A molecular and in vivo investigation of advanced prostate cancer: Deconstructing AMPK activity and developing an improved mouse model

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A molecular and \textit{in vivo} investigation of advanced prostate cancer: Deconstructing AMPK activity and developing an improved mouse model

by

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A molecular and in vivo investigation of advanced prostate cancer:
Deconstructing AMPK activity and developing
an improved mouse model

A Thesis
Presented to the Faculty of
The University of Texas
MD Anderson Cancer Center UTHealth
Graduate School of Biomedical Sciences
in Partial Fulfillment
of the Requirements
for the Degree of
Master of Science

by
Sandi Robyn Wilkenfeld, BS
Houston, Texas
December, 2022
Dedicated to my parents for their unconditional love and support
Acknowledgments

I would like to express my appreciation for everyone who has supported me through these years. To my mentor Dr. Daniel Frigo, who has always provided support and has helped me become a better scientist. To the members of my advisory committee who have provided guidance on my evolving project. To the members of the Frigo laboratory and the Department of Cancer Systems Imaging who have provided invaluable help and insightful discussions. To my many friends and colleagues whose support, both scientific and otherwise, got me through every struggle: Dr. Chenchu Lin, Dr. Sunada Khadka, Dr. Iman Sahnoune, Dr. Alyssa Pollard, Dr. Margie Sutton. To my partner Bryan Rogers for his unwavering support and encouragement. And finally to my family, who have never stopped believing in me, I would not be here without their support.
A molecular and in vivo investigation of advanced prostate cancer: Deconstructing AMPK activity and developing an improved mouse model

Sandi Robyn Wilkenfeld, BS

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ABSTRACT

Prostate cancer is one of the leading causes of cancer-related death in men. Prostate cancer is dependent on androgen receptor (AR)-mediated pathways, and AR is therefore targeted to treat advanced prostate cancer. Despite an initial response to current AR-targeted therapies, patients invariably relapse, due in large part to the reactivation of AR through a variety of mechanisms. My goal is to identify pathways downstream of AR that can be therapeutically targeted. We and others previously demonstrated that in prostate cancer, calcium/calmodulin-dependent kinase kinase 2 (CaMKK2) is a direct downstream target of AR, and can promote disease progression through the phosphorylation and activation of the 5' AMP-activated protein kinase (AMPK). AMPK is a major regulator of cell homeostasis. While it is well established that AMPK is required for processes like cell growth, development, and stress response, its role in cancer is enigmatic. AMPK can promote both oncogenic and tumor suppressive pathways in different contexts, making it challenging to target for cancer drug development. Interestingly, the α1 and α2 isoforms of the catalytic α subunit of AMPK have been shown to localize to distinct compartments within the cell, and molecular studies indicate that these
isoforms have non-redundant functions. Furthermore, subcellular populations of AMPK behave differently in response to stress. Together, these findings suggest that separate populations of AMPK within the cell may behave differently, challenging the dogma of AMPK existing as a single signaling molecule.

In the advanced stages of prostate cancer, the cancer spreads to local and distant lymph nodes and other organs. Prostate cancer metastasizes primarily to bone, with up to 90% of men who die of prostate cancer having bone metastases upon autopsy. A major limitation in the study of advanced prostate cancer is a lack of relevant preclinical models of disease progression. Rodents are the most commonly used animal models for studying prostate cancer, and many mouse models have been developed and are widely used in prostate cancer research. However, these models typically fail to recapitulate the full progression of prostate cancer in humans. In fact, there are currently no prostate cancer mouse models that reliably produce bone metastases at a similar rate to human disease. Given this issue, I sought to develop a tractable model of mouse prostate cancer metastasis that consistently metastasizes from the primary site to bone, as it does in human disease.
# TABLE OF CONTENTS

Approval.................................................................................................................. i
Title........................................................................................................................... ii
Dedication.................................................................................................................. iii
Acknowledgements................................................................................................... iv
Abstract.................................................................................................................... v
Table of Contents..................................................................................................... vii
List of Figures............................................................................................................ ix
List of Tables............................................................................................................. x
Chapter 1: Introduction........................................................................................... 1
Chapter 2: Materials and Methods.......................................................................... 12
  2.1: Cell lines.......................................................................................................... 12
  2.2: Xenografts....................................................................................................... 12
  2.3: Histology and immunostaining......................................................................... 13
  2.4: Expression vectors and sgRNA design................................................................. 13
  2.5: Generation of overexpression CRISPR/Cas9 KO cells..................................... 13
  2.6: Western blot.................................................................................................... 14
  2.7: Proliferation assays.......................................................................................... 14
  2.8: Immunofluorescence microscopy..................................................................... 15
  2.9: Bioluminescence imaging.................................................................................. 15
  2.10: Statistical analysis.......................................................................................... 15
Chapter 3: Results.................................................................................................... 16
  3.1: Different stimuli activate distinct subcellular AMPK populations................. 16
  3.2: Compartmentalized populations of AMPK have diverse functions.......... 20
3.3: Functional differences between AMPKα1 KO, AMPKα2 KO........... 22
3.4: AMPKα1 and AMPKα2 localize to distinct cell compartments......... 26
3.5: Bone metastases occur at a high rate in orthotopically injected, 
castrated NSG mice......................................................... 28
Chapter 4: Discussion and Future Directions..................................... 34
Bibliography...................................................................................... 38
Vita................................................................................................. 50
List of Figures

Figure 1. Different stimuli activate distinct subcellular AMPK populations…… 18

Figure 2. CAMKK2 knockdown decreases cytoplasmic AMPK activity………. 19

Figure 3. Inhibition of AMPK at different subcellular compartments has opposing effects on prostate cancer cell growth............................................. 21

Figure 4. AMPKα1 and AMPKα2 differentially activate known downstream AMPK targets................................................................. 24

Figure 5. AMPKα1 and AMPKα2 knockout cells have different responses to hypoxia................................................................. 25

