AWARD NUMBER: W81XWH-22-1-0059

TITLE: Functional Characterization of eRNA-Coregulator Interactions at AR-Bound Enhancers in Advanced Therapy-Resistant Prostate Cancer

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REPORT DATE: March 2024

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
March 2024	Annual	15Feb2023-14Feb2024

4. TITLE AND SUBTITLE

Functional Characterization of eRNA-Coregulator Interactions at AR-Bound Enhancers in Advanced Therapy-Resistant Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-22-1-0059

5c. PROGRAM ELEMENT NUMBER

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Prostate cancer (PCa) which has relapsed after first line androgen deprivation therapy (ADT), known as ADT-Recurrent PCa (ADT-RPCa) is incurable and the lethal form of the disease. Second generation antiandrogens, such as enzalutamide and apalutamide, are often used to treat ADT-RPCa but resistance to these drugs emerges within several months. Disease progression and the acquisition of therapy resistance in PCa is associated with changes in binding of the androgen receptor (AR) to its cis-regulatory enhancer elements. Enhancers, which regulate the rate of transcription by serving as nucleation sites for the binding of transcription factors, coregulators, RNA polymerase II and other regulatory proteins, were recently found to be transcribed, producing non-coding RNA molecules called enhancer RNA (eRNA) which are increasingly being recognized for their role in enhancer function. While eRNAs have been shown to be transcribed from critical AR-bound active enhancers, their role in AR-regulated gene expression and PCa progression remains largely unknown. This work seeks to address this issue by applying cross-platform genomic approaches to define roles for potentially oncogenic eRNA to sustain AR enhancer interactions and target gene expression. Here, we used Global run on (GRO) sequencing to identify differences in eRNA transcriptomes between isogenic pairs of enzalutamide sensitive and resistant prostate cancer cell lines. This was integrated with AR ChIP-seq and ChIA-PET datasets to annotate resistance-associated eRNAs potentially regulated by AR. Ongoing work aims to test the functional implications of expression of these eRNA on coregulator functions, AR signalling and prostate cancer cell growth. This work will ultimately provide new insights into functional interactions at enhancers most likely to contribute to therapy resistance in prostate cancer.

15. SUBJECT TERMS None listed.					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRDC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified	Unclassified	15	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Functional characterization of eRNA-coregulator interactions at oncogenic AR-bound enhancers in advanced therapy-resistant prostate cancer

1. Introduction

Androgen receptor (AR) signalling is the main driver of prostate cancer (PCa) and the main therapeutic target in this disease. The AR is targeted in advanced disease with androgen deprivation therapy (ADT), but recurrent and lethal disease often emerges in a therapeutically resistant state known as ADT-Recurrent PCa (ADT-RPCa). Although mechanisms vary, the AR remains an important driver of resistance and progression. The AR, a ligand-activated, DNA-binding transcription factor is the key mediator of responses to the male sex steroid hormones dihydrotestosterone and testosterone [1]. Upon binding to androgens, the AR translocates to the nucleus where it binds to recognition sequences in DNA, mainly within enhancers and to a lesser extent promoters [2]. At active enhancers, AR forms a complex with coregulators, epigenetic modifiers, structural proteins, enzymes and RNA polymerase II [3] to modulate transcription of target genes. More recently, it has emerged that transcription of the enhancer regions themselves, including those bound by AR, occurs by RNA polymerase II, producing small non-coding RNA molecules termed enhancer RNA (eRNA). Enhancer RNAs are increasingly being recognized for their role in enhancer function such as promoting recruitment and activity of transcription factors and/or coregulators, modifying chromatin accessibility and stabilizing enhancer-promoter interactions [4-6]. AR dependant enhancer dysfunction is a common occurrence in PCa with enhancer amplification, mutations and aberrant activation contributing to disease progression and therapy resistance [7-9]. An emerging area of importance to therapeutic resistance concerns which specific enhancers the AR is guided to by carcinogenic mechanisms. Recent genomic approaches have demonstrated that chromatin accessibility and looping events are all disrupted in PCa and the choice of enhancers bound by the AR represent a sub-set of the potential AR binding sites, and suggests there is considerable tailoring of the precise AR-genomic interactions that occur in PCa. Consistent with changes in enhancer usage, eRNA profiles also change in advanced PCa [10, 11] but current knowledge about eRNA roles in AR signalling and interactions with AR coregulators is limited. Understanding eRNA-coregulator functions at AR-dependant enhancers has the potential to improve our understanding of how enhancers work and their utility as potential therapeutic targets either alongside current ADT, to impede progression to ADT resistance, or after ADT failure to augment current chemotherapies, or as a novel therapy following ADT-RPCa.

