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Regulation of C-X-C Chemokine Receptor 7 by Androgen Receptor in Prostate Cancer

James Joseph Hoy

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REGULATION OF C-X-C CHEMOKINE RECEPTOR 7 BY ANDROGEN RECEPTOR IN PROSTATE CANCER

By

James Joseph Hoy

A DISSERTATION

Submitted to the Faculty
of the University of Miami
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Doctor of Philosophy

REGULATION OF C-X-C CHEMOKINE RECEPTOR 7 BY ANDROGEN
RECEPTOR IN PROSTATE CANCER

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The atypical C-X-C chemokine receptor 7 (CXCR7) is implicated in supporting aggressive cancer phenotypes in several cancers including prostate cancer. However, the mechanisms driving overexpression of this receptor in cancer are poorly understood. The aim of this current study was to investigate the role of CXCR7 in the progression to lethal castration resistant prostate cancer (CRPC).

CXCR7 expression was measured after androgen receptor (AR) signaling was modulated through androgen deprivation, siRNA-mediated depletion of AR or antiandrogen treatment. Chromatin immunoprecipitation (ChIP) assays were performed to investigate the interaction of AR with the CXCR7 promoter. Clustered regularly interspaced short palindromic repeats (CRISPR) - Cas9 technology was used to abrogate expression of CXCR7. Immunoblotting and real-time quantitative PCR were performed to analyze downstream signaling.

Androgen deprivation or chemical inhibition of AR significantly increased CXCR7 expression in androgen-responsive prostate cancer cell lines. Depletion of AR by siRNA prevented androgen-mediated suppression of CXCR7, indicating this phenotype is dependent on AR. Increased CXCR7 expression during
androgen deprivation facilitated enhanced epidermal growth factor receptor (EGFR)-mediated mitogenic signaling, promoting tumor cell survival through an androgen-independent signaling program. ChIP assays showed that AR binds to the CXCR7 promoter, demonstrating that AR directly down-regulates CXCR7 mRNA transcription, a suppressive mechanism which is disrupted when AR signaling is interrupted.

CRISPR-Cas9-mediated knockout of CXCR7 revealed that prostate cancer cells depend on CXCR7 for proliferation, survival and clonogenic growth potential. Loss of CXCR7 expression by CRISPR-Cas9 gene editing resulted in the halt of cell proliferation, severely impaired EGFR signaling, and the onset of cellular senescence. LNCaP cells expressing a CRISPR-Cas9-derived CXCR7 mutant (characterized by a 392 nucleotide internal deletion, but maintaining the reading frame) remained proliferative in culture, but exhibited altered interactions between CXCR7 and scaffold protein and reduced mitogenic signaling potential. A xenograft study showed that the mutant line had substantially reduced tumorigenicity and growth rate relative to the wild-type (WT) LNCaP cell line.

Together these results demonstrate that increased CXCR7 expression resulting from AR signaling inhibition drives EGFR-mediated, androgen-independent prostate cancer cell survival. Targeting CXCR7 expression as a combination treatment to androgen deprivation therapy may prevent androgen-independent growth and survival involved in therapeutic resistance and the progression to lethal CRPC.
This work is dedicated to my mother. Your commitment to family and ceaseless hard work has ingrained in me qualities of persistence, humility and humanity. I would not be the person I am without you, and know I can always count on you for your unwavering support in all of my future endeavors.
ACKNOWLEDGEMENTS

This work could not have been accomplished without the constant support of my research mentor Dr. Bal Lokeshwar. I am grateful for all of the helpful conversations, critiques, planning and troubleshooting that he always found time to provide. My growth as a scientist, perseverance, and interest in continuing to study cancer biology can all be attributed to his kind help and belief in my abilities which will never be forgotten.

Thanks to the members of our lab group, both past and present, for all of their help and support over the years. I thank Dr. Nicole Salazar, Dr. Lei Zhang, Dr. Shamaladevi Nagarajarao, Daniel Munoz and Maite Lopez for all of the discussions and suggestions throughout my work at the University of Miami which has helped me develop the skills and critical thinking necessary to succeed. I grateful to Dr. Georgios Kallifatidis for assisting me in all aspects of my work, from troubleshooting experiments to teaching me how to write scientific articles as well as his willingness to share his expertise and experience whenever needed. I also thank Dr. Jie Gao and Diandra Smith for assistance in the lab at Augusta University. I would also like to acknowledge Dr. Vinata Lokeshwar and her laboratory including Andre Jordan and Luis Lopez for assistance in various aspects of this work.

I thank my committee members: Dr. Karoline Briegel, Dr. Mark Gonzalgo, Dr. Guy Howard, and Dr. Mike Xu, for all of their support, patience and invaluable
suggestions over the years as my project developed and evolved to get to this point.

I am thankful to all of my friends for their support throughout this process, in particular Santas Rosario. Her helpful discussions, assistance with experimental procedures, advice and encouragement through both the best and worst times made a truly positive impact on me that cannot be overstated.

I am absolutely grateful and I thank my family for their support and encouragement throughout my life. To my mother, Ann Hoy for teaching me to apply myself, to work hard, and to always be good to others, and for always believing in me, sure I would reach any goal I had. My siblings: Mary Hoy, Steve Hoy and Danyelle Hoy, who were always my best friends and continue to cheer me on. And to my Grandfather, James O. Hoy, and Father, James M. Hoy, who taught me the value of hard work and ensured I made it to Miami to start this journey.
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<td>ADT</td>
<td>Androgen deprivation therapy</td>
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<td>AR</td>
<td>Androgen receptor</td>
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<td>AR-V</td>
<td>Androgen receptor mRNA splice variant</td>
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<td>ARE</td>
<td>Androgen receptor response element</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Cas9</td>
<td>CRISPR associated protein 9</td>
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<tr>
<td>CDFBS</td>
<td>Charcoal-Dextran stripped fetal bovine serum</td>
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<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered regularly interspaced short palindromic repeats</td>
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<td>CRPC</td>
<td>Castration-resistant prostate cancer</td>
</tr>
<tr>
<td>Ct</td>
<td>Threshold cycle</td>
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<tr>
<td>CXCL</td>
<td>C-X-C chemokine ligand</td>
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<tr>
<td>CXCR</td>
<td>C-X-C chemokine receptor</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DHT</td>
<td>Dihydrotestosterone</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>EBRT</td>
<td>External beam radiation therapy</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>ERK (MAPK)</td>
<td>Extracellular signal-regulated protein kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FD</td>
<td>Fold difference</td>
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<tr>
<td>GnRH</td>
<td>Gonadotropin-releasing hormone</td>
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<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
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<tr>
<td>gRNA</td>
<td>CRISPR guide ribonucleic acid</td>
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<tr>
<td>HIC1</td>
<td>Hypermethylated in cancer 1</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin-8</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>LBD</td>
<td>Ligand binding domain</td>
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<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
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<tr>
<td>NFDM</td>
<td>Non-fat dry milk</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor Kappa B</td>
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<tr>
<td>NHEJ</td>
<td>Non-homologous end joining</td>
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<tr>
<td>PAM</td>
<td>Protospacer adjacent motif</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
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<tr>
<td>PLA</td>
<td>Proximity ligation assay</td>
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<tr>
<td>PPIA</td>
<td>Peptidylprolyl isomerase A</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate specific antigen</td>
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<tr>
<td>R1881</td>
<td>Methyltrienolone (non-hydrolyzable androgen analog)</td>
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<td>RCA</td>
<td>Rolling-circle amplification</td>
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<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>RP</td>
<td>Radical prostatectomy</td>
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<td>RTK</td>
<td>Receptor tyrosine kinase</td>
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<td>RT-q-PCR</td>
<td>Real-time quantitative polymerase chain reaction</td>
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<td>SA-gal</td>
<td>Senescence-associated β-galactosidase</td>
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<td>SDF-1</td>
<td>Stromal cell-derived factor-1</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>shRNA</td>
<td>Short hairpin ribonucleic acid</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering ribonucleic acid</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline + Tween-20</td>
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<tr>
<td>TSS</td>
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PUBLICATION NOTE

Chapters 3 and 4:

Versions of some material in these chapters have been accepted for publication in: Scientific Reports (Nature publishing group).
Chapter 1: INTRODUCTION

1.1: Introduction to prostate cancer: incidence and prognosis:

Prostate cancer is among the most common malignancies diagnosed in men worldwide [1]. As of 2017, it is estimated that nearly 161,000 men in the United States will be diagnosed with prostate cancer, accounting for approximately 20% of all male cancer diagnoses, while nearly 27,000 will die from the disease [2]. Fortunately, early diagnosis is common; routine digital rectal examination in combination with detection of prostate specific antigen (PSA) in patient blood samples is highly effective in accurately diagnosing the presence of organ-localized prostate cancer [3]. The 5 year survival rate of prostate cancer patients is near 100% when treated with either surgery or radiation while tumors are localized to the prostate [4-6]. However, a subset of aggressive prostate cancer cases do disseminate to other organs. These metastatic cases are initially treated with hormone-deprivation therapies and chemical inhibition of androgen receptor (AR). Unfortunately, these therapeutic interventions invariably fail due to the development of hormone-depletion resistance, also known as castration-resistant prostate cancer (CRPC). Survival in patients with CRPC is significantly reduced, with fewer than 30% surviving past 5 years [7]. Even with early diagnosis and multiple treatment phases, aggressive prostate cancer cases continue to escape current therapeutic interventions and drive the mortality rate of this disease.

Prediction of aggressive cancer cases is vital to directing optimal treatment options (where they are needed), while avoiding these invasive and harsh therapies for patients with the more common, indolent disease. The well-
established clinical method for predicting patient risk considers three factors in identifying aggressive disease: blood PSA levels, Gleason score of prostate cancer tissue, and clinical staging of the disease [8, 9]. Elevated blood PSA (≥ 4 ng/mL) was established as a marker for prostate cancer detection in 1994, and has remained one of the most useful non-invasive tools to detect and monitor prostate cancer. Furthermore, measurement of blood PSA is an invaluable method to indicate cancer progression in patients and to identify recurrent disease after therapy [10]. PSA may also be useful as a predictive marker in men to assess their risk for developing prostate cancer [11, 12]. While PSA is accepted as an important marker for cancer detection, it cannot be used as an independent diagnostic or prognostic marker.

If a patient presents with elevated PSA and a routine digital rectal examination and/or transrectal ultrasonography reveal abnormalities in the prostate, core needle biopsies are performed to histologically confirm the presence of cancerous tissue. The Gleason score has become an accepted determinant of the metastatic potential of prostate cancer, and thus can be used for the diagnostic stratification of patients. The Gleason scoring method established in 1967 [13] has been the standard for clinically grading prostate tumor tissue. Although it is less quantitative and more invasive than PSA testing, prostate cancer prognosis, aggressiveness and therapeutic resistance can be accurately predicted using this method. The Gleason score considers the differentiation of the prostate tissue on a scale from 1 (well-differentiated, small, uniform glands) to 5 (poorly-differentiated, few if any glands, sheets of cells). In a tissue sample, the most
commonly occurring histological grade is added to the second most common to get a combined score from 2 to 10 [14]. The prognostic correlation to Gleason score has changed several times following studies on patient outcome. Currently, there are five risk categories for patients to develop aggressive metastatic disease or recurrent cancer following therapy. The low-risk cohort includes patients with Gleason scores ≤ 6. Those with Gleason scores of 7 are divided into two groups: 3+4 which is lower risk, and 4+3, higher risk. Next are those with Gleason scores of 8, then the highest risk patients with Gleason scores of 9-10 [15, 16]. These divisions are important to directing the proper course of therapeutic intervention, as these options can carry severe impacts to quality of life for prostate cancer patients [17-20].

While there are lingering uncertainties regarding the exact PSA [21-26] or Gleason score values [27-29] for determining clinical outcome, it is clear that these methods for prostate cancer diagnosis and prognosis are indispensable in directing the course of action in treatment of patients. However, there remain many unresolved challenges associated with current therapeutic options, particularly for eliminating aggressive, metastatic disease.

1.2: Therapeutic options and resistance mechanisms:

Treatment options for prostate cancer are determined from staging of the disease as well as the prognosis based on Gleason score and PSA. Early, localized disease can be very effectively treated with either surgical resection of the prostate (radical prostatectomy [RP]), or radiation therapy: external beam radiation therapy (EBRT) or internal radiation therapy (brachytherapy) [17, 30]. To
date, there are no randomized studies which directly compare the outcome of patients who have undergone RP versus those who received radiation therapy, and it remains contentious as to which option offers the most effective cancer-free survival rate for patients, while also minimizing adverse side effects [31-33]. Regardless, both options have proven to be effective at eliminating localized, non-metastatic prostate cancer.

Unfortunately, aggressive metastatic disease still evades all therapeutic options, leading to patient mortality. First line systemic therapies for metastatic disease have historically relied on the requirement for androgen to drive prostate cell proliferation. Under normal physiological conditions, circulating testosterone enters prostate cells, is converted to dihydrotestosterone (DHT) by 5α-reductase, and binds to and activates AR which mediates the balance between cell proliferation and apoptosis [34]. In a healthy prostate, DHT is required to maintain organ homeostasis; however, as prostatic neoplasia arises, androgen signaling becomes the primary driver of tumor cell proliferation [35, 36]. Androgen deprivation therapy (ADT) through either surgical (orchiectomy) or chemical castration (gonadotropin-releasing hormone [GnRH] agonists) has been the preferred first-line therapeutic approach to treat metastatic prostate cancer for decades [37]. However, some adverse effects and resistance to these treatments exist. Initially, treatment with GnRH agonists leads to a flare of testosterone release which can temporarily promote prostate cancer tumor growth before it becomes beneficial. Additionally, these compounds do not target alternative sources of androgens, such as those synthesized from the adrenal glands [38].
Adrenal androgens such as dehydroepiandrosterone (DHEA) and androstenedione (AD) could be converted to DHT in prostate cancer cells, allowing for disease recurrence under ADT [39]. Following biochemical recurrence (increasing blood PSA) in these patients, secondary options for ADT include compounds, such as abiraterone acetate, which block enzymes involved in steroidogenesis and have been utilized to more efficiently eliminate androgen synthesis, improving the efficacy of ADT [40, 41]. Unfortunately, despite these efforts to eliminate circulating androgens, metastatic prostate cancer often develops resistance to ADT with a mean time to recurrence of about 2-3 years, progressing to CRPC [42].