Figure 6. AMPKα1 and AMPKα2 isoform localization in prostate cancer cells. 27

Figure 7. Quantification of primary tumors in NSG mice............................. 30

Figure 8. Quantification of metastases in NSG mice................................. 31

Figure 9. Histological validation of bone metastasis................................. 33
List of Tables

Table 1: Metastasis profile of C4-2B-LT xenografts…………………………… 32
CHAPTER 1: INTRODUCTION

Steroid hormones

Steroid hormones are lipophilic molecules produced in one cell that can travel great distances within the body to elicit biological effects in another cell. In the absence of hormone ligand, steroid hormone nuclear receptors (NRs) are predominantly held in the cytoplasm in an inactive conformation by heat shock proteins (HSPs). Upon binding ligand, the receptor undergoes a conformational change that results in the dissociation of HSPs, translocation to the nucleus, dimerization, association with various co-regulators, and binding to specific sequences of DNA termed hormone response elements (HREs). This DNA-bound complex can then regulate the transcription of genes. It is now realized that steroid hormones can also activate signal transduction pathways and physiological changes independent of their actions in the nucleus, via a mechanism designated non-genomic signaling. Since this bypasses the process of gene transcription, non-genomic signaling often occurs on a faster time frame and is referred to as rapid or extranuclear signaling. Rapid signaling often involves NR trafficking to the plasma membrane, where they can activate kinase pathways either directly or indirectly. Importantly, non-genomic signaling can also regulate genomic pathways and vice versa. Hence, the final cellular effect of steroids is often the result of a convergence of events that began at separate locations.

Classical NR signaling
Steroid hormones are best known to mediate various physiological cell functions via genomic activity. In this regard, steroid hormones typically interact with their cognate receptor in the cytoplasm. This ligand binding results in a conformational change that leads to the dissociation of heat shock proteins, translocation of the ligand-bound receptor to the nucleus and homodimerization. Ligand-bound receptor can then bind to DNA at specific HREs to regulate gene transcription. Several NRs can also interact indirectly with DNA by tethering to other transcription factors⁵. While some steroid hormone-induced nuclear events can occur in minutes⁶, typically the genomic effects of steroid hormones take longer, with changes in gene expression occurring on the timescale of hours⁷,⁸.

Prostate cancer progression and treatments

Prostate cancer is the most diagnosed cancer in men, and one of the leading causes of cancer-related death in men in the United States⁹. 268,490 new diagnoses and 34,500 deaths are estimated to occur in 2022⁹. If diagnosed early, it can usually be treated with surgery, radiation therapy, or a combination of treatments, and the survival rate is high. However, once the cancer has progressed, it requires a form of hormone therapy called androgen deprivation therapy (ADT), which includes surgical or chemical castration¹⁰. ADT blocks the activation of the androgen receptor (AR), a nuclear receptor that is the main driver of prostate cancer, and which initially relies on androgens (ex. testosterone) to propagate its signal.

While ADT is currently the backbone of almost all treatment regimens for advanced prostate cancer, there are several problems associated with it. First,
resistance to ADT occurs in the majority of patients after several years. Second, ADT has side effects. ADT can lead to a decrease in bone density, reduced metabolic and cardiovascular activity, and/or muscle loss among a variety of other effects\textsuperscript{11}. This is a complicated issue as ADT improves the mortality rate related to prostate cancer, but increases it as a result of the side effects caused by this treatment. Fortunately, many of the side effects can be anticipated in advanced and co-treated proactively if necessary. The negative side effects of ADT as well as other AR-targeted therapies provide additional rationale to search for mechanistically novel treatments for CRPC.

ADT initially has a high success rate, which can be increased by the addition of the chemotherapeutic agent docetaxel or second-generation AR-targeting agents such as abiraterone and enzalutamide (Harris et al., 2009). However, patients eventually experience a relapse to ADT-based treatment within 2-3 years, after which death typically occurs within 18 months\textsuperscript{12,13}. This stage of prostate cancer is called recurrent or castration-resistant prostate cancer (CRPC), because the disease is no longer responsive to hormone therapy. Metastases also occur after the recurrence of disease following hormone deprivation, a stage called metastatic CRPC (mCRPC). Treatments for advanced prostate cancer include, but are not limited to, docetaxel as mentioned previously, the antiandrogens abiraterone, apalutamide, enzalutamide, and darolutamide\textsuperscript{14}, PSMA-targeting agents, PARP inhibitors, and others. The combination of therapies used for treatment of disease depends on several factors, including presence of metastases, Gleason score, presences of genetic alterations (ex. \textit{BRCA2}), or prostate specific androgen (PSA) score.
Structure of the androgen receptor

The androgen receptor is a transcription factor normally located in the cytoplasm that is translocated to the nucleus upon activation. The protein structure of androgen receptors include an N-terminal domain that is responsible for most of the transcriptional activity, a DNA-binding domain containing two zinc-finger motifs, and a hinge region that is involved with AR nuclear localization. It also contains a ligand-binding domain at the C-terminal end, which is the main mechanism of control of androgen signaling, as it enables binding of the steroid hormones. Once the AR has entered the nucleus and binds DNA via its DNA binding domain, AR regulates the expression of hundreds of genes that, in non-transformed tissues, are involved in normal cell functions, but in prostate cancer cells these genes include those involved in growth and proliferation\textsuperscript{15}.

AR signaling in advanced prostate cancer

There are several different mechanisms by which cancer cells can overcome ADT to progress into the castration-resistant stage. In human, castration alone, either surgical or medical, is not able to remove all of the androgens in the tumor. Even with this treatment, a certain base level of androgens will be present due in to the body’s ability to synthesize androgens in the adrenal glands or intratumorally\textsuperscript{16,17}. Another mechanism prostate cancer cells utilize to circumvent androgen ablation focuses on the androgen receptors themselves. AR expression can be increased to mitigate the decrease in androgen presence, as both higher levels of \textit{AR} mRNA expression and AR protein levels have been observed in
CRPC\textsuperscript{16}. Also, AR splice variants exist which are missing the ligand binding domain, suggesting the possibility of constitutively active AR even in the absence of androgens\textsuperscript{15}.

