REGULATION OF NUTRIENT UPTAKE IN PROSTATE CANCER

A Dissertation Presented to

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In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

By

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REGULATION OF NUTRIENT UPTAKE IN PROSTATE CANCER

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ABSTRACT

Prostate cancer is a hormone-driven malignancy that relies on the function of the androgen receptor (AR). AR is a transcription factor that regulates the expression of many downstream targets, some of which can facilitate an important hallmark of cancer; metabolic reprogramming. Metabolic reprogramming allows the cancer to maintain an aberrant metabolism that supports uncontrolled cellular growth and survival. This reprogramming if often initiated by signaling pathways essential for growth and survival. There are therapies available that target AR signaling but they inevitably fail. Therefore, I sought to identify new potential targets that are downstream of AR and other oncogenic signals in prostate cancer and define the mechanism through which they are regulated.

First, I investigated how two glutamine transporters, *SLC1A4* and *SLC1A5* (Solute Carrier Family 1A, members 4 and 5) were regulated in glutamine-addicted prostate cancer cells. I found that the transporters were hormone-responsive but not direct targets of AR. Downstream of AR they are regulated via mammalian target of rapamycin (mTOR) signaling and selectively regulated via MYC. Importantly I determined that *SLC1A4* and *SLC1A5* represented a central node of several oncogenic signaling pathways that controlled overall cell growth, making them promising targets for prostate cancer therapy.

Next, I investigated the regulation of glucose uptake through *SLC2A12* (GLUT12 (glucose transporter 12)). I found that *SLC2A12* is a direct target of AR and is required for prostate cancer cell growth. GLUT12 is also regulated through calcium/calmodulin-dependent protein kinase kinase 2 (CaMKK2)-5'-AMP-activated protein kinase (AMPK)

signaling. CaMKK2-AMPK activity promotes GLUT12 translocation to the plasma membrane via modulation of TBC1D4 (TBC1 Domain Family Member 4) and also regulation of *TBC1D4* expression.

Taken together my findings demonstrate that *SLC1A4, SLC1A5*, and *SLC2A12* all have the potential to be prostate cancer therapeutic targets due to their modulation by major oncogenic signaling pathways and their functional role in cancer cell growth. Their essential role in cancer cell growth and easily accessible location on the cell surface suggest these proteins may be readily druggable. Thus, my findings highlight the utility of targeting pathogenic metabolism as a therapy and provide potential starting points for future translational research.

TABLE OF CONTENTS

Page#

LIST OF FIGURES

Page#

LIST OF TABLES

Page#

CHAPTER 1: INTRODUCTION

1.1 The prostate and prostate cancer progression

The prostate is an accessory sex gland in males that is located in front of the rectum, below the bladder. It surrounds the base of the bladder and has two lobes that surround the urethra. The prostate gland is covered in connective tissue called the prostatic layer. The prostate can be divided into three parts, the peripheral, transition, and central zones [1]. The peripheral zone is located closest to the rectum and makes up the majority of the mass of the prostate. This is where majority of prostate cancers occur. The transition zone is the middle of the prostate, comprised of the peripheral and central zones. It surrounds the urethra and is the location of enlargement during benign prostatic hyperplasia (BPH). BPH is a condition characterized by an enlarged prostate that puts pressure on the urethra making urination difficult, it is not cancerous or cancer related. The central zone is in front of the transition zone and furthest from the rectum. The prostate itself is made up of glandular cells, muscle cells, and fibrous cells. These cells all work together to produce seminal fluid for semen and control the flow of urine which are the two major functions of the prostate.

During the lifespan of a male the prostate will undergo two growth phases. The first phase occurs at puberty and the second after age 40. This growth is stimulated by the presence of testosterone and androgen receptor (AR) signaling. Androgens are produced in the body via the hypothalamic-pituitary axis (HPA). Gonadotropin-releasing hormone is secreted by the hypothalamus to stimulate luteinizing hormone which acts on Leydig cells in the testes to induce androgen production [2, 3]. The HPA accounts for about 95%

of total circulating androgen in the body while the other 5% comes from the adrenal glands behind the kidneys. Once free testosterone enters the cell, it gets converted to 5-alpha-dihydrotestosterone (DHT) by 5-alpha reductase [2, 3] and binds to AR to promote the growth of the prostate. As the prostate grows it will secrete PSA (prostate specific antigen) which is only produced by prostate epithelium. PSA levels are closely related to prostate growth because it is indicative of the amount of prostate epithelium [4]. PSA levels are not a confirmation of BPH or prostate cancer, for that a biopsy of the prostate tissue is required.

Collection of prostatic tissue is used to identify high-grade prostatic intraepithelial neoplasia (HGPIN) lesions. These lesions are the pre-malignancy leading to prostate cancer. Pathologists identify this as the presence of prominent nucleoli within an existing duct structure [5]. The presence of PIN lesions confirms that the prostate cells and tissue has already begun to undergo malignant transformation. A Gleason score is assigned to the tumor tissue based on how much the tumor cells look like normal cells. The Gleason score is on a scale from 6 to 10, six being low-grade cancer and ten being high-grade cancer. The most common type of prostate cancer is adenocarcinoma but there are rare types such as small-cell carcinomas. Prostate cancer occurs in two main phases; hormone dependent and hormone refractory (castration resistant prostate cancer (CRPC)) over four stages.

Stage 1 prostate cancer presents with small tumors that are localized to the prostate in the peripheral zone. The tumor is slow growing and would have a low Gleason score (ex. 6) and low PSA serum levels. During this stage the tumor is hormone responsive, meaning that androgens stimulate growth. At this point, active surveillance is recommended or start of radiation therapy for patients. Patients also have the option of a

prostatectomy to remove part or the entirety of the prostate. At this stage, prostate cancer patients have the highest probability of survival. Stage 2 prostate cancer tumors are larger but have not spread beyond the prostate and the prostate itself is also getting larger. This presents with a higher PSA serum level and higher Gleason score. The tumor is still hormone responsive so at this point patients are given hormone therapies in combination with the radiation. Hormone therapies include AR antagonists such as Enzalutamide in addition to androgen synthesis inhibitors such as Abiraterone. These types of drugs can be given in combination as a hormone blockade. Another patient treatment option is chemical castration using gonadotropin-releasing hormone agonist or antagonist to disrupt the HPA.

Stage 3 prostate cancer tumors have grown outside the prostate but have not spread to distant organs, there is high reoccurrence at this stage. Treatment options at this stage include radiation and hormone blockade therapy which will shrink the tumor but inevitably the tumor will grow back after the treatments fail in most patients. When the tumor grows back, it will transition from hormone responsive to hormone refractory. The tumor will grow independent of androgen production and sometimes independent of AR expression. At this point patients transition to stage 4 cancer where it has spread beyond the prostate to distant organs such as the bladder, bone, or lymph nodes. Gleason scores for these tumor types are 8, 9, or 10. Stage 4 prostate cancer is treatable but there is no cure. Hormone therapy in combination with chemotherapy is the best approach because the cancer is moving throughout the body and requires a systemic approach. Throughout all four stages of prostate cancer progression, the androgen receptor is the key regulator. It plays a pivotal role in the progression and survival of the cancer even in the presence of therapeutic

intervention. Therefore, it is important to understand its function and role in prostate cancer.

1.2 Prostate cancer maintenance

AR has a structure similar to other steroid receptors in the nuclear receptor superfamily [6]. The N-terminus contains a transcriptional activation domain, the DNA binding domain is central, a hinge region is C-terminally next to it, and the ligand binding domain is on the C-terminus [6, 7]. The C-terminal domain is important to prostate cancer because it is where androgens bind to promote AR activity, it is also the area targeted by current hormone ablation therapies [7]. The DNA binding domain and hinge region are important for AR nuclear localization, dimerization of the receptor, and DNA binding [7]. All of these functions contribute to the overall function of AR during prostate cancer progression.

During hormone-responsive prostate cancer progression, testosterone freely diffuses through the cell where it is converted to DHT by 5-alpha reductase. DHT binds to the androgen receptor in the cytoplasm. In the cytoplasm, unliganded AR is bound by heat shock proteins to prevent its degradation. Binding of the ligand causes a conformational change that releases the heat shock proteins. AR is imported to the nucleus where it dimerizes and binds to stretches of DNA sequences called androgen response elements (AREs). Once AR binds there, it will promote cancer progression by both increasing the transcription of pro-cancer genes while decreasing the expression of anticancer genes. During hormone-refractory prostate cancer progression, AR is often reactivated through a

variety of mechanisms. For example, AR can be truncated at the C-terminus through alternative splicing. The lack of the C-terminal domain allows AR activity to become constitutively active and promote the expression of AR regulated genes [8]. AR gene amplification, promiscuous mutations of AR, alterations of co-regulators of AR, and the increased synthesis of intratumoral androgen are additional mechanisms to increase AR signaling and therefore facilitate-hormone refractory prostate cancer [9]. As AR activity drives the progression of prostate cancer from the initial stages to the late stages of the disease, it is now known that shifts in cellular metabolism are required to satisfy the growing cancer's energetic and biosynthetic demands. Prostate cancer cells utilize both glucose and glutamine for growth. Together, glutaminolysis and glycolysis provide carbon and nitrogen to the cell to support the cell's changing metabolism.

Glucose metabolism in normal cells rely on oxidative phosphorylation in the mitochondria to generate energy but cancer cells rely on aerobic glycolysis to generate energy. Otto Warburg was the first to document this and it is still not clear why cancer cells uptake more glucose and glutamine than they need and use it in a less efficient manner than normal cells. However, metabolism in the prostate and prostate cancer is slightly different.

As prostate cells transition from benign to malignant, their metabolism will undergo an additional change in citrate metabolism and zinc accumulation [10]. Citrate is important for energy production and survival of prostate cells. Metabolic changes that take place are found mostly in the peripheral zone of the prostate since it makes up majority of the mass of the prostate; this is also an area of high citrate concentration [10]. The function of prostate citrate production is promoted by the high activity of specialized glandular

epithelial cells that have the capability to accumulate and secrete citrate [10]. Unlike most mammalian cells, normal prostate cells utilize high aerobic glycolysis to produce high amounts of acetyl co-enzyme A for citrate synthesis and lipogenesis [10, 11]. In addition to high citrate levels, the prostate also exhibits the body's highest concentration of zinc. The cellular accumulation of zinc leads to high levels of mitochondrial zinc that inhibits mitochondrial aconitase activity and citrate oxidation. This inhibition gives the cells a truncated tricarboxylic acid cycle (TCA) which provides an efficient metabolic pathway for citrate production and secretion [10, 11].

As prostate tissues start to progress from normal to malignant; they accumulate significantly less zinc and citrate. It is thought that the loss of zinc accumulation is due to the loss in expression of the zinc transporter, *ZIP1* [10]. The low levels of zinc will no longer inhibit mitochondrial aconitase and thus allows citrate oxidation through the TCA cycle. This observation in the difference in metabolism between normal and malignant prostate tissues lead to the development of zinc as an inhibitor of invasion/migration and tumor growth [10].

One of the seven hallmarks of cancer, is cell autonomy, meaning cancer cells choose which nutrients to utilize [12]. Prostate cancer cells can utilize glucose or become glutamine addicted. For example, regarding the latter, oncogenic signaling will contribute to metabolic reprogramming that will shift prostate cancer metabolism to utilize glutamine. Glutamine is the most abundant amino acid in the body. It is a precursor for other amino acids, proteins, and nucleotides. Glutaminolysis is the breakdown of glutamine to contribute carbon and nitrogen to many metabolic endpoints, such as glutathione

production, amino acid synthesis, and fatty acid synthesis via citrate production [13, 14]. The rate-limiting step of glutaminolysis is the conversion of glutamine to glutamate by the enzyme, glutaminase [14]. Glutamate is then converted to α-ketoglutarate by glutamine dehydrogenase (GLUD1) or glutamic-oxaloacetic transaminase (GOT1). This is important because α-ketoglutarate is a key intermediate in the TCA cycle. The TCA cycle is the central hub for carbon metabolism and is used for the above-mentioned metabolic needs of the cell. What is unclear are how particular genes and pathways regulate cellular metabolism to maintain growth and survival.

1.3 AR responsive genes that maintain prostate cancer metabolism

AR signaling in the cell maintains prostate cancer survival and proliferation by supporting cancer metabolism via the direct or indirect regulation of downstream targets. Here the focus will be on three targets in particular; MYC, mTOR (mammalian target of Rapamycin), and AMPK (5' adenosine monophosphate-activated protein kinase). These genes control proliferation, protein synthesis, energy regulation, and nutrient uptake in the cell making them essential to maintain prostate cancer metabolism.

The C-terminal domain of MYC contains a dimerization motif, called the helix-loop-helix leucine zipper; here it binds to Max, an obligate protein binding partner [15]. Together MYC/Max dimers will bind to a specific DNA sequence called an E-box region to promote transcription of its downstream gene targets. Dividing cells express high levels of MYC as its expression increases due to a response to growth factors. This links MYC expression to proliferation in cells. MYC regulates cell cycle progression by modulating

cell cycle genes such as the cyclin dependent kinases (CDK) and inhibiting p27, a CDK inhibitor [15, 16]. In order to keep dividing cells from becoming cancerous, normal cells that overexpress MYC will activate a protective pathway that will induce apoptosis and prevent neoplastic changes [15, 16]. In the prostate, for unknown reasons, MYC overexpression does not lead to apoptosis but instead, MYC promotes cell transformation [17]. This was demonstrated by Ellwood-Yen et al., when they used two different types of transgenic mice expressing low and high MYC. They found that in a dose-dependent manner MYC can be a driver of prostate cancer progression over time. MYC deregulated cell cycle progression can lead to genomic instability that will lead to new mutations and is characterized by gene amplification and abnormal chromosome number [15]. Alternatively, MYC has many downstream targets that regulate aspects of both glucose and glutamine metabolism. MYC is able to transactivate genes involved with glycolysis in response to hypoxia, while also having the ability to promote glutamine metabolism through metabolic reprogramming [15, 18]. MYC silencing can lead to many unforeseen off-target effects in the cell because MYC controls a large number of genes. For this reason, MYC silencing in cancer patients is a controversial point because of the potential for massive side effects. Regardless, this option is still being explored.

mTOR proteins are members of the phosphatidylinositol kinase-related kinase (PIKK) family, characterized by a serine/threonine kinase at the c-terminus [19]. mTOR activity is regulated by PI3K (phosphoatidylinositol 3-kinase) signaling and has a well-known role in promoting translational events that regulate cell growth in response to external stimulus [19]. mTOR is a complex of proteins with two core units; mTORC1 and

mTORC2 (mechanistic target of rapamycin complex 1 and 2). mTORC1 activity is regulated by cellular growth stimuli and promotes protein synthesis in the cell [20]. This is achieved by mTORC1 phosphorylating ribosomal S6 kinases and eukaryotic initiation factor 4E (eIF4E)-binding proteins to allow for an initiation complex that allows for cap-dependent translation [20]. The mTORC2 complex is different from mTORC1, and as such phosphorylates a different set of substrates, leading to different cellular effects. In prostate cancer, it is common to find that there is a loss in *PTEN* (phosphatase and tensin homolog) expression. This results in highly active mTOR activity independent of external growth stimuli [20]. Increased mTOR activity contributes to prostate cancer cell growth through its role in nutrient uptake and signaling that promotes metabolic reprogramming. In tumors mTOR can enhance glucose metabolism in hypoxic conditions in the tumor microenvironment. Conversely, mTOR also has a role in glutamine metabolism by enhancing MYC translation and indirectly repressing inhibitors of glutamate dehydrogenase 1 [21, 22]. Currently there are drugs such as rapamycin that inhibit mTORC1 but not mTORC2 activity. The current second generation of drugs, mTOR kinase inhibitors, inhibit both mTORC1 and mTORC2 through an ATP (adenosine triphosphate) competitive mechanism [23].

AMPK is an energy sensor that maintains the ratio of AMP to ATP in the cell, changes in the ratio will activate AMPK [24, 25]. The purpose of its metabolic sensor function is to allow for adaptive changes in growth and metabolism in low energy conditions [24]. The structure of AMPK is heterotrimeric. Alpha is the catalytic subunit, beta and gamma are the regulatory subunits. Maximal activation requires the

phosphorylation of threonine 172 in its activation loop [24]. AMPK activation can occur in additional ways. Upstream of AMPK, CaMKK2 (calcium/calmodulin kinase kinase 2) can phosphorylate threonine 172 in response to calcium influx to activate AMPK. Cellular stress on the cell that affects the AMP/ATP ratio and the agonist AICAR (5-aminoimidazole-4-carboxamide-1-b-d-ribofuranoside) can both also activate AMPK [24]. Once AMPK is activated, it has many downstream targets capable of regulating metabolism and cell growth. AMPK activation or inhibition in cancer is unique because it has tumorigenic and anti-tumorigenic activity. Tumorigenic AMPK activation promotes fatty acid oxidation, migration, and cell survival [26], whereas tumorigenic AMPK inhibition promotes cell growth via mTOR signaling and reduced nutrient sensitive p53 activation [25]. Conversely, anti-tumorigenic AMPK activation promotes p53-dependent cell cycle arrest and downregulation of mTORC1, whereas anti-tumorigenic AMPK inhibition disrupts the cell's ability to create more ATP and impairs autophagy [25]. This makes AMPK a difficult target for a cancer therapy because it is unclear what its role may be when it is expressed in a particular cancer or context.

1.4 Glutamine and glucose transporters in prostate cancer

As cells respond to oncogenic signaling to increase growth by creating more biomass, nutrient uptake is essential. Glutaminolysis and glycolysis are not possible without glutamine and glucose uptake, respectively. In prostate cancer, downstream of oncogenic signaling and responsible for nutrient uptake are the glutamine and glucose transporters [27, 28].

The solute carrier 1A family (SLC1A) are high affinity glutamine, glutamate, and neutral amino acid transporters [29]. These transporters are expressed in a wide variety of tissues throughout the body. The SLC1A family of transporters function to uptake glutamate in addition to inorganic ions, this function is important to synaptic function in the brain. In other tissues, such as the lungs, kidneys, and testis, these transporters function to maintain glutamine/glutamate metabolism. Glutamine and glutamate are nutrient sources needed by the cell as a source of carbon and nitrogen. As a result, glutamine and glutamate contribute to amino acid synthesis and the balance of cataplerosis (usage of TCA cycle intermediates) and anaplerosis (replenishing TCA cycle intermediates) in the cell via glutaminolysis. Neutral amino acid uptake is another function of this family of transporters and this is important in the creation of essential amino acids and nucleic acid synthesis. This function is similar in cancer. In cancer, the expression of the transporters is increased in response to increased energy demands and uncontrolled cellular growth. In prostate and breast cancer, *SLC1A5* plays an important role in the progression of both malignancies [30, 31]. Glutamine transporters are promising cancer therapeutic targets due to their essential role in glutaminolysis for glutamine-addicted cancer cells [27].

The solute carrier 2A family (SLC2A) is a mixture of glucose transporters (GLUTs) and fructose transporters characterized by twelve membrane spanning helices and conserved sequence motifs [32]. There are fourteen GLUTs and they are expressed in the brain, liver, muscle, heart, and prostate. The function of GLUTs is to transport glucose into the cell, which is the first rate-limiting step of glycolysis. There is some sequence homology between GLUT4 and GLUT10 which lead to the discovery of GLUT12 [33].

GLUT12 has 40% sequence homology with GLUT10 and 29% sequence homology with GLUT4 [33]. It has been found that GLUT12 is needed for glucose transport in muscle, similar to GLUT4 [34]. GLUT12 expression in malignant tissues compared to normal tissues are dramatically increased, making it a potential therapeutic target.

Glutamine and glucose transporters together regulate overall nutrient uptake in cancer cells in response to oncogenic signaling. Current therapies focus mostly on individual oncogenic signaling pathways. Unfortunately, cancer cells will ultimately use alternative pathways to support survival. I hypothesized that targeting nutrient uptake could be a more effective solution because no matter which pathway the cancer cells utilize, glucose and/or glutamine are requirements for growth and survival. As such, the transporters could represent central meeting points for multiple oncogenic cascades. Hence, targeting these transporters may help prevent the emergence of compensatory oncogenic signals. Further, localization of the transporters on the cell surface may make these proteins more druggable.

CHAPTER 2: MATERIALS AND METHODS

2.1.1 Cell culture, plasmids, and reagents

LNCaP and VCaP human prostate cancer cell lines were obtained from ATCC (Manassas, VA, USA) and maintained and validated for androgen responsiveness just prior to experiments as previously described [26]. For all experiments, cells were first plated in their respective media but without phenol red and with charcoal-stripped fetal bovine serum (CS-FBS) for 72 hours to minimize endogenous hormone signaling. Then, cells were switched to and treated in a customized experimental media (Sigma, St. Louis, MO) that was lacking serum, non-essential amino acids, sodium pyruvate, additional glucose and HEPES buffer. This experimental media was supplemented with 2 mM L-glutamine unless otherwise noted.

Plasmids used for stable cell line creation were the pINDUCER10 construct with a short hairpin RNA targeting MYC. Additional lentiviral vectors have been previously described [35]. Stealth siRNAs were from Life Technologies (Grand Island, NY). Antibodies recognizing MYC, SLC1A4, SLC1A5, phospho-S6 and total S6 were obtained from Cell Signaling (Beverly, MA). The antibody recognizing glutaminase was from Abcam (Cambridge, MA). Antibody recognizing AR and GAPDH were from Santa Cruz (Dallas, TX) and Sigma, respectively. Compound 968, a glutaminase inhibitor, was obtained from EMD Millipore (Billerica, MA). Methyltrienolone (R1881, a synthetic

androgen) was from PerkinElmer (Waltham, MA) and the α-ketoglutarate enzymatic assay was from Sigma.