2. Keywords

prostate cancer, therapy resistance, enhancers, eRNA

3. Accomplishments

- What were the major goals of this project?

The *objective* of this project was to apply cross-platform genomic approaches to define roles for potentially oncogenic eRNA to sustain AR-enhancer interactions and target gene expression, and in this manner also serve as the experiential basis for advanced genomic training. I *hypothesized* that eRNAs contribute to the normal functioning of the AR, and that in androgen deprivation therapy recurrent prostate cancer (ADT-RPCa) the expression and

genomic binding of AR is altered such that aberrantly activated AR-bound enhancers induce eRNA that lead to the aberrant recruitment and/or activity of coregulators. I further hypothesize that identifying and targeting such eRNA would enable switching off specific oncogenic enhancers and their target genes, thereby limiting oncogenic signalling pathways. This has the potential to provide further therapeutic options for ADT-RPCa. The specific aims of this project were (1) To identify novel eRNA expression at aberrantly activated AR-bound enhancers in therapy resistant PCa (2) To test the functional implications of eRNA expression on coregulator functions, AR signalling and prostate cancer cell growth.

- What was accomplished under these goals?

Work during this reporting period has primarily focussed on completion of outstanding tasks from **Specific Aim 1:** To identify novel eRNA expression at aberrantly activated AR-bound enhancers in therapy resistant PCa and completion of part of **Specific Aim 2:** To test the functional implications of eRNA expression on coregulator functions, AR signalling and prostate cancer cell growth.

Accomplishments based on tasks in the approved statement of work are detailed below.

- ***Major Task 1****: Exploit a collection of ADT-sensitive and -resistant PCa cell lines to identify oncogenic AR-regulated eRNA and enhancer-promoter interactions associated with their expression*

Subtask 1: Perform Global Run on (GRO) sequencing in isogenic pairs of enzalutamide-responsive and enzalutamide-resistant cell lines ($C42_{Parental}$ and $C42_{EnzR}$, V16D and $MR49F_{EnzR}$ to identify differential eRNA expression between responsive and sensitive lines. (**Completed during previous reporting period)**

Subtask 2: ChIA-PET sequencing in isogenic pairs of enzalutamide responsive and enzalutamide-resistant cell lines ($C42^{Parental}$ and $C42^{EnzR}$, V16D and $MR49F_{EnzR}$ to identify AR enhancer-promoter interactions in between responsive and sensitive lines **(Contingency ChIP experiments ongoing)**

There were significant delays with optimizing the ChIA-PET protocol [12] proposed for this project, as reported in the annual report for year 1. ChIP-seq was listed as a contingency plan in the project proposal in case of problems with generating ChIA-PET datasets. We are currently generating material for ChIP-sequencing and have also used ChIP-PCR to confirm AR binding at eRNA expressing loci in cell lines used in this study (Figure 1).