This reactivation of AR signaling pathways, the success of second-generation AR-targeting agents such as abiraterone, enzalutamide, apalutamide, and darolutamide indicate that factors downstream of AR have a role in disease progression even in CRPC. However, the precise AR-mediated pathways driving prostate cancer are still poorly understood.

**AR-CAMKK2-AMPK signaling axis**

One of the genes regulated by AR is the calcium/calmodulin-dependent kinase kinase 2 (CAMKK2), a serine/threonine kinase which is required for prostate cancer progression\textsuperscript{18-21}. The protein CAMKK2 encoded by this gene activates various pathways involved in pro-tumor effects. CAMKK2 expression is increased as the disease progresses to the castration-resistant stage, while suppression of CAMKK2 activity results in decreased cancer cell growth and migration \textit{in vitro} and \textit{in vivo}\textsuperscript{18-21}. Furthermore, CAMKK2 expression and activity is increased in response to androgens in an AR-dependent manner in preclinical models and patients\textsuperscript{18-21}.

CAMKK2 is a serine/threonine kinase that has several known substrates that mediate its downstream effects. CAMKI and CAMKIV are two downstream targets that are similarly regulated\textsuperscript{22}. They both are expressed strongly in the brain, but while CAMKI is expressed throughout all tissue, CAMKIV has a more restricted expression profile\textsuperscript{22}. AMPK has been identified as another major target of CAMKK2.
Since it is both present in the prostate and dependent on CAMKK2 activity, it is being investigated as a downstream target of CAMKK2.

AMPK (5’ AMP-activated protein kinase) is a serine/threonine kinase with a major role in maintaining cellular homeostasis\textsuperscript{23,24}. When CAMKK2 is inhibited by STO-609, the androgen-mediated phosphorylation and activation of AMPK as well as subsequent cell growth are impaired. Furthermore, androgen-mediated cell growth is decreased when AMPK is inhibited by siRNA\textsuperscript{25}. Together, these data show that the AR-CAMKK2-AMPK signaling pathway is important in promoting prostate cancer progression.

**AMPK structure and function**

In response to cell stress, AMPK works to maintain homeostasis by promoting ATP-producing pathways and inhibiting ATP-consuming pathways. As the AMP/ADP:ATP ratio increases, indicating energetic stress, the regulatory regions of AMPK become phosphorylated, which increases kinase activity\textsuperscript{26}. Importantly, AMPK has a known role in cancer and can promote both tumor suppressive and oncogenic pathways\textsuperscript{27-31}. It was first described as a tumor suppressor due to its activation by the upstream tumor suppressor LKB1\textsuperscript{32,33}. However, recent data suggests that it may also have an oncogenic role in prostate cancer, as clinical samples show that its activation increases with disease progression, and knockdown of AMPK impairs prostate cancer cell growth\textsuperscript{25}. Furthermore, CAMKK2, not LKB1, is the primary upstream activator of AMPK in prostate cancer\textsuperscript{18,34}. Together, these findings highlight the complicated role that
AMPK has in cancer progression and emphasizes the importance of identifying the mechanism of AMPK activation and how it relates to downstream cancer biology.

AMPK is a heterotrimer composed of a catalytic alpha and regulatory beta and gamma subunits. The isoforms of each subunit (2 each for alpha and beta, 3 for gamma) allow for 12 complexes, and potentially more when splice variants are considered. Interestingly, the different isoforms of the AMPK subunits may have non-redundant functions with respect to metabolism, cell structure and function, and tumorigenesis, both in vitro and in vivo. The focus of this study is on the alpha subunit, as it is the catalytic subunit of AMPK. Several studies suggest distinct functions of the two alpha subunit isoforms (AMPKα1 and AMPKα2) in cell and mouse models, as well as in human tissue. Genetic analysis of human prostate tumor samples show that patients with a higher ratio of AMPKα1 relative to AMPKα2 had a significantly poorer prognosis. Furthermore, clinical data from cBioPortal show that across various types of cancers, genomic alterations of PRKAA1 (gene encoding AMPKα1) are most often amplifications, while genomic alterations of PRKAA2 (gene encoding AMPKα2) are more often mutations or deletions. Together, these data suggest that AMPKα1 may have a oncogenic role in prostate cancer, while AMPKα2 may act more as a tumor suppressor, and that this effect may be relevant across multiple cancer types beyond prostate cancer.

Compartmentalization of AMPK

Previous evidence from our lab and others shows that AMPK is located in distinct compartments throughout the cell. This localization may be dependent on the subunit composition of the heterotrimer. In particular, the
localization of the isoforms of the alpha subunit have been studied in various cell models. The AMPKα1 subunit is predominantly cytoplasmic, while the AMPKα2 subunit can shuttle between the cytoplasm and the nucleus. This is supported by the presence of a functional nuclear localization sequence only in the AMPKα2 subunit\textsuperscript{50}. Furthermore, isoform localization has been shown to change in response to certain stimuli\textsuperscript{51}. In particular, we have shown that AMPK spatial dynamics may change during the onset and progression of prostate cancer as demonstrated by IHC staining of a tissue microarray of human benign prostate and prostate cancer tissues\textsuperscript{25}. Staining of p-AMPK in benign tissue was weak, but increased in prostate cancer. This increase was driven by an enrichment of p-AMPK staining in the cytoplasm, suggesting that AMPK activation in the cytoplasm may have a role in prostate cancer progression. Interestingly, the IHC staining of the prostate cancer tissue microarray also showed possible enrichment of staining of p-AMPK in the plasma membrane. This is important in light of studies showing that AMPK can be associated with different membrane-bound compartments, for example the mitochondria, lysosome, or plasma membrane\textsuperscript{52-54}.

