NOVEL MECHANISMS OF ANDROGEN RECEPTOR SIGNALING IN PROSTATE CANCER

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Of the Requirements for the Degree

Doctor of Philosophy

By

Alicia M. Blessing

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NOVEL MECHANISMS OF ANDROGEN RECEPTOR SIGNALING IN PROSTATE CANCER

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This dissertation is dedicated to my loving parents,
Kurt and Kathleen Blessing,
who always put their own dreams on hold
so that I could achieve mine.

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Abstract

Androgens regulate the physiological development of the prostate and the pathology of prostate cancer. Androgen-receptor (AR)-mediated transcriptional activity is a driver of prostate cancer (PCa) progression. AR-induced transcriptional activity is a dynamic process that is regulated by the binding of ligands that induce distinct conformational changes in AR. These structural alterations lead to the differential recruitment of coregulators (coactivators or corepressors) that control the expression of specific subsets AR-regulated genes. Despite continual improvement in design and enhanced efficacy, PCa cells eventually become resistant to AR-antagonists and anti-androgen treatment. Therefore, in this dissertation, we have proposed and identified two novel mechanisms of harnessing AR-mediated gene transcription in PCa.

First, we show that a stretch of proline residues located within the N-terminus of AR is a *bona fide* coregulator binding surface, the disruption of which reduces the androgen-dependent proliferation and migration of prostate cancer cells. Using T7 phage display, we identified a novel AR-interacting protein, SH3YL1, whose interaction with the receptor is dependent upon this polyproline domain. As with mutations within the AR polyproline domain, knockdown of SH3YL1 attenuated androgen-mediated cell growth and migration. RNA expression analysis revealed that SH3YL1 was required for the induction of a subset of AR-modulated genes. Notable was the observation that ubinuclein1 (UBN1), a key member of a histone H3.3 chaperone complex, was a transcriptional target of the AR/SH3YL1 complex, correlated with aggressive prostate cancer in patients, and was necessary for the maximal androgen-mediated proliferation and migration of prostate cancer cells. Collectively, these data highlight the importance of an amino-terminal activation domain, its associated coregulator, and downstream transcriptional targets in regulating cellular processes of pathological importance in prostate cancer.

In the second approach of this dissertation, we studied the downstream AR targets that regulate autophagy. We have determined that 1) androgens regulate overall cell metabolism and cell growth, in part, by increasing autophagy in prostate cancer cells, 2) functional autophagy was clinically detected in metastatic, castration-resistant cancers but not treatment-naïve, localized tumors and 3) autophagy is required for prostate cancer progression in preclinical animal models. Inhibition of autophagy using molecular inhibitors significantly abrogated androgen-induced prostate cancer cell/tumor growth. Autophagy and subsequent cell growth is potentiated by androgen-mediated increases in the expression and activity of several core autophagy genes, including ULK1, ULK2, AT4B, ATG4D, and TFEB. We identify these five genes as direct targets of the androgen receptor (AR) in prostate cancer. Moreover, expression of these five genes is essential for maximal androgen-mediated autophagy and cell proliferation. These findings demonstrate a role for increased autophagy in prostate cancer and highlight the potential of targeting underexplored metabolic pathways for the development of novel therapeutics.

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1.1 On the Origin of Cancer

History

Thousands of years before President Richard Nixon of the United States of America declared a political "war" on cancer by signing into law the National Cancer Act of 1971, the ancient Egyptians had already recorded their own battle against cancer[1]. The "Edwin Smith" and "George Ebers" papyri that date back to at least 3000 B.C., along with the evidence of osteosarcomas and head and neck cancers found in the mummified bones of these ancients verify that our current battle against cancer is in fact, not a new war[2]. Indeed, cancer is a pathology characteristic of all vertebrates, including the lesser vertebrates, and has been plaguing life on earth for millions of years. The first well-document case of malignancy and the first known case of metastasis found in the fossil record occurred in the dinosaur *Allosaurus fraulis* and dates back to the Jurassic period [3]. The earliest known neoplasm in man occurred 1.5 million years ago, identified in the mandible of a *Homo erectus* found in Kanam, Kenya that displayed a lesion consistent with Burkitt's lymphoma or an ossifying sarcoma [4,5].

It was not until around 400 B.C., after observing tumors with long, finger-like projections, Hippocrates, the great Greek philosopher and "father of medicine" used the Greek word that refers to crabs, *carcinoma,* to describe ulcer-forming tumors[6]. More than 350 years later, the

Roman physician Galen, translated the Greek word for crab into the Latin, *cancer*, giving rise to the word still synonymously used world-wide to describe more than 100 related diseases.

The study of cancer and the field of scientific oncology dates back to the late eighteen century when the Italian Giovanni Morgagni began using autopsies to link the pathology to the clinical course of the disease. A few short years later, the Scottish surgeon John Hunter began surgically removing tumors, a now refined practice that is still considered to be one of the first lines of defense against solid tumors. The microscope, originally invented in the 1600s by Anton van Leeuwenhoek was technologically improved in the nineteenth century, further revolutionized the field of pathology; allowing for scientists and physicians to better diagnosis and enhance the efficacy of the surgery.

In this new era of surgery, the Scottish physician, Thomas Beatson first discovered that removal of the ovaries resulted in improved outcome for women with breast cancer. Shortly thereafter, Charles Huggins of Chicago found that surgical castration caused regression of metastatic prostate cancer[7]. Later, drugs that block the production of the sex hormones, and drugs that antagonize the female hormone, estrogen, or the male hormone, androgen, were developed and found to be equally effective in the treatment of these cancers.

In the early twentieth century, Marie Skoldowska Curie and her husband, Pierre Curie, isolated the first radioactive elements which they found to emit a natural form of X-ray. This discovery has since improved the survival of millions of cancer patients due to the risky experiment a Chicago medical student named Emil Grubbe performed on his cancer patient, Rose Lee. Dr. Grubbe became the first radiation oncologist after he irradiated Ms. Lee, who before radiation therapy, suffered from medically non-responding locally advanced breast cancer[8]. Throughout the twentieth and twenty-first centuries, new technology improved radiation therapy, making it a viable treatment option for more than half of cancer patients today[9].

More than 40 years after the war on cancer began, significant progress has been made to treat several forms of cancer; for example, the cure rate for childhood Acute Lymphoblastic Leukemia is nearly 90%[9]. However, to the disappointment of many, the overall mortality rate due to cancer in the United States has not significantly decreased since the inauguration of the National Cancer Act of 1971[9]. This perceived lack of results only underscores the tremendous amount of research that is still needed to better understand, target, treat, and eventually, win the battle against cancer.

Etiology

While there are many different types of cancers, they are all unified by the way in which they start: uncontrolled cellular proliferation that allows for clonal selection. Normal cells grow, divide, and eventually die in a very controlled and orderly fashion. Cancer cells differ from normal cells in that they continue to live and divide creating new cells that are also abnormal. These abnormally growing cells eventually invade surround tissues and often times, distant organs.

Cancer is generally thought to begin with alterations to the DNA. These changes to the genes (coding and noncoding) that control cell function can result from parental inheritance, environmental damage, or a combination of both. Not only does each person's cancer have unique genetic alterations, but in many cases, each tumor has multiple different genetic foci [10,11]. Moreover, mounting evidence suggests that metastatic lesions have genetically different mutations that the primary tumor within the same patient[12]; however, multiple metastasis within the same patient are often clonally related [13,14].

Altered genes that contribute to the initiation and progression of cancer can generally be categorized into three main groups based on their function: tumor suppressors, oncogenes, and DNA damage or repair. Tumor suppressors are genes that would normally control cell proliferation; these genes are frequently lost or have inactivating mutations in cancer cells.

Oncogenes are genes that are involved in normal cell growth and survival; however, in cancer these genes are altered or over-active causing the cells to grow, divide, and survive inappropriately. Mutations in genes that produce protein products that are involved in repairing DNA after damage often lead to further mutations in tumor suppressors or oncogenes.

Nomenclature

Cancers are typically named based on the organ in which they originate. They can be further classified based on the cell type in which they form. Carcinomas originate in the epithelium, cells that line the surfaces of blood vessels, cavities, and organs throughout the body, and are the most frequent form of cancer. Carcinomas can be further subdivided based on the specific type of epithelial cell. Adenocarcinoma refers to carcinomas that originate in the glandular epithelial cells. Most cancers of the breast, prostate, and colon are adenocarcinomas. Cancers that arise from the transitional epithelium or urothelium are called transitional cell carcinoma and include some cancers of the bladder, ureters and kidney. Basal cell and squamous cell carcinomas are the final two subtypes of carcinoma that both originate in different layers of the skin.

Cancers that arise in the bone and soft tissue including the muscle, fat, lymph vessels, and fibrous tissue are called sarcomas. Leukemias are cancers that arise in the blood forming tissue of the bone marrow. Lymphomas are cancers that begin in either the T-cells or B-cells (lymphocytes). Cancers that begin in the plasma cell of the immune system are called Multiple Myeloma. Melanoma, the most aggressive skin cancer arises from the melanocytes. Additionally, germ cell tumors arise in the cells that become eggs or sperm. Signals from the nervous system promote a small fraction of cells within different organs to release hormones into the blood. Cancers that arise from these cells are called neuroendocrine tumors.

1.2 Prostate Cancer

Statistics

Prostate cancer (PCa), being one of the most frequent cancers, accounts for more than 13% of all cancers diagnosed each year in the United States and is responsible for 4.7% of the deaths due to cancer each year[9]. If the cancer is found while it is still localized to the primary site of origin within the prostate, the 5 year survival rate is close to 100%. However, if the PCa is diagnosed only after it has spread regionally or to a distant site, sadly, only 28% of patients are expected to live 5 years past diagnosis. Men are most likely to receive this devastating diagnosis between the ages of 65-74. However, mounting evidence taken from studies of asymptomatic, otherwise healthy male patients in their 20s-40s with the histological foci of PCa suggests that PCa initiation may take place many years before diagnosis [15-18]. While PCa affects men from all ethnic backgrounds, African American men have the highest risk of developing PCa (214 men per 100,000) and their cancers tend to more aggressive[19]. In contrast, out of every 100,000 white men, 130 will develop PCa. Family history should also be taken into consideration as men with one first-degree relative with PCa have a 2-fold increased risk of developing PCa [20].

Diagnosis

Detection of the *KLK3* gene product, prostate-specific antigen (PSA), in the blood has had a profound impact on the detection, stage migration (the decrease overtime in the number of men with PCa who are found to have advanced PCa at the time of diagnosis), and mortality [21,22]. PSA is synthesized within the normal prostate epithelial cells and released into the seminal fluid, where its concentrations range from 0.3 -3mg/ml [23]. PSA plays a large role in the liquefying of the seminal fluid by proteolysis of semenogelin I into two smaller peptides thereby breaking down the gel matrix to allow the spermatozoa to move freely[24]. The normal architecture of the prostate keeps PSA within the prostate and seminal fluid, with very little leakage to the blood. In

the circulatory system, PSA has little or no catalytic activity, mainly due to excess of protease inhibitors which inactivate PSA[25-27]. PSA is thought to become elevated in the blood in response to breakdown of the normal architecture of the prostate; this can occur through various mechanisms including benign prostatic hyperplasia (BPH), prostatitis, inflammation, infection, trauma, age, body mass index, race, or cancer [21,22].

The FDA approved the use of PSA as a marker to monitor PCa patients in 1986 and for detection of PCa in 1994[21]. Before this blood biomarker was available, prostate cancer was detected in the clinic with a digital rectal exam (DRE). It has been demonstrated that using a combination of circulating PSA levels (above 4 ng/mL) and DRE is more accurate than DREs alone for the detection of PCa [28]. Using DREs alone misses 32-37.5% of PCa that are detectible by higher levels of serum PSA[28,29]. Screening for PCa with PSA is credited with lowering the incidence of men with PCa outside of the prostate at the time of diagnosis from 79.3% in 1984 to 24.7% in 2005 [30].

For many years, heightened serum levels of PSA along with a positive DRE signaled to the clinician to initiate a prostate biopsy, with which the diagnosis could be confirmed pathologically. However, there has never been a true consensus at which a single PSA level should warrant a biopsy. To further confound the situation, the optimal upper limit of the normal PSA range is also unclear. As mentioned above, PSA levels can be elevated in response to multiple different pathological issues arising within the prostate. Work validating PSA as a biomarker for PCa used serum levels above 4ng/mL as a lower threshold cutoff to recommend a biopsy. However, some men with well documented clinically aggressive PCa have had PSA levels well below the 4 ng/mL theshold [31]. Additionally, clinically insignificant or indolent PCa can also present with high PSA levels, further showing that PSA does not discriminate between low- and high-risk disease [21,22]. With recent changes in the health care system, agencies within the

United States are currently reevaluating whether screening for PSA is beneficial as it might result in overtreatment of some men. Since there are currently no methods available to distinguish between indolent and aggressive forms of PCa, any harm that is done from over-diagnosis leading to overtreatment because of elevated PSA must be carefully weighed with the benefit of early detection and treatment of lethal PCa. Nonetheless, while PSA has proven valuable in the diagnosis of PCa over the years, there is a clear need for a better biomarker that is specific for PCa, and not prostate disease in general, and can distinguish between high-and low-risk PCa.

A biopsy of the suspected prostate tumor allows for the histopathological grade to be assigned by Gleason scoring. Gleason scores range from 1, being the most differentiated, to 5 which are the least differentiated, and are reported as a sum of the two most common patterns found throughout the 12 different needle core biopsies from each patient. Therefore, the score reported falls in the range between 2 to 10 [9]. Patients are also given an additional diagnosis after surgery to further help physicians understand their disease using TMN scores. Pathological T describes the how the tumor was initially diagnosed (i.e. positive DRE) along with the confinement of the tumor to the prostate (T1) to fully invasive (T4). Pathological N addresses whether PCa cells were found in the lymph nodes (N1) or not (N0). Pathological M describes the presence and degree of metastasis (M0 and M1a-c) [32].

Current Treatment

PCa treatment has progressed significantly since the first known case of PCa was found in the artificial mummy of a 16th Century A.D. King of Naples [33]. If PCa is diagnosed, conventional treatment includes surgery to remove the prostate (radical prostatectomy) and/or irradiation (brachytherapy). In the case of advanced PCa, these methods are usually followed by or substituted with androgen deprivation therapy that will decrease the amount of circulating androgens or antagonize the androgen receptor (AR). This treatment will initially reduce AR

signaling, PSA levels, and cause regression of the tumor. However, most patients will eventually experience rising AR signaling simultaneous with increased PCa growth and metastasis. This is discussed in more detail below.

Current Challenges

One of the major challenges of PCa currently, is the inability to distinguish indolent and aggressive cancers at the time of diagnosis. The aggressiveness of PCa is inconstant between patients. Patients with Gleason scores above 8, often progress from localized disease to metastasis and succumb to death within a short time; whereas other patients, usually those with Gleason scores below 6, may remain asymptomatic with no evidence of disease progression for greater than 10 years [19]. To complicate the situation, a small fraction of patients who initially present with Gleason grade 3 tumors do progress rapidly and require immediate treatment [34]. This brings to light to major problems: overtreatment for some patients and too conservative treatment for others. On one hand, if the PCa is indolent, those patients would be best treated conservatively with "watchful waiting" so as to maintain a higher quality of life. Overtreatment of these patients can result in several serious side effects that could have potentially been avoided. However, not treating some patients aggressively enough in the initial stages of the disease can result in the unavoidable reality of a premature death. The ability to accurately distinguish between the indolent and aggressive forms of PCa is desperately needed in order to better treat patients.

A second major challenge to treating prostate cancer is the inability to further treat the patient after the cancer has reemerged following androgen deprivation therapy; this recurrent disease is commonly referred to as castration resistant prostate cancer (CRPC). Unfortunately, standard chemotherapy of docetaxel for metastatic CRPC (mCRPC) is not effective, even initially, for greater than 20% of men[35]. In order to improve survival for the men who suffer from

this essentially untreatable disease, it is necessary to understand the biology behind the progression from hormone-dependent PCa to mCRPC.

1.3 Androgen Receptor Signaling

Nuclear Hormone Receptors

The Androgen Receptor (AR) is a member of the nuclear receptor (NR) superfamily. The members of this superfamily are ligand-inducible transcription factors (TF) that control the transcription of target genes in response to receptor specific ligands. The NR superfamily includes three related but diverse sub-families. The classical nuclear steroid hormone receptors (NHR) are classified as type 1 receptors and include receptors such as AR, estrogen receptor (ERα and ERβ), progesterone receptor (PR), glucocorticoid receptor (GR), and the mineralocorticoid receptor (MR). Upon binding of their lipophilic ligands (androgen, estrogen, progesterone, glucocorticoids, or mineralocorticoids, respectively), these NHR typically homodimerize and bind to an inverted repeat NR response element (NRE)[36]. Type 2 NRs typically heterodimerize with the 9-*cis* retinoic acid receptor (RXR) and typically bind NREs that are direct repeats[36]. Type 2 receptors include the vitamin D3 receptor (VDR), thyroid hormone (TR), all-*trans* retinoic acid (RAR), peroxisome proliferator-activated receptor (PPARα, PPARγ, and PPARβ/δ), liver x receptor (LXRα and LXRβ), and the farnesoid x receptor (FXR). Type 3 receptors are the orphan NRs whose ligands are unknown, or were unknown at the time the receptor was first identified[37,38].

All NRs share two common features: a central DNA binding domain (DBD) and C-terminus ligand binding domain (LBD). The N-terminus, which contains the transactivation domain (A/B domain), the C-terminus, and the hinge region between the DBD and LBD lengths vary between the different receptors.

The DBD, a region which is present in all NRs, consists of two zinc fingers that recognize specific DNA consensus sequences. While NRs can exist as either homo- or hetero-dimers, they bind DNA through their DBDs at specific NR response elements (NREs). These NREs typically appear as half sites separated by variable length nucleotide spacers. As a homodimer, AR binds to the specific inverted androgen response elements (ARE); many unique sites have been described but the consensus ARE: GGTACAnnnTGTTCT remains prevalent [39-42].

AR, like the other NHRs has a ligand-dependent bipartite nuclear localization sequence (NLS) located in the hinge region (amino acids 617-633)[43]. A point mutation in this region (R617P) found in three separate clinical samples does not affect the ability of AR to bind its ligand or DNA but does appear to repress transcriptional activation; this mutation most likely suppresses the transcriptional activity of AR by preventing its translocation to the nucleus[44-46]. The N-terminus is the most varied region between receptors. There is no crystal structure of the AR N-terminus to date; it has been well postulated in the literature that this is due to the intrinsic disorder of this region[47]. The polyproline domain, the polymorphic polyglutamine region, and the surrounding sequence (amino acids 141-338) of the AR N-terminus are required for complete transcriptional activity of the AR [48-50]. The N-terminus activation function 1 (AF-1), is located adjacent to this region (amino acids 360-494) and is able to function in a ligand independent manner [51]. This region is thought to be responsible for the recruitment of the general transcription factors (GTF). A second activation function, AF-2, exists in the LBD of NHR. Mutation or loss of AR's AF-2 results in decreased ligand-induced transcriptional activity[52-57]. Moreover, post-translational modification of the NRs, and specifically AR, can dramatically alter the ligand-induced activity of the receptor [58-62].

Mechanism of Action

The lipophilic hormones are able to pass through the cell membrane and bind to the target receptor. This binding causes a conformation change in the NR which allows it to be displaced from a heat shock protein (HSP) complex, translocate to the nucleus, dimerize, recruit specific coregulators, and influence transcription of its specific target genes. In addition to forming the ligand binding pocket, the LBD of AR, stabilizes the interaction between unliganded-AR and the HSPs[63]. After binding of the ligand, the LBD interacts with the N-terminus to stabilize the bound androgen [55]. Crystal structures provide evidence that suggest upon ligand binding, NRs undergo a conformational change that results in the helix 12 and the AF-2 domain folding back across the ligand binding pocket [37,64].

Co-regulators

Historically, classical NR pharmacology has hinged on the concept of two types of ligands: agonists and antagonists. Based on this model, an agonist can bind to its receptor, causing a conformational change which results in receptor nuclear localization and the subsequent transcription of specified target genes [65]. Alternatively, an antagonist can bind to its receptor and cause the transcriptional repression of the specified target genes. With the identification of tissue-specific NR effects, a new and more complex hypodissertation was formed throughout the NR field. NR controlled transcription is a complex process in which different ligands have the ability to bind and create unique conformational changes in the NR. These conformational changes result in the recruitment of distinct coregulators (coactivators or corepressors) that ultimately regulate the expression of NR-regulated genes [66]. Thus, these coregulators are unique not only to the tissue in which they reside, but also to the NRs and, more specifically, the presented NR surfaces to which they bind [67].

Transcriptional co-regulators are a heterogeneous group of molecules that are functionally distinct. They can either bind directly to the TFs or bind through a multiprotein complex, and can help facilitate the transcriptional regulation of target genes. Based on whether they assist in promoting active gene transcription or transcriptional repression of target genes, they are grouped as co-activators or co-repressors. Co-regulators most well characterized functions include: transient modification of chromatin to allow for the TF to bind to the DNA, facilitation and regulation of proximal and distal "machineries" needed for processes of transcriptional initiation, elongation, and splicing, and being the specialized mediator of cellular signals, primarily through post translational modifications (PTM) to the locus of transcription [68]. Some co-regulators have also been characterized as having transcriptionally-unrelated cellular functions within the cytoplasm.

One mechanism in which some co-activators are thought to enhance gene transcription is by forming a bridge between the DNA-bound NR and the GTFs[69]. The members of the steroid receptor coactivator (SRC) family are some of the most studied co-regulators and SRC-1 is known to interact directly with two of the GTFs: TFIIB (transcription factor II B) and TBP (tata binding protein)[70]. The SRC family including SRC-1, SRC-3, and TIF-2, are also able to recruit additional NR co-regulators to enhance gene transcription [71-73]. AR does interact with the SRC family of coactivators; however, this interaction is weaker than the family's affinity for ER or GR [55,57]. It is also worth noting that while the SRC family members are able to enhance AR-mediated transcription, knockdown of any specific member does not cause androgen insensitivity, which suggests that there are multiple other coactivators that can compensate for the loss of a single coactivator [55,62,74,75].

Additionally, several co-regulators of AR are also known to be actin-binding proteins or have been characterized as part of an actin-binding complex[76-78]. The organization of f-actin in

the cytoskeleton is important for many cellular processes including motility, morphology, adhesion, apoptosis, as well as trafficking of TFs to the nucleus[79-85].

Most of the co-regulators thus far described in the literature have been shown to enhance NR-mediated transcriptional activity. Like the other classical steroid receptors, when not bound to ligand, AR is complexed with HSPs in the cytoplasm, thereby preventing DNA binding and rendering AR transcriptionally silent[86]. Other NRs, such as the thyroid receptor (TR), are able to bind DNA in the absence of ligand, resulting in transcriptional repression. Corepressors were originally identified as proteins interacting with this group of unlignanded, DNA-bound NRs[36]. However, with more invested research it soon became clear that there were multiple co-repressors that could act upon the other NRs as well. Nuclear receptor co-repressor 1 (NCoR) and silencing mediator of retinoid and thyroid hormone (SMRT or NCoR2) are the two most well-studied NR corepressors that have also been identified to interact with AR [87-91]. NCoR and/or SMRT are recruited to AR typically in the presence of an AR antagonist and aid in further transcriptional suppression of AR-target genes. While more corepressors of AR have been identified recently; as evidenced by the lack of breadth of literature on this topic, this is clearly an area in which more research needs to be conducted.

Prostate Cancer

In prostatic adenocarcinoma cells, the most abundant serum androgen, testosterone (T) is converted to dihydrotestosterone (DHT) by the enzyme 5-α-reductase[92]. AR has a higher affinity for DHT in the prostate then T. The important role of AR in PCa is highlighted by the clinical progression of PCa with current treatment (**Figure 1.1**). The androgen-deprivation and AR antagonist treatment can give patients reprieve from the disease for months or in some cases, up to years but ultimately, this treatment is not curative. Once patients fail androgen deprivation therapies, through the restoration of AR signaling pathways, their progressive disease is known as

castration resistant prostate cancer (CRPC). Once the disease has progressed to this CRPC, it is inevitably fatal.

Despite the initial spike in androgen production that can result in increased severity of symptoms, such as urinary obstruction or pain, with gonadotropin releasing hormone (GnRH) agonists; continual treatment with these GnRH agonists actually desensitize the pituitary and prevent the release of gonadotropins, ultimately suppressing the testicular production of androgens[93,94]. This decrease in androgens produced in the testes is initially effective in reducing AR activity as evidenced by decreases in circulating PSA and tumor regression. GnRH antagonists do not initially cause an increase in circulating testosterone levels; however, they also do not reduce the tumor burden more than the GnRH agonists and are therefore, are not widely prescribed[95,96]. Neither GnRH agonists nor antagonists are capable of completely eliminating androgens from the tumor, however.

The adrenal glands produce approximately 5% of the circulating androgens in the adult male and are not regulated via gonadotropins. Moreover, the tumors themselves express key enzymes that are necessary for intratumoral de novo steroidogenesis[97-99]. In fact, serum androgen depletion with GnRH agonists may actually select for the pathways needed to upregulated these key enzymes that are capable of converted the weak adrenal-derived androgens to T, as these therapies have been shown to only reduce intratumoral androgens by 75% when the circulating androgens remained at castrate (undetectable) levels[100]. Abiraterone acetate (Zytiga) is a selective CYP17 inhibitor and is newly FDA approved for refractory PCa[101]. Abiraterone inhibits the enzymes 17α-hydroxylase and C17,20 –lyase thereby blocking the production of T in both the testes and the tumor[102].

Additionally, post-translational modifications (PTM) of AR can lead to unwanted functions of this NR. Therefore, AR antagonists are frequently given in combination with

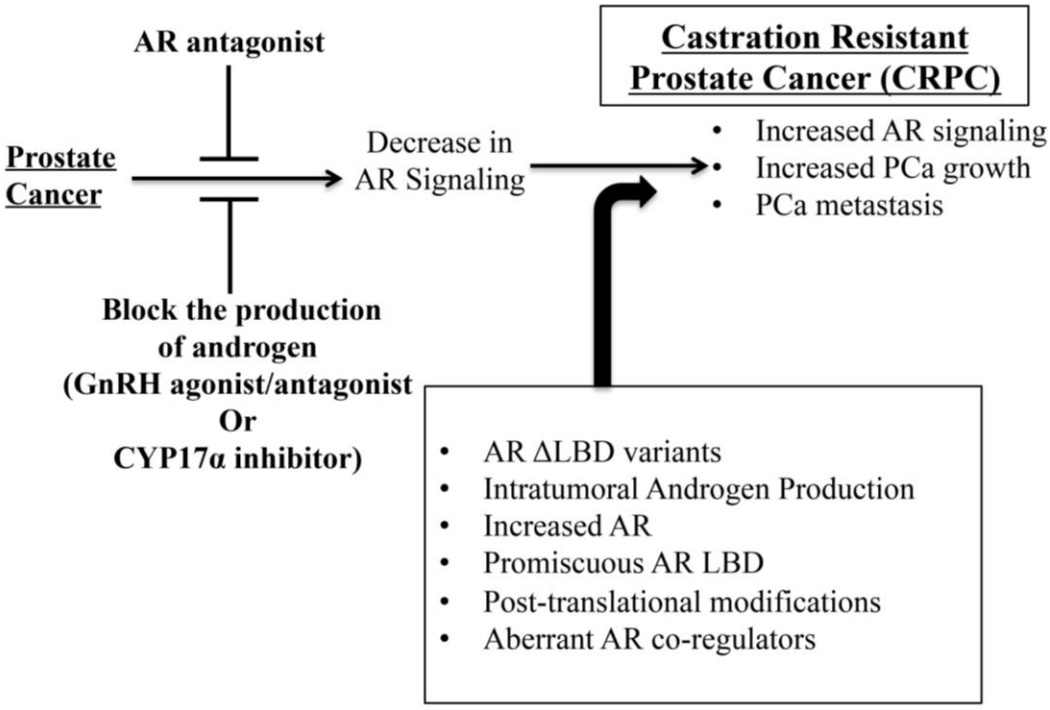


Figure 1.1 Prostate Cancer Treatment and Progression. After surgery and/or radiation therapy, the first targeted therapy available to prostate cancer (PCa) patients is an AR antagonist or a pharmaceutical that will globally (gonadotrophin releasing hormone, GnRH, agonist/antagonist) or locally (CYP17α inhibitor) block the production of androgens. This initially causes a decrease in AR signaling. However, after approximately 18 months, there will be reemergence of detectable AR signaling occurring concurrently with increased tumor burden and metastasis. This increase in AR signaling has been clinically shown to be enhanced via several methods outlined in this figure. This stage of the disease is known as castration resistant prostate cancer (CRPC).

the GnRH agonist/antagonist [103]. Bicalutamide (Casodex) is an anti-androgen that has been shown block AR activity both passively, by competing with agonist for binding, and actively, by recruiting corepressors and preventing the binding of coactivators[104,105]. Unfortunately, bicalutamide has a much weaker affinity than DHT for AR and is required to be administered in molar excess to be efficacious [103]. Bicaludamide resistance occurs rapidly in the clinic and as discussed above, the tumor has multiple mechanisms of restoring AR activity even under selective pressure. A newly FDA-approved anti-androgen, enzalutamide (Xtandi), was originally reported to be active in bicalutamide-resistant PCa and prevent both AR nuclear translocation and thus, DNA-binding[106,107]. Regrettably, some patients never respond to enzalutamide or abiraterone acetate treatment and those that do will ultimately become resistant to this therapy as well[108]. Trials aimed at optimizing the sequencing of or treating with a combination of abiraterone and enzalutamide are currently ongoing; however, these are also not curative currently and only marginally improve length of survival[109].

All of the current pharmacological methods used in the clinic to suppress AR activity focus on reducing ligand (DHT) binding. However, we now have evidence that AR can be alternatively spliced in PCa. This splicing event splices out the C-terminus LBD of AR rendering a constitutively active AR [110]. Ultimately, this is an obvious mechanism by which PCa cells can overcome the selective pressure of chemical castration and anti-androgens. This only highlights further the urgent need to develop N-terminus anti-androgens and to target some of the downstream gene targets of AR that drive disease progression.

1.4 Autophagy in Prostate Cancer

Overview of Autophagy

Autophagy, literally meaning “self-eating”, is the process in which components of a cell are sequestered in double-membraned vesicles, termed autophagosomes, then transported to and

fused with lysosomes for breakdown by resident hydrolases (**Figure 1.2**). Autophagy is necessary for maintaining cellular homeostasis and thereby, can be upregulated in the cell in response to extra- or intra-cellular stress stimuli such as starvation, pathogen infection, ER stress, growth factor deprivation, or in response to specific signaling events[111,112]. Autophagy is necessary for normal development and differentiation but numerous human pathologies are associated with dysfunctional autophagy including cancer, neurodegenerative disease, cardiovascular disease, and infectious disease[112-114].

The term autophagy refers to the general process in which cytoplasmic components are degraded by lysosomes but the word is colloquially used to describe a specific class of autophagy known as macroautophagy[115]. Macroautophagy is distinct from the other forms of autophagy in that it is believed to be non-selective bulk degradation of cytoplasmic components[115]. The other forms of autophagy include: mitophagy (the selective degradation of mitochondria)[116], ribophagy (the selective degradation of ribosomes) [117], pexophagy (the selective degradation of peroxisomes)[118], reticulophagy (the selective degradation of endoplasmic reticulum) [119], aggrephagy (the degradation of ubiquitin-labeled aggregated proteins)[120], chaperone-mediated autophagy (proteins that are specifically targeted for degradation and brought to the lysosome via chaperones)[121], microautophagy (the process in which the lysosomal membrane sequesters multiple cytosolic constituents, through invagination, forming intralysosomal vesicles)[122], xenophagy (autophagy in response to bacterial infection)[123], chromatophagy (the degradation of the chromatin)[124], and lipophagy (selective degradation of lipids)[125].

The formation and process of macroautophagy (hereafter referred to as autophagy) is highly conserved among species, highlighting its importance. This multistep process begins with cell signaling events under stressful conditions that allow for the biogenesis of the phagophore. The exact origin of the double-membraned phagophore is not known; however, many hypothesize

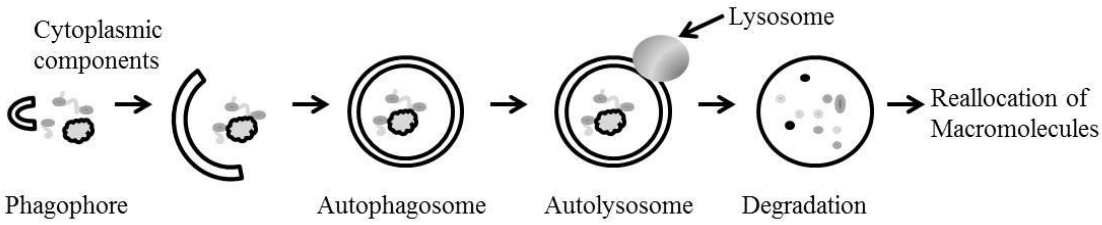


Figure 1.2 Schematic of Macroautophagy. The phagophore encircles cytoplasmic components, closes forming the autophagosome, fuses with the lysosome to become the autolysosome, allowing for degradation and reallocation of macromolecules.

that it is either a de novo assembly mechanism or formation from small sequestration of the endoplasmic reticulum, golgi apparatus, or mitochondria membranes[126]. After elongation, the phagophore will eventually seal around its cargo and be trafficked along microtubules to bring them into close proximity of the lysosomes[114].

The role of autophagy in cancer has been widely controversial and heavily debated in the recent years. The apparent paradox and perhaps entire controversy might in fact be answered by looking at the role of autophagy in the context of the stage of the disease. The literature appears to indicate that at early stages of cancer development, autophagy and the genes regulating it are *bona fide* tumor suppressors. It is generally believed and well accepted that autophagy can suppress tumor initiation. On the other hand, there exists a large body of literature suggesting that autophagy is essential in later stages of cancer, enabling the cancer cells to survive harsh conditions such as low oxygen or nutrient supply or chemotherapy. The role of autophagy in cancer can be further complicated as the specific genes that regulate autophagy and even the type of autophagy can differ by cancer type.