Despite the profound reduction in circulating testosterone during ADT, prostate cancer cells develop mechanisms of resistance. One such mechanism is the overexpression of AR. With increased AR abundance, even exceedingly low levels of androgens are sufficient to drive proliferative signaling [43]. Furthermore, alternative signaling pathways can lead to activation of AR in a ligand-independent manner. Receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs) activate downstream kinases which can phosphorylate AR, allowing it to signal even in the absence of DHT [43]. Furthermore, mutations within the AR ligand binding domain (LBD) can reduce specificity for ligands. For example, point mutations at amino acid 878 of AR (converting threonine to alanine, T878A) or at amino acid 702 (converting leucine to histidine, L702H) allow AR to be activated when binding estrogen, progesterone, or corticosteroids [44]. Variants of AR derived from alternative splicing of its mRNA are also sources of androgen-
independent signaling. Constitutively active AR splice variants (AR-Vs) lack the LBD, which results in ligand-independent AR signaling and CRPC cell proliferation [43]. These alternative mechanisms of AR signaling (summarized in Figure 1.2.1) significantly impact both the response to ADT as well as the approaches to treat recurrent disease.

**Figure 1.2.1: Alternative AR activation pathways:** Under normal AR signaling, dihydrotestosterone (DHT) will bind to the AR ligand binding region (LBD) and stimulate translocation of AR to the nucleus where it regulates gene transcription. Under ADT, alternative mechanisms can develop, driving resistance to therapy. Mutations in the AR LBD (AR-mut) can allow activation of AR by alternative steroid hormones such as estrogen, progesterone, and corticosteroids. AR can also be activated through ligand-independent mechanisms. Receptor tyrosine kinases (RTKs) activated by circulating growth factors (GF) mediate ERK/MAPK activation, which can phosphorylate and activate AR in a ligand-independent manner. Furthermore, RTKs can activate Phosphoinositide 3-kinase (PI3K), leading to Akt phosphorylation, which can also activate AR. Alternatively, G protein-coupled receptors (GPCRs) activated by circulating chemokines (CK) can also stimulate Akt activation and subsequent AR phosphorylation. Finally, constitutively active AR mRNA splice variants (AR-Vs) lacking LBDs mediate chronic AR signaling in a ligand-independent manner.
Chemical AR antagonists have also been developed to directly inhibit AR signaling and are used in combination with ADT. The first anti-androgen compounds approved for secondary treatment following failure of ADT included bicalutamide, nilutamide, and flutamide. Bicalutamide became the primary option, as it could competitively bind to AR with a greater affinity than the other compounds and prevented conformational changes to the receptor, thus preventing downstream signaling [45, 46]. The major failure of these compounds involved mutations to AR which rendered these compounds agonistic, leading to progression of prostate cancer [45, 47]. The T878A mutation in the AR LBD as well as a histidine to tyrosine mutation at amino acid 875 (H875Y) have been implicated in both receptor promiscuity and agonistic activation by anti-androgens such as flutamide [44]. Additionally, long-term treatment with antiandrogen compounds can lead to alternative mechanisms of resistance, including overexpression of AR, allowing for proliferative signaling from exceedingly low levels of androgens or recruitment of alternative transcription cofactors, driving agonistic activity of the compounds [48, 49]. The more recent AR antagonist, enzalutamide, has been developed to offer greater AR inhibition while eliminating some of the complications of agonistic activity exhibited by the earlier compounds. Enzalutamide is a non-steroidal antiandrogen which binds to AR with a greater affinity than bicalutamide. Furthermore, this compound greatly reduces nuclear translocation of AR and prevents recruitment of transcriptional cofactors, thus eliminating much of the agonistic potential that bicalutamide presented [50]. A clinical trial comparing the two compounds also revealed significantly reduced risk
for prostate cancer progression and death in patients treated with enzalutamide [51]. Unfortunately, advanced prostate cancer develops resistance to enzalutamide as well. Several AR-Vs have been identified which render AR constitutively active and capable of signaling in a ligand-independent manner [52, 53]. While enzalutamide offers substantial benefits over other AR inhibitors, and improved survival rates in patients, CRPC still escapes this therapeutic option through expression of AR-Vs [54].

In CRPC, AR remains a potent factor in driving prostate cancer growth, and is involved in various aspects of therapeutic resistances. Common modifications of AR include amplification of AR (allowing cells to respond to depleted but not eliminated androgens), loss of specificity of AR (response to alternative ligands, including receptor antagonists), and mutations or modifications to AR that lead to ligand-independent signaling [55]. These mechanisms eventually overcome all available options for treatment of metastatic disease. Beyond ADT and chemical AR inhibition, therapeutic options for metastatic prostate cancer are limited, particularly for CRPC. Systemic chemotherapeutic options available include Docetaxel and Cabazitaxel (after failure of Docetaxel) [42]. Other options are currently being investigated, such as inhibitors of alternative, androgen-independent signaling pathways (PI3K/Akt/mTOR, Ras/MAPK, FGFR, etc.) [42]. However, median survival times remain poor for these late-stage patients. Even with modern therapeutic interventions, these more aggressive and invasive cancers are responsible for the significantly lower 5-year survival rate (less than 28%) in these patients [17].
Few effective treatment options exist for these aggressive/late-stage prostate cancer cases. Furthermore, there are significant uncertainties regarding the mechanisms driving therapeutic resistance and metastasis in some cases while most cases remain indolent. These gaps in our understanding demand greater research into the underlying mechanisms involved in disease progression. Future research regarding prostate cancer should focus on the mechanisms which support resistance to therapy in metastatic disease, particularly in the transition from androgen dependence to castration resistance.

1.3: Role of chemokine receptors in prostate cancer:

Chemokines are small secreted proteins which are important mediators of development, inflammation, immune response and cell migration; however, chronically induced secretion of these factors can lead to the development and progression of cancer [56-58]. Chemokine receptors are members of a superfamily of seven-transmembrane spanning G protein-coupled receptors (GPCRs) (as depicted in Figure 1.3.1). These receptors mediate intracellular signaling through recruitment and activation of guanine nucleotide binding proteins (G proteins). In an inactive state, the Gα-Gβ-Gγ heterotrimeric G protein complex binds to a highly conserved ‘DRY’ (Asp-Arg-Tyr) motif near the third transmembrane spanning region of the chemokine receptor. Upon ligand stimulation, the receptor activates the Gα subunit which exchanges GDP for GTP and dissociates from the complex. Both the Gα and Gβγ subunits then mediate intracellular signaling programs [59].

Upon binding chemokine ligands, GPCRs can also transduce signals independent of canonical G protein pathway. Following ligand activation of the
receptor, G protein-coupled receptor kinases (GRKs) are recruited to the active receptor and phosphorylate C-terminal domains of the GPCR. This phosphorylation initiates β-arrestin binding to the receptor, which prohibits further G protein activation, and signals for receptor endocytosis and recycling [60]. However, β-arrestins act as scaffold proteins, and are associated with signal transduction alternative to the G protein pathways [61]. The β-arrestins may serve as adapter molecules to assemble multi-protein complexes which, in addition to receptor internalization and recycling, mediate downstream signaling events, including activation of extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinase (JNK), and p38, as well as Akt and phosphoinositide 3-kinase (PI3K) and their associated mitogenic signaling [62-65].

Chemokines and their associated receptors have been heavily investigated in cancer research due to their involvement in both normal and abnormal
physiological behaviors [66-68]. Chemokine receptors are grouped according to conserved cysteine motifs in the N-terminal region of their chemokine ligands. The family of GPCRs which recognize the cysteine-X-cysteine (CXC) motif containing chemokines have been shown to be important in the context of prostate cancer cell proliferation, migration and survival [67]. There are 7 members of the CXC-receptor (CXCR) family known to date, along with numerous chemokine ligands which have been shown to play a role in the survival of cancer cells, their proliferation, and/or in the migration of the cells to distant sites [67]. In the prostate cancer model, CXCR4 was found to have a strong influence on metastasis. Upon stimulation by its ligand, bone marrow stromal cell-derived factor-1 (SDF-1/CXCL12), CXCR4 enhanced the interaction of prostate cancer cells with bone cells [69]. Recent findings have shown that another member of this receptor family, CXCR7, enhances the phenotypes associated with CXCR4 [70], providing a new focus for this area of investigation.

1.4: C-X-C chemokine receptor 7:

CXCR7 is the most recently characterized member of the C-X-C family of chemokine receptors. It was first discovered during a screen for new GPCRs from a dog thyroid cDNA library [71]. Initially this receptor was considered an orphan receptor, with no known ligand. Later studies determined that this receptor did recognize specific chemokines with high affinity. These ligands were CXCL11 and CXCL12 [72, 73]. However, expression of this receptor alone failed to generate the same cellular responses as other members of the CXCR family. CXCR4 like other typical receptors in this family contains a conserved intracellular DRY motif (D-R-
Y-L-A-I-V) near the third transmembrane spanning region. This domain is necessary for interaction with the G protein subunit α (Gα) and initializes the G protein signaling pathways leading to chemotaxis, intracellular calcium mobilization and mitogenic pathway activation upon ligand binding [59]. Instead, CXCR7 has a modified DRY motif (D-R-Y-L-S-I-T) which does not interact with G proteins [74]. The CXCR4 and CXCR7 proteins and their respective DRY motifs are shown in the illustration in Figure 1.4.1.

Regardless of CXCR7’s lack of canonical GPCR signaling, it is noticeably upregulated and associated with aggressive tumor phenotypes in several cancer types, including colon cancer [75] breast cancer [76, 77], hepatocellular carcinoma [78] and prostate cancer [67, 79]. CXCR7 has also been shown as a prognostic marker for poor patient outcome in colorectal [80] and non-small cell lung cancers [81]. In the context of prostate cancer, immunohistochemistry of human prostate tissue revealed significantly increased CXCR7 expression in high grade prostate
tumor tissues and metastatic lesions compared to BPH tissue [79]. Furthermore, higher levels of CXCR7 significantly correlated with increased cell survival, motility and even tumor development in vivo [73].

1.5: CXCR7 and mitogenic signaling:

The role of CXCR7 in contributing to more aggressive prostate cancer phenotypes and therapeutic resistance may rely on alternative signaling mediated through β-arrestin recruitment [82, 83]. Published data has revealed that epidermal growth factor (EGF)-mediated activation of EGF receptor (EGFR) is significantly enhanced with CXCR7 overexpression. Furthermore, this CXCR7-EGFR signaling axis is supported independent of ligand binding to CXCR7. Overexpression of CXCR7 corresponded to increased EGFR phosphorylation, specifically at Tyrosine-1110 (Y1110), as well as enhanced downstream mitogenic signaling and phosphorylation of ERK 1 and 2 (ERK1/2), leading to tumor cell proliferation and survival; an effect that was reduced with depletion of CXCR7 [77, 84].

While these investigations revealed that CXCR7 interacted with EGFR, the transactivation of EGFR required differential expression of β-arrestin-1 (ARRB1) and β-arrestin-2 (ARRB2). The interaction of CXCR7 with EGFR in prostate cancer was shown to be inhibited ARRB2. In that study, it was demonstrated that the CXCR7-mediated EGFR transactivation was facilitated by Src recruitment. This signaling axis as well as downstream mitogenic signaling and cell proliferation was enhanced following depletion of ARRB2, while overexpression of ARRB2 yielded an opposing effect [83]. Other studies of β-arrestins and CXCR7 suggest an opposing role of ARRB1. Activation of EGFR in colorectal cancers leading to
increased mitogenic signaling is mediated by ARRB1 recruitment to CXCR7 [85]. Furthermore, ARRB1 is shown to facilitate CXCR7-mediated ERK signaling in glioma (in a SDF-1-dependent manner) [86] as well as migration and mitogenic signaling mediated by CXCR4-CXCR7 heterodimers [87]. Together these reports reveal an important link between increased CXCR7 expression and heightened mitogenic signaling and aggressive cancer phenotypes. However, the mechanisms which drive CXCR7 overexpression in prostate cancer, as well as the mechanisms of therapeutic resistance attributed to CXCR7, remain poorly understood.

1.6: Dysregulation of CXCR7 in prostate cancer:

While increased expression of CXCR7 is correlated with aggressive cancer phenotypes, the mechanisms of CXCR7 dysregulation in prostate cancer remain unclear. Three avenues of CXCR7 dysregulation in the context of prostate cancer have been suggested in recent years. Alterations to epigenetic regulation of gene expression in prostate cancer, particularly in the silencing of the tumor suppressor gene, hypermethylated in cancer 1 (HIC1) (a repressor of CXCR7 [88]), is one such avenue. Alternatively, it is established that inflammatory cytokines can alter expression of CXCR7 [84]. Finally, the least clear regulatory axis in CXCR7 which becomes disrupted during therapeutic intervention is that directed by AR. There is some early evidence that implicates AR in downregulation of CXCR7 [89]. However, the regulatory role of androgen over CXCR7 has not been previously investigated in the context of ADT as a mechanism of androgen-independent cell survival or proliferation.
The tumor suppressor HIC1 is located on the short arm of chromosome 17 and encodes a zinc-finger transcriptional repressor [90, 91]. A high level of methylation of HIC1 (reducing its expression) has been shown to correlate with more aggressive disease and poorer prognosis in renal, breast and prostate cancers [92-95]. In the prostate cancer cell lines PC3 and C4-2B (an osteotrophic metastatic isoform of LNCaP [96]), induction of HIC1 resulted in decreased tumor growth as well as reduction in the metastatic potential of these tumor cell lines [88, 95]. One proposed explanation for the observed phenotype is the depletion of CXCR7 in cells where HIC1 is overexpressed [95]. HIC1’s suppressive control over CXCR7 offers a potential explanation for the dysregulation of CXCR7 in more advanced cancers, where HIC1 is silenced. However, previous work in our lab using LNCaP cells showed that restoration of HIC1 expression failed to reduce CXCR7 gene transcription, suggesting that HIC1 may not be implicated in early CXCR7 dysregulation in castrate-sensitive prostate cancer cells.

The pro-inflammatory chemokine Interleukin-8 (IL-8/CXCL8) is normally released under conditions of injury or stress by activated macrophages and monocytes to direct the infiltration of other immune cells such as neutrophils, basophils and T-lymphocytes. This site-directed communication is vitally important for immune responses in the body, but is a transient signal which is highly regulated to prevent excessive activation [97]. Substantial research has investigated the link between chronic inflammation and the transformation of cells, therapeutic resistance, as well as pro-tumorigenic signaling in the tumor microenvironment. A subpopulation of tumor cells in the prostate produce IL-8
through stress-induced Nuclear factor Kappa B (NF-κB) activation as a response to chemotherapy [98]. Furthermore, inflammatory cytokines have also been observed to be significantly increased in patients undergoing ADT, and remain elevated 12 months after initiation of therapy and potentially longer [99]. Evidence has also linked IL-8 signaling with increased expression and ligand-independent activation of AR, which reduced the efficacy of common AR antagonists [43, 100]. Functionally this behavior can be attenuated via depletion of the IL-8 receptor CXCR1 as well as by inhibiting downstream signaling targets such as Src and Focal adhesion kinase (FAK) [101, 102]. Together, these results demonstrate a functional cross-talk between IL-8 and resistance mechanisms to therapeutic interventions for metastatic prostate cancer.