**Prostate cancer clinical progression**

In the advanced stage of prostate cancer, the cancer has spread to local and distant lymph nodes and other organs. Prostate cancer metastasizes primarily to bone, with up to 90% of men who die of prostate cancer having bone metastases upon autopsy. Besides being a highly painful metastatic site, the prognosis of prostate cancer patients with bone metastases is much poorer than patients without metastases\textsuperscript{55}. In prostate cancer, osteoblastic lesions are more common in bone
metastases. This induction of abnormal bone formation can lead to many different complications.

Preclinical models of prostate cancer

A major limitation in the study of advanced prostate cancer is a lack of relevant preclinical models of disease progression. Rodents are the most commonly used animal models for studying prostate cancer, and many mouse models have been developed and are widely used in prostate cancer research. However, these models typically fail to recapitulate the full progression of prostate cancer in humans. In fact, there are currently no prostate cancer mouse models that reliably produce bone metastases at a similar rate to human disease. Given this issue, I sought to develop a mouse model of prostate cancer metastasis that consistently metastasizes from the primary site to the bone, as it does in human disease.

Despite their benefits for use in human cancer research, mice have several important differences from humans that make it a challenging model for human prostate cancer. Mice rarely spontaneously develop prostate cancer, and in common genetically engineered mouse models (GEMMs), the cancer does not progress similarly to human disease, which often occurs late in life and is initially slow-progressing. An ideal prostate cancer GEMM would (1) develop as an adenocarcinoma, (2) be androgen dependent, and (3) metastasize, especially to bone.

Mouse and human prostates have significant differences anatomically, with the mouse prostate containing four defined lobes, and the human prostate divided histologically into four zones. The human prostate is a walnut shaped organ that
surrounds the urethra at the base of the bladder. It is divided histologically into three zones: the transition zone, the peripheral zone, and the central zone. In contrast, the mouse prostate is composed of four separate lobes surrounding the urethra: ventral, lateral, dorsal, and anterior. The dorsal and lateral lobes are often referred to collectively as dorsolateral. Despite being similar in molecular characteristics, the mouse and human prostates have significant anatomical and histological differences. Genomic data from microarray analysis suggests that the peripheral zone, from which the majority of prostate cancers arise in humans, is most similar to the dorsolateral prostate. Therefore, orthotopic mouse xenografts should be standardized by injection of cancer cells into the dorsolateral prostate to most accurately reflect human disease. However, even so, there is limited evidence that a dorsolateral origin of prostate tumors is, in fact, very similar to human prostate cancer.

In this study, I two hypotheses. The first is that different populations of AMPK are located at distinct compartments within the cell, and each of these populations can be activated by a distinct upstream stimulus. Furthermore, I hypothesize that this context-dependent response determines the net outcome of AMPK activity in cancer progression. I am studying this in several ways by: (1) investigating how distinct populations of AMPK are activated by different stimuli, (2) testing the functional role(s) of different subcellular AMPK populations and (3) demonstrating how the alpha subunit isoform present in the AMPK trimer determines its function in prostate cancer biology. My second hypothesis is that a mouse model that metastasizes reliably to the bone can be developed by orthotopic injection of prostate cancer cells into immune compromised, castrated mice. To do this, I compare organ tropism of
prostate cancer in castrated or intact immune compromised mice after orthotopic or subcutaneous injection of human prostate cancer cells. Together, the goal of these studies is to contribute to the advancement of prostate cancer research through targeting pathways activated in advanced disease, as well as improving the current landscape of mouse models of prostate cancer.
CHAPTER 2: MATERIALS AND METHODS

2.1: Cell lines and reagents

LNCaP and C4-2 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured according to ATCC protocols. C4-2B and C4-2B-LT (gift from Dr. Sue-Hwa Lin, UT MD Anderson Cancer Center) were cultured as previously described\(^59\). Cell lines were maintained without any addition of antibiotics, validated by STR profiling (University of Texas MD Anderson Cancer Center Cell Culture Core) and tested for mycoplasma upon thawing out fresh vials. The synthetic androgen methyltrienolone (R1881) was purchased from PerkinElmer (Naperville, IL, USA; Cat # NLP005005MG). Doxycycline hyclate (Cat # D9891), puromycin (Cat # P8833), and polybrene (Cat # TR-1003) were purchased from Sigma-Aldrich (St. Louis, MO, USA). D-luciferin sodium salt (cat # LUCNA-1G) was purchased from GoldBio, and 30mg/ml stock solution was maintained at -20°C.

2.2: Xenografts

All animal experiments were approved by and conducted under the Institutional Animal Care and Use Committee (IACUC) at the University of Texas MD Anderson Cancer Center (MDACC) according to NIH and institutional guidelines. Tumor volume was calculated by the formula: \(\text{length} \times \text{width}^2 / 2\). Xenografts were performed on 8-10-week old male NSG mice obtained from either The Jackson Laboratory (Bar Harbor, ME, USA; Cat # 005557) or MDACC Experimental Radiation Oncology Breeding Core. \(1 \times 10^6\) C4-2B-LT cells in Matrigel® 1:1 vol/vol (Corning, Corning, NY, USA; Cat # 356231) were injected either orthotopically (total
25ul cell solution) into the dorsolateral prostate, or subcutaneously (total 100ul cell solution) into the flank. Sham surgeries were performed on mice that did not require surgery.

2.3: Histology and immunostaining

Organs and tumors were immediately fixed in 4% PFA overnight at 4°C. For staining, samples were dehydrated and embedded in paraffin (MDACC Research Histology Core Laboratory). Paraffin slides were then rehydrated and further processed with antigen retrieval in citrate buffer (DAKO, Santa Clara, CA, USA; Cat # S169984-2). Peroxidase blocking was performed in 1% H2O2 plus 10% methanol solution. After washing with PBST (PBS with 0.02% Tween 20), secondary antibodies (Mouse-on-Mouse HRP Polymer, Biocare Medical, CA, USA, Cat#: MM620; SuperBoost™ Goat anti-Rabbit Poly HRP, Thermo Fisher Scientific, Waltham, MA, USA; Cat # B40962) were incubated for 30 minutes. Sections were developed by DAB (Vectorlabs, Burlingame, CA, USA; Cat # SK-4100) and imaged on a Nikon Eclipse Ci microscope.

