study is a model of acute inflammation that progresses to chronic inflammation. This is characterized by an early neutrophilic infiltrate that dominates in the first 2 days of induction, and progresses to a primarily lymphocytic and monocytic infiltrate in days 3–5. This later lymphocytic chronic stage of the inflammatory response has a cellular infiltrate and an expression profile consistent to what is observed in human chronically inflamed prostates. The time course of cellular infiltrate in this model exhibits remarkably little experimental variation error, demonstrating that the time course of the inflammatory response is highly reproducible and consistent. Further, inflammation in this model was accompanied by increased expression of several inflammatory mediators and gene products including IL-1 family members, IL-6, COX-2, IGF-1, and FGFs, commonly observed in chronic prostatic inflammation. Therefore it is not unexpected that an increase in survival protein expression

occurs during the chronic phase of this model, just as is observed in human chronically inflamed prostates.

Survivin was the most consistent and substantially induced of the survival proteins in our mouse study, and we sought to validate its responsiveness to inflammation in human specimens. While the expression of survivin is well-known to be induced in both BPH and prostate cancer [205-207], this is the first report we are aware of in which survivin localization is characterized juxtaposed to inflammation. We found that survivin expression does localize to areas of severe inflammation, but what was striking is how this localization is largely disease-independent. There was no difference in the number of survivin-positive cells in inflamed regions between BPH, cancer, and non-diseased regions; the primary discriminating factor between the pathological states is how widespread inflammation is in each state. Severe inflammation was present in over 80% of our BPH sections and over 70% of our prostate cancer specimens, but was only a feature in less than 15% of non-diseased sections. Inflammation was associated with reactive hyperplasia and stromal desmoplasia in all tissues where it was present, but there was no consistent formation of any pre-malignant epithelial lesions such as dysplasia in inflamed regions of non-diseased prostates. We propose that the previously published findings describing high

survivin localization in diseased prostates is in large part a factor of the increased inflammation in those specimens. It must be noted, however, that in those uncommon regions of prostate cancer that are not associated with inflammatory infiltrate, there is still an increased number of survivin-positive cells, independent of inflammation. Therefore we conclude that prostate cancer does have intrinsic survivin induction relative to benign epithelium, but this is still enhanced with inflammation. BPH specimens did not exhibit any increase, absent of inflammation.

There is a clear association of BPH with inflammation, both in proliferating cells and in association with symptoms. The prevalence of inflammation in BPH specimens is repeatedly reported to be found in between 75% and 100% of specimens. A well-characterized study by Nickel et al. reported substantial prostatic inflammation in 100% of 80 men undergoing prostatectomy for treatment of BPH [208], and histological examination of prostates from 8224 men enrolled in the REDUCE trial revealed inflammation in 78% of specimens [209]. Critically, histologically verified inflammation is the most tightly correlated histological finding to prostate symptomology in men with BPH [210]. Similarly, strong evidence links inflammation to the development, growth, and survival of cancer in the prostate [187]. Histopathology studies of human prostatectomy

specimens identified lesions characterized by proliferating epithelial cells and activated inflammatory cells (proliferative inflammatory atrophy, PIA) in juxtaposition to areas of neoplasia. Sustained cell proliferation in the inflammatory environment rich in growth factors, activated stroma, and DNA-damage-promoting agents, could potentiate, and/or promote neoplasia. Additionally, proliferative inflammatory atrophy (PIA) is characterized by proliferating epithelial cells and is found in association with prostatic intra-epithelial neoplasia (PIN) and prostate cancer. These findings have prompted the hypothesis that chronic inflammation is involved in the genesis and/or progression of prostate cancer.

Several aberrant molecular mechanisms in apoptosis pathways have been identified to result in prostate disease, and androgen axis modulators—a mainstay of our therapies against prostate cancer growth—rely heavily on induction of apoptotic mechanisms for their efficacy [211-214]. Multiple modifications to cell death and survival pathways have been discovered in prostate tumors, BPH, and prostate cell lines that may participate in either tumorigenesis, proliferation, or therapy resistance. Increased expression of the survival proteins survivin, Bcl-2, Bcl-xL, Bcl-ω, and Mcl-1 are associated with prostate cancer and BPH, and interestingly inhibiting their expression sensitizes

cells to cytotoxic therapies [215-216]. In addition, down-regulation or inhibition of these survival proteins results in increased chemosensitivity in prostate cell lines [217-219]. Finally, prostate cancer cells exhibit decreased death receptor expression and upregulated decoy death receptor expression, and this results in diminished apoptosis induction capacity [220-221]. Our present findings add to the understanding of the balance between pro-apoptotic and pro-survival signaling in prostate epithelial cells by demonstrating extrinsic control of both death factors and subsequent survival factor induction in a specialized cell population, by inflammation.

## Chapter 4: APE1/Ref-1 Redox-Specific Inhibition Decreases Survivin Protein Levels and Induces Cell Cycle Arrest in Prostate Cancer Cells

### Introduction

Prostate cancer is one of the most common male malignancies and the third leading cause of cancer-related death of men in the United States. [222-223] Small prostatic carcinomas exist in up to 29% of men in their thirties and 64% of men in their sixties, with most of these carcinomas being indolent and/or cured by surgery or radiation. [224-226] However, some men develop an aggressive phenotype that metastasizes and becomes incurable once colonizing the bone. [227-228] These bone metastases produce osteoblastic lesions that

are associated with high morbidity and high mortality [229] and attempts at delaying this tumor progression with chemotherapeutic agents have only prolonged survival a few months. [230-231] In order to create more effective treatments where conventional therapeutics have failed, a better understanding of the aggressive phenotype of the disease is of utmost importance and a great unmet medical need.

It is now well-established that reduction-oxidation (redox) regulation of critical transcriptional activators plays an essential role in cell proliferation and survival in a number of different cancers, including prostate cancer. [232-234] Apurinic/apyrimidinic endonuclease 1/redox factor 1 (APE1/Ref-1) is a multifunctional protein that participates in DNA repair and redox transcriptional regulation. [235-236] APE1/Ref-1 has been implicated in the development and progression of numerous cancer types, is conversely correlated to tumor radiation and chemotherapy sensitivity, and is overexpressed in prostate cancer. [237-241] APE1/Ref-1 redox regulation of transcriptional activators occurs through cysteine residues within the DNA binding or transactivation domain of the transcription factor and is essential for full activation of certain transcriptional activators including the oncogenic transcriptional activators AP-1, HIF-1α, NF-κB and STAT3. Treatment with small molecule inhibitors of the redox activity of

APE1/Ref-1, such as APX3330 has been shown to diminish the activity of these redox-regulated transcriptional activators. [242-244] Furthermore, the blockade of APE1/Ref-1's redox activity has been shown to reduce growth-promoting, inflammatory and anti-apoptotic activities in cells. [245-246]

The ability of cancer cells to overcome apoptotic signals is crucial for tumor progression. Survivin is an Inhibitor of Apoptosis (IAP) family member, and it is overexpressed in prostate cancer. Survivin has been implicated in resistance to various chemotherapeutic and pro-apoptotic agents. [247-249] Survivin is classically known as an inhibitor of caspases due to its single BIR (Baculovirus IAP Repeat) domain, but recently survivin has been found to be crucial in cell cycle progression as a member of the chromosomal passenger complex. [250] Previously as described in Chapter 3, our lab has demonstrated that survivin is juxtaposed to inflammation in human prostate cancer specimens. [251] Attempts at directly targeting survivin have ultimately failed in clinic, therefore new approaches or therapeutics that in some way block the expression or function of survivin are needed.

Accumulating evidence demonstrates that APE1/Ref-1 is a key regulator of cancer cell growth and survival signaling and is upstream of pathways that regulate survivin expression. [252] Here we report that inhibition of APE1/Ref-1

redox signaling activity decreases prostate cancer cell proliferation, decreases the transcriptional activity of NFκB, and downregulates survivin expression in prostate cancer cells in vitro and in vivo. This is the first report to our knowledge that mechanistically demonstrates that APE1/Ref-1 redox-specific inhibitors are a viable therapeutic option for prostate cancer treatment

**APE1/Ref-1 and survivin are overexpressed in human prostate cancer**

To confirm that APE1/Ref-1 and survivin expression is altered in prostate cancer, we performed immunofluorescence using human non-diseased and cancerous prostate specimens **(Figure 17A)**. We found that APE1/Ref-1 is overexpressed in prostate cancer compared to non-diseased control prostates and it co-localizes with survivin-expressing cells. Expression of both proteins was primarily found to be nuclear and localized in the epithelium. To verify if well-characterized prostatic cell lines displayed the same pattern, PC-3, C4-2, LNCaP and non-cancerous E7 cell lysates were collected and immunoblotting performed evaluating APE1/Ref-1 and survivin protein levels **(Figure 17B)**.

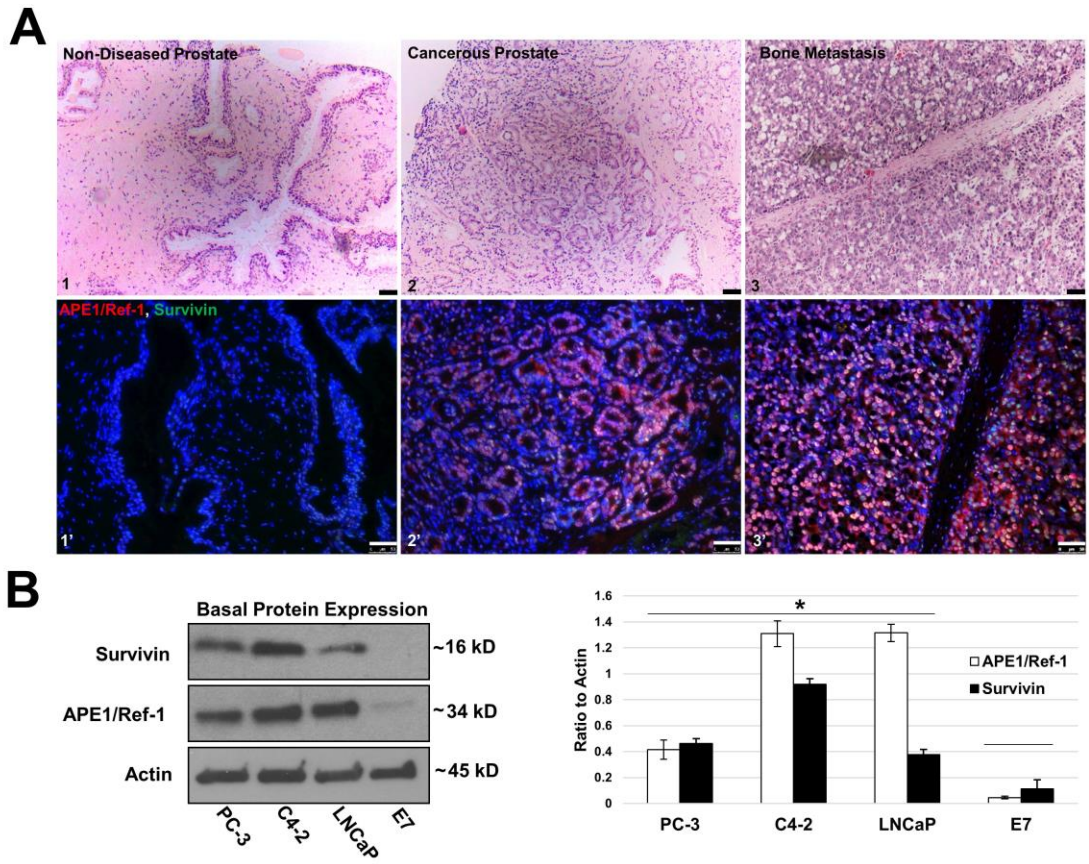



**Figure 17.** APE1/Ref-1 and survivin are overexpressed in human prostate cancer

**A:** Hematoxylin and Eosin staining representing non-diseased (peripheral zone taken from cystoprostatectomy) and cancerous human prostate specimens. Scale bar = 100 µM. Immunofluorescent images of stained non-diseased and cancerous sections for APE1/Ref-1 (red) and survivin (green). Scale bar = 50 µm. **B:** Immunoblot example of basal survivin and APE1/Ref-1 protein levels between the prostatic cell lines. Representative of three determinations with densitometry quantification, N=3.

**APE1/Ref-1 redox inhibition decreases prostate cancer cell proliferation**

To determine if inhibition of APE1/Ref-1's redox function affects cell proliferation, prostatic cell lines were treated with increasing concentrations of APE1/Ref-1 redox-specific inhibitors APX3330 and APX2009 for five days and cell number was measured via methylene blue assay **(Figure 18A-D)**. RN7-58, an inactive analogue of the APX3330 and APX2009 chemical families, was used as a negative control. [253] APX3330 and APX2009, inhibited cell proliferation in a concentration-dependent manner. Growth $IC_{25}$'s and $IC_{50}$'s were determined and arranged in **Table 2**. APX2009 was found to be 5-10 fold more efficacious than parent compound APX3330 in inhibiting cell proliferation, while the inactive analogue RN7-58 had no effect on cell growth in these assays.

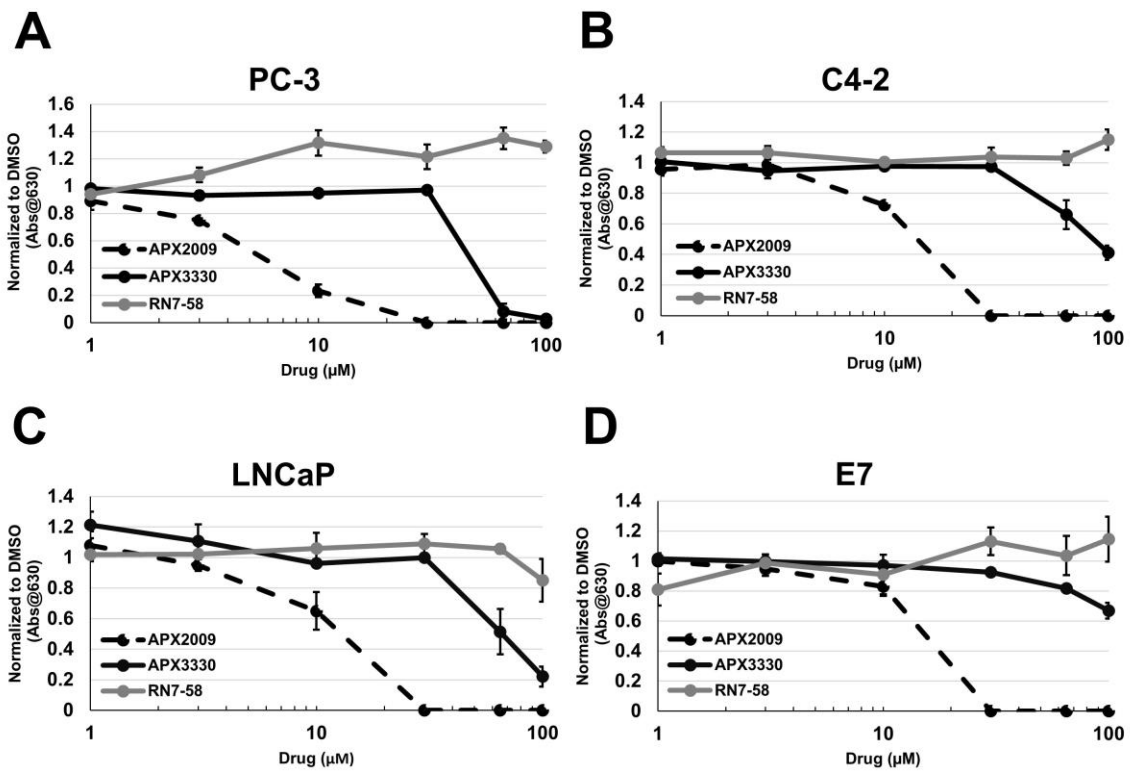



**Figure 18.** APE1/Ref-1 redox function specific inhibitors decrease cell number in a concentration dependent manner

**A:** PC-3, **B:** C4-2, **C:** LNCaP and **D:** E7 cell lines were treated with increasing concentrations of redox-specific inhibitor APX3330, more potent analogue APX2009, and inactive analogue RN7-58 for 5 days (N=3). The cells were fixed and stained with methylene blue and measured via spectrophotometry. $IC_{25}$ and $IC_{50}$ were determined as the concentrations of drug at which there was a 25% and 50% reduction in absorbance compared to vehicle control (DMSO) and were used for subsequent experiments.