Further investigations have also shown a correlation between IL-8 and CXCR7 expression. Attenuation of IL-8 signaling in the aggressive CRPC cell line PC3 leads to a decrease in CXCR7. Conversely, forced IL-8 overexpression or stimulation with exogenous IL-8 in androgen-dependent cell lines (LNCaP and LAPC-4) lead to an increase in CXCR7 mRNA and protein [84]. These results suggest a complex regulatory axis leading to overexpression of CXCR7 through inflammation. Thus, CXCR7 may be a central factor mediating resistance to therapeutic interventions.

The role of AR has been investigated comprehensively in studies of prostate cancer severity and progression, and is clearly a vital factor in nearly all stages of the disease. Modifications, mutations, and changes in AR expression during ADT have significant consequences on the recurrence and mortality of prostate cancer
Provided the essential function of AR in this disease and its involvement and dysregulation in CRPC, the interaction between AR and alternative signaling pathways is an active avenue of investigation. Chemokine receptors have indeed been implicated in modulating expression of AR as well as activation of AR even in the absence of androgens. One investigation revealed that CXCR4, following binding by CXCL12, leads to hormone-independent activation of AR and its associated transcriptional targets through involvement of PI3K and ERK signaling pathways [103]. Interestingly, another study determined that increased AR expression correlates with decreased CXCR4 in prostate cancer cell lines and leads to a subsequent reduction in metastatic potential [104]. CXCR7, which enhances CXCR4 signaling and related cancer phenotypes as described previously [70], is also downregulated by active AR [89].

From these studies, there seem to be contradictory behaviors regarding AR and the chemokine receptors. Activity of AR is clearly important throughout all stages of prostate cancer. The CXCR4/CXCR7/CXCL12 axis is also remarkably important in stimulating growth, survival and metastasis of tumor cells. However, there exists a dichotomy since chemokine receptors function synergistically with AR in advanced prostate cancer, while also being downregulated by AR. This hints at a complex cross-talk between pathways which remains to be elucidated.

1.7: Scientific premise:

From the current literature, there is substantial evidence for the role of chemokine receptors, particularly CXCR7, in facilitating the progression of aggressive prostate cancer. However, it remains unclear how CXCR7 becomes
dysregulated, particularly during ADT, in the transition of prostate cancer from a dormant, androgen-dependent state to CRPC. This project aims to determine if the loss of AR signaling derepresses CXCR7, allowing it to support alternative, androgen-independent signaling pathways associated with survival and proliferation and the development of castration resistance.

This study critically investigates the overall regulation of CXCR7 by AR. Utilizing ADT, AR inhibitors, and RNAi methods to deplete AR, I measured the changes in CXCR7 expression to investigate how it is dysregulated during therapeutic interventions targeting the AR signaling axis. Furthermore, this work investigates how the dysregulation of CXCR7 during ADT alters androgen-independent mitogenic signaling programs in androgen-dependent prostate cancer cells. RNAi-mediated depletion of CXCR7 is known to decrease prostate cancer cell proliferation under normal androgen stimulated conditions [84]. This work, for the first time, examines the alterations in prostate cancer cell proliferation and tumorigenicity following abrogation of wild-type (WT) CXCR7 using the clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR associated protein 9 (Cas9) gene editing method [105]. These CRISPR-altered cell lines were investigated for changes in mitogenic signaling, clonogenic and sphere formation potential, and their tumorigenicity in a xenograft model.

1.8: Hypothesis:

Based on the results in this study, I propose that loss of AR signaling, either through androgen depletion or AR inhibition results in significantly increased CXCR7 expression and an associated increase in EGFR transactivation and
mitogenic signaling. Furthermore, CXCR7 has a necessary role in supporting cell proliferation and survival in both androgen stimulated and androgen deprived prostate cancer cells. Altogether, CXCR7 mediates the transition from androgen dependence to castration resistance by supporting androgen-independent cell proliferation and survival signaling programs during ADT.

1.9: Impact:

In metastatic prostate cancer, mutations in AR, changes in its expression, and alternative splicing of AR mRNA are common mechanisms which overcome all available therapeutic options [49]. Because prostate cancer can develop resistance to all AR-targeting treatment options, there is a profound need to identify targets beyond AR to eliminate the androgen-independent mechanisms that support cell survival and proliferation, especially at the onset of ADT. The link between CXCR7 and growth factor-mediated mitogenic signaling is one such pathway. CXCR7 activates non-canonical GPCR signaling, mediated by β-arrestins and transactivates EGFR and downstream mitogenic signaling, and thus is a potential therapeutic target to inhibit alternative signaling programs. This study is a critical investigation of the dysregulation of CXCR7 during ADT as well as the role of CXCR7 in facilitating androgen-independent tumor cell survival and proliferation supporting the development of CRPC.
Chapter 2: MATERIALS AND METHODS

2.1: Cell lines and cell culture:

Human LNCaP (ATCC CRL-1740), CRW-22Rv1 (ATCC CRL-2505), and PC3 (ATCC CRL-1435) prostate cancer cell lines were cultured in RPMI-1640 (Corning cellgro) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals) and 10 μg/mL gentamicin sulfate (Sigma-Aldrich). C4-2B cells (ViroMed Laboratories), a castration resistant derivative of LNCaP [96] were cultured in T-medium prepared as described previously [106] with the exception of 10 μg/mL gentamicin sulfate in place of penicillin/streptomycin antibiotics. PC3 cells stably overexpressing AR (PC3-AR) were a kind gift from Dr. K. L. Burnstein (University of Miami) [107]. All cell lines were maintained in a humidified incubator at 37°C and 5% CO₂ for no more than 10 passages before returning to earlier passages, stored in liquid nitrogen. Cells were tested and determined to be clear of mycoplasma contamination regularly (Agilent MycoSensor PCR Assay Kit).

Androgen deprivation was carried out by incubating cells in charcoal-dextran stripped FBS (CDFBS) supplemented medium for 48 hours for RNA analysis or 72 hours for protein analysis. Androgen stimulation was carried out by pre-incubating cells for 48 hours in CDFBS, then supplementing CDFBS medium with 5 nM of the non-hydrolysable androgen analog, Methyltrienolone (R1881) (Sigma-Aldrich). For AR inhibition, cells cultured in FBS medium were treated with either 2 μM bicalutamide or 5 μM enzalutamide (MedChem Express) for 48 hours (RNA analysis) or 72 hours (protein analysis). For EGF stimulation cells were first serum-starved (media supplemented with 0.5% FBS, or 0.5% CDFBS ± R1881)
for 24 hours, followed by stimulation with 10 ng/mL human recombinant EGF (Gibco) for the times indicated in the respective figure legends. After incubation with EGF, culture medium was removed and the reaction was quenched by washing 2 times with ice-cold phosphate buffered saline (PBS). Total protein was isolated for western blot analysis.

2.2: Transient gene depletion with siRNA:

AR was depleted in LNCaP cells by transfection with SMARTpool ON-TARGETplus small interfering RNA (siRNA) targeting AR (siAR) using the Dharmacon DharmaFECT 2 siRNA transfection system (GE Healthcare, Dharmacon). Transfection with siGLO RISC-Free Control siRNA (siCtl) served as a negative control. Cells were seeded into 6-well culture plates (Corning Costar) and grown to a 50-60% confluency. Culture medium was replaced with 1.6 mL antibiotic free medium for transfection. DharmaFECT and siRNA were diluted separately in Opti-MEM reduced serum medium (Gibco). For each transfection reaction, 4 μL of the DharmaFECT reagent was diluted into 196 μL of the Opti-MEM (2 μL reagent per 1 mL of the final transfection volume). Separately, 25 μL siRNA stock (2 μM) was diluted into 175 μL of Opti-MEM (25 nM final transfection concentration) for each transfection reaction. Dilutions (DharmaFECT and individual siRNA targets) were incubated at room temperature for 5 minutes, then the DharmaFECT dilution was added to each siRNA dilution 1:1 (added directly to the liquid with gentle pipetting to mix). The transfection mix was incubated at room temperature for 20 minutes. 400 μL of the transfection mix was then added dropwise to the cells (avoiding contact with sides of well) and mixed gently by
swirling the plate. Cells were incubated with siRNA for 6-8 hours before changing to fresh complete medium. Cells were treated with or without androgen (CDFBS ± R1881) for 48 hours for RNA or 72 hours for protein analysis.

2.3: Stable gene depletion with shRNA:

Short hairpin RNA (shRNA) constructs for stable depletion of target genes were cloned into a pRS plasmid under the control of a U6 promoter (HuSh-29mer, Origene Technology Inc.). Constructs were obtained for depletion of CXCR7 (shCXCR7) or as scrambled non-specific control sequence shRNA (shCtl). Cells were cultured in 6-well plates to 60-70% confluency for transfection. Cell culture medium was replaced with 1.6 mL of antibiotic-free medium. For each reaction, 2 µL of Lipofectamine 2000 transfection reagent (Invitrogen Inc.) were diluted into 200 µL Opti-MEM media. Separately, 2 µg of shRNA plasmid DNA (1 µg per 1 mL final concentration) were diluted in 200 µL Opti-MEM for each transfection reaction. After 5 minutes, Lipofectamine reagent mix was added to the plasmid DNA dilution (1:1) and incubated 20 minutes at room temperature. Transfection mix (400 µL) was then added dropwise to each respective well. After 24 hours, the medium was changed to complete medium. After 48 hours from the start of transfection, cells were treated with 2 µg/mL puromycin sulfate (Sigma-Aldrich) selection antibiotic. Fresh medium with antibiotic was added frequently to remove dead, non-transfected cells from culture. After two weeks of selection, stable transfectants were evaluated for depletion of target genes by RT-q-PCR and immunoblotting.
2.4: Quantitative real-time PCR (RT-q-PCR):

Total RNA was isolated from cells with the Aurum Total RNA Mini Kit (Bio-Rad) according to manufacturer’s protocols, and stored at -80°C. From the total isolated RNA, 1 μg was reverse transcribed to complementary DNA (cDNA) with the iScript cDNA Synthesis Kit (Bio-Rad) according to manufacturer’s protocols, then diluted with nuclease-free H₂O (added 130 μL to the 20 μL cDNA reaction). RT-q-PCR reactions were prepared in 96-well clear Multiplate PCR Plates (Bio-rad) with 5 μL of the cDNA, 0.5 μL of forward and reverse primers (10 μM stock), 10 μL SsoFast EvaGreen supermix (Bio-rad), and 4 μL nuclease-free H₂O. Each target was measured in triplicate. Primers for RT-q-PCR detection of targets are listed in Table 2.1. The housekeeping gene peptidylprolyl isomerase A (PPIA) was used as an internal control. RT-q-PCR was performed in the CFX96 Real-Time PCR Detection System (Bio-rad): one cycle at 95°C for 30 seconds, then 40 cycles of: 95°C for 5 seconds, 67°C (optimal temperature for CXCR7 and PPIA) for 10 seconds, then a SYBR green signal detection step at the end of each cycle. Threshold cycle (Ct) values were determined with the CFX Manager software. Abundance of target mRNA relative to the housekeeping gene (PPIA) was calculated with the ΔCt method: $2^{\Delta \Delta Ct} = 2^{[-(Ct_{Target} – Ct_{PPIA})]}$. The ΔΔCt method was used to calculate the change in gene expression between treatments: $\Delta \Delta Ct = [(\Delta Ct \text{ of treatment}) / (\Delta Ct \text{ of control})]$, and represented as either a percent change or fold difference (FD) [108].
2.5: Immunoblotting and densitometric analysis:

Cell lysates were prepared in radioimmunoprecipitation assay (RIPA) buffer (150 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% IGEPAL CA-630, 1mM EDTA, 0.5% Na$_3$VO$_4$, 0.1% sodium dodecyl sulfate [SDS], 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 1X Protease Inhibitor Cocktail [Sigma-Aldrich]). Culture medium was removed, and cells were washed twice with ice-cold PBS. RIPA buffer was added at 100 $\mu$L per 1x10$^6$ cells. Cells were scraped from the culture container and transferred to 1.5 mL capped vials. Lysate was incubated on ice for 30 minutes, with vigorous vortexing every 10 minutes. Lysates were then centrifuged at 12,000 x g for 20 minutes at 4°C to clear insoluble cellular debris, and supernatant was transferred to a new vial for storage at -20°C. Total protein was quantified with the Pierce BCA Protein Assay Kit (Thermo Scientific). For nuclear/cytoplasmic protein analysis, cells were processed using the NE-PER Nuclear and Cytoplasmic Extraction Reagent kit (Thermo Scientific) according to manufacturer’s protocols.

For each immunoblotting procedure, equivalent protein from RIPA buffer lysates was transferred to new vials and adjusted with fresh RIPA buffer to equalize the protein concentration. Samples were then diluted 1:1 with 2X Lammeli sample buffer (4% SDS, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris-HCl); followed by addition of 1M dithiothreitol (DTT) to a final concentration of 100 $\mu$M. Samples were then boiled at 95°C for 5 minutes, loaded to polyacrylamide gels, and separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) for 1 hour at 200 V. Separated proteins in gels were then wet transferred to
polyvinylidene fluoride (PVDF) membranes in 20% methanol tris-glycine buffer for 1 hour at 100 V or overnight at 30 V. Ponceau S staining was applied to membranes immediately after transfer to confirm equal loading and then membranes were washed 3 times for 5 minutes in Tris-buffered saline with 0.1% Tween-20 (TBS-T). Membranes were blocked with 5% bovine serum albumin (BSA) or 5% non-fat dry milk (NFDM) in TBS-T for 1 h at room temperature. Primary antibodies were diluted in either BSA or NFDM according to manufacturer recommendations then added to membranes. Incubation was performed at 4°C with gentle agitation overnight. After primary incubation, membranes were washed in TBS-T for 10 minutes, 3 times. Secondary antibody diluted in NFDM was added to the membranes and incubated for 1 hour at room temperature with gentle rocking. Details for the antibodies utilized throughout this work are listed in Table 2.2.

Membranes were washed 3 times for 10 minutes in TBS-T. Chemiluminescence was detected with Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences). Membranes were then wrapped in clear plastic wrap (rolling out any bubbles) and placed in X-ray enhancer imaging cassettes. Luminescence was imaged on HyBlot Autoradiography Films and developed in an automatic X-ray film processor. For phosphorylated proteins, membranes were stripped and reprobed with antibodies to detect respective total proteins. Membranes were stripped in freshly prepared stripping buffer (2% SDS, 62.5 mM Tris-HCl pH 6.8, 100 mM β-mercaptoethanol), incubated at 50° C for 30 minutes with moderate shaking. Membranes were then
washed 3 times in TBS-T for 5 minutes, and blocked with BSA or NFDM. All
membranes were reprobed for β-Actin to confirm equivalent protein loading. Films
were then scanned at 600 dpi resolution with the Epson V30 Flatbed scanner.
Relative protein signal intensities were quantified by densitometry with LI-COR
Image Studio Digits version 3.1. Phosphorylated proteins were normalized to their
total protein band density, while other targets were normalized to the β-Actin
intensity reprobed on their respective membranes.