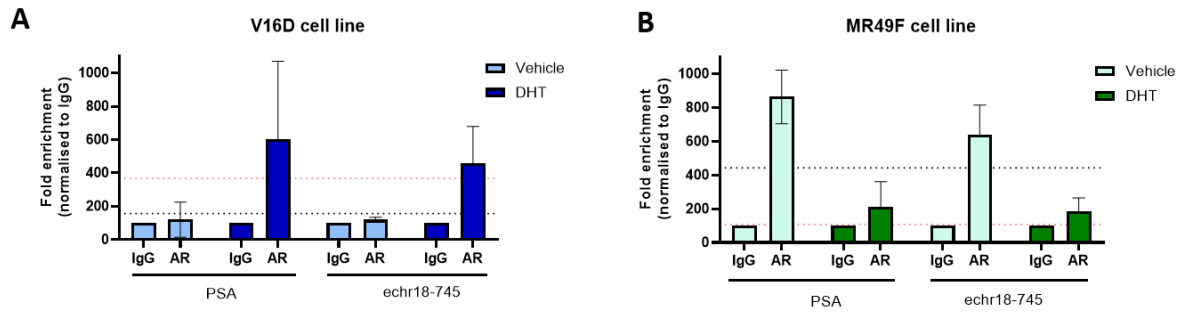
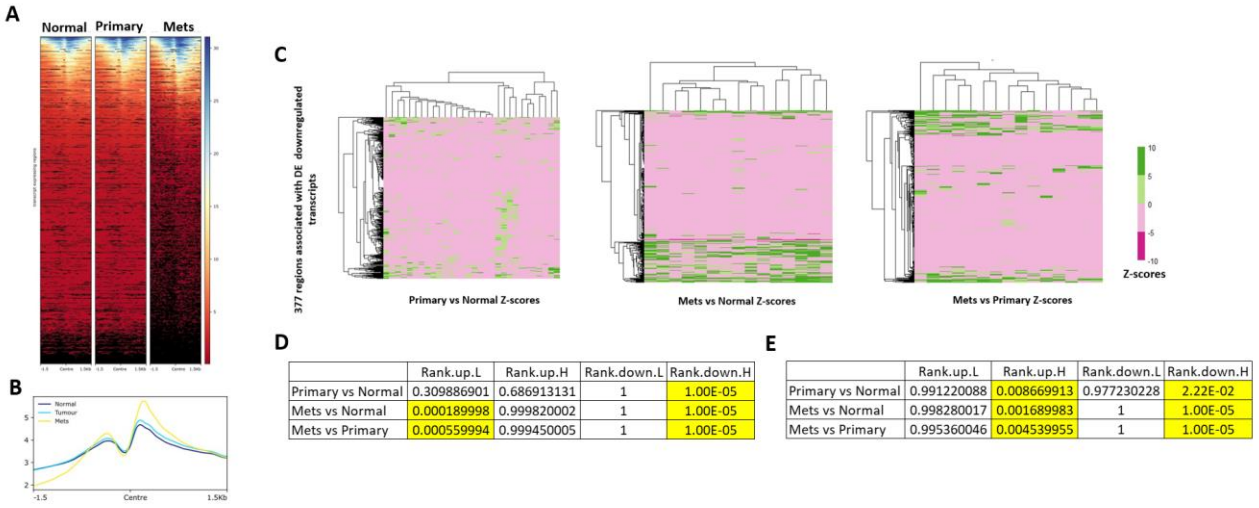


Figure 1*:* ChIP-qPCR to validate AR binding at eRNA expressing loci (A) ChIP for AR at PSA enhancer (positive control) and locus expressing eRNA echr18-745 locus in V16D cells treated with vehicle or DHT (B) ChIP for AR at PSA enhancer (positive control) and locus expressing eRNA echr18-745 locus in MR49F^{EnzR} cells treated with vehicle or DHT. AR enrichment was normalized to IgG antibody.

