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14. ABSTRACT

Androgen Deprivation Therapy (ADT) remains the first-line treatment option for patients with advanced metastatic prostate cancer. However, despite initial responses to ADT, the majority of patients develop resistance to ADT and progress to castration resistant prostate cancer (CRPC). The mechanisms of resistance to ADT are poorly understood and remains an urgent unmet need in prostate cancer research. It has been hypothesized that AR splice variants play a causal role in conferring endocrine resistance in prostate cancer. ARv7 is the most frequently expressed AR splice variant in CRPC and is associated with poor prognosis and resistance to ADT. The overarching goal of this study is to further delineate the role and mechanisms governing ARv7 activity in CRPC. We hypothesize that the use of AR N-terminal disrupting inhibitors may target the ARv7 and demonstrate increased effectiveness in the treatment of CRPC.

15. SUBJECT TERMS

Prostate Cancer, Androgen Receptor, Androgen Receptor variant 7, castration resistant prostate cancer, transcription

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1. Introduction

Androgen Deprivation Therapy (ADT) remains the first-line treatment option for patients with advanced metastatic prostate cancer. However, despite initial responses to ADT, the majority of patients develop resistance to ADT and progress to castration resistant prostate cancer (CRPC). The mechanisms of resistance to ADT are poorly understood and remains an urgent unmet need in prostate cancer research. It has been hypothesized that AR splice variants play a causal role in conferring endocrine resistance in prostate cancer. ARv7 is the most frequently expressed AR splice variant in CRPC and is associated with poor prognosis and resistance to ADT. The ARv7 variant retains the same N-terminal domain and DNA-binding domain (DBD) as the full-length AR, but importantly, lacks the LBD, the intended target for enzalutamide and other AR-targeting inhibitors. The overarching goal of this study is to further delineate the role and mechanisms governing ARv7 activity in CRPC. The biology of ARv7 activity remains poorly understood, and a better understanding of ARv7 activity could be critical in improving therapeutic options for patients with castration resistant prostate cancer.

2. Keywords

Prostate Cancer, Androgen Receptor, Androgen Receptor variant 7, castration resistant prostate cancer, transcription

3. Accomplishments

Major Goals:

Aim 1: Determine the ARv7-dependent essential genes responsible for driving CRPC growth through CRISPR/Cas9 genetic screening.
Aim 2: Determine if EZH2 is required for ARv7-dependent transcriptional repression in CRPC.
Aim 3: Determine if novel AR N-terminal disrupting inhibitors target ARv7 and decrease CRPC tumor growth.

Results

Aim 1: Determine the ARv7-dependent essential genes responsible for driving CRPC growth through CRISPR/Cas9 genetic screening.

In order to identify the key genes downstream of ARv7 that mediate cell proliferation, we performed a genome-wide CRISPR screen in shGFP, shARv7, shARfl LN95 cells. The LN95 model is a CRPC cell line model that expresses ARv7. These cells have been engineered to inducibly express short hairpins directed at either GFP (control), ARv7, or ARfl in the presence of Doxycycline (Figure 1). We then performed the genome-wide CRISPR screen in the presence and absence of Doxycycline (Figure 2). We then compared each +DOX condition to its corresponding -DOX condition to eliminate those genes that are pan essential. From this analysis, we were able to determine those genes that are

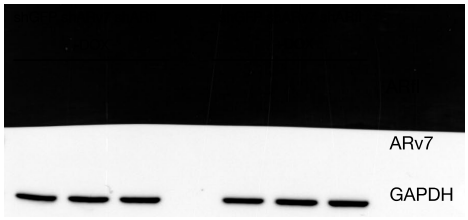

Figure 1. Expression of ARv7, ARfl, and GAPDH in shGFP, shARv7, shARfl LN95 cells.

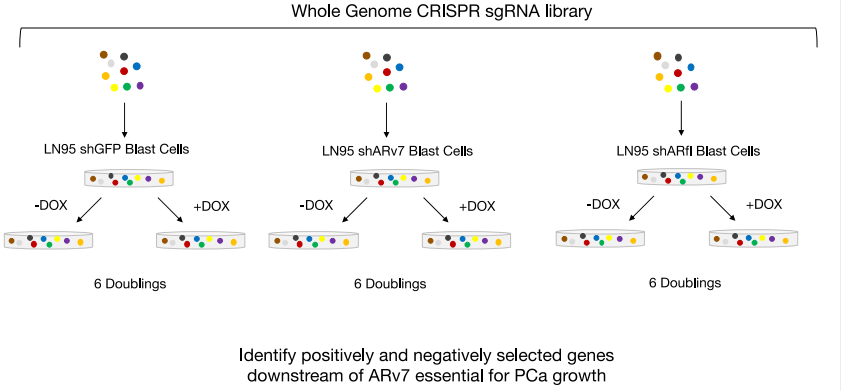

Figure 2. Overview of CRISPR screen.

specifically essential in the shARv7 +DOX and the shARfl +DOX conditions. We then overlapped these essential genes between the shARv7 and shARfl conditions to identify genes that are specifically essential only in the shARv7 condition, the shARfl condition, and essential in both (Figure 3A). We then performed gene ontology analysis to identify which pathways were enriched (Figure 3B). We identified that oxidative phosphorylation was an essential pathway in the shARv7 only, shARfl only, and overlapping genes.