2.1.2 Cell culture, plasmids, and reagents

LNCaP, C4-2, and 22Rv1 human prostate cancer cell lines were obtained from ATCC (Manassas, VA, USA). LNCaP and 22Rv1 were cultured in RPMI-1640 supplemented with 8% FBS, 1% sodium pyruvate. LNCaP were supplemented with 1% non-essential amino acids while 22Rv1 were supplemented with 0.5% glucose. C4-2 were maintained in improved-MEM with 8% FBS and L-glutamine. PC-3 cells were a gift from Dr. Anders Strom and maintained in RPMI-1640 supplemented with 8% FBS. Prior to all experiments, PC-3 cells were plated and incubated for 24 hours in charcoal-stripped FBS, while LNCaP, C4-2, and 22Rv1 cells were incubated for 72hours.

The plasmid used for the creation of the stable cell line was the pINDUCER20 construct with a *CaMKK2* coding sequence. Methyltrienolone (R1881) was purchased from PerkinElmer (Waltham, MA, USA). Enzalutamide was from SelleckChem (Houston, TX, USA). STO-609 was purchased from Tocris (Bristol, UK). Anti-AMPK and anti-p-AMPK T172 antibodies were purchased from Cell Signaling (Danvers, MA, USA). Anti-GLUT12 was purchased from BioSUSA (Woburn, MS, USA). Anti-TBC1D4, anti-p-TBC1D4 T642, anti-ATPase antibodies and the Plasma Membrane Protein Extraction Kit were purchased from Abcam (San Francisco, CA, USA). GLUT12 and TBC1D4 siRNAs, Universal siRNA negative control, and anti-GAPDH antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). AMPK siRNAs were purchased from Invitrogen

(Carlsbad, CA, USA). Anti-AR antibody was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA).

2.2.1 Proliferation assay

All cell lines were plated in their respective phenol red-free, CS-FBS containing media at a density of $5x10^3$ cells per well in 96-well plates for 3 days. After this, the media was switched to a serum-free media that contained a final concentration of ± 2 mM glutamine without sodium pyruvate, non-essential amino acids, HEPES or additional glucose (experimental media). LNCaP and VCaP cells were then treated and incubated for 3 or 7 days as indicated. At the end, cell numbers were quantitated using a fluorescent Hoechst-based DNA dye as previously described [36].

2.2.2 siRNA transfection

In the context of targeting the glutamine transporters with siRNAs, all cell lines were plated as stated above. Cells were then transfected with 100 nM final concentration siRNAs for 3 days. Afterwards, the cells were transfected a second time and treated as indicated and allowed to incubate an additional 4 days. Cell proliferation was then quantified as described above. All siRNAs used in this study are listed in Table 2.1.

Table 2.1: Chapter 3 siRNA Sequences

siRNA	Sequence
MYC #1	GGAACUAUGACCUCGACUA
MYC #2	CAGAGAAGCUGGCCUCCUA
SLC1A4 #1	GAGAUAGAAGGGAUGAACA
SLC1A4 #2	GACAUCAUCGUGCUGGUGA
SLC1A5 #1	GUCAGCAGCCUUUCGCUCA
SLC1A5 #2	CCAAGCACAUCAGCCGUUU

2.3.1 siRNA transfection

Transient transfection with 100nM final concentration of siRNAs was performed in the presence of DharmaFECT Transfection Reagent from Life Technologies (Carlsbad, CA, USA) following manufacturer protocol. Briefly, siCtrl or specific siRNAs targeting specific genes were mixed with DharmaFECT transfection agent in reduced serum Opti-MEM medium for 20 minutes. The cells were maintained in antibiotics-free conditions and 20% of the cell medium was replaced with the transfection medium. LNCaP cells were transfected once and treated with androgens. PC-3, C4-2, and 22Rv1 were treated once and then again 24 hours later. All siRNAs used in this study are listed in Table 2.2.

Table 2.2: Chapter 4 siRNA Sequences

siRNA	Sequence
siAR #1	CCCUUUCAAGGGAGGUUACACCAAA
siAR #2	UAGAGAGCAAGGCUGCAAAGGAGUC
siGLUT12 #1	CAGUUUAUCCUACACGGUU
siGLUT12 #2	GGAAGUACAUGUUUGGUCU
siTBC1D4 #1	GAGUUUCGGUCUCGGUGCA
siTBC1D4 #2	GGAAGAUAUUCAUACUCUU
siTBC1D4 #3	CAUCUACCUGCAGCAAUGA
siAMPK #1	CCCAUCCUGAAAGAGUACCAUUCUU
siAMPK #2	CCCUCAAUAUUUAAAUCCUUCUGUG
siAMPK #3	ACCAUGAUUGAUGAUGAAGCCUUAA

2.3.2 Proliferation assay

Cell proliferation assays were performed upon freeze-thaw cycles followed by measurements of cellular DNA content using a FluoReporter Blue fluorometric double-stranded quantitation kit from Life Technologies following the manufacturer's protocol.

2.4 Immunoblot analysis

Immunoblot analysis was performed as previously described [37]. All primary antibody concentrations were used at the manufacturer-recommended concentrations. Results shown are representative blots. Densitometry was performed using ImageJ software (National Institutes of Health (NIH), Bethesda, MD) and normalized to indicated controls. Results are presented as normalized mean values + SEM from experimental repeats ($n \geq 3$).

2.5 Creation of LNCaP-shMYC stable cell lines

Inducible stable cell lines were created using the pINDUCER10 system and G418 selection as previously described [35, 38]. The sequence for the MYC-targeting shRNA is: 5'−CTCGAGAAGGTATATTGCTGTTGACAGTGAGCGCACGACGAGAACAGTTG AAACATAGTGAAGCCACAGATGTATGTTTCAACTGTTCTCCGTCGTTTGCCTA CTGCCTCGGAATTC−3'

2.6 Creation of LNCaP-CaMKK2 stable cell lines

LNCaP cells that stably express *CAMKK2* under the control of a doxycycline-inducible promoter were made by first creating a pINDUCER20-*CAMKK2* lentiviral construct using the Gateway recombinase cloning system. To do this, the *CAMKK2* coding sequence (prostate splice variant) was shuttled from pOTB7-*CAMKK2* (American Type Tissue Culture) to pINDUCER20 (gift from Thomas Westbrook). LNCaP cells were then

infected with lentivirus expressing pINDUCER20-*CAMKK2* and cells were selected using G418 (Sigma).

2.7 α-ketoglutarate assays

Cells were plated at a density of $5x10^5$ cells/well and treated as described. The assay was performed using the coupled enzymatic assay according to the manufacturer's instructions (Sigma). Total α-ketoglutarate levels were normalized to cellular DNA content.

2.8 Glutamine uptake assays

LNCaP cells were plated at a density of $3x10^4$ cells per well, while VCaP cells were plated at $1.2x10^5$ cells per well in 24-well plates. After three days, the cells were switched to 2mM glutamine-containing experimental media and transfected and/or treated as indicated. Afterwards, the media was collected and glutamine levels were analyzed using a YSI 2700 Bioanalyzer (YSI Life Sciences, Yellow Springs, OH). Glutamine uptake levels were normalized to cellular DNA content.

2.9 Glucose uptake assays

LNCaP were plated at a density of $2.5x10^5$ cells per well in a 6-well plate while PC-3 cells were plated at a density of $1.5x10^5$ cell per well in a 6-well plate. LNCaP cells were transfected and treated with +/- 10nM R1881 in starvation media (phenol-red free

RPMI media, 10mM HEPES, and 0.1% BSA) for 24 hours, afterwards media was switched to phenol red free RPMI media for 2 hours. The media was collected and analyzed using a YSI 2700 Bioanalyzer (YSI Life Sciences, Yellow Springs, OH). Glucose uptake was determined by normalizing glucose concentrations to cellular DNA content. PC-3 cells were transfected in starvation media for 24 hours and transfected a second time for another 24 hours. The glucose uptake was determined as previously described for LNCaP cells.

2.10 Chromatin immunoprecipitation sequencing (ChIP-Seq)

ChIP-Seq analysis was performed as previously described [39, 40].

2.11 Reporter assay

Cells were transfected with plasmids using Lipofectamine 2000 (ThermoFisher Scientific) according to the manufacturer's instructions and then treated with hormones approximately 16 h before the assay. Luciferase and β-galactosidase (transfection normalization) activities were measured as previously described [41]. Each treatment was performed in triplicate and results are expressed as mean relative light units (RLUs) normalized to β-galactosidase transfection control + SE. Each experiment was repeated at least 3 times, with a representative experiment shown. The pGL4.26-*SLC2A12* (*SLC2A12* (GLUT12)-Luc) enhancer construct was created by PCR amplifying the sequence (forward primer: 5'-CAGTCGGTACCTACCCCTCCTGGATTCTAAAT-3'; reverse primer: 5'-CGAGGCTCGAGCTTTACATCCATATTCACATT-3') that encompassed the potential AR binding site identified using ChIP-Seq [39, 40] that was located within intron 2 of

SLC2A12. This fragment was then cloned into the pGL4.26 vector (Promega, Madison, WI, USA) using KpnI and XhoI restriction sites. Sequences were confirmed using restriction digests and sequencing. The *KLK3* (PSA)-Luc reporter plasmid was previously described [26].

2.12 Plasma membrane fractionation

Cells were plated at a density of $6x10^6$ on 45x120 mm plates. Three plates were used per treatment group to total ~$18x10^6$ cells. PC-3 cells were treated with vehicle (100mM NaOH) or STO-609 for 72 hours. LNCaP cells were treated with in a similar manner in addition to +/- 10nM R1881. The fractionation was performed according to manufacturer's instructions. Protein expression was determined with Western Blot analysis.

2.13 Bioinformatic analyses of gene expression in clinical datasets

For the gene expression signature comparisons, transcriptomic profiles of human prostate cancer cohorts were downloaded from The Cancer Genome Atlas (TCGA). Androgen-induced signatures (Hieronymus AR and Nelson AR) were generated from previously defined data [42, 43]. For each of the signatures, an activity score for each sample in each cohort was generated as described previously [44]. Briefly, gene expressions of prostate cancer cohorts were converted to z-scores with respect to normal samples. The activity score for each sample for a signature was evaluated by adding the z-scores of upregulated genes and subtracting the z-scores of downregulated genes.

Correlation between pairs of gene signature activity scores were evaluated using the Pearson Correlation Coefficient as implemented in the Python statistical library SciPy; significance was assessed at $P<0.05$.

2.14 RNA isolation, cDNA synthesis, and RT-qPCR

RNA isolation, cDNA synthesis and qPCR were performed as previously described using 36B4 as a control [26]. All primers used in this study are listed in Table 2.3.

Table 2.3: qPCR Sequences

qPCR Primer	Sequence
SLC1A4 FW	TGCCTATGGGGATCTTTTCA
SLC1A4 RV	TGGACTCTTTCCTCGACCTG
SLC1A5 FW	GGGCAAAGAGTAAACCCACA
SLC1A5 RW	CACCATGGTTCTGGTCTCCT
GLS FW	GGGAATTCACTTTTGTCACGA
GLS RV	TGACTTTACCCTTTGATCACCAC
GLUL FW	ACGCCACTCCAAAAAGAGAA
GLUL RV	AGTGGGAACTTGCTGAGGTG
MYC FW	CACCGAGTCGTAGTCGAGGT
MYC RV	TTTCGGGTAGTGGAAAACCA
36B4 FW	GGACATGTTGCTGGCCAATAA
36B4 RV	GGGCCCGAGACCAGTGTT

Table 2.3	**Continued**
TBC1D4 FW	AGATGGCCTGCCACGTT
TBC1D4 RV	CTCTTTCATGGCCGCTTTAG
FKBP5 FW	TGGGGCTTTCTTCATTGTTC
FKBP5 RV	GCGGAGAGTGACGGAGTC
CXCR4 FW	CTCACTGACGTTGGCAAAGA
CXCR4 RV	AGGAAGCTGTTGGCTGAAAA
CaMKK2 FW	TCCAGACCAGCCCGACATAG
CaMKK2 RV	CAGGGGTGCAGCTTGATTTC
GLUT1 FW	ACTCCTCGATCACCTTCTGG
GLUT1 RV	ATGGAGCCCAGCAGCAA
GLUT2 FW	ATCCAAACTGGAAGGAACCC
GLUT2 RV	CATGTGCCACACTCACACAA
GLUT3 FW	GATGGGCTCTTGAACACCTG
GLUT3 RV	GACAGCCCATCATCATTTCC
GLUT4 FW	CCCCAATGTTGTACCCAAAC
GLUT4 RV	CTTCCAACAGATAGGCTCCG
GLUT5 FW	TGACAGCAGCCACGTTGTA
GLUT5 RV	GCAACAGGATCAGAGCATGA
GLUT6 FW	AACATGATGCTCAGCTTCCG
GLUT6 RV	CTGACCTGCATCTGACCAAA
GLUT7 FW	TGTTGTTGATCAGCAGGGTC

Table 2.3	**Continued**
GLUT7 RV	TGCTGCTTCTATGGTCTTGC
GLUT8 FW	GAAGCACATGAGAAGCAGCA
GLUT8 RV	CTGTGTGCAGCTAATGGTCG
GLUT9 FW	GGTGCCTGCAATGATGAAG
GLUT9 RV	GAGTATCGTGGGCATTCTGG
GLUT10 FW	CAGCAAAGACACAGAGGCAC
GLUT10 RV	GGAAAGTTTGTCCGGCG
GLUT11 FW	AAACAGGATTGCTGCTGACA
GLUT11 RV	CGTGTCTCTGTATCCCCTGG
GLUT12 FW	ACGAGCCATGGCTTTAACTT
GLUT12 RV	CATGGCAGGCCAATAAGAT
GLUT13 FW	AGCCAGCCATATTGCAAGTC
GLUT13 RV	TGTGGCCTACAAATGTTCCA
GLUT14 FW	ATGGCAAAGATCAGAGCTGG
GLUT14 RV	AGGATAGCAGAGAGATGGACAA

2.15 Statistical analysis

Unless otherwise noted, two-sample comparisons were performed using Student's t tests. Multiple comparisons were performed using one-way ANOVA and *post hoc* Dunnett's test with GraphPad Prism, Version 5 (GraphPad Software, La Jolla, CA, USA). Statistically significant changes were determined at the $P<0.05$ level.

CHAPTER 3: The Glutamine Transporters SLC1A4 and SLC1A5 are Downstream Targets of Multiple Oncogenic Signaling Pathways in Prostate Cancer

Despite the known importance of androgen receptor (AR) signaling in prostate cancer, the processes downstream of the receptor that drive disease development and progression remain poorly understood. This knowledge gap has thus limited our ability to treat the cancer. Here, we demonstrate that androgens increase the metabolism of glutamine in prostate cancer cells. This metabolism was required for maximal cell growth under conditions of serum starvation. Mechanistically, AR signaling promoted oncogenic glutamine metabolism by increasing the expression of the glutamine transporters *SLC1A4* and *SLC1A5*, genes commonly overexpressed in prostate cancer. Correspondingly, gene signatures of AR activity correlated with SLC1A4 and SLC1A5 mRNA levels in clinical cohorts. Interestingly, MYC, a canonical prostate cancer oncogene and previously described master regulator of glutamine metabolism, was only a context-dependent regulator of SLC1A4 and SLC1A5 levels. In contrast, rapamycin was able to decrease the androgen-mediated expression of *SLC1A4* and *SLC1A5*, indicating that mechanistic target of rapamycin complex 1 (mTORC1) was needed for maximal AR-mediated glutamine uptake and prostate cancer cell growth. Taken together, these data indicate that three established oncogenic drivers in prostate cancer, AR, MYC and mTOR, function in part by converging to collectively increase the expression of *SLC1A4* and *SLC1A5*, thereby promoting glutamine uptake and subsequent prostate cancer cell growth.

3.1 Introduction

Prostate cancer is the second most commonly diagnosed malignancy amongst men in Western countries [45]. Since the 1940s, it has been known that the development and progression of prostate cancer relies heavily on androgens [46]. Androgens function by binding to and activating a ligand-inducible transcription factor called the androgen receptor (AR). In the context of prostate cancer, AR then, in combination with additional oncogenic signals, promotes prostate cancer cell proliferation and survival [46]. Despite AR's established role in prostate cancer, it is still not completely understood which AR-mediated downstream processes, either alone or in combination with other oncogenic cascades, drive the disease.

Altered cellular metabolism is now recognized as one of the hallmarks of cancer [12]. Although the majority of metabolic cancer research focuses on glucose metabolism, it has become clear that cancer cells also readily metabolize glutamine to fulfill their metabolic needs [47, 48]. In this context, glutamine metabolism can be used to balance the influx and efflux of carbon and nitrogen during the tricarboxylic acid (TCA) cycle. When cells convert glutamine to α-ketoglutarate, a key intermediate of the TCA cycle, to subsequently replenish intermediates of the TCA cycle in part for biosynthetic purposes, this is called glutaminolysis [49]. The oncogene MYC is a known regulator of the initial steps of glutaminolysis, during which MYC up-regulates mitochondrial glutaminase as well as glutamine transporters, promoting glutamine influx and subsequent metabolism [50]. In prostate cancer, MYC alone can function as a transformative factor. In the mouse prostate, *Myc* overexpression promotes prostatic intraepithelial neoplasia (PIN) followed

by invasive adenocarcinoma in a dose-dependent manner [17]. Interestingly, recent work has demonstrated that AR signaling can increase glutamine metabolism in prostate cancer cells [51]. Additionally, AR has been demonstrated to modulate *MYC* expression in a context-dependent manner [52-54]. Given MYC's previously described role in glutamine metabolism, we hypothesized that androgens promoted prostate cancer cell growth in part through augmenting MYC-mediated glutamine metabolism.

3.2 Androgens promote glutamine-mediated prostate cancer cell growth

The majority of cancers depend on increased glucose uptake and glycolysis as first described by Otto Warburg in the 1920s [55]. It is now recognized that many cancers additionally exhibit an increased affinity for the amino acid glutamine, a metabolic shift that is likely a result of altered oncogenic and/or tumor suppressive signaling events that are to date not completely defined. Given AR's predominant role in prostate cancer, we tested whether androgens could augment prostate cancer cell growth in part through increasing glutamine consumption. We hypothesized that this intersection of hormone signaling and glutamine metabolism might be most pronounced under conditions of limited nutrient availability. To test this, we first assessed the effects of androgen treatment (100 pM R1881) on prostate cancer cell growth in the presence or absence of glutamine under conditions with no additional non-essential amino acids, sodium pyruvate or serum. Glucose was still required for cell seeding and survival. In both AR-positive, hormone-responsive LNCaP and VCaP cells, glutamine was consistently required for maximal androgen-mediated prostate cancer cell growth (Fig. 1A). To confirm a requirement for

glutamine metabolism in androgen-mediated prostate cancer cell growth, we next treated cells with or without androgen and with increasing concentrations of compound 968, an inhibitor of glutaminase, a rate-limiting step of glutamine metabolism. Addition of the glutaminase inhibitor significantly decreased androgen-mediated prostate cancer cell growth in both LNCaP and VCaP cells (Fig. 1B). Interestingly, compound 968 had limited effect, particularly in VCaP cells, on basal prostate cancer cell growth, suggesting some specificity to androgen-mediated signaling. Given that androgens appeared to increase glutamine utilization, we then tested whether androgens increased cellular glutamine uptake. As shown in Fig. 1C, androgens significantly increased glutamine uptake in both LNCaP and VCaP cells at the same concentrations that stimulated cell growth. Consistent with these findings, androgens also increased the intracellular levels of the TCA cycle metabolite α-ketoglutarate, a key intermediate of glutamine-mediated anaplerosis/glutaminolysis (Fig. 1D). These results are consistent with our previous mass spectrometry findings that androgen treatment increased intracellular levels of all the TCA intermediates including α-ketoglutarate [37, 51]. Taken together, these results suggest that AR signaling increases glutamine uptake and metabolism to increase prostate cancer cell growth.