- ***Major Task 2:*** *Data integration of results from Major Task 1 with publicly available datasets*

Subtask 1: Integrate the eRNA and ChIP-PET data sets **(Complete)**

Due to delays in generating of ChIA_PET datasets to match the GRO-seq data in this study, publicly available ChIP-seq and ChIA-PET datasets have been used to identify eRNA/novel transcript expressing regions that are potentially bound by AR and that interact with other genomic loci (as reported in annual report for year 1). In addition to the datasets used previously, we have now also incorporated additional prostate cancer ChIP-seq datasets into our analysis by using CistromeDB toolkit [13] and assessed association of eRNA expressing regions with H3K27Ac (Fig 2A,B), FOXA1 and AR binding changes in previously published patient datasets - GSE130408, comprising normal, primary and metastatic prostate cancer samples [9, 14] and GSE161948-comprising adenocarcinoma and neuroendocrine prostate cancer [15]. In the case of H3K27Ac, which is a mark of active enhancers, we observe more enrichment of this mark in metastatic tissues compared to normal or primary tissues (Fig 2B). To determine if eRNA expressing regions were significantly associated with changes in H3K27Ac levels, we generated average H3K27Ac enrichment values across 13746 eRNA/novel transcript expressing regions (identified from cell lines) in the patient H3K27Ac ChIP-seq dataset using BigWigAverageOverBed. These values were then used to calculate Z-scores for normal-primary, normal-metastasis or primary-metastasis comparisons and bootstrapping analysis was used to test if regions associated with differentially expressed eRNAs/transcripts in the cell line pairs (e.g n=717 in the V16D/MR49F pair) were significantly changed in terms of H3K27Ac enrichment in different disease states (Figure 2C).



	Rank.up.L	Rank.up.H	Rank.down.L	Rank.down.H
Primary vs Normal	0.309886901	0.686913131	1	1.00E-05
Mets vs Normal	0.000189998	0.999820002	1	1.00E-05
Mets vs Primary	0.000559994	0.999450005	1	1.00E-05

	Rank.up.L	Rank.up.H	Rank.down.L	Rank.down.H
Primary vs Normal	0.991220088	0.008669913	0.977230228	2.22E-02
Mets vs Normal	0.998280017	0.001689983	1	1.00E-05
Mets vs Primary	0.995360046	0.004539955	1	1.00E-05

Figure 2: Integration of eRNA expressing regions with patient ChIP-seq datasets (A) Heatmaps showing average H3K27Ac binding density at eRNA/novel transcript expressing loci (n=13746) in normal (n=36), primary (n=32) and metastatic (n=15) prostate cancer samples from GSE130408 [9] (B) Overlaid H3K27Ac signal distribution plots for normal, primary and metastatic samples (C) H3K27Ac enrichment Z-scores for 377 regions associated with downregulated eRNAs in normal-primary, normal-metastatic or primary-metastatic comparisons (D) Outcome of bootstrapping analyses for regions associated with downregulated transcripts (E) Outcome of bootstrapping analyses for regions associated with upregulated eRNA/transcripts. Rows/columns marked in yellow indicate significant differences than expected by chance, $p < 0.05$

Based on bootstrapping analysis, regions associated with downregulated eRNAs had significantly less H3K27Ac enrichment (Rank.up.L) than expected by chance in the mets-normal and mets-primary comparison and more loss of H3K27Ac enrichment (Rank.down.H) (Figure 1D) in all comparisons than expected by chance. This suggests that eRNAs that were identified as downregulated in resistant cell lines in this study are likely associated with enhancers that lose the active mark on disease progression in patients. Additionally, regions associated with upregulated eRNAs had significantly more H3K27Ac enrichment (Rank.up.H) than expected by chance in all comparisons but more loss of H3K27Ac enrichment (Rank.down.H) (Figure 1E) in all comparisons than expected by chance, suggesting that the set of resistance upregulated eRNAs may need to refined further to identify those most likely to be also be associated with disease progression. No significant differences were found when comparing adenocarcinoma and neuroendocrine prostate cancer using the GSE161948 dataset.