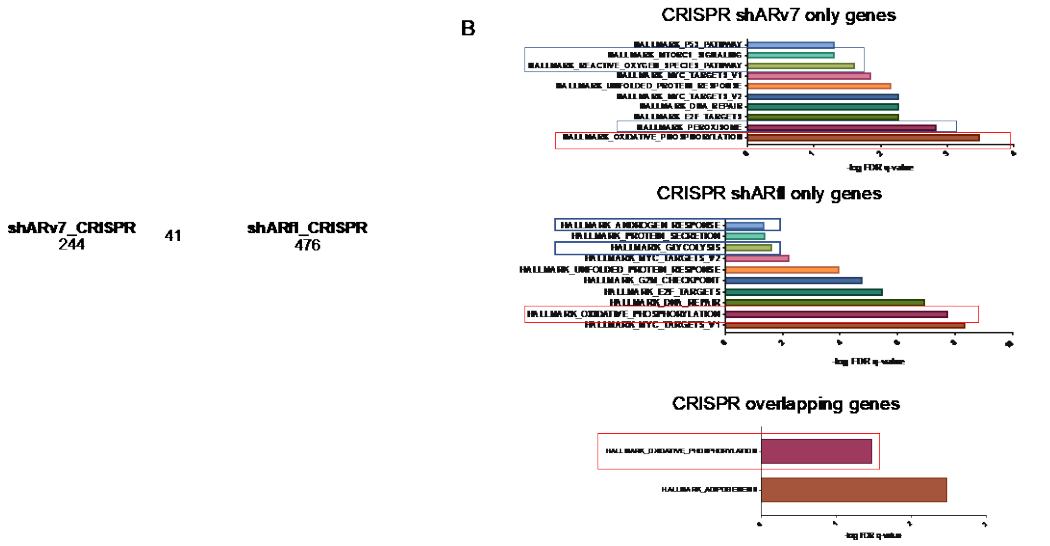

Figure 3. Comparison of shARv7 and shARfl essential genes. A) Venn diagram of essential genes from the shARv7 and shARfl conditions. B) Gene ontology analyses for shARv7 only, shARfl only, and overlapping essential genes.

In the shARv7 only essential genes, we identified the Hallmark reactive oxygen species pathway and the mTORC1 signaling pathway were specifically enriched, potentially indicating therapeutic vulnerabilities to those pathways in ARfl-only prostate cancer cells. In the shARfl only essential genes, we identified the Hallmark androgen response and glycolysis pathways were enriched, perhaps indicating therapeutic vulnerabilities to those pathways in ARv7-only prostate cancer cells. The enrichment of the androgen response in the shARfl only essential genes seems somewhat paradoxical, but this result could indicate that ARv7 is continuing to drive an AR transcriptional program in the absence of ARfl, consistent with what has been previously reported.

In an effort to validate the results of the CRISPR screen, we sought to inhibit these enriched pathways using either small molecule inhibitors or CRISPR-mediated genetic knockout. However, we encountered a number of issues with the LN95 prostate cancer cell line models that we had previously engineered. The cells appeared to arrest in culture and ceased to grow after a number of passages. We attempted to troubleshoot the cell proliferation issues, but could not come up with a workable solution during the reporting period of this award.

Aim 2: Determine if EZH2 is required for ARv7-dependent transcriptional repression in CRPC.

Defining the ARv7 cistrome

In an effort to better understand the mechanism of action of ARv7, we have defined the ARv7 cistromes in a number of CRPC cell lines: 22RV1, LN95, and VCaP-16. VCaP-16 cells are CRPC cells developed by our collaborator, Dr. Steve Balk, that are resistant to the AR antagonist, enzalutamide. We have performed ChIP-seq of both the ARfl and ARv7 in VCaP-16 cells to better understand the dynamics of AR recruitment to its regulatory regions. Similar to what we have previously observed in LN95, the majority of ARv7 binding sites in VCaP-16 overlap with ARfl binding sites (Figure 4). We then overlapped the VCaP-16 ARv7 binding sites with the cistromes of ARv7 in 22RV1 and LN95 cells, we found that ARv7 binding is quite heterogenous (Figure 5). In the Vehicle treatment group, only 255 binding sites were common among all three cell lines. In each cell line, the majority of ARv7 binding sites were unique to each cell line and not shared. We then performed Cistrome Toolkit GIGGLE similarity analysis which queries publicly available ChIP-seq data and returns datasets that are most similar to the input dataset. We input the unique ARv7 binding sites for each cell line and queried the datasets that are most similar to identify potential members of the ARv7 transcriptional complex (Figure 6). We found that each cell line had unique datasets that were most similar to the ARv7 dataset, potentially indicating

that ARv7 is regulated distinctly in each cell line. For each cell line, the AR dataset was either the top or among the topmost similar datasets. For LN95, CHD1, NKX3.1, and HOXB13 were among the most similar datasets. For 22RV1, SRC1 and SOX2 were among the top-ranking datasets; SOX2 is not a well-known cofactor of AR and it has been implicated in promoting castration resistant prostate cancer and stemness. Finally, for VCaP-16, EZH2 and ERG factors rank highly amongst the most similar datasets. Characterization of the ARv7 cistromes reveal that ARv7 binding sites are varied and heterogenous in CRPC cell lines, however, it appears that ARv7 utilizes the same coregulators at its regulatory regions as ARfl.