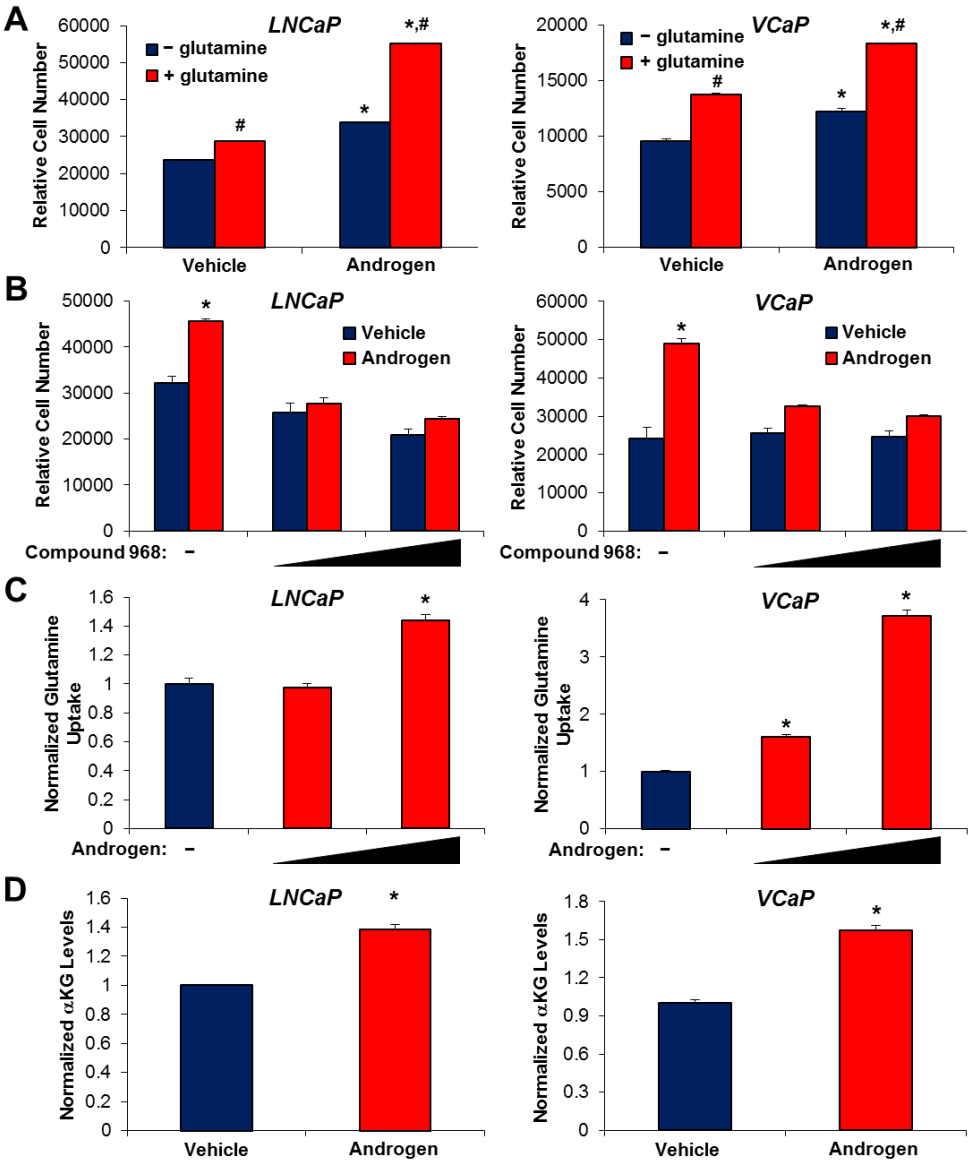

Figure 1. Androgens and glutamine increase prostate cancer cell growth. A, indicated cells were treated with vehicle (ethanol) or androgen (100 pM R1881) for 7 days in serum starved media ± 2 mM glutamine. Cells were lysed and relative cell number was measured using a Hoechst-based DNA dye. B-D, cells were grown in serum starved media supplemented with 2 mM glutamine. B, cells were treated with vehicle (DMSO), 10 μM or 20 μM of Compound 968, a glutaminase inhibitor, followed treatment ± androgen (100 pM R1881) for 7 days. Relative cell numbers were then quantitated as in A. C, cells were treated for 3 days with vehicle, 10 pM or 100 pM androgen (R1881). Spent media was then collected and analyzed for glutamine levels using a bioanalyzer and normalized to cellular DNA content. D, cells were treated for 3 days with vehicle or androgen (100 pM R1881). Intracellular levels of α-ketoglutarate were then quantitated using an enzymatic assay and values were normalized to cellular DNA content. *, significant (P<0.05) changes from vehicle. #, significant (P<0.05) changes from no glutamine.

3.3 AR signaling increases the expression of the glutamine transporters SLC1A4 and SLC1A5

Since androgens increased glutamine uptake, we next tested whether AR signaling increased the expression of glutamine transporters. We focused on the major glutamine transporters *SLC1A4* (also called ASCT1) and *SLC1A5* (commonly referred to as ASCT2) because they were commonly upregulated in prostate cancer in multiple clinical datasets (Table 3.1) while other reported transporters were not 1) expressed in our prostate cancer models, 2) upregulated in prostate cancer clinical datasets or 3) regulated by androgens (ex. *SLC7A5* and *SLC38A5*)[44, 56-62]. In LNCaP cells, androgens increased SLC1A5 mRNA and protein levels (Fig. 2A). While *SLC1A4* was expressed at a high basal level in LNCaP cells, its expression was not further changed following androgen treatment (Fig. 2A). Conversely, both SLC1A4 and SLC1A5 were significantly increased by androgens in VCaP cells (Fig. 2B). To assess whether AR could also regulate these genes in patients, we leveraged two different previously published, curated AR gene signatures of identified AR target genes (genes that were increased in response to androgens and modulated by AR antagonists)[42, 43]. Using a bioinformatics approach, we determined that these AR gene signatures positively correlated with increased mRNA transcript levels of *SLC1A4* and *SLC1A5* in the TCGA clinical dataset (Figs. 2C and D, $R>0$, $P<0.05$), suggesting AR may also regulate the expression of these genes in patients. Of note, while other groups have observed dramatic regulation of glutaminase (*GLS*) by additional oncogenic cascades such as MYC [50], we did not detect a robust, androgen-mediated change in GLS protein levels in either cell model despite the apparent androgen-mediated increase in GLS mRNA levels

in VCaP cells. In addition, the AR gene signatures described above did not correlate with *GLS* expression in patients (P>0.05) nor was *GLS* overexpressed in clinical datasets (data not shown). However, it is important to note that while GLS protein levels did not change significantly in response to androgens, its basal expression was high unlike the expression for *GLUL*, the gene encoding glutamine synthetase (Figs. 2A and B). This is important because glutamine synthetase carries out the reverse reaction of glutaminase. The combined presence of high glutaminase levels and undetectable levels of glutamine synthetase indicates that any increase in glutamine uptake will subsequently lead to the rapid forward movement through glutaminolysis, consistent with our observed increase in α-ketoglutarate levels (Fig. 1D).

Mechanistically, *SLC1A4* and *SLC1A5* appeared to be secondary targets of AR. In support of this, treatment of LNCaP cells for shorter time periods (16 hours compared to the 72 hour treatment shown in Figs. 2A and B), while sufficient to increase the expression of known primary AR target genes such as *FKBP5*, was not sufficient to increase *SLC1A4* or *SLC1A5* expression (Fig. 3A). Likewise, 16-hour androgen treatment did not increase *SLC1A5* expression in VCaP cells, but did increase FKBP5 mRNA levels (Supplementary Fig. S1B). Although androgens increased *SLC1A4* expression at 16 hours posttreatment, this induction was blocked by an inhibitor of protein translation, cycloheximide. In contrast, cycloheximide had no effect on androgen-mediated *FKBP5* expression (Fig. 3B). Collectively, these results indicate that AR signaling increases the expression of the glutamine transporters *SLC1A4* and *SLC1A5* via an indirect mechanism.

Table 3.1. Fold increased expression of the glutamine transporters *SLC1A4* and *SLC1A5* in prostate cancer samples compared to benign controls in clinical datasets.

Transporter	Dataset	Fold Change	*P* value	# of samples
SLC1A4	Vanaja et al.	1.687	7.37E-4	40
	Holzbeierlein et al.	1.175	.011	54
	Taylor et al.	1.123	.003	185
	Welsh et al.	1.405	.004	34
	Wallace et al.	1.486	.027	89
	Singh et al.	1.476	.034	102
	Arredouani et al.	1.513	.012	21
SLC1A5	Magee et al.	1.518	.018	15
	Singh et al.	2.106	3.24E-4	102
	Wallace et al.	1.745	5.11E-4	89
	Welsh et al.	1.399	.007	34

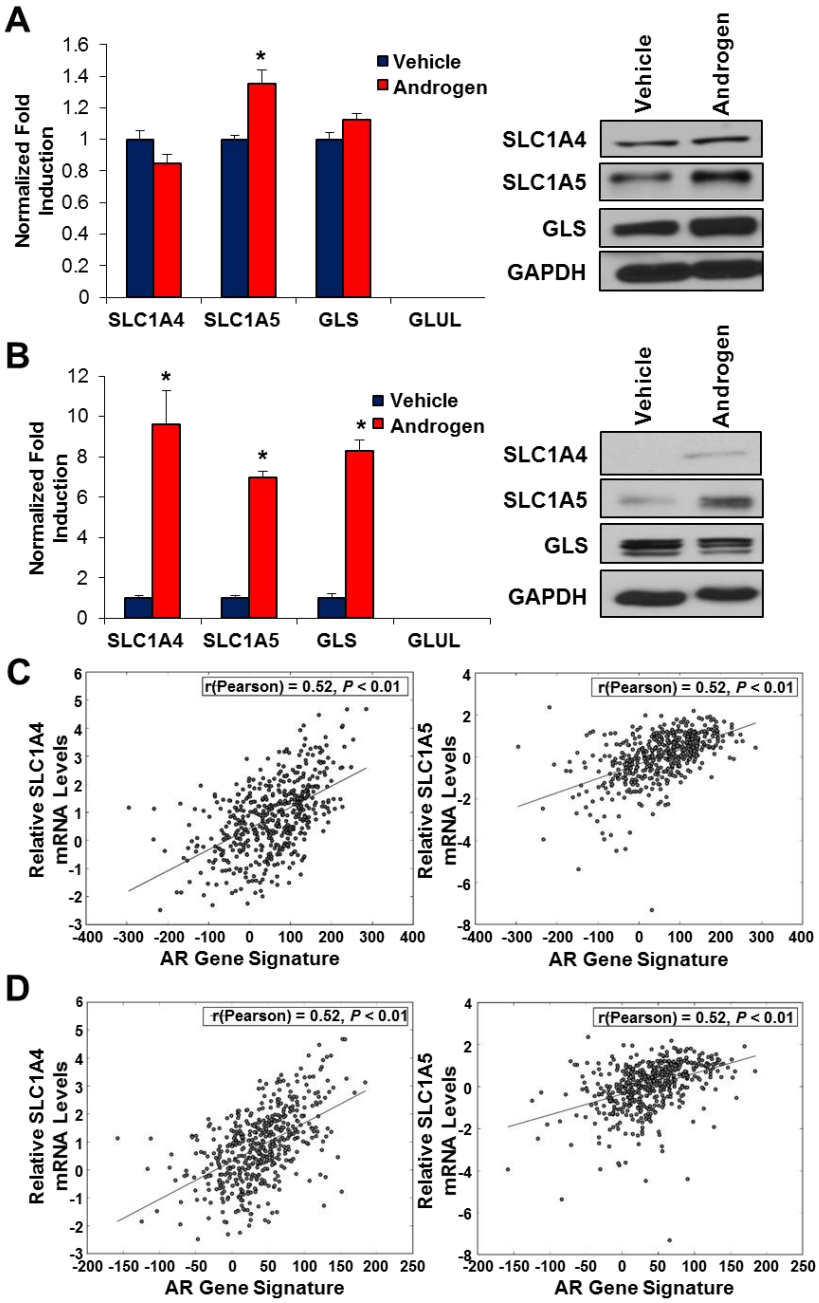

Figure 2. Androgen receptor signaling increases the expression of the glutamine transporters *SLC1A4* and *SLC1A5*. LNCaP (A) and VCaP (B) cells were treated for 3 days with either vehicle or androgen (100 pM R1881) in serum starved media containing 2 mM glutamine. *Left*, qPCR was used to quantify gene expression and normalized to 36B4 mRNA levels and vehicle control. Note, *GLUL* expression was not detected. *, significant (P<0.05) changes from vehicle. *Right*, Western blot analysis was done on whole cell lysates. GAPDH was used as a loading control. C and D, expression of *SLC1A4* or *SLC1A5* correlated significantly with two, distinct, previously described AR gene signatures (C, Hieronymus et al(18) and D, Nelson et al(19)) in transcriptomic profiles of prostate cancer patients from TCGA. Similar results were obtained using these AR activity signatures across multiple clinical cohorts.

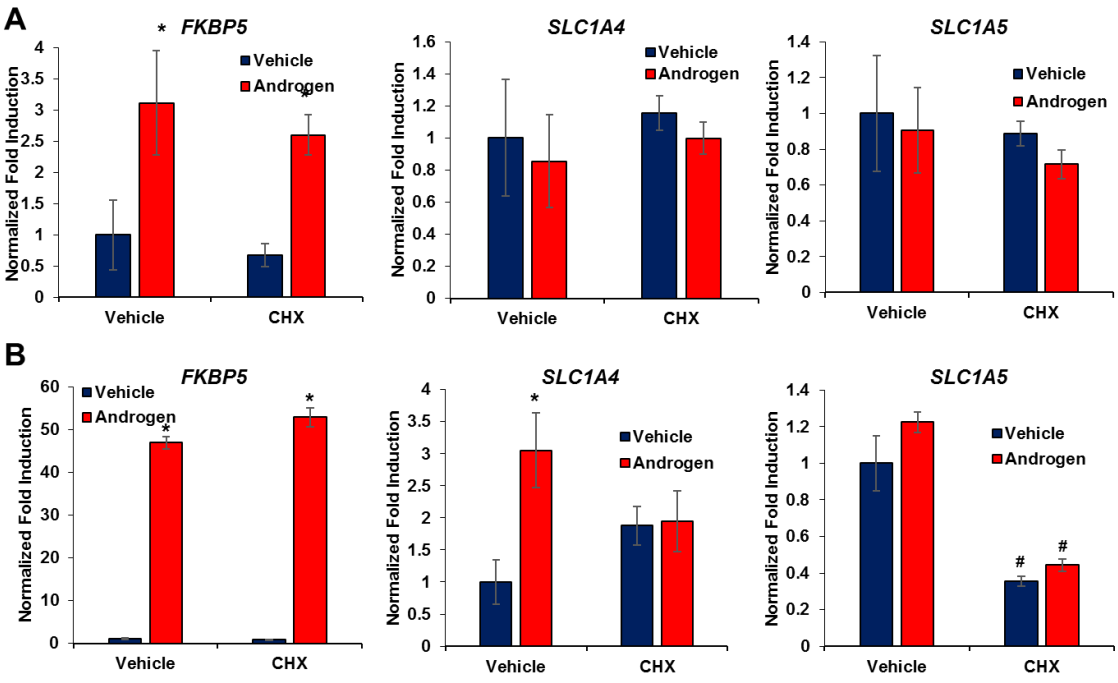

Figure 3. *SLC1A4* and *SLC1A5* are likely secondary targets of AR in prostate cancer cells. LNCaP (A) and VCaP (B) cells were treated with 1 µg/ml cycloheximide (CHX) and either vehicle or androgen (100 pM) for 16 hours. Cells were lysed and cDNA collected for qPCR to analyze the mRNA levels of the indicated genes. Results were normalized to 36B4 mRNA levels. *, significant (P<0.05) changes from vehicle. #, significant (P<0.05) changes from no cycloheximide.

3.4 Functional role of SLC1A4 and SLC1A5 in hormone-sensitive prostate cancer cells

Given the AR-mediated regulation of *SLC1A4* and *SLC1A5* (Fig. 2) and the requirement for glutamine for maximal androgen-mediated prostate cancer cell growth (Fig. 1), we next wanted to test the functional roles of these glutamine transporters. To do this, we assessed the impact of silencing *SLC1A4* or *SLC1A5* expression in prostate cancer cells (Fig. 4A and Fig. 5A) on glutamine uptake (Fig. 4B) and cell growth (Fig. 4C). Knockdown of *SLC1A5* consistently decreased androgen-mediated glutamine uptake (Fig. 4B) and cell growth (Fig. 4C) in both LNCaP and VCaP cells. Again, there were modest effects on basal cell growth, indicating some specificity for androgen-mediated signaling. Knockdown of *SLC1A4* with siRNA #1 also decreased both androgen-mediated glutamine uptake (Fig. 4B) and cell growth (Fig. 4C) in VCaP cells. Unfortunately, despite multiple attempts, we were unable to get effective knockdown of *SLC1A4* with siRNA #2 in VCaP cells at either the mRNA (Fig. 5B) or protein level (Fig. 3A). Correspondingly, this siRNA then functioned as an additional negative control as no effect was observed on either glutamine uptake or cell growth as would be expected. Surprisingly, knockdown of *SLC1A4* (Fig. 4A and Fig. 5A) decreased glutamine uptake (Fig. 4B) and cell growth (Fig. 4C) in LNCaP cells. This was unexpected because androgens did not increase *SLC1A4* expression in LNCaP cells (Fig. 2A and Fig. 5A). Thus, it appears that in a cell-type dependent manner AR signaling may potentiate SLC1A4 activity through additional mechanisms, unknown at this time, beyond gene expression (ex. posttranslational modifications, etc).

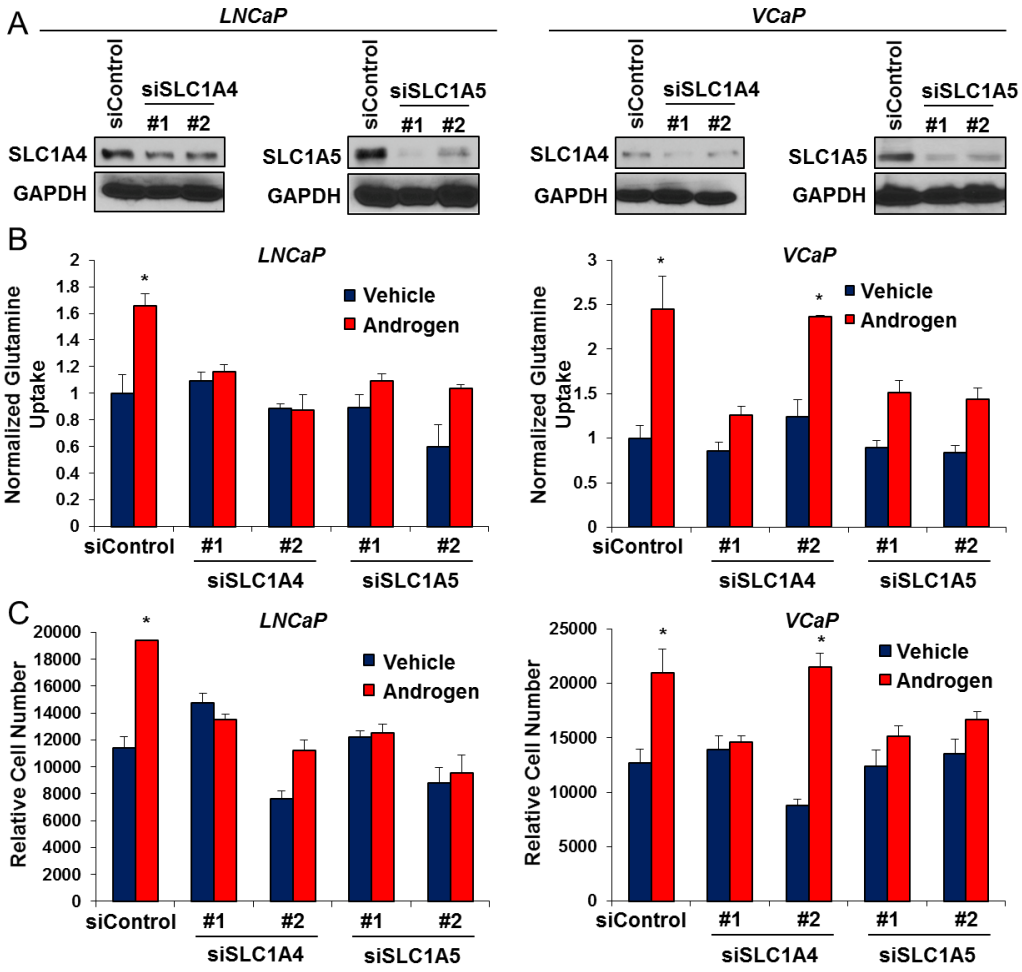

Figure 4. SLC1A4 and SLC1A5 are required for maximal androgen-mediated prostate cancer cell growth. A, prostate cancer cells were transfected for 3 days with indicated siRNAs. Cells were then harvested and lysates were subjected to Western blot analysis. B and C, prostate cancer cells were transfected with indicated siRNAs and treated for 7 days with vehicle or 100 pM R1881 (androgen). Then, glutamine uptake (B) or cell numbers (C) were assessed as described in Figure 1. *, significant (P<0.05) changes from vehicle.