2.6: Transcription factor binding prediction and promoter cloning:

AR response elements (AREs) are promoter DNA regions which are
recognized and bound by activated AR [109]. To identify any potential AREs in the
CXCR7 promoter, a DNA sequence including 6,000 nucleotides upstream of the
transcription start site (TSS) of CXCR7 was queried using the online transcription
factor prediction PROMO-ALGGEN algorithm [110, 111]. A cluster of 6 potential
AREs were predicted within 2,600 nucleotides of the TSS. To assess whether this
region is sufficient to model the androgen-mediated transcriptional activity of
CXCR7, promoter-luciferase assays were performed. The promoter fragment
containing the putative AREs was PCR amplified from CalTech Human BAC
Library D (CTD) clone 2207H15 plasmid DNA (pBeloBAC11 plasmid containing
100 kb of human chromosome 2 [GRCh38.p2 primary assembly] genomic DNA
including the CXCR7 gene locus). The CTD-2207H15 clone was obtained as a
glycerol stock (ThermoFisher Scientific), and amplified for mini-prep with PureLink
HiPure Plasmid Miniprep Kit in combination with the PureLink HiPure BAC Buffer
Kit (Invitrogen) according to manufacturer’s protocols. A 2,721 nucleotide CXCR7
promoter fragment (2,549 nucleotides upstream and 172 nucleotides downstream of the TSS) was amplified with primers specific within the CTD-2207H15 clone DNA sequence, which also added KpnI (5’) and XhoI (3’) restriction enzyme linker sequences to the PCR product (primer sequences listed in Table 2.1). The purified promoter fragment and the pGL4.20 [Luc2/Puro] promoterless luciferase reporter plasmid (Promega) were restriction enzyme digested (KpnI-HF and XhoI enzymes at 37°C for 1 hour with CutSmart digestion buffer [New England Biolabs Inc.]) then agarose gel purified. The digested promoter fragment (45 ng) and linearized plasmid (60 ng) were ligated with T4 DNA ligase (New England Biolabs, Inc.) for 10 minutes at room temperature, followed by heat inactivation (60°C for 10 minutes). Competent E. coli were transformed with the ligated reporter construct plasmid reaction (4 μL plasmid ligation were added to 50 μL competent E. coli for 30 minutes on ice, heat shocked at 42°C for 30 seconds, and incubated on ice 2 minutes). Transformed bacteria were incubated in S.O.C. medium for 1 hour at 37°C with vigorous shaking. The bacterial culture was streaked onto Luria broth (LB)-agar plates infused with 100 μg/mL carbenicillin disodium salt (Sigma-Aldrich), and incubated at 37°C overnight. Isolated colonies were selected and amplified in 5 mL of sterile carbenicillin treated LB medium for 8 hours at 37°C with vigorous shaking. A glycerol stock of each colony was prepared by adding 500 μL of each bacterial colony to 167 μL of glycerol and stored at -80°C. For maxi-prep amplification, 1 mL of the starter bacterial culture was inoculated into 500 mL of sterile carbenicillin-LB medium. Culture was incubated at 37°C for 16 hours with vigorous shaking. The PureYield Plasmid Maxiprep System (Promega) was used
to purify plasmid DNA according to manufacturer’s protocols. Plasmid DNA was transfected into cells (Lipofectamine 2000, ThermoFisher), and selected with 2 μg/mL puromycin (Sigma-Aldrich) until a stably transfected population was obtained.

2.7: Promoter luciferase assay:

Cells transfected with promoter-luciferase constructs were plated into 96-well opaque tissue culture plates. Androgen deprivation and R1881 stimulation were carried out as previously described. Medium was removed from each well followed by one wash with PBS. Cells were incubated in 20 μL of kit-supplied lysis buffer for 10 minutes at room temperature. Following lysis, 80 μL of luciferase detection reagent was added to each well. The plate was covered in foil and incubated for 5 minutes at room temperature. Luciferase activity was detected with the Promega GloMax 96 Microplate Luminometer with luminescence compared between treatment and control groups. Luciferase activity was measured for each treatment condition in triplicate.

2.8: Chromatin immunoprecipitation (ChIP) assay:

LNCaP cells were cultured to 75% confluence in 10 cm cell culture dishes (at least 2x10^6 cells per ChIP target). All cells were androgen deprived for 48 hours. Medium was then changed with one set of cells maintained under continued ADT (CDFBS containing media alone), while the other plates were treated with CDFBS supplemented with 5 nM R1881. After 24 hours, cells were fixed for 10 minutes in a final concentration of 1% formaldehyde (Thermo Fisher, 16% [w/v] methanol free). Cells were rinsed twice with ice-cold PBS. One mL of PBS was then added
to each plate, and cells were scraped and collected. Cells were lysed with the Pierce Agarose ChIP Kit (Thermo Scientific, 26156) according to the manufacturer's protocols. Chromatin was optimally digested with kit-supplied Micrococcal DNAse at 20 Units per plate of collected cells. Following incubation with nuclear lysis buffer, brief sonication was performed to disrupt nuclei and improve the yield of digested chromatin (MicroSonicator probe: 20% amplitude for 3 cycles of 10 seconds on and 10 seconds off, in ice). For a total input control, 10% of the digested chromatin from each treatment group was isolated and stored at -20°C (10% input control). The remaining chromatin was equally distributed and incubated with either 1 μg anti-AR rabbit IgG (ChIPAb+, Millipore) or 1 μg non-specific isotype rabbit IgG control overnight at 4°C with gentle rocking. Antibody bound chromatin was pulled down with kit supplied A/G agarose beads according to the manufacturer's protocol. Antibody-purified DNA was recovered according to the manufacturer's protocol.

Recovered DNA was analyzed by RT-q-PCR relative to the 10% input controls with primer sets designed to amplify 50-100 nucleotide DNA sequences containing each predicted ARE in the CXCR7 promoter (Table 2.1). The Ct values for the 10% input samples were adjusted to estimate total abundance of each target (Ct-10%-Input – 3.32). Percent of input (%-input) was calculated for all incubated samples relative to the adjusted 10% input control (ΔCt method). The %-input of the AR precipitated samples were then set relative to the non-specific IgG samples (fold enrichment over background). The fold enrichment of the cells incubated with androgen were compared to the cells under ADT to determine whether AR was
bound to the predicted CXCR7 promoter AREs in an androgen-dependent manner. As a positive control for AR binding, the enhancer region of the PSA gene promoter was analyzed for enrichment with or without androgen.

2.9: CRISPR-Cas9 design and directed gene editing:

The CRISPR-Cas9 gene editing technology was derived from an adaptive immunity mechanism of *Streptococcus pyogenes*. With this method, 20 nucleotide genomic DNA sequences followed immediately by a three nucleotide protospacer adjacent motif (PAM) sequence (NGG) can be selectively targeted for editing. A CRISPR guide RNA (gRNA) sequence is designed as a complement to the 20 nucleotide target genomic DNA sequence. When the gRNA recognizes and binds to the target genomic DNA, the complex recruits the Cas9 nuclease which induces a double strand break three nucleotides upstream of the PAM sequence. The cell relies on non-homologous end joining (NHEJ) to repair the DNA damage, which has a very high risk for producing insertion or deletion mutations (InDels). An InDel in a gene coding region can result in a shift in the translation reading frame (frameshift), introducing a premature stop codon or nonsense protein sequence downstream of the mutation, thus eliminating protein expression in the cell [112]. This process is illustrated in Figure 2.9.1.

CRISPR gRNA sequence design and plasmid preparation were provided by Santa Cruz Biotechnologies, utilizing the Genome-Scale CRISPR Knock-Out (GeCKO) v2 library [113]. Three CRISPR-Cas9 gRNA plasmids containing a GFP transfection reporter gene were supplied as a combined pool: sc-403187A1 (CXCR7-gRNA 1), sc-403187A2 (CXCR7-gRNA 2), and sc-403187A3 (CXCR7-
gRNA 3). The 20-nucleotide target sequences (Table 2.1) along with the PAM sequences were confirmed to be specific to CXCR7 (Basic Local Alignment Search Tool [BLAST] search; National Center for Biotechnology Information [NCBI]) and further confirmed with the CasFinder algorithm [114]. The pooled plasmid DNA was transfected into LNCaP and C4-2B cells using the Lipofectamine 3000 Transfection Reagent (Invitrogen) according to manufacturer’s protocols. GFP positive cells were sorted using the BD FACSaria II (Augusta University flow cytometry core), and plated at single cell dilutions into 96-well tissue culture plates for clonal expansion. Surviving clones were harvested and amplified further in 48-well plates, then transferred to 25 cm² tissue culture flasks (Corning). Proliferating colonies were tested for CXCR7 knockout via immunoblotting and through Sanger sequencing of genomic DNA (GENEWIZ).
2.10: Cell viability assay:

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay method. This method employs the NADH-NAD\(^+\) reduction mechanism available in live cells to reduce a soluble yellow tetrazolium salt to insoluble purple formazan [115]. Cells were plated in 48-well culture plates and treated in triplicate. Three wells contained medium alone (± treatment vehicle) as background normalization controls. Following treatment and incubation, MTT reagent was added to culture medium 1:10 (50 \(\mu\)L of 5 mg/mL MTT reagent per 500 \(\mu\)L of culture medium) and incubated for 3 hours. After incubation, medium was removed and the precipitated purple formazan crystals (formed within viable cells) were dissolved in 250 \(\mu\)L dimethyl sulfoxide (DMSO). The plate was then incubated at room temperature for 30 minutes with moderate rocking. The Bio-Rad Benchmark Plus microplate spectrophotometer was used to measure the colorimetric absorption of the formazan product (absorption wavelength of 570 nm) compared to a reference wavelength (650 nm). The reference adjusted absorption values were averaged for each treatment group then the mean background absorbance was subtracted. Reduced absorbance in treatment groups relative to controls was a measure of either cytotoxicity or slower cell proliferation (reduced overall viable cell number at the endpoint).

2.11: Clonogenic survival assay:

Clonogenic survival or colony-formation assays of cell lines is a measure of the ability to establish a colony of cells from a single progenitor. The abundance of established colonies (defined as a group of at least 50 cells) is a test of how many
cells in the sample are capable of unlimited proliferation. This method can assay for efficacy of treatments in reducing the number of colony progenitors in the total cell population [116]. Cells were seeded at 500 cells per well in 6-well culture plates in triplicate for each treatment group. Cultures were allowed to proliferate for 10 days before assessment of colony formation. At the endpoint of the assay, medium was removed and cells were washed twice with ice-cold PBS. Cells were then fixed in ice-cold 100% methanol for 10 minutes. Methanol was removed then cells were stained with crystal violet solution (25% methanol, 0.5% crystal violet [w/v]) for 10 minutes at room temperature with gentle rocking. The staining solution was aspirated from the wells and the residual stain was rinsed by submerging plates in a basin of cool water under a continuous running tap until water ran clear. Plates were gently tapped on paper towels to remove excess water and allowed to air dry. Total colonies in each well were counted and an average count of the three wells per treatment was calculated. Mean counts were compared between control and treatment groups to assess clonogenic potential after treatment.

2.12: Chemotactic migration assay:

Chemotactic assays are utilized to test a cell line’s potential to migrate along a chemoattractant gradient. An SDF-1 concentration gradient has been used to test for CXCR4- and CXCR7-mediated migration [117]. Cell cultures were serum starved (0.5% FBS) for 24 hours. Following starvation, cells were harvested with a non-enzymatic cell dissociation solution (Gibco), and 2.5x10^5 cells were seeded in triplicate onto 6.5 mm transwell permeable supports (Corning Costar, 8 μm pore membrane) over a 24-well culture plate. Starvation medium was added to the lower
well and supplemented with recombinant human SDF-1α (R&D Systems) to a final concentration of 100 ng/mL. Cells were incubated for 16 hours, then media in the top and bottom wells were treated with MTT reagent (1:10). After 3 hours, medium was removed from the top and bottom wells. Filter paper was used to wipe the migrated cells from the bottom of the insert membrane and placed into the lower well of the plate. This step was performed to pool together the cells that migrated into the lower well with those still attached to the lower side of the membrane. The insert was separated to a new 24-well plate. The formazan precipitate in the insert (upper well) and the lower well (including the wipe) were both dissolved in 250 mL of DMSO for 30 minutes. 200 μL of the DMSO solution from the inserts and bottom wells was transferred to a 96-well plate for colorimetric absorbance analysis. The percentage of migrated cells was calculated: \[
\frac{(\text{Absorbance Bottom})}{(\text{Absorbance Top} + \text{Absorbance Bottom})} \times 100
\]
The percent of migrated cells were then compared between the treatment and control groups to assess changes to the migratory potential of cells after treatment.

2.13: Senescence associated β-galactosidase staining:

In determining if there is potential for recurrence of cell proliferation following therapeutic interventions, it is imperative to identify non-proliferating cells as dormant but capable of renewed proliferation (quiescence) or irreversibly arrested (senescence). One classical biochemical assay to measure cellular senescence is the detection of senescence-associated β-galactosidase (SA-gal), which is active at pH 6.0 and not detectable in quiescent cells [118]. SA-gal activity was determined by analyzing the cleavage of a galactose-indole molecule, X-gal
(Sigma-Aldrich). Following β-galactosidase-mediated cleavage of the molecule, the indole group dimerizes and produces an insoluble blue product [119].

To test for SA-gal activity, culture medium was removed from proliferation-arrested cells (maintained with no proliferation for more than one month in culture) and washed two times with PBS. Cells were fixed with 4% formaldehyde (in PBS) for 3 minutes at room temperature, then rinsed twice with PBS. SA-gal staining solution was prepared fresh, according to established protocols [120] and added to the fixed cells. Cells were incubated in the staining solution overnight. Staining solution was then removed and replaced with PBS for direct imaging of cells. As a positive control, normally proliferating cells were treated with 10 μM H_2O_2 for 96 hours to initiate oxidative stress-induced cellular senescence [121].