Subtask 2: Annotation of eRNA-stabilized AR-dependent loops to genes **(Completed during previous reporting period)**

Specific Aim 2 To test the functional implications of eRNA expression on coregulator functions, AR signalling and prostate cancer cell growth.

- ***Major Task 3****: Functional testing of eRNA effects on AR signaling and growth*

Subtask 1: Functional characterization of eRNAs (**Ongoing, ~65% complete**)

Custom siRNAs were designed and tested against differentially expressed candidate eRNAs to determine their effects on growth. eRNAs for knockdown experiments were selected based

on differential expression, binding of AR and presence of H3K27Ac within or near eRNA expressing loci in cell lines (Figure 3A, Figure 4A) or patient ChIP-seq datasets and/or association with chromatin interaction from ChIA-PET data. Of the siRNAs tested, echr1_1375 and echr13_360 were found to inhibit growth to a greater extent in the resistant cells compared to parental (sensitive) cells (Figure 2C,D, Figure 3B,C).

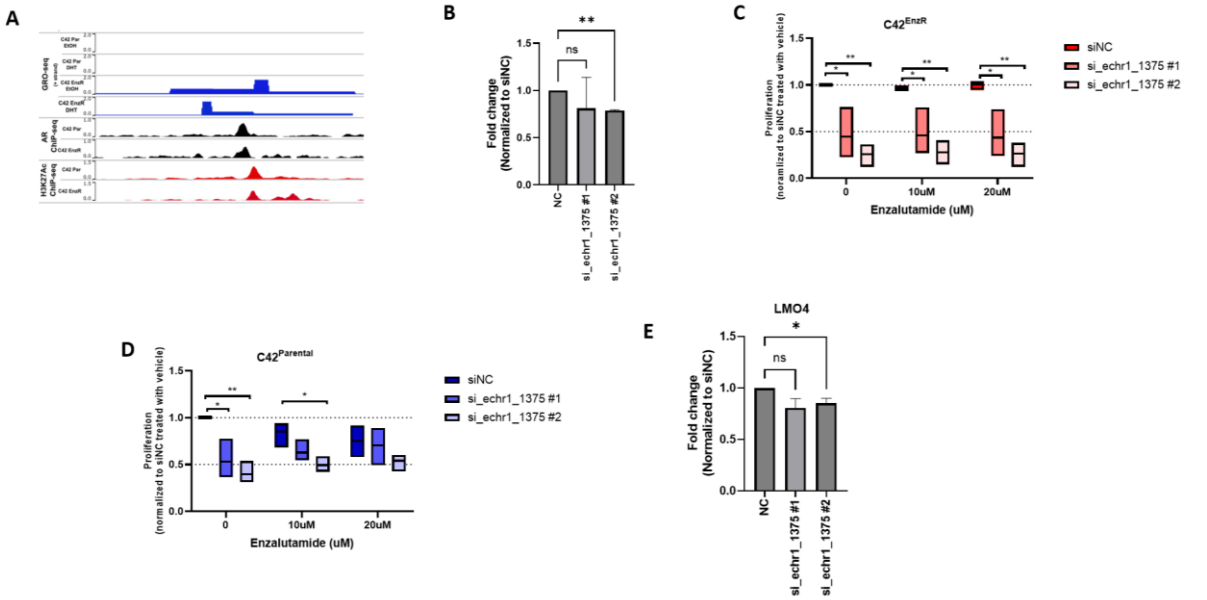


Figure 3: Enzalutamide resistant cells are more sensitive to knockdown of echr1_1375 (A) GRO-seq tracks (this study) and Chip-seq tracks (GSE136128) showing expression of chr1_1375 in the enzalutamide resistant cells and AR and H3K27Ac presence in both cell lines (B) Confirmation of chr1_1375 knockdown by siRNA in the $C42^{EnzR}$ cell line (C) Knockdown of echr1_1375 supresses growth of $C42^{EnzR}$ cells (D) Knockdown of echr1_1375 supresses growth of $C42^{Par}$ cells, however this is to a lesser extent than the $C42^{EnzR}$ cells (E) echr1_1375 knockdown results in downregulation of the LMO4.