Pre-initiation Complex

The autophagic machinery is regulated via the upstream kinases 5′-AMP-activated protein kinase (AMPK) and mechanistic target of rapamycin (mTOR: formally mammalian target of rapamycin). Under optimal, full-nutrient conditions mTOR will phosphorylate unc-51 like autophagy activating kinase 1 (ULK1), a homolog of the yeast ATG1 at serine 757. Phosphorylation of ULK1 at serine 757 inhibits AMPK from interacting with ULK1 and thereby leaving the pre-initiation complex inactive[127]. If this pre-initiation complex, which is composed of ULK1, unc-51 like autophagy activating kinase 2 (ULK2), FAK family kinase-interacting protein of 200kDa (FIP200), and autophagy-related protein 13(ATG13), is inactivated by mTOR signaling, under most conditions this will repress the initiation of autophagy in the cell[128].

Alternatively, when the cell is stressed or starved of nutrients, AMPK will be activated. AMPK is activated first via phosphorylation at threonine 172 by liver kinase B1 (LKB1), transforming growth factor β-activated kinase-1 (TAK1), or Ca^{2+}-activated Ca^{2+}/calmodulin-dependent kinase kinase β (CAMKK2 or CAMKKβ)[129](**Figure 1.3)**. AMP binding allosterically to the γ-subunit of AMPK further enhances the activity of AMPK[130]. Phosphorylated (activated) AMPK will phosphorylate ULK1 at serine 317, 555, and 757[131]. ULK1 can then phosphorylate the other members of the pre-initiation complex: ULK1 directly phosphorylates ULK2 and Atg13 facilitates the interaction of phosphorylated ULK1 with FIP200 allowing for FIP200 phosphorylation[128]. This allows for this complex to then phosphorylate the initiation complex, ultimately leading to the induction of autophagy.

AMPK is activated in PCa and correlates with disease progression; functioning as an oncogene rather than as a tumor suppressor. Despite being a well-described tumor suppressor in multiple other tissues and the dominant kinase responsible for AMPK phosphorylation throughout the body, LKB1 appears to be inconsequential in the phosphorylation of AMPK in PCa[129]. The gene encoding TAK1, *MAP3K7*, is often deleted in PCa and is therefore, most likely not responsible for the increase in phosphorylated AMPK observed in PCa either. Recently, CAMKK2 was found to be a direct transcriptional target of AR that tracts with increased AMPK phosphorylation, migration, proliferation, and clinical PCa progression[132-134]. p-AMPK can potentiate autophagy directly through the phosphorylation of ULK1 and VPS34, a member of the initiation complex and indirectly, by inhibiting mTOR signaling via TSC2 and Raptor phosphorylation [130,135-137]. To further highlight the role of the autophagy pre-initiation complex in PCa, increased expression of ULK1 has been shown to be correlated in clinical samples with increased biochemical reoccurrence (raising PSA levels)[138].

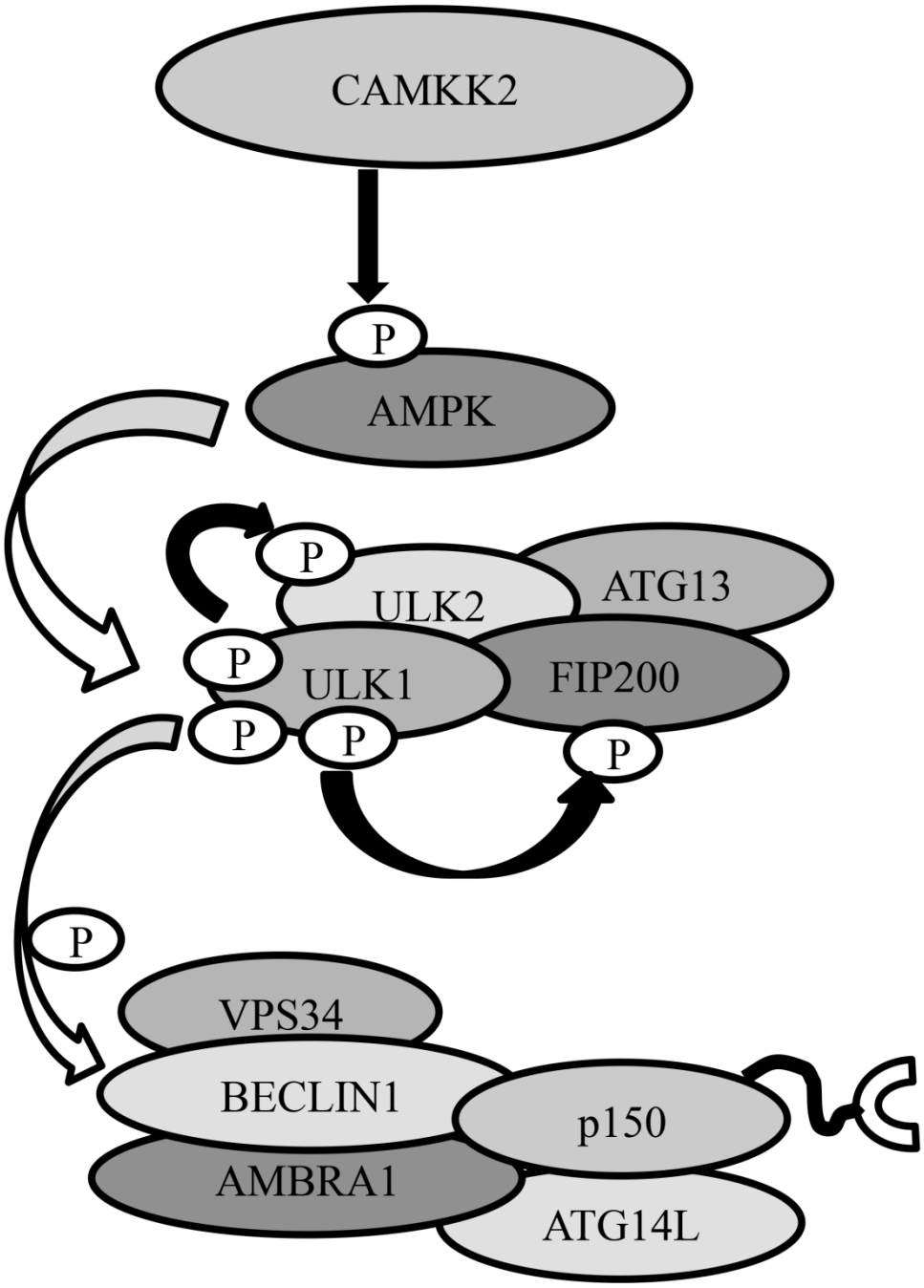


Figure 1.3 Overview of the autophagic pre-initiation complex. CAMKK2 phosphorylates AMPK, which phosphorylates ULK1 leading to the phosphorylation of other complex components and finally phosphorylation of downstream targets.

Initiation Complex

The pre-initiation complex phosphorylates a second key complex: the Beclin 1-class III phosphatidylinositol 3-kinase (PI3KC3) complex (or initiation complex)[139]. The core members of the initiation complex include the lipid kinase, vacuole protein sorting protein 34 (Vsp34), phosphoinositide 3-kinase p150 subunit (p150 or Vps15 or PIK3R4), and Beclin-1. This complex is responsible for suppling the autophagy specific phosphatidylinositol 3-phosphate (PIP3) which is needed for the formation of the phagophore and recruitment of other effector proteins [140-142]. Inhibitors of PI3K, wortmannin or 3-methyladenine, also block autophagy by inhibiting this complex, highlighting its essential nature for this basic biological process[143-145]. Additionally and importantly, Beclin1 interacts with multiple other proteins forming a complex interaction network that allow autophagy to be regulated temporally and spatially by multiple signals within the cell[140]. Both UVRAG, a known tumor suppressor, and Atg14L (Barkor) compete for binding to Beclin-1's coil-coil domain, thereby creating two separate and exclusive complexes[143]. Interestingly, while both complexes appear to preclude the binding of the other, they both promote the initiation complex activity and autophagosome formation[143,146]. On the other hand, RUN domain and cysteine-rich domain containing, Beclin-1 interacting protein (rubicon) binds the initiation complex through interactions with UVRAG and Beclin-1, decreasing Vps34 lipid kinase activity thereby decreasing autophagosome formation[144,145,147]. Some of the most well-characterized interacting partners of Beclin-1 include the anti-apoptotic Bcl-2 family members which prevent Beclin-1 from interacting with and forming the complex with Vps34 and p150[148,149].

PCa, similar to breast and ovarian cancers, frequently have allelic loss the Beclin-1 gene[150]. Beclin-1 halpoinsufficent mice have increased incidence of tumor formation suggesting that it is a tumor suppressor[151]. However, if these cells or tissues in addition to being *beclin1*$^{+/-}$

also have deficiencies in their apoptotic signaling, their overall tumorigenicity is greatly increased compared to the *beclin1*$^{+/+}$ apoptotic deficient counterparts[152].

The Autophagosome

The formation of the phagophore requires two conjugation systems. The first conjugation system consists of Atg12, Atg5, and Atg16. Atg12 is conjugated to Atg5 byAtg7, which functions as an E1-ubiquin-like-modifier-activating-enzyme and Atg10 which functions as an E2-ubiquitin-like modifier-activating-enzyme[153]. The Atg12-Atg5 conjugate then forms a complex with Atg16 and is localized to the phagophore.

The second conjugation system results in the lipidation of the eight mammalian homologs of the yeast Atg8: microtubule-associated protein 1 light-chain 3A (MAP1LC3A or LC3A), LC3B, LC3C, gamma-aminobutyric acid receptor-associated protein (GABARAP), GABARAP-like protein 1(GABARAPL1), and GABARAP-like protein 2 or golgi-associated ATPase enhancer (GABRARAPL2 or GATE-16). Despite having 8 homologs, LC3B is the only one that has been extensively studied and is currently used a marker of autophagy. After translation, pro-Atg8 homologs are cleaved in the C-terminus to reveal a glycine residue by Atg4A, Atg4B, Atg4C, or Atg4D[154](**Figure 1.4)**. After this initial cleavage by Atg4, LC3B is known as LC3B-I. Upon induction of autophagy, LC3B-I is conjugated to phosphatidylethanolamine (PE), a lipid moiety that allows LC3B to attach to the phagophore, via Atg7 (E1), Atg3 (E2), and Atg12-Atg5-Atg16 (E3)[155]. After conjugation to the PE, LC3B-I is known as LC3B-II. Both the LC3 and GABARAP subfamilies are necessary for autophagy; LC3 family appears to be crucial for the elongation of the growing phagophore while GABARAP family members are crucial for the closure of the phagophore[156].

To study autophagy, LC3B is commonly used as a molecular marker. LC3B-I and II can be separated on a sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE)

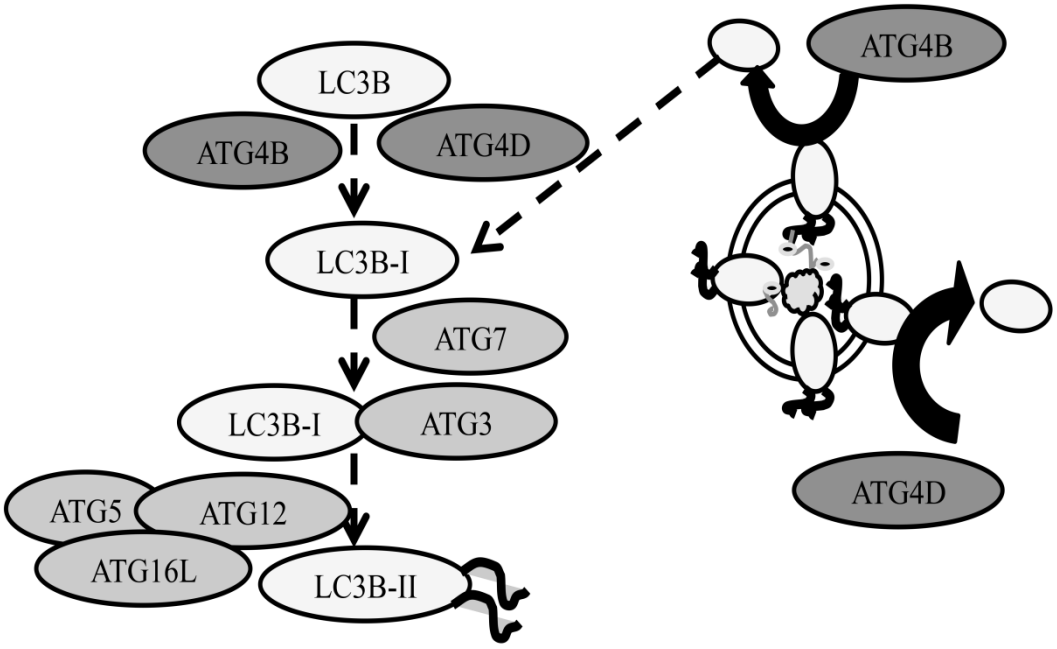


Figure 1.4 Overview of LC3B processing in autophagy. Pro-LC3B is cleaved by an ATG4 isoform into LC3B-I, which then goes through a series of ubiquin-like conjugations to eventually allow a phosphoethanolamine to be added forming LC3B-II. LC3B-II can then interact with the phagophore. Additionally, ATG4 isoforms can recycle LC3B-II back to LC3B-I.

gel[157]. Despite, LC3B-II having a greater molecular weight, it is more hydrophobic and therefore is more mobile on the gel giving the appearance of a smaller molecular weight. Moreover, LC3B conjugated to a green florescent protein (GFP) allows for tracking of autophagy within the physical cellular space as GFP-LC3B-I is more diffuse around the cells, GFP-LC3B-II forms punctate as it localizes to the phagophore and autophagosome.

Lysosomal Regulation and TFEB

The lysosome was first discovered by Nobel laureate Christian de Duve in the 1950s and is responsible for the majority of degradation that occurs in eukaryotic cells[158]. The lysosome is important for multiple cellular degradation pathways in addition to autophagy and contains more than 50 acid hydrolases[159]. The late stages of autophagy, the maturation and degradation stages require the fusing of the autophagosome to a lysosome; however, for many years the field of autophagy focused on the initiation of autophagy, ignoring the role of the lysosome. In recent years it has become more evident that the lysosomal regulation is an important aspect of autophagy and may be a way to pharmacologically regulate autophagy.

Once the autophagosome has fused with the lysosome but before the lysosome has released its acidic hydrolases the vesicle is termed an autolysophagosome; after the lysosome has released its acidic hydrolases to digest the cytoplasmic components of the autophagosome, the vesicle is them known as an autolysosome.

Lysosomal biogenesis, function, and role in autophagy is governed by the transcription factor E-box (TFEB) [160](**Figure 1.5**). TFEB controls the transcriptional regulation of a specific gene network known as coordinated lysosomal expression and regulation (CLEAR which include many of the genes necessary for lysosomal biogenesis, maintaining the low lysosomal pH, and general autophagy[160]. TFEB is normally sequestered in the cytoplasm, in a phosphorylated state,

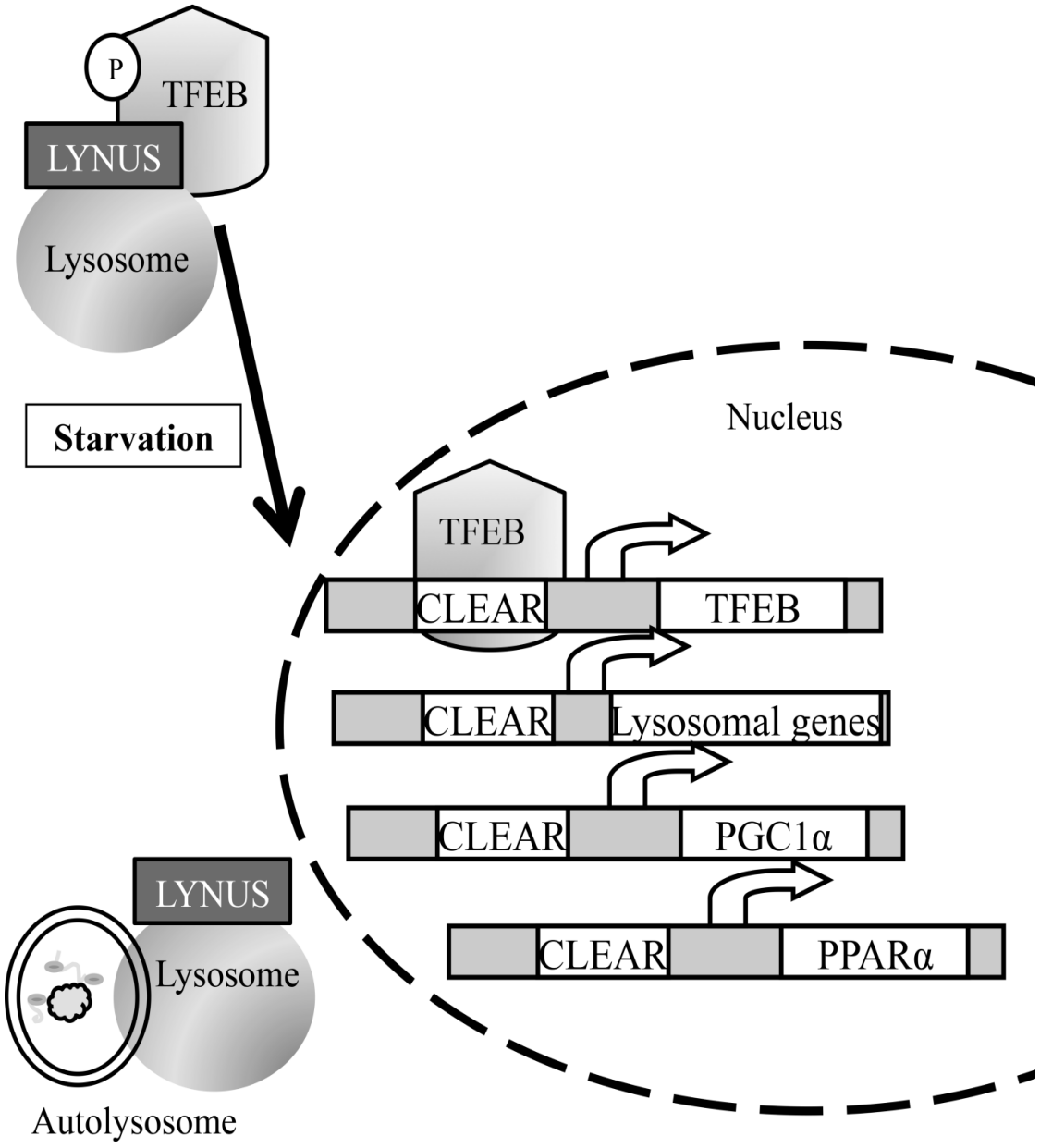


Figure 1.5 Overview of the role and regulation of TFEB. Under normal nutrient conditions, TFEB is phosphorylated by mTOR, a member of the lysosomal nutrient sensing (LYNUS) complex and sequestered to the lysosomal. Upon starvation or other signaling events, TFEB is dephosphorylated, translocates to the nucleus and enhances transcription of multiple genes. Simultaneously, lysosome is able to fuse with the autophagosome forming the autolysosome.

TFEB, includes mTOR complex 1 (mTORC1) which is responsible for the phosphorylation of TFEB at serines 142 and 211, vacuolar-type H^+ ATPase (V-ATPase), ATP-sensitive Na^+-permeable channel ($LysoNa_{ATP}$), and Ragulator[160,164,165].

Amino acids must accumulate in the lysosomal lumen in order for mTORC1 to dock at the lysosomal surface and be activated [166]. Therefore, after the completion of autophagy or during normal to high nutrient conditions, when amino acid levels are high and the threat of starvation has passed, mTORC1 will bind to the lysosomal lumen, phosphorylate TFEB and inhibit further flux through autophagy [167]. When TFEB is phosphorylated, it will remain in the cytoplasm, precluding it from initiating transcription of the CLEAR network of genes.

During cellular stress or starvation, mTORC1 will become inactivated and disassociate from the lysosomal membrane [167]. Furthermore, Ca^{2+} levels in the cytoplasm raise, activating calcineurin, a phosphatase that can dephosphorylate TFEB[168]. Once TFEB is no longer phosphorylated, it will translocate to the nucleus where it will activate the transcription of itself in a positive feedback loop and the CLEAR network of genes[160,165].

Overexpression of TFEB in cell culture resulted in increased autophagosomes and autolysosome, and enhanced lysosomal degradation of substrates[160,161]. TFEB is a member of microphthalmia-associated transcription factor (MiTF) subfamily of transcription factors that also includes MiTF, transcription factor binding to IGHM enhancer 3 (TFE3), and transcription factor E-box C (TFEC) [169]. Members of this family have been have been heavily implicated in poor prognosis for renal tumors and melanoma but have not up until now been studied in PCa [170,171].

In the Clinic

Targeting autophagy in the clinic has recently come into vogue for multiple cancers, including PCa[172]. Pre-clinical models have indicated that decreasing functional autophagic activity pharmacologically decreases PCa cell survival [173-175]. Moreover, some studies have

proposed that targeting autophagy would reduce the tumor burden in enzalutamide-resistant CRPC[176].

While pre-clinical research takes advantages of molecular mechanisms such as small interfering RNA (siRNA) to regulate autophagy, this is not currently possible in the clinic and thus, inhibitors are also widely used. Inhibitors are classified based on whether they on whether they inhibit the initial stages of phagophore or autophagosome formation or whether they inhibit the later stages of lysosomal function. Most of the inhibitors that are being transitioned to the clinic take inhibit the latter[177]. Chloroquine and its derivative hydroxyclorquine are lysosomotropic amines that were originally used to treat malaria and rheumatoid arthritis are the most highly studied compounds currently. These compounds are already FDA approved and are fairly inexpensive facilitating their easy approval for clinical trial[177]. Despite high hopes for chloroquine in clinical trials, it has become apparent recently that 1) chloroquine/hydroxychloroquine affects multiple processes within the body in addition to raising the lysosomal pH, 2) high and multiple daily doses of chloroquine/hydroxychloroquine are needed to inhibit autophagy [178,179]. Therefore, there are multiple other clinical trials in Phase I or Phase II aiming to alternatively target autophagy[180]. The results of these ongoing clinical trials show great promise thus far and could prove pivotal in the fight against PCa.

Chapter II:

Materials and Methods

Cell culture and reagents

LNCaP, C4-2, CR22, 22Rv1, PC-3, HeLa, CV-1 and HEK293 cell lines were obtained from American Type Culture Collection (Manassas, VA). Androgen-sensitive LAPC4 cells were a gift from Charles L Sawyers (Memorial Sloan Kettering Cancer Center). HEK293TS cells were a generous gift from Christopher Counter (Duke University School of Medicine). Cells were maintained and validated as previously described132,181-183. Methyltrienolone (R1881) was purchased from PerkinElmer (Waltham, MA). Cycloheximide (cat#: C7698), doxycycline hyclate (cat#: D9891), anti-GAPDH (cat#: G8795), anti-flag (cat#: F3165), and anti-v5 tag antibodies (cat#: V8012) were obtained from Sigma (St. Louis, MO). Anti-SH3YL1 (cat#: ab122141), anti-UBN1 (cat#: ab101282), anti-ULK2(cat#: ab97695), and anti-ATG4D(cat#: ab137621) antibodies were purchased from Abcam (Cambridge, MA). Anti-ULK1(cat#:4773), anti-p-ULK1 ser555(cat#:5869), anti-ATG4B(cat#:13507), anti-LC3B(cat#:2775), and anti-TFEB(cat#:4240) antibodies were purchased from Cell Signaling technologies. Agarose A/G beads (cat#: sc-2003) and anti-AR antibody (cat#: sc-816) were from Santa Cruz Biotechnology (Santa Cruz, CA).

Creation of inducible stable cell lines using retroviruses or lentiviruses

Stable cell lines were created with standard retroviral and lentiviral approaches as previously described 132.

For the retrovirus, MSCV-AR-IRES-GFP and MSCV-LC3B-GFP constructs were made using standard cloning techniques. These constructs were cotransfected (FuGENE, Roche

Applied Science, Indianapolis, IN) with a packaging vector pCL10A1 into the 293TS packaging cell line.

For the lentivirus, commercially available shRNAs in the pGIPZ backbone were obtained from Thermo Scientific (Lafayette, CO). An shRNA that we determined in preliminary experiments (data not shown) to give the best knockdown of SH3YL1 and a non-silencing control were cloned into the pINDUCER11 backbone, a generous gift from Thomas Westbrook (Baylor College of Medicine) and used to produce lentivirus as previously described [184].

In both cases, viral supernatants were filtered, supplemented with 8 µg/ml polybrene, and used to replace the media on top of the target cells for two serial 24-hour infections. GFP positive cells were sorted through three rounds of flow cytometry. Each cell line was validated with qPCR and western blot.

Cell proliferation and migration assays

Cell proliferation assays were carried out as previously described by measuring the cellular DNA content using a FluoReporter Blue fluorometric double-stranded DNA Quantitation kit (Life Technologies, Grand Island, NY) [184].

Boyden dual-chamber migration assays were carried out as previously described [185]. For the high-throughput scratch-wound assays, LNCaP cells were seeded in 96-well plates 72 hours post doxycyline treatment. Twenty-four hours after seeding, cells were scratched as previously described [186] and the media changed. With the new media, cells were given a second dose of doxycyline. Analysis of the migration was performed using an IncuCyte™ Zoom (Ann Arbor, Michigan) and done as previously described [186].

Small interfering RNA (siRNA) transfection

Stealth and Silencer Select siRNA (Life Technologies) transfections were carried out as previously described [183], with the exception that Silencer Select siRNAs were transfected at a final concentration of 10 nM. The sequences of the siRNAs are listed in **Table 2.1**.

Plasmid transfection and reporter gene assays

Unless otherwise noted, for all experiments cells were first steroid-starved for 72 h in phenol red-free medium containing 8% charcoal-stripped fetal bovine serum (CS-FBS). Plasmids were then transfected using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. Cells were treated with hormones approximately 16 hours before the assay. Luciferase and β-galactosidase (transfection normalization) activities were measured as previously described [185]. Each treatment was performed in triplicate, and results are expressed as mean ± SE. Each experiment was repeated at least three times, with a representative experiment shown.

Immunoblot analysis

Immunoblotting was conducted as previously described [184]. Densitometry was performed using ImageJ software (National Institutes of Health, Bethesda, MD) and samples were normalized to GAPDH (loading control).

RNA isolation, cDNA preparation, and quantitative RT-PCR (qPCR)

RNA isolation, cDNA preparation, and qPCR were carried out as previously described using 36B4 as an internal control [132]. The sequences of the primers are listed in **Table 2.2**.

T7 phage display

Protein interactions with full-length, ligand-bound AR tethered to DNA was identified using a high-throughput T7 phage display screen as previously described [67,187].

Co-immunoprecipitation

LNCaP cells were transfected with v5-SH3YL1 or flag-SH3YL1 48 hours prior to androgen treatment. Sixteen hours post-treatment, cells were harvested in radioimmunoprecipitation assay (RIPA) buffer as previously described [188]. Lysates were incubated in RIPA at 4ºC followed by centrifugation at 14,000 rpm for 15 minutes. Protein supernatant was transferred to a new pre-chilled tube, measured for protein concentration, and 500 µg incubated with 2 µg anti-v5, anti-flag or IgG control antibody and 20 µL agarose A/G beads at 4ºC. Beads were washed and subjected to western blotting as previously described [188]

GST pull-down assay

The GST pull-down assay was performed as previously described using full-length human SH3YL1 cloned into the pDEST15 vector to be expressed as a GST-fusion protein and whole-cell extracts of LNCaP cells treated ± R1881 [189]. MemCode™ (Thermo Scientific, Rockford, IL) stained blots were used as a control.

Microarray

LNCaP cells stably expressing the pINDUCER11-shSH3YL1 construct were treated with vehicle (ethanol), 100 pM R1881, or 10 nM R1881 for 24 or 72 hours ± doxycycline (DOX) before RNA collection. Samples were hybridized to Illumina Human HT-12 arrays and scanned on an Illumina® HiScan BeadArray (San Diego, CA). We then preprocessed the data with the IlluminaExpressionFileCreator module in GenePattern [190]. We checked for data integrity issues by generating boxplots that show the distribution of expression values, as well as PCA plots to identify potential outlier samples (data not shown). We normalized the data by quantiles. Using a t test, we found genes that were differentially expressed between pairs of conditions with a 1.5-fold change and a p-value < 0.05. We tested a total of 12 pairs of conditions: vehicle vs 100 pM R1881, vehicle vs 10 nM R1881, 100 pM R1881 vs 10 nM R1881, vehicle vs DOX, 100 pM

Table 2.1 siRNA sequences.

siRNA	Sequence
siAR #1	5’-CCCUUUCAAGGGAGGUUACACCAAA-3’
siAR #2	5’-UAGAGAGCAAGGCUGCAAAGGAGUC-3’
siSH3YL1 #1	5'-CAUAGUUCUCUUGUGAACACUUCAA-3'
siSH3YL1 #2	5'-AACAGCUCCCUUCUAAAGACACGCC-3'
siUBN1 #1	5'-GAAUAUCCGAGGGAAGGUAtt-3'
siUBN1 #2	5'-CCUUCAUCGAUAACUCUGAtt-3'
siUBN1 #3	5'-GGACCGGAUUUGUUCGGAUtt-3'
siAR 3'UTR	5'-CAGAUGUCUUCUGCCUGUUAUACC-3'
siATG4B #1	5'-ACGCAUUCAUCGACAGGAAtt-3'
siATG4B #2	5'-GGAUACUGGGUAGAAAAUAtt-3'
siATG4D #1	5'-CGACACUCACUGUACUUCAtt-3'
siATG4D #2	5'-CGCCUGGUGGUGUACGUUUtt-3'
siULK1 #1	5'-GCAUCGGCACCAUCGUCUtt-3'
siULK1 #2	5'-GCAUGGACUUCGAUGAGUUtt-3'
siULK2 #1	5'-GCUCGUUACCUACAUAGUAtt-3'
siULK2 #2	5'-GAAUCUGAACGAACGAUAUtt-3'
siTFEB #1	5'-ACAUCAAUCCUGAAAUGCAtt-3'
siTFEB #2	5'-AGGAGACGAAGGUUCAACAtt-3'

Table 2.2 qPCR primer sequences

Primer	Sequence
36B4 Forward	5'- GGACATGTTGCTGGCCAATAA-3'
36B4 Reverse	5'-GGGCCCGAGACCAGTGTT-3'
3' UTR Forward	5'-CCATGGCACCTTCAGACTTT-3'
3' UTR Reverse	5'-ACTGGGCCATATGAGGATCA-3'
CXCR4 Forward	5'-TGGCCTTATCCTGCCTGGTAT-3'
CXCR4 Reverse	5'-AGGAGTCGATGCTGATCCCAA-3'
FKBP51 Forward	5'-CGGAGAACCAAACGGAAACG-3'
FKBP51 Reverse	5'-CTTCGCCCACAGTGAATG-3'
SH3YL1 Forward	5'-GAACTCTGGCTCTCAAAGCAA-3'
SH3YL1 Reverse	5'-ATTCAAATCCCCAGGCTGCT-3'
UBN1 Forward	5'-TGGGCACCTGACTTCAATCC-3'
UBN1 Reverse	5'-AGGCTTCGTAGACGATTCCTTC-3'
AMBRA1 Forward	5'-ACCCAGACCCAGCGAGATTA-3'
AMBRA1 Reverse	5'-AGACGGTTCTGTTGGTAGCG-3'
ATG3 Forward	5'-CCAAAAATGTGCCGTGCTAT-3'
ATG3 Reverse	5'-TATCTACCCATCCGCCATCA-3'
ATG4A Forward	5'-CTTCAAACCAGAGTGACGAGC-3'
ATG4A Reverse	5'-CAGGCAATGGAAAGTCTGGTC-3'
ATG4B Forward	5'-TCGCTGTGGGGTTTTTCTGT-3'
ATG4B Reverse	5'-CACCTCCAAGCAGAGACAGC-3'
ATG4C Forward	5'-TGTGTGGGTATTATTGGTGGC-3'
ATG4C Reverse	5'-GGGCAGTGGAATGTCTCAAG-3'
ATG4D Forward	5'-GGGCGAGGGTGACATACAG-3'
ATG4D Reverse	5'-ACAGTCCGAGGTCAGGCA-3'
ATG5 Forward	5'-GAGTAGGTTTGGCTTTGGTTGA-3'
ATG5 Reverse	5'-CGTCCAAACCACACATCTCG-3'
ATG7 Forward	5'-GCATCCAGAAGGGGGCTATG-3'
ATG7 Reverse	5'-AGGCTGACGGGAAGGACAT-3'
ATG9A Forward	5'-CGGGTCGCTGTTCCTGA-3'
ATG9A Reverse	5'-TCCACCTTGACCACCAGC-3'
ATG9B Forward	5'-CAGTGCCAGGGTCGTGC-3'
ATG9B Reverse	5'-CCAGGAGGGAGACCGCT-3'
ATG10 Forward	5'-GCTACCCTTGGATGATTGTG-3'
ATG10 Reverse	5'-AAGGTCTCCCATCTAAAAAGC-3'
ATG12 Forward	5'-AATCAGTCCTTTGCTCCTTCCC-3'
ATG12 Reverse	5'-TACCATCACTGCCAAAACACTCA-3'
ATG16L1 Forward	5'-CGAGATAAGGAGGCGGCAAG-3'
ATG16L1 Reverse	5'-CTGATGGCTCGCACAGGAG-3'
ATG16L2 Forward	5'-GGAATGTTGTGGGAAGTCGC-3'
ATG16L2 Reverse	5'-GAGGGGTCAAAGTCCACACT-3'
BECN1 Forward	5'-GCGATGGTAGTTCTGGAGGC-3'
BECN1 Reverse	5'-VAGACCCTTCCATCCCTCAGC-3'

DRAM1 Forward	5'-TTCATCCAAGATTTCCAGAGTGTC-3'
DRAM1 Reverse	5'-CCCTGTCTGTCCTCTGTAGC-3'
FAM176A Forward	5'-AACCTCTACTGGCTGATGC-3'
FAM176A Reverse	5'-CTCCTTCTTCTCTTCTGGGG-3'
GABARAP Forward	5'-GGTGATAGTAGAAAAGGCTCCCA-3'
GABARAP Reverse	5'-AAGTAGAACTGACCAACTGTGAGA-3'
GABARAPL1 Forward	5'-GGTCCCCGTGATTGTAGAGA-3'
GABARAPL1 Reverse	5'-ACAGTAAGGTCAGAGGGCAC-3'
GABARAPL2 Forward	5'-CCCACAGTCCAGCCTAACTA-3'
GABARAPL2 Reverse	5'-CCAAAAGTGTTCTCTCCGCT-3'
IRGM Forward	5'-CTCTCCCTCACTTCAGTTGG-3'
IRGM Reverse	5'-TGGAGTAAGCTCATCAGGC-3'
MAP1LC3A Forward	5'-GCTTCCGAGTTGCTGACTGA-3'
MAP1LC3A Reverse	5'-GCGGTCGGCTGGGTC-3'
MAP1LC3B Forward	5'-AGCAGCATCCAACCAAAATC-3'
MAP1LC3B Reverse	5'-CTGTGTCCGTTCACCAACAG-3'
RAB24 Forward	5'-GCCATCGTCTGCTATGACCT-3'
RAB24 Reverse	5'-CGCAGTTCCTTCACCCAGAA-3'
RGS19 Forward	5'-GTGGTAGACGAGAAGGCGA-3'
RSG19 Reverse	5'-GGCTCACCTCCTTGGGG-3'
ULK1 Forward	5'-CAGAGACCGTGGGCAAGT-3'
ULK1 Reverse	5'-CTCCAAATCGTGCTTCTCGC-3'
ULK2 Forward	5'-GTATTGAGAGAAGACTGTCGGC-3'
ULK2 Reverse	5'-TTCCCCTCTTCCTCACGTT-3'
TFEB Forward	5'-TAGAGAATGATGCCTCCGCA-3'
TFEB Reverse	5'-CTGGCTCCCAGCCTGA-3'
LAMP1 Forward	5'-TCACGAAGGCGTTTTCAGTC-3'
LAMP1 Reverse	5'-CACTCCTCCACAGAGCCAAA-3'
PGC1α Forward	5'- AGTACAACAATGAGCCTTCAA-3'
PGC1α Reverse	5'- CATCAAATGAGGGCAATC-3'
MCOLN1 Forward	5'-TCTTCCAGCACGGAGACAAC-3'
MCOLN1 Reverse	5'-GCCACATGAACCCCACAAAC-3'
GLBN1 Forward	5'-AACGCCATCCAGACATTACCT-3'
GLBN1 Reverse	5'-GAGAGGCTTCATCTTGGGCA-3'
ATP6AP1 Forward	5'-TCACAGGCAACGATGAGGTC-3'
ATP6AP1 Reverse	5'-GCCGCTGTGTATGGGACAT-3'
SQSTM1 Forward	5'-CCATTGCGGAGCCTCATCTC-3'
SQSTM1 Reverse	5'-AGTCCCCGTCCTCATCCTTT-3'
MITF Forward	5'-CCTTCCCAACATAAAAAGGGAGC-3'
MITF Reverse	5'-TCGTTCAATCAGGTTGTGATTGTC-3'
TFE3 Forward	5'-GCCTGAACTCTTTGCTTCCG-3'
TFE3 Reverse	5'-TCCTGGAGCCCCCTTGA-3'
TFEC Forward	5'-CCCTTCTGGCATGGTGCATC-3'
TFEC Reverse	5'-CTGGGACCAGCAATGAGTGG-3'

R1881 vs 100 pM R1881+DOX, 10 nM R1881 vs 10 nM R1881+DOX; each comparison at 24 and 72 hours. We generated the heatmap using custom-developed software and leveraged Cluster 3.0 for clustering the genes (using default parameters). SH3YL1 levels were significantly decreased with doxycycline treatment as was verified by microarray and qPCR (data not shown). GEO accession number is GSE64885.