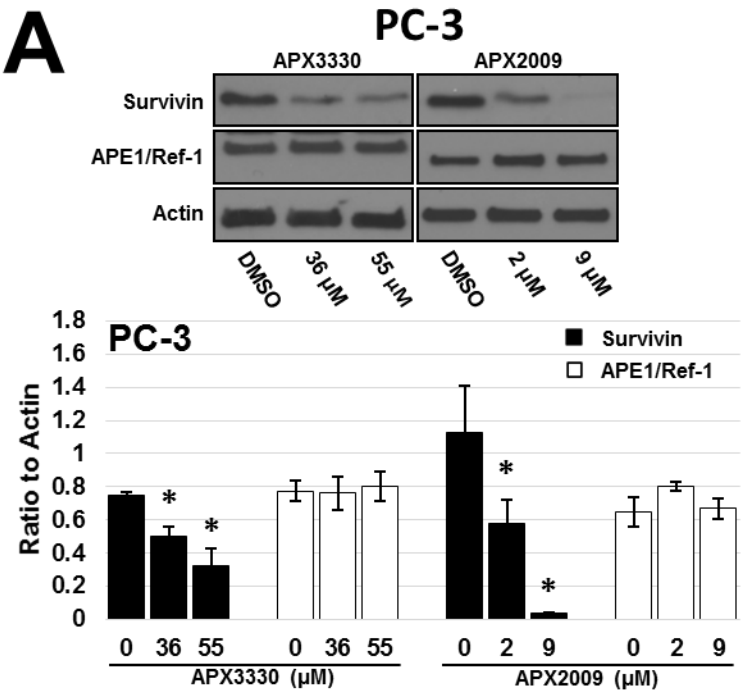
| | | APX3330 | APX2009 |
|---|---|---|---|
| PC-3 | $IC_{25}$ | 36.0 +/- 1.0 | 2.2 +/- .5 |
| | $IC_{50}$ | 54.7 +/- 1.6 | 8.9 +/- .7 |
| C4-2 | $IC_{25}$ | 57.4 +/- 3.8 | 7.6 +/- .2 |
| | $IC_{50}$ | 89.5 +/- 7.8 | 14.2 +/- .3 |
| LNCaP | $IC_{25}$ | 43.8 +/- 4.2 | 6.3 +/- 1.6 |
| | $IC_{50}$ | 71.9 +/- 7.2 | 13 +/- 1.2 |
| E7 | $IC_{25}$ | 82.7 +/- 8.7 | 9.2 +/- .7 |
| | $IC_{50}$ | >100 | 16.1+/- .8 |

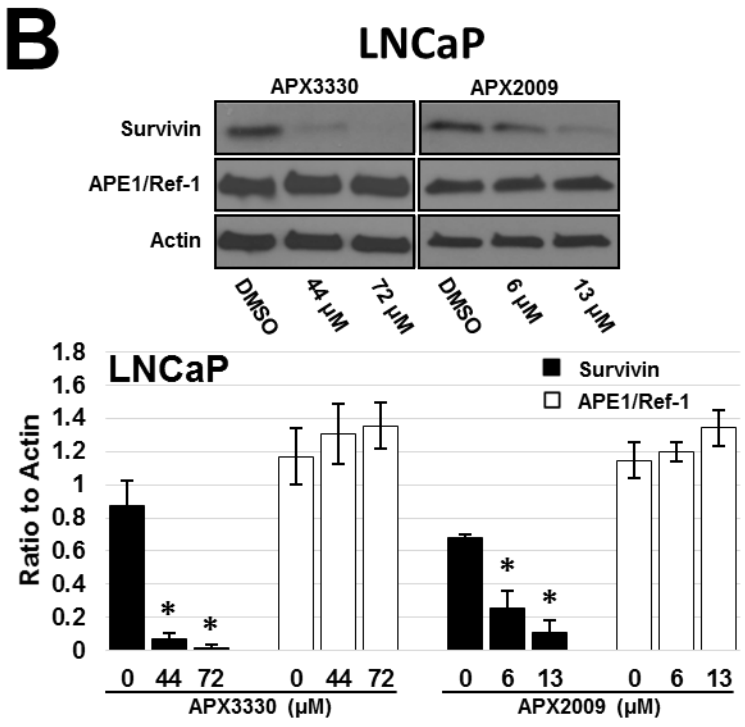
**Table 2.** Growth $IC_{25}$ and $IC_{50}$'s were determined for each cell line using the 3 growth curves for APX2009 and APX3330

Data is presented as mean ± s.e.m.

**APE1/Ref-1 redox-specific inhibitors decrease survivin protein levels**

Survivin plays an important role in prostate cancer cell proliferation and survival. Since survivin is controlled by APE1/Ref-1-regulated transcription factors in other organ systems such as the pancreas and liver [254-255], we hypothesized that treatment with APE1/Ref-1 redox-specific inhibitors APX3330 and APX2009 would decrease survivin protein levels at least partially explaining the reduction in proliferative capacity. Prostate cancer cells treated with the respective growth inhibitory $IC_{50}$ drug concentrations of APX3330 and APX2009 (as determined in **Figure 18**) exhibited a significant decrease in survivin protein expression within 48 hours compared to DMSO treated controls **(Figure 19A-D)**. In contrast, prostate cancer cell total APE1/Ref-1 protein levels were not significantly altered with treatment.


A
PC-3
APX3330
APX2009
Survivin
APE1/Ref-1
Actin
DMSO
36 μM
55 μM
DMSO
2 μM
9 μM
PC-3
Survivin
APE1/Ref-1
Ratio to Actin
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 36 55 0 36 55
APX3330 (μM)
0 2 9 0 2 9
APX2009 (μM)


B
LNCaP
APX3330
APX2009
Survivin
APE1/Ref-1
Actin
DMSO
44 μM
72 μM
DMSO
6 μM
13 μM
LNCaP
Survivin
APE1/Ref-1
Ratio to Actin
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 44 72 0 44 72
APX3330 (μM)
0 6 13 0 6 13
APX2009 (μM)

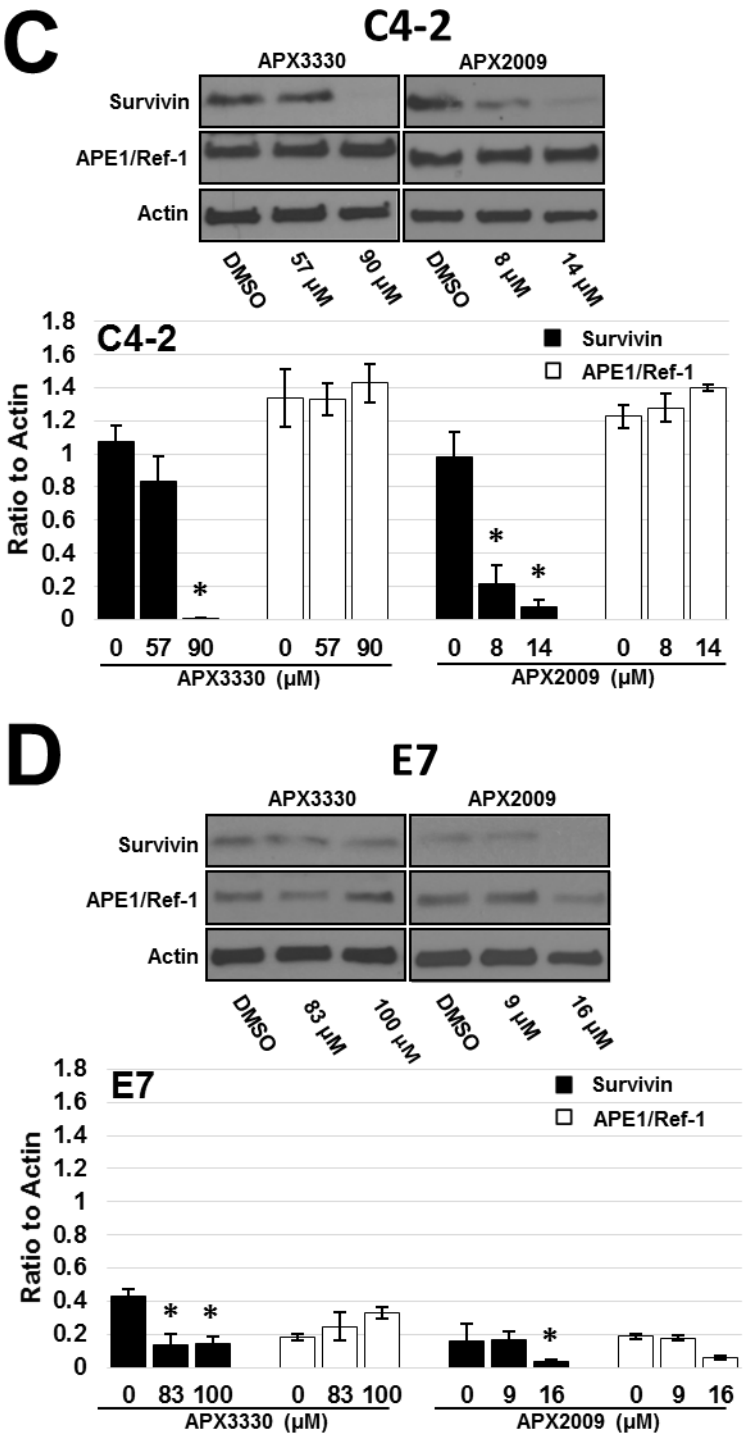



**Figure 19.** Treatment with APX3330 and APX2009 decreases survivin protein levels

**A-D:** PC-3, C4-2, LNCaP and E7 cell lines were treated with vehicle (DMSO), IC25 and IC50 drug concentrations for 48 hours. Immunoblotting for survivin, APE1/Ref-1 and Actin as labeled. Representative of three determinations with densitometry quantification, N =3, *-denoting $p<0.05$ (DMSO vs. IC25 and IC50 Drug Concentrations) within ANOVA.

**APX2009 reduces survivin mRNA expression and perturbs NFκB activity**

Based on the observation that inhibition of APE1/Ref-1 reduces survivin protein levels, we sought to determine the mechanism by which APE1/Ref-1 regulates survivin expression, and ultimately, cell growth. We hypothesized that APE1/Ref-1's redox control of transcription factors such as NFκB would decrease survivin transcript levels. Based on the increased potency of APX2009 over APX3330 in prostate cancer cells, we focused on the second generation compound, APX2009 for the remainder of our molecular studies. C4-2 cells were treated with vehicle or APX2009 $IC_{50}$ (14 μM) for 12 hours. RNA was collected and RT-qPCR was performed using a primer/probe set for survivin (BIRC5) and HPRT1 for the reference gene **(Figure 20A)** using the conditions suggested by the SuperScript III Platinum One-Step qRT-PCR System (Invitrogen). Survivin mRNA was significantly reduced upon treatment with the relative quantity (RQ) value of <0.5. Survivin has been shown in other cancers to be regulated by NFκB, and NFκB is regulated by APE1/Ref-1 redox signaling. [256-259] Therefore, we evaluated the ability of these two proteins to interact physically with each other. In **Figure 20B**, we demonstrate via co-immunoprecipitation that APE1/Ref-1 interacts with NFκB subunit p65 when using an APE1/Ref-1 antibody and in reverse experiments using a p65 antibody. In addition, we assessed the

cellular localization of both NFκB and APE1/Ref-1 upon treatment with APX2009 **(Figure 20C)**. p65 and APE1/Ref-1 were found to be co-localized in the nucleus however upon treatment with APX2009, p65 nuclear localization was diminished suggesting altered NFκB protein trafficking. To determine if NFκB signaling is regulated by APE1/Ref-1 redox activity, we transfected C4-2 cells with NFκB-driven luciferase constructs. Inhibition of APE1/Ref-1 redox activity with APX2009 resulted in a significant 2-fold decrease in NFκB-driven luciferase activity (**Figure 20D**). Finally as a positive control, PCa cells were treated with increasing concentrations of NFκB-selective inhibitor ammonium pyrrolidinedithiocarbamate (PDTC). Inhibition of NFκB with PDTC also resulted in the reduction of survivin protein levels (**Figure 20E**) further confirming a role for NFκB in regulating survivin.

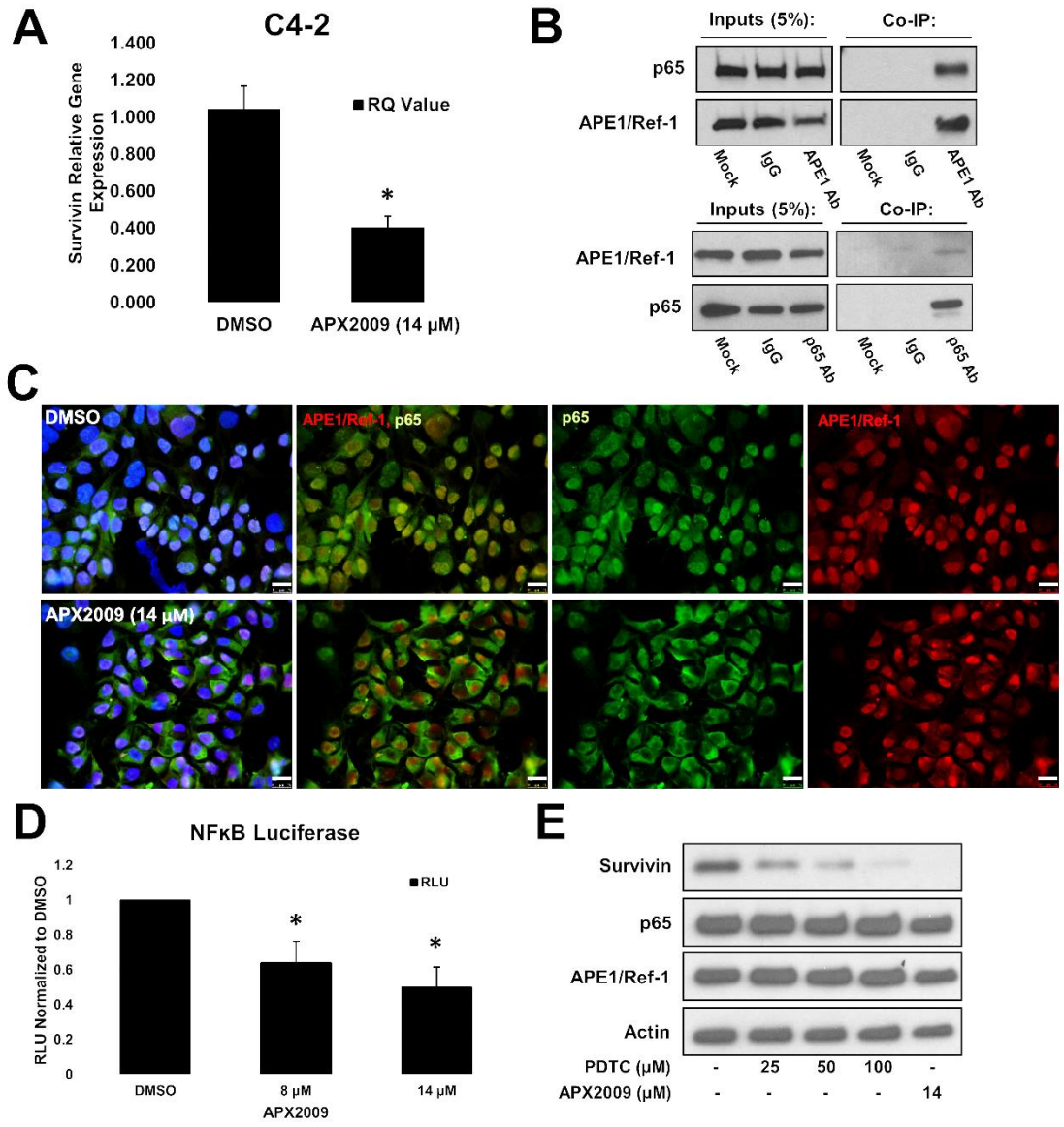



**Figure 20.** APE1/Ref-1 redox inhibition decreases survivin protein levels via NFκB

**A:** C4-2 cell line was treated with DMSO or APX2009 (14 μM) for 12 hours. RNA was isolated and RT-PCR for survivin was performed with HPRT1 as the reference gene. **B:** Immunoblot validation of APE1/Ref-1 and p65 Co-Immunoprecipitation (Co-IP) reactions. The input and IP were loaded for each reaction. Mock beads and generic IgG were used as negative controls. **C:** C4-2 cell line was treated with DMSO or APX2009 for 48 hours and immunofluorescence was performed using antibodies for APE1/Ref-1 (Red) and

NFκB subunit p65 (Green). Representative images were taken. Scale bar = 50 μM. **D:** C4-2 cells were transfected with NFκB–Luc construct and co-transfected with a Renilla vector, pRL-TK. After 16 hours, cells were treated with increasing concentrations of APX2009 for 24 hours, and Firefly and Renilla luciferase activities were assayed using Renilla luciferase activity for normalization. All transfection experiments were performed in triplicate and repeated 3 times in independent experiments. Data are expressed as Relative Luciferase Units (RLU) normalized to DMSO showing the mean ± SEM. N=3, *-denoting $p<0.05$ within ANOVA. E: C4-2 cell line was treated with NFκB-selective inhibitor PDTC (25, 50 and 100 μM) and APX2009 (14 μM) for 24 hours. Immunoblotting was performed with antibodies for survivin, p65, APE1/Ref-1 and Actin as labeled.