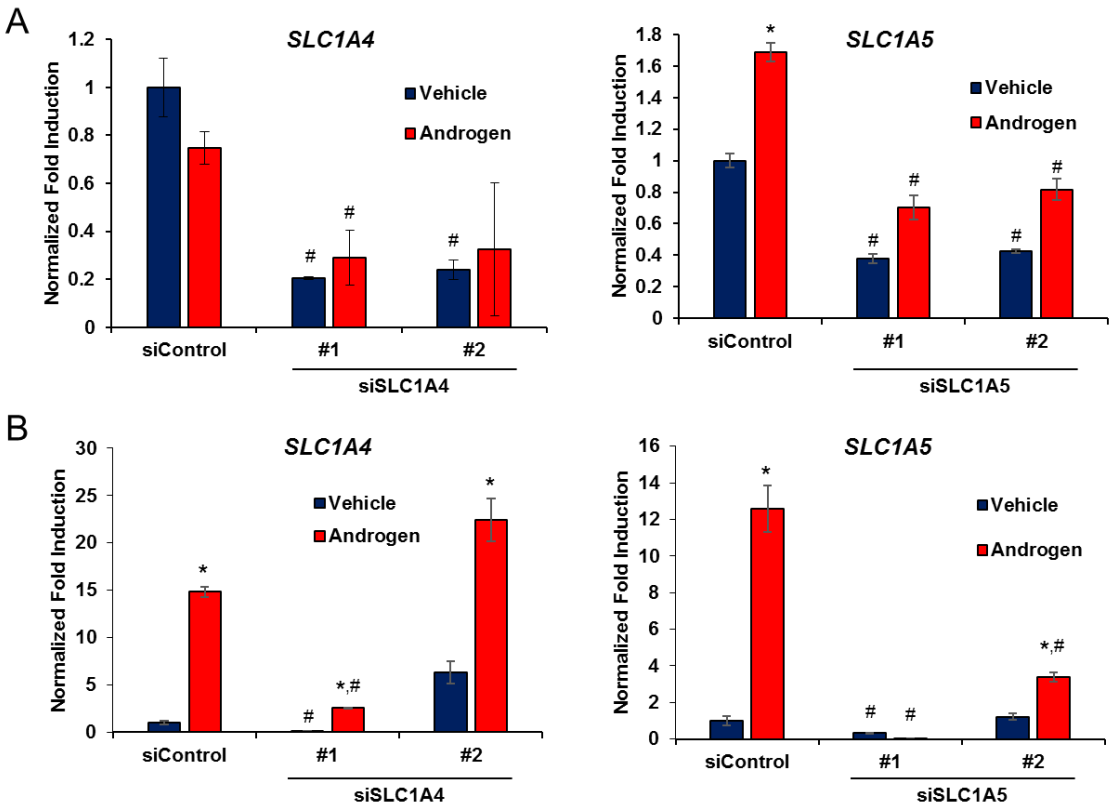

Figure 5. **qPCR knockdown controls for Figure 4.** LNCaP (A) or VCaP (B) prostate cancer cells were transfected for 3 days with indicated siRNAs and treated ± androgen (100 pM R1881). RNA was then extracted and subjected to qPCR analysis. Results are normalized to 36B4 levels. *, significant (P<0.05) changes from vehicle. #, significant (P<0.05) changes from siControl.

3.5 MYC is a contextual regulator of SLC1A5 in prostate cancer cell models

A master regulator of oncogenic glutamine metabolism is MYC [47, 49, 50, 63], a canonical oncogene in prostate cancer [17, 64, 65]. Previous work has suggested that AR signaling could modulate *MYC* (c-MYC) expression [66-68]. As such, we hypothesized that androgens promoted prostate cancer cell growth through MYC-dependent glutaminolysis. Specifically, we sought to determine what role MYC played, if any, in the regulation of *SLC1A4* and *SLC1A5* expression and function under our conditions of serum starvation. To facilitate these studies, we created stable derivatives of LNCaP cells that could inducibly express an shRNA targeting *MYC* in the presence of doxycycline (LNCaP-shMYC)(Fig. 6). Here, androgens increased the protein levels of MYC and SLC1A5 but not SLC1A4 (Fig. 7A), consistent with our earlier results (Fig. 2A). Doxycycline-mediated knockdown of *MYC* decreased androgen-mediated SLC1A5 protein levels but had no effect on SLC1A4 or basal SLC1A5 levels (Fig. 7A). In contrast to previous work done in PC-3 prostate cancer cells [50], silencing of *MYC* also had no impact on GLS protein levels. Regardless, *MYC* knockdown decreased both glutamine uptake (Fig. 7B) and prostate cancer cell growth (Fig. 7C). The significant decrease in basal glutamine uptake and trend towards decreased baseline cell growth following *MYC* knockdown indicate that MYC likely has additional functions in LNCaP cells besides the regulation of *SLC1A5* that contribute to glutamine uptake and, perhaps not surprisingly given MYC's known role in proliferation, cell growth.

Unfortunately, we were unable to create stable derivatives of VCaP cells using the same lentiviral approach as we have found that these cells are particularly resistant to lentiviral modulation. As an alternative, we silenced *MYC* expression using two different siRNAs and assessed the effect of *MYC* knockdown on *SLC1A4* and *SLC1A5* expression and androgen-mediated glutamine uptake and cell growth. As previously reported [69], androgen treatment reduced MYC protein levels in VCaP cells (Fig. 7D). Similar to LNCaP cells, *MYC* knockdown had no consistent effect on SLC1A4 or GLS protein levels (Fig. 7D). In direct contrast to the regulation we observed in LNCaP cells (Fig. 7A), knockdown of *MYC* had no effect on androgen-mediated SLC1A5 levels in VCaP cells (Fig. 7D). Consistent with these findings, depletion of MYC in VCaP cells did not change basal or androgen-mediated glutamine uptake (Fig. 7E) or cell growth (Fig. 7F). Thus, MYC appears dispensable for glutamine uptake and cell growth in VCaP cells but is required for androgen-mediated *SLC1A5* expression, glutamine uptake and cell growth in LNCaP cells under our conditions of limited nutrient availability. Together, these data indicate that MYC acts as contextual regulator of glutamine metabolism in prostate cancer cells.

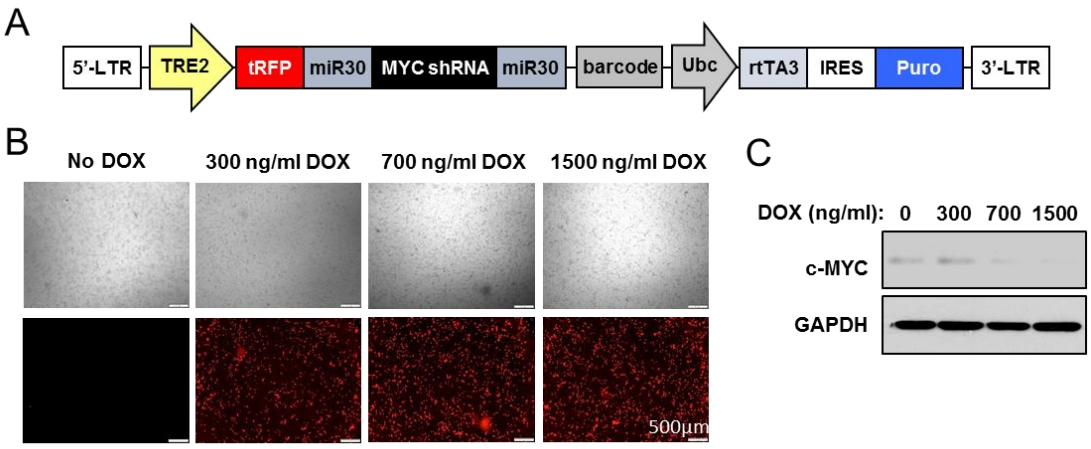

Figure 6. Creation of stable LNCaP cells that express an shRNA targeting MYC under the control of a doxycycline (DOX)-inducible promoter. A, construct used to created LNCaP-shMYC inducible stable cell line. B and C, validation of LNCaP-shMYC stable cell line. Cells were cotreated for 3 days with increasing concentrations of doxycycline. Cells were then (B) imaged using phase contrast (*top*) or immunofluorescence (*bottom*) microscopy or (C) lysed and subjected to Western blot analysis. White bars indicate a distance of 500µm.

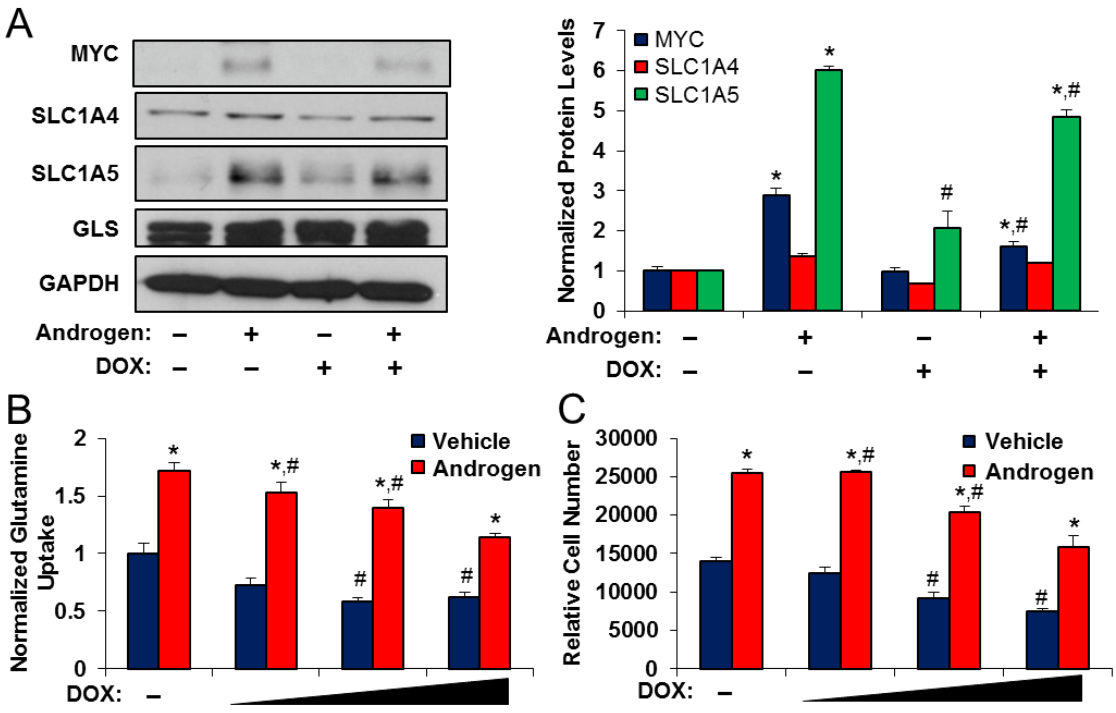

Figure 7. Regulation of MYC levels by AR and regulation of glutamine transporter levels by MYC are cell-type dependent. A, LNCaP stable cells that inducibly express an shRNA targeting MYC (LNCaP-shMYC) following doxycycline (DOX) treatment were treated for 3 days ± 700 ng/ml DOX with vehicle or 100 pM R1881 (androgen). Cells were then lysed and subjected to Western blot analysis. *Left*, representative blots. *Right*, densitometry summary of Western blot repeats (n =3). Data are normalized to experimental GAPDH loading control. B-C, LNCaP-shMYC cells were treated with a dose response of DOX (0, 300, 700, 1500 ng/ml) ± androgen (100 pM R1881) for 3 days and then assayed for glutamine uptake (B) or proliferation (C) as described in Figure 1. A-C, *, significant (P<0.05) changes from vehicle (no androgen). #, significant (P<0.05) changes from no DOX.

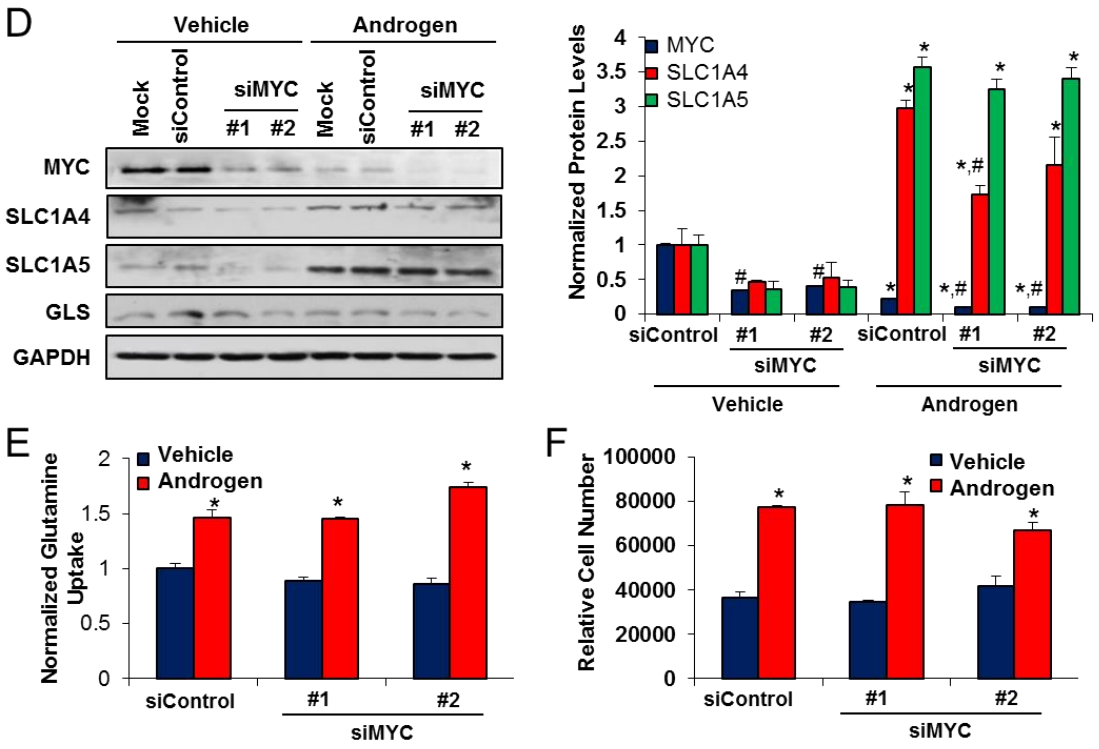

Figure 7 (continued). Regulation of MYC levels by AR and regulation of glutamine transporter levels by MYC are cell-type dependent. D-F, VCaP cells were transfected with mock or siRNAs targeting scramble control or MYC (#1 and #2) and then treated ± androgen (100 pM R1881) and subjected to Western blot analysis (D) or assessed for glutamine uptake (E) or proliferation (F). D-F, *, significant (P<0.05) changes from vehicle (no androgen). #, significant (P<0.05) changes from siControl.

3.6 mTOR stimulates expression of the glutamine transporters SLC1A4 and SLC1A5

Given MYC's previously described role as a master regulator of glutamine metabolism, it was surprising to us that MYC did not have a more pronounced role in our prostate cancer cell models. Hence, we suspected additional pathways that are 1) hyperactivated in prostate cancer and 2) known to be influenced by AR signaling could regulate *SLC1A4* and *SLC1A5* and therefore oncogenic glutamine metabolism. The mechanistic target of rapamycin (mTOR), formerly known as the mammalian target of rapamycin, is one of the most commonly activated oncogenic proteins in prostate cancer and has previously been shown to be regulated by AR signaling [36, 70, 71]. Its role as a sensor for amino acid levels made it an ideal candidate to test. As shown in Figs. 8A and B, treatment with androgens increased the expression of *SLC1A5* in LNCaP cells and *SLC1A4* and *SLC1A5* in VCaP cells, consistent with our results described in Fig. 2. As previously reported, androgens also increased mTOR signaling in prostate cancer cells as assessed by the phosphorylation of S6, a well-characterized downstream target of mTOR signaling [36, 71]. Co-treatment with rapamycin, a selective inhibitor of the mTORC1 complex, decreased both basal and androgen-mediated *SLC1A5* expression in LNCaP cells and suppressed the androgen-mediated induction of *SLC1A4* and *SLC1A5* in VCaP cells (Figs. 8A and B). This effect appeared to not be due to any changes in MYC (Fig. 9). The effects of rapamycin on basal *SLC1A5* expression are likely due to the fact that LNCaP cells have high basal mTOR signaling as a result of a mutation in phosphatase and tensin homolog (*PTEN*) that renders this upstream tumor suppressor inactive [72]. Conversely,

VCaPs express wild-type *PTEN* and do not have constitutively active phosphoinositide 3-kinase (PI3K)/Akt signaling [73]. To our knowledge, this is the first description of mTOR regulation of *SLC1A4* or *SLC1A5* expression in prostate cancer. Consistent with this regulation and with the described roles for SLC1A4 and SLC1A5 above, rapamycin also blocked both androgen-mediated glutamine uptake (Fig. 8C) and cell growth (Fig. 8D).

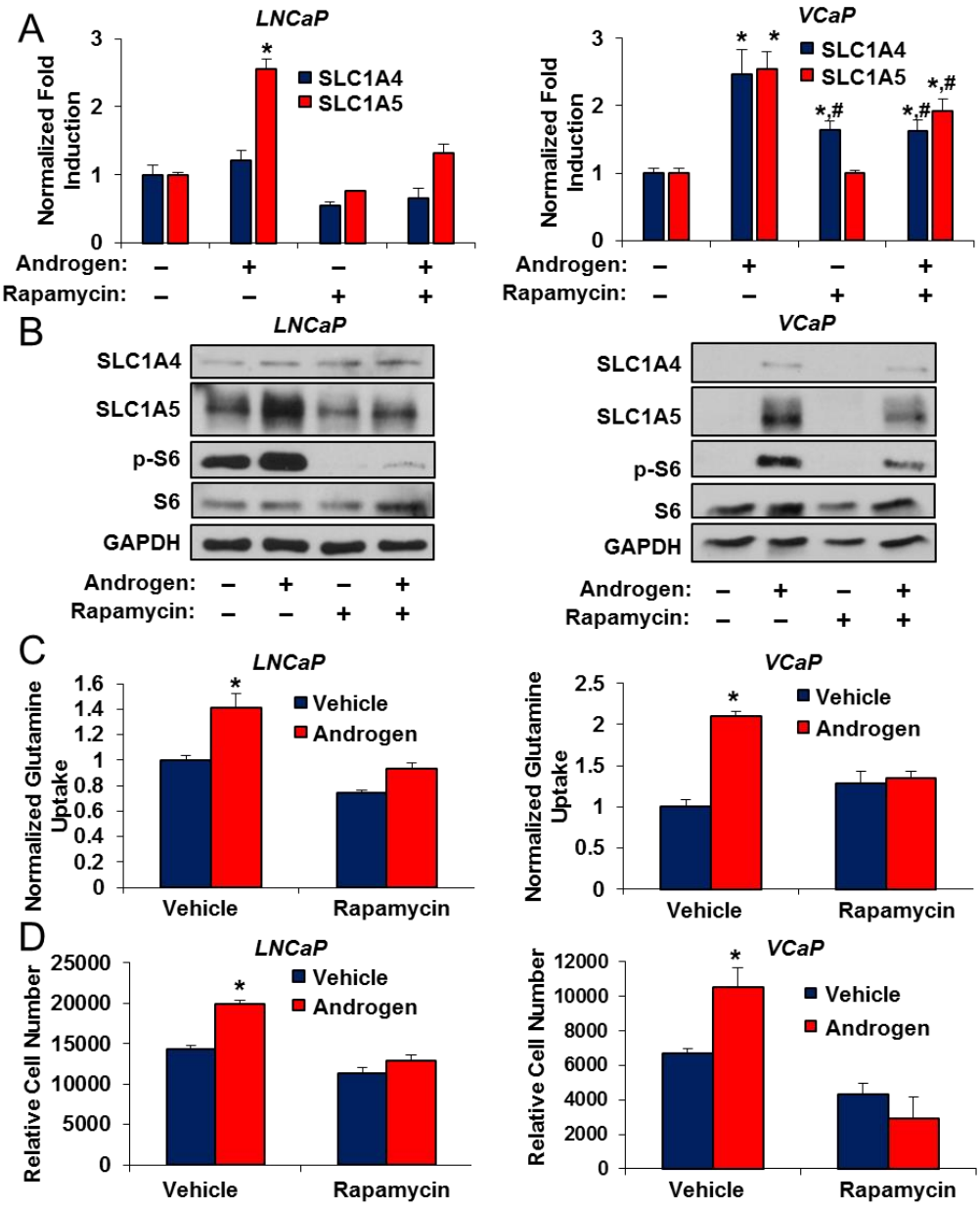

Figure 8. mTOR activity increases *SLC1A4* and *SLC1A5* expression, glutamine uptake and cell growth. A and B, prostate cancer cells cells were treated with vehicle or 10 nM rapamycin in addition to vehicle or androgen (100 pM R1881) for 3 days in serum-starved media with 2 mM glutamine. Cells were then lysed and subjected to qPCR (A) or Western blot (B) analysis. *, significant (P<0.05) changes from vehicle (no androgen). #, significant (P<0.05) changes from vehicle (no rapamycin). C and D, cells were treated as in A and B. C, glutamine uptake was then quantitated and normalized as described in Fig. 1. D, cell numbers were then also quantitated as described in Fig. 1. *, significant (P<0.05) changes from vehicle (no androgen).

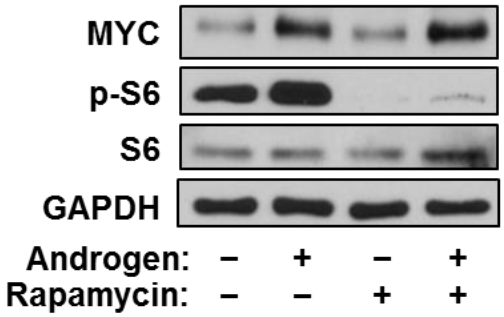

Figure 9. Androgens increase MYC levels independent of mTOR signaling in LNCaP cells. LNCaP cells were cotreated ± 10 nM rapamycin and either vehicle or androgen (100 pM R1881) for 3 days. Whole cell lysates were collected and subjected to Western blot analysis.

3.7 Discussion

Prostate cancer has an atypical metabolism. Benign prostate is characterized by the existence of a truncated TCA cycle that occurs as a result of high zinc levels in prostatic epithelial cells [11, 74]. Zinc inhibits mitochondrial aconitase, shunting carbons that entered the TCA cycle out in the form of secreted citrate [11]. One of the first transformation events that occurs during the evolution of prostate cancer is a drop in intracellular zinc levels due to the decreased expression of zinc transporters [11, 74]. This decreased zinc leads to a derepression of aconitase that ultimately increases forward flux through the TCA cycle and augments oxidative phosphorylation (OXPHOS). To date, the majority of attention has focused on glucose's contribution to cancer metabolism. However, it is now recognized that glutamine metabolism may also contribute to oncogenesis under certain circumstances [27, 31, 47]. Here, we demonstrate that under conditions of serum starvation, prostate cancer cells readily increase glutamine uptake and metabolism that is required for maximal cell growth (Fig. 1).