Chromatin-immunoprecipitation-sequencing (ChIP-Seq)

ChIP-Seq analysis was performed as previously described [191,192].

Analysis of UBN1 association with clinical variables using the TCGA data

A Kaplan-Meier survival plot was generated in March 2015 using data from *The Cancer Genome Atlas (TCGA)* available at www.cancergenome.nih.gov [193,194]. Prostate adenocarcinoma tumors with mRNA and *gene(s) of interest* expression greater than 1.5 fold above the mean were compared with the remaining samples. We evaluated the association of mRNA levels for the gene with a number of reported clinical variables for the Prostate Cancer patient cohort collected and profiled by TCGA (https://tcga-data.nci.nih.gov/tcga/). Gene expression profiles were downloaded for the entire patient cohort and quantile normalized; we then evaluated the association between each clinical variable and the gene mRNA levels using an ANOVA test and further applied multiple hypodissertation testing correction ($q<0.1$) using the R statistical system.

Xenografts

Male NSG mice were castrated at 7 weeks of age. One week post-op, mice were subcutaneously injected with 2 x 10^6 22Rv1 cells stably expressing a pINDUCER10 plasmid with an inducible shCAMKK2. Immediately after xenograft, mice were fed a diet of control chow or 625mg/kg DOX supplemented chow diet. Mice were palpated daily for tumor growth.

LysoTracker and Lysosomal Quantification

LNCaP cells were seeded on coverslips in charcoal stripped media and allowed to adhere for 72 hours before transfection and treatment. After the last treatment, 1uL/mL of LysoTracker was added to each well and allowed to incubate at 37° C for 30 minutes before cells were fixed, stained with DAPI and mounted onto slides for imaging. Each coverslip was repeated in triplicate and at least 10 randomly selected images per coverslip were taken. Images were analyzed in Image J with a script provided by Dr. Fatima Merchant.

MTT Assays

MTT assays were performed as previously described.[195]

Chapter III:

Identification of a Novel Coregulator, SH3YL1, that Interacts with the Androgen Receptor N-terminus[1]

3.1 Introduction

Androgens act by binding to the androgen receptor (AR), a member of the steroid hormone receptor subfamily of nuclear receptors (NR). The binding of androgens to AR causes its dissociation from heat shock protein complexes, translocation to the nucleus, homodimerization, binding with coregulators (commonly still referred to as cofactors) and recruitment to regulatory regions of AR target genes [196]. It has been demonstrated that the pharmacology of AR agonists, antagonists and selective androgen receptor modulators (SARMs) is determined by the impact of the bound ligands on receptor structure and the effect that this has on coregulator recruitment [67,197-199]. Thus, depending on the relative and absolute expression of functionally distinct coregulators the same AR-ligand complex can manifest different biological activities in different cells. Despite the beneficial physiological effects that androgens have on promoting sexual differentiation and increased bone and muscle mass, AR signaling also has deleterious pathological effects; promoting prostate and prostate cancer (PCa) growth [18].

[1] This work was previously published as Blessing, AM**,** G Sathya, . Rajapakshe, YY Sung, LR Bollu, Y Shi, E Cheung, C Coarfa, JT Chang, DP McDonnell, and DE Frigo. Identification of a novel coregulator, SH3YL1, that interacts with the androgen receptor N-terminus. *Molecular Endocrinology.* 2015:29(10):1426-39. Used by permission from the Endocrine Society.

When diagnosed early PCa can often be treated successfully with surgery and/or radiation alone [18]. However, a significant number of patients progress to the advanced stages of PCa. Since AR is a primary driver of PCa growth and metastasis, patients with advanced disease are generally treated with systemic hormone therapy to prevent the spread of the disease [200]. While androgen ablation therapy is the standard of care for advanced PCa, most tumor cells develop resistance to this therapy. Interestingly, relapse of the disease is often associated with increased AR signaling [18]. Several mechanisms have been proposed to explain the development of resistance to endocrine therapy although the most prevalent are *AR* overexpression, aberrant expression and/or activity of coregulators, and the expression of constitutively active, C-terminally truncated AR splice variants [18,200,201]. Hence, while the ligand-binding domain (LBD) is the target of existing endocrine therapeutics it now appears as if other regions of AR, particularly the N-terminal domain, are crucial for the malignant progression of PCa.

To date, the N-terminus of AR has been poorly understood. This is due in large part to the intrinsically disordered structure of this region which has precluded its crystallization [202]. Within this region there exists a polyproline domain that is thought to be important in AR action [203-205]. Although the role of the analogous domain in the progesterone receptor (PR) is well established, the role of this domain in AR-function remains enigmatic [204-212]. In the case of PR, the polyproline domain facilitates the interaction of the receptor with the SH3 domain of Src kinase; which has also been reported to interact with AR in a trimer complex with estrogen receptor α (ERα) [204,205,208,210,211]. However, others have questioned such a role for the AR polyproline domain [203]. The goal of this study, therefore, was to define the mechanism(s) by which the polyproline domain influences AR action and how this impacts androgen action in processes of pathological importance in cancer.

3.2 Results

The polyproline domain of AR is required for maximal androgen-mediated prostate cancer cell proliferation and migration

Studies of AR structure/function have indicated that sequences within both the amino terminus and the carboxyl terminus of the receptor are required for maximal transcriptional activity [213,214]. Whereas the canonical coregulator binding site denoted as AF-2 within the carboxyl terminus of AR has been studied extensively, considerably less effort has been focused on defining the roles of the amino-terminal regions in receptor function. Of particular interest to us was a polyproline region located in the amino terminus of the receptor that exhibits the structural features of an SH3-interacting domain and which we considered was likely to function as a protein-protein interaction surface **(Figure 3.1A)**. Thus, the first goal of these studies was to define the impact of disrupting this domain within AR on the biology of androgens in cellular models of PCa.

As a first step, we developed a strategy to study the activity of mutations within the polyproline region of AR in relevant PCa cells without interference from the endogenous, wild-type (wt) receptor. To this end, a retroviral approach was used to create LNCaP cells stably overexpressing a GAL4 control protein, v5-tagged wild-type AR (v5-ARwt), polyproline-deleted AR (v5-ARΔpro) or a DNA-binding domain (DBD) mutant (v5-AR(C562S)) **(Figure 3.1B)**. Simultaneously, the levels of endogenous AR were depleted using siRNAs directed towards the 3'UTR of the receptor mRNA **(Figure 3.1B)**. Knockdown of the endogenous receptor mRNA, and appropriate expression of the exogenously expressed receptor variants, was confirmed by qPCR and immunoblot **(Figure 3.1C; Figure 3.2)**. As expected, depletion of endogenous AR attenuated androgen-stimulated cell proliferation in the GAL4 control cell line, an effect that

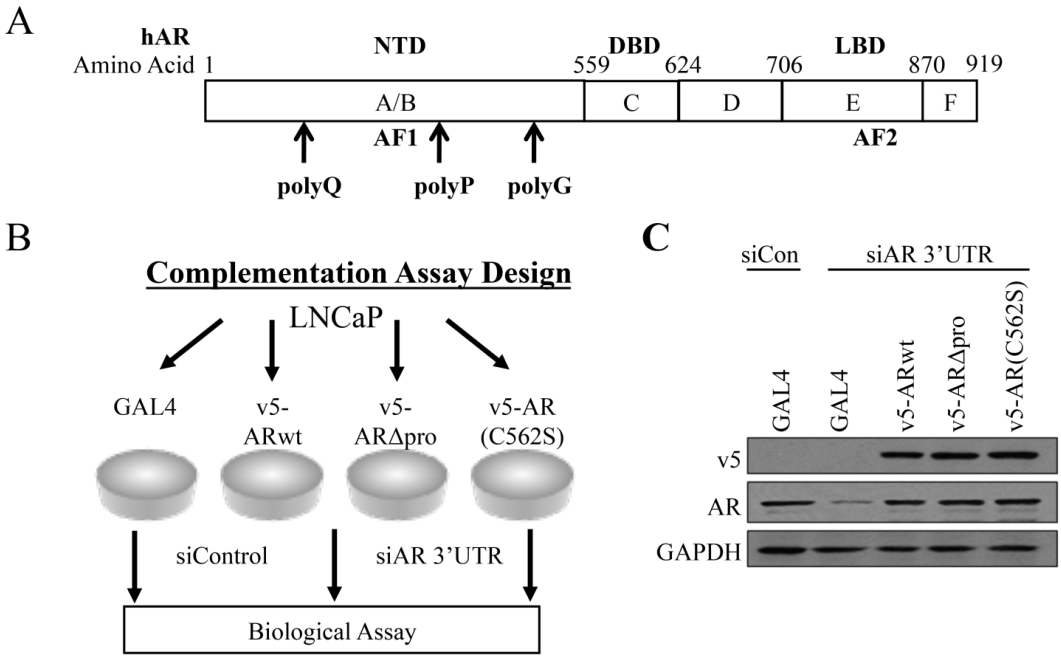


Figure 3.1 The AR polyproline domain. A, schematic of the human AR with the polyglutamine (polyQ), polyproline (polyP), and polyglycine (polyG) regions indicated. B, schematic of the AR replacement strategy. LNCaP cells were retrovirally infected to create stable cell line derivatives with plasmids expressing GAL4 (control) or v5-tagged versions of AR (wild-type (wt), a stretch of 6 proline residues deleted in the polyproline domain (Δpro) or DNA-binding domain mutant (C562S)) linked to an IRES-GFP. Cells were selected using flow cytometry. Subsequently, GFP-positive cells are transfected with chemical siRNAs targeting either a control sequence (siControl/siCon) or the 3'-untranslated region of AR (siAR 3'UTR) after which various biological assays were performed. C, expression of the endogenous and exogenous AR was confirmed with western blot.

could only be reversed by the expression of v5-ARwt but not v5-ARΔpro **(Figure 3.3A)**. However, similar to what others have previously reported and highlighting the fidelity of our complementation approach, overexpression of the v5-ARΔpro construct (or the GAL4 or v5-ARwt) in the presence of the endogenous AR (siControl) had no significant effect on LNCaP cell proliferation. These results indicate that the polyproline domain within AR is required for maximal androgen-dependent PCa cell proliferation.

We next wanted to determine the impact of disrupting the AR polyproline domain on androgen-regulated LNCaP cell migration. Using a previously described Boyden dual-chamber migration assay (see Materials and Methods), we confirmed that androgen treatment led to a expression of a DNA-binding deficient AR mutant (v5-AR(C562S)) and importantly, was only significant increase in LNCaP migration and that this was attenuated upon AR knockdown **(Figure 3.3B)**. Reexpression of v5-ARwt restored the migratory capacity of cells in which endogenous wtAR had been depleted. Such complementation was not accomplished by partially rescued by expression of the v5-ARΔpro mutant. Thus, as with cell proliferation, an intact polyproline domain is needed for maximal AR-mediated cell migration.

Disruption of the polyproline domain within AR impacts it transcriptional activity

A comparative assessment of the activity of wild-type AR and AR variants in a co-transfection assay was performed to define the importance of the polyproline domain in transcriptional activation. Interestingly, in AR-negative CV-1 cells, the activity of exogenously expressed, wild-type AR or polyproline-deleted AR were comparable when evaluated on the MMTV promoter **(Figure 3.4A)**. Using the complementation assay described above, we next assessed the relative activity of wild-type AR and the polyproline mutant AR on the expression of the endogenous AR target genes, *FKBP51* and *KLK3* (PSA), in LNCaP cells **(Figures 3.4B and C)**. No differences were noted between the ability of the wild-type and polyproline mutant-

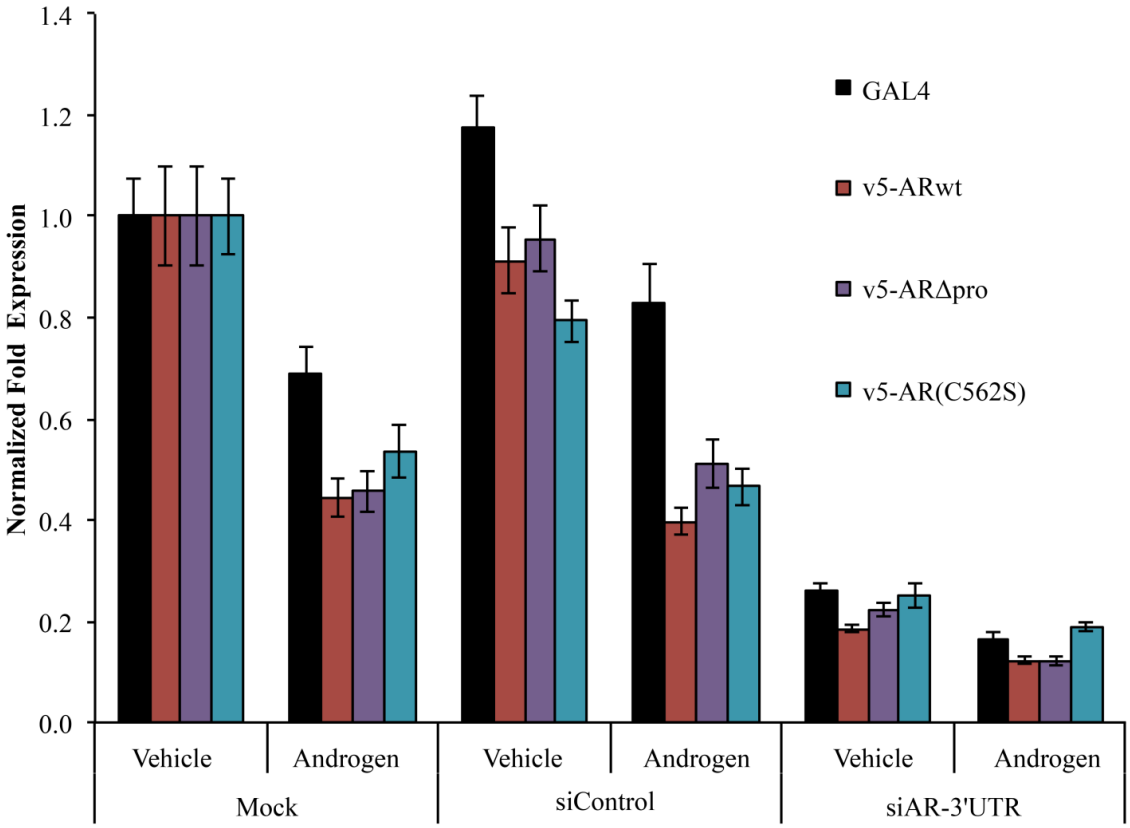


Figure 3.2 mRNA expression of endogenous AR in complementation assay. LNCaP cells were retrovirally infected to create stable cell line derivatives with plasmids expressing GAL4 (control) or v5-tagged versions of AR (wild-type (wt), a stretch of 6 proline residues deleted in the polyproline domain (Δpro) or DNA-binding domain mutant (C562S)) linked to an IRES-GFP. Cells were selected using flow cytometry. Subsequently, GFP-positive cells are transfected (or not, Mock) with chemical siRNAs targeting either a control sequence (siControl) or the 3'-untranslated region of AR (siAR 3'UTR) after which expression of endogenous AR was confirmed with qPCR using primers directed towards the 3'UTR of AR.

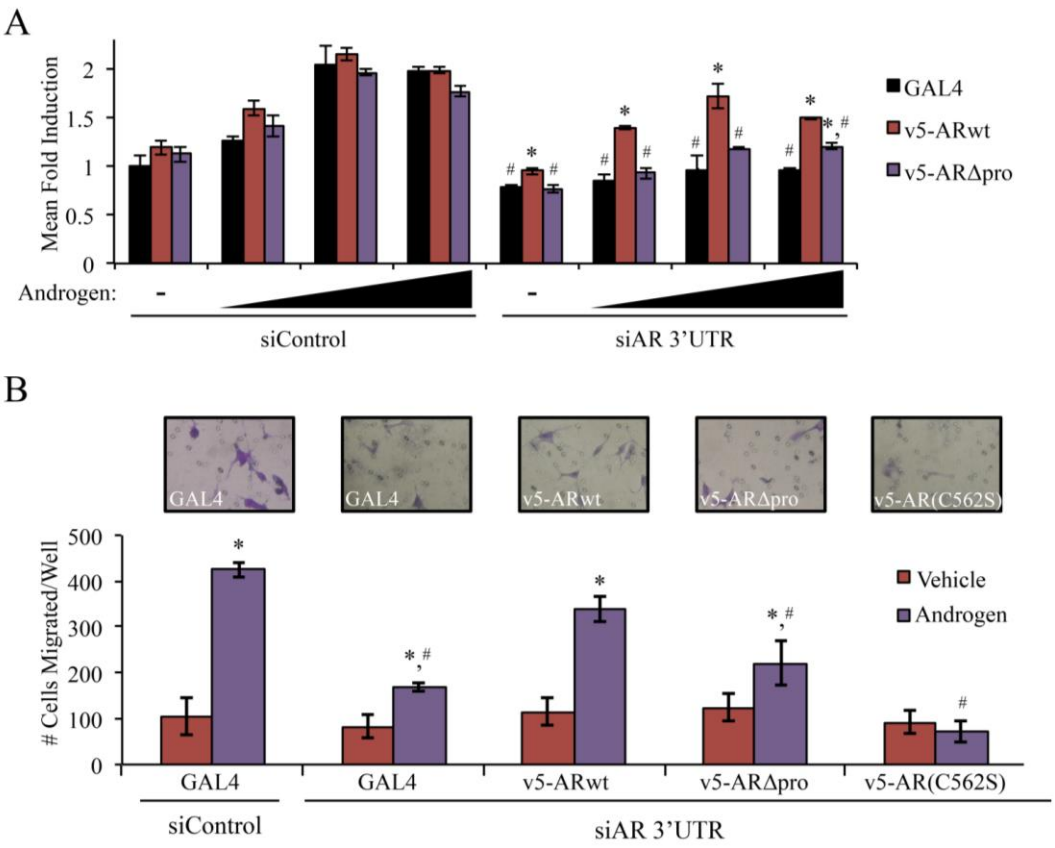


Figure 3.3 The AR polyproline domain is required for maximal androgen-mediated prostate cancer cell growth and migration. A, derivative cells were treated for 7 days with vehicle or increasing concentrations (.01, .1, 1 nM) of the synthetic androgen R1881. Cells were then lysed, and the relative number of cells was quantified using a fluorescent DNA-binding dye. Each sample was performed in triplicate. Results are expressed as mean relative fold induction ± SE (n = 3). *, significant ($p<0.05$) changes from GAL4; #, significant ($p<0.05$) changes from v5-ARwt. B, derivative cells were treated with vehicle or 1 nM R1881 for 16 hours before they were counted (50,000 cells/chamber) and seeded for a Boyden dual chamber migration assay. After 24 hours, the cells were fixed and stained with crystal violet. Inserts were visualized under a light microscope (inset) and the cells were quantitated by counting the number of cells in three randomly selected microscopic fields at x40 magnification. The number of cells in all three fields was added together and an average of three inserts ± SE was determined. *, significant ($p<0.01$) changes from Vehicle; #, significant ($p<0.01$) changes from GAL4 siControl.

containing receptors to regulate *FKBP51* **(Figure 3.4B)**. However, we did observe a reduction in PSA transcript levels in cells expressing v5-ARΔpro when compared to the cells expressing wild-type AR **(Figure 3.4C)**. Together these data indicate that the polyproline domain of AR is necessary for the transcription of a subset of androgen-mediated genes, a likely consequence of the differential requirement for coregulators that interact with this specific region of the receptor.

Identification of a novel coregulator that binds to the polyproline domain of AR

Previously, we described the use of a high-throughput protein-protein interaction screen using T7 phage display to identify, in an unbiased manner, proteins interacting with full-length, ligand-bound AR tethered to DNA [67]. Among the proteins identified in this manner was SH3YL1 (also called SH3-domain containing, Ysc84-like 1), an SH3 domain-containing protein that we hypothesized may have the ability to interact with the AR polyproline domain **(Figure 3.5A)**. Using both co-immunoprecipitation and GST-pull down assays we confirmed that indeed SH3YL1 interacts with AR **(Figures 3.5B and C)**. Further, a mammalian 2-hybrid assay was used to demonstrate that the androgen-dependent interaction of SH3YL1 with AR requires an intact polyproline domain **(Figure 3.6A)**. This requirement for the polyproline domain was further validated using co-immunoprecipitation **(Figure 3.6B)**. Not surprisingly, SH3YL1 also interacted with agonist-bound PR, the only other NR with a polyproline domain **(Figure 3.7)**; suggesting SH3YL1 may also be involved in PR action and modulate some progesterone-mediated effects, an area of ongoing investigation.

SH3YL1 is necessary for maximal androgen-mediated prostate cancer cell proliferation and migration

The data generated thus far suggests that SH3YL1 may be a mediator of the functional activities of the AR polyproline domain. To address this possibility, we evaluated the impact of SH3YL1 knockdown on androgen-mediated proliferation in two different hormone-sensitive

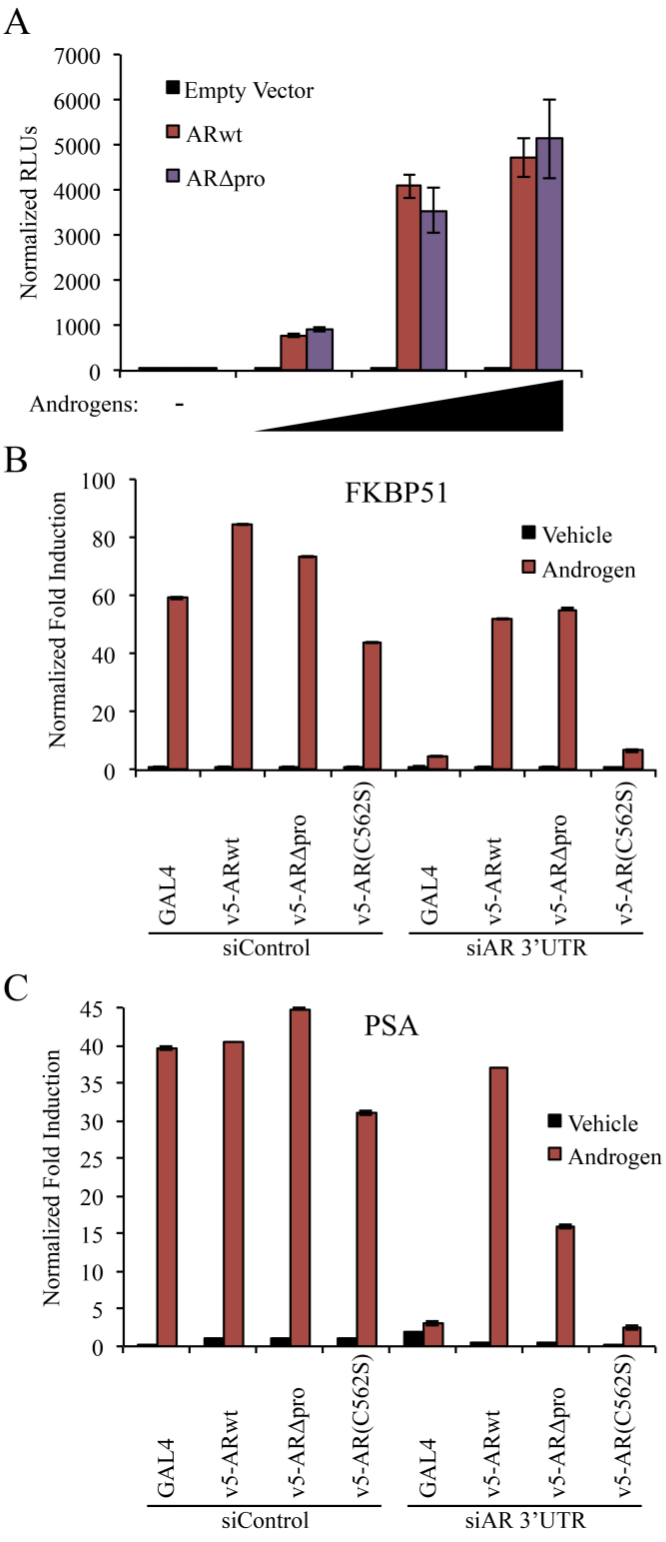

A
7000
6000
5000
4000
3000
2000
1000
0
Normalized RLUs
Empty Vector
ARwt
ARΔpro
Androgens: -
B
FKBP51
100
80
60
40
20
0
Normalized Fold Induction
Vehicle
Androgen
GAL4
v5-ARwt
v5-ARΔpro
v5-AR(C562S)
GAL4
v5-ARwt
v5-ARΔpro
v5-AR(C562S)
siControl
siAR 3'UTR
C
PSA
45
40
35
30
25
20
15
10
5
0
Normalized Fold Induction
Vehicle
Androgen
GAL4
v5-ARwt
v5-ARΔpro
v5-AR(C562S)
GAL4
v5-ARwt
v5-ARΔpro
v5-AR(C562S)
siControl
siAR 3'UTR

Figure 3.4. The polyproline domain of AR controls the expression of a subset of androgen-mediated genes. A, CV-1 cells were transfected with pcDNA empty vector, pcDNA-ARwt, or pcDNA-ARΔpro expression vectors in combination with an MMTV-Luciferase reporter plasmid. After transfection, cells were treated with vehicle or increasing concentrations of R1881 (.01, .1, 1 nM) for 24 hours. Cells were harvested and assayed for luciferase activity. All luciferase values were normalized to β-galactosidase transfection controls. Data are expressed as mean relative light units (RLUs) ± SE. B, LNCaP cells expressing GAL4, v5-ARwt, v5-ARΔpro, or v5-AR(C562S), were transfected with siControl or siAR 3'UTR followed by treatment with vehicle or 10 nM R1881 for 16 hours. RNA was then collected and transcript levels of the AR target genes *FKBP51* and *KLK3* (PSA) were assessed using qPCR. Data are normalized to 36B4 and expressed as mean ± SE.

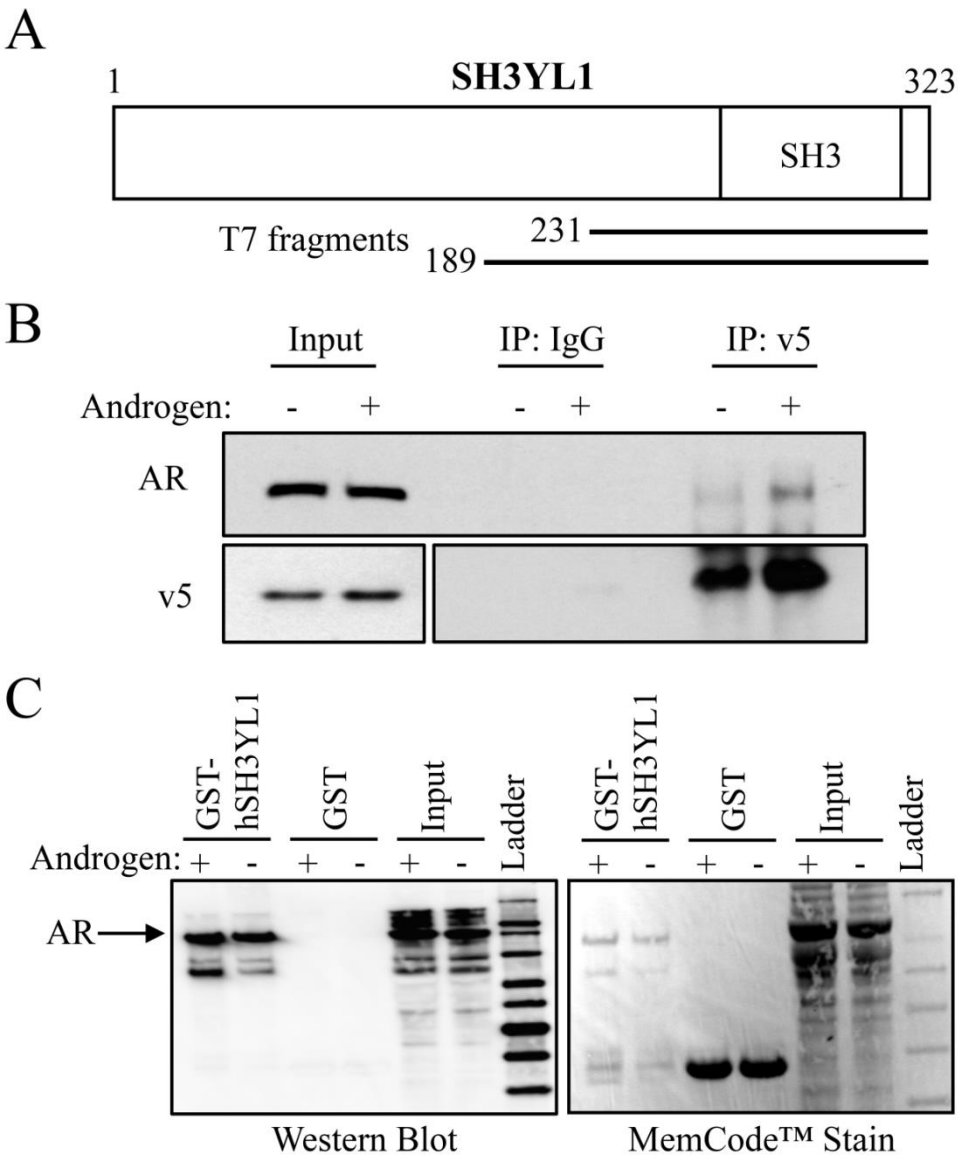


Figure 3.5 AR interacts with SH3YL1. A, schematic of human full-length SH3YL1 and the corresponding T7 phage display fragments that were found to be interacting with full-length, ligand-bound AR. B, LNCaP cells were transfected with a v5-SH3YL1 construct and treated with vehicle or androgen (.1 nM R1881) for 24 hours. After lysis, cells were immunoprecipitated with anti-v5 (SH3YL1) and immunoblotted to detect interaction with AR. C, GST pull-down was performed using whole-cell extracts from LNCaP cells treated with vehicle or 10 nM R1881 (androgen) and either GST alone or GST-human SH3YL1. Western blots were then used to detect pull-down of endogenous AR (*left*). Total protein-stained blots are shown as a control to demonstrate equivalent GST expression (*right*).

prostate cancer cell lines. Here, siRNA-mediated depletion of SH3YL1 resulted in a significant decrease in the androgen-mediated proliferation of both LNCaP and LAPC4 cells **(Figures 3.8A and B)**. This activity was confirmed in cells engineered to express an inducible shRNA directed against SH3YL1 **(Figures 3.9A and B)**. To explore potential roles for SH3YL1 in advanced prostate cancer, we generated a model in which conditional knockdown of SH3YL1 could be accomplished in the castration-resistant PCa (CRPC) cell line 22Rv1. This cell line expresses AR splice variants that retain the amino terminus and DBD and exhibit constitutive activity [201]. Importantly, knockdown of SH3YL1 in this cell line also resulted in a significant decrease in proliferation **(Figure 3.9C)**. Interestingly, in the AR-negative prostate cancer cell line, PC-3, siRNA-mediated depletion of SH3YL1 resulted in variable decreases in basal cell proliferation **(Figures 3.10A and B)**, suggesting that like many other NR coregulators, SH3YL1 may have additional roles beyond AR, an area we are actively pursuing.