**Treatment with APX2009 induces G1 cell arrest but not cell death**

To determine if inhibition of APE1/Ref-1 via APX2009 results in cell death due to loss of survival signaling, PC-3 and C4-2 cells were treated with either DMSO or previously-determined $IC_{50}$ concentrations of APX2009 (9 µM in PC3 and 14 µM in C4-2) for 48 hours (**Figure 21A**) and cell lysates were collected for immunoblotting (**Figure 21B**). After APX2009 treatment, both PC-3 and C4-2 cells displayed an altered, flattened cellular morphology. However, treatment with these compounds did not induce cell death as determined by both a lack of increased caspase 3 cleavage (**Figure 21B**) and TUNEL labeling (data not shown). Because no increase in apoptosis was detected and cell cycle proteins Cdc2 and Cyclin B1 were dramatically decreased by APE1/Ref-1 inhibition, cell cycle analysis was performed using Propidium Iodide (PI) staining. PC-3 and C4-2 cells were treated with APX2009 (9 µM and 14 µM, respectively) for 48 hours, stained with PI, and analyzed by flow cytometry. We found that the percentage of cells in G1 significantly increased, $p<0.05$ via Student's t-test, from 58 to 68% and 63 to 74% in PC3 and C4-2 cells, respectively, indicating G1 arrest of prostate cancer cells in response to APE1/Ref-1 inhibition. These effects on the cell cycle progression are similar to other recent reports of APE1/Ref-1 redox inhibition in cancer. [260-261]

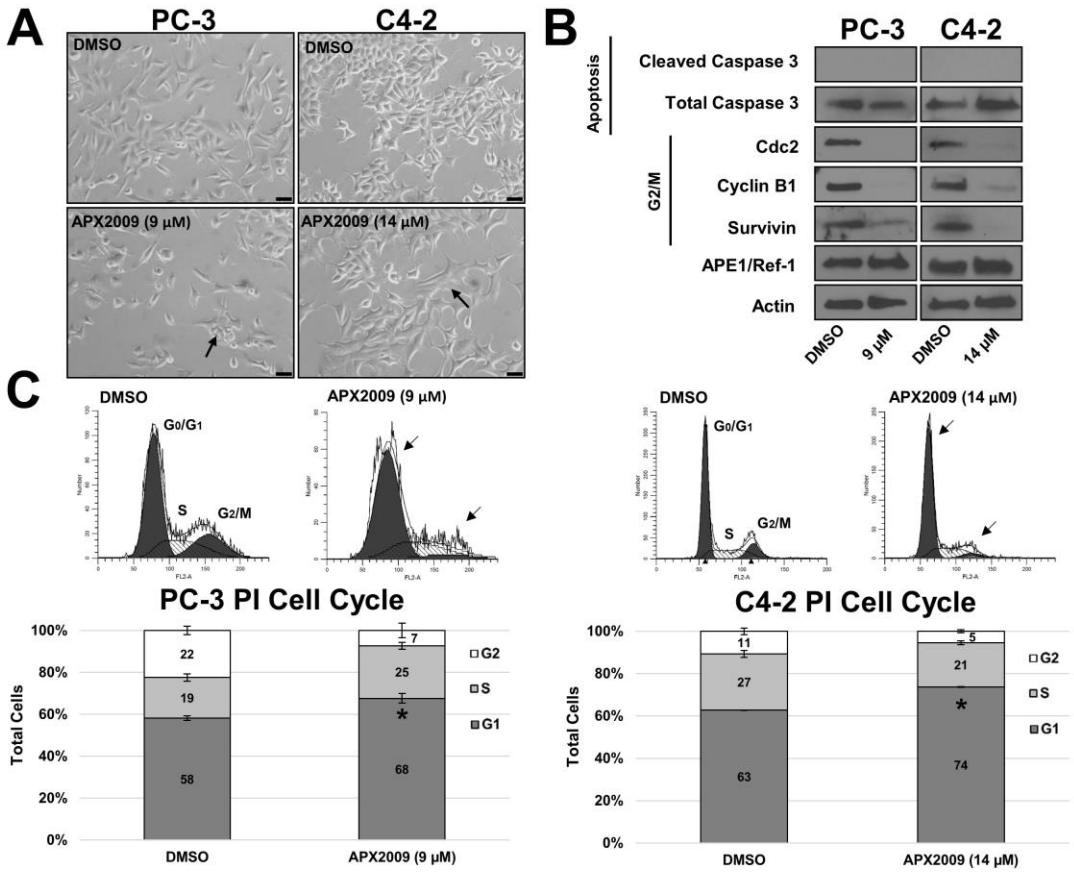



**Figure 21.** APE1/Ref-1 redox inhibition induces G1 cell arrest

**A:** PC-3 and C4-2 cell lines were treated with DMSO or APX2009 (9 and 14 μM, respectively) for 48 hours. Representative images were taken at 20X Magnification. Scale bar = 50 μm. **B:** Immunoblotting was performed and membranes were probed with antibodies for Cleaved Caspase 3, Total Caspase, Cyclin B1, Cdc2, survivin and Actin as labeled. **C:** PC-3 and C4-2 cells were treated with DMSO or APX2009 (9 and 14 μM, respectively) for 48 hrs and then collected and stained with RNAse/PI wash. Flow Cytometry was then performed. N =3, *-denoting $p<0.05$ within Student's t-Test.

**APE1/Ref-1 redox inhibition decreases survivin protein levels and cell proliferation *in vivo***

Based on the in vitro data, we expanded our studies of the role of APE1/Ref-1 redox activity in cell proliferation and survivin protein levels in vivo using C4-2 subcutaneous xenografts. Animals were treated with either APX2009 (25 mg/kg bid) or Vehicle (PKT) for 5 days and then tumors were harvested. Total survivin protein via immunoblotting was significantly reduced (**Figure 22A**) when compared to control tumors. Survivin and APE1/Ref-1 localization via immunofluorescence remained nuclear with survivin co-localizing with the chromatin during mitosis (**Figure 22B**). Furthermore, BrdU incorporation was significantly reduced from 8.2% to 5.1% in the treatment group demonstrating that inhibition of APE1/Ref-1 redox activity reduces tumor cell proliferation (**Figure 22C**). Similar results were seen with APX3330 (data not shown).

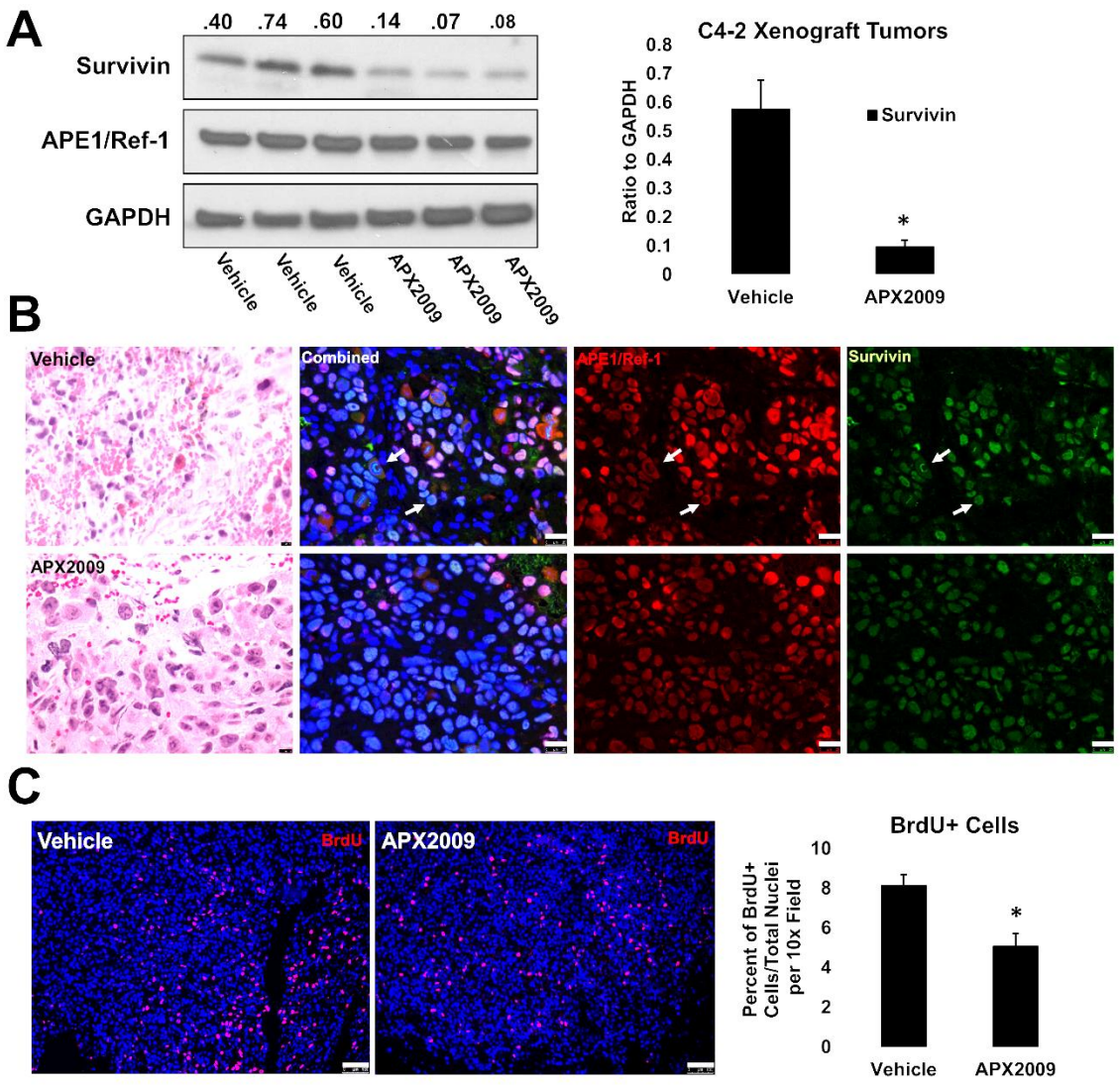



**Figure 22.** In vivo treatment with APX2009 reduces survivin protein levels and BrdU incorporation in C4-2 xenograft tumors

C4-2 xenograft tumors were treated with Vehicle (PKT) or APX2009 (25 mg/kg, IP bid) for 5 days (N=3). **A:** APE1/Ref-1 and survivin protein levels were measured using immunoblotting as labeled. **B:** Immunofluorescence was performed using APE1/Ref-1 (red) and survivin (green) specific antibodies on vehicle and APX2009 groups. Representative images were taken. White arrows are depicting survivin nuclear staining patterns. Scale bar = 50 µm. **C:** Mice were injected with BrdU 2 hours prior to sacrifice and tumors were collected and

stained for BrdU incorporation (red). Scale bar = 100 μm. ImageJ Nucleus Counter was used to quantify number of BrdU+ nuclei and total nuclei per image. N =3, *-denoting $p<0.05$ within Student's t-Test.

## Discussion

Prostate cancer is one of the leading causes of cancer-related death in American men, and challenges remain in targeting key drivers of the aggressive phenotype despite recent advances in prostate cancer treatment. Androgen deprivation therapies and microtubule-targeting agents prolong survival but resistance to these therapeutics is inevitable. It is thought that this resistance is driven in part by aberrant survival signaling and the induction of survival proteins which allows for the cancer to evade cell death. [262-263] Survivin is a bifunctional protein that has been shown to be overexpressed in a number of different cancers including prostate cancer. Survivin has anti-apoptotic and pro-proliferative functions in cancer cells. Inhibition of survivin is a logical therapeutic strategy, however directly targeting survivin has been difficult. In this study, we took a novel approach to survivin targeting; we provide evidence that targeting the redox-signaling regulator APE1/Ref-1 with small molecule inhibitors effectively suppresses survivin protein levels and inhibits cell proliferation.

APE1/Ref-1 is a multifunctional protein that was initially discovered as an enzyme in the base excision repair (BER) pathway, but has emerged as a redox-signaling regulator of a number of transcription factors known to be involved in cancer, namely NFκB, AP-1, and HIF1$\alpha$, STAT3. [264] These transcription

factors have been shown to be important in the initiation and progression of prostate cancer, as well as other cancers. [265-267] In this way, inhibiting the redox activity of APE1/Ref-1 effectively targets multiple different pathways at once and may therefore represent an advantageous therapeutic strategy.

The data presented in our studies further support the rationale for APE1/Ref-1 as a viable target in prostate cancer. Our results indicate that APE1/Ref-1 and survivin are overexpressed in primary and metastatic tumors as previously reported by Kelley et al [268]. APE1/Ref-1 was found to be primarily nuclear localized but cytoplasmic staining was also present in the tumors. Similar to other cancer cell lines, we found that APE1/Ref-1 siRNA knockdown decreased cell proliferation and survivin protein levels (**Figure 23**). Additionally, we demonstrate that inhibition of APE1/Ref-1 redox activity halts prostate cancer cell growth and induces G1 cell arrest in prostate cancer, consistent with recent reports in other cancers. APE1/Ref-1 is crucial in moving cells from G1 to S, and redox inhibition induces key cyclin-dependent kinase inhibitors (CDKi's) like p21 and p27. [269-270] This is a translationally relevant finding, as the first-generation APE1/Ref-1 small molecule inhibitor APX3330 used in this study is now approved for phase 1 clinical trials [Investigational New Drug (IND) application number 125360]. APX3330, and the second generation molecule

APX2009, is known to bind to APE1/Ref-1 in the redox active region of the protein, cause unfolding of the APE1/Ref-1 protein and block the redox active cysteine 65 from functioning thus effectively inhibiting its transcriptional regulatory activity of growth signaling pathways. [271-275] APX3330 has been shown to decrease cell proliferation in other cancers including pancreatic and ovarian, and here we show it has similar affects in prostate cancer.