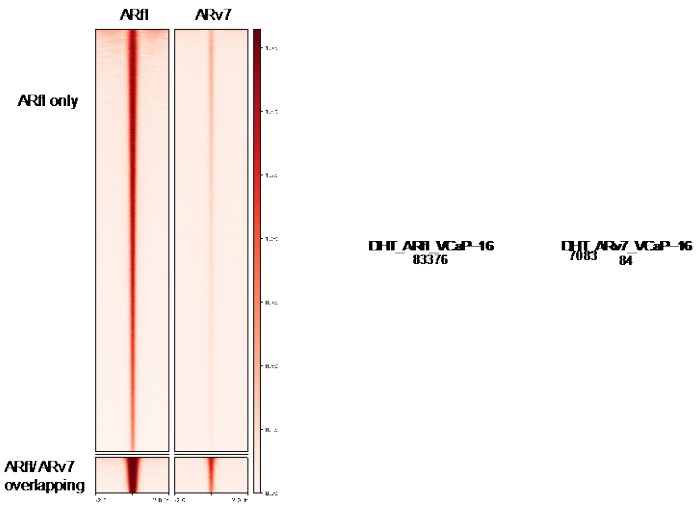

Figure 4. The majority of ARv7 binding sites overlap with ARfl in VCaP-16 cells.

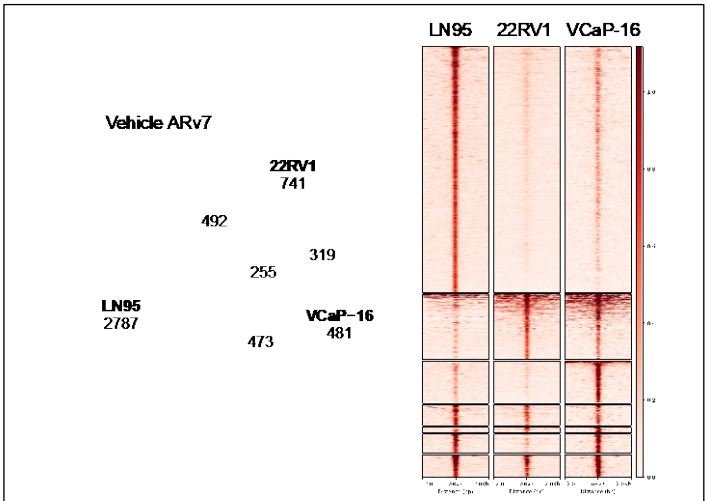

Figure 5. ARv7 binding sites in LN95, 22RV1, and VCaP-16.

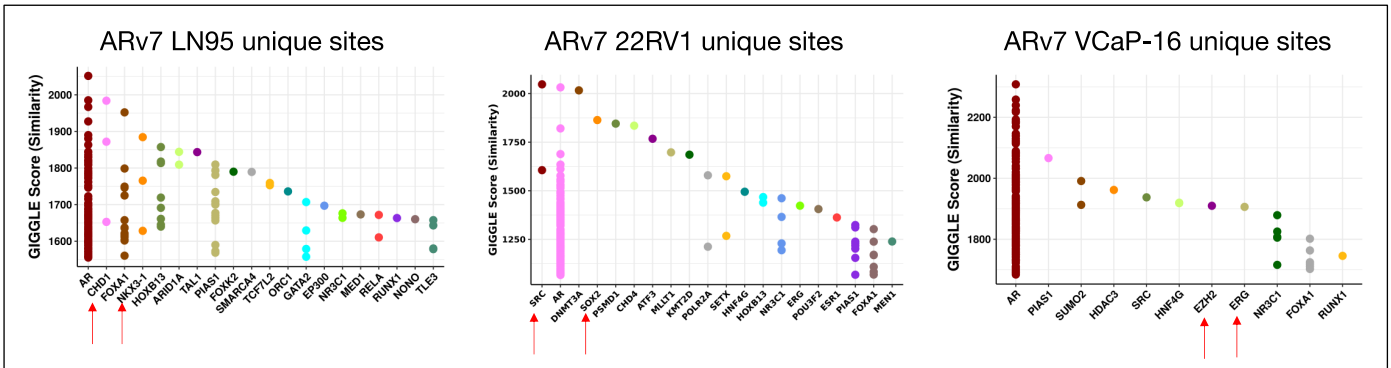

Figure 6. Cistrome Toolkit GIGGLE similarity analysis of LN95, 22RV1, VCaP-16 unique ARv7 binding sites.

Exploring the relationship between EZH2 and ARv7

Previously, we hypothesized that EZH2 may play a co-regulator role for ARv7-dependent transcriptional activity. It has been established that EZH2 and ARfl are co-localized at regulatory regions in CRPC cells, however, no such relationship has been established between EZH2 and ARv7. From our CRISPR screen analysis, we were able to determine that EZH2 and other PRC2 family members SUZ12 and EED, are essential in all conditions (Figure 7). EZH2 appears to be most essential in the shGFP condition while SUZ12 and EED appear to be more essential in the shARv7 condition compared to the other conditions.