While many of the oncogenic pathways that govern sugar metabolism have been elucidated (ex. PI3K-Akt), those controlling glutamine metabolism are still emerging. Previous work has demonstrated that AR increases glutaminolysis in prostate cancer cells [51]. Here, we demonstrate that AR-mediated glutamine metabolism is also augmented by the increased uptake of the amino acid through indirectly increasing the expression of two transporters, *SLC1A4* and *SLC1A5* (Figs. 1-4). Interestingly, AR promoted *SLC1A4* and *SLC1A5* expression in a cell-type specific manner through several mechanisms including MYC- and mTOR-dependent as well as -independent pathways (Fig. 10). Further, both

MYC and mTOR signaling are prevalent oncogenic cascades in prostate cancer that can be stimulated through AR-independent mechanisms [70, 75]. Hence, SLC1A4 and SLC1A5 appear to serve as functional, downstream conduits for AR, MYC and mTOR.

Analyses of several cancer types indicate that the oncogene MYC could function as a master regulator of glutamine metabolism through directly increasing the expression of *SLC1A5* and indirectly increasing the levels of GLS [47, 49, 50]. The MYC-mediated modulation of GLS occurs through the suppression of miR-23a/b [50]. Although we also observed MYC-mediated expression of *SLC1A5* in LNCaP cells, we did not detect significant changes in GLS protein levels in either LNCaP or VCaP cells (Fig. 7). This work contrasts previous work in PC-3 prostate cancer cells that demonstrated that MYC was required for stabilizing GLS protein levels [50]. These variances may be due to the differences in the cell types as PC-3 cells more closely resemble small cell-like or neuroendocrine-like prostate cancer cells whereas LNCaP and VCaP cells are classical adenocarcinoma cells that are more prevalently observed in the clinic [76]. Previous studies suggest a complex relationship between AR and MYC in the prostate [53, 54, 66-68]. Evidence suggests that in the normal/benign prostate, AR inhibits *MYC* expression [53, 54]. Conversely, as prostatic epithelial cells become transformed, the AR-mediated downregulation of MYC is either lost or reversed [54]. In this regard, the AR/MYC relationship in VCaP cells appears to still resemble what is observed in the benign prostate while the connection appears to have already switched in LNCaP cells where AR increases MYC (Figs. 7 and 10). What exactly causes this regulatory switch is still poorly understood.

Because of mTOR's 1) established role in amino acid metabolism [77] and 2) known regulation by AR [36, 71], we postulated that AR may also influence glutamine uptake through mTOR. Consistent with this idea, we found that rapamycin decreased androgen-mediated SLC1A5 mRNA and protein levels (Fig. 8). In addition, rapamycin impaired the androgen-mediated *SLC1A4* expression in VCaP cells (Fig. 8). These data indicate that mTOR, and more specifically the mTORC1 complex, can also potentiate glutaminolysis. Interestingly, others have shown that glutamine flux through the SLC1A5 transporter activates mTOR signaling in breast cancer [78]. Taken together, mTOR signaling and glutamine uptake may form a positive feedback loop.

We suspect that our findings may have translational significance. There is current interest in blocking glutamine metabolism in cancer [47]. To that end, inhibitors of glutaminase such as CB-839 are in early phase clinical trials (NCT02071927, NCT02944435, NCT02071888, NCT02861300, NCT02771626, NCT02071862). Targeting glutamine transporters may offer an alternative therapeutic approach. This approach would be advantageous because it targets the potential pathological meeting point of three driver cascades (AR, MYC and mTOR). Further, as cell surface molecules, these transporters may be more readily druggable. Accordingly, novel inhibitors of SLC1A5 have recently been described [79]. In addition, several groups are evaluating glutamine analogs for their value in positron emission tomography (PET) imaging of cancer [80]. Our data here could inform radiologists regarding specific cellular signaling events that may influence results. Earlier this year (May 2016), the U.S. Food and Drug Administration approved Axumin™, also known as fluciclovine or anti-1-amino-3-18F-

fluorocyclobutane-1-carboxylic acid (FACBC), for PET imaging of men with suspected prostate cancer recurrence. Fluciclovine is an amino acid analog that has been reported to be taken up into cells in part by SLC1A5-mediated transport [81]. The uptake of fluciclovine appears to correlate with the levels of PSA/*KLK3*, an AR-regulated biomarker. Our results shown here would strongly suggest that the mechanistic explanation for this phenomenon is due in part to the AR-mediated expression of *SLC1A5* and possibly *SLC1A4*. In future, it would be of interest to determine whether other regulators of these transporters such as mTOR signaling also track with increased fluciclovine PET imaging sensitivity.

Our study examined the regulation and role of two transporters, SLC1A4 and SLC1A5, in the earliest steps of glutaminolysis, namely glutamine uptake. It still remains to be determined how glutamine is subsequently metabolized by the cancer cell. Glutamine can be used in anaplerotic reactions to refill TCA cycle intermediates [47, 80]. Accordingly, proliferating cells often metabolize glutamine to restore parts of the TCA cycle in part for biosynthetic purposes [49]. Carbons and nitrogens are syphoned off throughout this process to contribute to the synthesis of nucleic acids, other amino acids and hexosamines, the latter of which can contribute to posttranslational modifications. Additionally, glutamine, via its metabolism through glutamate, can be used for the biosynthesis of glutathione and therefore help modulate oxidative stress. Alternatively, nitrogens can also be released in the form of ammonia. Certainly, future studies using stable isotope tracing will help delineate how glutamine is further metabolized and for what it is being used.

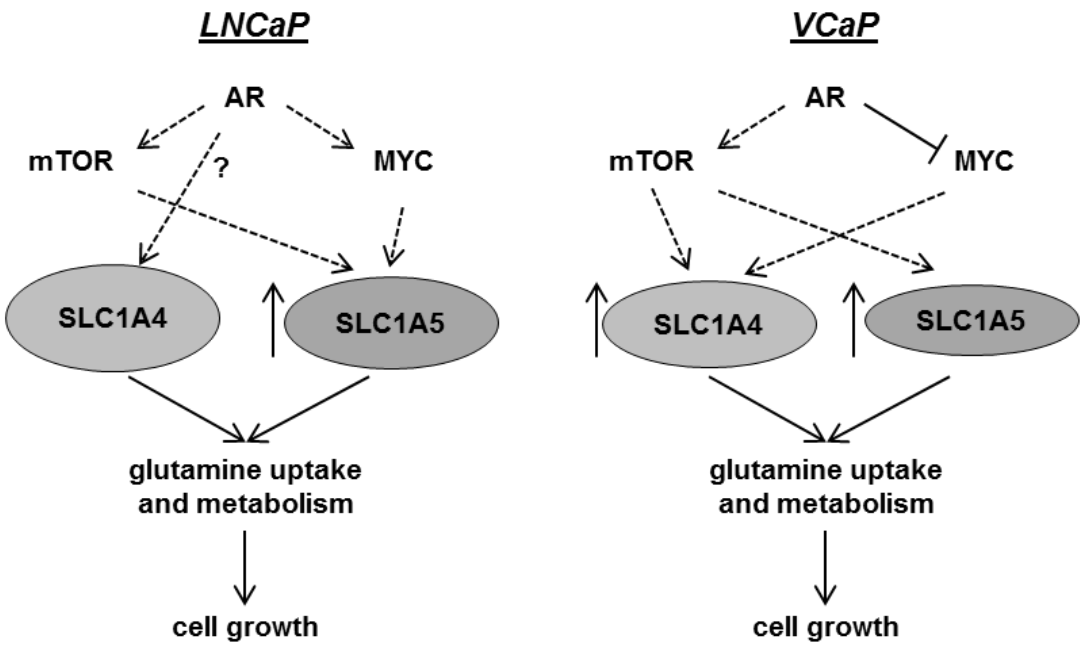

Figure 10. Working model of the regulation of the SLC1A4 and SLC1A5 glutamine transporters by AR, mTOR and MYC signaling in prostate cancer cells. Prostate cancer cells can augment cell growth by increasing glutamine metabolism. This increased metabolism can be initiated by various oncogenic signaling cascades that, in a cell-type dependent manner, increase the expression of *SLC1A4* and *SLC1A5*, two of the primary glutamine transporters. In addition, AR may also increase SLC1A4 function through an unknown mechanism.

CHAPTER 4: GLUT12 promotes prostate cancer cell growth and is regulated by androgens and CaMKK2 signaling

Despite altered metabolism being an accepted hallmark of cancer, it is still not completely understood which signaling pathways regulate these processes. Given the central role of androgen receptor (AR) signaling in prostate cancer, we hypothesized that AR could promote prostate cancer cell growth in part through increasing glucose uptake via the expression of distinct glucose transporters. Here, we determined that AR directly increased the expression of *SLC2A12*, the gene that encodes the glucose transporter GLUT12. In support of these findings, a gene signature of AR activity correlated with *SLC2A12* expression in multiple clinical cohorts. Functionally, GLUT12 was required for maximal androgen-mediated glucose uptake and cell growth in LNCaP cells. Knockdown of GLUT12 also decreased the growth of C4-2, 22Rv1 and AR-negative PC-3 cells. This latter observation corresponded with a significant reduction in glucose uptake, indicating that additional signaling mechanisms could augment GLUT12 function in an AR-independent manner. Interestingly, GLUT12 trafficking to the plasma membrane was modulated by calcium/calmodulin-dependent protein kinase kinase 2 (CaMKK2)-5'-AMP-activated protein kinase (AMPK) signaling, a pathway we previously demonstrated to be a downstream effector of AR. Inhibition of CaMKK2-AMPK signaling decreased GLUT12 translocation to the plasma membrane by inhibiting the phosphorylation and, surprisingly, expression of TBC1D4, a known regulator of glucose transport. Further, expression of *CAMKK2* correlated with *TBC1D4* expression in prostate cancer patient samples. Taken together, these data demonstrate that prostate cancer cells can increase the functional levels

of GLUT12 through multiple mechanisms to promote glucose uptake and subsequent cell growth.

4.1 Introduction

Altered cell metabolism is now acknowledged as one of the emerging hallmarks of cancer [12]. Common amongst most cancer cells is an increased ability to take up and metabolize large amounts of glucose to help meet the growing cells' energetic and anabolic demands. The first rate-limiting step in glucose metabolism is the cellular uptake of the sugars [82]. This is done primarily by a family of facilitative glucose transporters (GLUTs). To date, fourteen glucose transporters have been identified in humans [83]. While many of these transporters have known functions in basic physiology (e.g., GLUT4 functions in muscle), growing evidence indicates roles for some of these transporters in cancer [83-85]. To that end, multiple members of the glucose transporter family are overexpressed in various cancer types (reviewed in [83]). Not surprisingly, several oncogenic and tumor suppressive signaling pathways have been shown to regulate glucose uptake [86]. The delineation of which specific transporters play a functional role in the disease and the determination of how they are regulated are needed to improve our understanding of this important aspect of pathogenic cell biology.

Prostate cancer is the most commonly diagnosed non-cutaneous cancer in US men [45]. One of the central drivers of the disease is the androgen receptor (AR). Despite AR's established role in prostate cancer [46], our understanding of the specific downstream events that are regulated by AR and that promote the disease is not complete. Recent work from

both our laboratory as well as others has demonstrated that androgen signaling increases glucose metabolism [36, 37, 67, 87, 88]. This augmented carbohydrate metabolism was mediated in part through a signaling cascade involving the calcium/calmodulin-dependent protein kinase kinase 2 (CaMKK2) and the 5'-AMP-activated protein kinase (AMPK)[37, 87]. Given the requirement for initial glucose uptake prior to its subsequent metabolism, we hypothesized that AR signaling promoted glucose metabolism by increasing the expression and/or activity of select glucose transporters in prostate cancer. We further speculated that AR could increase glucose uptake in part through CaMKK2-AMPK signaling. Here, we sought to determine whether androgens increased the expression of any of the glucose transporters that were overexpressed in prostate cancers relative to benign tissue. In addition, we wanted to determine the mechanism(s) behind this regulation to aide future efforts to treat the disease.

4.2 Identification of *SLC2A12* as a direct AR-regulated gene that is overexpressed in prostate cancer

We first sought to determine if AR signaling, a major driver of prostate cancer [46], increased the expression of members of the glucose transporter family. To test this, AR+ LNCaP prostate cancer cells were treated for 24 or 72 hours with vehicle or the synthetic androgen R1881. Cells were then lysed and subjected to qRT-PCR analysis to quantitate the mRNA levels of the 14 known GLUT family members. Of the 14 members, only two (*SLC2A2* (GLUT2) and *SLC2A14* (GLUT14)) could not be detected at the mRNA level

(Fig. 11A). Three family members (*SLC2A3* (GLUT3), *SLC2A10* (GLUT10) and *SLC2A12* (GLUT12)) were found to be androgen responsive (Fig. 11A). While androgen treatment increased *SLC2A3* mRNA levels, this observation did not correspond to an increase in the functional protein levels of GLUT3 as siRNA-mediated knockdown of *SLC2A3* had no effect on androgen-mediated glucose uptake (D Frigo, unpublished observations). These data are consistent with previous reports of high *SLC2A3* mRNA levels being detected in the absence of GLUT3 protein expression [89]. Like *SLC2A3*, androgens also increased the mRNA levels of *SLC2A10* twofold (Fig. 11A). However, the baseline mRNA levels of *SLC2A10* were low (Ct values ~31-32 cycles) and, more importantly, *SLC2A10* expression did not correlate with prostate cancer in clinical samples [44, 56, 58, 90, 91]. Conversely, androgens increased the expression of *SLC2A12* (Fig. 11A), the expression of which was also significantly elevated in prostate cancer patient samples relative to benign controls in multiple clinical cohorts (Table 4.1) [44, 56, 58, 90, 91]. Consistent with these findings, androgen treatment increased the expression of *SLC2A12* in additional AR+ prostate cancer cell models (LAPC4 and VCaP; Figure 12). Androgens also increased the protein levels of GLUT12 with peak expression being observed ~24 h after initial treatment (Fig. 11B), indicating that androgens promoted a pulse of GLUT12 synthesis. To assess whether AR could also regulate *SLC2A12* in patients, we leveraged a previously published, curated AR gene signature of identified AR target genes (genes that were increased in response to androgens and modulated by AR antagonists) [42]. Using an informatics approach, we determined that this AR gene signature positively correlated with increased *SLC2A12* mRNA transcript levels in a well-known clinical prostate cancer cohort (Fig. 11C),

suggesting AR also regulates the expression of *SLC2A12* in patients [44]. In support of this, similar results were obtained using this AR activity signature as well as an additional AR activity signature [43] across multiple clinical cohorts [44, 91] including The Cancer Genome Atlas (TCGA) [92].

To determine how androgens increased *SLC2A12* expression, we first evaluated the role of AR to verify that this was a receptor-mediated event. Knockdown of *AR* using two separate, validated siRNAs [36, 41, 93] blocked the androgen-mediated expression of *SLC2A12* (Fig. 13A). As further validation, co-treatment of prostate cancer cells with the antiandrogen enzalutamide suppressed androgen-mediated *SLC2A12* expression (Fig. 13B and Fig. 14). Enzalutamide's inhibition of androgen-mediated *SLC2A12* expression was overcome by saturating concentrations of the agonist, confirming AR's role (Fig. 13B).

Next, we wanted to determine whether *SLC2A12* was a direct AR target. To do this, we initially co-treated prostate cancer cells ± androgen in the presence or absence of cycloheximide, an inhibitor of translation (Fig. 13C). As previously described, cycloheximide treatment did not block androgen induction of *FKBP5*, an established direct target of AR [93, 94]. Conversely, cycloheximide did inhibit the androgen-mediated expression of *CXCR4*, a known indirect target of AR [94]. Similar to *FKBP5*, cycloheximide treatment did not impair the androgen-mediated expression of *SLC2A12* (Fig. 13C), suggesting that *SLC2A12* was also a direct AR target. Mining of existing chromatin-immunoprecipation sequencing (ChIP-Seq) datasets from several prostate cancer cell models indicated that AR directly bound to an intronic region of *SLC2A12* in the presence of dihydrotestosterone (DHT) (Fig. 13D and Fig. 15) [39, 40]. To confirm that

the identified AR binding site was a functional androgen response element and thus primary AR target, we cloned out the genomic region surrounding the identified binding site and tested its ability to confer androgen responsiveness to an enhancerless luciferase reporter gene. Indeed, the identified AR binding site conferred androgen responsiveness, indicating that *SLC2A12* is a direct AR target (Fig. 13E).

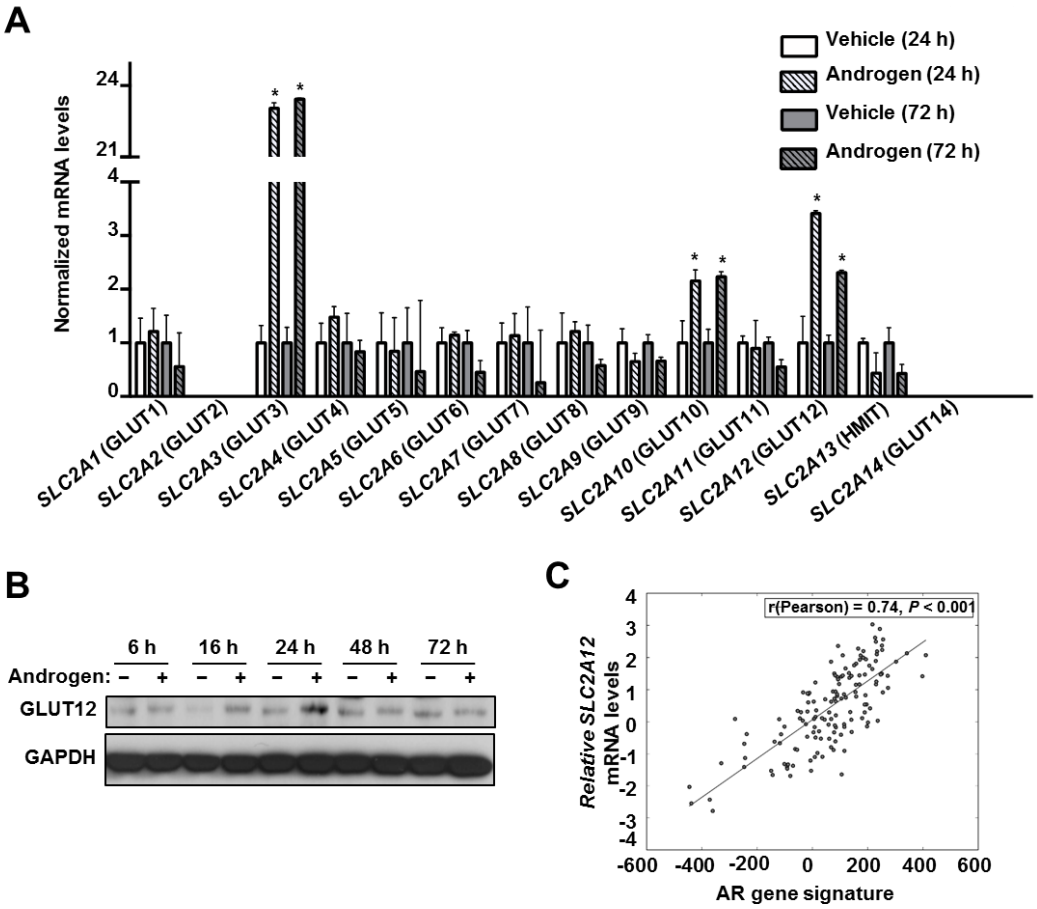

Figure 11. Identification of SLC2A12 (GLUT12) as an AR-regulated gene in prostate cancer. (A) LNCaP cells were treated ± androgen (10 nM R1881) for 24 or 72 h. Total RNA was then extracted and subjected to qRT-PCR analysis to detect the mRNA levels of indicated glucose transporters. Data were normalized to 36B4 (RPLP0) levels. *, significant (P<0.05) changes from vehicle treatment. (B) LNCaP cells were treated ± androgen (10 nM R1881) for indicated time points. Whole cell lysates were then extracted and analyzed via Western blot. GAPDH was used as a loading control. (C) Expression of SLC2A12 correlated significantly with a previously described AR gene signature (Hieronymus et al. 2006) in transcriptomic profiles of prostate cancer patients from the Taylor et al (2010) prostate cancer clinical cohort (Taylor et al. 2010). Similar results were obtained using this AR activity signature as well as an additional AR activity signature (Nelson et al. 2002) across multiple clinical cohorts (Grasso et al. 2012; Taylor et al. 2010) including The Cancer Genome Atlas (TCGA) (Cerami et al. 2012).

Table 4.1. Fold increased expression of *SLC2A12*, the gene that codes for GLUT12, in prostate cancer samples compared to benign controls in clinical datasets.

Gene	# of samples	Fold Change	*P* value	Dataset
SLC2A12	34	1.516	3.05E-5	Welsh et al.
	19	1.559	.020	Varambally et al.
	185	1.294	.008	Taylor et al.
	122	1.448	.007	Grasso et al.
	40	1.315	.039	Vanaja et al.