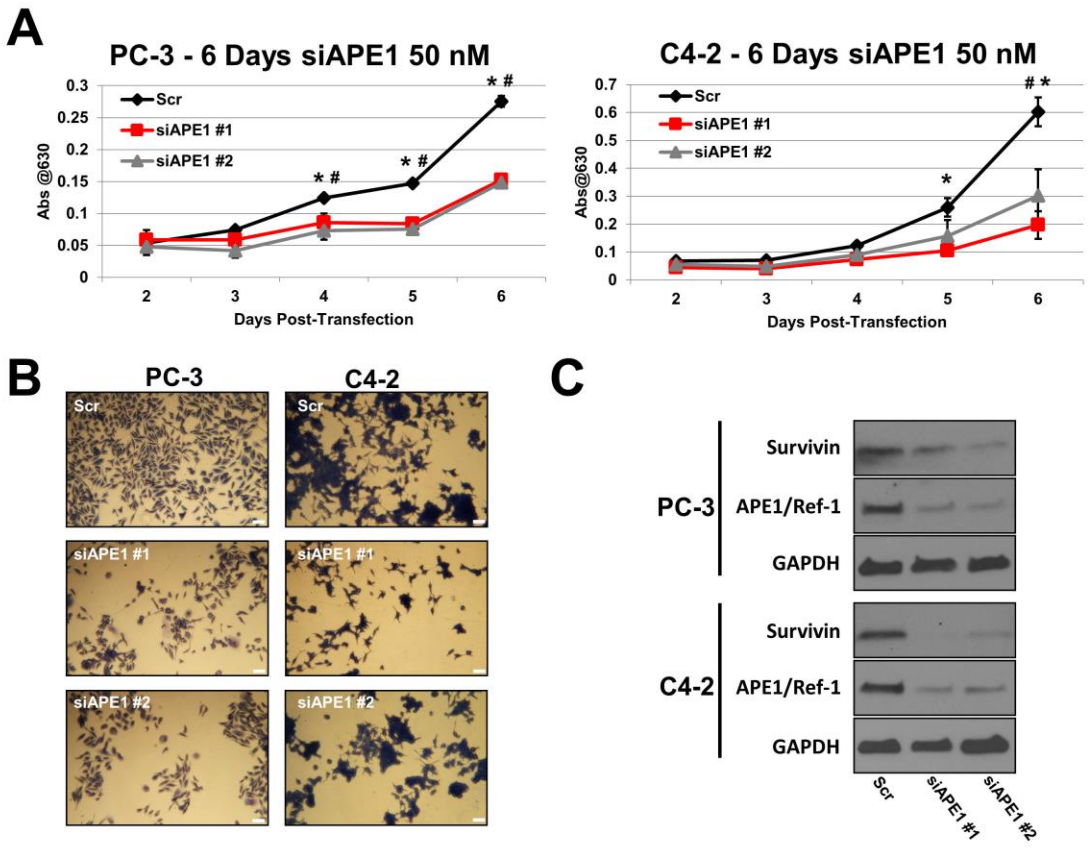



**Figure 23.** APE1/Ref-1 siRNA knockdown decreases cell proliferation and surviving protein levels

**A:** PC-3 and C4-2 cell lines were transfected with 50 nM APE1/Ref-1 siRNA (verified >70% knockdown by immunoblotting) and growth was compared to scrambled siRNA-transfected cells. **B:** Representative pictures of fixed and methylene blue stained C4-2/PC-3 scrambled siRNA (Scr), survivin siRNA #1 (siAPE1 #1) and #2 (siAPE1 #2). **C:** Immunoblotting was performed using antibodies for APE1/Ref-1, survivin and Actin as labeled. N =3, *-denoting $p<0.05$ within Paired Student's t-Test (Scr vs siAPE#1), #- denoting $p<0.05$ within Paired Student's t-Test (Scr vs siAPE#2).

Survivin is known to be differentially regulated in various tissues and in response to external stimuli. [276] Survivin is transcriptionally regulated by a number of transcriptional activators including STAT3 and NFκB. NFκB-driven survivin protein expression was interrogated here due to our observation that APE1/Ref-1 inhibition is effective in PC-3 cells despite their lacking the gene coding for STAT3. All four cell lines express functional NFκB signaling. [277] We provide evidence that survivin is transcriptionally regulated by NFκB. Upon APE1/Ref-1 redox inhibition, survivin mRNA levels are reduced and IP experiments demonstrate a strong interaction between APE1/Ref-1 and NFκB subunit p65. Following modulation of APE1/Ref-1 signaling with APX2009, NFκB signaling was decreased as assessed by NFκB-driven luciferase activity. In addition to this decrease in NFκB activity, we also demonstrated that p65 nuclear localization was disrupted upon APE1/Ref-1 redox inhibition. [278] This could occur due to diminished paracrine signaling factors such as, IL-6 or IL-8, which activate NFκB. It is also possible that APE1/Ref-1 inhibition disrupts APE1/Ref-1/p65 nuclear trafficking due to altered protein conformation. Treatment with NFκB inhibitor PDTC, which has also been shown to disrupt p65 translocation, was found to decrease survivin protein levels in a concentration-dependent manner, further supporting NFκB role in survivin transcription. [279] Additional experiments are needed to identify the underlining mechanism of the disrupted

p65 translocation. Nevertheless, our data do not preclude that multiple transcription factors could be contributing to survivin protein levels, and future studies will be directed at carefully assessing the role of each potential transcriptional activator in APE1/Ref-1-mediated prostate cancer cell growth and survivin expression.

In summary, our data indicate that APE1/Ref-1's redox function plays a role in regulating the proliferative capacity of prostate cancer cells by perturbation of NFκB transcriptional activity and survivin protein levels in human prostate cancer cell lines and in vivo in tumors **(Figure 24)**. Survivin plays an important role in prostate cancer survival, progression and therapeutic resistance. Thus, inhibition of APE1/Ref-1's redox function in combination with the current therapeutics like docetaxel or cabazitaxel may prove to be novel treatment strategy in advanced prostate cancer.

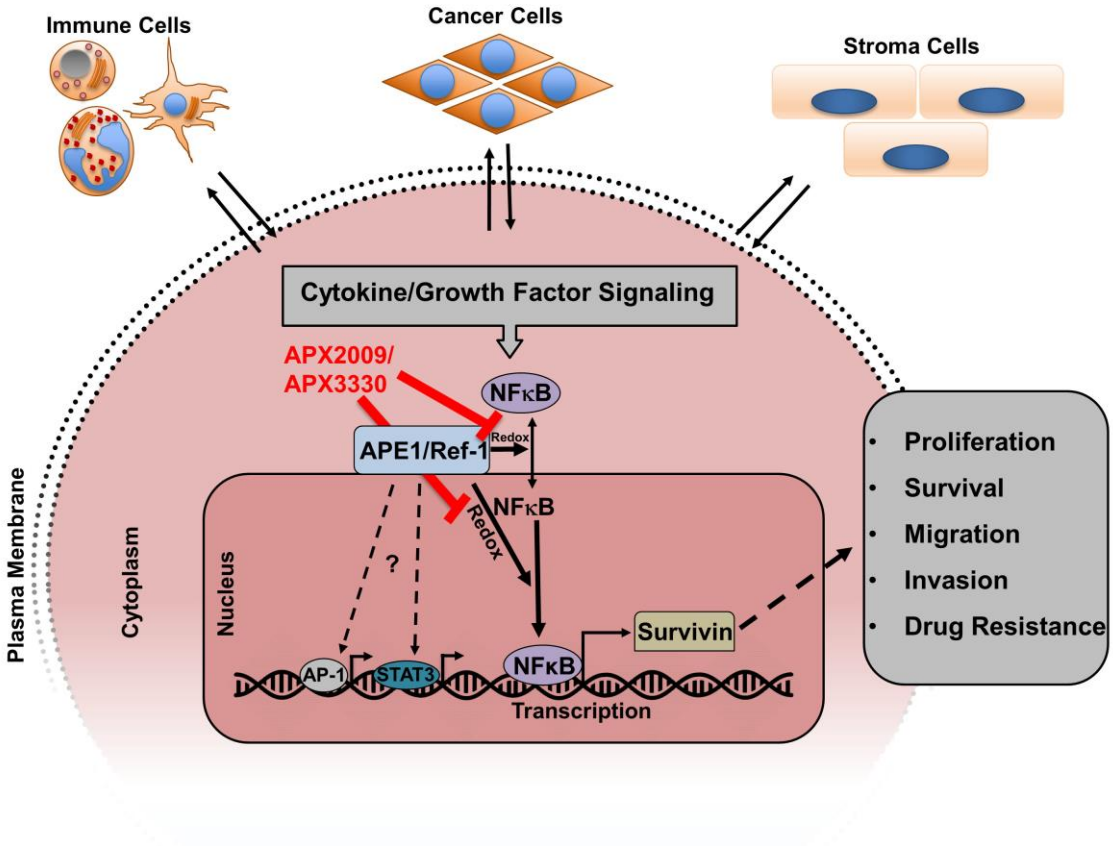



**Figure 24.** Model showing how cytokine/growth factor signaling induces survivin protein expression and at what points where APX2009/APX3330 disrupts this.

# Chapter 5: Docetaxel-Resistant Prostate Cancer

## Introduction

Metastatic castrate-resistant prostate cancer treatment has been historically difficult with very few successes over the years. Docetaxel, a member of the taxanes drug class, was the first chemotherapeutic therapy with survival benefits for men with metastatic castrate-resistant prostate cancer. [280] Unfortunately, half of all patients do not respond to docetaxel innately and all patients eventually develop resistance at some point. [281] That said, docetaxel is still an effective therapy and continues to be used in treatment plans to this day. Overcoming taxane-resistance remains of paramount of importance with more research needed to understand the mechanisms of resistance and to develop new therapeutic strategies of treating metastatic castrate-resistant prostate cancer. APE1/Ref-1 redox inhibition is an unexplored treatment option for taxane-resistance mechanisms with potential of curbing metastatic prostate cancer progression.

One well described mechanism of resistance is the upregulation of drug efflux transporters and loss of docetaxel accumulation within the cell. [282] Drug efflux transporters are intermembrane proteins belonging to the adenosine triphosphate binding cassette (ABC) family of transporters. The most famous of

these transporters is known as P-glycoprotein (P-gp) which is encoded by the mdr1/ABCB1 gene. [283] Expression of P-gp is weak in normal prostatic tissue but is increased in prostate cancer with regards to tumor stage and grade. [284] It was found to also be expressed by primary prostate cancer cell cultures of those with taxane-resistance [285]. Genetic variation within the mdr1/ABCB1 has also been shown to correlate to clinical outcome in patients with metastatic prostate cancer receiving docetaxel [286]. Recently, it has been shown that APE1/Ref-1 is instrumental in protein recruitment to mdr1 gene promoter and depletion of APE1/Ref-1 results in the decreased expression of P-gp. [287]

Apoptosis is a conserved process that plays an essential role in development and tissue homeostasis. Apoptotic defects have long since been tied to therapy resistance and this is also the case in prostate cancer with the upregulation of anti-apoptotic proteins as another well described mechanism of taxane-resistance. [288] Emerging evidence suggests the Bcl-2 and IAP families play an essential role in preventing apoptosis caused by chemotherapeutics and intrinsic/extrinsic factors. [289]

The Bcl-2 family proteins are divided into three groups based on their apoptotic action and how many Bcl-2 Homology (BH) domains they possess. Anti-apoptotic Bcl-2 family proteins like Bcl-2, Bcl-xL and Mcl-1 display 4 of these

BH domains and are known to be regulated by the NFκB pathway. [290] These Bcl-2 family proteins have critical roles in autophagy, calcium handling and mitochondrial dynamics/energetics and when dysregulated can contribute to disease.

The IAP family of proteins all contain one to three BIR domains and these BIR domains allow the proteins to bind caspases effectively inhibiting apoptosis. c-IAP1, c-IAP2 and XIAP can directly bind to activated caspase-3, caspase-7 and caspase-9. These proteins also have an E3 ligase activity by which they can ubiquitinate caspases. Survivin only has one BIR domain and has been found to be overexpressed in metastatic castrate-resistant prostate cancer and correlated to taxane-resistance. [291] It is a unique member meaning not only is it anti-apoptotic but is also pro-mitotic participating as a member of the chromosomal messenger complex and its microtubule-binding abilities. [292-293] Recently, it has also been shown to participate in cytokinesis but it does lack the ability to ubiquitinate other proteins. The IAP family is also known to be directly regulated by the NFκB pathway. [294]

Our preliminary experiments have shown APE1/Ref-1 is necessary for full transcriptional activation of NFκB. We hypothesize that APE1/Ref-1 redox function promotes cell survival and taxane-resistance in prostate cancer via

increased expression of survival proteins. This is the first time to our knowledge that the mechanism of APE1/Ref-1's redox function in prostate cancer cell survival during drug-resistance will be studied.

**Docetaxel-resistant metastatic castrate-resistant prostate cancer cell line generation**

To determine if APE1/Ref-1 redox activity mediates taxane-resistance two docetaxel-resistant cell lines were generated; DocR PC-3 and DocR C4-2. This was done by sequential dosing of increasing concentrations of docetaxel over a period of a year with DocR PC-3 being maintained at 10 ng/mL and DocR C4-2 being maintained 5 ng/mL. Both docetaxel-resistant cell lines portrayed altered cellular morphology compared to the parental cell lines which may be due to a difference in cellular adhesion **(Figure 25)**.

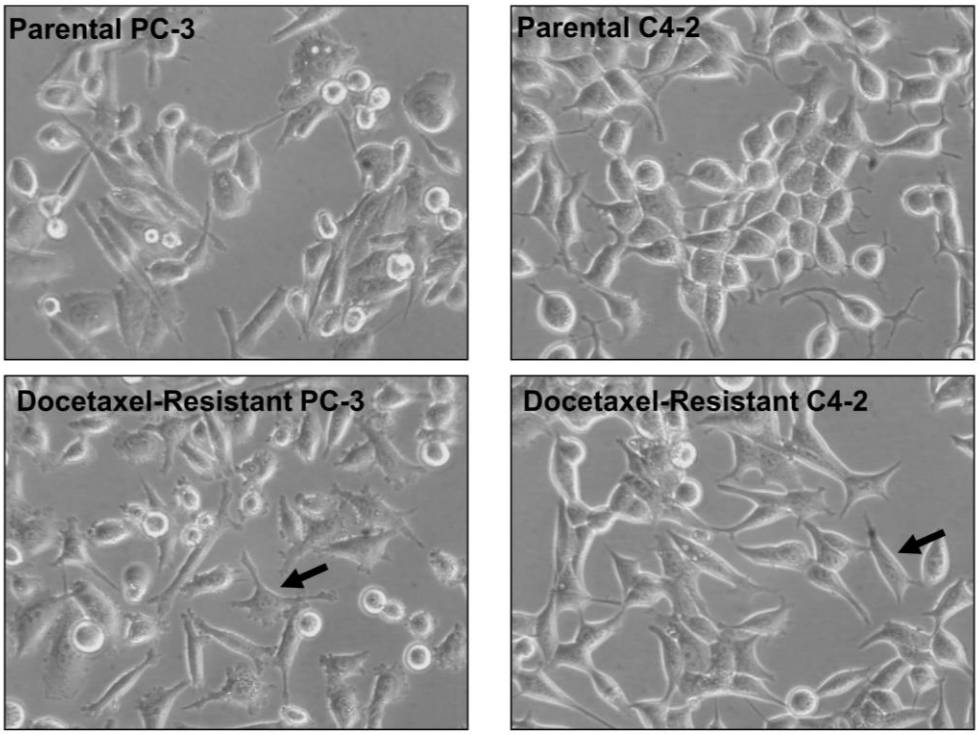



**Figure 25.** Docetaxel-Resistance induces altered cellular morphology

Images of Parental (Par) PC-3 and C4-2 **(TOP)** and Docetaxel-Resistant (DocR) PC-3 and C4-2 **(Bottom)**. Two docetaxel-resistant cell lines were generated using sequential dosing of increasing concentrations of docetaxel; DocR PC-3 and DocR C4-2. DocR PC-3 is maintained at 10 ng/mL docetaxel and DocR C4-2 is maintained 5 ng/mL docetaxel. Both docetaxel-resistant cell lines portrayed altered cellular morphology compared to the parental cell lines **(Black Arrows)**.

**Docetaxel-resistant C4-2 and PC-3 cell lines are less sensitive to docetaxel than parental cell lines**

To confirm the sensitivity of docetaxel-resistant cell lines to the parental cell lines, a docetaxel concentration response curve was performed for 72 hours. Both DocR C4-2 and DocR PC-3 were less sensitive to docetaxel than the parental cell lines with docetaxel $IC_{50}$'s around 20 ng/mL and 30 ng/mL, respectively. **(Figure 26)**. Parental C4-2 and PC-3 IC50's were around 2 ng/mL and 10 ng/mL, respectively.