2.4: Expression vectors and sgRNA design

CRISPR/Cas9 cells were developed with the lentiCRISPRv2 construct60. The gRNAs targeting PRKAA1 and PRKAA2 were developed using ChopChop (https://chopchop.cbu.uib.no/).

2.5: Generation of overexpression CRISPR/Cas9 KO cells
LentiCRISPRv2 was co-transfected with lentiviral packaging plasmids into 80% confluent HEK293T cells using Lipofectamine 2000. After 6 hours, media was changed to DMEM with 10% FBS and 1% BSA. After 48 hours, supernatants were harvested, filtered, and added to target cells with 7μg/ml polybrene. After 24 hours, fresh media with 1μg/ml puromycin was used to select for sgRNA expressing target cells. Then, single clones were isolated to establish knockout cells. Clones were validated by sequencing and western blot.

2.6: Western blot

Cells were harvested in RIPA lysis buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with Complete Protease Inhibitor Cocktail (Sigma, Cat # 11697498001) and PhosSTOP phosphatase inhibitor (Roche, Cat # 4906845001). Immunoblotting was performed with the following primary antibodies: ULK1 (Cat # 4773), p-ULK1(S555), AMPK (Cat # 2793), p-AMPK(T172) (Cat #: 2535), CAMKK2 (Cat # 16810), ACC (Cat # 3676), p-ACC (Cat # 3661), KU80 (Cat # 2180) from Cell Signaling Technology; CAMKK2 (Cat #: HPA017389), GAPDH (Cat #: G8795) from Sigma; AR (Cat # sc-7305) from Santa Cruz, AMPKα1 (Cat # MAB3197), AMPKα2 (Cat # AF2850) from R&D.

2.7: Proliferation assays

Proliferation assays were carried out as previously described by measuring the cellular double-stranded DNA content using a fluorescent DNA stain61. Transfection
was done with Lipofectamine 2000 tranfection reagent in OptiMEM media. Fluorescence was read at excitation 360 and emission 460.

2.8: Immunofluorescence microscopy

Cells were fixed with 4%PFA and permeabilized with 0.1% Triton. Images were captured using the Olympus BX51 fluorescence microscope and cellSense imaging software. Images were compiled and analyzed in ImageJ. Immunofluorescence staining was performed with the following primary antibodies: AMPK\(\alpha\)1 (Cat # MAB3197) and AMPK\(\alpha\)2 (Cat # AF2850) from Cell Signaling Technologies; and the following secondary antibodies: Donkey anti-mouse AlexaFluor 568 (Cat # A10037) and Donkey anti-goat AlexaFluor 568 (Cat # A11057) from Thermo Fisher Scientific. Nuclei were counterstained with DAPI (4’,6-diamidino-2-phenylindole).

2.9: Bioluminescence imaging

5-10 minutes before imaging, live mice were injected with 10ul/g of total body weight of 15mg/ml of D-luciferin in sterile PBS, and imaged on IVIS (in vivo imaging system) (Perkin Elmer). Dissected organs and tumors were placed in a 24-well plate and covered with D-luciferin solution and imaged on IVIS.

2.10: Statistical analysis

One-way ANOVA with Tukey’s post-hoc test for multiple comparisons were performed. The data were analyzed using GraphPad Prism 9.0. All data is presented as mean +/- standard error of the mean, and \(P\) values are <0.05.
CHAPTER 3: RESULTS

3.1: Different stimuli activate distinct subcellular AMPK populations

Previous studies suggest that distinct populations of AMPK behave differently in response to stimuli. Organelle-targeted AMPK activity reporters (AMPKARs) were used to measure AMPK activity at different subcellular compartments\textsuperscript{62}. AMPKAR is a peptide reporter of Förster resonance energy transfer (FRET), which measures energy transfer between two fluorescent chromophores, upon activation by AMPK. AMPKAR has been optimized from its original form\textsuperscript{63} and has been genetically encoded with organelle targeting sequences to either the plasma membrane, cytoplasm, nucleus, mitochondria, endoplasmic reticulum, Golgi apparatus, or lysosomes\textsuperscript{62} (collectively called organelle specific AMPKAR, or osAMPKAR). This study reported that different organelle-localized populations of AMPK behaved differently in response to diverse metabolic perturbations\textsuperscript{62}.

I used these genetically encoded AMPKARs to measure spatially and temporally distinct activation of intracellular AMPK. These reporters have previously shown that the dynamics of AMPK activation vary between cell compartments in response to stress\textsuperscript{62}. To investigate this in prostate cancer, I used AMPKAR targeted to either the cytoplasm (Cyto-AMPKAR) or nucleus (Nuc-AMPKAR) to measure AMPK activity at these compartments in response to mechanistically different stimuli: the glycolytic inhibitor 2-deoxyglucose (2-DG), and thapsigargin, which increases intracellular calcium by blocking calcium uptake into the endoplasmic reticulum (Figure 1A). I transfected 22Rv1 cells, a model of CRPC,
with the Cyto-AMPKAR or Nuc-AMPKAR and observed that thapsigargin rapidly and transiently activated AMPK activity in both the cytoplasm and the nucleus, while 2-DG caused a gradual, sustained activation of cytoplasmic AMPK, with no effect on nuclear AMPK (Figure 1B).