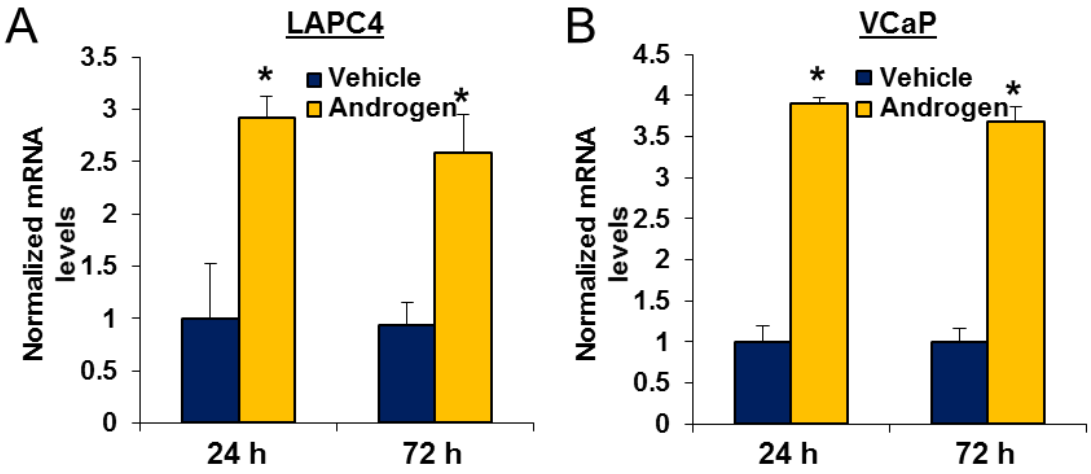

Figure 12. Identification of *SLC2A12* (GLUT12) as an androgen-regulated gene in prostate cancer cells. LAPC4 (A) or VCaP (B) cells were treated ± androgen (10 nM R1881) for 24 or 72 h. Total RNA was then extracted and subjected to qRT-PCR analysis to detect the mRNA levels of *SLC2A12*. Data were normalized to 36B4 (*RPLP0*) levels. *, significant (*P*<0.05) changes from vehicle treatment.

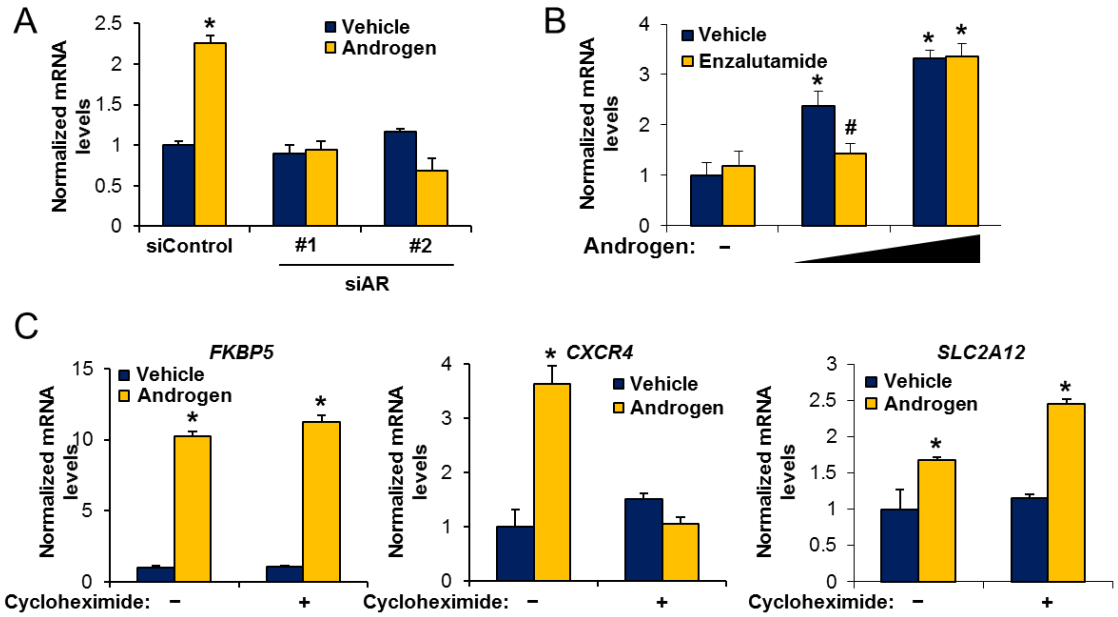

Figure 13. SLC2A12 is a direct AR target. (A) LNCaP cells were transfected with siRNAs targeting a scramble sequence (siControl) or AR and then treated ± androgen (10 nM R1881) for 72 h. SLC2A12 relative mRNA expression was quantified via qRT-PCR and data were normalized to 36B4 levels. *, significant (P<0.05) changes from vehicle treatment. (B) LNCaP cells were co-treated ± 10 μM enzalutamide (antiandrogen) with increasing concentrations of androgen (0, 100 pM or 10 nM R1881) for 72 h. SLC2A12 mRNA levels were quantified using qRT-PCR and normalized to 36B4 levels. *, significant (P<0.05) changes from vehicle (no androgen) treatment. #, significant (P<0.05) changes from vehicle (no enzalutamide) treatment. (C) LNCaP cells were treated for 16 h ± 1 μg/μl cycloheximide ± androgen (10 nM R1881). FKBP5 is a direct transcriptional target of AR (Frigo et al. 2009). CXCR4 is an indirect transcriptional target of AR (Frigo et al. 2009). Data are normalized to 36B4 levels. *, significant (P<0.05) changes from double vehicle.

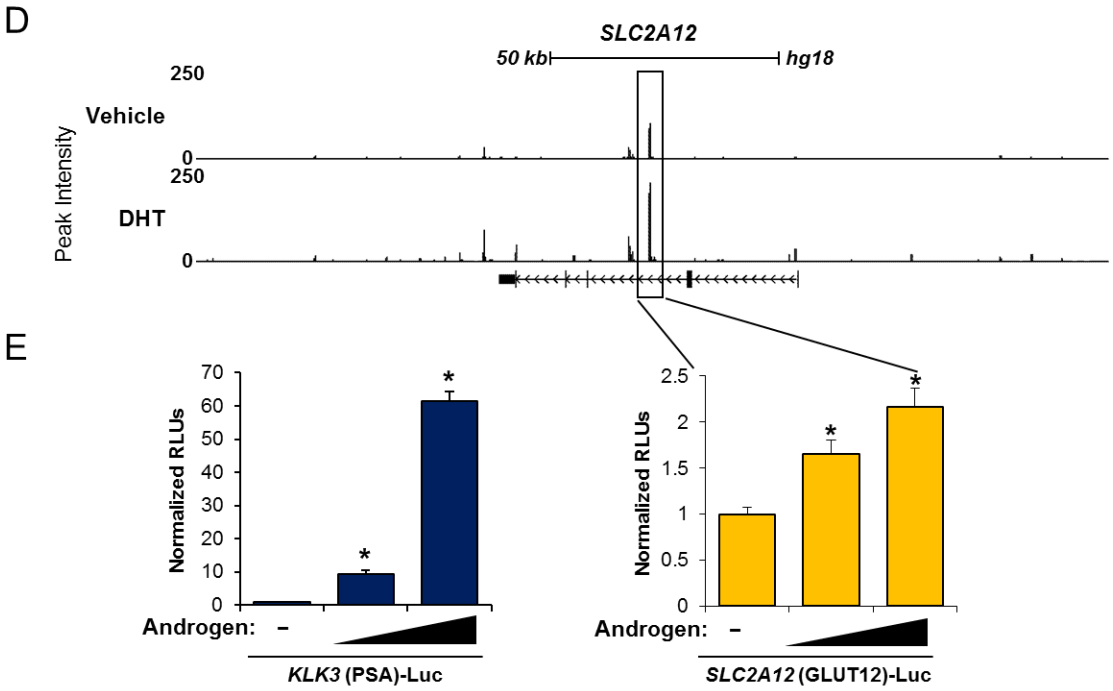

Figure 13 (continued). SLC2A12 is a direct AR target. (D) ChIP-Seq tracks of LNCaP cells treated with vehicle or DHT for 2 h. An AR binding site located within an intronic region of SLC2A12 is highlighted. Similar data for VCaP and C4-2B cells are presented in Fig. 15. (E) Enhancer luciferase reporter constructs including the AR binding site identified in (D; right graph) or a known enhancer of the KLK3 (PSA) gene (left graph; positive control) were transfected into LNCaP cells and treated overnight with 0, 0.1 or 10 nM androgen (R1881). After treatment, cells were harvested and assayed for luciferase activity. Luciferase values were normalized to the β-galactosidase transfection control. Data are the mean relative light units (RLUs) + SEM for one representative experiment conducted in triplicate (n=3). *, significant ($P<0.05$) changes from vehicle-treated cells.

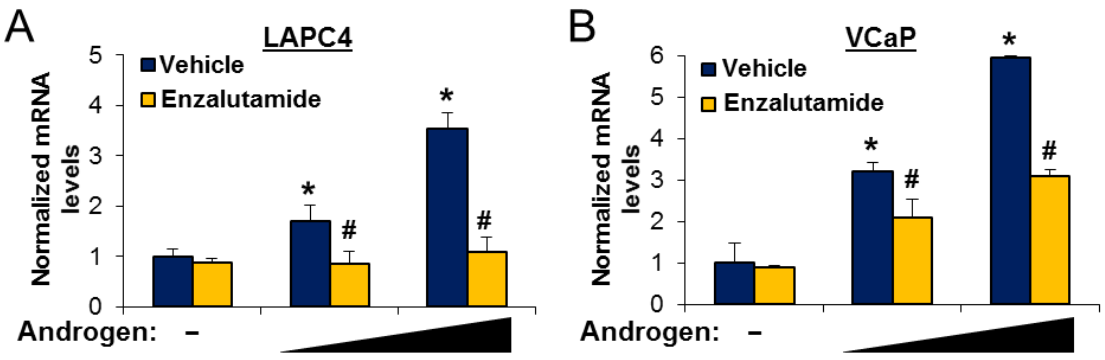

Figure 14. *SLC2A12* is an AR target. LAPC4 (A) or VCaP (B) cells were cotreated ± 10 μM enzalutamide (antiandrogen) with increasing concentrations of androgen (0, 100 pM or 10 nM R1881) for 72 h. *SLC2A12* mRNA levels were quantified using qRT-PCR and normalized to 36B4 levels. *, significant (P<0.05) changes from vehicle (no androgen) treatment. #, significant (P<0.05) changes from vehicle (no enzalutamide) treatment.

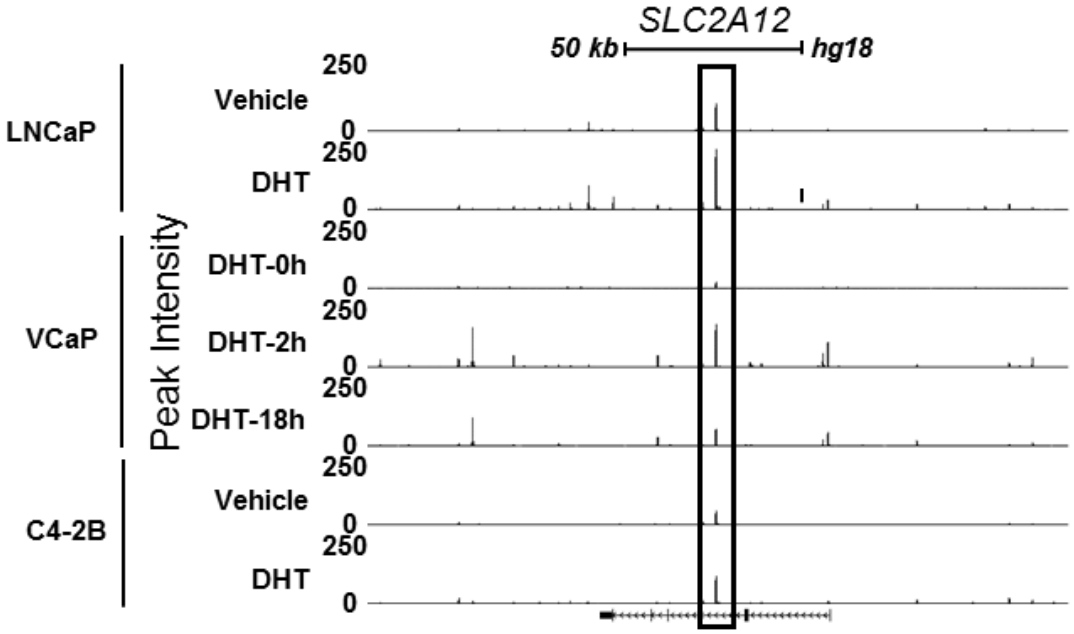

Figure 15. Identification of an AR binding site in *SLC2A12* intron. ChIP-Seq tracks of LNCaP, VCaP and C4-2B cells treated with vehicle or DHT for 0, 2 or 18 h. The AR binding site in the intronic region of *SLC2A12* is highlighted.

4.3 GLUT12 is required for maximal glucose uptake and cell growth in prostate cancer cells

To investigate the functional importance of GLUT12, we depleted GLUT12 levels using siRNAs in diverse prostate cancer cell models (Fig. 16A). Interestingly, GLUT12 was still expressed in the AR-negative PC-3 cells, suggesting that in this aggressive prostate cancer model, additional, AR-independent mechanisms maintain GLUT12 expression. GLUT12 knockdown blocked androgen-mediated glucose uptake (Fig. 16B) and proliferation (Fig. 16C) in LNCaP cells. Knockdown of GLUT12 also decreased glucose uptake (Fig. 16B) and proliferation (Fig. 16C) in PC-3 cells. These effects were not unique to LNCaP and PC-3 cells as GLUT12 knockdown impaired the growth of additional castration-resistant prostate cancer (CRPC) cell models including C4-2 and 22Rv1 (Fig. 16C). Collectively, these results demonstrate that GLUT12 is required for maximal glucose uptake and cell growth in prostate cancer cells.

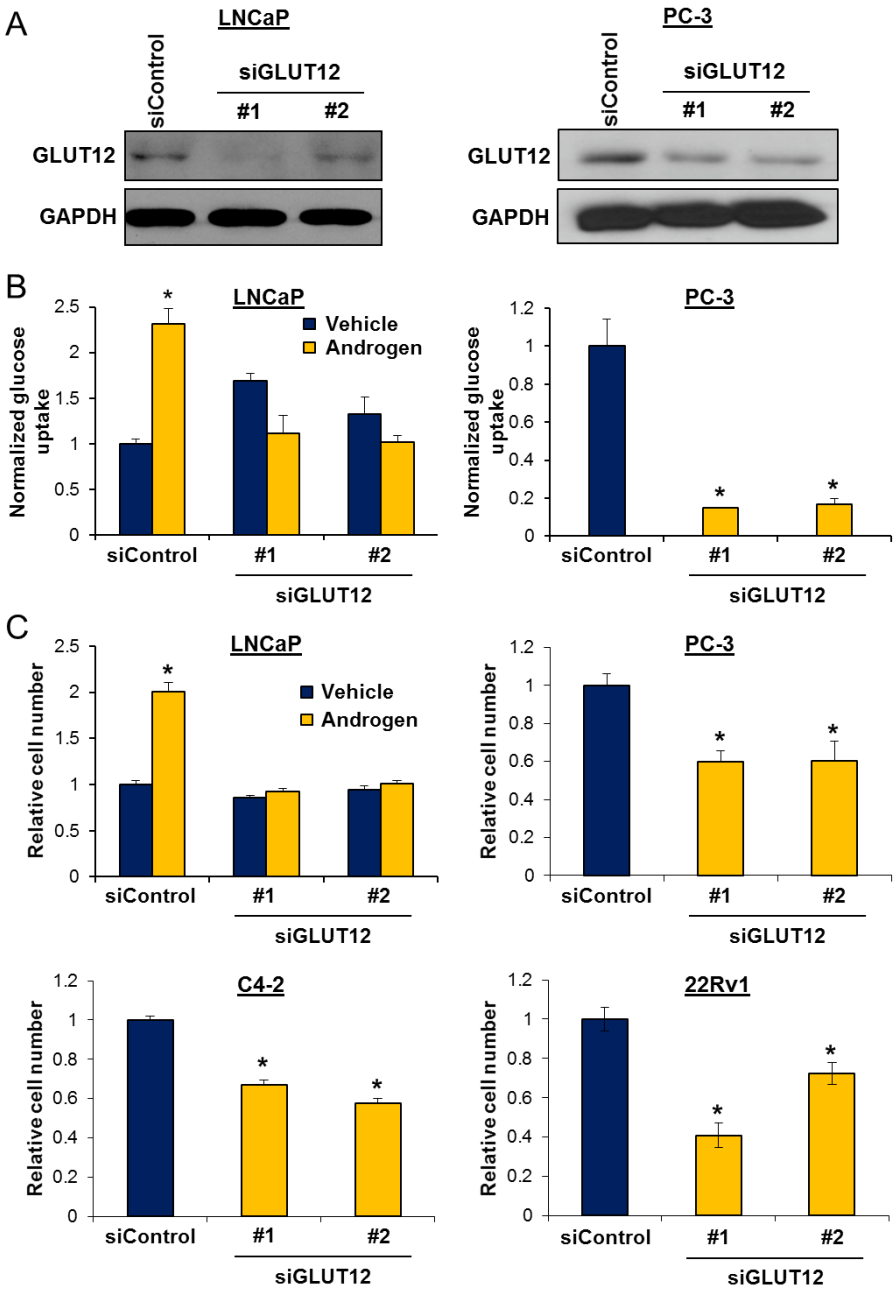

Figure 16. GLUT12 is required for maximal glucose uptake and cell growth in prostate cancer cells. (A) Indicated prostate cancer cells were transfected with siRNAs targeting a scramble sequence (siControl) or SLC2A12 (siGLUT12). After 72 h, whole cell lysates were extracted and probed for GLUT12 and GAPDH (loading control) using Western blot analysis. (B) Indicated prostate cancer cells were transfected as in Fig. 16A. LNCaP cells alone were also treated for 72 h ± androgen (R1881). Glucose uptake was then measured using a bioanalyzer and data were normalized to cellular DNA content that was measured using a fluorescent DNA stain. *, significant ($P<0.05$) changes from vehicle-treated (LNCaP) or siControl (PC-3) cells. (C) Indicated prostate cancer cells were transfected and/or treated as in Fig. 16B. Relative cell numbers were then measured using the fluorescent DNA stain. *, significant ($P<0.05$) changes from vehicle-treated (LNCaP) or siControl (PC-3, C4-2, 22Rv1) cells.

4.4 TBC1D4 is regulated by AR and CaMKK2-AMPK signaling

While *SLC2A12* is a direct AR target, the activity of several glucose transporters can be controlled by additional regulatory mechanisms [83]. Previous work from our laboratory and others demonstrated that androgens increased glucose metabolism in part through a CaMKK2-AMPK-mediated mechanism [37, 87]. Consistent with these findings, inhibition of CaMKK2 using the antagonist STO-609 or depletion of AMPK using siRNAs decreased androgen-mediated LNCaP prostate cancer cell growth (Figs. 17A and B) and normal cell growth in AR-negative PC-3 cells (Figs. 18A and 18B). Of interest, AMPK has been shown to augment glucose uptake through the phosphorylation and regulation of tre-2/USP6, BUB2, cdc16 domain family member 4 (TBC1D4; also called Akt substrate of 160 kDa (AS160)), a protein that controls the trafficking of GLUT-containing vesicles [95, 96]. To determine whether this regulation also occurred in prostate cancer, we treated cells ± STO-609 ± androgen and quantified the levels of T642 phosphorylated TBC1D4 (known AMPK target site [95, 96]). As predicted, androgens increased AMPK signaling and p-TBC1D4 levels, an effect that was inhibited by STO-609 (Fig. 17C). Surprisingly, androgens also increased the total protein levels of TBC1D4 (relative to GAPDH loading control) in a CaMKK2-dependent manner (Fig. 17C), a CaMKK2-mediated effect that was also observed in PC-3 cells (Fig. 18C). This was of further importance because the expression of *TBC1D4* was elevated in samples from prostate cancer patients in multiple clinical cohorts (Table 4.2) [44, 56, 58, 59, 90, 91, 97]. To verify the effects on TBC1D4 expression, we created stable LNCaP derivatives that could inducibly overexpress *CAMKK2*, and thus increase AMPK signaling, in the presence of doxycycline (Figs. 17D

and E and Fig. 19). Here, expression of *CAMKK2* was sufficient to increase AMPK activity as previously described [26]. In addition, *CAMKK2* expression increased the T642 phosphorylation of TBC1D4 (relative to total TBC1D4) and increased the total levels of TBC1D4 (relative to GAPDH loading control) (Fig. 17E). However, it should be noted that the CaMKK2-mediated increase in TBC1D4 levels was more moderate than that with androgen treatment (Fig. 17E), suggesting that additional AR-regulated pathways likely also govern TBC1D4 expression.

Because the CaMKK2 regulation of TBC1D4 levels had never been described before, we next wanted to determine whether this was due to effects on mRNA expression. Using qRT-PCR, we found that STO-609 decreased *TBC1D4* expression (Fig. 17F) while overexpression of *CAMKK2* (Fig. 17E and Fig. 19) was sufficient to increase *TBC1D4* mRNA levels (Fig. 17G). Consistent with this, depletion of AR, a transcriptional activator of *CAMKK2*, decreased both basal and androgen-mediated TBC1D4 expression (Fig. 20). Finally, we determined that AR gene signatures (Fig. 17H), as well as *CAMKK2* (Fig. 17I), positively correlated with *TBC1D4* mRNA levels in multiple clinical cohorts [44, 90, 91] including TCGA [92]. Taken together, these data indicate that AR and CaMKK2 signaling increase the activity and the expression of TBC1D4.