We next evaluated the role of SH3YL1 in androgen-mediated LNCaP migration. Using the Boyden dual-chamber migration assay described above, it was observed that knockdown of SH3YL1 dramatically reduced the number of migrating cells under basal conditions and following 12-hour treatment with an androgen **(Figure 3.11A)**. This activity was confirmed using a high-throughput microscopy imaging system (IncuCyte™ Zoom) to analyze the role of SH3YL1 on LNCaP migration in a scratch test assay **(Figure 3.11B; Figure 3.12A)**. Notably, only cells expressing an shRNA directed against SH3YL1 (shSH3YL1 + DOX) exhibited impaired androgen-mediated cell migration. Inducible knockdown of SH3YL1 in the CRPC cell line 22Rv1, or chemical siRNA-mediated knockdown in the AR-negative cell line PC-3, reduced the number of cells migrating under basal conditions, further highlighting the importance and possible multiple roles of SH3YL1 in prostate cancer **(Figures 3.12B and C)**.

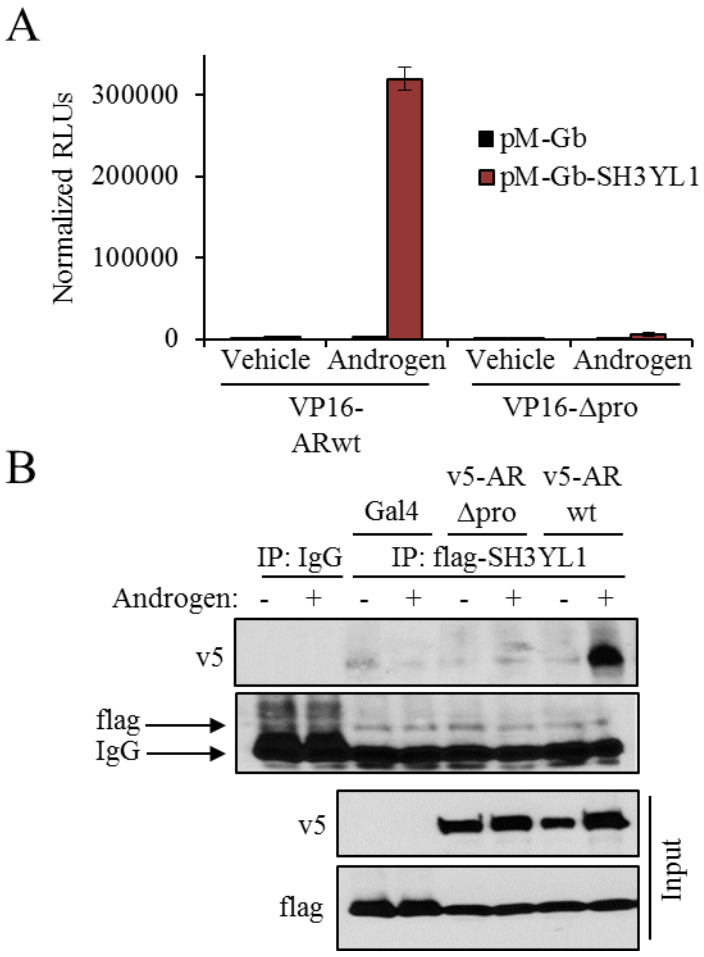


Figure 3.6 AR interacts with SH3YL1 through AR's polyproline domain. A, for mammalian 2-hybrid assays, HeLa cells were transfected VP16-ARwt or VP16-ARΔpro and pM-Gb empty vector or pM-Gb-SH3YL1 (full length) expression vectors in combination with a 5x-GAL4-TATA-Luc reporter plasmid. After transfection, cells were treated with vehicle or androgen (10 nM R1881) for 24 hours. Cells were harvested and assayed for luciferase assay; all luciferase values were normalized to β-galactosidase controls. Data are expressed as mean RLUs ± SE. B, LNCaP cells stably expressing GAL4, v5-ARwt, v5-ARΔpro, or v5-AR(C562S) were transfected with a flag-SH3YL1 construct and treated with vehicle or androgen (.1 nM R1881) for 24 hours. After lysis, cells were immunoprecipitated with anti-flag (SH3YL1) and immunoblotted to detect interaction with v5-AR.

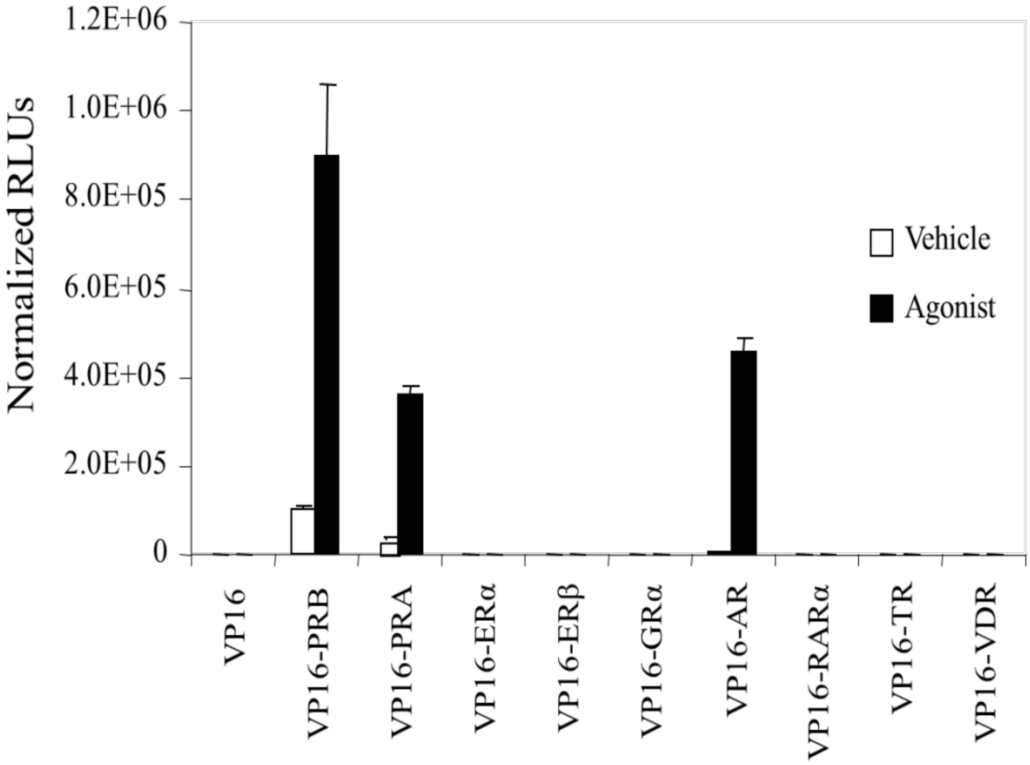


Figure 3.7. SH3YL1 binds to agonist-bound AR and PR in mammalian 2-hybrid assays. HeLa cells were transfected with various VP16-fused nuclear receptors, a GAL4(DBD)-fused full-length SH3YL1, and a 5xGAL4-luciferase reporter construct along with a β-galactosidase transfection control plasmid. Transfected cells were treated with NR-specific agonists (ex.dihydrotestosterone (DHT) for AR, 17β-estradiol for ERα and ERβ, etc) for 24 hours and then harvested and assayed for luciferase activity. All luciferase values were normalized to β-galactosidase transfection controls. Data are expressed as mean normalized relative light units (RLUs) ± SE.

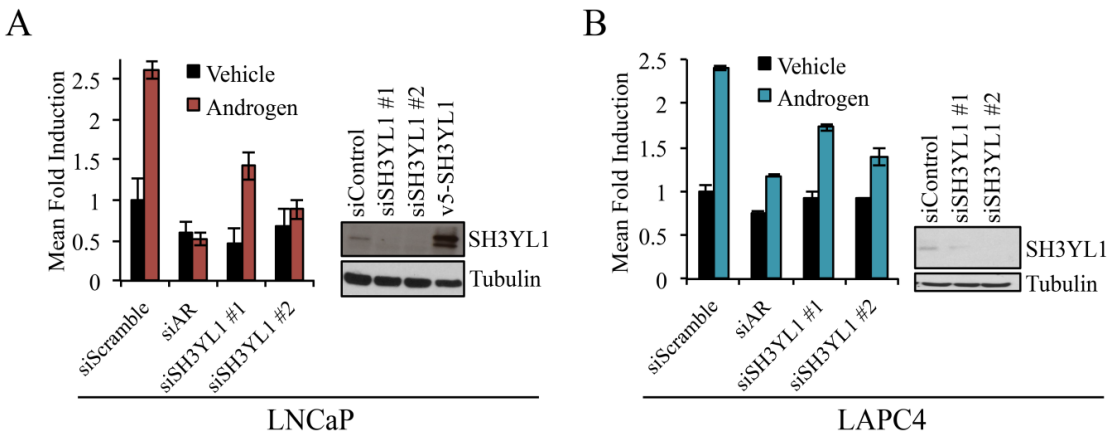


Figure 3.8 SH3YL1 is necessary for maximal androgen-mediated cell proliferation. LNCaP (A) and LAPC4 (B) cells were transfected with siRNAs directed against AR or SH3YL1 and treated with vehicle or .1 nM R1881. Cells were then lysed, and the relative number of cells was quantified using a fluorescent DNA-binding dye. Each sample was performed in triplicate. Results are expressed as mean relative fold induction ± SE (n = 3). Knockdown was confirmed with western blot (*right*). *, significant ($p<0.05$) changes from siControl + androgen.

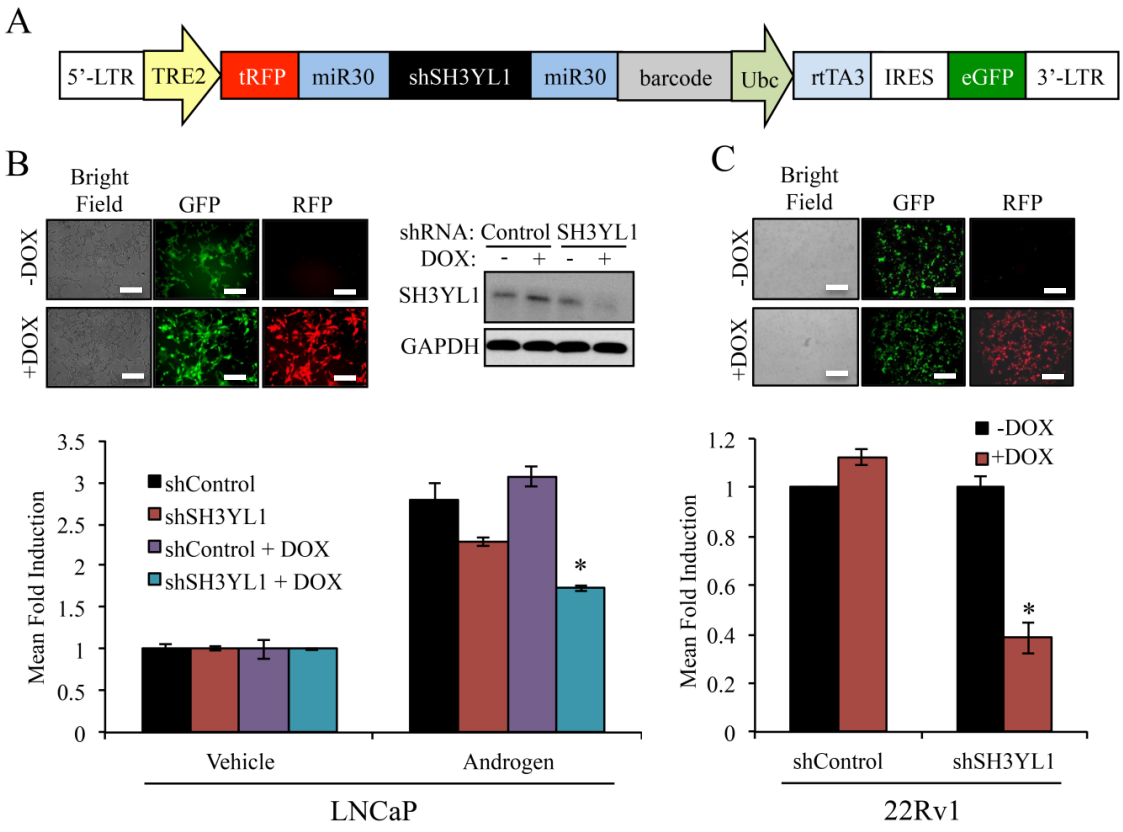


Figure 3.9 SH3YL1 is necessary for maximal androgen-mediated cell proliferation in inducible model. A, schematic of the pINDUCER11 construct used. LNCaP (B) and 22Rv1 (C) cells were lentivirally transduced with the pINDUCER11 construct containing a constitutively expressed turbo GFP and an inducible shRNA against SH3YL1 or a shControl (scramble). Cells were sorted twice for GFP-positive cells using flow cytometry. Inducible expression was confirmed with fluorescence microscopy and western blot. Cells were treated for 7 days. The number of cells was assayed using the fluorometric dsDNA dye assay described in Figure 8A. Data are expressed as mean fold induction ± SE. *, significant ($p<0.05$) changes from (B) other androgen treated groups or (C) from

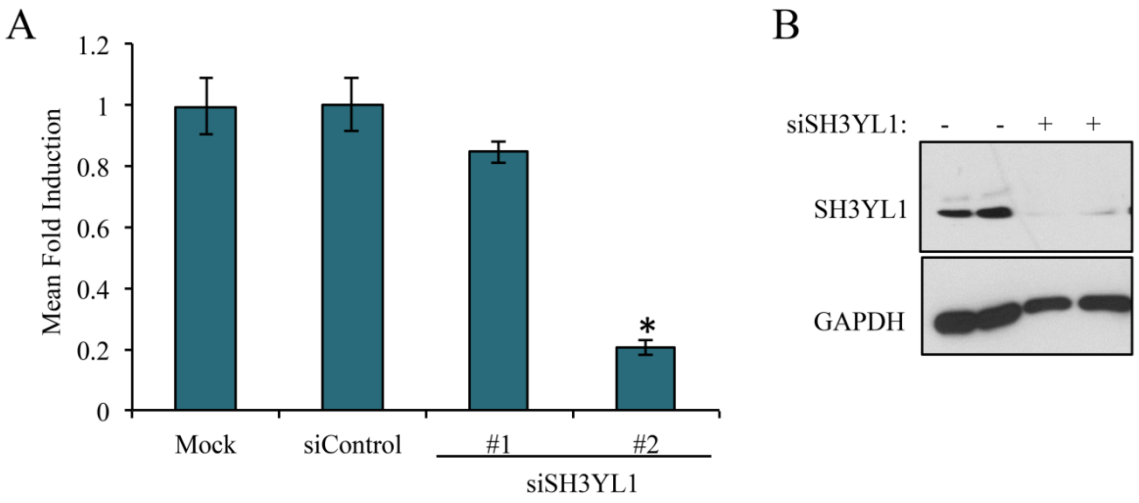
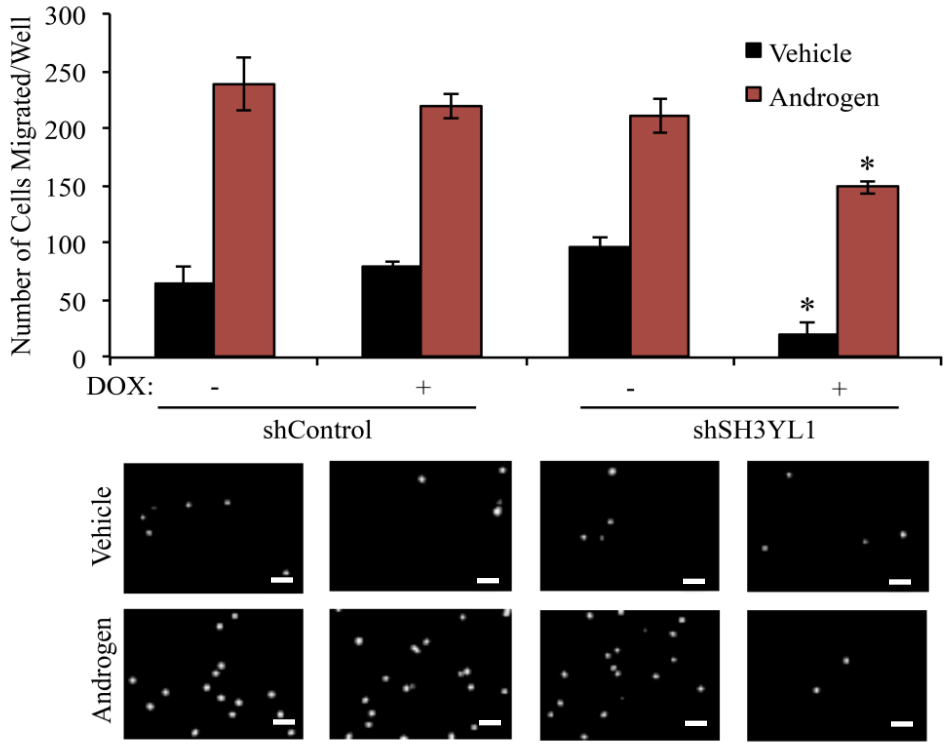


Figure 3.10 SH3YL1 is necessary for maximal proliferation in AR-negative prostate cancer cells. PC-3 cells were transfected with siRNAs directed against SH3YL1. Cells were then lysed, and the relative number of cells was quantified using a fluorescent DNA-binding dye. Each sample was performed in triplicate. Results are expressed as mean relative fold induction ± SE (n = 3). Graph shown is a representative of three biological repeats. Knockdown was confirmed with western blot.

***UBN1* is a transcriptional target of AR and SH3YL1**

Given that SH3YL1 modulated the effects of androgens on cancer cell proliferation and migration, we reasoned that SH3YL1 could be controlling a subset of androgen-regulated genes involved in these processes. To identify these genes, we took advantage of our inducible LNCaP system to knock down SH3YL1 in the presence or absence of androgens and looked for changes in gene expression using a microarray **(Figure 3.13A and B; Table 3.1)**. Similar to what we observed with the deletion of the AR polyproline domain, microarray analysis revealed that FKBP51 levels were not altered by knockdown of SH3YL1 while KLK3 (PSA) levels were reduced, although not significantly (data not shown). However, siRNA-mediated knockdown in combination with a shorter androgen treatment (16 hours) revealed that knockdown of SH3YL1, similar to deletion of the AR polyproline domain, resulted in a significant reduction of PSA mRNA levels **(Figure 3.14A)**. This was in contrast to FKBP51 mRNA levels, which were again not significantly altered by the knockdown of SH3YL1 **(Figure 3.14B)**. While here it appeared at first that AR polyproline disruption decreased FKBP51 expression, suggesting a broader role for this domain in AR-dependent, SH3YL1-independent transcription, this effect was likely due to the fact that the v5-ARs in general could not fully rescue the effects of our endogenous AR knockdown (**Figure 3.4**, compare v5-ARwt and v5-ARΔpro). Interestingly, we did still observe a significant fold androgen induction of PSA compared to vehicle in SH3YL1 knockdown cells ± AR polyproline disruption (15-fold (-AR polyproline mutation) and 235-fold (+AR polyproline mutation), respectively). However, we suspect this may be a mathematical artifact that resulted from the nearly complete loss of detectable basal PSA mRNA levels following SH3YL1 knockdown and/or AR polyproline domain disruption (i.e. leaving a miniscule denominator to calculate fold induction, a scenario susceptible to wide variations in fold induction calculations). Regardless, at this time we cannot rule out the possibility that additional SH3YL1-dependent and

A


Number of Cells Migrated/Well
300
250
200
150
100
50
0
Vehicle
Androgen
*
*
DOX:
-
+
-
+
shControl
shSH3YL1
Vehicle
Androgen

B

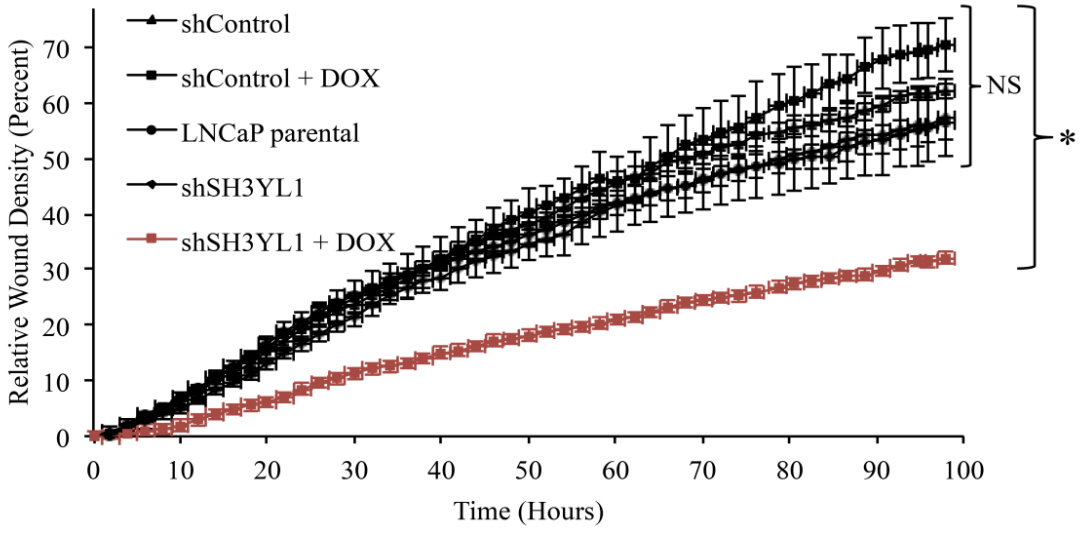

shControl
shControl + DOX
LNCaP parental
shSH3YL1
shSH3YL1 + DOX
Relative Wound Density (Percent)
0
10
20
30
40
50
60
70
0
10
20
30
40
50
60
70
80
90
100
Time (Hours)
NS
*

Figure 3.11 SH3YL1 is important for androgen-mediated prostate cancer cell migration. A, LNCaP stable cells were treated with vehicle or 10 nM R1881 (androgen) ± doxycycline (DOX) for 72 hours before they were counted (20,000 cells/chamber) and seeded in a Boyden dual-chamber as described in Figure 1. After 16 hours, the cells were then fixed and stained with 4',6-diamidino-2-phenylindole (DAPI). Inserts were visualized under a fluorescence microscope (*bottom*), and the cells were quantitated (*top*) by counting the number of cells in three randomly selected microscopic fields at ×40 magnification. The number of cells in all three fields was added together and an average of three inserts ± SE was determined. *, significant ($p<0.01$) changes from shControl. B, LNCaP stable cells were treated with vehicle or 1 nM R1881 ± DOX for 48 hours before they were counted (5,000 cells/well) and seeded in 96-well plates. The Essen Wound Maker™ was used to create a scratch wound in the center of each well 24 hours after seeding. Media was replaced and plates were monitored by the IncuCyte™ Zoom for 96 hours. NS, not significant; *, significant ($p<0.01$) changes from control.

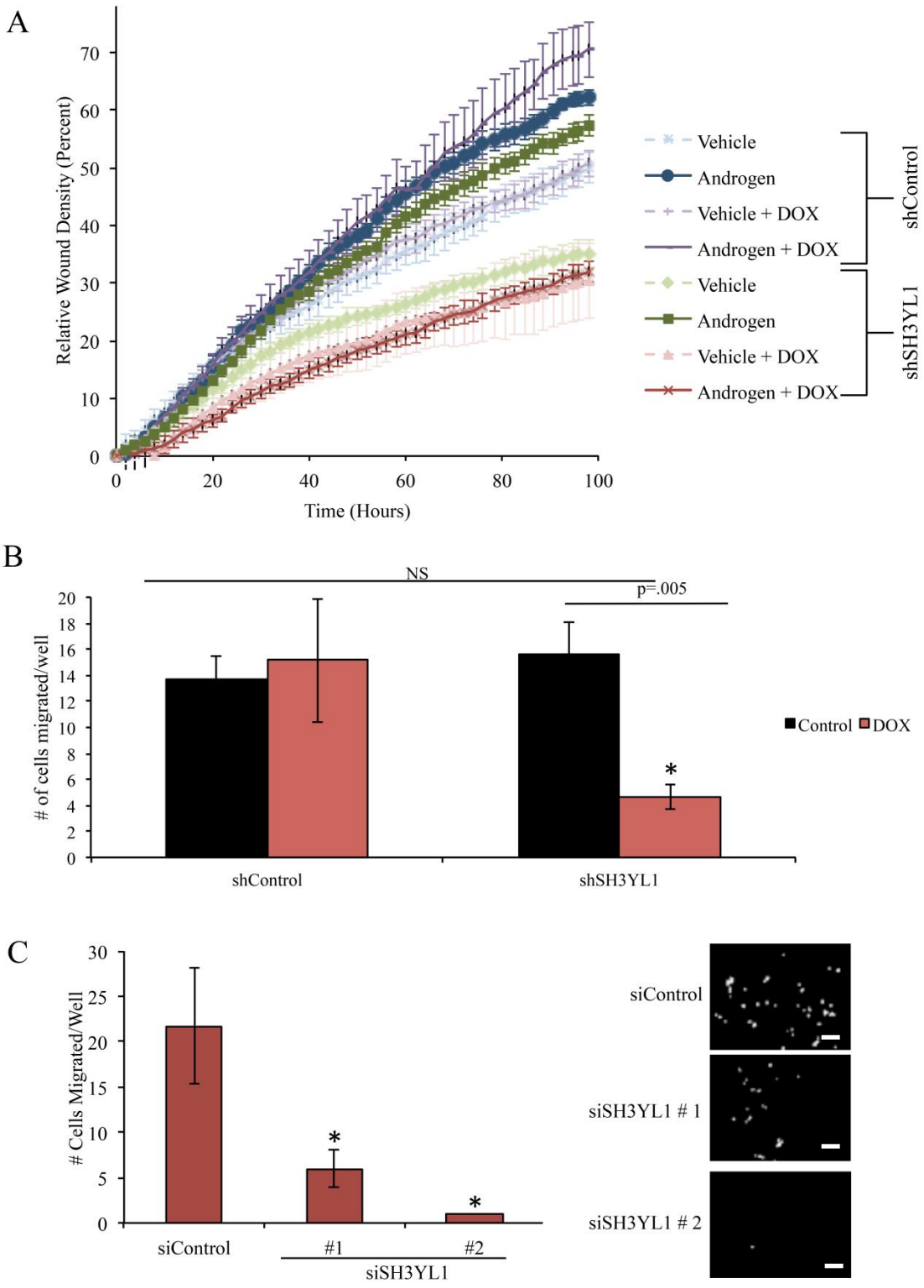

A
Relative Wound Density (Percent)
Time (Hours)
Vehicle
Androgen
Vehicle + DOX
Androgen + DOX
Vehicle
Androgen
Vehicle + DOX
Androgen + DOX
shControl
shSH3YL1
B
NS
p=.005
# of cells migrated/well
Control
DOX
shControl
shSH3YL1
C
Cells Migrated/Well
siControl
#1
#2
siSH3YL1
siControl
siSH3YL1 # 1
siSH3YL1 # 2

Figure 3.12 SH3YL1 is important for androgen-mediated, castration resistant and androgen-independent prostate cancer cell migration. A**,** LNCaP stable cells were treated with vehicle or 1 nM R1881 (androgen) ± doxycycline(DOX) for 48 hours before they were counted (5,000 cells/well) and seeded in 96-well plates. The EssenWound Maker™ was used to create a scratch wound in the center of each well 24 hours after seeding. Media was replaced and the plate was monitored by the IncuCyte™ Zoom for 96 hours. B, 22Rv1 stable cells described in Figure 14, were treated ± doxycycline (DOX) for 72 hours before they were counted (20,000 cells/chamber) and seeded in a Boyden dual-chamber as described in Figure 8. After16 hours, the cells were then fixed and stained with 4',6-diamidino-2-phenylindole (DAPI). Inserts were visualized under a fluorescence microscope (*bottom*), and the cells were quantitated (*top*) by counting the number of cells in three randomly selected microscopic fields at ×40 magnification. The number of cells in all three fields was added together and an average of three inserts ± SE was determined. *, significant (p=0.005) changes from shControl. C, PC-3 cells were counted (20,000 cells/chamber) and seeded in a Boyden dual-chamber as described in Figure 1. After 16 hours, the cells were then fixed and stained with 4',6-diamidino-2-phenylindole (DAPI). Inserts were visualized under a fluorescence microscope (*bottom*), and the cells were quantitated (*top*) by counting the number of cells in three randomly selected microscopic fields at ×40 magnification. The number of cells in all three fields was added together and an average of three inserts ± SE was determined. Graph show in a representative of two biological repeats. *, $p<0.05$.

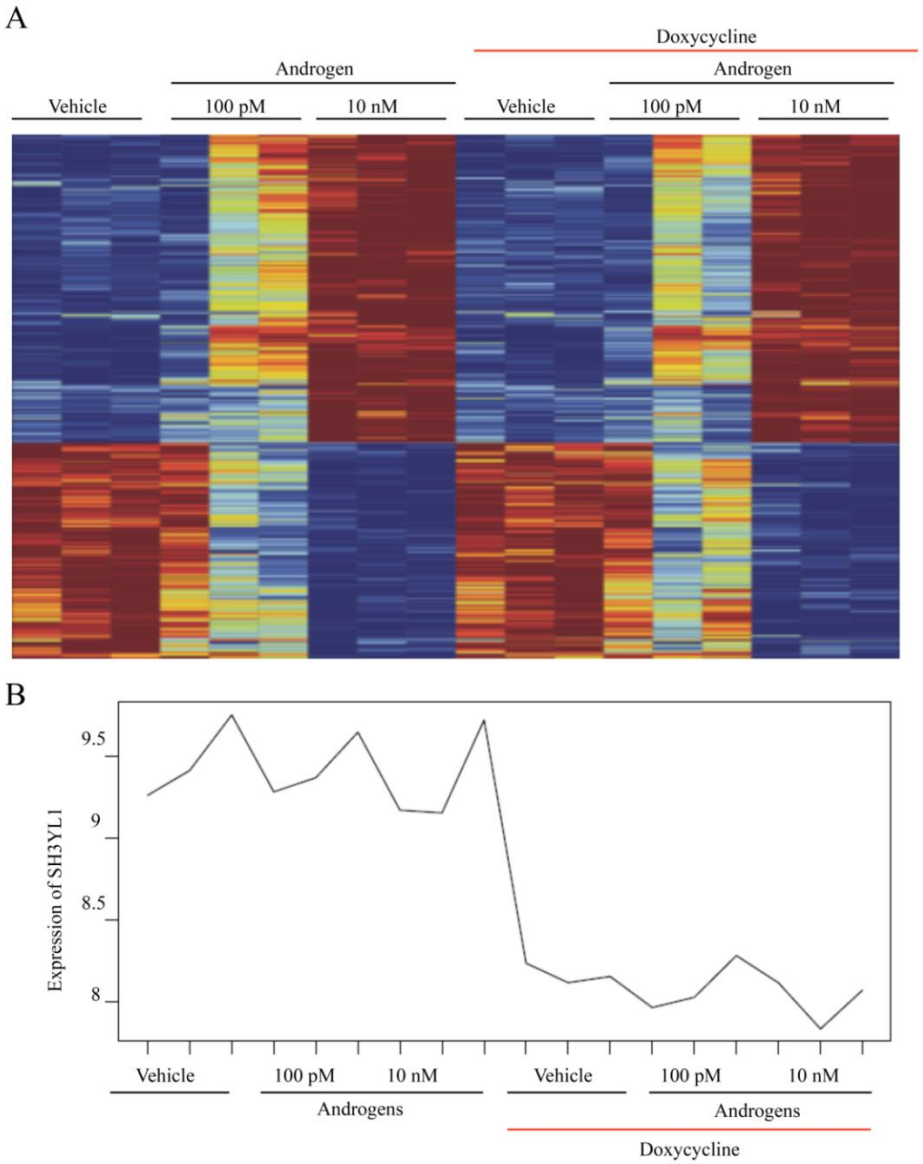


Figure 3.13 SH3YL1 regulates the expression of a subset of androgen-mediated genes. A, inducible LNCaP stable cells were treated ± doxycycline (DOX) with Vehicle, 100 pM or 10 nM R1881. RNA was harvested after 72 hours from triplicate samples and used in an Illumina® microarray to assess changes in gene expression. Data was analyzed to identify differentially-regulated genes between the 12 different treatment groups and shown in a heat map. Only genes with at least a 1.5-fold change in expression between classes were considered (see Table 3 for complete list; significance was determined using a two-tailed t test and a $p<0.05$). B, *SH3YL1* expression was significantly decreased in all doxycycline-treated samples ($p < 0.05$).

Table 3.1 Genes changed following combined androgen and doxycycline treatment.