2.14: Sphere formation assay:

To assess anchorage-independent survival and proliferation, tumor spheroid formation assays were performed. Sphere-formation assays provide evidence of self-renewal and offer in vitro evidence for the tumor initiation potential of cancer cells [122, 123]. Sphere-formation assays were performed by seeding 1x10^3 cells into low-adhesion, 35 mm cell culture dishes. Cells were maintained in SpheroMax medium and supplement (PromoCell) for 10 days. First generation spheres were collected, dissociated, and 1x10^3 cells were reseeded into low-adhesion dishes. Second-generation spheres were counted in each plate as a measure of clonogenic and stem-like potential.
2.15: Proximity ligation assay:

The Proximity Ligation Assay (PLA) is an antibody-based method to detect co-localization of two proteins in a cell. Fixed cells are incubated with primary antibodies for each target protein (from different host species). Secondary antibodies conjugated to PLA probe oligonucleotides are then applied. A ligation solution containing additional oligonucleotides is then added to the cells. If the target proteins are within 40 nm of each other, the oligonucleotides hybridize and form a circular DNA construct. One arm of the PLA probe acts as a primer for rolling-circle amplification (RCA) of the circularized complex. When polymerase is added, RCA of the circular complex will synthesize a repeating DNA product. Fluorescently-tagged oligonucleotides hybridize with the repetitive DNA product, producing a red-fluorescent spot. This process is depicted in the illustration in figure 2.15.1.

![Figure 2.15.1: PLA principle and reaction](image.png)

**Figure 2.15.1: PLA principle and reaction:** Cells are incubated with primary antibodies (ab) specific to target proteins (different host species [i.e. rabbit or mouse]), followed by secondary PLA probe oligonucleotide-conjugated antibodies. Ligation buffer containing additional oligonucleotides is added. If target proteins are within 40 nm of each other, the oligonucleotides hybridize to form a circular complex. Polymerase is added and rolling-circle amplification (RCA) produces a repeating sequence. Fluorescent-tagged oligonucleotides hybridize with the product, producing a red-fluorescent spot.
PLA was performed to assess co-localization between CXCR7 and ARRB1 or ARRB2 proteins within the cell (Duolink In Situ Red Starter Kit, Mouse/Rabbit, Sigma Aldrich). Cells were seeded into 8-chamber slides (Nunc Lab-Tec, chambered cover glass). Cell preparation, washing, incubation, and reagent preparations were performed according to the manufacturer’s protocols and as previously reported [77, 83]. Antibody details used in these assays are provided in Table 2.2. Cell nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI) supplied in the mounting medium (PLA kit supplied). Red fluorescent spots indicate that the two target proteins co-localized within 40 nm of one another.

2.16: Xenograft tumorigenicity and tumor growth:

Seven-week-old male athymic nude mice from the same litter (Envigo) were obtained for the xenograft study. Two groups of animals (n=10 each) were implanted with either wild-type (WT) CXCR7 LNCaP or CRISPR-mutated CXCR7 (CX7-mut) LNCaP cells. 100 µL of cell suspension (2x10^6 cells in RPMI medium mixed 1:1 with Matrigel [Corning]) was injected subcutaneously into the right flank of the mice. Tumor volume was measured three times weekly with a handheld manual caliper (Tumorimeter and RECIST Caliper [Cancer Technologies Inc.]) taking the diameter of the long and short axes of the tumor. Tumor volumes were estimated using the ellipsoid volume formula (π/6 x L x W x H) [124] with the assumption the height is equal to the short diameter measurement: [(π/6) x (short axis)^2 x (long axis)]. Animal weight was measured weekly. All animals were sacrificed 12 weeks after implantation of tumor cells unless humane endpoint conditions were reached earlier (e.g. excessive weight loss [≥ 10% decrease from
starting body mass], tumor burden [tumor volume ≥ 1,000 mm³], ulceration of tumor, severe mobility impairment, or behavioral evidence of illness or pain).

2.17: Statistical analyses:

All statistical analyses were carried out using Prism v.4.03 (GraphPad software). Data sets (unless otherwise stated in the figure legends) are representative of the mean of 3 independent replicates ± standard error of the mean (SEM). Significance between the means of individual treatment groups and controls is calculated using the unpaired, two-tailed Student’s t-test. For multiple treatment conditions (i.e. siCtl vs. siAR ± R1881), One-way ANOVA was used with the secondary Tukey’s multiple comparisons test to determine significance between treatment groups. Tumor volumes in the LNCaP CX7-mut cells were not normally distributed (Shapiro-Wilk test for normality) so the nonparametric Mann-Whitney two-tailed, U-test was used to calculate significance between groups. Significance was considered achieved with $p<0.05$ and is designated in the figures as: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, and ns = no significance.
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<td>CXCR7 Reverse</td>
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Table 2.1: Primer sets and CRISPR gRNA sequences: Sequences of primer sets utilized in this study and gRNA target sequences for CRISPR-Cas9 experiments.
### Table 2.2: Antibody information

#### Western blot

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<th>Catalog number</th>
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#### Chromatin Immunoprecipitation (ChIP) Assay

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#### Proximity Ligation Assay (PLA)

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**Table 2.2: Antibody information**: Details for antibodies used throughout this study. NFDM = non-fat dry milk, BSA = bovine serum albumin
Chapter 3: REGULATION OF CXCR7 BY ANDROGEN RECEPTOR

3.1: CXCR7 expression decreases with androgen stimulation:

Microarray studies of gene expression in LNCaP cells revealed that CXCR7 mRNA expression was among the set of genes which decreased in the presence of androgens [89]. To determine the role of androgens in the regulation of CXCR7, I investigated four prostate cancer cell lines with varying androgen responsiveness. The cell lines used throughout this study were androgen-dependent LNCaP cells; LNCaP-derived, androgen-independent C4-2B cells, the CRPC cell line CRW-22Rv1 (22Rv1), and the AR-null CRPC cell line PC3. Of these four lines under standard culture conditions (shown in Figure 3.1.1), LNCaP cells had the highest expression of CXCR7 mRNA relative to the internal housekeeping control gene PPIA, followed by C4-2B cells, then by PC3. The 22Rv1 cell line yielded the lowest baseline CXCR7 mRNA transcription of the tested prostate cancer cell lines. These results indicated that the expression of CXCR7 is greatest in androgen-dependent prostate cancer cells: CXCR7 mRNA abundance in LNCaP, C4-2B, 22Rv1, and PC3 prostate cancer cell lines relative to PPIA. Means of 3 independent experiments, ± standard error of the mean (SEM).
cancer cells, while it is substantially downregulated in CRPC cells. From this finding, I next aimed to determine if this differential expression was regulated by AR signaling; as evidence for the dysregulation of CXCR7 during ADT or AR inhibition.

To determine if there were alterations in the overall expression of CXCR7 in these prostate cancer cells during ADT, I measured the change in CXCR7 mRNA transcription in androgen deprived conditions relative to the normal culture conditions. Cell were incubated in androgen deprived medium (supplemented with hormone depleted CDFBS) to model ADT, while a control group was stimulated with the androgen analog R1881. Shown in Figure 3.1.2, CXCR7 mRNA was significantly increased in both LNCaP and C4-2B cells in CDFBS supplemented medium relative to CDFBS-medium + 5 nM R1881. A slight but insignificant increase was observed in 22Rv1 cells, while in the AR-null PC3 cell line, there was no change in the CXCR7 mRNA expression with or without R1881. These results indicate that in the cell lines which retain responsiveness to androgen (LNCaP and C4-2B), the absence of androgens leads to a significant upregulation of CXCR7 transcription. In the 22Rv1 cell line expressing constitutively active AR-Vs [54], CXCR7 expression is maintained at an exceedingly low level regardless of androgen stimulation. PC3 cells with no expression of AR showed no androgen-mediated regulation over CXCR7. Given the results showing androgen-mediated suppression of CXCR7, the next investigation was to clarify whether expression of AR would be sufficient to restore androgen-mediated CXCR7 suppression in AR-null CRPC cell lines.
To test if AR could restore androgen regulation of CXCR7, PC3 cells stably expressing exogenous WT AR (PC3-AR) were treated with or without androgen, followed by RT-q-PCR assessment of CXCR7 expression. PC3-AR cells were confirmed to express AR, as shown in the western blot panel in Figure 3.1.3A. Furthermore, to confirm the exogenous AR is transcriptionally active, a PSA promoter-driven luciferase reporter construct was transfected into the PC3-AR cell line. As shown in Figure 3.1.3B, stimulation with androgen resulted in significantly increased PSA-luciferase activity compared to androgen deprived PC3-AR cells. However, as shown in Figure 3.1.3C, stimulation with androgen did not alter overall CXCR7 mRNA expression in PC3-AR cells relative to the androgen deprived cells.

To test if AR could restore androgen regulation of CXCR7, PC3 cells stably expressing exogenous WT AR (PC3-AR) were treated with or without androgen, followed by RT-q-PCR assessment of CXCR7 expression. PC3-AR cells were confirmed to express AR, as shown in the western blot panel in Figure 3.1.3A. Furthermore, to confirm the exogenous AR is transcriptionally active, a PSA promoter-driven luciferase reporter construct was transfected into the PC3-AR cell line. As shown in Figure 3.1.3B, stimulation with androgen resulted in significantly increased PSA-luciferase activity compared to androgen deprived PC3-AR cells. However, as shown in Figure 3.1.3C, stimulation with androgen did not alter overall CXCR7 mRNA expression in PC3-AR cells relative to the androgen deprived cells.

Figure 3.1.2: CXCR7 expression during ADT: CXCR7 mRNA transcription in androgen deprived cells relative to androgen stimulated cells. N=3, mean ± SEM. *p<0.05, ns = no significance; unpaired, two-tailed Student’s t-test
These results indicate that restoration of transcriptionally active AR in AR-null CRPC cell lines does not restore androgen regulation of CXCR7.

The results from these experiments indicate that androgen stimulation in androgen-sensitive, but not androgen-independent cell lines significantly suppresses the expression of CXCR7. Expression of constitutively active AR splice variants (22Rv1) or AR-independent growth programs (PC3) seem to maintain suppression of CXCR7 in CRPC cells, which cannot be alleviated through ADT.
This may reveal an important mechanism for CXCR7 overexpression in early, castrate-sensitive prostate cancer cells which is unnecessary in advanced CRPC.

From these results, I next aimed to determine whether AR signaling inhibition could mimic the ADT response, to determine the necessity of AR in the regulation of CXCR7.

3.2: AR signaling inhibition increases CXCR7 expression:

To investigate whether AR is necessary for the androgen-mediated suppression of CXCR7, cells maintained in normal culture medium (with FBS) were treated with sub-lethal doses of AR inhibiting compounds (2 µM bicalutamide or 5 µM enzalutamide). Shown in Figure 3.2.1, bicalutamide (BiC) treatment relative to untreated (UT) controls, resulted in significantly increased CXCR7 mRNA transcription in LNCaP and to a greater extent in C4-2B cells but not 22Rv1.

![Figure 3.2.1: CXCR7 expression after AR inhibition by bicalutamide](image)

*Figure 3.2.1: CXCR7 expression after AR inhibition by bicalutamide.* CXCR7 mRNA in cells treated with 2 µM bicalutamide (BiC) relative to untreated (UT) cells. N = 3, mean ± SEM. *p<0.05, **p<0.01, ns = no significance; unpaired, two-tailed t-test.
cells. The more robust AR inhibitor enzalutamide (Enz) (Figure 3.2.2) shows a similar, significant increase in CXCR7 mRNA in both LNCaP and C4-2B cells, whereas treatment of 22Rv1 cells resulted in a mild but insignificant increase in CXCR7 mRNA relative to UT cells.

These results show that direct inhibition of AR signaling can mediate a similar increase in CXCR7 expression as observed with ADT. This indicates that the androgen-mediated suppression of CXCR7 was facilitated via AR signaling and not alternative mechanisms altered by R1881 treatment.

3.3: AR is required for androgen-mediated CXCR7 suppression:

Both ADT and AR signaling inhibition in LNCaP and C4-2B cells resulted in significantly increased CXCR7 expression, whereas androgen re-stimulation in androgen deprived cells resulted in significant suppression. To further confirm that AR is necessary for the observed androgen-mediated suppression of CXCR7, AR
was depleted in LNCaP cells with siRNA and CXCR7 transcription was measured with and without androgen. Figure 3.3.1 shows a western blot panel confirming depletion of AR by targeted siRNA both in the presence and absence of androgens. AR protein expression is maintained in control scramble-siRNA (siCtl) transfected cells, and was observed to increase when cells were stimulated with androgen. In cells transfected with AR-specific siRNA (siAR), AR protein was not detectable in androgen deprived cells, while a faint band could be observed following androgen stimulation (depleted but not completely abrogated). All cells maintained in androgen-deprived conditions showed increased expression of CXCR7. Importantly, in the siCtl transfected cells stimulated with androgen, CXCR7 expression substantially decreased. Conversely, in the siAR transfected cells, CXCR7 protein expression is not decreased following treatment with R1881.

RT-q-PCR analysis further confirmed that the androgen-mediated suppression of CXCR7 is abrogated following AR depletion. Shown in Figure 3.3.2, R1881 stimulation of siCtl cells resulted in a strong suppression of CXCR7 mRNA. Following AR depletion (siAR), there was a slight but insignificant decrease in
CXCR7 mRNA with R1881 stimulation relative to the androgen deprived controls. Thus, depletion of AR abrogated the androgen-mediated suppression of CXCR7 mRNA and protein (Figure 3.3.1), consistent with the results from treatment with AR-inhibiting compounds. These results confirm that AR is essential for the androgen-mediated suppression of CXCR7 in androgen-sensitive prostate cancer cells.

3.4: Identifying CXCR7 promoter elements involved in AR regulation:

From the previous results, androgen-mediated depletion of CXCR7 is shown to rely on AR signaling in androgen-sensitive prostate cancer cells. However, it is unclear if this response was due to direct or indirect transcriptional regulation by AR. I next investigated the presence of AR-responsive, transcription factor binding elements (AREs) in the CXCR7 promoter, which would suggest direct transcriptional repression of CXCR7 by AR. Utilizing the online PROMO-ALGGEN transcription factor binding element prediction algorithm [110, 111],
6,000 nucleotides upstream of the TSS in the CXCR7 promoter (NCBI reference DNA sequence: Homo sapiens chromosome 2, GRCh38.p7 Primary Assembly) was queried for potential AREs. The output of the search revealed a cluster of 6 possible AREs located between 2,600 and 1,000 nucleotides upstream of the TSS (illustration of the locations of the AREs are represented in Figure 3.4.1).

![Diagram of the CXCR7 promoter region including the 6 putative AREs tested for AR binding](image)

**Figure 3.4.1: Predicted AREs in the CXCR7 promoter**: Diagram of the CXCR7 promoter region including the 6 putative AREs tested for AR binding

After identification of putative AREs in the CXCR7 promoter, I investigated whether this region of the CXCR7 promoter was required for the androgen mediated suppression of CXCR7 as observed in the RT-q-PCR experiments. A shorter CXCR7 promoter-luciferase reporter (< 1,000 nucleotides upstream of the TSS of CXCR7 [88]) could not model the androgen-mediated suppression of CXCR7 (Figure 3.4.2). Since the predicted AREs are upstream of this original construct, a new reporter construct was developed with the longer CXCR7 promoter fragment. The CXCR7 promoter-driven luciferase reporter plasmid was generated to contain 2,549 nucleotides upstream to 172 nucleotides downstream
of the TSS of CXCR7, which contained all 6 predicted AREs. The promoter construct was stably transfected into C4-2B cells.