eRNA echr1_1375 is upregulated in the $C42^{EnzR}$ cells compared to $C42^{Par}$ cells and is associated with AR binding and H3K27Ac marks in both cell lines (Figure 2A). eRNA chr1_1375 is also expressed from a locus downstream of the LMO4 gene, which has been reported to be repressed by the AR agonist R1881 and is downregulated in metastases [16]. On knocking down chr1_1375 we observed a significant decrease in LMO4 mRNA expression with one of the siRNAs used, suggesting that this gene may be regulated by eRNA chr1_1375 and its constituent enhancer. This locus is also annotated as a super-enhancer in the seDB2.0 super-enhancer database. Similarly, knockdown of eRNA chr13-360 has a more pronounced effect on growth of the EnzR cells then in the parental cells (Fig 4 B, C) although its target genes remain unknown. We observed similar results on growth for eRNA chr10-1201, however other candidates such as echr18-434, had no effects on cell growth.

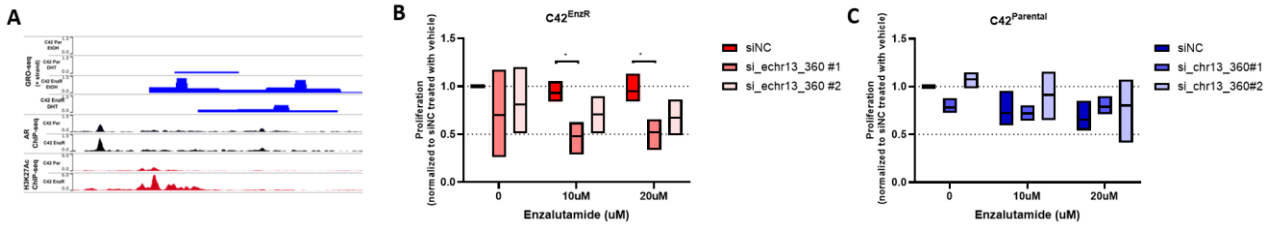


Figure 4: Enzalutamide resistant cells are more sensitive to knockdown of echr13_360 (A) GRO-seq tracks (this study) and Chip-seq tracks (GSE136128) showing expression of echr13_360 in the enzalutamide resistant cells and AR and H3K27Ac presence in both cell lines (B) Knockdown of echr13_360 supresses growth of $C42^{EnzR}$ cells (D) Knockdown of echr13_360 supresses growth of $C42^{Par}$ cells, however this is to a lesser extent than the $C42^{EnzR}$ cells.

We are currently generating plasmids for GAL4-BoxB tethering luciferase assay [17] to assess ability of candidate eRNAs to activate gene expression.

Subtask 2: Proteomic analyses of eRNA activity (**Ongoing**)

We are currently generating plasmids for identifying proteins interacting with candidate eRNAs using the CRISPR-biotinylated dCas9 based CAPTURE method [18].

- ***Major Task 4:*** Functional validation of eRNA-coregulator interactions

Subtask 1: Institutional review board approval for use of patient organoid Models (**Completed during previous reporting period**)

Subtask 2: HRPO approval for using patient organoid models (**Completed during previous reporting period**)

Subtask 3: Characterize the ability of eRNA to recruit coregulators and facilitate their activity (**Pending**)

Subtask 4: Validate results in near patient organoid models (**Ongoing, ~10% complete**)