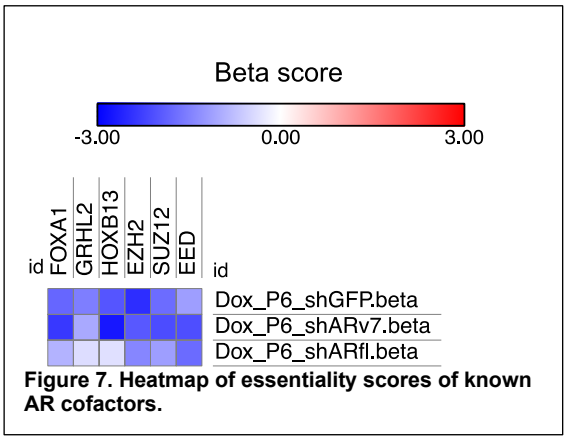

Figure 7. Heatmap of essentiality scores of known AR cofactors.

This led us to interrogate the potential relationship between EZH2 and ARv7. We performed EZH2 and H3K27me3 ChIP-seq studies to determine if there is significant overlap with ARv7 binding sites. We interrogated H3K27me3 for two reasons: PRC2 complexes are responsible for methylation of H3K27 and ARv7 was previously demonstrated by our laboratory to repress H3K27ac (Cato et al, *Cancer Cell*, 2019). We hypothesized that ARv7 represses H3K27ac through recruitment of EZH2 and PRC2 complexes resulting in enrichment of H3K27me3 at ARv7 binding sites.

We were able to successfully ChIP both EZH2 and H3K27me3 in shGFP, shARv7, shARfl +/- DHT in LN95 cells (Table 1).

Sample Name	ChIP Antibody	Binding Sites
shGFP VC LN95	EZH2	3,120
shGFP DHT LN95	EZH2	6,262
shARv7 VC LN95	EZH2	5,034
shARv7 DHT LN95	EZH2	3,911
shARfl VC LN95	EZH2	2,936
shARfl DHT LN95	EZH2	3,046
shGFP VC LN95	H3K27me3	42,394
shGFP DHT LN95	H3K27me3	43,107
shARv7 VC LN95	H3K27me3	33,247
shARv7 DHT LN95	H3K27me3	26,721
shARfl VC LN95	H3K27me3	42,404
shARfl DHT LN95	H3K27me3	40,619

Table 1. Summary of EZH2 and H3K27me3 ChIP-seq data.

We then sought to determine if EZH2 and/or H3K27me3 coincided with ARv7 binding sites. We had previously identified ARv7 binding sites in LN95 cells and used this dataset to overlap with the EZH2 and H3K27me3 cistromes. We found that very few ARv7 binding sites overlapped with either EZH2 or H3K27me3 binding sites (Table 2). We additionally plotted the EZH2 and H3K27me3 ChIP signal intensities centered upon the ARv7 cistromes with and without DHT (Figures 8 and 9). The heatmaps revealed that EZH2 ChIP signal is quite low at ARv7 binding sites (Figure 8). In the H3K27me3 heatmap, it appears that H3K27me3 flanks ARv7 binding sites but is absent at the center of the ARv7 binding peaks. These results indicate that EZH2 does not play a direct co-regulatory role in ARv7 transcriptional activity. Furthermore, it appears that ARv7 does not drive transcriptional repression through recruitment of PRC2 complexes and enrichment of H3K27me3.

Sample Name	ChIP Antibody	ARv7 overlapping binding sites
shGFP VC LN95	EZH2	1
shGFP DHT LN95	EZH2	18
shARv7 VC LN95	EZH2	7
shARv7 DHT LN95	EZH2	11
shARfl VC LN95	EZH2	4
shARfl DHT LN95	EZH2	13
shGFP VC LN95	H3K27me3	1
shGFP DHT LN95	H3K27me3	21

shARv7 VC LN95	H3K27me3	1
shARv7 DHT LN95	H3K27me3	9
shARfl VC LN95	H3K27me3	5
shARfl DHT LN95	H3K27me3	21

Table 2. Summary of EZH2 and H3K27me3 sites that overlap with ARv7 binding sites.