Next, I knocked down CAMKK2 using a doxycycline-inducible shRNA system (Figure 2A-C). CAMKK2 is the main upstream kinase of AMPK, and it is known to be a predominantly cytoplasmic protein. With the knockdown of CAMKK2, there was a significant decrease in cytoplasmic AMPK activity, with no change in nuclear AMPK activity (Figure 2D). This is in accordance with our understanding of CAMKK2 mainly interacting with the cytoplasmic population of AMPK. Together, these data support my hypothesis that different populations of AMPK (in this case, nuclear vs. cytoplasmic) are differentially activated depending on the upstream signal.
Figure 1. Different stimuli activate distinct subcellular AMPK populations. (A) Fluorescent microscopy confirming localization of AMPKAR to cytoplasm (left) or nucleus (right) (B) AMPK activity quantification by FRET output of cyto-AMPKAR (left) and nuc-AMPKAR (right). Cells were treated with either thapsigargin (blue) or 2-DG (orange) at 5 minutes after FRET reading began. SEM is shown.
Figure 2. CAMKK2 knockdown decreases cytoplasmic AMPK activity. (A) pINDUCER construct used for CAMKK2 knockdown. (B) Doxycycline-inducible shCAMKK2 22Rv1 cells were treated with vehicle (PBS) or doxycycline (DOX) for 72 hrs and cell lysates were subject to immunoblot analysis. (C) Fluorescent microscopy confirming localization of AMPKAR to cytoplasm (left) or nucleus (right). (D) 22Rv1 shCAMKK2 cells were treated ± 800 µg/ml doxycycline for 3 days and transfected with either Cyto-AMPKAR or Nuc-AMPKAR. FRET/CFP intensity was averaged. *p<0.05.
3.2: Compartmentalized populations of AMPK have diverse functions

Next, I investigated how different spatially located subpopulations of AMPK contribute to cell growth. I first used an AMPK inhibitor peptide (AIP) fused to various organelle targeting sequences to perturb AMPK activity at specific subcellular locations (Figure 3A). The AIP also contains an mCherry sequence which was used by imaging flow cytometry to confirm correct expression and subcellular localization of the peptide within the cell (Figure 3B). I transfected LNCaP cells with either the mCherry backbone, the AIP peptide, or AIP targeted specifically to the cytoplasm or nucleus, and quantified cell proliferation in response to R1881, a synthetic androgen. I observed that LNCaP cell proliferation increased upon inhibition of nuclear AMPK relative to cells with cytoplasmic AMPK inhibition (Figure 3C). This data further supports the hypothesis that nuclear AMPK is more tumor suppressive, while cytoplasmic AMPK promotes oncogenic effects.
Figure 3. Inhibition of AMPK at different subcellular compartments has opposing effects on prostate cancer cell growth. (A) Constructs were developed containing an AMPK inhibitor peptide (AIP) sequence fused to an organelle-targeting sequence. Schematic of organelle-localized AIP plasmids is shown. (B) Imaging flow cytometry analysis shows localization of nuclear and cytoplasmic AIP constructs. (C) LNCaP cells were transfected with mChF, mChF-AIP, Nuc-mChF-AIP, or Cyto-mChF-AIP for 24 hours and then treated with vehicle or androgen for 7 days. Relative cell number was analyzed with a DNA stain.
3.3: Functional differences between AMPKa1 KO, AMPKa2 KO

I have developed AMPKa1 and AMPKa2 CRISPR/Cas9 knockout models of LNCaP, a hormone-sensitive prostate cancer cell line, and the CRPC cell lines 22Rv1 and C4-2. These cell lines represent early and advanced stages of prostate cancer, respectively. Using the lentiCRISPR v2 vector\textsuperscript{60} containing sgRNA sequences for either \textit{PRKAA1} or \textit{PRKAA2}, I knocked out either gene in these cell models.

Single cell cloning was performed to create cell lines with no residual protein, something that is possible in the pooled knockout cell lines. I then confirmed the \textit{PRKAA1} or \textit{PRKAA2} knockout cell lines (hereafter referred to as “\(\alpha1\) KO or \(\alpha2\) KO,” respectively) by western blot (Figure 4A). Given the localization of each subunit to distinct compartments, I wanted to test the hypothesis that the different isoforms are involved in activating distinct populations of downstream substrates. I tested this by measuring the activation of AMPK downstream targets that are known to localize to either the nucleus (p300) or the cytoplasm (ULK, ACC, and RAPTOR). I observed a decrease in phosphorylation in the cytoplasmic proteins in the \(\alpha1\) KO cell lines, and a decrease in the nuclear p300 protein in the \(\alpha2\) KO cell lines (Figure 4B).

Next, I investigated the how the different KO clones behaved in response to hypoxia. Hypoxia activates AMPK by inhibiting oxidative phosphorylation\textsuperscript{64}, thereby increasing the ratio of AMP:ATP within the cell. Parental LNCaP cells, \(\alpha1\) KO cells, and \(\alpha2\) KO cells were treated with either R1881 or vehicle, and then incubated in either 5% O2 (normal oxygen) or 1% O2 (low oxygen) in a dedicated hypoxia chamber. A proliferation assay showed that after 7 days, the \(\alpha1\) KO cells had a significantly lower cell number relative to the \(\alpha2\) KO cells (Figure 5A-B). This
suggested that AMPKα1 may have more of a role in prostate cancer cell growth, while AMPKα2 may suppress prostate cancer cell growth under conditions of hypoxia.
Figure 4. AMPKα1 and AMPKα2 differentially activate known downstream AMPK targets. (A) Western blot validating knockout of AMPKα1 and AMPKα2 in CRISPR knockout cells. (B) Western blot showing the effect on phosphorylation of AMPK downstream targets in AMPKα1 and AMPKα2 knockout cells.
Figure 5. AMPKα1 and AMPKα2 knockout cells have different responses to hypoxia. AMPKα1 or AMPKα2 knockout LNCaP cells were treated with either vehicle or androgen and incubated either in normoxia (A) or hypoxia (B) for 7 days. Relative cell number was analyzed with a DNA stain. p<0.05
3.4: AMPKα1 and AMPKα2 localize to distinct cell compartments

Next, I treated LNCaP cells with either vehicle or R1881 and performed cell fractionation using centrifugation to isolate the nuclear and cytoplasmic cell compartments. A western blot showed that AMPKα1 was present in the cytoplasm, while AMPKα2 was in the cytoplasm, and to a lesser extent in the nucleus (Figure 6A). This supports my hypothesis that AMPKα1 is more cytoplasmic, while AMPKα2 may also shuttle to the nucleus.