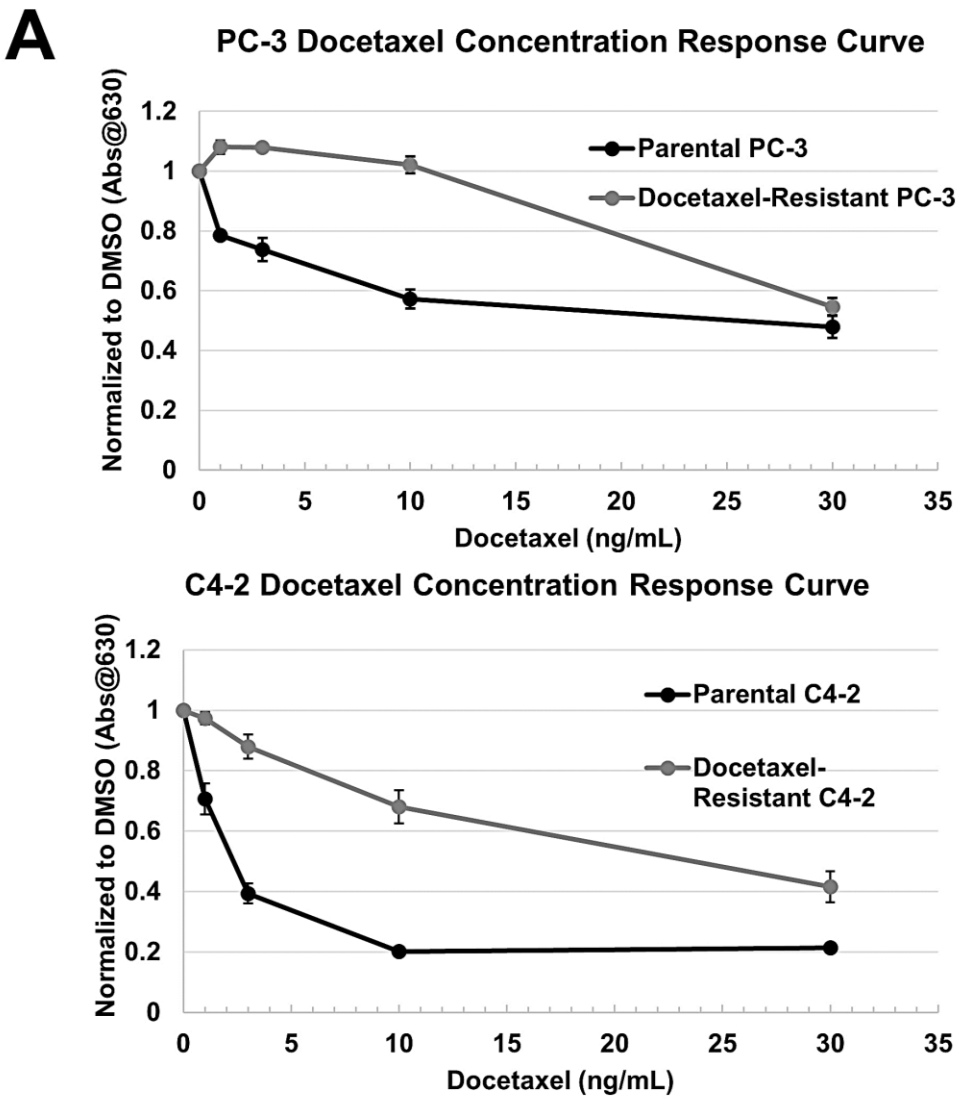



**Figure 26.** Parental PC-3 and C4-2 cell lines are more sensitive to docetaxel-induced cell growth inhibition

**A:** Both parental and docetaxel-resistant PC-3 **(Top)** and C4-2 **(Bottom)** cell lines were treated with increasing concentrations of docetaxel for 72 hours. The cells were methanol fixed and stained with methylene blue and absorbance was measured via spectrometry (N=3).

**APE1/Ref-1 redox inhibition decreases cell proliferation in parental and docetaxel-resistant cell lines**

To determine relative sensitivity to APE1/Ref-1 redox inhibition, a 72 hour APX3330 concentration response curve was performed on all 4 lines. Both DocR C4-2 and DocR PC-3 were more sensitive to APX3330 inhibition than compared to the parental cell lines. The $IC_{50}$'s were then determined and those concentrations used in the subsequent experiments **(Figure 27)**.

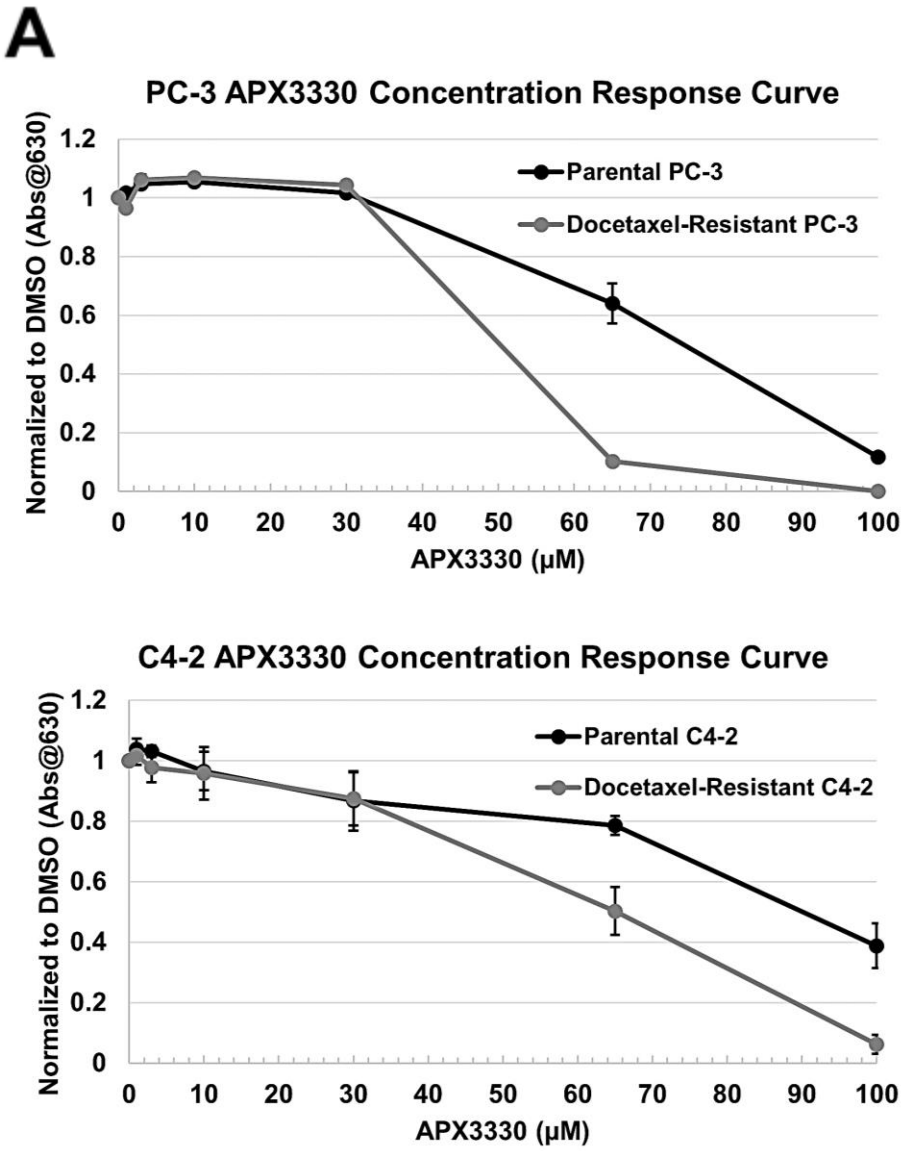



**Figure 27.** Docetaxel-resistant PC-3 and C4-2 cell lines are more sensitive to APX3330-induced cell arrest

**A:** Both parental and docetaxel-resistant PC-3 **(Top)** and C4-2 **(Bottom)** cell lines were treated with increasing concentrations of APX3330 for 72 hours. The cells were methanol fixed and stained with methylene blue and absorbance was measured via spectrometry (N=3).

**Combinational treatment with APX3330 and docetaxel does not induce synergy**

To determine if APX3330 could re-sensitize the docetaxel-resistant cell lines back to parental docetaxel concentrations, a combination experiment with the respective $IC_{25}$'s and $IC_{50}$'s of docetaxel and APX3330 was performed **(Figure 28)**. DocR C4-2 cells were pretreated with 40 or 60 μM APX3330 or 10 or 30 ng/mL of docetaxel for 24 hours. A concentration response curve was then performed using increasing concentrations of APX3330 **(Top)** or docetaxel **(Bottom)**. Combination treatment did overall decrease the proliferation of DocR C4-2 but failed to induce synergy.

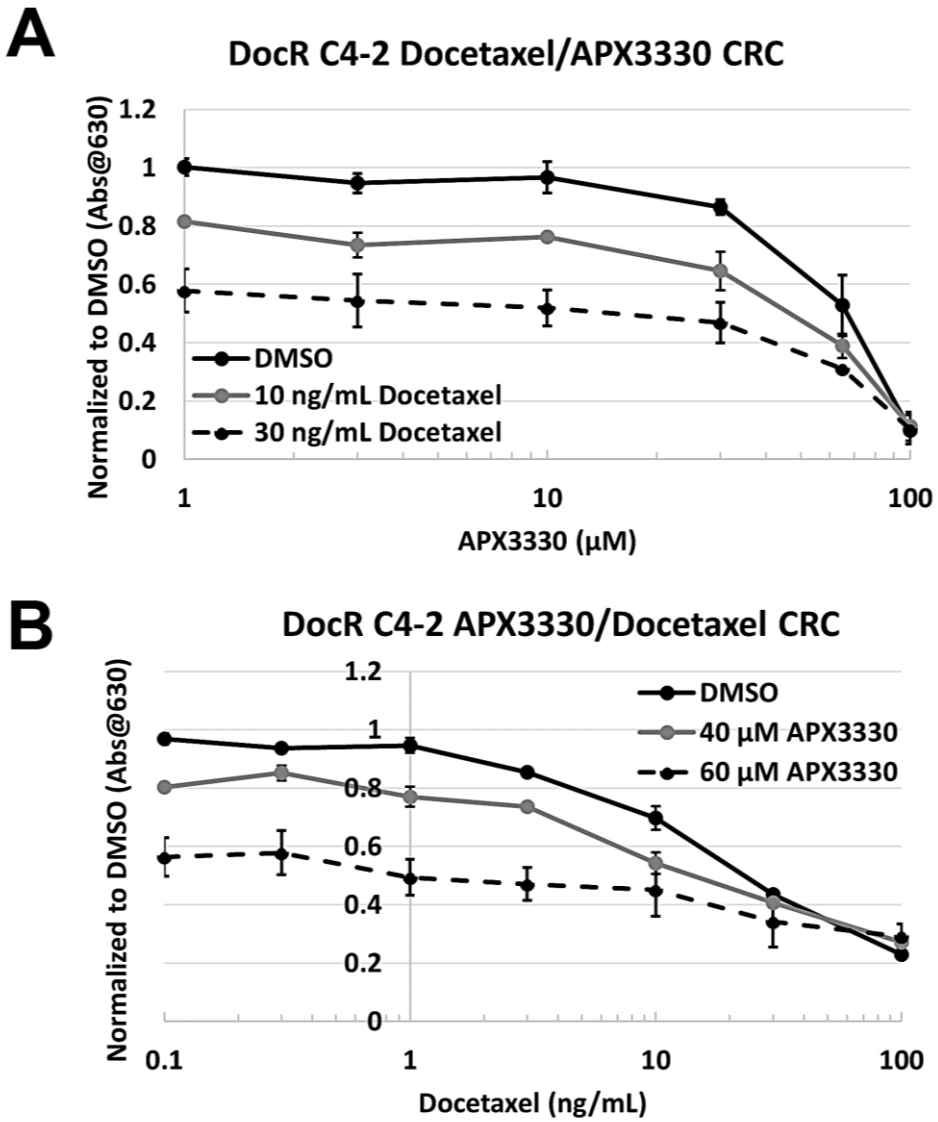



**Figure 28.** Combinational treatment with APX3330 and docetaxel does not induce synergy

**A:** Docetaxel-resistant C4-2 cells were pretreated with either 10 ng/mL or 30 ng/mL docetaxel for 24 hours and then increasing concentrations of APX3330 for 72 hrs. Cells were then fixed and stained with methylene blue and absorbance was measured via spectrometry (N=2). **B:** Docetaxel-resistant C4-2 cells were pretreated with wither 40 µM or 60 µM APX3330 for 24 hours and then increasing

concentrations of docetaxel for 72 hours. Cells were then fixed and stained with methylene blue and absorbance was measured via spectrometry (N=2).

**Combinational treatment with APE1 siRNA and docetaxel does not induce synergy**

Recently it has been shown that a pro-longed mitotic phase induces DNA damage. To determine if the DNA repair function of APE1/Ref-1 contributes to taxane-resistance APE1/Ref-1 knockdown (50 nM siRNA) was performed and after 48 hrs a concentration response curve with docetaxel was performed. The cells were fixed after 72 hours. Knockdown of APE1/Ref-1 decreased basal cell proliferation but did not induce synergy reflecting what we saw with the redox inhibitor **(Figure 29)**. Mutant APE1/Ref-1 C65A will be used in future experiments.

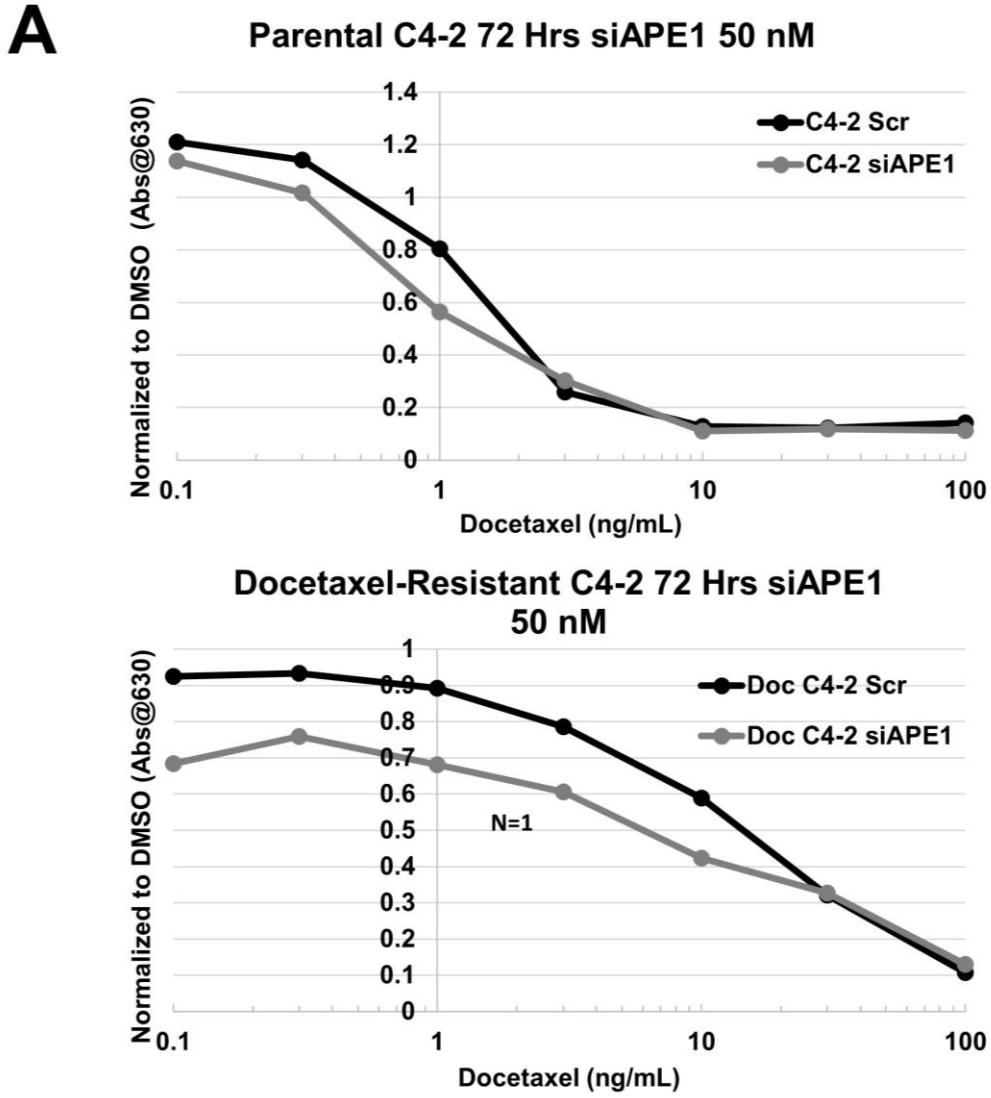



**Figure 29.** Combinational treatment with APE1 siRNA and docetaxel does not induce synergy

**A:** Parental **(Top)** and docetaxel-resistant **(Bottom)** C4-2 cells were either transfected with 50 nM siAPE1 or 50 nM scrambled siRNA and then treated with increasing concentrations of docetaxel for 72 hours. Cells were then fixed and stained with methylene blue and absorbance was measured via spectrometry (N=1).

**Docetaxel-Resistance does not increase basal survival protein levels**

To characterize the molecular mechanisms of resistance cell lysates were collected from the four cell lines and a western blot was performed to measure survival protein levels **(Figure 30)**. No difference was detected in Bcl-2, Bcl-xL and Mcl-1 protein levels. Survivin protein levels were decreased in the resistant cell lines compared to the parental cells. Interestingly, APE1/Ref-1 was reduced in the DocR PC-3 cell line.