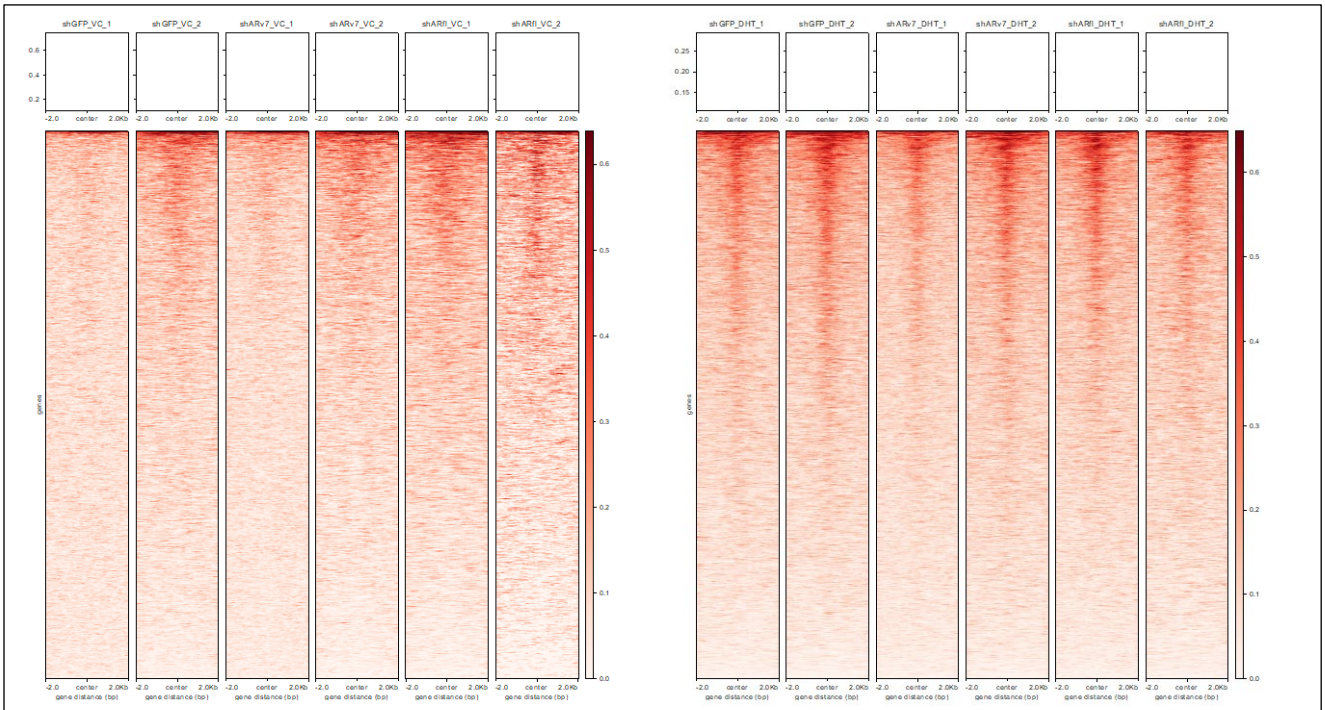

Figure 8. ARv7 binding sites overlapped with EZH2 signal intensity in Vehicle treated (left panel) and DHT treated (right panel) LN95 cells.

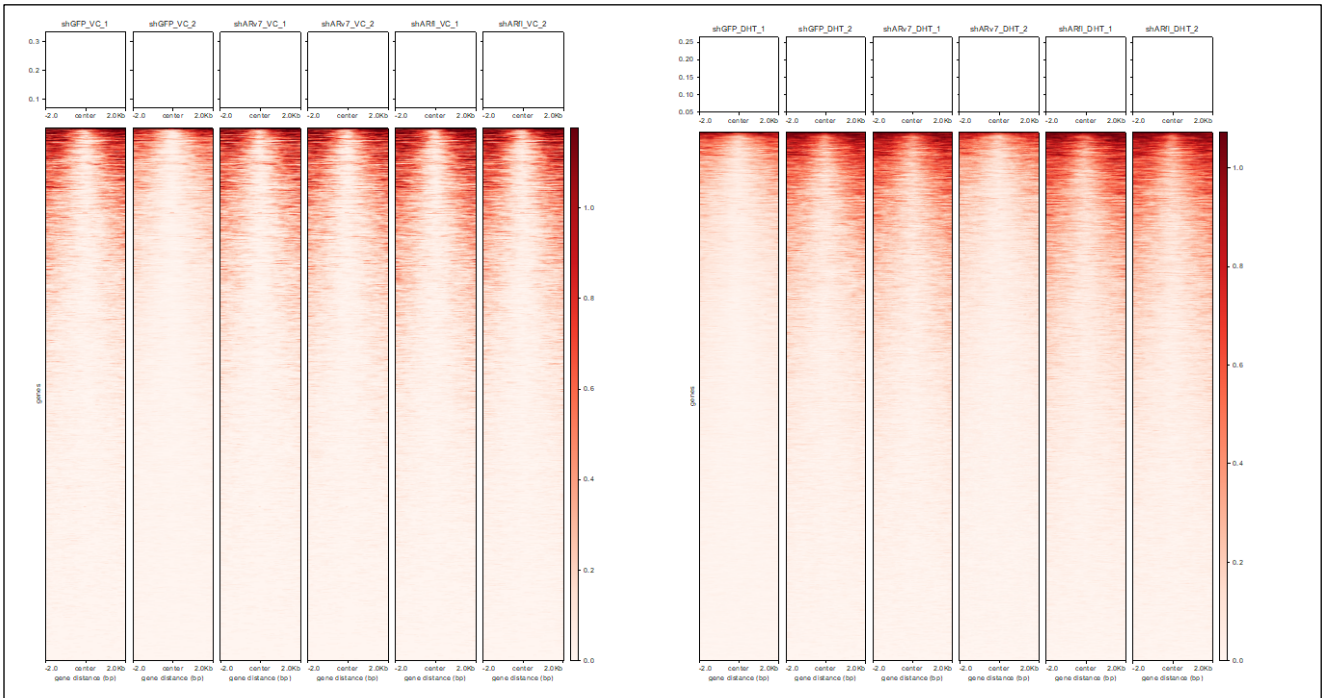

Figure 9. ARv7 binding sites overlapped with H3K27me3 signal intensity in Vehicle treated (left panel) and DHT treated (right panel) LN95 cells.

Our results indicate that EZH2 is unlikely to play the co-regulator role of ARv7 transcriptional activity and repression. From our CRISPR screen results, EZH2 is quite essential for the growth of CRPC cells,

however, our results indicate that the activities of EZH2 may be independent of ARv7. Further interrogation into the factors governing ARv7 activity is needed to better understand the role of ARv7 in CRPC. Unfortunately, validation of other putative co-regulatory factors of ARv7 could not be completed during the reporting period of this award.