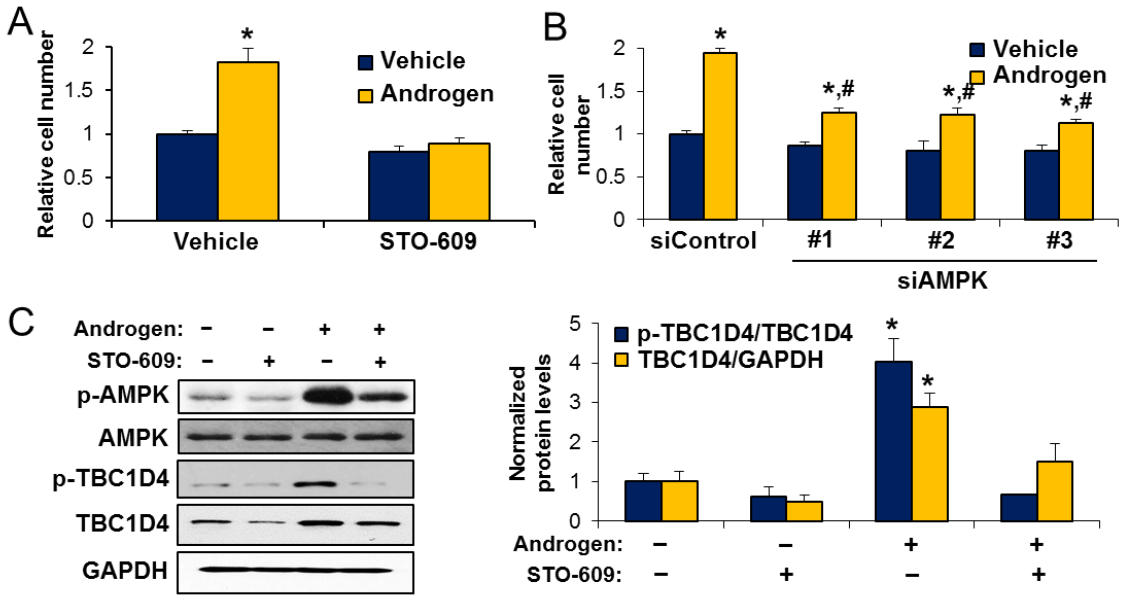

Figure 17. TBC1D4 is regulated by AR and CaMKK2-AMPK signaling. (A) LNCaP cells were co-treated ± 30 μM STO-609 ± 10 nM R1881 (androgen) for 72 h. Relative cell number was then measured using a fluorescent DNA stain. *, significant (P<0.05) changes from double vehicle-treated cells. (B) LNCaP cells were transfected with siRNAs targeting a scramble sequence (siControl) or AMPK and treated ± androgen (R1881) for 72 h. Relative cell number was then measured as in Fig. 17A. *, significant (P<0.05) changes from vehicle treatment. #, significant (P<0.05) changes from siControl. (C) LNCaP cells were treated for 72 h ± STO-609 ± androgen (R1881) after which whole cell lysates were analyzed via Western blot. *Left*, representative images. *Right*, Western blot analysis was subjected to densitometry. Total TBC1D4 levels were normalized to GAPDH levels and p-TBC1D4 levels were normalized to total TBC1D4 levels. *, significant (P<0.05) changes from double vehicle-treated cells (n=3).

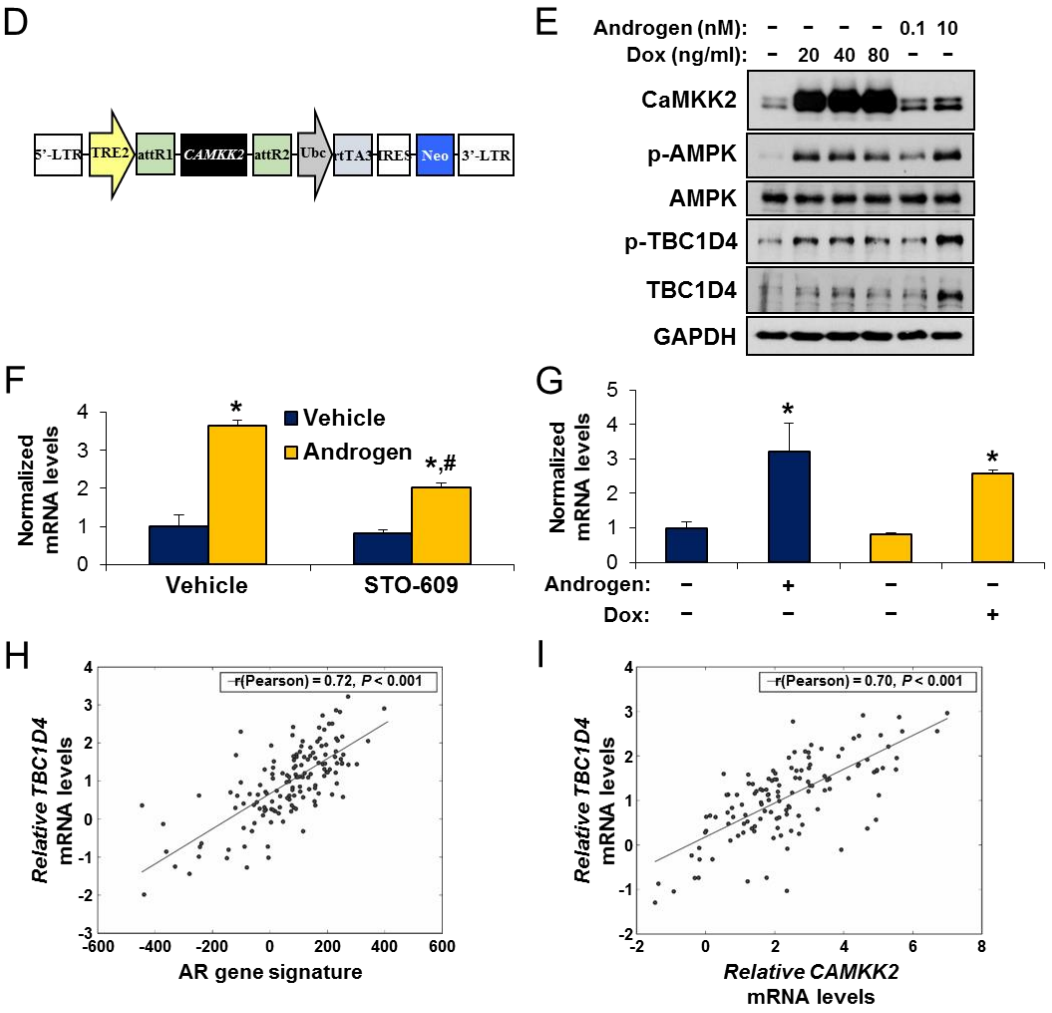

Figure 17 (continued). TBC1D4 is regulated by AR and CaMKK2-AMPK signaling. (D) Diagram of the construct used to create the LNCaP-CaMKK2 stable cell line in which CAMKK2 can be overexpressed in the presence of doxycycline. (E) LNCaP-CaMKK2 stable cells were treated for 72 h with increasing concentrations of doxycycline (Dox) or androgen (R1881). Whole cell lysates were then analyzed via Western blot. (F) LNCaP cells were co-treated as in Fig. 4A. RNA was then extracted and the mRNA levels of TBC1D4 were quantified by qRT-PCR and normalized to 36B4 levels. *, significant (P<0.05) changes from vehicle (no androgen) treatment. #, significant (P<0.05) changes from vehicle (no STO-609). (G) LNCaP-CaMKK2 stable cells were treated for 72 h ± androgen (10 nM R1881) or ± 20 ng/ml doxycycline (Dox). RNA was then extracted and the mRNA levels of TBC1D4 were quantified by qRT-PCR and normalized to 36B4 levels. *, significant (P<0.05) changes from vehicle treatment. (H) Expression of TBC1D4 correlated significantly with a previously described AR gene signature (Hieronymus et al. 2006) in transcriptomic profiles of prostate cancer patients from the Taylor et al (2010) prostate cancer clinical cohort (Taylor et al. 2010). Similar results were obtained using this AR activity signature as well as an additional AR activity signature (Nelson et al. 2002) across multiple clinical cohorts (Grasso et al. 2012; Taylor et al. 2010) including TCGA (Cerami et al. 2012). (I) Correlation analysis of Taylor et al (2010) was performed as in Fig. 4H to demonstrate that CAMKK2 expression positively correlated with TBC1D4 expression. Similar results were observed across multiple clinical cohorts (Cerami et al. 2012; Grasso et al. 2012; Varambally et al. 2005).

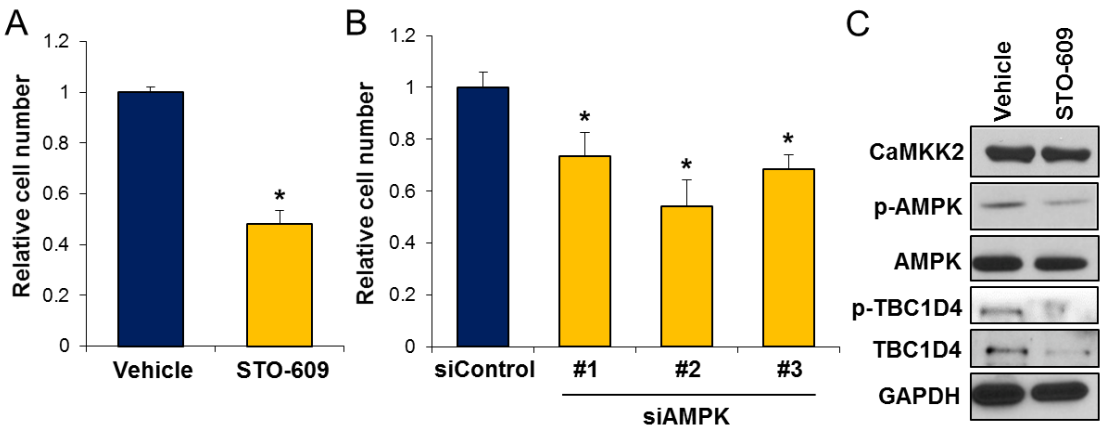

Figure 18. TBC1D4 is regulated via CaMKK2-AMPK signaling in AR-negative PC-3 cells. (A) PC-3 cells were treated ± 10 μM STO-609 for 72 h. Cells were lysed and relative cell numbers were measured using a fluorescent DNA stain. *, significant (P<0.05) changes from vehicle. (B) PC-3 cells were transfected with siRNAs targeting AMPK. After 72 h, relative cell numbers were measured as described in Fig. 18A. *, significant (P<0.05) changes from siControl. (C) PC-3 cells were treated the same as in Fig. 18A. Whole cell lysates were then extracted and analyzed via Western blot.

Table 4.2 Fold increased expression of *TBC1D4* in prostate cancer samples compared to benign controls in clinical datasets.

Gene	# of samples	Fold Change	*P* value	Dataset
TBC1D4	19	2.58	1.54E-4	Varambally et al.
	89	1.837	2.95E-4	Wallace et al.
	122	1.864	1.35E-7	Grasso et al.
	40	1.524	3.38E-5	Vanaja et al.
	34	2.24	1.78E-4	Welsh et al.
	185	1.467	8.65E-6	Taylor et al.
	30	1.495	.018	Luo et al.

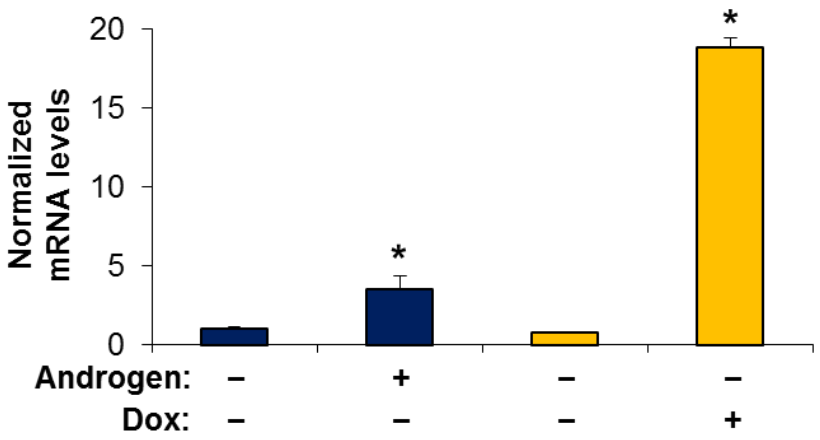

Figure 19. Validation of LNCaP-CaMKK2 stable cells that inducibly express *CAMKK2* following treatment with doxycycline (Dox). LNCaP-CaMKK2 stable cells were treated for 72 h ± androgen (10 nM R1881) or ± 20 ng/ml Dox. RNA was then extracted and the mRNA levels of *CAMKK2* were quantified by qRT-PCR and normalized to 36B4 levels. *, significant (P<0.05) changes from vehicle treatment.

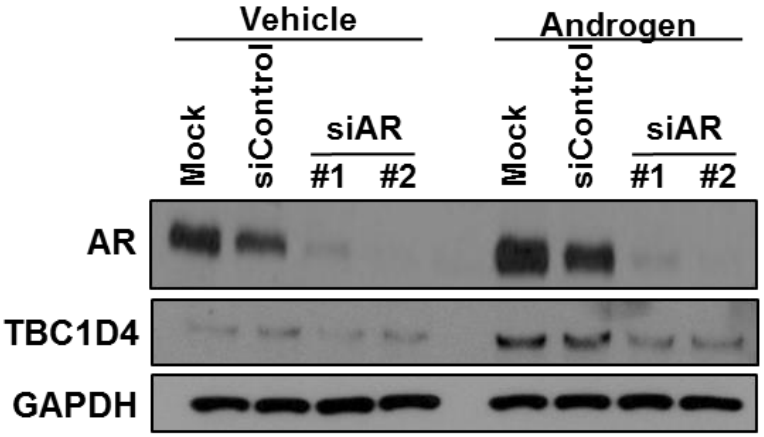

Figure 20. TBC1D4 expression is regulated by AR. LNCaP cells were transfected with mock control or siRNAs targeting a scramble sequence (siControl) or *AR* (siAR) and treated ± androgen (R1881) for 72 h. Whole cell lysates were then analyzed via Western blot.

4.5 TBC1D4 regulates glucose uptake via GLUT12 trafficking to the plasma membrane

Given TBC1D4's known role in glucose uptake, we next wanted to test its functional role in prostate cancer cells. To test this, we depleted TBC1D4 levels using siRNAs (Fig. 21A) to impair TBC1D4-mediated vesicle trafficking. Knockdown of TBC1D4 blocked androgen-mediated glucose uptake in LNCaP cells and basal glucose uptake in PC-3 cells (Fig. 21B). The impaired glucose uptake corresponded with decreased cell growth (Fig. 21C). We next assessed the role of TBC1D4 in GLUT12 translocation to the plasma membrane. To do this, we depleted TBC1D4 using siRNA, treated cells ± androgen (AR+ LNCaP cells only), and collected protein from whole cell lysates (WCL) or following isolation of the plasma membrane fraction (PM). Knockdown of TBC1D4 did not affect the total levels of GLUT12 (WCL samples), but did decrease GLUT12 in the plasma membrane fraction (PM samples) (Fig. 21D). These results indicated that TBC1D4 was required for maximal GLUT12 plasma membrane localization. Consistent with a role for CaMKK2 signaling in this process, treatment with the CaMKK2 inhibitor STO-609 blocked the expression of GLUT12 specifically at the plasma membrane (Fig. 22). Surprisingly, androgen treatment did not result in an increased level of GLUT12 at the plasma membrane in LNCaP cells (Fig. 21D and Fig. 22A). This was unexpected because androgens increased total TBC1D4 and p-TBC1D4 levels (Fig. 21D) as well as glucose uptake (Fig. 21B) at this same time point. Hence, androgen signaling may promote GLUT12 translocation to the plasma membrane through a CaMKK2-AMPK-TBC1D4-

mediated mechanism but simultaneously cause its turnover at this same location through additional mechanisms that are unknown at this time.

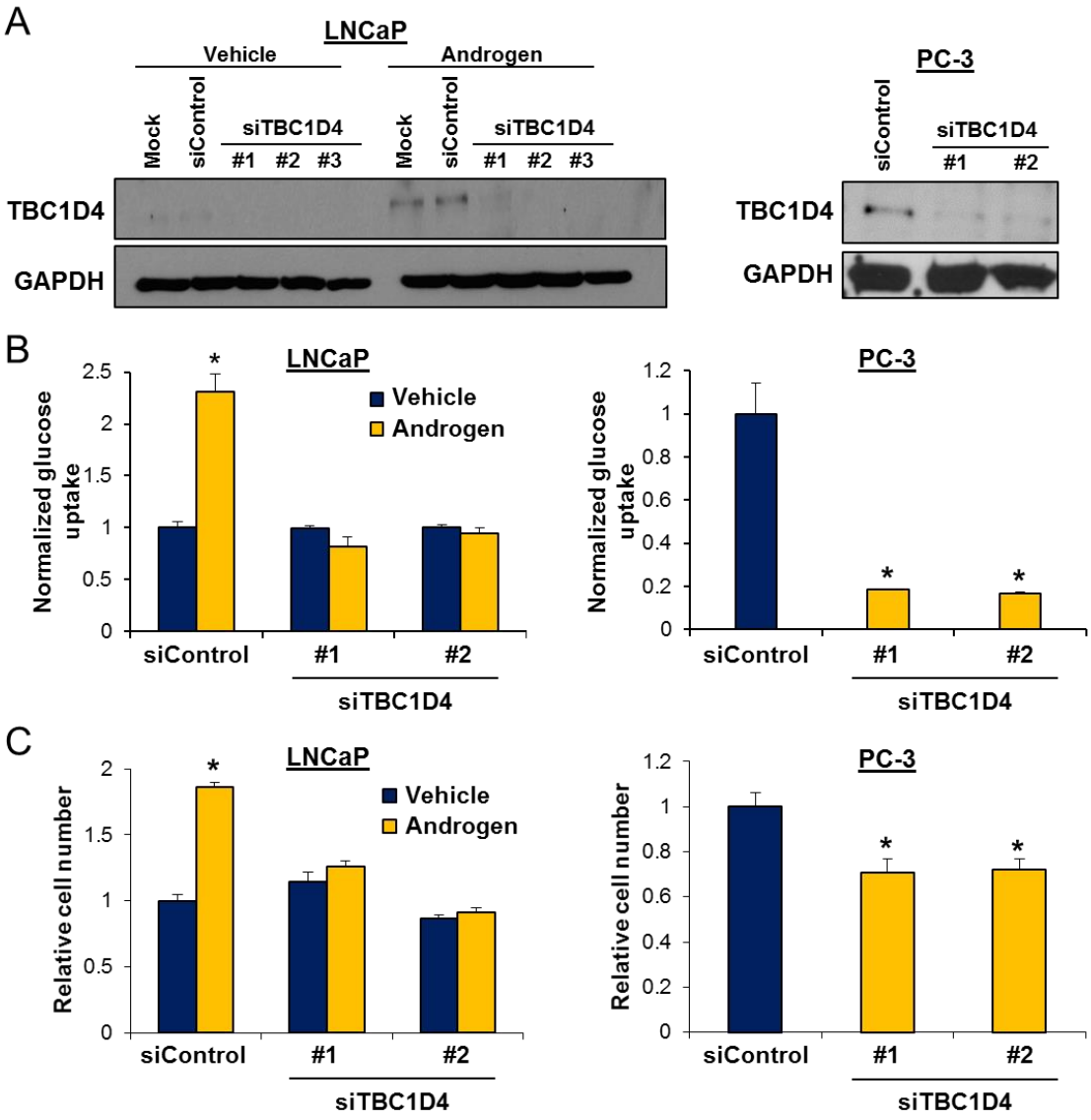

Figure 21. TBC1D4 regulates glucose uptake by modulating GLUT12 trafficking to the plasma membrane. (A) Indicated prostate cancer cells were transfected with mock or siRNAs targeting scramble control (siControl) or TBC1D4 (siTBC1D4). LNCaP cells were also treated for 72 h ± androgen (10 nM R1881). Whole cell lysates were extracted and analyzed by Western blot. (B) Indicated prostate cancer cells were transfected and/or treated as in Fig. 5A. Glucose uptake was measured using a bioanalyzer and data were normalized to cellular DNA content that was assessed using a fluorescent DNA stain. *, significant ($P<0.05$) changes from vehicle-treated (LNCaP) or siControl (PC-3) cells. (C) Indicated prostate cancer cells were transfected and/or treated as in Fig. 5B. Relative cell number was then measured using the fluorescent DNA stain. *, significant ($P<0.05$) changes from vehicle-treated (LNCaP) or siControl (PC-3) cells.

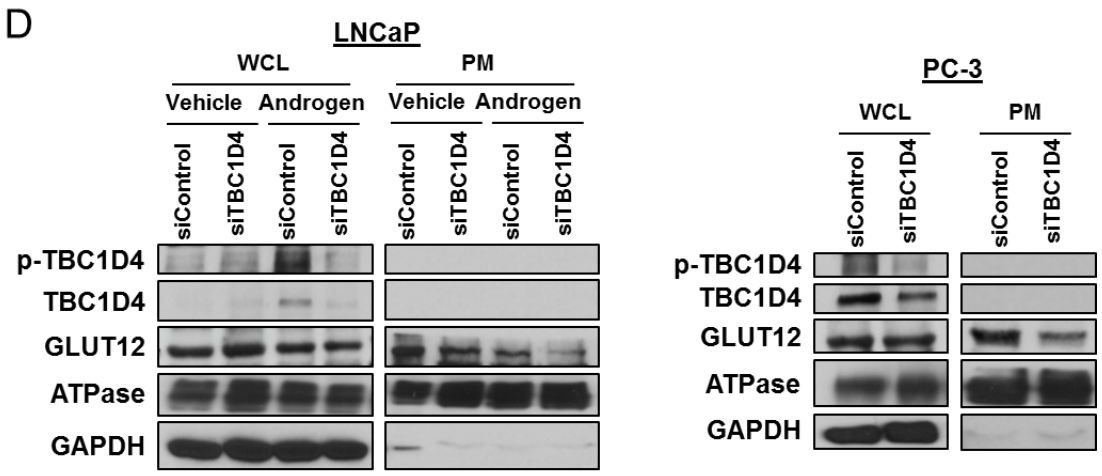

Figure 21 (continued). TBC1D4 regulates glucose uptake by modulating GLUT12 trafficking to the plasma membrane (D) Indicated prostate cancer cells were transfected and/or treated as in Fig. 21A. Afterwards, whole cell lysates (WCLs) were extracted and analyzed by Western blot. Duplicate cells were also subjected to a plasma membrane (PM) fractionation where the PM fraction was extracted and analyzed by Western blot. ATPase serves as a positive control for the PM fraction. GAPDH serves as a loading control for the WCL and a negative control for the PM fraction.