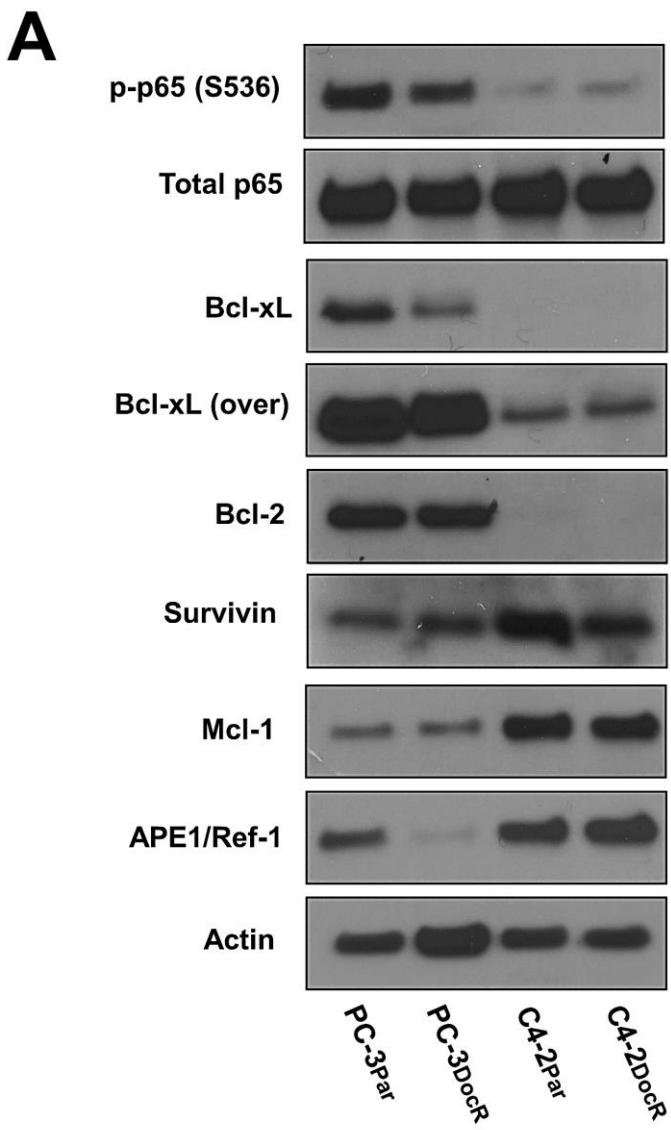



**Figure 30.** Docetaxel-Resistance does not increase basal survival protein levels

**A:** Parental and docetaxel-resistant PC-3 and C4-2 cell lysates were collected and immunoblotting was performed. The membrane was probed for p-p65, total p65, Bcl-xL, Bcl-2, surviving, Mcl-1, APE1/Ref-1 and actin as labeled (N=2). Survival protein levels were not found to basally increase in the docetaxel-resistant cell lines and APE1/Ref-1 protein levels were found to be decreased in the docetaxel-resistant PC-3 cell line.

**Treatment with docetaxel decreases APE1/Ref-1 levels in docetaxel-resistant PC-3**

The reduction in APE1/Ref-1 protein levels were detected 24 hours after the maintenance dose of concentration was administered. To confirm if APE1/Ref-1 protein levels would return after the initial decrease, DocR PC-3 were treated with maintenance concentrations of docetaxel for 24 and 48 hours and cell lysates were collected for western blot **(Figure 31)**. APE1/Ref-1 protein levels were found rebounding after 48 hours suggesting that docetaxel was transiently altering intracellular APE1/Ref-1 protein levels.

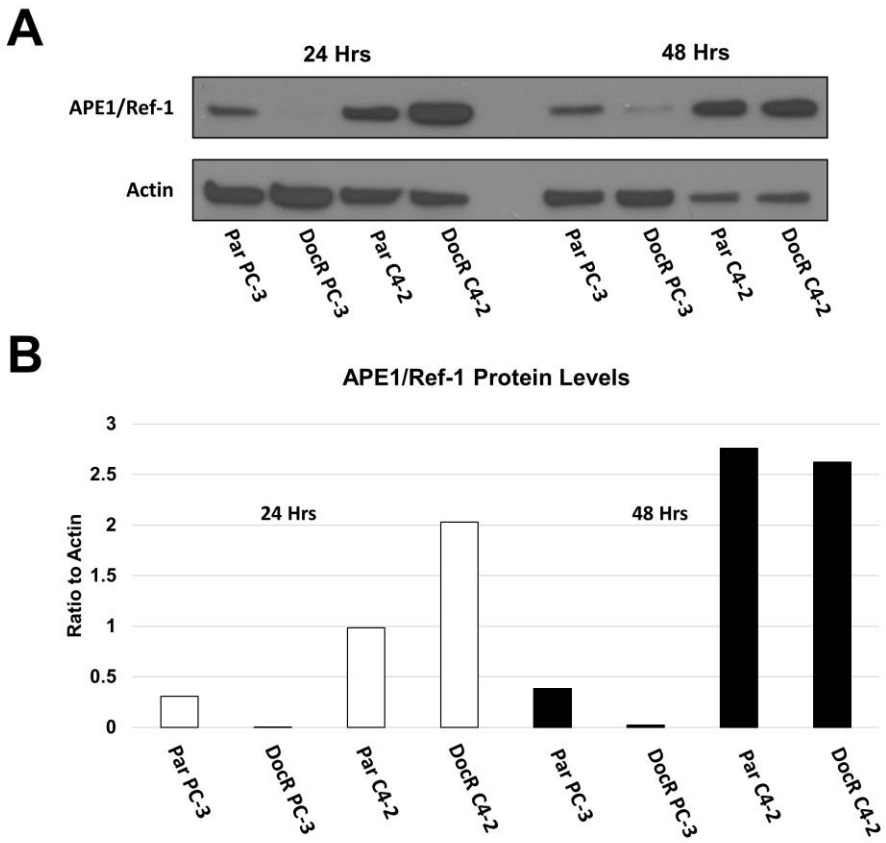



**Figure 31.** Treatment with docetaxel decreases APE1/Ref-1 levels in docetaxel-resistant PC-3

**A:** Docetaxel-resistant PC-3 and C4-2 cells were treated with maintenance doses of docetaxel (10 ng/mL and 5 ng/mL docetaxel, respectively) and parental cell lines were treated with DMSO. Cell lysates were collected at 24 and 48 hrs and an immunoblot was performed. The membrane was probed with antibodies for APE1/Ref-1 and actin as labeled (N=1). **B:** Densitometry was performed and a quantified graph showing the ratio of APE1/Ref-1 to actin for both time points.

**Treatment with docetaxel causes an increase in extracellular APE1/Ref-1**

Recently APE1/Ref-1 has been classified as a non-classically secreted protein. To determine if APE1/Ref-1 is secreted and if this secretion is regulated by docetaxel, DocR PC-3 and DocR C4-2 cells were dosed with 15 ng/mL Docetaxel for 0, 3, 6, 12 and 24 hrs. The supernatant and the cell lysates were collected at the respective time points for western blot. DocR PC-3 cells were cultured in media that did not contain docetaxel for 5 days prior to this treatment in order to allow intracellular APE1/Ref-1 levels to return to basal. DocR PC-3 and DocR C4-2 cells were both found to secrete APE1/Ref-1 into the extracellular space **(Figure 32)**. Interestingly, it was found that in the DocR PC-3 cells there was a clear time-dependent decrease in APE1/Ref-1 protein levels in the cell lysates and time-dependent increase in the supernatant. This suggests that it is possible for APE1/Ref-1 to be released into the extracellular compartment and that docetaxel may induce that secretion.

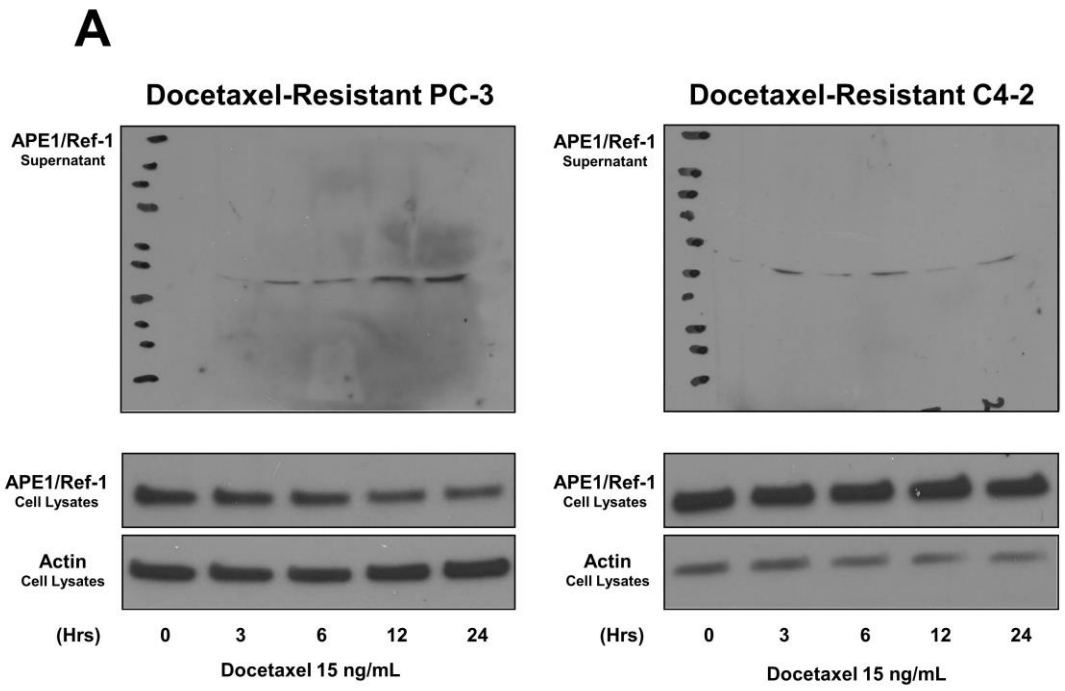



**Figure 32.** Treatment with docetaxel causes an increase in extracellular APE1/Ref-1

**A:** Docetaxel-Resistant PC-3 **(Left)** and C4-2 **(Right)** cells were cultured without docetaxel for 5 days prior to experiment. The cells were then treated with 15 ng/mL docetaxel and the supernatant and cell lysates were collected at 0, 3, 6, 12 and 24 hours. The proteins were concentrated out of the supernatant using -20 ºC acetone and immunoblot was performed. The membranes were probed with antibodies for APE1/Ref-1 and Actin as labeled (N=1).

## Discussion

There is no effective therapy to treat metastatic castrate-resistant prostate cancer with taxanes only extending patient life a few months. Taxane resistance is found innately in 50% of patients and those who respond ultimately develop acquired resistance. Multiple mechanisms including unfavorable tumor microenvironment, drug efflux transporters, and alterations in microtubule structure/function and apoptotic defects have been shown to contribute to taxane resistance. APE1/Ref-1 is known to regulate inflammatory processes, drug efflux transporter protein levels and survival protein levels in cancer. The data presented in this study investigates APE1/Ref-1 redox inhibition as a possible therapeutic option in docetaxel-resistant prostate cancer.

To test this two docetaxel-resistant prostate cancer cell lines were generated; DocR PC-3 and DocR C4-2. These two specific cell lines were chosen because they are both considered castrate-resistant cell lines and C4-2 cells have the androgen receptor while PC-3 cells do not. The DocR C4-2 cells were remarkably more sensitive to docetaxel than the DocR PC-3 cells and it was necessary to start dosing them in the picomolar concentration. It has been shown that prostate cancer cells with a functional p53, like C4-2 cells, are more

sensitive to docetaxel than those with mutant or null p53 expression, like PC-3 cells. [295]

Relative $IC_{50}$'s of docetaxel and APX3330 for all docetaxel-resistant cell lines were determined for future combination experiments. It was found that the docetaxel-resistant cell lines were less sensitive to docetaxel than the parental cell lines which was expected. Interestingly, the docetaxel-resistant cells lines were more sensitive to APE1/Ref-1 redox inhibition than the parental cell lines. This could be due to increased signaling pathways regulated by redox signaling, DNA damage induced by taxanes or altered cellular adhesion. In the combination experiments, an initial decrease in cell number by the pretreatment with APX3330 or docetaxel occurred as expected but when combined the drugs did not induce synergy and the curves converged at the higher doses. This was contrary to what was hypothesized. This may be due to the cell phase at which these cells are entering cell arrest. It is known that docetaxel does not affect non- or slow-growing cells and arrests cancer cells in G2/M. APE1/Ref-1 redox inhibition causes G1 arrest and this could explain why the two drugs are not complimenting each other *in vitro* as both actions occur in different phases.

Recently, it has been shown in endothelial and immune cells that APE1/Ref-1 is non-classically secreted into the microenvironment. [296] We

found that APE1/Ref-1 protein expression was lower in DocR PC-3 cells after maintenance docetaxel treatment. This lead to the hypothesis that APE1/Ref-1 was being released into the extracellular space upon treatment with docetaxel. This was found to be true in DocR PC-3 and C4-2 cells. The function of extracellular APE1/Ref-1 has not been fully elucidated but appears to be associated with its redox domain. In endothelial cells, extracellular APE1/Ref-1 was found to reduce the TNFα receptor altering its signaling pathway. [297] Something of this affect could be happening within our system.

# Chapter 6: Conclusions and Future Directions

## Conclusions

Inflammation is a natural process that protects the body against infection and promotes wound healing. Without an active immune system, the body succumbs to injuries and disease which is often described in immunocompromised individuals. However, in some cases the immune system triggers an overactive inflammatory response causing damage to its own tissues. It is not known entirely how cells survive and sometimes thrive in this noxious inflammatory environment. The data in Chapter 3, Coordinated Induction of Cell Survival Signaling in the Inflamed Microenvironment of the Prostate, indicate that inflammation induces a profile of cell death and cell survival-inducing factors, coordinated such that death factors (TNFα, TWEAK, TRAIL, and FasL) and induction of cell death cellular mechanisms (caspase cleavage and DNA fragmentation) occurs first, followed by expression of survival factors (IL-1 family members, IL-6, COX-2, IGF-1, and FGFs ) and survival signaling pathways (survival proteins).

These survival signaling pathways correspond to the activation and upregulation of survival proteins from the IAP and Bcl-2 families like survivin, Mcl-1, and Bcl-2. We concluded from this study that a population of epithelial cells

residing in the prostatic epithelium respond to inflammatory signals by inducing survival proteins like survivin, and repopulates the tissue after the initial cell death cascade. This is significant because survivin is rarely expressed in normal differentiated tissue but is often expressed in diseases like cancer. This was also the first report in which survivin localization was characterized juxtaposed to inflammation. We found that survivin expression localizes to areas of severe inflammation and that the localization is largely disease-independent. 80% of our BPH sections and over 70% of our prostate cancer specimens displayed severe inflammation but only less than 15% of non-diseased sections did. We found that the inflammation was also associated with reactive hyperplasia and stromal desmoplasia and that most likely high survivin expression in diseased prostates was caused by increased inflammation in those specimens.

Uncontrolled cell proliferation in an inflammatory environment rich in growth factors and activated stroma could potentiate and/or promote neoplasia. Increased expression of the survival proteins survivin, Bcl-2, Bcl-xL and Mcl-1 are associated with prostate cancer and inhibiting their expression sensitizes cells to cytotoxic treatments. In addition, down-regulation or inhibition of these survival proteins results in increased chemosensitivity in prostate cancer cell lines. These

findings have prompted the hypothesis that chronic inflammation is involved in the genesis and/or progression of prostate cancer.

The immune cell make up of this *E. coli* induced inflammation matches that of human infectious inflammation with early neutrophilic infiltrate that dominates in the first 2 days of induction, and progresses to a primarily lymphocytic and monocytic infiltrate in days 3–5. Further, inflammation in this model showed increased expression of several inflammatory mediators including IL-1β, IL-6, COX-2, IGF-1, and FGFs, commonly observed in chronic prostatic inflammation. This data lead us to Chapter 4, APE1/Ref-1 Redox-Specific Inhibition Decreases Survivin Protein Levels and Induces Cell Cycle Arrest in Prostate Cancer Cells. APE1/Ref-1 regulates a number of transcription factors activated by these inflammatory mediators.