To further investigate these results, I examined AMPKα1 or AMPKα2 subcellular localization using immunofluorescence microscopy. Using antibodies specific for either the AMPKα1 or AMPKα2 subunit, I observed that in LNCaP α1 KO cells, the fluorescence staining appeared throughout the cells, while in the α2 KO cells, the fluorescence appeared more nuclear (Figure 6B). Future studies are needed to confirm the specificities of these antibodies in immunofluorescence microscopy.
Figure 6. AMPKα1 and AMPKα2 isoform localization in prostate cancer cells.

(A) LNCaP cells treated with androgen for 72 hours were subjected to biochemical fractionation to isolate cytoplasmic and nuclear fractions. (B) LNCaP cells were immunofluorescently labeled with anti-AMPKα1 (top) or anti-AMPKα2 (bottom) and DAPI.
3.5: Bone metastases occur at a high rate in orthotopically injected, castrated NSG mice

Immunodeficient male mice (NOD-\textit{scid} IL2Rgamma\textsuperscript{null}) (“NSG”) were castrated and orthotopically injected (into the dorsolateral lobe of the prostate) with C4-2B prostate cancer cells labeled with tdTomato and luciferase reporters. C4-2 castrate-resistant cells are derived from LNCaP prostate cancer cells, and C4-2B is a CRPC cell line derived from cells that have metastasized to bone after castration. After 6 weeks, the mice were dissected, and isolated organs (brain, heart, liver, kidneys, lungs, femur, spleen, and lymph nodes) were imaged on the IVIS (\textit{in vivo} imaging system) to view presence of metastases. In all 3 mice, bone metastases were observed. This was a surprising observation as there are no mouse models that consistently metastasize from the primary site to the bone. I wanted to investigate this further because the development of a mouse model metastasizing reliably to bone would be useful in prostate cancer metastasis studies.

To further investigate, I created 4 groups of NSG mice with either orthotopic or subcutaneous injection, and either castrated or intact (group 1: orthotopic, castrated; group 2: orthotopic, intact; group 3: subcutaneous, castrated; group 4: subcutaneous, intact). This would allow me to test the impact of both castration and route of inoculation on the incidence of bone metastases. The mice were injected with luciferin and imaged on the IVIS weekly for 6-7 weeks or when the primary tumor reached 1.5\text{cm}^3 (\textit{Figure 7A}). During this time, the primary tumor was quantified by IVIS as well as caliper measurements for mice with subcutaneous flank tumors (\textit{Figure 7B}). Then, the mice were dissected, and the organs were imaged in a luciferin solution on the IVIS (\textit{Figure 8}). Several mice died before end
point, so several groups had fewer than 10 mice. A threshold for a positive metastatic signal was set at $1 \times 10^4$ photons/second (total flux) based on previous reports\textsuperscript{65}. I observed that 7/8 mice from group 1 (orthotopic, castrated) and 9/10 mice from group 4 (subcutaneous, intact) had bone metastases (Table 1). These groups had the highest rates of metastasis to bone. The orthotopic, castrated group is more physiologically relevant to advanced prostate cancer research as it reflects the castrate-resistant status of advanced disease and dissemination from the primary site (prostate), so I pursued the development of this bone metastatic mouse model.

To validate the results from bioluminescent imaging, organs, and bones with and without observable signal were embedded in paraffin and analyzed by immunohistochemistry. Samples were stained for human-KU80, a nuclear protein involved in double strand DNA break repair. This was used to identify the presence of human cancer cells within the surrounding mouse organ cells. Hematoxylin was used to counterstain the nuclei. I observed positive staining for h-KU80 in tissues with positive bioluminescence signal, and negative staining in tissues with negative bioluminescence (Figure 9).
Figure 7. Quantification of primary tumors in NSG mice. (A) Representative images of primary tumor bioluminescence. (B) Average radiance (p/s/cm²/sr) was measured once per week for 6 weeks.
Figure 8. Quantification of metastases in NSG mice. Total flux (p/s) was measured for each organ. NTB=non-tumor bearing (mice which were injected with cancer cells but did not develop a primary tumor).
<table>
<thead>
<tr>
<th>Route of injection</th>
<th>Castrated or intact</th>
<th>Visceral organs</th>
<th>Bone</th>
<th>Number of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subcutaneous</td>
<td>Intact</td>
<td>9 (90%)</td>
<td>9 (90%)</td>
<td>10</td>
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<tr>
<td>Subcutaneous</td>
<td>Castrated</td>
<td>7 (78%)</td>
<td>6 (67%)</td>
<td>9 (7 TB)</td>
</tr>
<tr>
<td>Orthotopic</td>
<td>Intact</td>
<td>5 (100%)</td>
<td>2 (40%)</td>
<td>5</td>
</tr>
<tr>
<td>Orthotopic</td>
<td>Castrated</td>
<td>8 (100%)</td>
<td>7 (88%)</td>
<td>8</td>
</tr>
</tbody>
</table>

**Table 1: Metastasis profile of C4-2B-LT xenografts.** Presence of metastases was measured by IVIS. Organs with total flux above threshold ($1 \times 10^4$ p/s) were considered metastatic. Visceral organs include: brain, heart, lung, liver, kidneys, spleen, and lymph nodes. TB = tumor-bearing.
Figure 9. Histological validation of bone metastasis. Femurs from tumor bearing and non-tumor bearing mice were analyzed by immunohistochemistry and stained for the human protein KU80.
CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