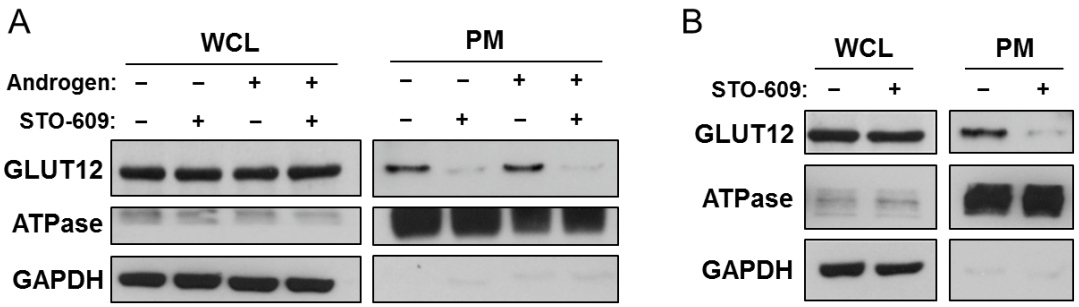

Figure 22. STO-609 blocks GLUT12 translocation to the plasma membrane. (A) LNCaPs were treated for 48 h ± 10 μM STO-609 ± androgen (10 nM R1881). Whole cell lysates (WCLs) were extracted and analyzed via Western blot. The cells were also subjected to a plasma membrane (PM) fractionation and the PM fraction was analyzed via Western blot. ATPase serves as a positive control for the PM fraction. GAPDH serves as a loading control for the WCL and a negative control for the PM fraction. (B) PC-3 cells were treated for 48 h ± 10 μM STO-609. WCLs were extracted and analyzed via Western blot. The cells were also subjected to a PM fractionation and the PM fraction was analyzed via Western blot. ATPase serves as a positive control for the PM fraction. GAPDH serves as a loading control for the WCL and a negative control for the PM fraction.

4.6 Discussion

The data presented here suggest a working model, depicted in Fig. 23, for GLUT12 regulation in prostate cancer. *SLC2A12*, the gene encoding GLUT12, can directly be targeted by AR. This leads to a pulse of new GLUT12 synthesis. Simultaneously, androgens can directly increase the expression of *CAMKK2* [26]. CaMKK2 can then later phosphorylate and activate the master metabolic regulator AMPK [98-100]. Interestingly, as shown here in PC-3 cells (Fig. 18) and by others in DU145 cells [101], CaMKK2-AMPK signaling is maintained in AR-negative prostate cancer cells, indicating that other transcription factors beyond AR promote CaMKK2-AMPK signaling. Regardless, CaMKK2-AMPK signaling will in turn increase GLUT12 translocation to the plasma membrane by modulating both the expression and activity of TBC1D4, a known regulator of vesicle trafficking [95, 96]. In addition to CaMKK2 signaling, our data (Fig. 17, 19 and 20) suggest that androgens may promote the expression of *TBC1D4* through additional, CaMKK2-independent mechanisms (indicated as the expression of gene *X*, unknown at this time, that codes for protein X). While the regulation of TBC1D4 by AMPK has been well described [95, 96], to our knowledge the androgen and CaMKK2-AMPK modulation of *TBC1D4* expression has not been previously described. Ultimately, this combined series of transcriptional and posttranscriptional events converge to increase the levels of functional GLUT12 at the plasma membrane where it can promote glucose uptake and subsequent cell growth.

GLUT12, as it name implies, was the 12th of 14 known glucose transporters of the *SLC2A* family to be identified [33]. As such, there is relatively little known about its

regulation and function compared to many other GLUTs such as GLUT1 and GLUT4. Interestingly, GLUT12 was first discovered in MCF-7 breast cancer cells [33]. Since then, it has been found to be expressed in a number of malignancies including rhabdomyosarcomas, oligodendrogliomas, oligoastrocytomas, astrocytomas as well as breast, lung, colorectal and prostate cancers [28, 33, 102-104]. Hence, a potential oncogenic role for this transporter has been emerging. In agreement with the mRNA expression data listed in Table 4.1, immunohistochemical staining for GLUT12 revealed the transporter's protein expression in malignant but not benign prostatic hyperplasia tissue [28]. In estrogen receptor-positive (ER+) MCF-7 breast cancer cells, 24 hour treatment with the steroid hormone estrogen was shown to increase GLUT12 protein but not mRNA levels, suggesting estrogen increased GLUT12 levels through promoting translation or protein stability [105]. They also demonstrated that GLUT12 protein could not be detected in ER-negative breast cancer cells, further supporting a model of ER regulation. The authors additionally stated (but did not show the data) that 24 hour DHT treatment increased GLUT12 protein levels in MCF-7 cells, which express AR [105]. It was not reported whether DHT increased mRNA expression. Like ER signaling in breast cancer cells, our data demonstrate that AR signaling increased GLUT12 expression in prostate cancer (Fig. 11, 12 and 14). However, our findings indicate that, unlike ER signaling in breast cancer, *SLC2A12* is a direct AR target in prostate cancer cells (Figs. 13 and 15). Further, AR-negative PC-3 prostate cancer cells express GLUT12, indicating that AR is not the only transcription factor that regulates *SLC2A12* (Figs. 16, 21, and 22).

GLUT transporters are grouped into three classes based on their sequence homology and structure [83, 106]. GLUT12 belongs to class III while many of the originally identified GLUTs including the insulin-regulated GLUT4 belong to class I. Despite belonging to two different classes, GLUT12 was originally identified by homology to GLUT4 [33]. Like GLUT4, evidence suggests that GLUT12's subcellular location is also regulated by signals such as insulin [33, 34]. In this regard, it is of note that GLUT12 possesses the motifs found in GLUT4 that facilitate this transporter's subcellular trafficking [33]. To that end, it has been speculated that GLUT4 and GLUT12 might even be colocalized to the same vesicles under baseline conditions. Our data here suggest that like GLUT4 [107, 108], GLUT12 translocation is also regulated by TBC1D4 (Fig. 21).

Under physiological conditions in muscle or fat, TBC1D4 can be phosphorylated by Akt or AMPK [34, 108-110]. This phosphorylation inhibits its Rab GTPase-activating activity and promotes GLUT4 and, as our data suggest here, GLUT12 translocation. Consistent with this, inhibition of CaMKK2-AMPK kinase signaling by STO-609 decreased GLUT12 plasma membrane levels (Fig. 22) and glucose uptake [87]. The existence of this regulatory mechanism for GLUT12 is consistent with the presence of the conserved motif, described above, in both transporters that is known to be responsible for insulin-mediated GLUT4 translocation. As this phosphorylation is an inhibitory event, it is not unexpected that knockdown of *TBC1D4* or its paralog *TBC1D1* have been described before to increase cell surface GLUT4 levels and glucose uptake in muscle and fat cells [111-113]. Paradoxically, silencing of *TBC1D4* in prostate cancer cells decreased the plasma membrane-localized levels of GLUT12 while having no effect on total GLUT12

levels (Fig. 21D). These findings suggest that TBC1D4 might be required to establish basal GLUT12 vesicle trafficking that can then be further regulated in response to cues such as CaMKK2 signaling. Future experiments will be critical to determine whether this regulation is specific to GLUT12 and/or prostate cancer.

At this time, we cannot exclude the possibility that androgens or CaMKK2-AMPK signaling could promote glucose uptake through additional, GLUT12-independent mechanisms. As described above, TBC1D4 was first demonstrated to regulate the translocation of GLUT4 [95, 96], a transporter that is also expressed (albeit lower) in the prostate (Fig. 11) [114]. In addition, AMPK has been reported to increase GLUT1 levels through a variety of mechanisms [115, 116]. Further, AMPK can directly phosphorylate two of the four isoenzymes of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphate 2-phosphatase (PFKFB), an enzyme that represents the rate-limiting step of glycolysis [117-119]. These phosphorylation events on PFKFB2 and PFKFB3 increase their kinase activity and, therefore, promote forward flux through glycolysis. Because glucose uptake through the facilitative transporters is regulated by the concentration gradient, increased cellular catabolism of glucose would stimulate the uptake of more sugar by other GLUTs that might also function independently of TBC1D4. Likewise, androgens and CaMKK2-AMPK signaling have also been demonstrated to increase glucose metabolism through the tricarboxylic acid (TCA) cycle, the hexosamine biosynthetic pathway and the pentose phosphate pathway [36, 37, 67, 87]. Collectively, these effects suggest that GLUT12 only represents one of potentially several mechanisms downstream of androgen and CaMKK2 signaling that could increase glucose uptake. Given prostate cancer's heterogeneous

makeup [120], it is likely that some subtypes may favor GLUT12- and/or TBC1D4-independent glucose uptake.

This study examined the regulation and role of the transporter GLUT12 in glucose uptake. It still remains to be determined how glucose is then used by the cancer cells. As described above, androgen and CaMKK2 signaling have previously been demonstrated to promote glucose metabolism through the hexosamine biosynthetic pathway, the pentose phosphate pathway, glycolysis, and the TCA cycle in prostate cancer [36, 37, 67, 87]. In addition, androgens are known to stimulate the formation of intracellular fat depots, thought to promote growth, in prostate cancer cells [35, 121]. These fat reservoirs are likely created in part through the breakdown of sugars and shuttling of their carbons into *de novo* lipogenesis. Future studies using isotopic-tracing techniques will undoubtedly help determine the exact contribution of each one of these pathways to the growing tumor.

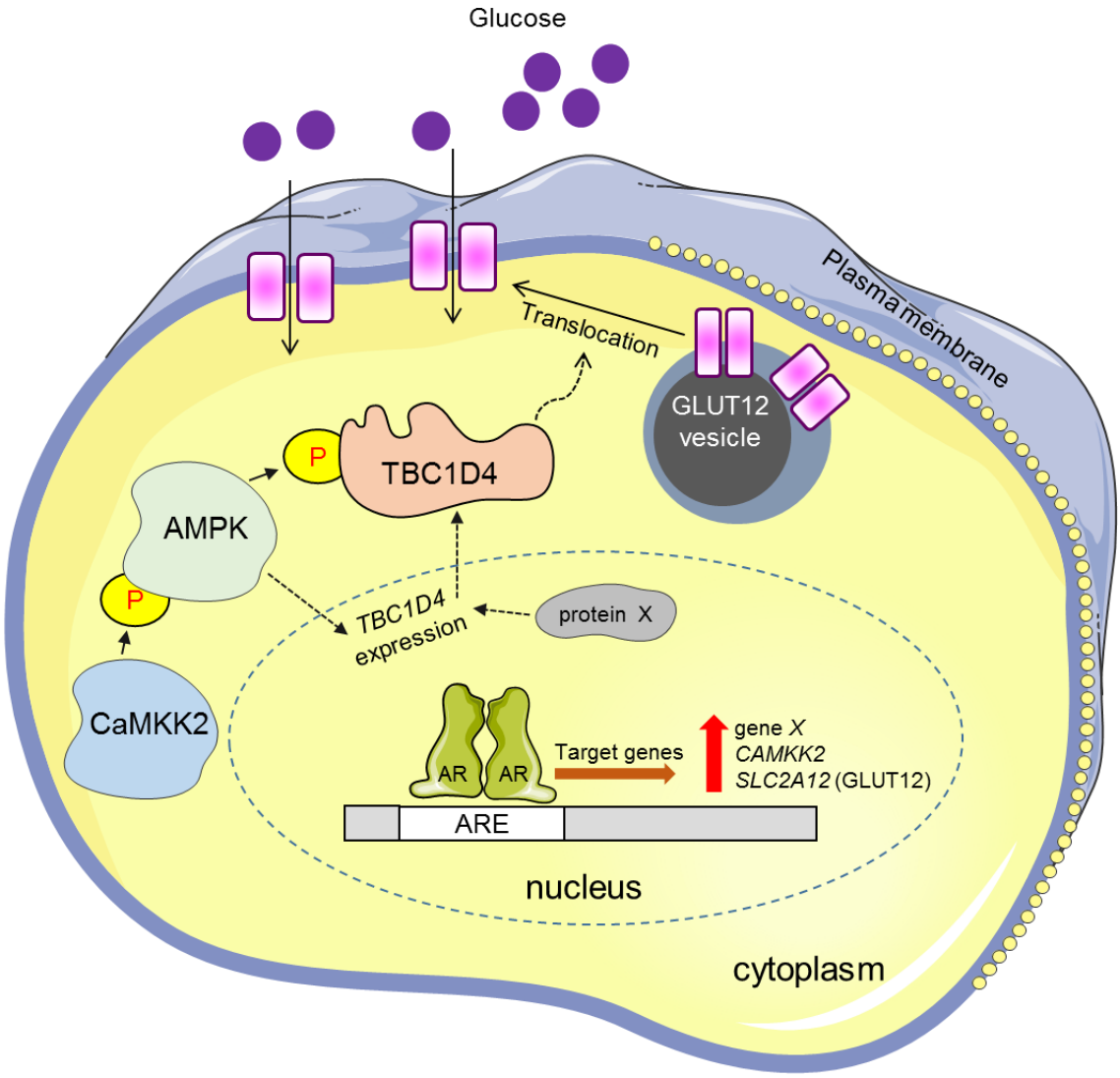

Figure 23. Working model of GLUT12 regulation and function in prostate cancer. AR directly increases the expression of *CAMKK2*, *SLC2A12* (GLUT12), and an additional gene, *X*, unknown at this time. CaMKK2 signaling also promotes GLUT12 function through the AMPK-mediated phosphorylation of TBC1D4, promoting GLUT12 translocation to the plasma membrane through a posttranslational mechanism similar to that described for GLUT4. Interestingly, CaMKK2 signaling also appears to modulate GLUT12 translocation by regulating the expression of *TBC1D4* through a still poorly defined mechanism (indicated by the dashed lines). Further, AR can increase *TBC1D4* expression in a CaMKK2-independent manner (indicated by the expression of gene *X* that codes for protein X). Together, these combined events ultimately function to increase the level of plasma membrane-localized GLUT12 and therefore promote cellular glucose uptake and subsequent metabolism.

CHAPTER 5: FUTURE DIRECTIONS AND CONCLUDING REMARKS

My studies taken together have found that *SLC1A4* and *SLC1A5* are regulated via AR, mTOR and selectively by MYC to augment glutamine metabolism. In addition, AR and CaMKK2-AMPK signaling promotes *SLC2A12* function to increase glucose uptake. Cancer reoccurance during the later stages of the maliganancy is the result of the tumor cells overcoming therapeutic treatments by utilizing new pathways for survival. This puts more emphasis on targeting nutrient uptake than cell signaling because mulitple pathways work through increasing metabolic changes. Signaling in the cell can change rapidly, there is cross-talk between signals, and an unpredictable nature trying to figure which pathway the cell will utilize. Targeting nutrient uptake is more advantageous as this may circumvent the issues of redundancy when targeting cellular pathways. Future directions of this work should include overepressing *SLC1A4*, *SLC1A5*, and *SLC2A12* transporters to see which one can potentaite prostate cancer survival in the presence of chemotherapeutic drugs. It would be interesting also to look for changes in known pathways that regulate the glutamine and glucose transpoter(s) to see of there is any change in activity or expression. This would show that the transporters not only potentiate survival but the cell is able to regulate them using different pathways in the presence of chemotherapeutic drugs. This would expand our work into possible new regulatory pathways of glutamine and glucose transporters. My work as it is, already suggests that there are alternate pathways of regulation of the transporters. It is still unclear what regulates the expression and activity of *SLC1A4* and also the identity of “gene X” that regulates TBC1D4.

The alternate regulatory pathways could be hidden in the intersection between the glutamine and glucose metabolism. For example, both metabolic pathways come together to regulate hexosamine biosythesis and glycosylation reactions [122]. Glutamine is required for the formation glucosamine-6-phosphate needed for N-linked glycosylation. This reaction is catalyzed by glutamine-fructose-6-phosphate amidotransferase (GFAT) [122]. N-glycosylation of *SLC1A5* is required for translocation to the plasma membrane [123]. The formation glucosamine-6-phosphate and the N-glycosylation reactions require carbons from glucose and the movement of the amine group from glutamine catabolism. Lacking in the literature and in our own studies is a readout of how each metabolic pathway contributes to this aspect of SLC1A5 regulation. This could be assessed by using a ^{15}N-labeled glutamine to trace the nitrogen group thoughout the cell as it is being metabolized. The amount incorporated into glucosamine-6-phosphate and subsequently used for N-linked glycosylation, could correlate with glutamine uptake into the cell since N-glycosylation is required for translocation of *SLC1A5*.

Another approach to finding new regulatory pathways could be to sensitize cells to either glutamine or glucose and assess which genes and cellular pathways are being changed. I observed that VCaP cells express higher androgen-mediated levels of glutaminase than LNCaP cells. This is of interest because it is not known whether or not rate-limiting enzymes of metabolism play a role in determining metabolic switches in the cell. For example, LNCaP cells would be a good model to demonstrate this. My work has shown them to have the ability to be glutamine-addicted (Fig. 1) as well as glucose sensitive (Fig. 16), so they serve as a model for both aspects of metabolism. To test this in future

experiments, one could overexpress glutaminase in the cell to sensitize them to glutamine. To assess their sensitiviy, I would treat them with increasing concentrations of glutamine and expect their growth to be comparible to androgen-mediated growth. After this, I would collect samples and subject them to microarray analysis and perform pathway analysis. To assess changes I would compare parental LNCaP cells to those overexpressing glutaminase and ascertain which pathways or genes are being up or down-regulated. These experiments would help determine whether the metabolic switch in cancer cells are due to overexpression of rate-limiting enzymes or the avalibility of glucose or glutamine.

Finding new regulatory pathways is an important step in cancer research but more important than that is the target that is being regulated. I have identified *SLC1A4, SLC1A5,* and *SLC2A12* as potential targets. Overlooked in my studies is the potential of *TBC1D4* as a prostate cancer target. I found that *TBC1D4* knockdown decreased glucose uptake and proliferation in prostate cancer cells (Fig. 21). Not shown here is functionally whether the phosphorylation status of TBC1D4 has any affects glucose uptake or proliferation. In normal muscle TBC1D4 is phosphorylated at theronine 642 which is an inhibitory action to allow GLUT4 vesicle trafficking to the plasma membrane. In prostate cancer it is unclear if the phosphorylation event will inhibit GLUT12 translocation. To assess this, I would create a mutant TBC1D4 with a point mutation at the threonine 642 phospho-site, changing it to an alanine. To determine if the mutation worked, I would use an *in vitro* kinase assay, comparing mutant versus the wild-type and the ability of CaMKK2/AMPK signaling to phosphorylate each. After validation of the mutant, I would overexpress it in prostate cancer cells and measure proliferation, glucose uptake, and use a cellular membrane

fractionation to see how much GLUT12 protein expression is there. If it is determined that TBC1D4 phosphorylation status plays a role in the cell, this will further validate TBC1D4 as a prostate cancer target and strengthen the rationale for targeting CaMKK2 and/or AMPK.

Understanding the translational significance of *SLC1A4, SLC1A5,* and *SLC2A12* is the next logical step to my research to date. There is an extensive amount of data on the use of SLC1A5 as an effective target *in vivo* [27, 124] that have lead to the creation of new SLC1A5 inhibitors [79, 125]. Future experiments would use xenograft models with intact and castrated mice to validate SLC1A4 and SLC2A12 as *in vivo* targets throughout all stages of the malignancy because this is what is currently lacking in the field. I want to determine how effective the knockdown of SLC1A4 or SLC2A12 would be at tumor cell proliferation and survival. I expect that knockdown of both transporters simulaneously could yeild a greater effect on tumor growth in both intact and castrated mice. These studies will demonstrate the need for inhibitors that block both glucose and glutamine metabolism to be used a nutrient uptake blockades.

Current prostate cancer hormone therapies work well initially but fail because the drugs do not target the total cell population of the tumor. The tumor is made up of multiple populations of cells with different varied expression of AR and/or AR splice variants. I think the key to less reoccurence in prostate cancer patients is maintaning a great amount of pressure on cancer cells to survive with limited resouces. My strategy would be to use a nutrient uptake blockade in combination with a hormone blockade therapy. I think this approach will stress the cells enough to force them into apoptotic signaling or remain

quiescent. I would administer the nutrient blockade throughout all stages of prostate cancer. I think this would keep the tumor small enough to aviod surgery in early stage pateints. In addition, it would prolong the regrowth of castration-resistant prostate cancer and possibly make chemotherapy more effective in patients in the later stages of the cancer. Realistically, I expect for this type of treatment to prolong survival significantly but not be a cure.

CHAPTER 6: REFERENCES

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