Determining the role of ARv7 in mediating genome-wide transcriptional repression

Previously, we demonstrated that ARv7 is required for the repression of a set of enhancers, through decreasing H3K27ac levels (Cato et al, *Cancer Cell*, 2019). We inquired whether ARv7 may impact the global epigenetic landscape in LN95 cells and lead to a more repressive epigenetic state. We performed ATAC-seq in shGFP, shARv7, shARfl LN95 cells +/- DHT to address this question. We hypothesized that knock down of ARv7 would lead to an increase in accessible chromatin regions. We performed differential peak analysis to determine the peaks that demonstrate differential regulation between shGFP, shARv7, and shARfl. Our results indicate that knock down of ARv7 leads to a global decrease in accessible chromatin regions (26,180 peaks) compared to shGFP in the Vehicle Control treatment (Figure 10). In the presence of DHT, there were no peaks that were significantly differentially regulated between shGFP and shARv7. In contrast, knock down of ARfl leads to no significant differential peaks in the Vehicle treatment, but in the presence of DHT, there were 4,251 downregulated peaks and 144 upregulated peaks compared to shGFP.

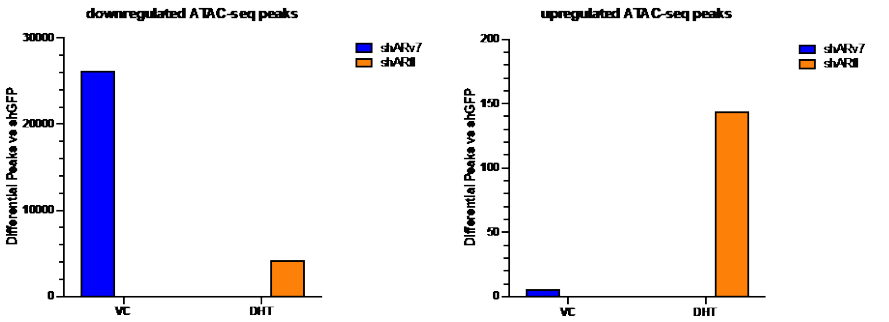

Figure 10. Differential ATAC-seq peaks summary.

We then focused on the 26,180 downregulated accessible chromatin regions in the shARv7 VC vs shGFP VC comparison. We confirmed that there was a marked decrease in signal at these regions in the shARv7 VC group compared to the shGFP VC group (Figure 11A). Hierarchical clustering of all the accessible regions revealed clusters of sites that demonstrated differential accessibility in the shARv7 VC group compared to the others (Figure 11B). We examined the motifs that were enriched in the 26,180 downregulated accessible chromatin regions and identified a number of known AR co-factor motifs among the most highly ranked, including FOXA1, Jun-AP1, ETS, and GRHL2 (Table 3). We then explored the genes and pathways that were enriched among the shARv7 VC downregulated accessible regions. Gene set enrichment analysis indicated that Hallmark UV response down, Hallmark Androgen response, and Hallmark KRAS signaling up gene sets were most highly negatively enriched (Figure 12). These results indicate that ARv7 may be mediating the activation of these pathways in LN95 cells.

Motif Name	p-value
FOXA3	$1x10^{-696}$
FOXA1	$1x10^{-622}$
SP1	$1x10^{-602}$
CTCF	$1x10^{-562}$
Jun-AP1	$1x10^{-441}$
ETS	$1x10^{-393}$
GRHL2	$1x10^{-359}$
ARE	$1x10^{-259}$

Table 3. Motifs enriched in the downregulated chromatin regions in the shARv7 VC vs shGFP VC comparison.

Taken together, our ATAC-seq results indicate that ARv7 may play a very prominent role in increasing chromatin accessibility in LN95 CRPC cells. From our previous study, we demonstrated that ARv7 plays a key role in repressing the expression of key tumor suppressor genes through decreasing H3K27ac at enhancer regions. The ARv7-dependent interplay between increasing chromatin accessibility and decreasing enhancer activation suggests a context-dependent role for ARv7 and further investigation into this complex regulation is warranted.