Time	Treatment	Gene	Regulation
24 hours	EtOH	PPAP2C	Up
		LOC644880	Up
		PLEKHG5	Up
		SH3YL1	Down
		CHTF8	Up
		OTP	Down
		LOC388160	Up
		UBC	Up
		ANKRD13D	Up
		FGFBP3	Down
		LOC642644	Up
		LOC100134560	Up
		DNASE1L1	Up
		LOC644014	Up
		ADAM9	Up
	100 pM R1881	LOC645010	Up
		LOC643834	Up
		SIPA1L3	Up
		SH3YL1	Down
		COX7B2	Up
		UBC	Up
		C20orf175	Up
		C16orf86	Up
		LOC100128202	Up
		CD1C	Up
		SLC28A1	Up
	10 nM R1881	SH3YL1	Down
		MGC57359	Up
		UBC	Up
		LOC651991	Up
		LOC644014	Up
		AMDHD2	Up
		SLC28A1	Up

Time	Treatment	Gene	Regulation
72 hours	EtOH	LOC645010	Up
		LOC643834	Up
		SIPA1L3	Up
		SH3YL1	Down
		COX7B2	Up
		UBC	Up
		C20orf175	Up
		C16orf86	Up
		LOC100128202	Up
		CD1C	Up
		SLC28A1	Up
	100 pM R1881	UBN1	Down
		LOC390282	Down
		TMEM38B	Down
		SH3YL1	Down
		KIAA1751	Down
		LOC648169	Down
		CTGF	Down
		LOC730024	Up
		LOC730024	Up
		USH1G	Up
		ERC1	Down
		UBC	Up
		LOC100130311	Down
		LOC340357	Down
		LOC440905	Up
		IGF2AS	Down
		CLK2	Up
		FLJ46836	Down
		FLJ30428	Up
		SCMH1	Up
		LOC388931	Up
		LOC440905	Up
		LOC100131071	Down
		LOC100132506	Down
		FLJ30428	Up
		LOC100132106	Down
	10 nM R1881	CXorf48	Down
		GPR1	Down
		LOC339804	Down
		SCARNA3	Up
		CXCR7	Down
		SH3YL1	Down
		PRLR	Down
		BRWD1	Up
		BRSK2	Up
		PCTK3	Up

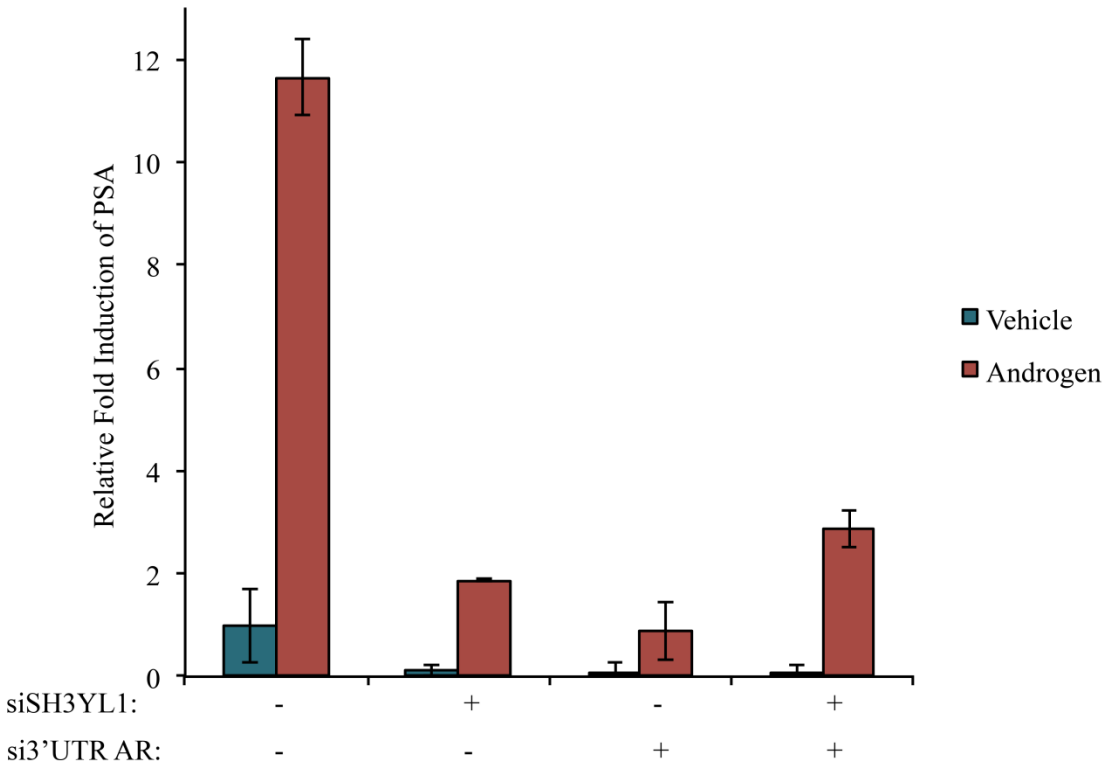


Figure 3.14 The AR polyproline domain and SH3YL1 affect the partially regulate the transcription of KLK3(PSA). LNCaP cells expressing v5-ARΔprowere transfected with siControl, siAR 3'UTR, or siSH3YL1 followed by treatment with vehicle or 10 nM R1881 for 16 hours. RNA was then collected and transcript levels of the AR target gene *KLK3* (PSA) were assessed using qPCR. Data are normalized to 36B4 and expressed as mean ± SE.

possibly AR-independent regulatory mechanisms, stimulatory or inhibitory, could be regulating this gene.

Within the subset of genes that were jointly regulated by AR and SH3YL1, *ubinuclin 1* (*UBN1*), a member of the histone H3.3 chaperone complex [215-217], was sensitive to androgen treatment and was down-regulated in the absence of SH3YL1. The change in gene expression observed in the microarray was confirmed using both qPCR **(Figure 3.15A)** and by western immunoblot **(Figure 3.15B and C)**. We also confirmed that *UBN1* expression was increased in response to androgens in another hormone-sensitive cell model, VCaP **(Figure 3.15D)**. Further, the androgen-mediated increase in *UBN1* expression was blocked by the competitive inhibitor enzalutamide **(Figure 3.15E)**, confirming that its expression was AR-dependent. Increases in *UBN1* expression four hours post-androgen treatment or in the presence or absence of cyclohexamide suggest that *UBN1* is a direct target of AR **(Figure 3.16A and B)**. In support of this, the results of AR ChIP-Seq experiments in several prostate cancer cell lines indicate that AR binds, albeit weakly, to an intronic region of *UBN1* **(Figure 3.16C)**. AR's binding peak in *UBN1* was dwarfed in comparison to a well-defined direct transcriptional target, *CAMKK2*, potentially explaining why this AR target had not been described until now [132]. Nonetheless, several androgen response element (ARE) half sites within the *UBN1* AR-binding region were identified. Importantly, knockdown of either SH3YL1 or both the full-length and constitutively-active splice variants of AR in the CRPC 22Rv1 cell model resulted in decreased *UBN1* protein expression **(Figure 3.17A and B)**. Moreover, LNCaP cells expressing polyproline domain-mutated AR, unlike wild-type AR, could not rescue *UBN1* expression, similar to cells lacking endogenous AR (Gal4 control) or expressing the DBD-mutated AR (v5-AR(C562S)) **(Figure 3.18)**.

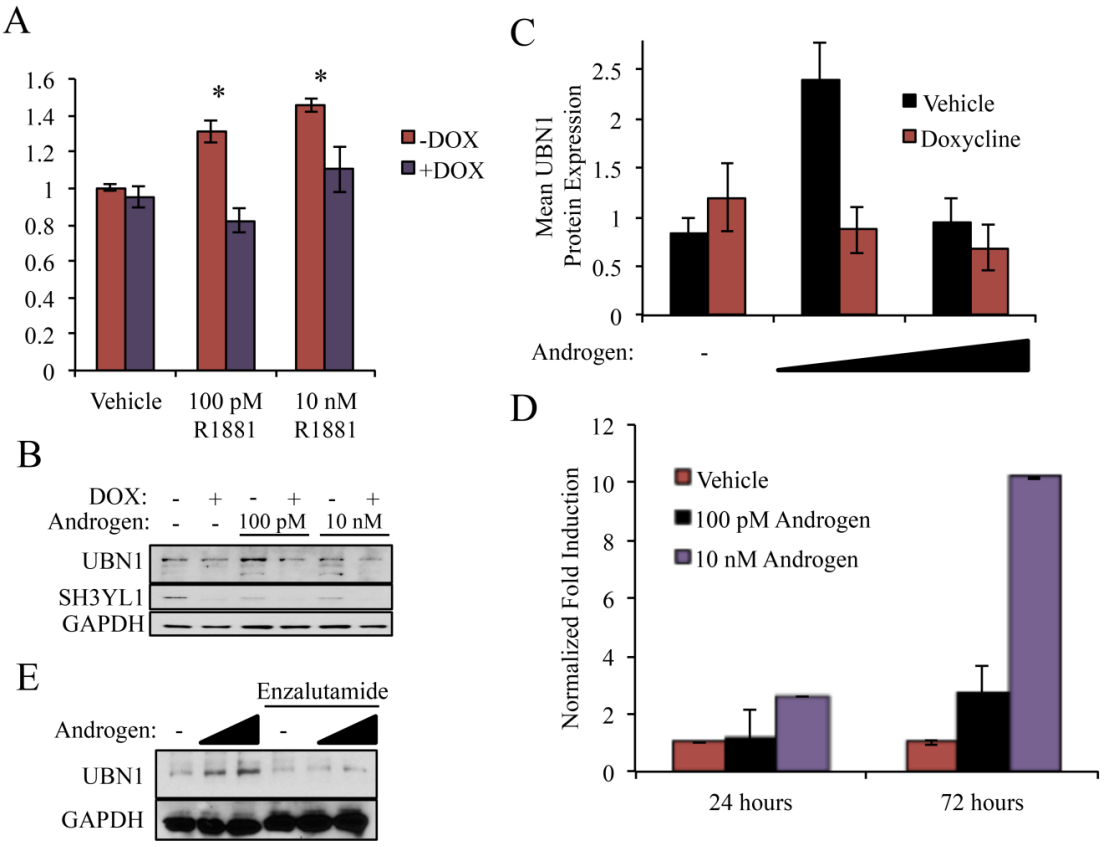


Figure 3.15 SH3YL1 regulates the expression of UBN1. A, RT-qPCR confirmation of *UBN1* expression normalized to the housekeeping gene, *36B4*. B, *UBN1* protein expression from samples treated under the same conditions as A. C, *UBN1*expression (qPCR) after treatment with androgens (R1881) ± doxycycline (SH3YL1 knockdown). C, *UBN1* expression increases as assessed by qPCR in response to androgens (R1881) in a second androgen- sensitive prostate cancer cell model, VCaP. E, LNCaP cells were pretreated with vehicle or 10 mM of the FDA-approved, competitive AR antagonist enzalutamide one hour prior to treatment with vehicle, .1 or 10 nM R1881. Protein levels were then assessed via western blot.

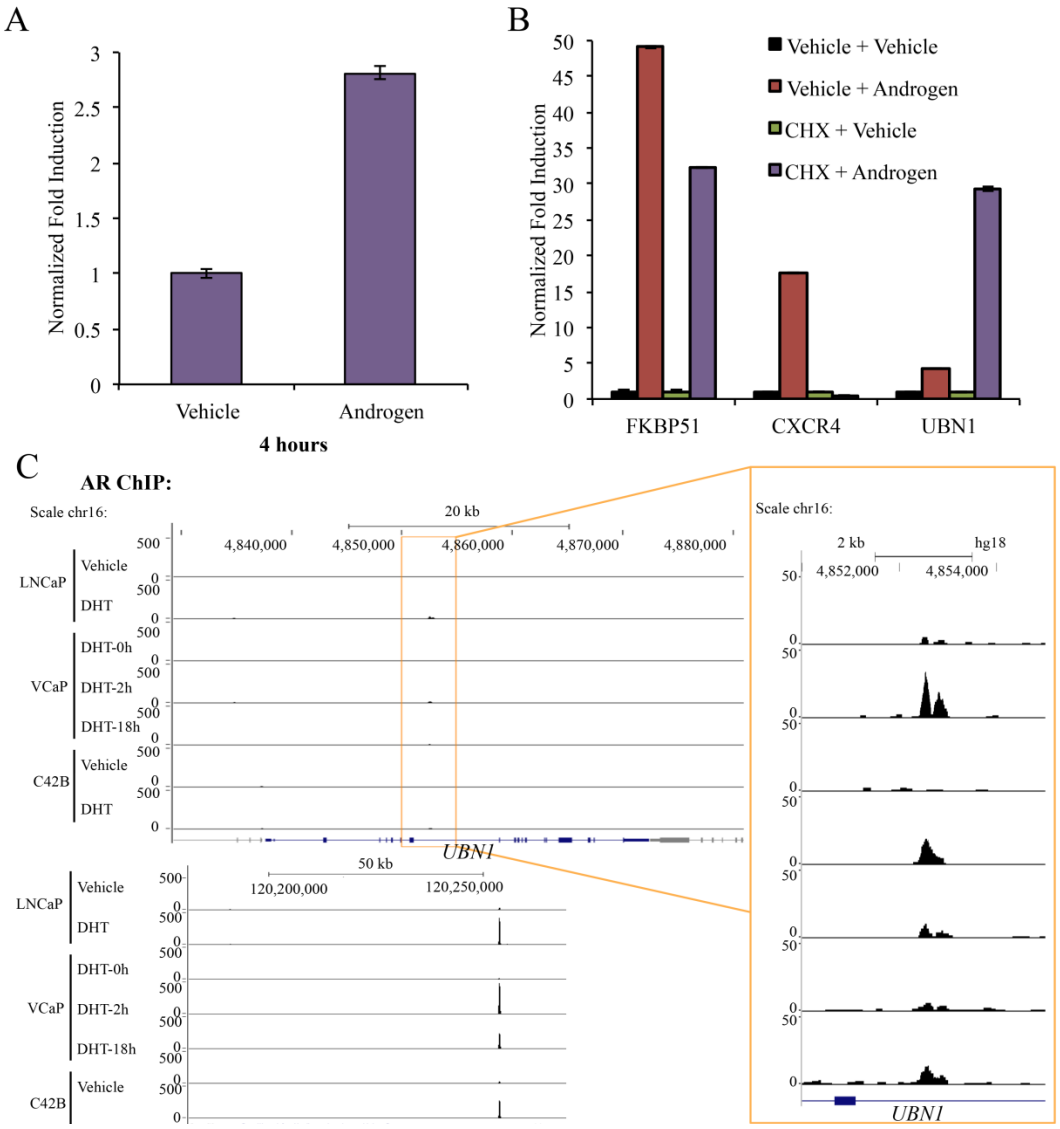


Figure 3.16 *UBN1 is* a direct target of ligand-bound AR and SH3YL1. A, LNCaP cells were treated ± R1881 (Androgen) for four hours before RNA was collected and subjected to qPCR analysis to assess *UBN1* expression. B, LNCaP cells were treated with 1 µg/mL cyclohexamide (CHX) for one hour before treatment with vehicle or 10 nM R1881 for 16 hours. As controls, also shown are FKBP51, a direct target of AR, and CXCR4, an indirect target of AR. All data are normalized to 36B4, expressed as normalized fold induction ± SE and are a representation of 3 biological replicates. C, ChIP-Seq tracks of LNCaP, VCaP, and C4-2B cells treated with vehicle or DHT for 0, 2, or 18 hours. Potential AR binding sites in the intronic region of *UBN1* are highlighted and enlarged on the right. For comparison, a well-characterized AR binding site located ~2 kb upstream of the *CAMKK2* gene is shown below.

UBN1 expression correlates with disease progression and poor prognosis in patients with prostate cancer

Analysis of clinical data derived from The Cancer Genome Atlas (TCGA) demonstrated that high UBN1 transcript levels correlated with poor patient prognosis **(Figure 3.19A)**. Correspondingly, UBN1 levels also significantly correlated with Gleason score **(Figure 3.19B)**, biochemical (PSA) recurrence **(Figure 3.19C)**, new tumor occurrence after initial treatment **(Figure 3.19D)**, tumor stage **(Figure 3.19E)** and metastasis **(Figure 3.19F)**. Taken together, these observations indicate that UBN1 may play a role in the pathobiology of advanced prostate cancers.

UBN1 is necessary for maximal androgen-mediated cell proliferation and migration

To determine if the impact of AR/SH3YL1 signaling on PCa cell proliferation and migration could be mediated through UBN1, we next assessed the role of UBN1 in these processes. Of note, knockdown of *UBN1* expression **(Figure 3.20A and B)** resulted in a significant decrease in androgen-mediated LNCaP proliferation **(Figure 3.21A)**. Moreover, UBN1, like SH3YL1, was also necessary for maximal androgen-mediated migration in a Boyden dual-chamber assay **(Figure 3.21B)**, confirming its functional role in multiple AR-mediated processes of pathological significance in PCa. Importantly, transient overexpression of *UBN1* rescued the impaired androgen-mediated proliferation of LNCaP cells expressing only the ARΔpro mutant (**Figure 3.21C**), indicating that UBN1 is a downstream mediator of the effects of the AR/SH3YL1 complex on PCa cell proliferation and migration.

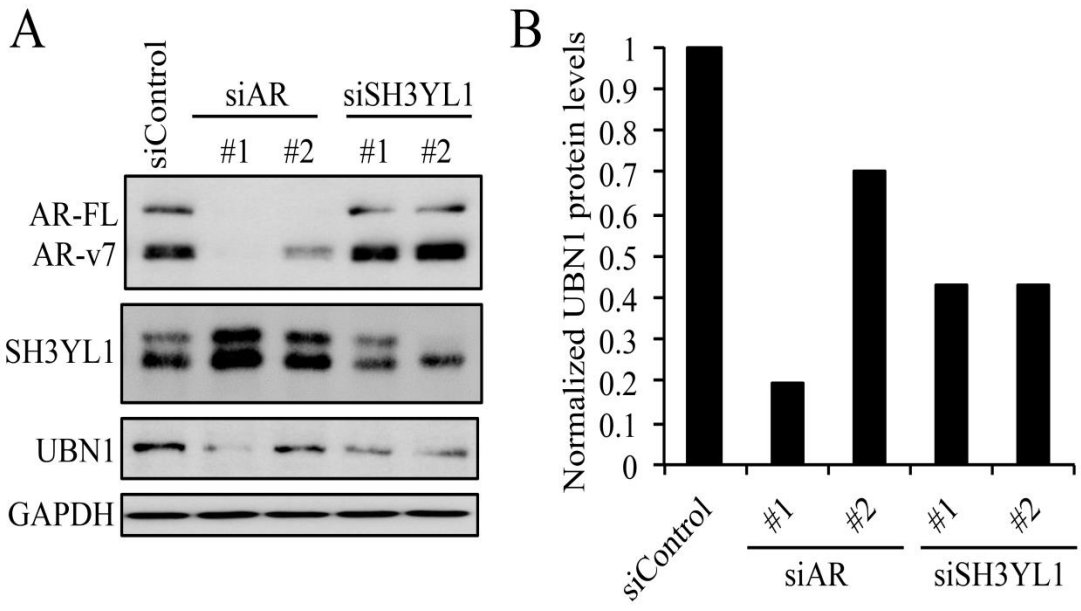


Figure 3.17 *UBN1* is a regulated by AR and SH3YL1 in CRPC. A, 22Rv1 cells, a CRPC cell model, were transfected with siRNAs targeting scramble control (siControl), the AR full-length and the constitutively active (ΔLBD) ARv7 jointly (siAR #1), AR full-length only (siAR #2), or SH3YL1 (siSH3YL1s #1 and #2). Protein lysates were probed for AR, SH3YL1, UBN1, and GAPDH (loading control). B, Densitometry was performed using Image J. *UBN1* expression was normalized to GAPDH and graphed.

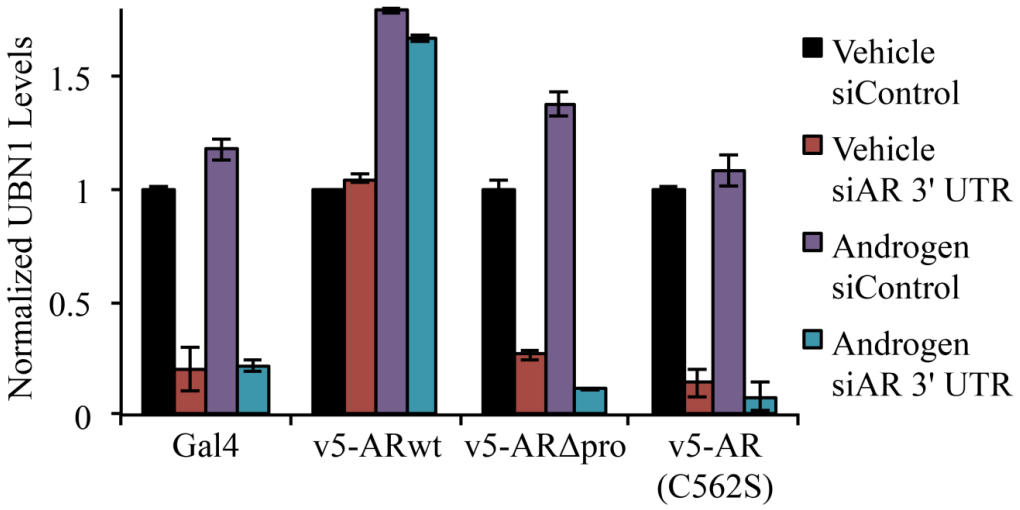


Figure 3.18 The androgen receptor polyproline domain is necessary for *UBN1* transcription. LNCaP stably cells expressing GAL4, v5-ARwt, v5-ARΔpro, or v5-AR(C562S) were transfected with siControl or siAR 3'UTR followed by treatment with vehicle or 10 nM R1881 for 24 hours. RNA was then collected and transcript levels of UBN1 were assessed using qPCR. Data are normalized to 36B4 and expressed as mean ± SE. *, significant ($p<0.05$) androgen induction.

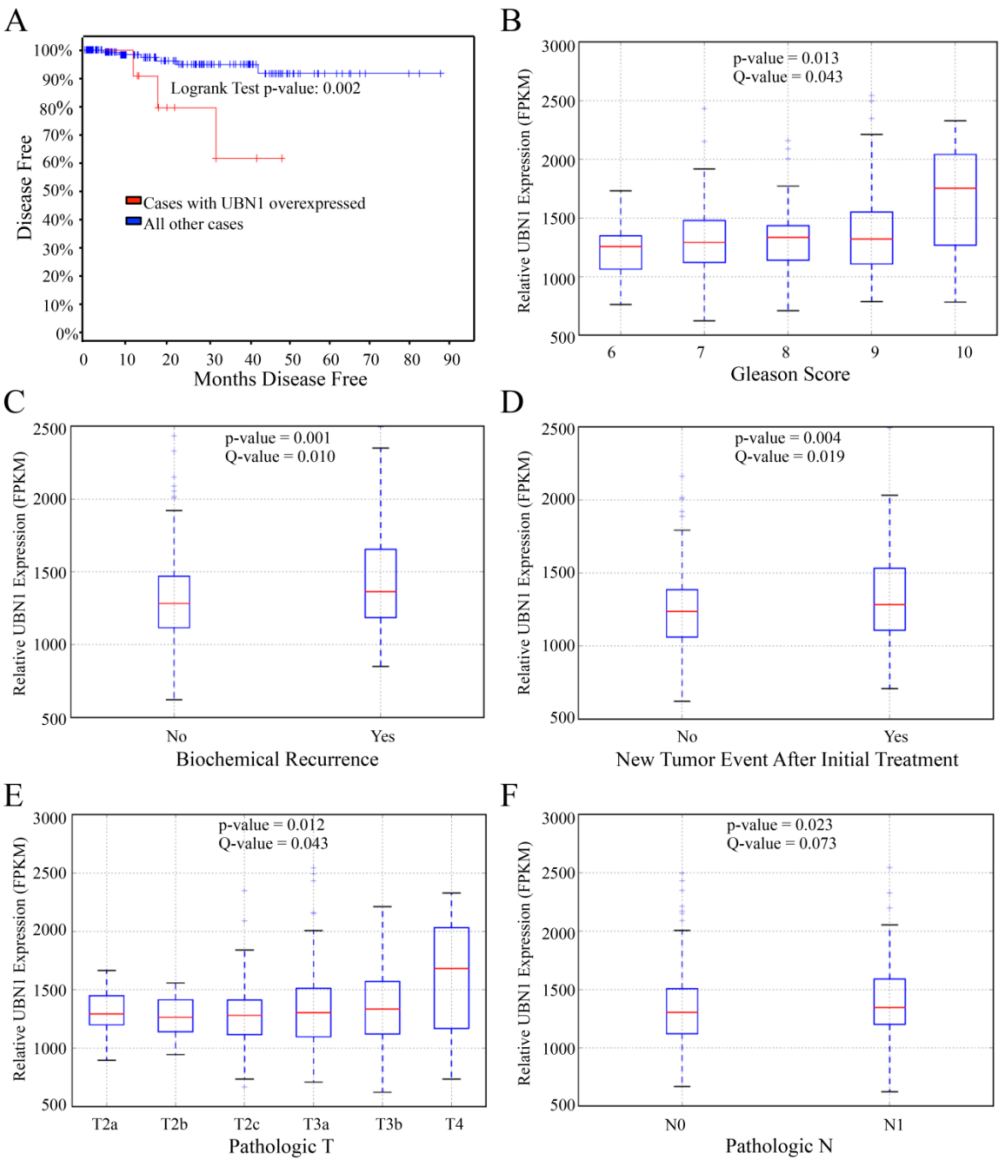


Figure 3.19 *UBN1* expression correlates with disease progression and poor patient prognosis. A, Kaplan-Meier analysis of *The Cancer Genome Atlas* (*TCGA*) demonstrating UBN1 mRNA levels predict poor prognosis in prostate cancer patients. B, high UBN1 levels correlate significantly with Gleason score. C, UBN1 levels increase in patients with detectable PSA levels after targeted molecular therapy. D, patients incurring new tumors after initial treatment correlate with increased *UBN1* expression. E-F, increased UBN1 levels associate with tumor grade (E) and lymph node metastasis (F).

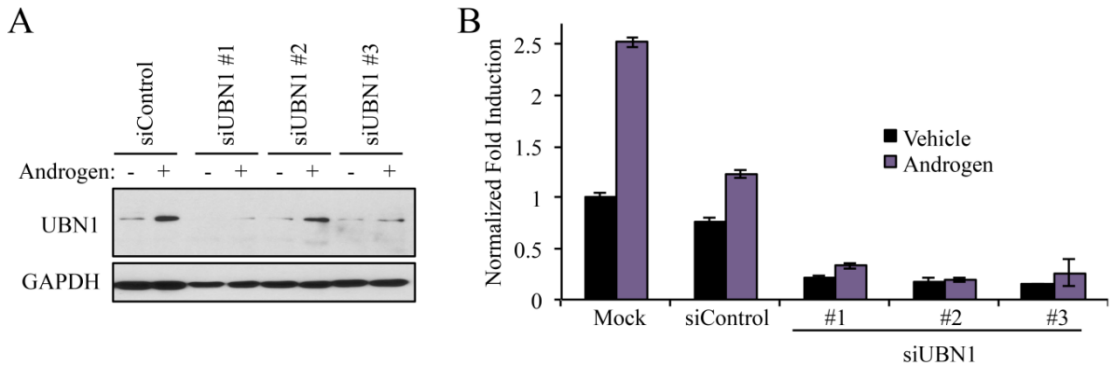
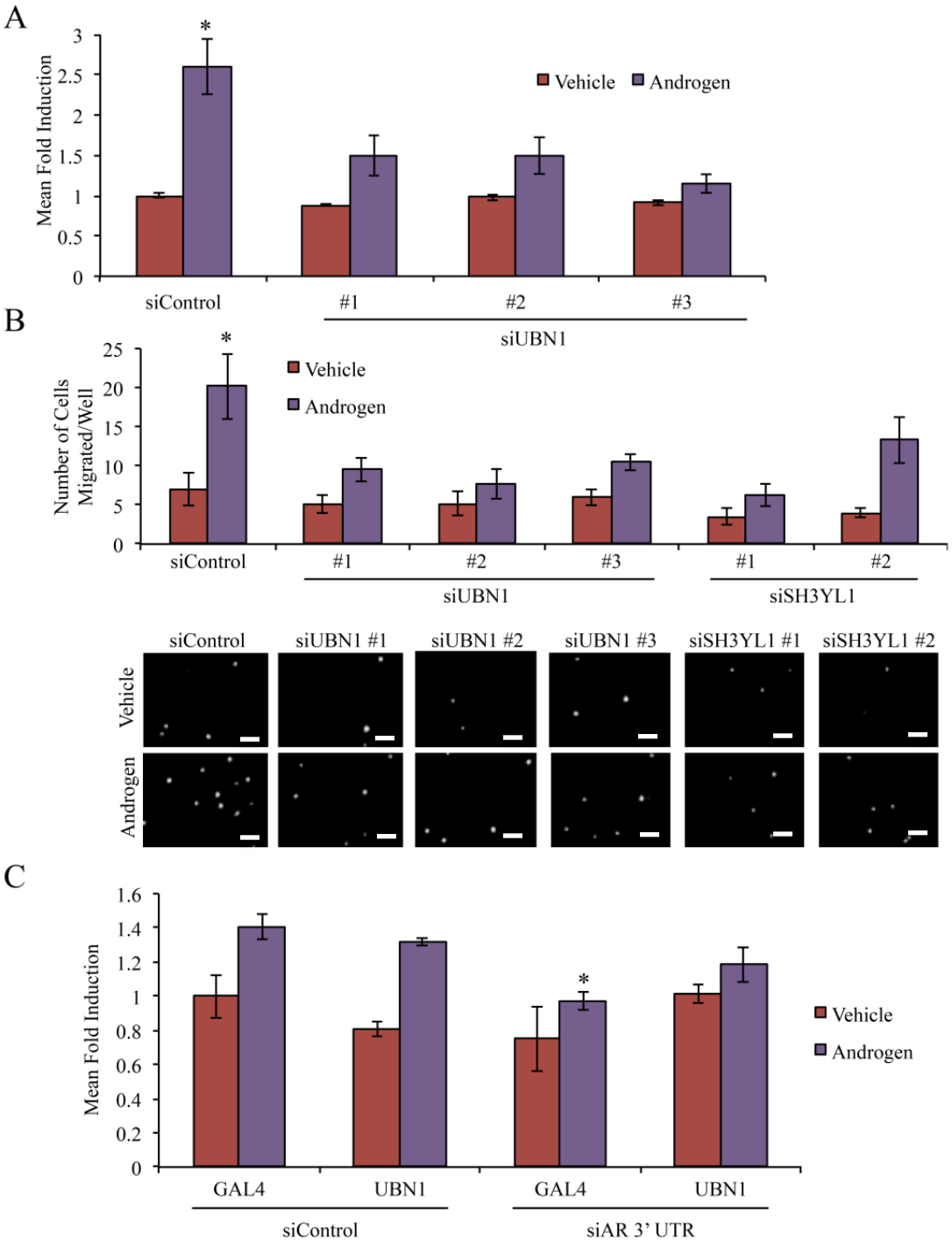


Figure 3.20 Confirmation of UBN1 knockdown with siRNAs. A, LNCaP cells were treated for 3 days with vehicle or 100 pM R1881 in combination with siControl or three different siRNAs targeting UBN1. Cells were then lysed and knockdown was confirmed via western blot and B, qPCR.


A
Mean Fold Induction
3
2.5
2
1.5
1
0.5
0
*
Vehicle
Androgen
siControl
#1
#2
#3
siUBN1
B
Number of Cells Migrated/Well
25
20
15
10
5
0
*
Vehicle
Androgen
siControl
#1
#2
#3
#1
#2
siUBN1
siSH3YL1
siControl
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siUBN1 #3
siSH3YL1 #1
siSH3YL1 #2
Vehicle
Androgen
C
Mean Fold Induction
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Vehicle
Androgen
GAL4
UBN1
GAL4
UBN1
siControl
siAR 3' UTR

Figure 3.21 UBN1 is necessary for maximal androgen-mediated cell proliferation and migration. A LNCaP cells were treated for 7 days with vehicle or 100 pM R1881 in combination with siControl or three different siRNAs targeting UBN1. Cells were then lysed and the relative number of cells was quantified using a fluorescent DNA-binding dye. Each sample was performed in triplicate. Results are expressed as relative mean fold induction ± SE (n = 3). *, significant ($p<0.05$) changes from siControl + Vehicle. B, LNCaP cells were transfected with siRNAs directed against UBN1, SH3YL1, or negative control and treated with vehicle or 10 nM R1881 for 16 hours prior to seeding in a Boyden chamber. Migration was then assessed as described in Figure 8B. *, significant ($p<0.01$) changes from siControl + Vehicle. C, LNCaP v5-ARΔpro cells were transfected with siControl or siAR 3'UTR in combination with pcDNA3.1-GAL4 or pcDNA3.1-UBN1 followed by a 3-day treatment with vehicle or 100 pM R1881. The relative number of cells was quantified using a fluorescent DNA-binding dye. Each sample was performed in triplicate. Results are expressed as relative mean fold induction ± SE (n = 3). *, significant ($p<0.05$) changes from siControl.

3.3 Discussion

The results of the studies outlined in this report confirmed the importance of the AR polyproline domain in processes of pathological importance in PCa. To date, several approaches have been used to study the polyproline domain of AR, but have yielded conflicting results. For example, NR domains are often studied by exogenously transfecting various wild-type or mutant NR constructs into NR-negative cell lines (e.g. transfecting AR-negative PC-3 cells with AR). This approach thus makes the assumption that the newly created NR transcriptional complex will be identical to that which forms in its native environment. However, this assumption carries caveats in that the NR-negative cell types have clearly evolved to no longer depend on that NR. Hence, this could be a highly artificial approach. To study the AR polyproline domain, some have transfected cells with SH3-domain containing peptides (SH3 domains canonically interact with proline-rich regions) to block its function [210]. While this would indeed block AR polyproline signaling, it also inhibits the activity of other polyproline domain-containing cellular proteins, of which there are many. Other studies have been reported which have failed to observe a significant role of the AR polyproline domain in prostate cancer [203]. However, in most of these studies the AR mutants used to evaluate the activity of the polyproline domain have been overexpressed in prostate cancer cells and evaluated in the background of high levels of the wild-type receptor. Hence, unless the mutant AR had a strong dominant-negative phenotype or functioned as a significant hypermorph, its activity would be masked by the endogenous, wild-type AR. To circumvent this problem, we used an AR replacement strategy to demonstrate the importance of the polyproline domain within AR on prostate cancer cell proliferation, migration, and on the transcription of a subset of AR-mediated genes **(Figure 3.22)**.