In this study we investigated APE1/Ref-1's role in regulating survivin protein levels in prostate cancer cells. Inflammatory signaling is notorious in cancer and prostate cancer cells are known to express IL-8 and IL-6 autocrine/paracrine signaling mimicking inflammatory signaling from the microenvironment. Not surprisingly, we found that both survivin and APE1/Ref-1 were overexpressed in primary and metastatic prostate tumors as previously reported in other publications. This upregulation was also found to be true in our

prostatic cell lines. Survivin and APE1/Ref-1 are known to be upregulated in response to inflammatory signaling.

Survivin is transcriptionally regulated by a number of transcriptional activators including Sp1 as well as STAT3 and NFκB, which are regulated by APE1/Ref-1. We investigated NFκB-driven survivin protein expression due to our observation that APE1/Ref-1 inhibition is effective in PC-3 cells despite their lacking the gene coding for STAT3. All four cell lines express functional NFκB signaling though it should be noted that survivin has been shown to be regulated by other APE1/Ref-1 regulated transcription factors like AP-1, HIF1α and p53. Similar to other cancer cell lines, we found that APE1/Ref-1 siRNA knockdown decreased cell proliferation and survivin protein levels. Also treatment with redox specific inhibitors APX3330 and APX2009 decreased cell proliferation, survivin mRNA and survivin protein levels. Additionally, we demonstrated that redox inhibition of APE1/Ref-1 redox activity induces G1 cell arrest in prostate cancer, consistent with recent reports of APE1/Ref-1 inhibition in other cancers. APE1/Ref-1 is crucial in moving cells from G1 to S, and redox inhibition induces key cyclin-dependent kinase inhibitors (CDKi's) like p21 and p27 and both of this CDKi's have been shown to downregulate survivin.

Survivin plays an important role in prostate cancer survival, progression and therapeutic resistance. Thus, inhibition of APE1/Ref-1's redox function in a viable therapeutic strategy to shutting down inflammatory signaling and reducing survivin protein levels.

Multiple mechanisms including unfavorable tumor microenvironment, drug efflux transporters, and alterations in microtubule structure/function and apoptotic defects have been shown to contribute to taxane resistance. Taxane resistance is found innately in 50% of metastatic castrate-resistant prostate cancer patients and those who respond ultimately develop acquired resistance. APE1/Ref-1 is known to regulate inflammatory processes, drug efflux transporter protein levels and survival protein levels in cancer. This led us to investigate APE1/Ref-1 redox inhibition as a possible therapeutic option for docetaxel-resistance in prostate cancer in Chapter 5.

We generated two docetaxel-resistant prostate cancer cell lines to address this question; DocR PC-3 and DocR C4-2. These two specific cell lines are considered castrate-resistant, meaning they do not need androgens to survive, and they differentially express the androgen receptor (AR); C4-2 cells have the AR while PC-3 cells do not. The DocR C4-2 cells were remarkably more sensitive to docetaxel than the DocR PC-3 cells. This was most likely due to having an

intact p53 as it has been shown that prostate cancer cells with a functional p53, like C4-2 cells, are more sensitive to docetaxel than those with mutant or null p53 expression, like PC-3 cells.

We found that the docetaxel-resistant cell lines were less sensitive to docetaxel than the parental cell lines as expected. Interestingly, the docetaxel-resistant cells lines were more sensitive to APE1/Ref-1 redox inhibition than the parental cell lines. This could be due to increased signaling pathways regulated by redox signaling (increased inflammatory signaling), DNA damage induced by taxanes (due to a prolonged mitotic phase) or altered cellular adhesion (FAK kinase phosphorylation). [298] In the APX3330 and docetaxel combination experiments, we observed an initial decrease in cell number by APX3330 or docetaxel pretreatment was but when combined the two drugs did not induce synergy and the curves converged at higher doses. This was contrary to what was hypothesized. This may be due to the cell phase at which these cells are entering cell arrest. It is known that docetaxel does not affect non- or slow-growing cells and primarily arrests cancer cells in G2/M. [299] We have shown that APE1/Ref-1 redox inhibition causes G1 arrest and this could explain why the two drugs are not complimenting each other *in vitro* as both actions occur in different phases. It could also be the length of the experiment as we only did 72

hours and if these cells are entering autophagy than we need a longer experimental window to visualize the differences in cell viability.

Interesting we found that APE1/Ref-1 protein levels decreased with treatment of docetaxel in DocR PC-3 cells. It has recently been shown in endothelial and immune cells that APE1/Ref-1 is non-classically secreted into the microenvironment. This lead to the hypothesis that APE1/Ref-1 was being released into the extracellular space upon treatment with docetaxel (cell stress). This was found to be true in both docetaxel-resistant cell lines. The function of extracellular APE1/Ref-1 has not been fully elucidated but appears to be associated with its redox domain. Extracellular APE1/Ref-1 may be playing a role in receptor-mediated signaling which is important in a number of different conditions. This discovery is novel in the prostate field and more research is needed to determine if the extracellular is just a biomarker or something with fundamental activity for prostate cancer survival.

Together, these three chapters intimately tie inflammation with survivin with APE1/Ref-1 being a master regulator of the survival signaling necessary for survivin transcription in prostate cancer.

**Future Directions**

**APE1/Ref-1 mediating Androgen Receptor activity**

APE1/Ref-1 is known to redox regulate a number of inflammatory transcription factors but its role in regulating steroid receptors has yet to be fully elucidated. Recently it has been shown that APE1/Ref-1 and the Estrogen Receptor Alpha (ERα) interact and that APE1/Ref-1 enhances ERα binding to estrogen-response elements (ERE). [300] Furthermore, the authors showed that treatment with APX3330 decreased ERα enrichment of ERE's in PR and pS2 genes suggesting that ER activity is regulated by redox signaling. Since all nuclear steroid receptors share significant homology, I hypothesis that APE1/Ref-1 directly interacts with the AR and redox regulates its activity on androgen-response elements (ARE). This is especially significant in metastatic prostate cancer since castrate-resistance is ultimately inevitable.

To determine if the AR and APE1/Ref-1 are co-expressed in prostate cancer, first I would perform immunofluorescence on cancerous and non-cancerous human prostate specimens. Once I confirmed that, I would collect cell lysate from AR+ prostatic cell lines, LNCaP, C4-2 and E7, which we culture in our lab and run an immunoblot to see if the pattern matches the human prostate specimen data. A prostatic cell line that is AR- , like PC-3 or DU145, could be used as a negative control. Furthermore, to verify interaction between the AR and

APE1/Ref-1 I would perform co-immunoprecipitation after treatment with either ethanol or 1 nM DHT. DHT is being used to activate the AR and induce nuclear translocation. Cellular fractionation could also be used to verify AR nuclear translocation and APE1/Ref-1 localization in response to DHT. I would expect that the AR would be immunoprecipitated with APE1/Ref-1 and APE1/Ref-1 immunoprecipitated with the AR under the presence of DHT.

If APE1/Ref-1 immunoprecipitates with the AR, I would then investigate the functional consequences of this interaction on AR-mediated transcription. First I would knockdown APE1/Ref-1 with siRNA and treat with Ethanol or DHT and then via western blot measure AR-mediated proteins PSA and NKX3.1. PSA and NKX3.1 are under androgen-dependent transcription in prostatic luminal epithelial cells. If I see a decrease in PSA and NKX3.1 protein levels under APE1/Ref-1 knockdown, I would repeat this experiment and run RT-PCR. To measure steady state mRNA. If PSA and NKX3.1 relative gene expression is altered, this would suggest that APE1/Ref-1 is regulating AR activity.

The ability of APE1/Ref-1 to interact with the AR and influence AR-mediated transcription suggests that APE1/Ref-1 might associate with regulatory regions of androgen-responsive genes to influence transcription. To determine this, I would perform ChIP using the AREs in the PSA and NKX3.1 genes in the

presence and absence of DHT. I would hypothesize that with increasing concentrations of DHT and time, the APE1/Ref-1/ AR complex's association with the ARE's would increase though it is not necessary for APE1/Ref-1 to be part of the transcriptional complex and still regulate the AR's ability to bind DNA. I would validate this using gel mobility shift assay to show that APE1/Ref-1 influences receptor-DNA complex formation. I would hypothesize that as increasing amounts of purified APE1/Ref-1 were added to, the ability of the purified AR to bind to ARE-containing oligos increased in a concentration-dependent manner and that the addition of AR or APE1/Ref-1 antibodies would produce a supershift.

Given that decreased Ape1/Ref-1 expression altered expression of androgen-responsive genes and that Ape1/Ref-1 was able to increase AR-ARE DNA complex formation, I would next investigate whether inhibiting the DNA repair or redox activity of Ape1/Ref-1 might affect endogenous androgen-responsive gene expression. First, I would repeat past experiments and instead of knocking down APE1/Ref-1 I would treat with increasing concentrations of APX3330 and measure PSA and NKX3.1 relative gene expression and protein levels. I would then do another gel mobility shift assay to measure whether APE1/Ref-1 redox activity regulates AR-ARE complex formation. For these experiments, I would utilize increasing amounts of APX3330 and the redox dead

APE1/Ref-1 mutant C65A to further support AR redox regulation. Finally, I would then repeat the previous ChIP experiments with APX3330 to investigate if APE1/Ref-1 redox inhibition decreases APE1/Ref-1 and AR enrichment of ARE's in prostatic cell lines.

This would show that AR transcriptional activity is regulated by APE1/Ref-1's redox activity **(Figure 33)**. This would be novel and of high significance. Future studies could incorporate enzalutamide/abiraterone resistance and possible new therapeutic strategies for metastatic prostate cancer.

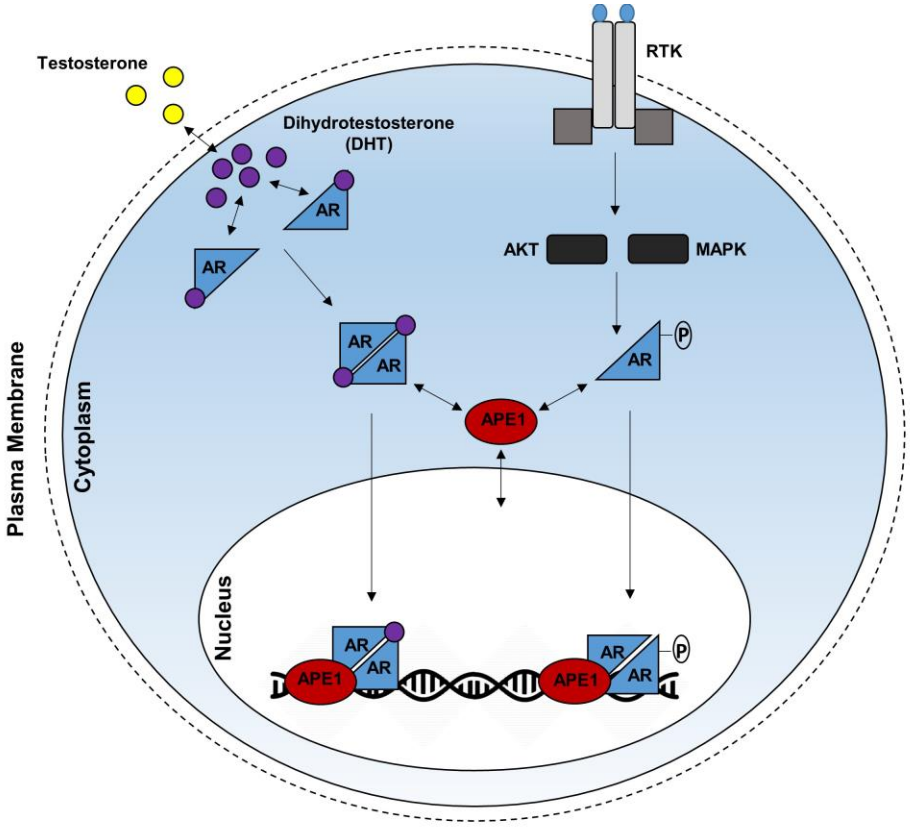



**Figure 33.** APE1/Ref-1 mediating Androgen Receptor activity

The androgen receptor (AR) can be activated two ways; ligand-mediated and kinase-mediated. The canonical AR signaling pathway involves the ligand dihydrotestosterone (DHT) binding to an AR monomer causing it to dimerize with another AR monomer and then translocating to the nucleus. The non-canonical AR signaling pathway involves the transactivation of an AR monomer by a kinase (AKT or MAPK) resulting in the dimerization and nuclear translocation of the receptor. APE1/Ref-1 could potentially redox regulate the AR in the cytoplasm or nucleus and also function as a cofactor in the nucleus.

**Taxane-resistance mechanisms**

Taxane-resistance occurs in all metastatic castrate resistant prostate cancer patients overtime. The mechanisms for taxane-resistance are most likely multifaceted with multiple different pathways contributing. Some possible mechanisms include induction of survival proteins (IAP and Bcl-2 family of proteins), drug efflux/intake transporters (ATP-binding cassette transporters (ABC's) and Solute carrier transporters (SLC's), metabolic shifts (aerobic glycolysis to oxidative phosphorylation) and exosome/oncosome/microvesicle secretion. ABC transporters, like p-glycoprotein (P-gp), are upregulated in response to docetaxel and play a role in docetaxel's extrusion from the cell. Despite this, directly targeting these transporters have yet to yield any successful therapeutics. Recently it has been shown that the downregulation of certain intake transporters, specifically SLCO1B3, may contribute to docetaxel-resistance. [301] I hypothesize that the upregulation of ABC transporters and the downregulation of SLC transporters are needed for taxane-resistance.

To determine if P-gp is upregulated and SLCO1B3 is downregulated in response to docetaxel resistance I would first collect cell lysate from both the parental and docetaxel-resistant PC-3/C4-2 cell lines and measure protein expression via immunoblotting. If there was a difference in parental and

docetaxel-resistant cell levels of these two proteins I would then do a concentration response curve with docetaxel to determine if this difference is transient or permanent and what time peak levels occurs. I would also couple this with RT-PCR to see if the changes in protein levels are in result to altered transcription or protein stability.

If P-gp and SLCO1B3 are altered at both the transcript and protein levels with respect to docetaxel-resistance, I would then proceed to modify the levels of the endogenous proteins and perform a concentration response curve to investigate changes to the docetaxel $IC_{50}$. I would use siRNA to knock down P-gp and I would overexpress SLCO1B3 using a transient plasmid. I would hypothesis that alone P-gp siRNA knockdown or SLCO1B3 overexpression would decrease the docetaxel $IC_{50}$ and this effect would be enhanced when the two methods were combined. I would also measure the cellular efflux and uptake of docetaxel using a [14C] labeled-docetaxel to further validate the change of intracellular docetaxel concentration.

The ability of P-gp knockdown and/or SLCO1B3 overexpression to alter intracellular docetaxel concentrations suggests that dual targeting these proteins is a logical therapeutic strategy. To validate this *in vivo*, I would infect the docetaxel-resistant cells with an inducible SLCO1B3 construct and P-gp siRNA

and then xenotransplant these cells into a nude mouse prostate. I would then treat these animals with docetaxel and the inducible construct drug and at the end of 6 weeks measure tumor size and local invasion. I would hypothesize that in those animals where SLCO1B3 is overexpressed and P-gp is downregulated the tumors would experience significant cell growth inhibition compared to those with single protein and no protein alterations.

This would show that P-gp and SLCO1B3 are potential targets in taxane-resistant prostate cancer **(Figure 34)**. This would be novel and of high significance and future studies could incorporate inhibitors/gene therapy as potential therapeutic strategies. Other cancers that use taxanes as the standard of care, like breast cancer, could benefit from this discovery.