C4-2B cells stably expressing the promoter construct were maintained under ADT, then stimulated with R1881. Luciferase activity was measured, comparing control cells maintained under ADT (CDFBS alone) to those in CDFBS+R1881. Shown in Figure 3.4.3, cells stimulated with R1881 yielded significantly reduced CXCR7 long-promoter-driven luciferase activity when compared to androgen deprived control cells. This result shows that the longer CXCR7 promoter region contains the response elements required for androgen-mediated suppression of CXCR7.

**Figure 3.4.2: CXCR7 Short-promoter-luciferase androgen response:** The shorter, < 1,000 nucleotide CXCR7 promoter-driven luciferase activity in C4-2B cells cultured in CDFBS ± R1881, relative to CDFBS control. N = 3; mean ± SEM; ns = no significance: two-tailed, unpaired t-test.

**Figure 3.4.3: CXCR7 Long-promoter-luciferase androgen response:** The longer, > 2,600 nucleotide CXCR7 promoter-driven luciferase activity in C4-2B cells cultured in CDFBS ± R1881, relative to untreated control. N = 3; mean ± SEM; ***p<0.001: two-tailed, unpaired t-test.
These results determined that the regulatory elements driving the androgen-mediated suppression of CXCR7 were located more than 1,000 nucleotides upstream of the TSS. Prediction of 6 potential AREs indicates that AR may regulate CXCR7 transcription by binding to this promoter region. To confirm AR binding to one or more of the predicted AREs, ChIP assays were performed to probe for direct AR binding to the CXCR7 promoter.

### 3.5: AR directly regulates CXCR7 transcription:

To probe for direct AR binding to the CXCR7 promoter in an androgen-dependent manner, ChIP assays were performed on LNCaP cells in the presence and absence of androgen. RT-q-PCR primer sets were designed to specifically amplify the regions of the CXCR7 promoter containing the AREs, numbered sequentially from the most distal to the most proximal to the TSS (Figure 3.4.1). AREs 4 and 5 are only separated by 7 nucleotides and could not be resolved with this method, so the region containing both was probed (ARE4/5). Cells were androgen deprived for 48 hours then cultured in the presence or absence of R1881 for 24 hours prior to the ChIP assay. Crosslinked, digested chromatin from androgen deprived and R1881 stimulated cells was precipitated with either isotype control IgG or AR specific IgG antibodies. The enhancer region of the PSA promoter was used as a positive control for AR binding.

RT-q-PCR of the promoter DNA enriched by the antibodies was performed for the androgen deprived and androgen stimulated samples. Target DNA abundance was calculated relative to the 10% input chromatin control. The %-input values for the AR-enriched DNA was then calculated relative to the control, non-
specific IgG pulled-down samples to obtain enrichment over background. The results from the ChIP assays are presented in Figure 3.5.1. The positive control PSA RT-q-PCR shows a significant enrichment of PSA promoter DNA from cells stimulated with androgen compared to androgen deprived cells. RT-q-PCR was performed using five primer sets for the CXCR7 promoter DNA to measure AR-pulldown enrichment of the putative AREs. A significant increase in AR-enriched promoter DNA was observed for the CXCR7 promoter region containing ARE 2 and the region containing the closely spaced AREs 4 and 5 (ARE 4/5). The regions containing ARE 1 and ARE 3 were also enriched in the androgen stimulated cells compared to androgen deprived controls; however, these increases did not achieve significance. There was no noticeable enrichment with androgen in the ARE 6 region. From these results, enrichment of two ARE-containing regions in the CXCR7 promoter indicated that AR directly binds to the CXCR7 promoter.

Figure 3.5.1: ChIP assay for AR binding to the CXCR7 promoter: ChIP assay for AR pulldown of CXCR7 promoter regions containing predicted AREs in LNCaP ± R1881. The PSA enhancer region served as a positive control for AR binding. N = 3, mean ± SEM; *p<0.05, ns = no significance; unpaired, two-tailed t-test.
Together, these data reveal that androgen-mediated suppression of CXCR7 is dependent on AR, and that the transcriptional regulation is directly mediated by AR binding to the CXCR7 promoter.

3.6: CXCR7 potentiates mitogenic signaling during androgen deprivation:

Previous work has shown that increased CXCR7 expression is strongly correlated with the transactivation of EGFR, particularly through phosphorylation at the tyrosine residue 1110 (Y1110) in EGFR. This enhanced activation of EGFR results in a subsequent increase in the activation of the downstream mitogenic signaling pathway leading to phosphorylation of ERK1/2 and an associated increase in proliferation [84]. Furthermore, the results of this study revealed that CXCR7 expression increases during ADT or through chemical AR inhibition. Based on these findings, I aimed to determine whether the increase in endogenous CXCR7 expression during ADT can facilitate increased EGF-mediated EGFR activation and downstream mitogenic signaling. LNCaP cells were treated in CDFBS with or without 5 nM R1881 for 72 hours, followed by EGF stimulation for 2 or 5 minutes. Western blot analysis of phosphorylated EGFR and ERK1/2 relative to their respective total protein, as well as the expression changes to CXCR7 and AR are shown in Figure 3.6.1A. The associated densitometric analysis (Figure 3.6.1B) is representative of the protein bands in the individual western blot panel shown (two independent experimental repetitions yielded similar results). Under androgen deprived conditions, CXCR7 protein is increased while AR protein expression is decreased relative to R1881 stimulated cells. The phosphorylation of EGFR at Y1110 (pEGFR) relative to total EGFR protein is substantially greater
in CDFBS treated cells compared to R1881 stimulated cells at both 2 minutes and 5 minutes of EGF stimulation. Downstream ERK1/2 phosphorylation (pERK1/2) relative to total ERK1/2 is also increased in CDFBS treated cells compared to R1881 stimulated cells at 2 and 5 minutes of EGF stimulation.

The results of these experiments reveal evidence of alternative, androgen-independent mitogenic signaling in androgen-dependent prostate cancer cell lines. Through the loss of direct repression of CXCR7 by AR resulting from ADT or chemical AR-inhibition, alternative mitogenic signaling programs become enhanced. The increase in alternative signaling through EGFR reveals evidence
for early androgen-independent growth and survival signaling mediated by increased CXCR7 expression.

3.7: Discussion:

Factors which are dysregulated during ADT that support early androgen-independent cell survival or proliferation are vital targets for preventing prostate cancer progression. In the first aim of this study, I have focused on the atypical chemokine receptor CXCR7 due to its significant association with prostate cancer cell proliferation and survival [77, 84]. From the results presented here, I provide novel evidence supporting the association between loss of AR signaling, the increase in CXCR7 expression and the potentiation of EGFR-mediated mitogenic signaling in androgen sensitive prostate cancer cells.

Previous studies have determined that EGFR activation and mitogenic signaling programs are involved with androgen-independent prostate cancer proliferation and survival, and is one mechanism facilitating development of CRPC during ADT [125, 126]. While I have shown here that relative pEGFR to total EGFR is substantially increased in androgen deprived cells, the total EGFR expression seems strongly reduced in the androgen deprived samples. Consistent with our results, it was previously reported that AR regulates both EGFR mRNA and protein expression in LNCaP cells, and that androgen deprivation results in decreased EGFR compared to androgen stimulated cells [127]. While I observe noticeable depletion of total EGFR protein under ADT, there was no comparable loss in phosphorylated EGFR on the western blots. The conclusion from this result is that the increase in CXCR7 during ADT is capable of at least maintaining the same
EGFR phosphorylation potential as that observed with androgen stimulation. However, the downstream activation of ERK1/2 is clearly increased, suggesting that sustained EGFR phosphorylation leads to more robust mitogenic signaling pathway activation during ADT. Together, this work shows that CXCR7 can enhance overall mitogenic signaling during ADT, supporting androgen-independent cell survival. The potentiation of androgen-independent mitogenic signaling by overexpressed CXCR7 reveals a mechanism supporting hormone-refractory phenotypes leading to CRPC.

Literature evidence clearly implicates CXCR7 expression in imparting survival and growth advantages to prostate cancer cells. However, from the results of this work, it is surprising that CRPC cell lines (C4-2B and 22Rv1) exhibit lower CXCR7 expression than androgen-dependent cells (LNCaP). This phenomenon may be explained by the mechanisms driving CRPC. One mechanism that C4-2B cells rely on to overcome ADT is increased expression of AR. Overexpression of AR in C4-2B cells allows them to respond to greatly reduced circulating androgen levels compared to what is required by LNCaP cells to proliferate [128]. Because AR is shown in this work to suppress CXCR7 expression, increased AR expression and the resulting increase in AR activity in C4-2B cells would result in reduced CXCR7 compared to LNCaP cells under the same culture conditions. Regarding the 22Rv1 cell line, the mechanism of resistance to ADT and AR inhibition involves the constitutively active AR variants. These cells proliferate completely independent of androgens by expressing constitutively active, ligand-independent AR-Vs which are capable of maintaining constitutive AR signaling [129].
Furthermore, the AR-Vs expressed in 22Rv1 are also implicated in therapeutic failure of AR inhibitors, as they are resistant even to the more potent AR inhibiting compound, enzalutamide [54]. Due to the constitutive AR-V signaling in 22Rv1 cells, the expression of CXCR7 is potently suppressed by AR, which explains why there was no significant increase in CXCR7 expression with either ADT or AR inhibition. While AR signaling remains the primary factor driving prostate cancer proliferation, even during the progression to CRPC [130], the results of this investigation demonstrate that CXCR7 may potentiate prostate cancer cell survival during ADT, and may be a critical factor meditating the transition to castration resistance.
Chapter 4: CXCR7 IS INVOLVED IN PROSTATE CANCER CELL SURVIVAL

4.1: CRISPR-Cas9-mediated knockout of CXCR7 results in cellular senescence:

From the previous results, CXCR7 was shown to be directly repressed by AR and that ADT-mediated CXCR7 overexpression facilitates androgen-independent mitogenic signaling. Based on these findings, the next aim was to determine the importance of CXCR7 in facilitating the transition to CRPC. To date, the role of increased CXCR7 expression during ADT has not been investigated in potentiating the transition to CRPC. The goal of this aim was to abrogate CXCR7 expression and monitor the long-term survival and proliferative capacity of LNCaP and C4-2B cells maintained under ADT.

To maintain depletion of CXCR7, the initial method was to stably express shRNA plasmids to reduce CXCR7 mRNA abundance. Unfortunately, these experiments failed to yield long-term suppression of CXCR7 due to the loss of efficacy of the shRNA depletion over time, even with maintenance of antibiotic selection of the transfected cells. Figure 4.1.1 shows the RT-q-PCR analysis of CXCR7 mRNA expression in early (1 passage after establishing stable lines) and later (after 5 passages in culture) shCXCR7-LNCaP cells. The initial suppression of CXCR7 is significant compared to the shCtl cells in the early cultures; however, after multiple passages, the depletion of CXCR7 is attenuated. One possible explanation is selective silencing of the shRNA expression over time, leading to
restored CXCR7 expression. To overcome this limitation, I utilized the CRISPR-Cas9 gene editing technology to completely abrogate CXCR7 expression.

Both LNCaP cells and C4-2B cells were transfected with a pool of three CRISPR-gRNA constructs specifically targeting CXCR7. CRISPR-Cas9 editing relies on the high probability for random insertion or deletion mutation events (InDels) inherent in the non-homologous end joining (NHEJ) DNA repair mechanism. Due to the random nature of these InDel events, transfected cells were plated at single cell dilutions for clonal expansion to obtain a population of cells with identical DNA mutations. The first transfection experiment yielded one C4-2B colony with complete CXCR7 knockout. Figure 4.1.2A shows western blot confirmation of CXCR7 protein knockout in the C4-2B clone (CX7-KO) relative to control, non-specific CRISPR gRNA transfected C4-2B cells (Ctl). The knockout was confirmed with a second western blot experiment probing with an alternative, N-terminal specific CXCR7 antibody (Figure 4.1.2B).
The CX7-KO cell line exhibited exceedingly slow proliferation under normal culture conditions compared to control cells. Viable cell counts over 96 hours (Figure 4.1.3) showed the Ctl cells proliferate significantly faster than the CX7-KO cells, with a nearly static number of CX7-KO cells observed over 96 hours. To determine the cause of this exceedingly slow proliferation, even under normal androgen stimulation, CX7-KO cells were stimulated with EGF to investigate the impact on mitogenic signaling mediated
through the CXCR7-EGFR transactivation axis. C4-2B Ctl and CX7-KO cells were stimulated for 2 or 10 minutes with EGF after 24 hours of culture in low serum medium. The western blot panel and associated densitometric analysis (Figure 4.1.4) show that the CX7-KO cell line exhibited reduced pEGFR relative to total EGFR compared to Ctl cells after 2 minutes of EGF stimulation. After 10 minutes of EGF stimulation, the pEGFR to total EGFR ratio in Ctl cells declined but remained greater than that of the CX7-KO cells. The pERK1/2 to total ERK1/2 ratio was potently reduced in the CX7-KO cells compared to Ctl cells at both 2 minutes and 10 minutes of EGF stimulation.

**Figure 4.1.4: Mitogenic signaling in CX7-KO C4-2B cells:** Western blots of EGFR and ERK1/2 activation in control (Ctl) and CXCR7-knockout (CX7-KO) C4-2B cells and densitometry showing relative phosphorylated protein to total protein. Representative western blot panel of two independent experiments. Densitometry is measured from the individual western blot panel shown.
The clonogenic potential of the CX7-KO cells was also significantly reduced, as revealed by clonogenic survival assays. Figure 4.1.5A shows a representative image of the colonies formed by the Ctl and CX7-KO cells. The CX7-KO cells formed fewer and smaller colonies than the Ctl cells. Figure 4.1.5B shows the average count of colonies formed from 500 seeded cells.