In this study, we determined that SH3YL1 interacted with the AR-polyproline domain and functioned as a *bona fide* AR coregulator. While relatively little is known about SH3YL1, it, through its SH3 domain, interacts with the polyproline domains of several proteins [218,219]. Thus, it is likely that SH3YL1 has activities beyond its role as a mediator of AR action. However, in PCa cells, our data reveal a necessary role for SH3YL1 in AR-mediated growth and migration. Interestingly, SH3YL1 is expressed in several different prostate cancer cell lines and in some cell lines examined we have determined that it migrates in western immunoblots as a doublet, suggesting that its expression and activity may be regulated by splicing events and/or posttranslational modifications **(Figure 3.17A)**. Follow-up studies are currently underway to determine how SH3YL1 activity is regulated. We have noted that SH3YL1 is a highly conserved protein with homologs identified in fungi, plants, and vertebrates [220]. It has previously been shown to play important roles in meiosis, hair follicle formation, and dorsal ruffle formation [219-221]. Further, it has recently been shown that SH3YL1 also interacts with the proline-rich region of Dock4, promoting Rac1 activation and cell migration in the breast cancer cell line, MDA-MB-231 [218]. This could explain why SH3YL1 also affected the migration of AR-negative PC-3 cells. Little is currently known about the other binding partners of SH3YL1; however, we suspect that there are several that enable AR-independent phenotypes. Nonetheless, the impact of SH3YL1 knockdown on proliferation and migration in hormone-sensitive, castration-resistant and AR-negative prostate cancer cells further highlights the importance of this molecule in prostate cancer. Taken together with our data presented here, this may indicate that SH3YL1 could have an important role in multiple cancer types.

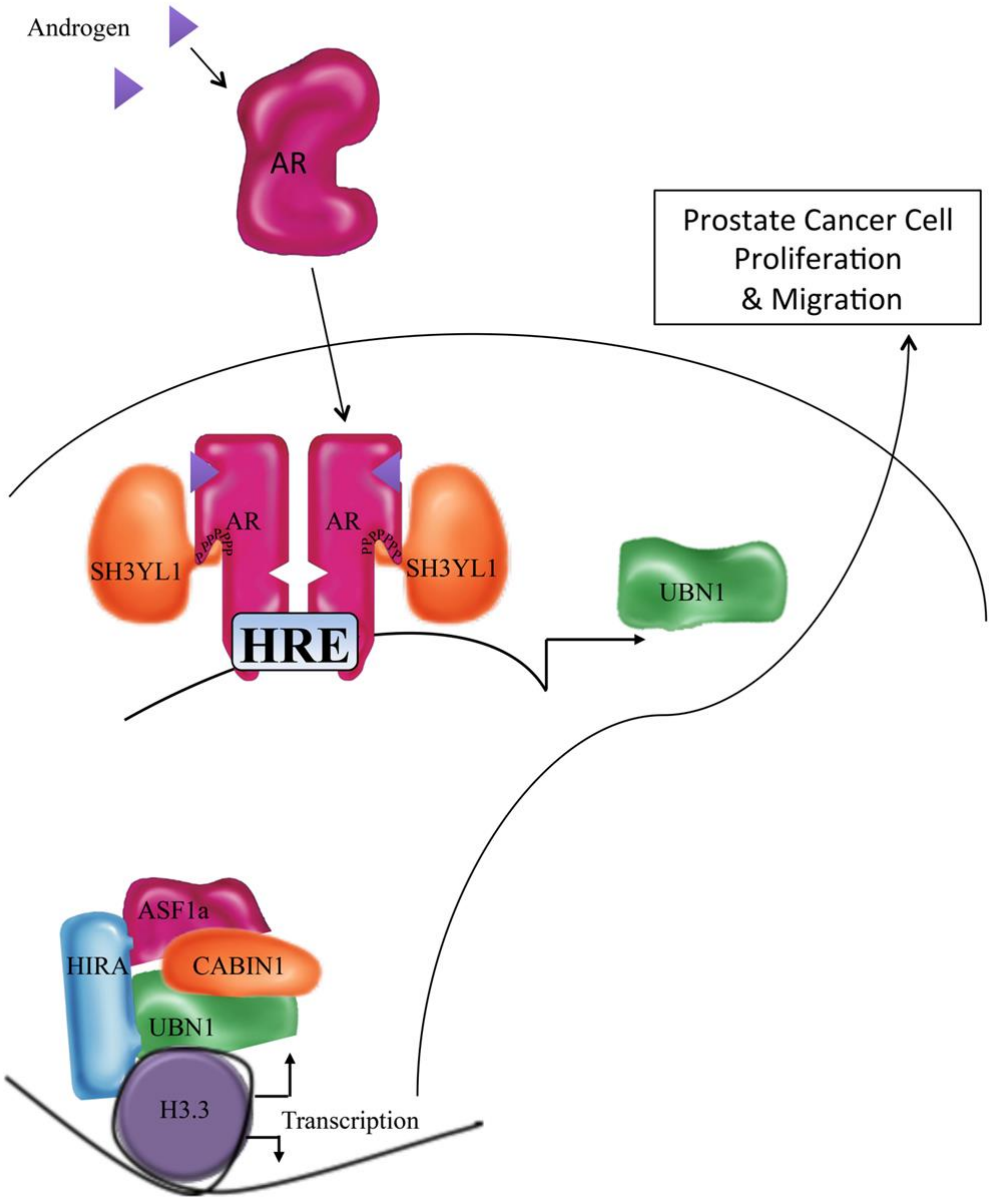

Figure 3.22 Working model of the mechanism of action of AR's polyproline domain in prostate cancer. Upon AR activation, the receptor undergoes a conformational change that allows the polyproline domain to interact with SH3YL1. Together in the nucleus, this complex promotes the transcription of a specific subset of AR-modulated genes, which includes *UBN1*. Changes in *UBN1* expression are then responsible for an increase in androgen-mediated prostate cancer cell proliferation and migration potentially through the modulation of a second set of chromatin alterations.

In this study, we also identified *UBN1* as a transcriptional target of AR/SH3YL1 in PCa cells and that its expression correlates with clinical outcome. UBN1 is a multifunctional protein involved in several important cellular processes and, not surprisingly, is widely expressed in different cell types and throughout development [222]. UBN1 has been found to compete for binding to AP-1 consensus sites by interacting with the basic domains of the transcription factors EB1 and c-Jun [222]. Furthermore, UBN1 forms a complex with HIRA, CABIN1, and ASF1a; a histone H3.3 chaperone complex that is localized to active promoters as well as active and weak/poised enhancers **(Figure 3.22)** [216,217,223]. The localization of this so-called "HUCA" complex at promoters correlates with gene expression [216]. Hence, our work here may have uncovered the AR-mediated regulation of a new transcriptional network via control of UBN1. However, it should be noted that UBN1 may also manifest its regulatory activities in a non-genomic manner. To that end, UBN1 has been described as a component of the nuclear and adherent junction complex (NACo) protein family that interacts with the tight junction protein ZO-1, suggesting that it could also function through transcription-independent mechanisms to facilitate its role in androgen-mediated migration [224,225]. Certainly, future studies focused on the UBN1-mediated transcriptional and non-transcriptional processes, in the context of AR signaling in PCa, are warranted.

Because of our interest in transcriptional regulation, we focused on defining the role of AR/SH3YL1 on the regulation of *UBN1* expression. However, as shown in **Figure 3.13** and **Table 3.1**, this specific AR complex appeared to regulate other genes that could also contribute towards the observed phenotypes. For instance, another AR/SH3YL1-regulated gene was the *prolactin receptor*. Prolactin has been shown to exhibit mitogenic activities in the prostate [226,227]. Hence, it is likely that the ultimate impact of the AR/SH3YL1 complex on prostate cancer biology is an amalgam of multiple signaling events.

Recently, the ability to selectively decrease androgen signaling in the prostate while maintaining it throughout the rest of the male body, particularly in the bone and muscle, has led to the pursuit of SARMs as potential pharmaceuticals for the treatment of prostate cancer, cachexia, sarcopenia, and other muscle wasting syndromes [198,228,229]. However, to date, no SARMs have attained FDA-approval. The data presented here could help guide the next generation of SARMs. For example, SARMs that lead to the recruitment of SH3YL1 would not be desirable since they would then promote prostate cancer cell growth and migration. As such it would be prudent to screen against AR ligands that facilitate SH3YL1 recruitment. However, whether this interaction can be uncoupled from the desired anabolic effects in the muscle and bone remains to be seen.

In PCa, the continued importance of AR in the advanced stages of the disease is reflected by the recent FDA approvals of several new drugs (ex. enzalutamide and abiraterone acetate) targeting AR's LBD activity. While patients treated with these promising new drugs live slightly longer, they too eventually succumb to disease relapse and eventual mortality. These extremely resistant disease states are again largely due to residual AR activity. One emerging mechanism of continued resistance is altered AR mRNA splicing [100]. This aberrant splicing leads to the generation of constitutively active AR variants that lack the C-terminal LBD and hence, are completely insensitive to all existing AR-targeted drugs. As such, there is a major need to identify which regions of the truncated receptor facilitate pathological processes and determine whether these regions represent new therapeutic targets. We think our findings here could have significant therapeutic implications given the increased interest in AR's N-terminus, an area we are actively pursuing.

In summary, our data support the concept that AR conformation, coregulator recruitment and biology are intimately linked. Through this work, we have a better understanding of

specifically how the AR polyproline domain affects androgen-mediated PCa cell proliferation, migration and transcription. This study adds fundamental new knowledge to the field of AR-coregulator biology by focusing on a specific domain and elucidating its role. Importantly, this work could also aid in the rational development of improved SARMs and highlights potential new targets for the treatment of PCa.

Chapter IV:

Androgen Receptor Mediated Regulation of Autophagy in Prostate Cancer

4.1 Introduction

As a member of the nuclear receptor (NR) family of hormone-regulated transcription factors (TF), the androgen receptor (AR) plays a crucial role in both the development and maintenance of the prostate gland and is an overall regulator of male secondary sexual traits and reproductive function[196]. Beyond these functions, AR has also been implicated in with the development and progression of prostate cancer (PCa)[100]. Upon binding its preferred ligand, an androgen known as dihydrotestosterone (DHT), AR translocates to the nucleus where it dimerizes, interacts with regulatory regions of the DNA, and recruits coregulators and transcriptional machinery[36]. These transcriptional targets of AR potentiate the progression of PCa by altering several cellular processes that ultimately allow the cell to rapidly proliferate, migrate, and withstand adverse conditions.

If PCa is diagnosed early, it can often be treated successfully with surgery and/or radiation alone [18]. However, a significant number of patients progress to the advanced stages of PCa and PCa remains the second leading cause of cancer-related deaths in American men[9]. AR is a primary driver of PCa growth and metastasis, and as such, patients with advanced disease are generally treated with systemic hormone therapy to prevent the spread of the disease [200]. However, despite continuous androgen ablation therapy, which is the standard of care for

advanced PCa, most tumor cells develop resistance to this therapy and is henceforth known as castration resistant PCa (CRPC). Interestingly, relapse of the disease is often associated with increased AR signaling and upregulation of specific subsets of AR target genes[18]. Currently, after developing CRPC, the treatment options are very limited.

Macroautophagy (referred to as autophagy henceforth) is a process in which cytoplasmic components are encircled by a growing double membrane known as a phagophore, which upon maturation (autophagosome), fuses with a lysosome (autolysosome) that allows for the reallocation of the macromolecules produced by the degradation of the said cytoplasmic components[111,112]. Autophagy has long been implicated in cancer. It is generally believed and well accepted that autophagy can suppress tumor initiation. On the other hand, there exists a large body of literature suggesting that autophagy is essential in later stages of cancer, enabling the cancer cells to survive harsh conditions such as low oxygen or nutrient supply or chemotherapy.

We have previously shown that autophagy increases with androgen treatment in PCa in part by increasing reactive oxygen species (ROS)[184]. Moreover, molecular or pharmacological inhibitors of autophagy significantly reduce androgen-mediated PCa cell growth. However, this androgen-mediated increase in ROS could only account for part of the increased autophagy we observed in response to androgens in PCa cells. We therefore hypothesized that AR could be transcriptionally regulating core autophagy components that were responsible for the upregulation of autophagy in PCa cell in response to androgens.

4.2 Results

Androgens Increase phosphorylation of ULK1 at Serine 555

We have previously shown that CAMKK2 is a direct transcriptional target of AR and that this kinase is primarily responsible for the phosphorylation of AMPK within the context of prostate cancer[132]. AMPK is a master regulator of cellular metabolism and a critical regulator of the

autophagy pre-initiation complex[129]. We hypothesized that one mechanism in which androgens/AR might be increasing cellular autophagy in PCa cells is through the CAMKK2 -> p-AMPK -> p-ULK1 signaling node. To test this hypodissertation, we used an inhibitor of CAMKK2, STO-609, in the androgen-sensitive PCa cell line, LNCaP. Concurrent with what we have previously published, treatment with androgens increases p-AMPK levels, a phenomenon that is abrogated with STO-609 **(Figure 4.1)**. ULK1, a member of the autophagy pre-initiation complex and activated by phosphorylation at Serine 555 by AMPK, displays increased phosphorylated in response to androgens. This increase in p-ULK1 at SER555 occurs simultaneously with an

increase in the functional marker of autophagy, LC3B-II. However, androgens fail to induce phosphorylation of ULK1 at SER555 and therefore, do not induce autophagy as evidenced by LC3B-I to LC3B-II conversion, when cells are co-treated with the CAMKK2 inhibitor.

To further explore the role that this signaling cascade might have in advanced PCa, we used cells engineered to express an inducible shRNA directed against CAMKK2 in the castration resistant prostate cancer (CRPC) cell model, 22Rv1 **(Figure 4.2A)**. These cells express constitutively active splice variants of AR that retain the N-terminus and the DNA binding domain but lack the ligand binding domain. Importantly, in these cells, knockdown of CAMKK2 also resulted in decreased phosphorylation of AMPK and ULK1 at SER55 and decreased LC3BII conversion **(Figure 4.2B)**. This indicates that androgens promote autophagy in prostate cancer by increasing CAMKK2 which phosphorylates AMPK; AMPK in turn phosphorylates ULK1 at SER555, ultimately leading to increases in autophagy. Moreover, subcutaneous xenografts with this inducible stable cell line revealed that knockdown of CAMKK2 results in decreased tumor burden over time and increased survival (**Figure 4.3A-D; Figure 4.4**).

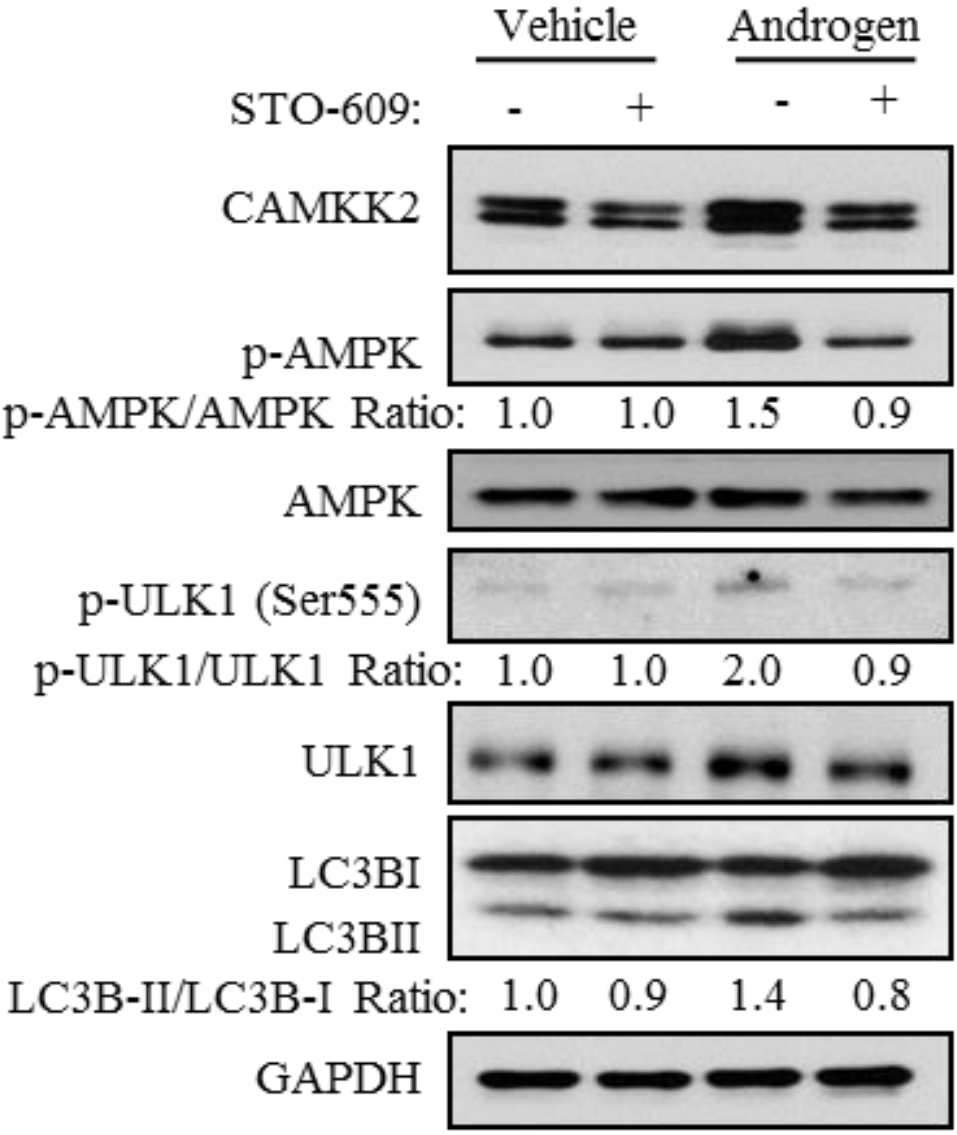

Figure 4.1 Pharmacological inhibition of CAMKK2 activity reduces androgen-mediated autophagy. Chemical inhibitor of CAMKK2, STO-609, was co-treated with vehicle or 10nM R1881 for 72 hours. Cell lysates were subjected to western blot.

Androgens regulate transcription of several core autophagy genes

Since AR primarily functions as a transcription factor, we hypothesized that AR could be controlling transcription of several of the core autophagy machinery components thereby further, increasing functional autophagy. Androgen treatment in two different androgen-sensitive PCa cell lines resulted in increased mRNA (**Figure 4.5**) and protein (**Figure 4.6**) expression of four core autophagy genes: *ULK1, ULK2, ATG4B*, and *ATG4D*. Increases in *ULK1, ULK2, ATG4B, and ATG4D* expression four hours post-androgen treatment or in the presence or absence of cyclohexamide suggest that all four genes are direct target of AR (**Figure 4.7A and B**). In support of this, the results of AR ChIP-Seq experiments in several prostate cancer cell lines indicate that AR binds to intronic regions of *ULK1 and ULK2 (***Figure 4.7C**). Nuclear receptors such as AR have been frequently shown to regulate transcriptional activity of genes far away from transcriptional start site; therefore, it is not surprising, that AR was not found to bind to the intronic regions of *ATG4B or ATG4D.*

Androgen-induced core autophagy genes are necessary for maximal androgen-mediated autophagy

To assess whether the increases in these androgen-mediated genes functionally affected autophagy, we took advantage of a well-described marker of autophagy and quantified the conversion of LC3B-I to LC3B-II[157]. Using an SDS-PAGE gel to separate LC3B-I and LC3B-II, when treated with androgens, as expected, there are substantial increases in LC3B-II conversion (**Figure 4.8**). Two different siRNAs were used to target each of the four genes: *ATG4B, ATG4D, ULK1,* and *ULK2.* In each case, knockdown of these four genes resulted in decreased LC3B-II conversion. Interestingly, we did not observe any compensation by in ATG4B protein levels when we knocked down ATG4D nor vice versa. This lack of compensation was also missing between ULK1 and ULK2.

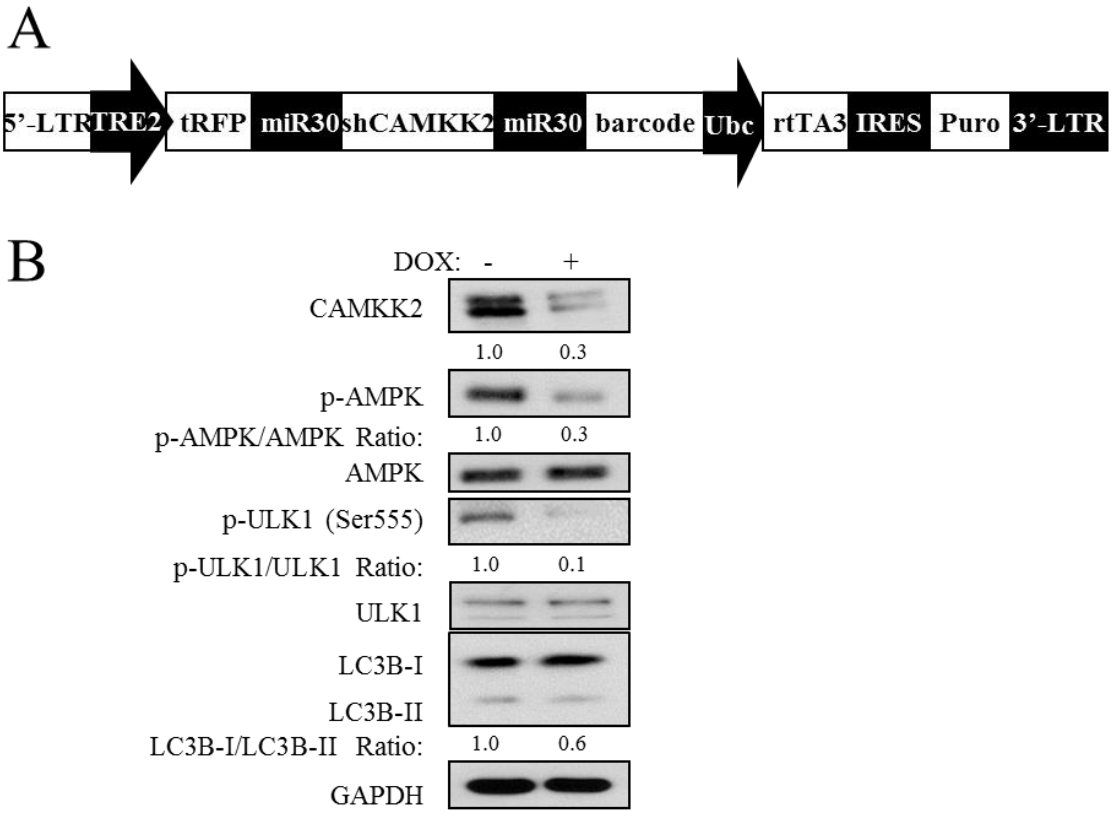

Figure 4.2 Molecular knockdown of CAMKK2 in Castration Resistant Prostate Cancer (CRPC)reduces phosphorylation of ULK1. A, Ischematic of the pINDUCER10 construct used. B, 22Rv1 cells were lentivirally transduced with the pINDUCER10 construct containing a constitutively expressed puromycin (Puro) resistant gene and an inducible shRNA against CAMKK2. Cells were selected with puromycin for 4 weeks. Inducible expression was confirmed with western blot.

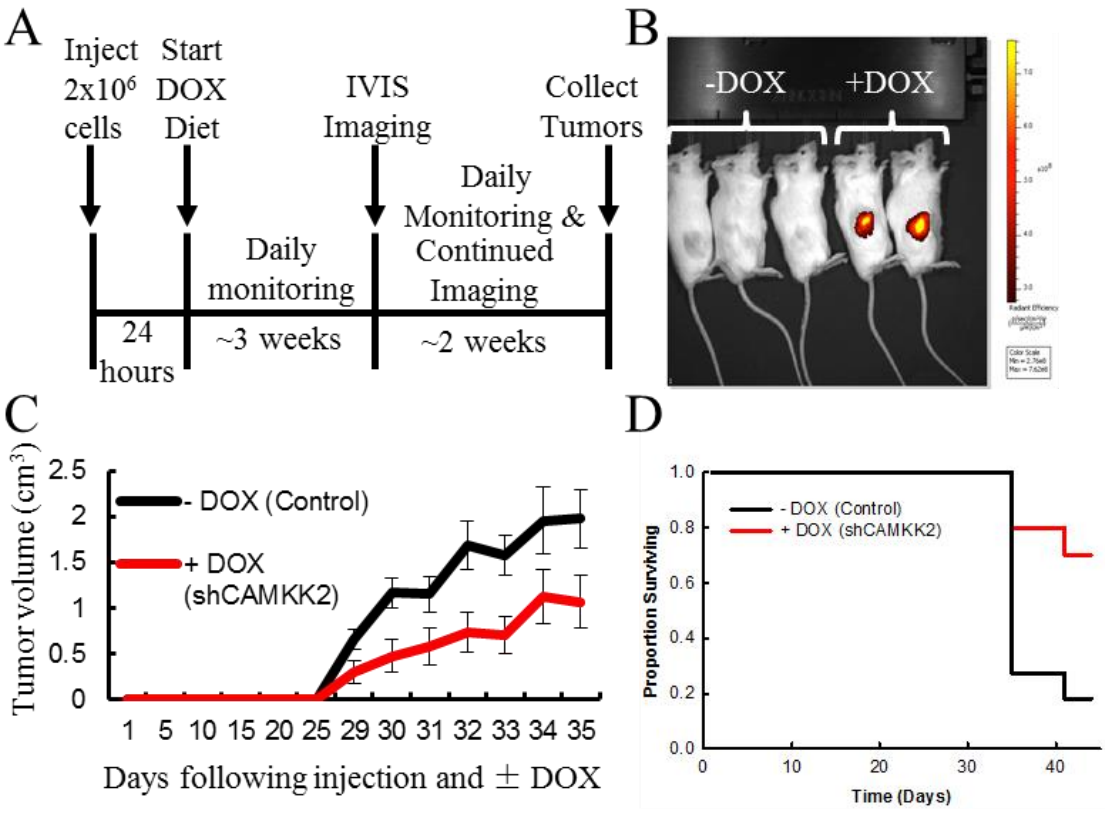

Figure 4.3 CAMKK2, a direct target of AR and upstream initiator of autophagy is required for Castration Resistant Prostate Cancer (CRPC) growth *in vivo*. A, schematic of CRPC xenograft study. Here, castrated male NSG mice (6 weeks old) were subcutaneously injected with 2 x 10^6 22Rv1-shCaMKK2 cells and half of the mice were switched to 625 mg/kg DOX-containing chow to induce expression of shCAMKK2 and the tRFP surrogate marker. B, fluorescence imaging of 5 sample mice (3 on normal chow, 2 on DOX-containing chow) confirming the DOX diet increases expression *in vivo*. C, tumors were allowed to propagate for 8 weeks and were measured daily . *, $P < 0.05$. D,Kaplan-Meier survival curve comparing treatment groups.

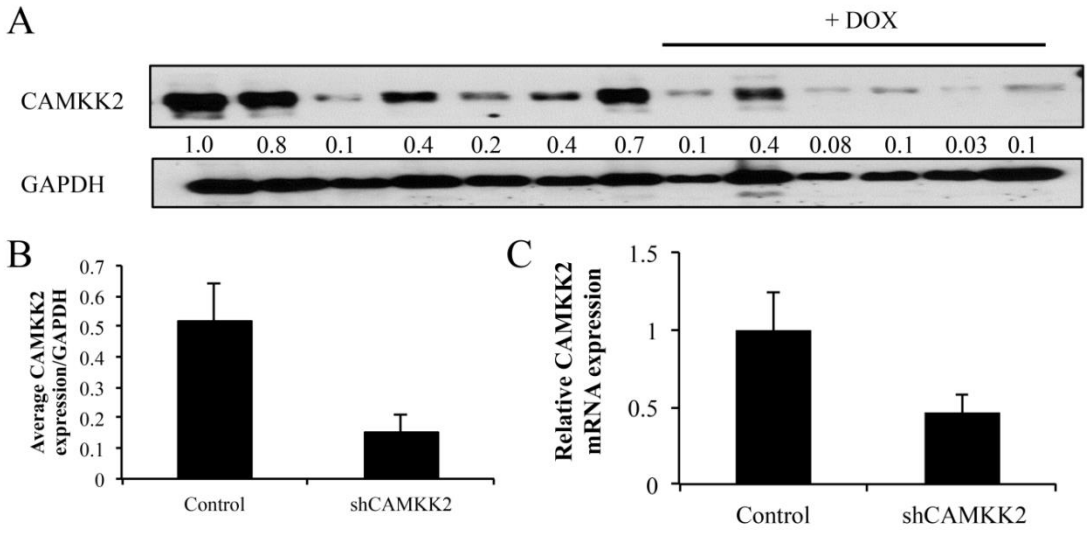

Figure 4.4 Confirmation that CAMKK2, a direct target of AR and upstream initiator of autophagy is required for Castration Resistant Prostate Cancer (CRPC) growth *in vivo*. A, Lysates from NSG mice (6 weeks old) that subcutaneously injected with 2 x 10^6 22Rv1-shCaMKK2 cells. Confirmation that 625 mg/kg DOX-containing chow induced expression of shCAMKK2. Quantification of band intensity normalized to GAPDH level is written beneath CAMKK2 blot. B, Average expression of CAMKK2 expression normalized to GAPDH expression for each group. C, Average mRNA expression of CAMKK2 for each group.

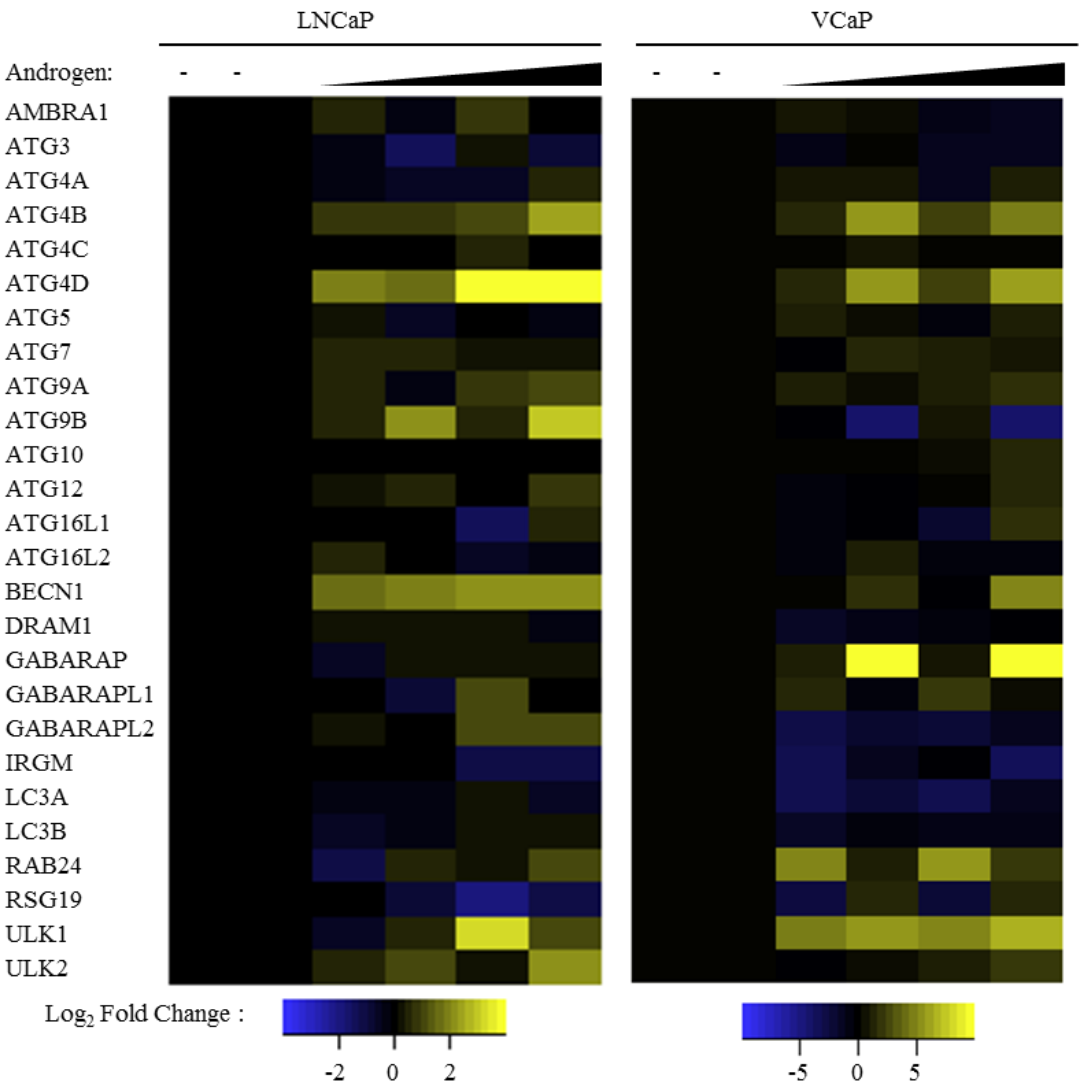

Figure 4.5 AR-mediated signature of core autophagy genes. Prostate cancer cell models were treated for 24 hours with vehicle (EtOH) or Androgen (100 pM or 10 nM R1881). RNA extracts were then subjected to qPCR-based arrays to assess changes in the expression of 26 core autophagy genes.