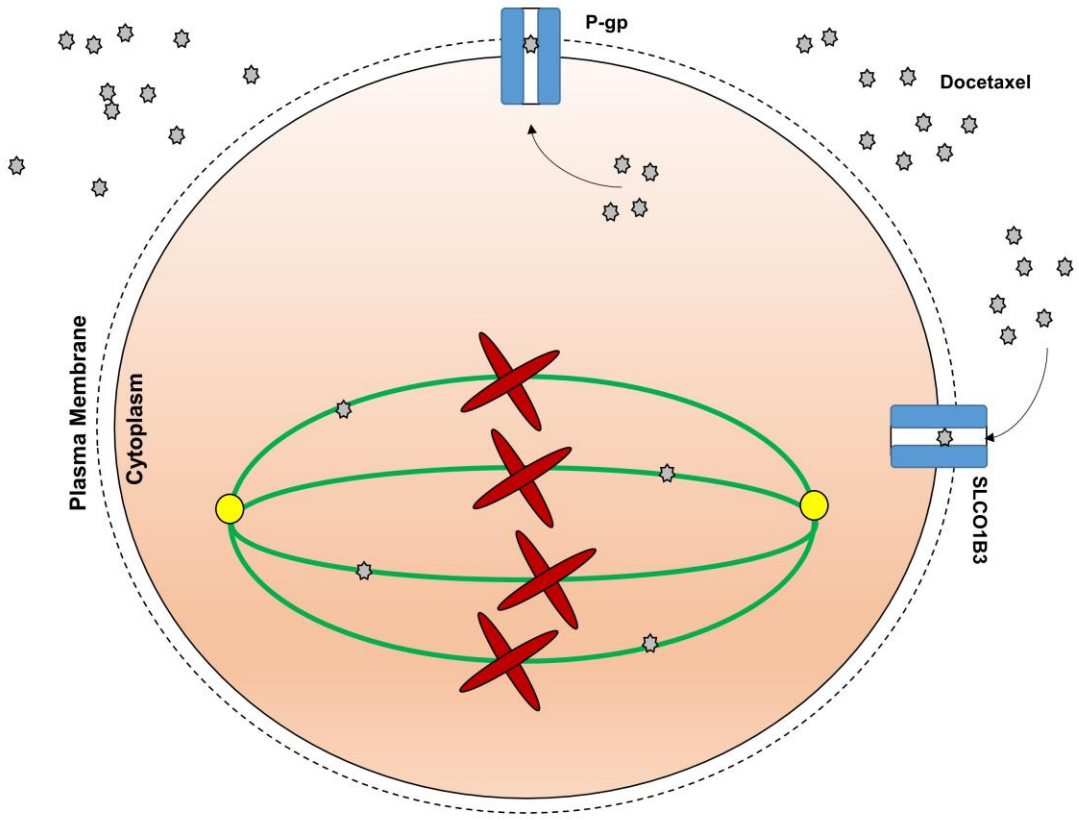



**Figure 34.** Taxane-resistance mechanisms

ABC transporters, like p-glycoprotein (P-gp), are upregulated in response to docetaxel and play a role in docetaxel's extrusion from the cell. Recently it has been shown that the downregulation of certain intake transporters, specifically SLCO1B3, prevents the accumulation of docetaxel within the cell. The less docetaxel within the cell equals less microtubule disruption. Together, these two transporters may be responsible for acquired and innate docetaxel-resistance.

**Extracellular APE1/Ref-1**

APE1/Ref-1 has recently been identified as a non-classically secreted protein and has yet to be identified as secreted by the prostatic cells. Secreted APE1/Ref-1 has been found in the serum and urine in patients with bladder cancer and serum APE1/Ref-1 autoantibodies have been found in patients with lung cancer suggesting its use as a possible biomarker. [302-303] Underlining mechanisms of APE1/Ref-1 secretion are just now being discovered and APE1/Ref-1's extracellular roles have yet to be elucidated. Treatment with histone deactylase inhibitor trichostatin A has been found to cause the nuclear to cytoplasmic translocation of APE1/Ref-1 and extracellular release in HEK293 cells without changing cell viability. Interestingly, the authors found that mutating lysine 6 and 7 to arginines prevented secretion suggesting protein acetylation might be necessary for excretion. APE1/Ref-1 is acetylated in most cancers. [304] In endothelial cells, secreted APE1/Ref-1 has found to be anti-inflammatory and alter TNFα receptor activity but in immune cells the addition of extracellular APE1/Ref-1 appears to be pro-inflammatory altering IL-6 signaling. [305] I hypothesis that APE1/Ref-1 is secreted by prostate cancer and that extracellular APE1/Ref-1 contributes to docetaxel resistance through activating autocrine/paracrine or immune cell-inflammatory signaling **(Figure 35)**. It would

be necessary to obtain serum and urine from prostate cancer patients to verify that APE1/Ref-1 can be extracellularly located.

To investigate whether APE1/Ref-1 is secreted and if its secretion is induced by docetaxel, which is a form of cell stress, I would treat parental and docetaxel-resistant cells with their respective docetaxel $IC_{50}$'s and then collect the supernatant and cell lysates at various time points. I would precipitate out the supernatant proteins using the cold acetone method and measure the levels of APE1/Ref-1 via immunoblotting. Once I found the optimal time where APE1/Ref-1 secretion is at its highest I would then treat the cells with increasing concentrations of docetaxel to see if the secretion is both time and concentration dependent. Taken together this should demonstrate that APE1/Ref-1 secretion is stimulated by docetaxel. The exact amount of secretion would be measured via ELISA.

Recently it has been shown that purified recombinant APE1/Ref-1 increased IL-6 production and secretion in monocytic cell line THP-1. I hypothesize that docetaxel-induced APE1/Ref-1 secretion increases IL-6 in the surrounding microenvironment causing an IL-6-dependent inflammatory response. To determine this I would treat parental and docetaxel-resistant prostate cancer cells with recombinant APE1/Ref-1 and look at IL-6 transcription

(RT-PCR) and IL-6 secretion (ELISA). Next I would treat prostate cancer cells with docetaxel to see if the IL-6 pathway is activated and if neutralizing APE1/Ref-1 with an antibody would perturb that activation. If the IL-6 pathway is disrupted, I would move onto a 3-D culture and repeat the previous experiments to see if the results hold up. 3-D culture allows for the co-culturing of different cell lines and it has been shown in previous work by Dr. Melissa Fishel that the IL-6 signaling is hyper activated in this system. It is possible that this secreted APE1/Ref-1 is meant for other cell types in the tumor microenvironment. Co-culturing with cancer-associated fibroblasts or an immortalized immune cell line could help answer that question.

IL-6 has long been implicated in the initiation and progression of prostate cancer. Knowing the mechanism of how IL-6 is upregulated is of paramount importance in the search of new therapeutic targets in drug-resistant prostate cancer. If extracellular APE1/Ref-1 is implicated in the activation of the IL-6 pathway potential neutralizing antibodies for both proteins could be used to shut down that inflammatory response.

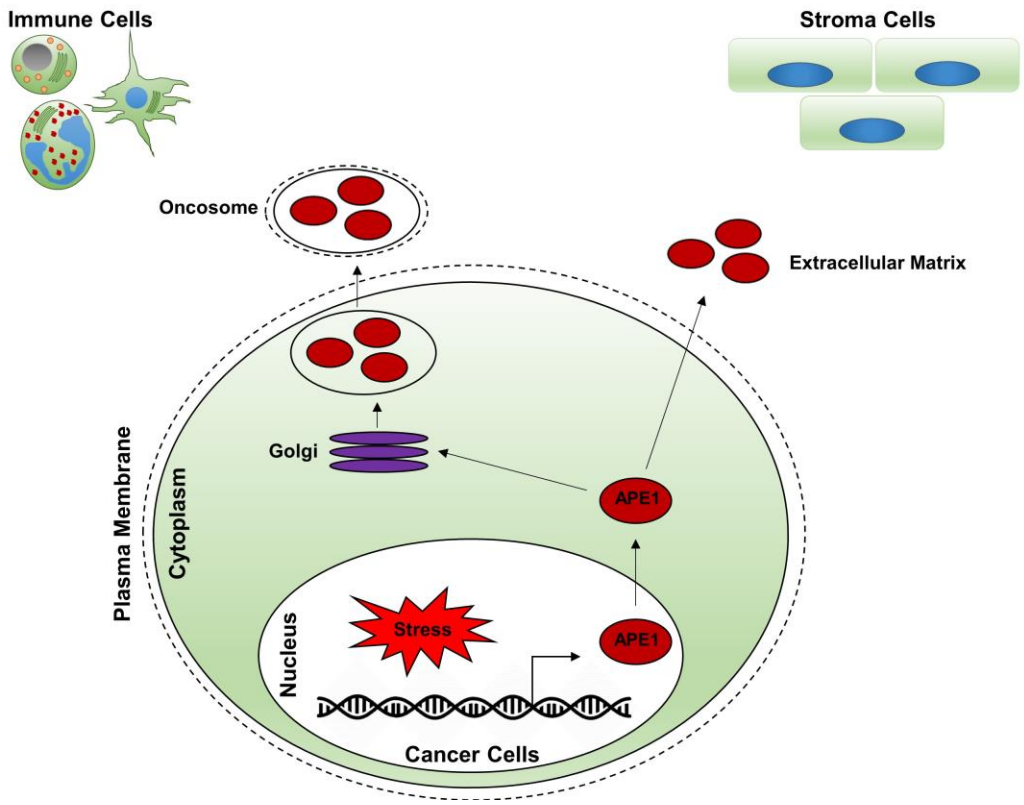



**Figure 35.** Extracellular APE1/Ref-1 in prostate cancer

APE1/Ref-1 is a non-classically secreted protein. Secreted APE1/Ref-1 has been found in the serum in patients with bladder cancer and serum APE1/Ref-1 autoantibodies have been found in patients with lung cancer. Underlying mechanisms of APE1/Ref-1 secretion are unknown but could be the result of oncosomal packaging or direct release into the extracellular space. The function of extracellular APE1/Ref-1 is also not known but could play a role in the inflammatory tumor microenvironment.

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# CURRICULUM VITAE

David W. McIlwain

## EDUCATION

Ph.D. in Pharmacology 2017

Indiana University, Indianapolis, IN

B.S. in Neurobiology & Physiology 2011

Purdue University, West Lafayette, Indiana

## ACADEMIC AND PROFESSIONAL HONORS

Howard Hughes Medical Institute Undergraduate Research Internship

May 2008 – September 2008

Podium Selection: 2014 John P. Donohue Visiting Professor Series & First Annual Indiana Basic Urological Research Symposium. “APE1/Ref-1 regulates survivin-mediated drug resistance in prostate cancer cells” February 28, 2014, Indianapolis, IN, USA.

Indiana-CTSI Predoctoral Training Award in Translational Research

July 2014 – July 2016

Poster Award Winner (KL2, TL1) – Indiana Clinical and Translational Sciences Institute Sixth Annual Meeting; September 26, 2014, Indianapolis, IN, USA.

Paradise Travel Award Recipient - Society of Basic Urological Research 2015 Fall Symposium. “APE1/Ref-1 regulates survivin protein expression in prostate cancer cells” November 12-15, 2015, Fort Lauderdale, FL, USA.

SBUR Travel Award Recipient – Society for Basic Urological Research 2015 Fall Symposium. “APE1/Ref-1 regulates survivin protein expression in prostate cancer cells” November 12-15, 2015, Fort Lauderdale, FL, USA.

Podium Selection: 2016 John P. Donohue Visiting Professor Series & Third Annual Indiana Basic Urological Research Symposium. “APE1/Ref-1 regulates survivin protein expression in castrate-resistant prostate cancer” February 26, 2016, Indianapolis, IN, USA.

Burroughs Wellcome Fund Trainee Travel Award Recipient – Association for Clinical and Translational Science (ACTS) 2016 Meeting. “APE1/Ref-1 regulates survivin protein expression in castrate-resistant prostate cancer” April 13-15, 2016, Washington DC, USA.

Oral Presenter (1 of 10 TL1 Trainees Selected) – Association for Clinical and Translational Science (ATCS) 2016 Meeting. “APE1/Ref-1 regulates survivin protein expression in castrate-resistant prostate cancer” April 13-15, 2016, Washington DC, USA.

## PROFESSIONAL SOCIETIES

Student National Pharmaceutical Association (SNPhA), 2007-2008

Bath University Biosciences Society, University of Bath, England, 2008-2009

Biology Club, Purdue University, 2008 -2011

Society for Basic Urological Research (SBUR), 2013 – Present

Association for Clinical and Translational Science (ACTS), 2014 - Present

## PUBLICATIONS AND PRESENTATIONS

Publications

**McIlwain DW**, Zoetemelk M, Myers JD, Edwards MT, Snider BM, Jerde TJ. 2016. Coordinated Induction of Cell Survival Signaling in the Inflamed Microenvironment of the Prostate. *The Prostate.* 76:722-734. (Published online 24 Feb 2016)

**McIlwain DW**, Fishel ML, Kelley MR, Jerde TJ. 2017. APE1/Ref-1 Redox-Specific Inhibition Decreases Survivin Protein Levels and Induces Cell Cycle Arrest in Prostate Cancer Cells. *Oncotarget.* 2017.

Presentations

**McIlwain DW**, Fishel ML, Wang L, Snider BM, Zhang JT, Kelley MR, Jerde TJ: APE1/REF-1 regulates survivin-mediated drug resistance in prostate cancer cells. Presented at the Annual meeting of the American Urological Association, San Diego, CA, May 6, 2013.

**McIlwain DW**, Fishel ML, Wang L, Snider BM, Zhang JT, Kelley MR, Jerde TJ: APE1/REF-1 regulates survivin-mediated drug resistance in prostate cancer cells. Presented at the Annual IU Simon Cancer Center “Cancer Research Day 2013” IUPUI, Indianapolis, IN, May 22, 2013

**McIlwain DW**, Fishel ML, Wang L, Snider BM, Zhang JT, Kelley MR, Jerde TJ: APE1/REF-1 regulates survivin-mediated drug resistance in prostate cancer cells. Presented at the Annual meeting of the Society for Basic Urological Research (SBUR) “10th World Congress on Urological Research”, Nashville, TN, November 21-24, 2013

**McIlwain DW**, Fishel ML, Wang L, Zhang JT, Kelley MR, Jerde TJ: Redox Factor 1 regulates drug resistance in prostate cancer cells via survival protein induction. Presented at the American Association for Cancer Research-Prostate Cancer Foundation Special Conference on Advances in Prostate Cancer Research, San Diego, CA, January 18-21, 2014

**McIlwain DW**, Fishel ML, Wang L, Snider BM, Zhang JT, Kelley MR, Jerde TJ: APE1/REF-1 regulates survivin-mediated drug resistance in prostate cancer cells. 2014 John P. Donohue Visiting Professor Series & First Annual Indiana Basic Urological Research Symposium, Indianapolis, IN, February 28, 2014

**McIlwain DW**, Fishel ML, Wang L, Snider BM, Zhang JT, Kelley MR, Jerde TJ: APE1/REF-1 regulates survivin-mediated drug resistance in prostate cancer cells. Presented at the Annual IU Simon Cancer Center “Cancer Research Day 2014” IUPUI, Indianapolis, IN, May 29, 2014

**McIlwain DW**, Fishel ML, Wang L, Snider BM, Zhang JT, Kelley MR, Jerde TJ: APE1/REF-1 regulates survivin-mediated drug resistance in prostate cancer cells. Presented at The Indiana Clinical and Translational Sciences Institute Sixth Annual Meeting, Indianapolis, IN, September 26, 2014

**McIlwain DW**, Fishel ML, Wang L, Snider BM, Zhang JT, Kelley MR, Jerde TJ: APE1/REF-1 regulates survivin-mediated drug resistance in prostate cancer cells. Presented at the Annual meeting of the Society for Basic Urological Research (SBUR), Dallas, TX, November 13-16, 2014

**McIlwain DW**, Fishel ML, Wang L, Snider BM, Zhang JT, Kelley MR, Jerde TJ: APE1/REF-1 regulates survivin-mediated drug resistance in prostate cancer

cells. Presented at the Association for Clinical and Translational Science (ACTS) 2015 Meeting, Washington, DC, April 16 -18, 2015

**McIlwain DW**, Fishel ML, Wang L, Snider BM, Zhang JT, Kelley MR, Jerde TJ: APE1/REF-1 regulates survivin-mediated drug resistance in prostate cancer cells. Presented at the Annual IU Simon Cancer Center "Cancer Research Day 2015" IUPUI, Indianapolis, IN, May 21, 2015

**McIlwain DW**, Fishel ML, Wang L, Snider BM, Zhang JT, Kelley MR, Jerde TJ: APE1/REF-1 regulates survivin protein expression in castrate-resistant prostate cancer. Presented at the Annual meeting of the Society for Basic Urological Research (SBUR), Fort Lauderdale, FL, November 12-15, 2015.

**McIlwain DW**, Fishel ML, Wang L, Snider BM, Zhang JT, Kelley MR, Jerde TJ: APE1/REF-1 regulates survivin protein expression in castrate-resistant prostate cancer. 2016 John P. Donohue Visiting Professor Series & Third Annual Indiana Basic Urological Research Symposium, Indianapolis, IN, February 26, 2016

**McIlwain DW**, Fishel ML, Wang L, Snider BM, Zhang JT, Kelley MR, Jerde TJ: APE1/REF-1 regulates survivin protein expression in castrate-resistant prostate cancer. Presented at the Association for Clinical and Translational Science (ACTS) 2016 Meeting, Washington, DC, April 13-15, 2016