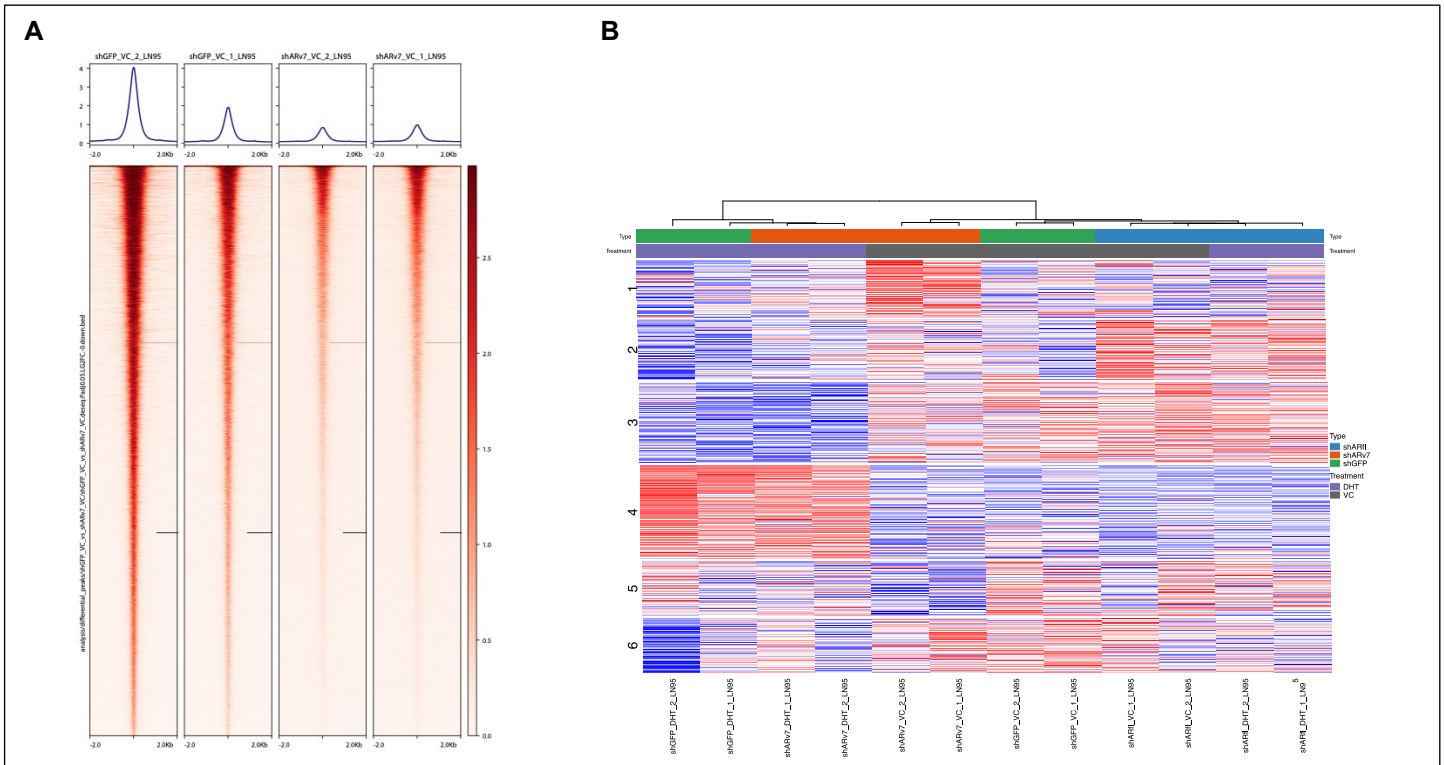

Figure 11. shARv7, but not shARfl leads to increased accessible chromatin regions compared to shGFP. A) ATAC-seq signal intensity heatmap showing decreased signal in shARv7 VC compared to shGFP VC. B) Hierarchical clustering demonstrates regions of differential accessibility in shGFP, shARv7, and shARfl LN95 cells.

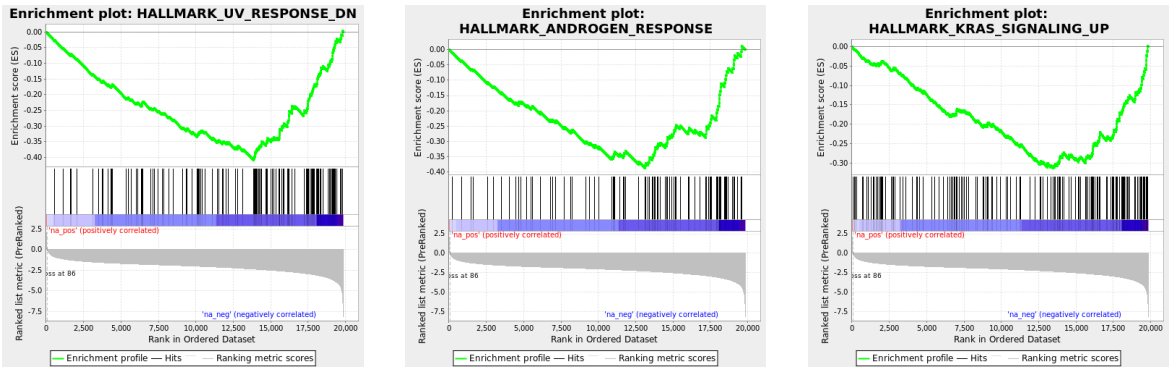

Figure 12. GSEA analysis of shARv7 VC downregulated chromatin regions.

Aim 3: Determine if novel AR N-terminal disrupting inhibitors target ARv7 and decrease CRPC tumor growth.

In order to identify a potential therapeutic strategy for targeting the ARv7, we have tested next-generation Thio-2 derivatives. Thio-2 has been shown to disrupt the interaction between AR and its coactivator/cochaperone, Bag-1L. Depletion of Bag-1L has been previously demonstrated to impair AR transcriptional activity and decrease prostate cancer cell growth. Bag-1L binds to AR at the AR N-

terminus and therefore, could theoretically also bind AR variants that lack the ligand binding domain, such as ARv7. In collaboration with Dr. Andrew Cato, we have assessed a number of next-generation Thio-2 derivatives for their ability to inhibit prostate cancer cell growth. We have identified one promising derivative, A4B17, which appears to decrease AR+ prostate cancer cell line growth but not AR- prostate cancer cell lines (Figure 13).