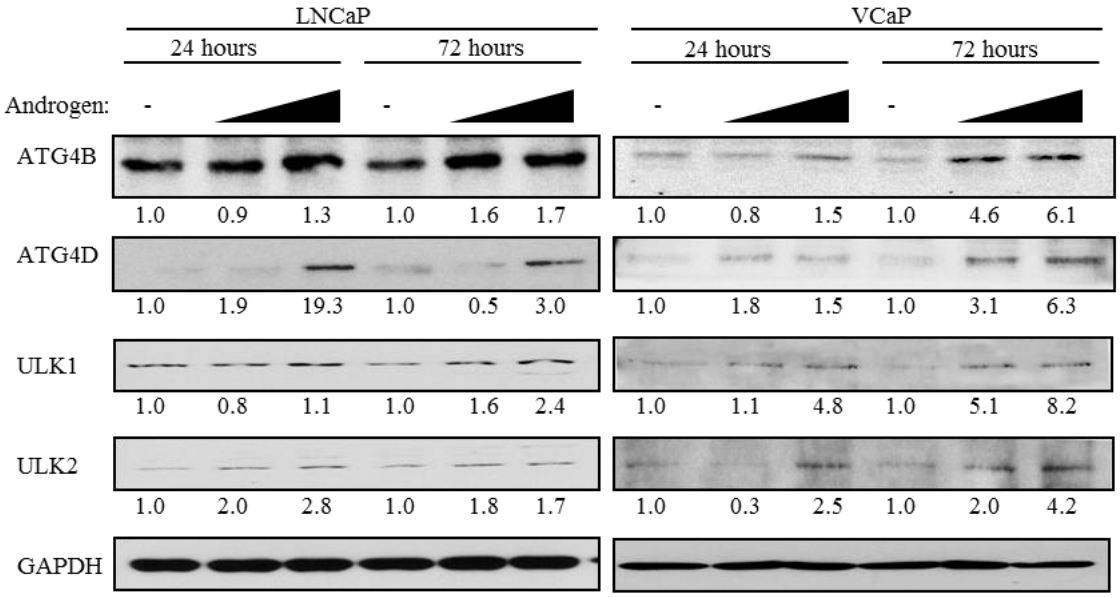

Figure 4.6 Four core autophagy genes increase in response to androgens. Prostate cancer cell models were treated for 24 or 72 hours with vehicle (EtOH) or Androgen (100 pM or 10 nM R1881). Protein lysates were then probed for increases in the core autophagy genes ATG4B, ATG4D, ULK1, and ULK2. Quantification of the increase of protein expression normalized to GPADH loading control is written below each blot.

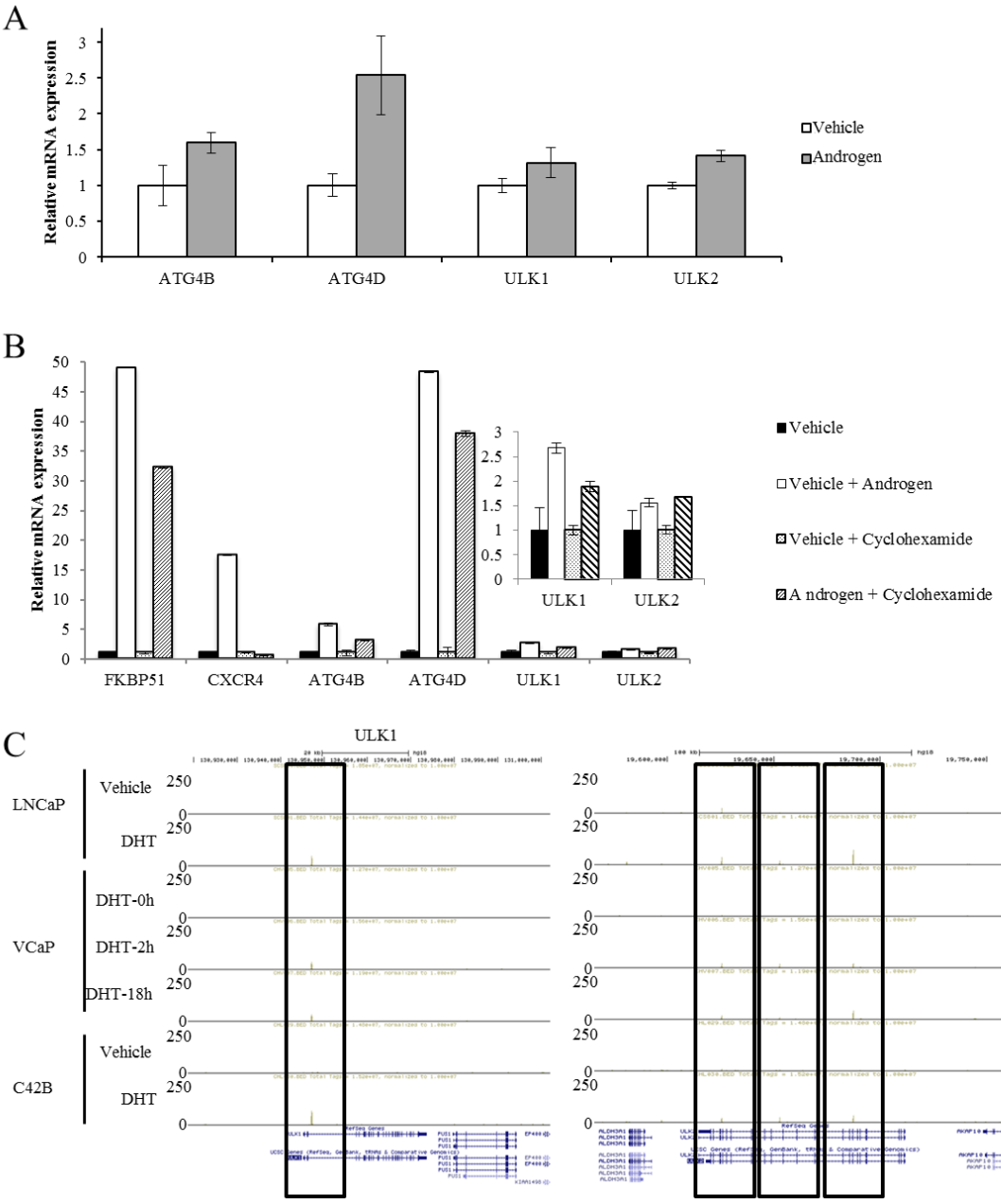

Figure 4.7 *ATG4B, ATG4D, ULK1,* and *ULK2* are direct transcriptional targets of AR. A, LNCaP cells were treated with Vehicle (Ethanol) or Androgen (100 pM R1881) for 3 hours before RNA was collected and subjected to qPCR. B, LNCaP cells were treated with Vehicle (Ethanol) or Androgens (100pM R1881) in the presence or absence of 1ug/uL cyclohexamide. *FKBP51* is a direct transcriptional target of AR, *CXCR4* is an indirect transcriptional target of AR. C, ChIP-Seq tracks of LNCaP, VCaP, and C4-2B cells treated with vehicle or DHT for 0, 2, or 18 hours. Potential AR binding sites in the intronic region of *ULK1* and *ULK2* are highlighted.

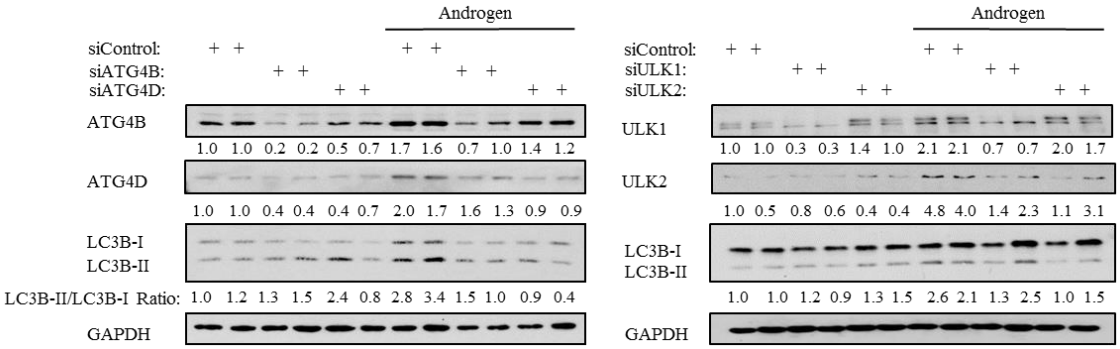

Figure 4.8 *ATG4B, ATG4D, ULK1,* and *ULK2* are necessary for maximal androgen-mediated autophagy. LNCaP cells were transfected with siRNAs then treated with Vehicle (Ethanol) or Androgen (10nM R1881) for 72 hours before cells were lysed and probed for protein expression. Quantification of band density normalized to GAPDH expression is written below each lane.

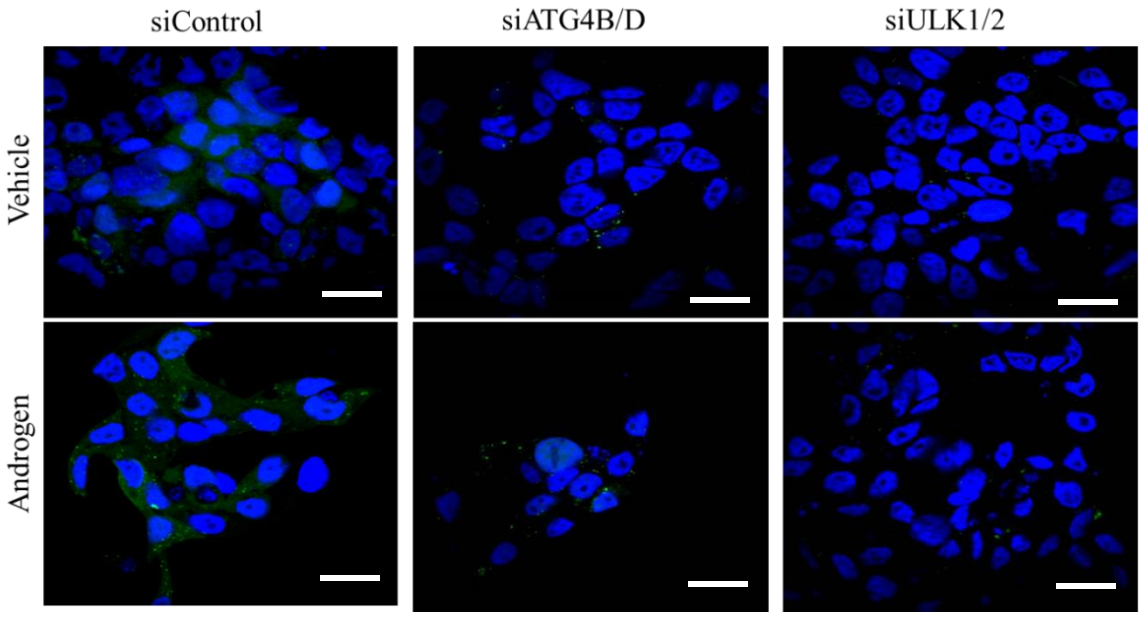

Figure 4.9 *ATG4B/D and ULK1/2* are necessary for maximal androgen-mediated GFP-LC3B-II. LNCaP cells were transfected with siRNAs then treated with Vehicle (Ethanol) or Androgen (10nM R1881) for 72 hours before cells were fixed and stained with DAPI. Experiment was performed in triplicate and 10 random images from each treatment group were taken. One representative image of each is shown above.

When expressed within the cell, the GFP-LC3B plasmid in the pre-lipidated, LC3B-I form, will appear throughout the cell as a diffuse GFP signal. However, once the GFP-LC3B has had the phophatidylethanolamine (PE) moiety attached, it will locate to the growing phagophore and be visible as GFP punctate. Using this construct in the hormone sensitive LNCaP cells, we observed an increase in the number of GFP-positive punctate per cell when treated with androgens in comparison to control, similar to what we have previously reported (**Figure 4.9**). siRNA mediated knockdown of *ATG4B* and *ATG4D* or *ULK1* and *ULK2* resulted in a significantly reduced number of GFP-positive punctate per cell. Taken together this data indicates that ATG4B, ATG4D, ULK1, and ULK2 are necessary for maximal androgen-mediated increases in autophagy.

Androgen-induced core autophagy genes clinically correlate with AR activity and poor patient prognosis

A previously published curated AR gene signature identified AR target genes that increased in response to androgens and was modulated by AR antagonists[230]. This gene signature positively correlated with increased mRNA transcript levels of *ATG4B, ATG4D, ULK1,* and *ULK2* in publicly available PCa cohort[231](**Figure 4.10A**). Moreover, these four genes correlated with poor patient prognosis using the clinical data from *The Cancer Genome Atlas (TCGA)*(**Figure 4.10B**).

The Androgen Receptor increases TFEB expression and potentiates its activity

Thus far, we have identified that four core autophagy genes (*ULK1, ULK2, ATG4B,* and *ATG4D*) are transcriptional targets of AR and that one of those targets, ULK1, is further modified post-translationally via an AR signaling cascade; all of which results in increased induction of autophagy. However, we know that treatment with androgens not only increases the induction of autophagy but also increases the flux through autophagy[184]. Recently, AR has been shown to exogenously interact with transcription factor E-box (TFEB), a master regulator of lysosomal

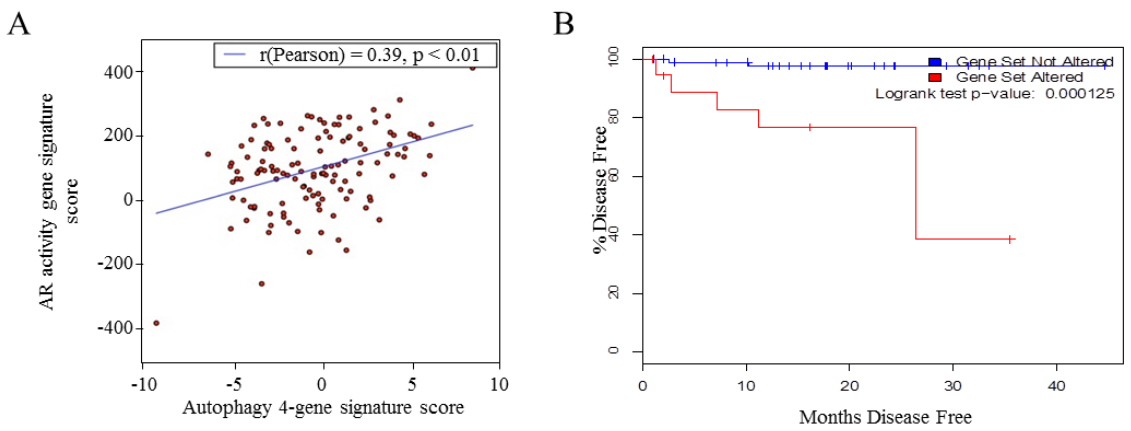

Figure 4.10 AR-mediated signature of core autophagy genes predicts disease-free survival. A, A gene signature of the four autophagy genes (*ATG4B*, *ATG4D*, *ULK1*, *ULK2*) correlated significantly with a previously described AR gene signature (*Hieronymus et al 2006*) in prostate cancer patients from the *Taylor et al 2010* clinical cohort. Similar results were obtained using additional AR activity signatures across multiple clinical cohorts. B, The AR-regulated autophagy gene signature of *ATG4B*, *ATG4D*, *ULK1* and *ULK2* was subjected to Kaplan-Meier analysis using clinical data from *The Cancer Genome Atlas (TCGA)*.

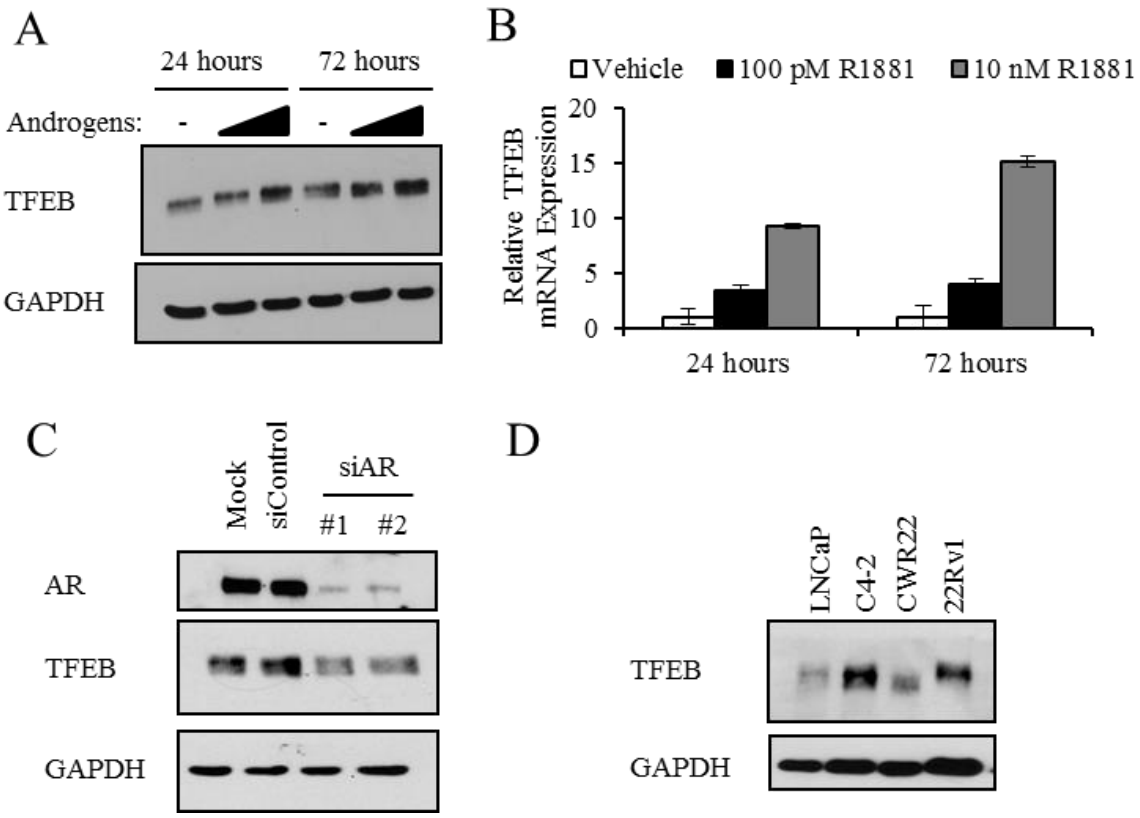

Figure 4.11 TFEB expression is regulated by AR. A, LNCaP cells treated with Vehicle (Ethanol) or Androgens (100pM or 10nM R1881) for 24 or 72 hours were subjected to western blot or B, qPCR. C, C4-2, a castration resistant prostate cancer (CRPC) cell line derivative of LNCaPs, were transfected with siRNAs targeting AR or controls, lysed and probed for TFEB protein expression. D, Hormone-sensitive cell lines LNCaP and CWR22, and their CRPC derivatives, C4-2 and 22Rv1, were probed for basal TFEB expression.

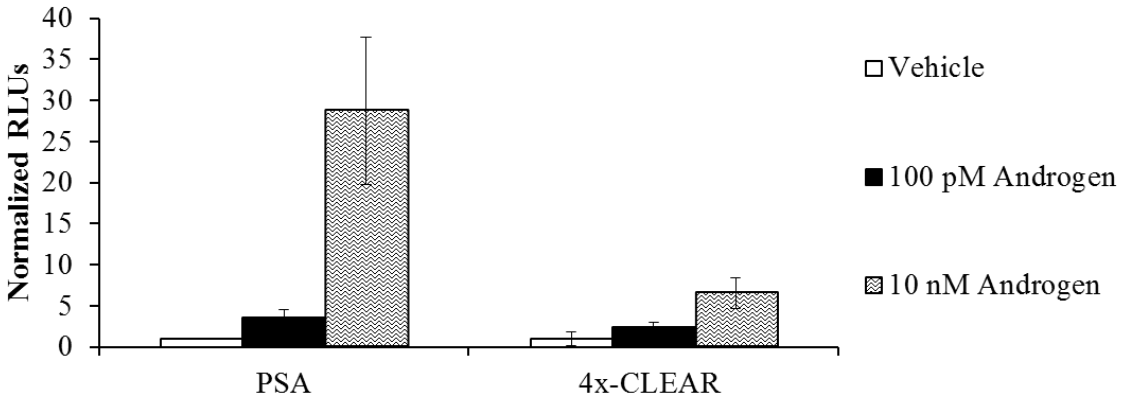

Figure 4.12 Androgens increase TFEB transcriptional activity. LNCaP cells were transfected with a PSA-luciferase reporter plasmid or a 4x-CLEAR (TFEB DNA binding sequence)-luciferase reporter plasmid. After transfection, cells were treated vehicle or increasing concentrations of R1881 for 24 hours. Cells were harvested and assayed for luciferase activity; all luciferase values were normalized to β-Gal controls. Data are expressed as ± SE.

function and biogenesis[232]. Although we were not able to repeat this endogenous interaction in PCa cell models, we did observe an increase in TFEB mRNA and protein expression in the hormone sensitive LNCaP cells in response to androgens (**Figure 4.11A and B**). Furthermore, siRNA mediated knockdown of AR in the CRPC cell model, C4-2 significantly reduced TFEB protein levels (**Figure 4.11C**). Interestingly, hormone sensitive cell lines, LNCaP and CWR22, expressed lower levels of TFEB than their CRPC derivatives, C4-2 and 22Rv1 (**Figure 4.11D**). To further support these findings, ChIP-Seq analysis experiments in multiple PCa cell lines indicate that AR binds to the intronic regions proximal regions of *TFEB* indicating that *TFEB* is a direct transcriptional target of AR (**Figure 4.12**).

We next assessed the functional activity of this androgen-mediated increase in TFEB expression in PCa cells. To do so, we first took advantage of a luciferase reporter plasmid that harbored a TFEB binding site (4x coordinated lysosomal expression and regulation sequence) upstream. Androgens were able to increase the luciferase activity in the cells transfected with a 4X-CLEAR reporter in a dose-dependent manner (**Figure 4.13**). This is similar to the dose dependent manner in which androgens increase the luciferase activity of a well characterized target of AR, PSA[233]. The transcriptional targets of TFEB have been well curated.[165] We therefore checked if androgen treatment could increase the mRNA expression of several of the TFEB target genes. All six TFEB target genes (*ATP6AP1*, *LAMP1a, PGC1α, GLBN1*, *MCOLN1*, and *SQSTM1*) that were checked were transcriptionally increased in response to androgens (**Figure 4.14**). Taken together, our data indicates that *TFEB* is transcriptionally regulated by AR in PCa and this transcriptional increase in response to androgens results in increased TFEB transcriptional activity.

function and biogenesis[232]. Although we were not able to repeat this endogenous interaction in PCa cell models, we did observe an increase in TFEB mRNA and protein expression in the

hormone sensitive LNCaP cells in response to androgens (**Figure 4.11A and B**). Furthermore, siRNA mediated knockdown of AR in the CRPC cell model, C4-2 significantly reduced TFEB protein levels (**Figure 4.11C**). Interestingly, hormone sensitive cell lines, LNCaP and CWR22, expressed lower levels of TFEB than their CRPC derivatives, C4-2 and 22Rv1 (**Figure 4.11D**). To further support these findings, ChIP-Seq analysis experiments in multiple PCa cell lines indicate that AR

TFEB is necessary for androgen-mediated flux through autophagy in PCa

In addition to transcriptionally regulating lysosomal biogenesis and function, TFEB has also been shown to regulate autophagy in general. Knockdown of TFEB using two different siRNAs resulted in decreased androgen-mediated LC3B-II accumulation in the hormone-sensitive cell line LNCaP and decreased basal LC3B-II accumulation in the CRPC cell line, C4-2 (**Figure 4.15**).

To study flux through autophagy, a mCherry-GFP-LC3B plasmid was used. When this LC3B-II is associated with the phagophore or autophagosome, both the mCherry and GFP will be expressed resulting in a merged yellow signal. Once fused with the lysosome, the GFP signal will be lost in the presence of the acidic hydroxylases, resulting in only a visible mCherry-LC3B-II. Similar to what we have previously reported, addition of androgens to cells expressing this plasmid resulted in increased LC3B-II overall expression as well as mCherry-LC3B-II expression (**Figure 4.16**). siRNA mediated knockdown of TFEB resulted in decreased total LC3B-II levels as well as mCherry-LC3B-II levels. This indicated that the AR transcriptional target, TFEB is responsible for the androgen-mediated flux through autophagy.

Since TFEB is the master regulator of lysosomal biogenesis and the lysosome is the necessary final step for the completion of autophagy, we hypothesized that TFEB might be regulating flux through autophagy by modulating the volume of lysosomes per cell. Again, we knocked down TFEB using siRNAs in the presence or absence of androgens and assessed the

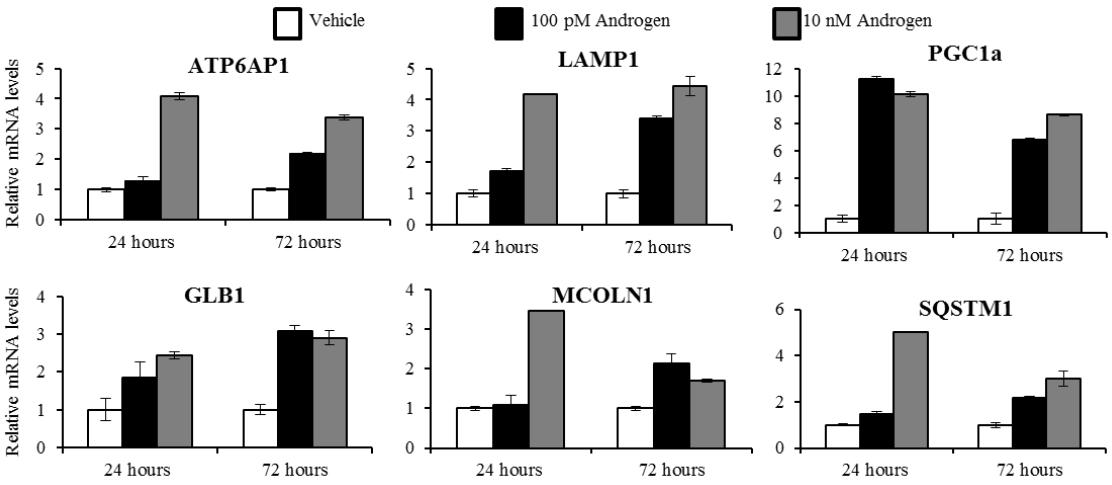

Figure 4.13 Androgens increase mRNA expression of TFEB-target genes. q-PCR of six already characterized transcriptional targets of TFEB: *ATP6AP1*, *LAMP1*, *PGC1α*, *GLB1*, *MCOLN1*, and *SQSTM1* increase in response to ± androgen treatment for 24 or 72 hours in VCaP cells.

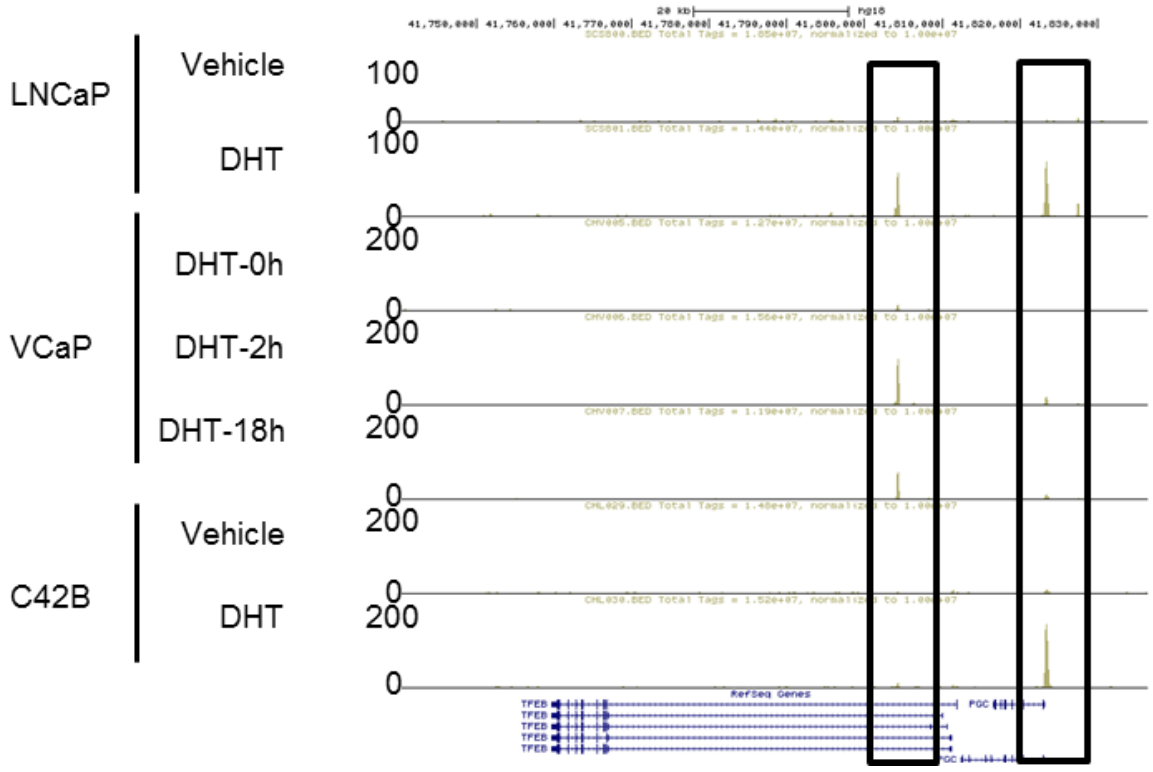

Figure 4.14 ***TFEB*** **is a direct transcriptional target of AR.** ChIP-Seq tracks of LNCaP, VCaP, and C4-2B cells treated with vehicle or DHT for 0, 2, or 18 hours. Potential AR binding sites in the intronic region and direct uprstream region of *TFEB* are highlighted.

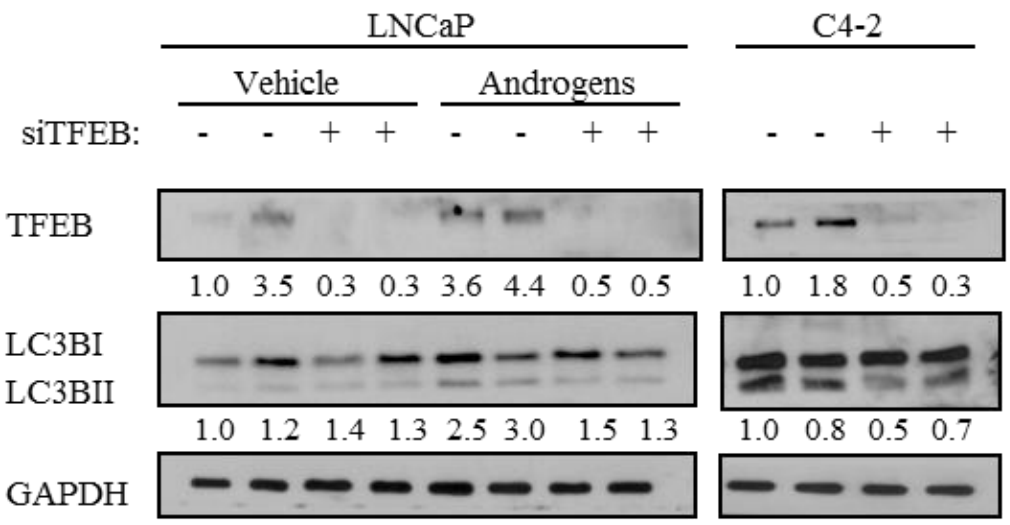

Figure 4.15 TFEB is necessary for maximal androgen-mediated autophagy. LNCaP or C4-2 cells were transfected with siRNAs then treated with Vehicle (Ethanol) or Androgen (10nM R1881) for 72 hours before cells were lysed and probed for protein expression.

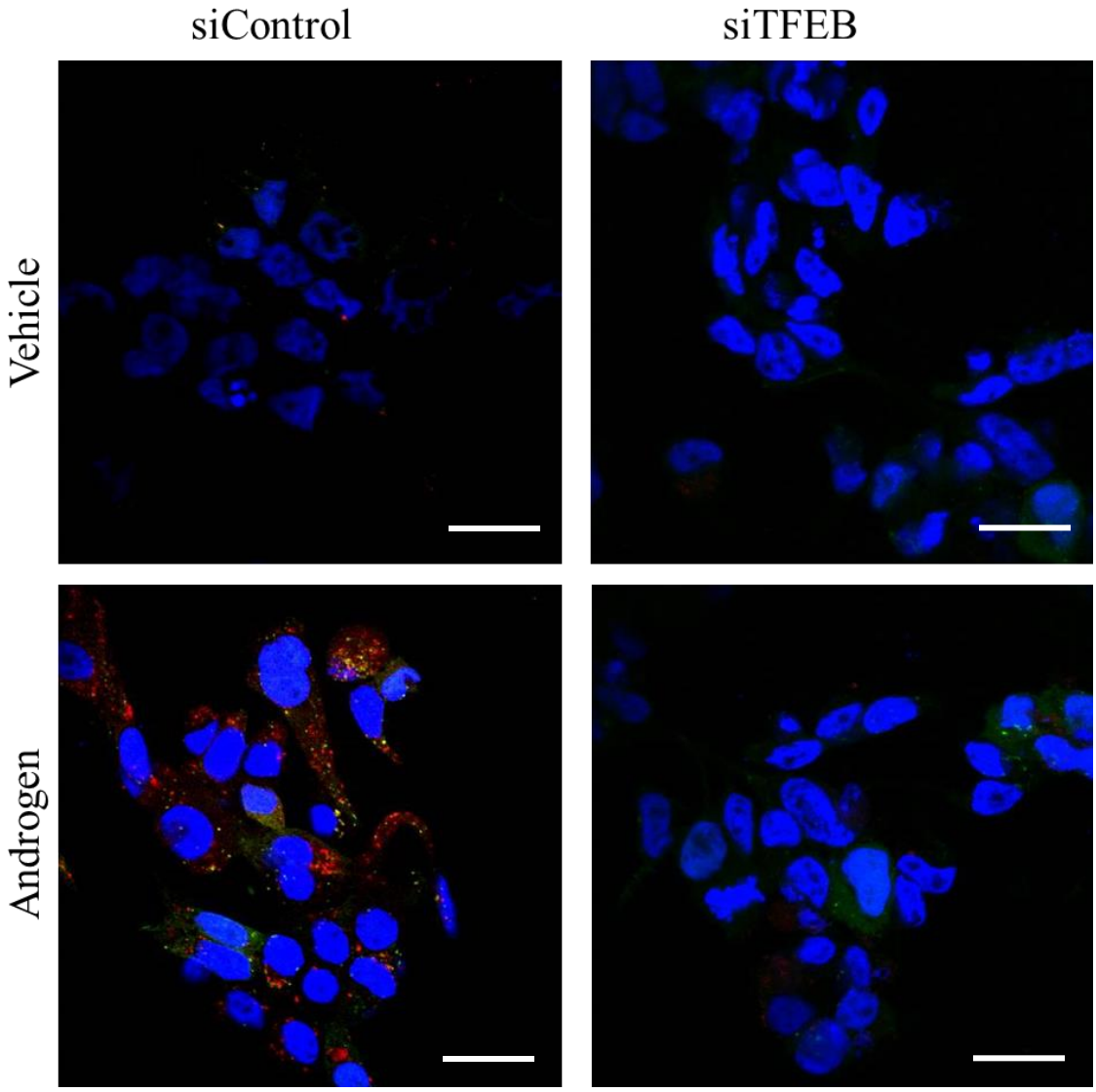

Figure 4.16 TFEB is necessary for maximal androgen-mediated flux through autophagy. LNCaP stably expressing an mCherry-GFP-LC3B construct were transfected with siRNAs then treated with Vehicle (Ethanol) or Androgen (10nM R1881) for 72 hours before cells were fixed, nuclei stained with DAPI (BLUE) and imaged.

total volume of lysosomes stained with LysoTracker per the total volume of nuclei (DAPI). As expected, androgens dramatically increased the total volume of lysosomes (**Figure 4.17**). Additionally, we observed a robust decrease in both basal and androgen-mediated lysosomal volume in the absence of TFEB indicating that the androgen-mediated increase in lysosomal volume is mediated through TFEB.