In this study, we provide evidence that AMPK is localized in distinct compartments within the cell, and furthermore, that these populations are functionally distinct and non-redundant. This is in consensus with existing evidence from our lab and others\textsuperscript{34,62,66}. Moreover, we provide evidence to support our hypothesis that subcellular localization of AMPK is related to the isoform composition of the trimer. We have shown by western blot and immunofluorescence microscopy that AMPKα1 appears in the cytoplasm, while AMPKα2 appears in the cytoplasm and nucleus. Shuttling of AMPK between the nucleus and cytoplasm requires a nuclear localization sequence (NLS) or nuclear export sequence (NES) to transport the protein through the nuclear pore complex. The AMPKα2 subunit contains both a known NLS and NES\textsuperscript{50,67}. In addition, the AMPKα1 subunit contains a putative NES\textsuperscript{50,67}.

We are interested in further understanding if and when AMPKα1 and AMPKα2 shuttle between compartments and how this affects the function of the complex in prostate cancer progression. To do this, we can use site-directed mutagenesis followed by live-cell imaging to observe the effects of mutating the NES and NLS sites, thereby sequestering the isoforms in either the nucleus or cytoplasm.

Our studies using the AMPK inhibitor peptide (AIP) suggest differences in functional effects between the cytoplasmic and nuclear populations of AMPK. Specifically, we show that inhibition of the nuclear population resulted in an increase in cell growth, while an inhibition of the cytoplasmic population lead to a decrease. This suggests that nuclear AMPK may behave as more of a tumor suppressor,
while cytoplasmic AMPK may be more oncogenic. Next, BrdU (a thymidine analogue that is incorporated into DNA during S phase) will be used to analyze changes in cell growth by quantification of replicating cells through high-throughput fluorescent cell imaging.

Our studies using the AMPKAR targeted to the cytoplasm or nucleus showed us that these populations of AMPK are differentially activated in response to different stresses, in both a spatial and temporal manner. We will continue to investigate this using AMPKAR targeted to other organs: ER, Golgi, lysosome, mitochondria, and plasma membrane. We will activate AMPK by mechanistically distinct stimuli by using 2-DG, thapsigargin, and oligomycin (an ATP synthase inhibitor), ± androgen, and ± CaMKK2 knockdown or overexpression. AMPK has known downstream targets that are associated with each of these organelles; therefore, it is important to understand how and when AMPK activates each of these targets. This may allow us to potentially target those AMPK downstream pathways activated in prostate cancer, while leaving the tumor suppressive pathways undisturbed.

CRISPR knockout of each of the AMPK alpha subunit isoforms showed us that each isoform may preferentially activate distinct groups of downstream targets. This is in line with our hypothesis that each isoform has separate functions in prostate cancer progression. To investigate this further, we are developing double knockout cell lines (PRKAA1 and PRKAA2) that, when coupled with genetic addbacks to preclude off target effects, will allow us to dissect the role of AMPKα1 and AMPKα2 in prostate cancer cells. Furthermore, we would like to establish knockout mouse models to observe changes on prostate cancer progression in
immune intact mice with genetic knockout of either gene. These mice will be established in the TRAMP (transgenic adenocarcinoma of the mouse prostate) mouse model in C57BL/6 male mice. While AMPKα1-/- AMPKα2-/- double knockout mice are embryonic lethal, single knockout mice are viable, albeit with certain metabolic dysfunctions, often affecting glucose or insulin intolerance. The bone metastatic mouse model we are developing will be immensely important to preclinical studies of prostate cancer progression. We found that several of the groups tested had high rates of metastasis to bone, which is normally not common in mouse prostate cancer models. Moving forward, we want to further dissect what exactly causes this effect. In our experiment, we compared groups with either castrated or intact mice, and either orthotopically or subcutaneously injected tumors. We are of the opinion that the castrated, orthotopic model is the most accurate as it mimics the hormone-sensitivity of advanced disease in men, and the prostate injection site allows the primary tumor to grow in a microenvironment most similar to clinical disease. Furthermore, the routes that the metastatic cancer cells travel to reach the metastatic sites will be mimicked more accurately. Intravenous or cardiac tumor cell injections are also commonly used in metastasis studies, with an obvious drawback being that the cells do not travel the same routes as in the organic development metastases. With all of this in consideration, there is no perfect mouse representation of human disease, as there are differences in the mouse prostate microenvironment as well as the microenvironment of the potential metastatic sites.

The IVIS imager is able to measure very small quantities of cancer cells that may be present anywhere throughout the organ. Other methods of measuring micrometastases such as fluorescent imaging or immunohistochemistry may miss
the presence of many of these metastases. By imaging the whole organ with bioluminescence imaging and then validating the positive signal with immunohistochemistry, we can ensure that we quantify all metastases.

Another important aspect of metastatic tropism that should be investigated is the immune microenvironment. Disseminated tumor cells can travel to bone marrow and live there dormant for years before forming metastases. NK cells mature and differentiate in the bone marrow, and can remain there until recruited to a site of inflammation. This suggests that they may have an important role in the bone pre-metastatic niche. There is a known crosstalk between NK cell and bone/immune cells in the bone premetastatic niche that can affect metastasis. Furthermore, the presence of high levels of NK cells correlates with a good prognosis in metastatic castration resistant prostate cancer patients. Our study used NSG mice, which are severely immune compromised, and are deficient in B and T cells as well as NK cells. Prior studies using C4-2B cells in SCID mice do not have a high rate of bone metastasis when injected orthotopically or subcutaneously. We would like to do a comparison study between NSG and SCID (severe combined immunodeficient) mice, which while being B and T cell deficient, do contain functional NK (natural killer) cells. Given the role of NK cells in bone metastasis, we expect NK deficient mice (NSG) to have a greater rate of metastasis, which would suggest that NK depletion will increase mouse prostate cancer to bone.
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