Unfortunately, after 2 passages under normal culture conditions, cells from this CX7-KO clone failed to proliferate further. Attempts to restore proliferation (standard culture medium supplemented with conditioned medium and additional growth factors) could not recover this cell population. Also of note, the morphology of the CX7-KO cells was altered dramatically compared to control cells (Figure 4.1.6). However, after the multiple attempts to restore proliferation, this population was lost.
Figure 4.1.6: CX7-KO morphological changes: A. Control C4-2B cells expressing WT CXCR7. B. Representative image of CX7-KO C4-2B cells after proliferation arrest.
CRISPR-Cas9 editing in C4-2B cells was repeated to obtain new CX7-KO colonies. Very few proliferative colonies were established after three repeated attempts to obtain viable CX7-KO cells. Some colonies did proliferate to an extent allowing isolation of sufficient genomic DNA for validation of CXCR7 mutations. However, even those colonies eventually exhibited the same proliferative arrest and morphological changes as observed previously. These colonies were passaged once, with 10% of the cells isolated for Sanger sequencing of their genomic DNA. The remaining cells were returned to culture. After one month in culture, no further proliferation could be observed, and all cells had developed a broad, flattened morphology with numerous long protrusions. These morphological changes along with arrest in proliferation indicated that the cells had undergone cellular senescence [118, 121, 131]. To confirm these observations are associated with senescence and not quiescence, I stained for SA-gal expression and activity. Figure 4.1.7 shows the results of these tests from one representative colony of CRISPR modified C4-2B cells. As controls for SA-gal staining, normally proliferating C4-2B cells expressing wild-type (WT) CXCR7 were stained. The WT C4-2B cells were incubated in the presence or absence of 10 µM H$_2$O$_2$ (oxidative damage-induced cellular senescence) for 96 hours. Untreated cells (negative control: upper left panel of Figure 4.1.7) and treated cells (positive control: upper right panel of Figure 4.1.7) were stained for SA-gal activity. In proliferation-arrested CRISPR-Cas9-mutated C4-2B colonies, strong blue staining was observed in nearly all cells remaining in culture. Representative images are shown in the middle-left, middle-right, and lower-left panels of Figure 4.1.7. Genomic DNA was
harvested from each colony at the first passage, and submitted for Sanger sequencing of the CXCR7 protein coding region. The lower-right panel of Figure 4.1.7 shows the Sanger sequencing chromatogram of CXCR7 at the site targeted by one CRISPR-gRNA construct (CXCR7-gRNA 1). The overlapping traces in the chromatogram downstream of the Cas9 cleavage site (3 nucleotides upstream of the PAM sequence targeted by the gRNA) indicate unique InDel mutations in CXCR7. While the exact mutations cannot be discerned from this chromatogram, analysis of the overlapping sequences revealed that any potential combination of each overlapping peak would yield a frameshift mutation, and loss of WT CXCR7 expression.

In parallel experiments, I also used the CRISPR-Cas9 technology to abrogate CXCR7 in LNCaP cells. Out of four independent experiments, one continuously proliferating colony (three weeks to establish, further discussed in subchapter 4.2) and two slow-growing colonies (two months to establish) were obtained. As with C4-2B cells, cultures were passaged once, with 10% of the cells harvested for genomic DNA sequencing. Remaining cells were returned to culture. After passaging cells once, no proliferation could be observed in the two initially slow-growing LNCaP colonies, and the cells developed a broad, flattened morphology. These proliferation-arrested colonies were tested for SA-gal activity. The upper two panels of Figure 4.1.8 show WT-CXCR7 LNCaP cells ± 10 µM H₂O₂. H₂O₂ treated LNCaP cells showed strong blue staining indicating SA-gal expression which was not observed in UT control cells.
The middle-left, middle-right, and lower-left panels in Figure 4.1.8 are representative images of the non-proliferating CRISPR-Cas9 LNCaP cells, exhibiting a broad, flattened morphology, and dark blue X-gal staining (indicative of SA-gal expression), which are hallmarks of cellular senescence [118, 121]. Genomic DNA sequencing results for the senesced clones (representative chromatogram in the lower-right panel of Fig. 4.1.8), shows overlapping sequences beginning at the Cas9 cleavage site targeted by the CXCR7-gRNA 1 construct. The overlapping signal is indicative of unique InDel mutations. These results, together with the observations in C4-2B cells suggest that loss of WT CXCR7 through frameshift mutations leads to severely inhibited proliferation and the onset of cellular senescence. The Sanger sequencing chromatograms of CXCR7 in the CRISPR-Cas9 edited clones revealed three overlapping sequence traces. A clone derived from a single cell should only exhibit up to two overlapping sequences due to unique InDel events (two CXCR7 alleles). These observed events may be due to residual Cas9 nuclease activity during the first few mitotic divisions after plating. If a second InDel event occurred on one CXCR7 allele in one of the daughter cells, the resulting cell population would exhibit three alternative sequences. While the sequencing cannot determine the exact individual mutations, no combination of the three potential sequences represent WT CXCR7, or inframe mutations.
Figure 4.1.7: Frameshift mutations of CXCR7 in C4-2B cells and detection of senescence: Upper-left panel: C4-2B cells with WT CXCR7 expression untreated (UT) (negative control). Upper-right panel: WT C4-2B cells treated with 10 μM H₂O₂ (positive control). Middle-left, middle-right, and lower-left panels: representative images of CXCR7-CRISPR-mutated cells. Lower-right panel: Sanger sequencing chromatogram at the CXCR7-gRNA 1 target site, showing overlapping unique InDel mutations of the CXCR7 protein coding region.
Figure 4.1.8: Frameshift mutations of CXCR7 in LNCaP cells and detection of senescence: Upper-left panel: WT LNCaP UT (negative control). Upper-right panel: WT LNCaP + 10 μM H₂O₂ (positive control). Middle-left, middle-right, and lower-right panels: representative images in CXCR7-CRISPR-mutated cells. Lower-right panel: Sanger sequencing chromatogram at the CXCR7-gRNA 1 target site, showing overlapping unique InDel mutations of the CXCR7 protein coding region.
4.2: Characterization of a CRISPR-Cas9-mediated mutation of CXCR7:

One continuously proliferating CRISPR-Cas9 modified LNCaP clone did not undergo proliferative arrest or exhibit the phenotypes indicative of cellular senescence. PCR amplification of the CXCR7 protein coding region performed with both the genomic DNA and the mRNA-derived cDNA from these cells indicated a significantly shorter product (close to 400 nucleotides shorter than WT CXCR7). A representative image of the PCR product is shown in Figure 4.2.1. While the truncated band is the dominant PCR product, a faint upper band is apparent in the CXCR7-mutant DNA PCR. Both the upper and lower bands were submitted for Sanger sequencing. Sequencing of the upper PCR band revealed an overlapping trace profile (similar to what was observed in the senesced clones) beginning at the site of Cas9 cleavage targeted to the CXCR7-gRNA 1 construct (Figure 4.2.2 middle panel). As a reference, the WT CXCR7 sequence trace is shown in the upper panel of Figure 4.2.2. Sanger sequencing of the shorter PCR band revealed a large internal deletion event which was flanked by the maintained WT CXCR7 sequence (Figure 4.2.2 lower panel).
Aligning the DNA sequence of the shorter CXCR7 protein coding region (CXCR7-mutant) to the WT CXCR7 DNA sequence revealed a 394 nucleotide deletion between two CRISPR gRNA target sites (CXCR7-gRNA 1 and CXCR7-gRNA 3) (corresponding to amino acids 114 to 243 of the CXCR7 protein). Furthermore, the protein translation reading frame for CXCR7 was maintained.

**Figure 4.2.2: CXCR7-mutant Sanger sequencing chromatograms:** Sanger sequencing chromatogram of the CXCR7 PCR fragments from LNCaP WT and CXCR7-mutant genomic DNA. **Top:** WT CXCR7 sequence. **Middle:** Upper band from the CXCR7-mutant PCR (the CRISPR-gRNA target site is indicated above the sequence). **Bottom:** Lower band from the CXCR7-mutant PCR. The CRISPR-gRNA target sites are indicated above the sequence; the 5’ CXCR7 sequence (black arrow) and the 3’ CXCR7 sequence (red arrow) flanking the deletion are indicated below the chromatogram.
downstream of the deleted region, suggesting the N and C termini of the CXCR7 protein could still be translated. The illustration in Figure 4.2.3 depicts the secondary structure of CXCR7 (predicted domains: UniProtKB - P25106 [ACKR3_HUMAN]). The red spheres indicate the segment of the CXCR7 protein which would be deleted based on the sequence resulting from the CRISPR-mediated mutation. Western blot analysis of the CXCR7-mutant clone confirms a shorter protein, showing a CXCR7 band at a lower molecular weight compared to WT CXCR7 (Figure 4.2.4). Furthermore, as controls to show the specificity of the antibody, the WT LNCaP cells were treated with control siRNA (siCtl) or CXCR7-specific siRNA (siCX7). The blot shows depleted CXCR7 protein signal in the siCX7 treated cells, confirming specificity of the antibody, and the recognized
shorter CXCR7 band in the LNCaP CXCR7-mutant (CX7-mut) cells. These results suggest that this cell line, unlike all other senesced clones, still expresses CXCR7 protein, albeit a truncated mutant, which may maintain the proliferative potential of these LNCaP cells.

As this CX7-mut LNCaP cell line was the only viable CRISPR-edited clone which continued to proliferate, I next characterized alterations to mitogenic signaling and survival phenotypes. Unlike the other LNCaP clones with CXCR7 frameshift mutations, this clone did not exhibit signs of cellular senescence.

To determine if the deletion of the internal CXCR7 sequence was necessary for CXCR7-mediated mitogenic signaling, cells were stimulated with EGF and phosphorylation of EGFR and ERK1/2 were investigated and compared to controls (WT-CXCR7). The western blot panel and its associated densitometry shows that pEGFR relative to total EGFR is greater in WT cells than in CX7-mut cells at 2 minutes and 5 minutes after EGF stimulation (Figure 4.2.5). The difference in the activation of ERK was more pronounced between the cell lines (compared to
EGFR phosphorylation). pERK1/2 relative to total ERK1/2 in CX7-mut cells is substantially decreased compared to WT cells at 5 minutes of EGF stimulation.

Previous work has shown that CXCR7-mediated EGFR phosphorylation, downstream mitogenic signaling, and tumor cell proliferation are inhibited by ARRB2 [83]. Others have shown ARRB1 mediates CXCR7 transactivation of EGFR [85, 86]. Our preliminary experiments regarding ARRB1 and EGFR transactivation corroborate the findings in the literature. Depletion of ARRB1 with stable shRNA expression in C4-2B cells resulted in decreased EGFR and ERK phosphorylation compared to control shRNA expressing cells (Figure 4.2.6).
These results demonstrate that ARRB1 plays a role in the transactivation of EGFR. To determine if the CXCR7-mutation altered CXCR7-ARRB1 or CXCR7-ARRB2 interactions leading to reduced EGFR transactivation, PLAs were performed to detect co-localization of CXCR7 with either ARRB1 or ARRB2 (Figure 4.2.7). The CX7-mut cell line exhibited substantially reduced CXCR7-ARRB1 and increased CXCR7-ARRB2 interactions compared to WT CXCR7 LNCaP cells. This alteration in the interaction with the β-arrestins may offer evidence to the importance of the internal protein region of CXCR7 (amino acids 114 to 243) in regulating the ARRB-EGFR-CXCR7 signaling axis.

![Western blot panel for phosphorylated EGFR and ERK relative to total protein in C4-2B cells stably expressing either control (shCtl) or ARRB1-specific (shARRB1) shRNA.](image)
**Figure 4.2.7:** CXCR7 interactions with ARRB1 and ARRB2 in LNCaP cells. Fluorescence imaging of PLA between CXCR7 and ARRB1 or ARRB2. **Top:** CXCR7-Isotype (negative control). **Middle:** CXCR7-ARRB1 co-localization in WT (Left) or CX7-mutant (Right) cells. **Bottom:** CXCR7-ARRB2 co-localization in WT (Left) or CX7-mutant (Right) cells. Cell nuclei stained with DAPI (blue); red spots indicate target protein co-localization within 40 nm of one another.
Based on the results showing an overall reduction in mitogenic potential in the CX7-mut line, I next investigated whether these cells would be more sensitive to long-term ADT than WT LNCaP cells. Both lines were seeded in androgen deprived conditions (CDFBS) and maintained for 6 weeks, with a viable cell count performed each week (Figure 4.2.8). During the first week, there was an overall increase in cell number in both cell lines. At the second week of ADT, the number of viable cells in the CX7-mut line began to decline, while those in the WT line increased slightly. Between 2 and 4 weeks, both cell lines experienced an overall decline in viable cells, before reaching a steady residual population by week 6. The viable cell number at the end of the experiment was significantly lower in the CX7-mut than in the WT cell line, which may be due to the initial delay before the WT cells exhibited declining viable cell numbers. This result suggests the CX7-mut cells may be more sensitive to early ADT than the WT cell line.

![Figure 4.2.8: Viable WT and CX7-mut cells surviving during ADT: 2.5x10^6 cells were seeded in cell culture flasks and viable cell numbers were determined once per week for 6 weeks (trypan blue exclusion). N = 2, mean ± SEM. *p<0.05, two-tailed unpaired t-test.](image)
4.3: CXCR7 mutation decreases metastatic phenotypes:

It is well understood that the CXCR family is involved in metastatic phenotypes in cancer [67]. In prostate cancer, bone is a disproportionally common site of metastasis which is directed in part by CXCR4-mediated migration along SDF-1 (CXCL12) chemokine gradients [132]. CXCR7, as described before, is implicated in enhancing the overall CXCR4 signaling following binding of ligand [73]. Consistent with these discoveries, a previous study determined that cell motility driven by SDF-1 is dependent on the CXCR4-CXCR7-AR regulatory axis [133]. Based on these reports, I investigated whether the CRISPR-mediated mutation of CXCR7 disrupted the migratory or metastatic phenotypes in LNCaP cells. To determine if there were alterations in migration along to SDF-1 gradients, transwell migration assays in Boyden chambers were performed. WT and CX7-mut LNCaP cells were seeded in the upper chambers, in SDF-1-free medium and allowed to migrate through a semi-permeable membrane towards SDF-1 supplemented medium in the lower chamber. The results of these assays (Figure 4.3.1) revealed a decrease in the migration of the CX7-mut cells; however, the difference in migration did not achieve statistical significance.

![Figure 4.3.1: SDF-1-mediated migration of LNCaP WT and CX7-mut cells](image-url)

**Figure 4.3.1:** SDF-1-mediated migration of LNCaP WT and CX7-mut cells: Transwell migration assay of LNCaP WT and CX7-mut cells towards an SDF-1α gradient. N = 2, mean ± SEM; ns = no significance; two-tailed, unpaired t-test.
The migration along a chemoattractant gradient is only one aspect of the metastatic potential of cancer cells. Another necessary mechanism is anchorage-independent cell survival during transport through circulation [134]. The sphere formation assay has been used to indicate this aspect of metastatic potential of a cancer cell line [135]. To determine the sphere-forming potential and anchorage-independent cell survival, WT and CX7-mut cells were seeded in low-adherence plates and allowed to establish spheres for 10 days. The first generation of spheres was harvested, disrupted and cells were reseeded for analysis of second generation spheres. Figure 4.3.2A shows the overall count of spheres formed from each cell line. The second generation sphere forming potential of the CX7-mut was significantly lower compared to WT cells. Additionally, the size of the spheres that did form were generally smaller in the CX7-mut line compared to WT cells, as depicted in the representative images in Figure 4.3.2B.