An AR antagonist, Enzalutamide, is able to block the induction of autophagy in hormone- sensitive and castration resistant prostate cancer cell models

Enzalutamide is the most recently FDA approved AR antagonist. We wanted to test whether this increase in androgen-mediated autophagy could be blocked in two cell models of prostate cancer with enzalutamide. Enzalutamide decreases LC3B-II conversion in the hormone-sensitive LNCaP cell line in the presence or absence of Chloroquine (**Figure 4.18A and B)**. Chloroquine raises the lysosomal pH, thereby blocking flux through autophagy. Moreover, in the CRPC derivate of LNCaP, C4-2, Enzalutamide in addition to Chloroquine blocked the dose dependent increase in LC3B-II conversion observed in the androgen and Chloroquine treated samples (**Figure 4.18C**). This indicates that Enzalutamide is capable of decreasing autophagy *in vitro.*

Androgen-induced core autophagy genes and TFEB clinically correlate with AR activity and metastasis

The same curated AR gene signature used above was positively correlated with increased mRNA transcript levels of *ATG4B, ATG4D, ULK1, ULK2,* and *TFEB* in publicly available primary PCa and metastatic PCa cohorts[231] (**Table 4.1**). As expected, two different curated AR gene signatures are significantly increased and correlated with progression from normal prostate to primary PCa in three different clinical cohorts[230,231,234-236] (Figure **4.19A-B, D-E, and G-H**). However, despite the fact that AR remains crucial in the transition from primary PCa to

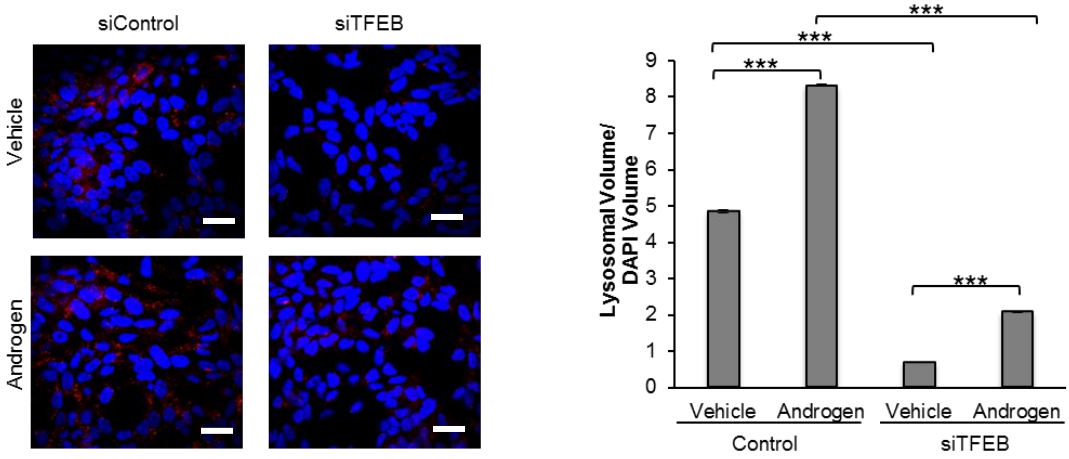

Figure 4.17 Androgens regulate Lysosomal Content via TFEB. LNCaP cells were transfected with siRNAs targeting TFEB or a control sequence. Twenty-four hours post-transfection, cells were treated ± androgen and cultured for 72 hours. LysoTracker was added to the culture for 30 minutes before fixation. Total lysosomal volume (red) per image was taken as a fraction of nuclear volume (DAPI= blue) using an Image J script written specifically for these images(left).

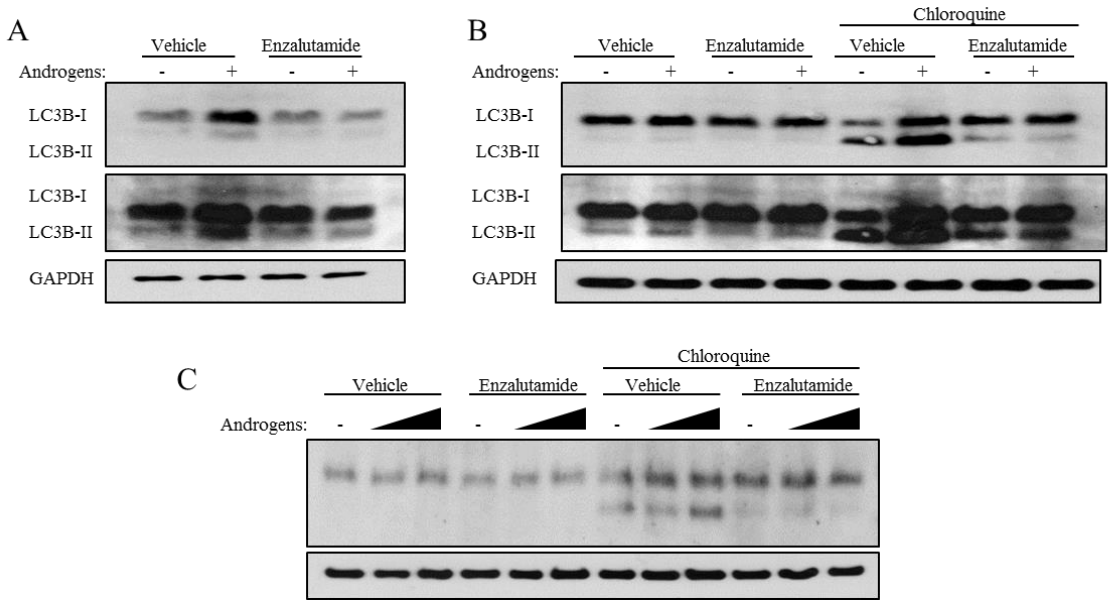

Figure 4.18 Enzalutamide, an AR antagonist, blocks androgen-mediated increases in hormone-sensitive and castration resistant prostate cancer. A-B, LNCaP cells and C, C4-2 cells were treated with Vehicle (Ethanol) or Androgen (10 nM R1881) along with 10uM Enzalutamide for 72 hours. B-C, 48 hours post-treatment, cells were treated with 40uM Chloroquuine. After 24 hours, cells were lysed and probed for LC3B expression.

Table 4.1 Correlation of AR activity with autophagy gene signature in patients. The expression of a previously annotated AR activity gene signature (*Hieronymus et al 2006*) was correlated with an autophagy gene signature consisting of *ATG4B*, *ATG4D*, *ULK1*, *ULK2* and *TFEB* in primary cancers and metastatic cancers from the *Taylor et al 2010* prostate cancer clinical dataset.

	Pearson's r correlation	**p-value**
Primary cancers	**0.32**	**< 0.05**
Metastatic cancers	**0.50**	**< 0.05**

metastatic lesions; both of these AR gene signatures are significantly decreased in the same clinical cohorts between the primary PCa and the metastatic cancer. This indicates that while AR signaling remains crucial to this metastatic progressive phenotype, not the entire AR gene signature is not necessary or utilized for the cancer cell to achieve this goal. Of importance, while the autophagy gene signature consisting of *ATG4B, ATG4D, ULK1, ULK2,* and *TFEB* does not significantly correlate with the progression of normal to primary PCa, this signature does significantly correlate with metastatic cancer in all three clinical cohorts (**Figure 4.19 C, F, and I**). Moreover, approximately 50% of samples from metastatic lesions collected as part of a rapid autopsy program express detectable levels of LC3B-II, indicating functional autophagy (**Figure 4.20**). Taken together this data highlights the important role that androgen-mediated autophagy is playing in PCa metastatic cancer.

Androgen-induced core autophagy genes along with TFEB promote androgen-mediated PCa cell proliferation

siRNA-mediated knockdown of *ATG4B, ATG4D, ULK1,* or *ULK2,* for 7 days in the hormone-sensitive LNCaP or VCaP cell lines results in significantly decreased androgen-mediated proliferation (**Figure 4.21A and B**). Moreover, knockdown of *ATG4B* and *ATG4D, ULK1* and *ULK2*, or *TFEB* significantly decreases androgen-mediated autophagy as measured by DNA or mitochondrial content (**Figures 4.22 A and B**). This indicates that androgen-mediated autophagy is necessary for androgen-mediated proliferation in prostate cancer cell models.

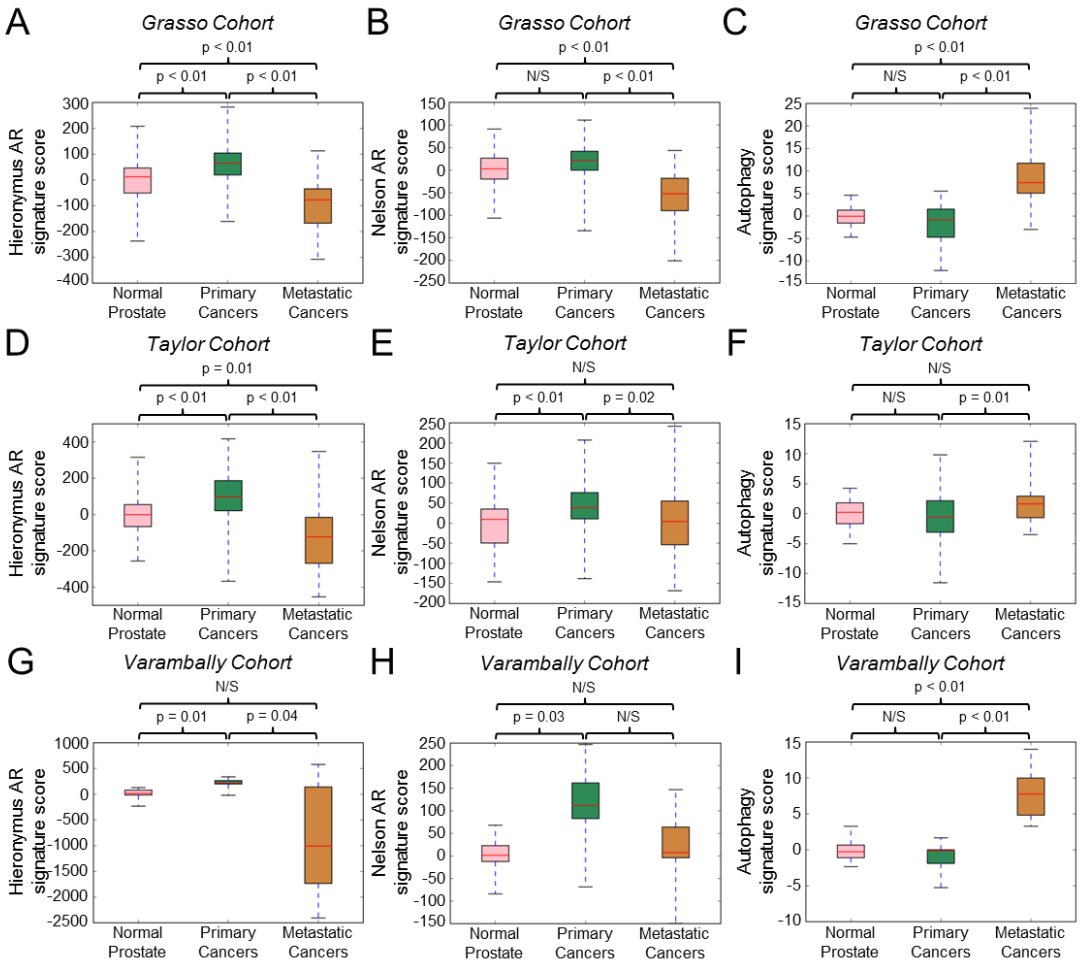

Figure 4.19 An autophagy gene signature correlates with the transition to prostate cancer metastasis in multiple clinical cohorts, unlike general AR activity gene signatures. Bioinformatic analysis of three separate prostate cancer clinical cohorts (**A-C**: Grasso et al 2012; **D-F**: Taylor et al 2010; **G-I**: Varambally et al 2005). The expression of two previously described AR activity gene signatures (*Hieronymus et al 2006*: **A**, **D**, **G** and *Nelson et al 2002*: **B**, **E**, **H**) or an autophagy gene signature consisting of *ATG4B*, *ATG4D*, *ULK1*, *ULK2* and *TFEB* were compared between samples from normal prostates, primary cancers and metastatic cancers in each cohort.

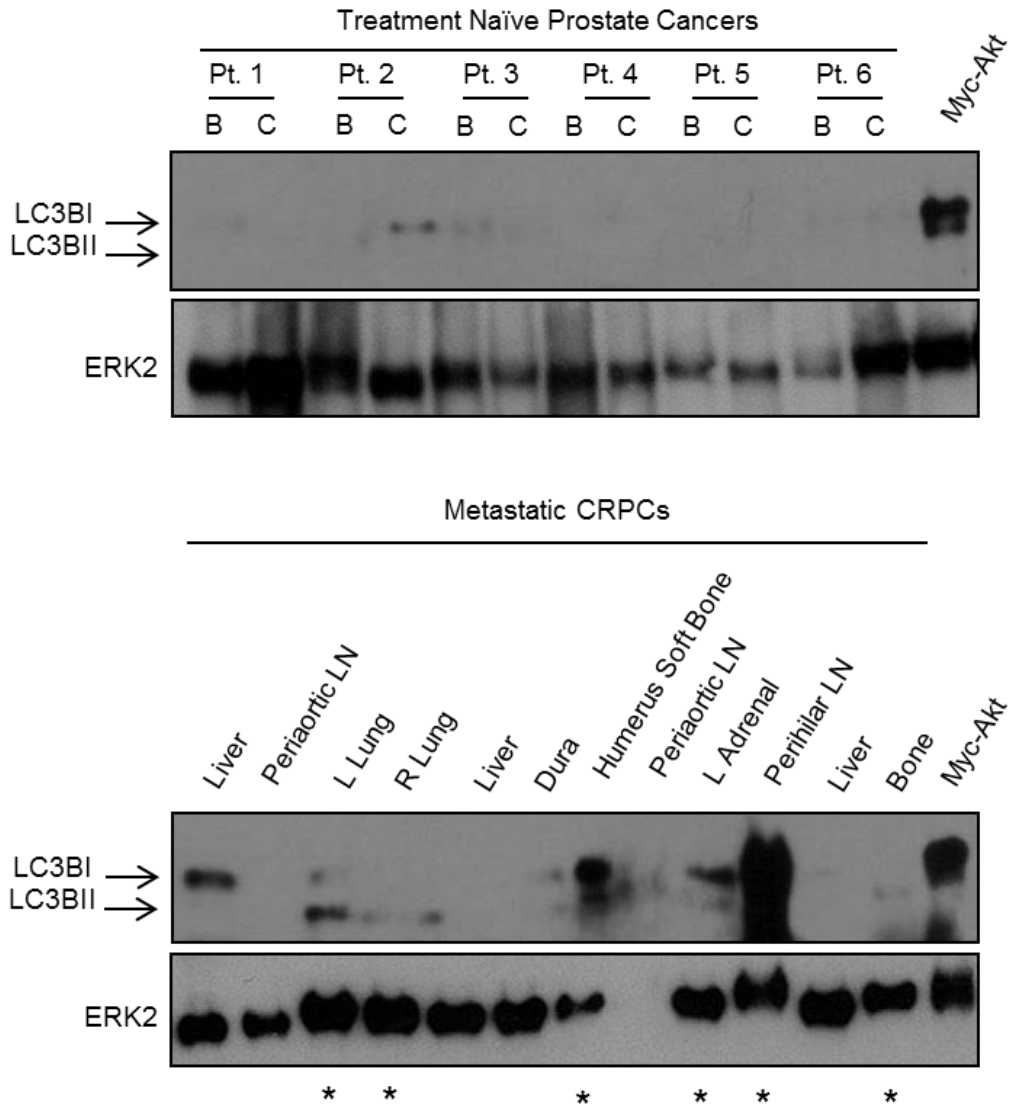

Figure 4.19 Autophagy is increased in patients with metastatic CRPC. Protein lysates were obtained via a rapid autopsy program from tumors derived from patients with either hormone-naïve prostate cancer or metastatic CRPC. Levels of LC3B conversion (functional readout of autophagy) or ERK2 (loading control) were then assessed by Western blot analysis. B = benign region of the prostate. C = cancer region of the prostate. LN = lymph node. Myc-Akt = human recombination assay with Myc- and Akt-expressing tumor used to help normalize the blot exposures between the two gels. *, indicate patients with detectable LCB-II.

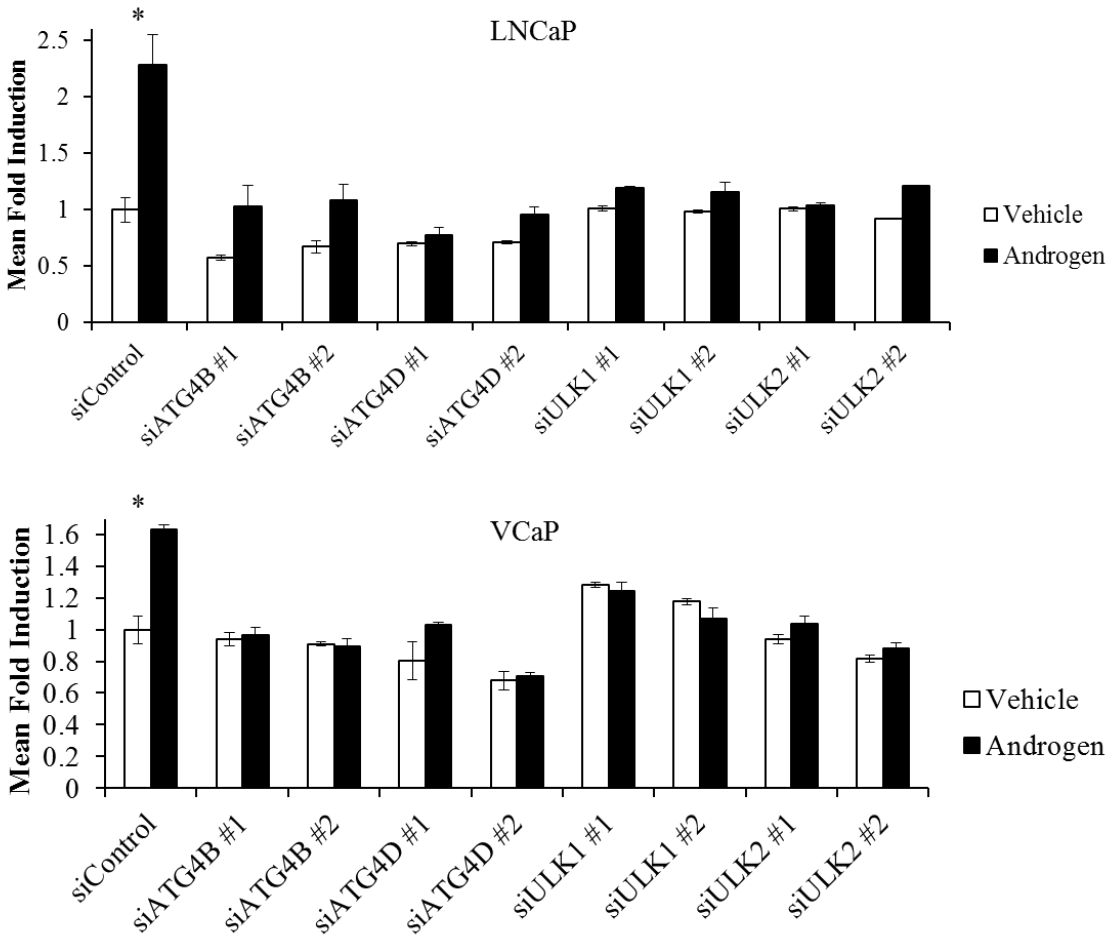

Figure 4.21 Androgen-regulated core autophagy genes are necessary for maximal androgen-mediated proliferation. A, LnCaP and B, VCaP cells were transfected with siControl or siRNAs directed against ATG4B, ATG4D, ULK1, or ULK2 then treated with vehicle or androgens for 7 days. Cells were then lysed, and the relative number of cells was quantified using a fluorescent DNA-binding dye. Each sample was performed in triplicate. Results are expressed as mean relative fold induction ± SE.

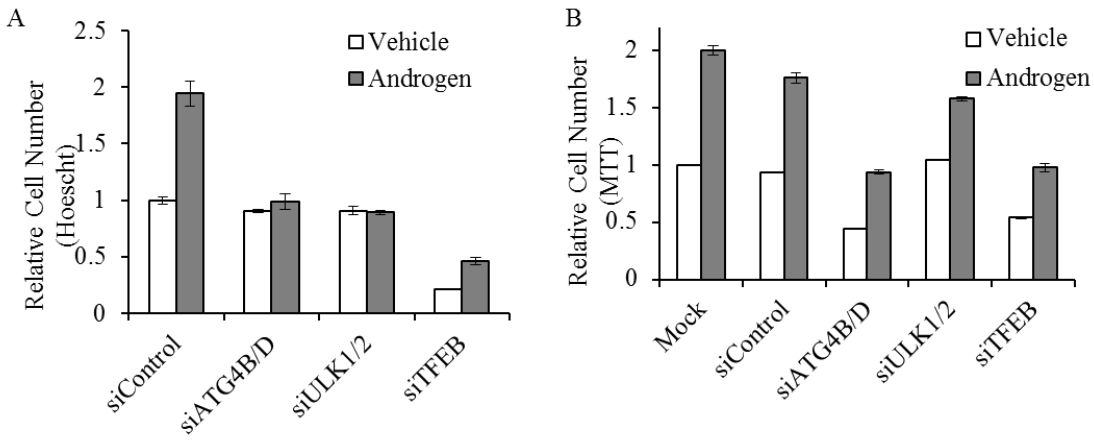

Figure 4.22 Androgen-mediated autophagy is necessary for maximal androgen-mediated proliferation. A-B, cells were transfected with siControl or siRNAs directed against ATG4B and ATG4D, ULK1 and ULK2, or TFEB then treated with vehicle or androgens for 7 days. A, Cells were then lysed, and the relative number of cells was quantified using a fluorescent DNA-binding dye. B, Cells were incubated in MTT Reagent at 37° C for 2 hours before florescence was quantified. Each sample was performed in triplicate. Results are expressed as mean relative fold induction ± SE.

4.4 Discussion

The results outlined in this report highlight the importance of androgen-mediated autophagy in PCa. Here, we have identified four core autophagy genes: *ULK1*, *ULK2*, *ATG4B*, and *ATG4D*, and a transcription factor that is the master regulator of lysosomal function, biogenesis, and autophagy: *TFEB*, which are transcriptionally regulated by AR in PCa (**Figure 4.23**). Knockdown of each of these genes results in a decrease in androgen-mediated PCa and decreased cell proliferation, indicating that in PCa autophagy is promoting tumor growth. To further support the hypodissertation that autophagy is supporting tumor growth, in clinical samples, all five of these AR-target genes were upregulated in metastatic lesions.

Targeting AR in the metastatic CRPC remains a challenge in the clinic; therefore, we rationalize that targeting the downstream targets of AR could be an alternative and viable pharmacological option for men suffering from this aggressive and devastating disease. We have identified a subset of AR-regulated genes that are upregulated in the metastatic lesions; targeting these five genes in combination or individually could hold promise for advanced stage PCa patients. A derivative of chloroquine, hydroxychloroquine, is currently in two separate phase II clinical trials for men with mCRPC[237]; the results of both clinical trials are highly anticipated.

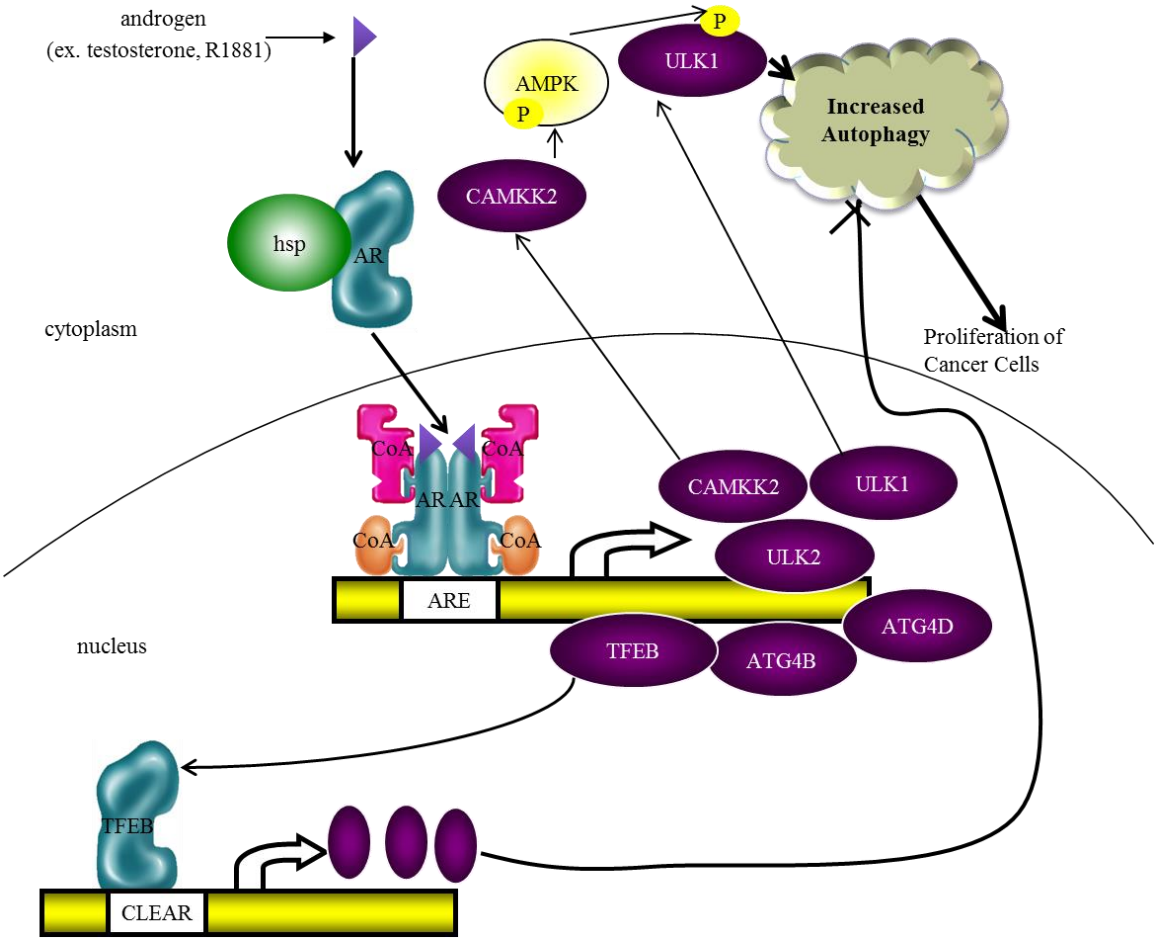

Figure 4.23 Proposed working model of androgen-mediated autophagy. Androgen Receptor regulates transcription of *CAMKK2*, *ATG4B*, *ATG4D*, *ULK1*, *ULK2*, and *TFEB*. CAMKK2 phosphorylates AMPK, which in turn phosphorylates ULK1 leading to the initiation of autophagy. ULK1, ULK2, ATG4B, and ATG4D all are essential core autophagy genes in mammals. TFEB is a master regulator of lysosomal biogenesis and function; TFEB increases transcription of lysosomal genes and lysosomal volume. Altogether, all of these AR-transcriptional targets result in increased autophagy and increased prostate cancer proliferation.

However, there are many drawbacks with using a pan-lysosomal inhibitor such as a chloroquine derivative. Lysosomes play important roles throughout the body and reduced lysosomal function has been shown to play an important role in neurodegeneration; while the side effects of low dosage chloroquine treatment are minimal, high dosage, long term studies have not been reported. Moreover, based on xenograft studies from our own laboratory, the conservative dose used in these clinical trials might not be fully efficacious for the patients. A more-specific inhibitor of a gene that is upregulated specifically in PCa would be more desirable. An ATG4B antagonist had promising effects in blocking autophagy and inhibiting osteosarcoma tumor formation *in vivo*; whether this antagonist will be beneficial for PCa patients remains should be further investigated[238]. Our data suggests that androgen-mediated autophagy is high in PCa metastatic lesions, a point at which current AR-targeted therapy has failed. However, we also show *in vitro* that the AR antagonist, Enzalutamide, is able to block autophagy based on LC3B-II conversion. Taken together, this could possibly indicate that treating patients with Enzalutamide earlier could greatly reduce the androgen-mediated autophagy and thereby greatly reduce the androgen-mediated proliferation.

In agreement with what others have previously reported, our data supports the hypodissertation that autophagy is promoting not just cell survival but proliferation as well. We have clearly outlined the mechanism by which AR directly upregulates transcription of autophagy genes and also controls the phosphorylation of ULK1 via the CAMKK2 signaling axis. This works significantly adds to the knowledge of PCa, linking for the first time a role for AR-mediated autophagy in the metastatic lesions. Importantly, this work highlights the potential for the rational development of improved pharmaceutical agents targeting autophagy for the treatment of PCa.

Chapter V:

Concluding Remarks and

Proposed Future Work

Prostate cancer remains one of the great health concerns in the twenty-first century despite decades of research. In the past several years, as a field we have gained much insight into the different types of PCa (indolent or aggressive), the different drivers of PCa (usually alterations that affect AR activity), its tendency to metastasize to the bone, and methods for its detection. In this dissertation, we have attempted to further the field of prostate cancer biology by:

1) Identifying a novel coregulatory of AR, SH3YL1, and characterizing its role in PCa progression,

2) Uncovering a novel transcriptional target of AR, UBN1 that correlates with disease progression and could potentially be used a PCa biomarker or therapeutically,

3) Identifying a signaling node by which AR via its transcriptional target CAMKK2, leads to the phosphorylation of ULK1 by AMPK resulting in the initiation of autophagy,

4) Identifying four core autophagy genes: *ULK1, ULK2, ATG4B,* and *ATG4D* which are direct transcriptional targets of AR and are all necessary for androgen-mediated autophagy in PCa,

5) Describing for the first time the role and regulation of TFEB by AR in PCa .

Taken together, this work will be valuable to the field and hopefully, to the patients.

It will be crucial going forward to follow up the initial studies presented here with more precise experiments to fully understand these observations within a larger body context. For example, I predict that the post translational modifications of SH3YL1 will determine its activity and affinity for AR in different contexts within the cell. Other coregrulators are known to be modified by phosphorylation, methylation, ubiquitylation, sumoylation, and acetylation. It would therefore be highly plausible that SH3YL1 could also be similarly modified and that a combination of such modifications could lead to functionally distinct activities for the same protein. Since such modifications would unlock the different physiological functions of SH3YL1, it is imperative that we understand what and how the 'coding' of these modifications affect their diverse functions. Furthermore, the upstream signaling cascades that control these events and impart these PTMs should be studied as they are probably differentially regulated based on tissue, cell type, and pathological status.

Moreover, the role of UBN1 in PCa should be further investigated. As a histone chaperone, it has the potential to transcriptionally control many genes within the cell including oncogenes or tumor suppressors. I propose that the following experiments should be done in the immediate future to verify UBN1's role in PCa: 1) RNA-Seq or gene microarrays of cells with normal levels of UBN1 compared to decreased or increased UBN1 expression and 2) Xenograft studies with UBN1 overexpressed or knockdown compared to normal cellular levels. In both cases, creation of stable inducible cell lines that modulate UBN1's activity will be preferable.

Additionally, further *in vivo* studies should be carried out to better understand the role of autophagy in the varying stages of PCa. Since autophagy is reported as both a tumor suppressor and an oncogene, it is quite imaginable that targeting autophagy in the clinic would only be

advantageous for patients at certain points of PCa progression; however, this should be shown empirically *in vivo* first.

PCa remains a highly lethal disease with many hurdles still blocking the path to a cure. While this dissertation has only uncovered a small portion of PCa biology, it will still be significant in laying the foundation for further dissecting the roles of 1) SH3YL1 in PCa 2) UBN1 in PCa and 3) Autophagy in PCa. All three of which, I believe will be crucial on our crusade to better treat and prevent PCa in the future.

"Success in life has nothing to do with what you gain in life or accomplish for yourself. It's what you do for others." –Danny Thomas

Chapter V: Works Cited

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