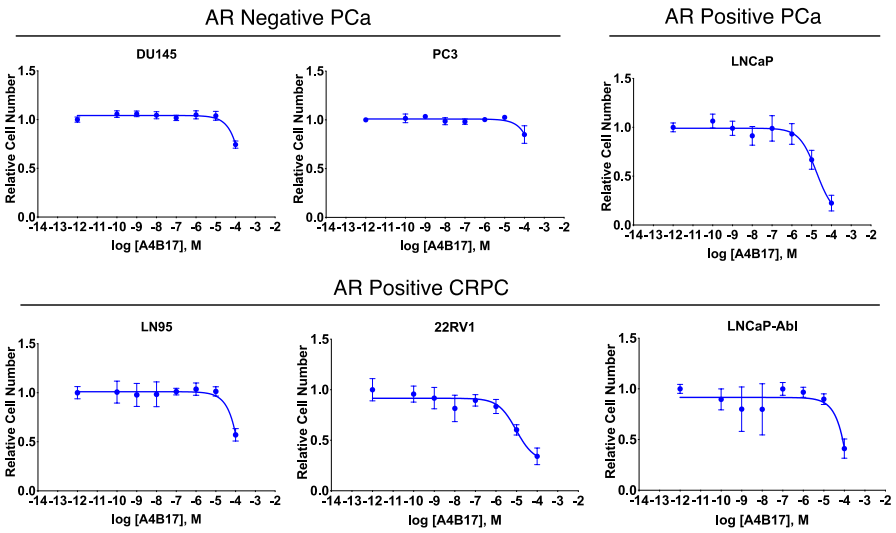

Figure 13. Prostate cancer cell line growth following treatment with Thio-2 derivative, A4B17.

However, further examination of these compounds demonstrated that very high doses of the compounds were required in order to achieve meaningful responses (> 10 μM). Therefore, the decision was made to not move forward with these compounds in animal studies. Other methods to disrupt ARv7 activity remain an active area of interest in the laboratory.

Training Opportunities

Professional Development Meetings

1. DFCI-BIDMC Monthly SPORE Meetings
2. DFCI-BIDMC Monthly P01 Meetings
3. DF/HCC Prostate Program Retreat

Results Disseminated

Nothing to Report

Future Studies

Nothing to Report

4. Impact

Impact on the Development of Principle Discipline

This study begins to address the DOD's Overarching Challenges to "Develop treatments that improve outcomes for men with lethal prostate cancer" and "Define the biology of lethal prostate cancer to reduce death." Our study aims to further explore the requirement of Androgen Receptor in castration resistant prostate cancer (CRPC) and the role that the splice variant ARv7 plays in promoting growth and survival of CRPC cells. Resistance to Androgen Deprivation Therapy (ADT) remains a significant obstacle in the treatment of prostate cancer patients. The majority of patients treated with ADT will at some point, develop resistance to this therapy and progress to CRPC which is lethal. Understanding the mechanisms of ADT resistance will be imperative to determine how to overcome resistance to ADT therapies. We hypothesized that targeting the splice variant, ARv7, is the key to overcoming ADT resistance. Our results have indicated that ARv7 seems to regulate distinct essential gene pathways from ARfl and further examination of these pathways could potentially exploit additional therapeutic vulnerabilities in CRPC. Additionally, we have shown that ARv7 binding to its regulatory regions is diverse and dependent upon its cellular context. Our results also indicate that EZH2 is not required for ARv7 transcriptional activity. Finally, we demonstrated that ARv7 plays a critical role in mediating

chromatin accessibility in CRPC cells, further confirming the critical role that ARv7 plays in CRPC cells. While our project did not fully delineate the mechanism of action of ARv7, our results help to expand and deepen our understanding of ARv7 biology in CRPC. We hope that our contributions will lead to a deeper understanding of ADT resistant CRPC and perhaps lead to novel therapeutic approaches to treat men with lethal prostate cancer.

Impact on Other Disciplines
Nothing to Report

Impact on Technology Transfer
Nothing to Report

Impact on Society
Nothing to Report

5. Changes/Problems

Changes in Approach
Nothing to Report

Actual/anticipated Problems or Delays
Nothing to Report

Changes that had a significant impact on expenditures
Nothing to Report

Significant changes in use of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to Report

6. Products

Journal Publications

Kuznik, N.C., Solozobova, V., **Lee, I.I.**, Jung, N., Yang, L., Nienhaus, K., Ntim, E. A., Rottenberg, J.T., Muhle-Goll, C., Kumar, A.R., et al. (2022). A chemical probe for BAG1 targets androgen receptor-positive prostate cancer through oxidative stress signaling pathway. *iScience* 25, 104175.

Lee, II, Kuznik, N.C., Rottenberg, J., Brown, M., and Cato, ACB. (2019). Bag-1L: a promising therapeutic target for androgen receptor-dependent prostate cancer. *J Mol Endocrinol, 62*(4), R289-R299.

Other Products
Nothing to Report

7. Participants and Other Collaborations

Individuals who have worked on project
Name: Irene Lee, PhD
Project Role: PI
Research Identifier (ORCID): 0000-0002-5749-9389
Nearest person month worked: 12

Contribution to project: Dr. Lee has conceived of the project, performed the experiments, data analysis, and data collection

Name: Myles Brown, MD
Project Role: Mentor
Nearest person month worked: 1
Contribution to project: Dr. Brown has provided guidance, support, and critical feedback of the project

Change in active support
Nothing to Report

Other organizations involved as partners
Organization Name: Beth Isreal Deaconness Medical Center
Location of Organization: Boston, MA, USA
Partner's contribution to project: collaboration

Organization Name: Karlsruhe Institute of Technology (KIT)
Location of Organization: Karlsruhe, Germany
Partner's contribution to project: collaboration

8. Special Reporting Requirements
Nothing to Report

9. Appendices
Nothing to Report