**Figure 4.3.2:** Sphere formation assay of LNCaP WT and CX7-mut cells: A. Count of second generation spheroids formed per plate from 10,000 seeded WT and CX7-mut cells (N = 2, mean ± SD; *p<0.05; two-tailed, unpaired t-test). B. Representative image of second generation spheres formed from each cell line.
Together, these in vitro assays suggest a decrease in the overall metastatic potential of the CX7-mut line, in addition to its substantially reduced mitogenic signaling potential and potentially decreased resistance to early ADT. However, the sphere formation assay offers additional evidence to how these mutant cells may function in vivo. The sphere formation assay has been classically utilized to enrich for and potentially identify the existence of cancer stem cells (formation of a sphere from a single progenitor) [122, 123, 136]. From the observation of reduced sphere formation potential, as well as the overall smaller size of spheres generated, the next investigation was to measure the tumorigenicity and tumor growth potential of the CX7-mut cells in vivo.

4.4: CXCR7 mutation decreases tumorigenic potential and tumor growth:

The in vitro observations demonstrated reduced sphere formation, disrupted mitogenic signaling, and potentially increased sensitivity to ADT in the CX7-mut LNCaP cell line. The next aim was to determine if WT CXCR7 is necessary for tumorigenicity of LNCaP cells. To investigate the hypothesis that CXCR7 is necessary for the survival and growth of prostate cancer cells, I performed xenograft studies with WT and CX7-mut LNCaP cells. For this analysis, FOXN1 deficient, athymic, male nude mice were injected subcutaneously with 2x10^6 cells into the right flank. Animals were injected with either WT or CX7-mut LNCaP cells and tumors were allowed to establish and grow over 12 weeks unless a humane endpoint condition was achieved.
Over the course of 12 weeks, the WT LNCaP cells established tumors and grew to significantly greater volumes than the CX7-mut cell line. The difference in tumor growth in the WT group was profoundly greater than observed in the CX7-mut implanted mice. Shown in Figure 4.4.1, the average tumor volume measured three times weekly, up to 61 days after implantation, was greater in the WT group than the CX7-mut group (a difference that was highly significant). After 60 days, several WT tumor bearing mice experienced humane endpoint criteria and had to be euthanized. However, surviving mice were maintained up to 12 weeks (84 days). In the WT group, all but 4 animals remained alive to the end of the study: 1 animal had no tumor, 2 animals had tumors with volumes less than 20 mm³, while the last animal had a tumor of approximately 500 mm³. Within the CX7-mut group, all 10 animals remained alive until the end of the study, with 4 animals bearing no tumors, 5 with tumors less than 20 mm³, and 1 animal with a tumor of approximately 50 mm³.

This preliminary xenograft analysis of the

![Figure 4.4.1: WT and CX7-mut LNCaP xenograft tumor growth](image)

- **Tumor volume (mm³):** Average tumor volume from WT and CX7-mut cell lines implanted subcutaneously in athymic, male, nude mice. Non-linear regression used to generate best fit exponential growth curves. Tumors volume was measured three times weekly up to 61 days after implantation. **p<0.001, Mann-Whitney U-test, two-tailed.**
CX7-mut LNCaP cell line compared to WT LNCaP cells clearly supports the hypotheses based on the in vitro evidence. The tumorigenicity and tumor growth potential in vivo is significantly inhibited in cells with the CRISPR-mediated mutation to CXCR7. Together with the in vitro characterization of this mutant line, the role of CXCR7 is shown to have a critical role in androgen-sensitive prostate cancer cell lines for cell survival and proliferation, resistance to therapy, and tumor initiation and growth.

4.5: Discussion:

The recent CRISPR-Cas9 genomic DNA editing tool was utilized in this project to eliminate CXCR7 expression in androgen-sensitive prostate cancer cell lines. The results from these experiments revealed a dependence on CXCR7 expression for proliferation, even under normal androgen stimulation. After multiple repetitions of the experiment, only a small number of colonies could be established from the CXCR7-CRISPR edited cells. All but one LNCaP clone from these experiments exhibited cell proliferation arrest and onset of cellular senescence compared to control CRISPR-Cas9 transfected cells. The importance of CXCR7 in prostate cancer cell proliferation has already been shown with RNAi methods to deplete CXCR7 in prostate cancer cell lines under normal culture conditions, where CXCR7 depletion resulted in significantly reduced cell proliferation and inhibited cell cycle progression [84]. However, RNAi methods could not completely eliminate CXCR7 expression. This current work confirmed the dependency on CXCR7 in these cell lines, as CRISPR-Cas9-mediated abrogation of CXCR7 significantly enhanced the phenotypes observed with RNAi
depletion. These observations support the critical role of CXCR7 in maintaining cell proliferation even with normal androgen stimulation.

Regarding the senesced LNCaP clones, cells that experienced frameshift mutations in the CXCR7 protein coding region required nearly two months to establish colonies and, after one passage, exhibited complete proliferation arrest. The one colony expressing an inframe mutation (maintaining both the N- and C-terminal sequences flanking the deletion site) continued to proliferate. Analysis of this clone revealed substantial reduction in EGFR transactivation and mitogenic signaling. The reduced EGFR transactivation in this clone may be explained through differential interactions between CXCR7 and the β-arrestins. Previous work has shown that the ARRB2-CXCR7 interaction inhibits EGFR-Y1110 phosphorylation and downstream ERK1/2 activation in prostate cancer cells [83]. Alternatively, investigations in other cancer models show that ARRB1 enhances the transactivation of EGFR leading to increased mitogenic signaling [85, 86]. Furthermore, ARRB1 enhances ligand-dependent mitogenic and migration signaling mediated by CXCR4-CXCR7 heterodimers [87]. Preliminary results shown in this study also support the role of ARRB1 in regulating the CXCR7-EGFR transactivation axis in C4-2B cells. The PLA performed to investigate CXCR7 and ARRB1 or ARRB2 interactions determined the CXCR7 mutation alters these protein-protein interactions. Mutant CXCR7 exhibited increased ARRB2 and reduced ARRB1 interactions compared to WT CXCR7, corresponding to reduced EGFR and ERK1/2 signaling.
Consistent with the observations in vitro, the in vivo characterization of the CRISPR-mutated CXCR7 LNCaP cells revealed profoundly reduced tumorigenicity and tumor growth potential compared to WT CXCR7 cells. While it remains unclear if the mutant line is more susceptible to ADT or chemical AR inhibition in vivo, the tumor initiation is clearly compromised in the mutant line. The sphere-forming assay provides some insight into these observations. While the assay provides evidence for anchorage-independent survival, which is characteristic of metastatic cells during transport in the circulation, it also suggests differences in stem-like properties of a cell line. Like colony formation assays, the sphere-forming assay provides information on the proportion of cells in a population capable of unlimited growth potential. However, the sphere forming assay also has been used to enrich for cancer stem cells by harvesting later generation spheres from culture [122, 123, 136]. The significant reduction in spheres formed in the mutant cell line suggested a reduced stem-like potential compared to WT LNCaP cells. The reduction in cancer stem cells is directly related to a decrease in tumorigenicity, consistent with the observations in this study. Studies in breast cancer [137], glioblastoma [138], and lung cancer models [139] support a link between CXCR7 and stem-like properties of cancer cells. While it is not completely clear how CXCR7 may regulate the stem-like properties in prostate cancer, the results of this analysis in vitro and in vivo indicate a disruption in self-renewal, proliferation, and establishment and growth of tumors from the CX7-mut cell population.
Chapter 5: OUTLOOK

5.1: Summary:

This study was an investigation into the role of CXCR7 in facilitating androgen-independent growth and survival in prostate cancer. Taken together, these data support a critical role for CXCR7 in androgen-independent mitogenic signaling programs as well as in the overall survival of prostate cancer cells. These results reveal CXCR7 to have a substantial impact on both efficacy and resistance to current therapeutic interventions. This investigation has shown CXCR7 to be actively regulated by AR, a repressive mechanism lost during ADT or AR inhibition. Together with CXCR7’s role in enhancing alternative signaling pathways (CXCR4 and EGFR), increased expression during early ADT underlies mechanisms of CRPC recurrence. Furthermore, complete abrogation of CXCR7 in androgen-sensitive prostate cancer cells results in cellular senescence, even under normal growth conditions. Alternatively, a mutation that disrupted interactions with β-arrestins profoundly altered the mitogenic signaling, metastatic, and tumorigenic phenotypes of prostate cancer cells. From this study, the role of CXCR7 has been shown to be critical to both androgen-independent and androgen-dependent prostate cancer proliferation, survival, and potentially the progression to CRPC.

Initially this investigation evaluated the role of AR on the regulation of CXCR7. Some literature evidence suggested a link between AR and CXCR7; however, it was unclear whether this was direct or indirect regulation. Through application of ADT and the AR inhibiting compounds bicalutamide and enzalutamide, I was able to determine that AR signaling significantly suppresses
CXCR7 in androgen-sensitive but not androgen-independent prostate cancer cells. Furthermore, through siRNA depletion of AR, I determined that AR is necessary for the observed androgen-mediated downregulation of CXCR7. To determine the direct or indirect regulation by AR in these prostate cancer cells, I designed and tested a CXCR7 promoter-driven luciferase reporter construct to identify the promoter region necessary for the observed transcriptional regulation. These assays revealed the regulatory region of the CXCR7 promoter (that could mimic the mRNA results) was contained within 2,600 nucleotides of the TSS. Within that region, six potential AR binding sites were identified. Through ChIP assays to detect AR binding to those predicted regions, I demonstrated that AR directly binds two regions on the CXCR7 promoter containing the predicted AREs in an androgen-dependent manner. Finally, the functional importance of CXCR7 overexpression during ADT was investigated. During androgen deprivation, the increase in CXCR7 expression substantially enhanced the overall EGF-mediated activation of EGFR and downstream mitogenic signaling through ERK1/2. Together, these results revealed that the dysregulation of CXCR7 through loss of AR signaling could promote alternative, androgen-independent signaling programs potentially involved in development of CRPC (this model is illustrated in Figure 5.1.1).
The second aim of this study was to investigate the requirement for CXCR7 in the long-term survival and eventual resistance to ADT. However, the results of this work indicated more profound implications for the role of CXCR7 supporting overall prostate cancer cell proliferation. Utilizing the latest CRISPR-Cas9 gene editing method, I completely abrogated CXCR7 from C4-2B and LNCaP cells. The results from these studies resulted in few viable colonies after transfection, and all but one LNCaP clone eventually arrested proliferation and underwent cellular senescence. These results demonstrate that CXCR7 is a critical factor in prostate cancer cell proliferation, even with normal androgen stimulation. However, one
LNCaP clone from these experiments did continue to proliferate in culture. Characterization of this clone revealed an inframe mutation to CXCR7, maintaining the sequence of the N- and C-termini of the protein. While the frameshift mutations in all other clones resulted in the onset of cellular senescence, the mutant CXCR7 seemed to maintain cell viability in this clone. In vitro functional assays revealed the mutant CXCR7 altered the protein interactions between CXCR7 and ARRB1 or ARRB2. The PLA results showing a preferential recruitment of ARRB2 to mutant CXCR7 and reduced interaction with ARRB1 (relative to the WT cells), supported the observation of reduced EGF-mediated mitogenic signaling compared to WT cells. Additionally, the CX7-mut cell line showed moderately reduced SDF-1-mediated migration as well as significantly lower sphere formation potential compared to the WT cells. Based on the significant phenotypic changes in the CX7-mut LNCaP cell line compared to WT-CXCR7, I next evaluated the tumorigenicity and tumor growth profile in vivo. Subcutaneous xenograft studies revealed a highly significant reduction in tumor initiation and growth rate in the CX7-mut cell line. Assessing the in vitro and in vivo results from these CXCR7 knockout studies, it is clear that CXCR7 has a profound role on prostate cancer cell proliferation in both androgen-dependent and androgen-independent conditions.

5.2: Conclusions:

The data presented in this study reveal a critical role of CXCR7 in promoting survival and proliferation of prostate cancer tumor cells by potentiating alternative, androgen-independent mitogenic signaling pathways. This investigation
demonstrates that inhibition of AR signaling results in increased CXCR7 expression and an associated enhancement of downstream mitogenic pathway activation. Furthermore, complete abrogation of CXCR7 results in cellular senescence, suggesting a dependence on the overall CXCR7-mediated signaling program in prostate cancer cells, even in the presence of androgens. Finally, in vivo xenograft studies show that tumorigenicity and tumor growth is disrupted with a CXCR7 mutation compared to WT CXCR7, further supporting the role for this atypical chemokine receptor in driving aggressive cancer phenotypes. Together, these results demonstrate CXCR7 as a potential target for therapy in conjunction with ADT to prevent progression to CRPC.

5.3: Future directions:

Overall this work has revealed a potent link between overexpressed CXCR7 and ADT and AR inhibition therapies and opens an avenue of investigation into combination therapies targeting CXCR7. However, it is unclear at this moment what overall therapeutic benefit could be achieved with CXCR7 inhibition. While this work reveals a link between CXCR7 and alternative signaling programs involved with development of CRPC, it is still unclear whether CXCR7 can be effectively targeted. This is evident in the loss of CXCR7 signaling with advanced CRPC. As shown in 22Rv1 and PC3 cells, there is exceedingly low expression of CXCR7, suggesting it may be an effective target at the onset of ADT, but loses efficacy in relapsed prostate cancer cases. The therapeutic potential is one direction which still needs further elucidation.
There is a lack of patient-derived tumor tissues taken between the onset of ADT and the recurrence of CRPC. Thus, the expression profile of CXCR7 is not clear during this therapeutic window. The current project would benefit greatly from mRNA analyses of primary, castrate-sensitive prostate cancer cells during early ADT. To further confirm the role of CXCR7 in the transition to CRPC, future studies could also investigate the expression of CXCR7 in circulating tumor cells isolated from patients undergoing ADT, prior to disease recurrence.

Finally, the in vivo analysis revealed a highly significant difference between the tumorigenicity of WT and CX7-mut cells under normal androgen signaling (uncastrated male mice). These preliminary results could greatly benefit from a full analysis of the sensitivity to ADT in an animal model. A further study comparing the tumor initiation between castrated and non-castrated animals will offer stronger support for the role of CXCR7 and ADT resistance. In vivo assays could also benefit through conditional knockout of CXCR7 (i.e. Tet-inducible Cas9 expression) to achieve abrogation of CXCR7 in established tumors. Furthermore, the mutation to CXCR7 can still provide insight into the functional domains of CXCR7 driving tumorigenicity and susceptibility to ADT. Future mutational analyses of the regions contained within this deleted protein sequence may reveal novel targets to disrupt CXCR7-mediated alternative signaling programs.

From these results, it is clear that CXCR7 plays a critical role in the survival, proliferation, and therapeutic resistance in prostate cancer cells. Future investigations will further clarify the role for CXCR7 in supporting ADT resistance,
and potentially generate therapeutic options to target CXCR7 in combination with ADT to prevent CRPC in high-risk patients.
REFERENCES