The final aspect of this project is to validate results from cell lines in organoid models of therapy-resistant CRPC. I have access to organoid models from the MURAL (Melbourne Urological Research Alliance) [19] cohort which include diverse PCa phenotypes, including primary, CRPC and enzalutamide resistant CRPC . These organoids, which are generated from patient derived xenografts and grown in 3D culture, maintain genetic and epigenetic concordance with corresponding patient tumours. To identify organoids best suited for functional studies, we have assessed level of eRNAs of interest in PDXs from which the organoids are derived. We used a subset of 21 PDXs from encompassing patients across the disease trajectory of prostate cancer from treatment-naïve to metastatic castrate-resistant disease, and included samples with different pathological subtypes, treatments, and androgen

receptor status. Our results indicate that eRNAs are expressed at different levels in patient PDXs and may be associated with clinical or pathological features. Of the 6 eRNAs tested so far, one candidate was found to be significantly associated with disease progression and treatment status (Figure 4A,B). echr12-1082 was identified as downregulated in $C42^{EnzR}$ compared to $C42^{Parental}$ cells in our GRO-seq data. In the PDX cohort, we observe downregulation of echr12-1082 in metastatic samples compared to samples from localized tumours (Fig 5A) and downregulation in samples treated with enzalutamide compared to untreated samples (Fig 5B), which is consistent with cell line results. eRNA expression results were also integrated with available PDX transcriptomic data to identify potential target genes (Figure 4C) for echr12-1082 and other eRNAs using Spearman correlation. We will be assessing level of additional eRNAs in this cohort and will be using this data to select organoid models that will be best suited for functional studies.

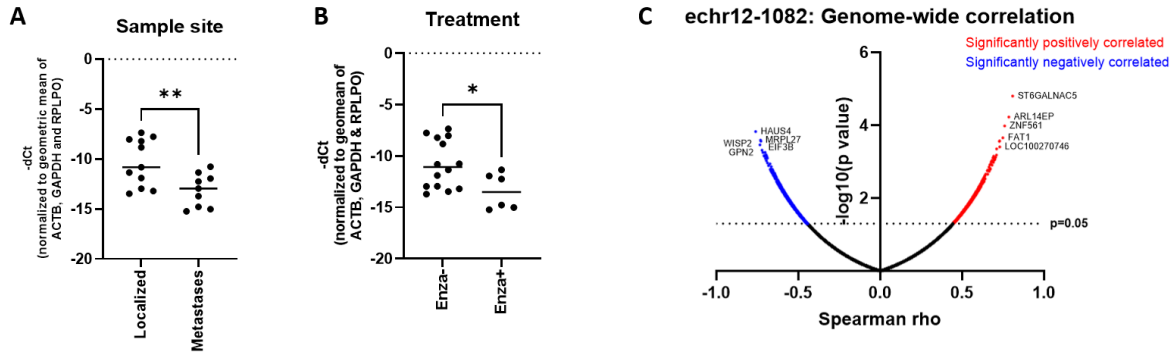


Figure 5: chr12-1082 expression in patient derived PDX samples (A) echr12-1082 is downregulated in metastatic samples compared to samples from localized tumours (B) echr12-1082 is downregulated in PDXs derived from patients treated with enzalutamide compared to PDX samples from patients who did not receive enzalutamide (C) Genome-wide correlation analysis between expression of echr12-1082 and all expressed genes in PDX samples, using Spearman correlation. Spearman correlation ccoefficient (rho) is plotted on the x axis and -log10 pvalue on the y axis.

- **Opportunities for training and development**

This project has continued to provided training opportunities for developing my bioinformatics skills. In addition, during this reporting period, I have had the opportunity for professional development through attending courses and workshops provided by the Postdoc and Fellows Development Centre at Imperial College London. This includes courses on fellowship applications, writing lay summaries, one-on-one voice coaching for effective presentations, and participating in the UNIque development program for early career researchers.

Data generated from this project was used as preliminary data for a successful application for the Society for Endocrinology/ Endocrine Society of Australia Exchange Award. This award enabled me to work with patient derived xenograft and organoid models at Monash University, Australia for 4 weeks between Nov-Dec 2023. These models will be used in Subtask 4 of Major Task 4.

- **How were the results disseminated to communities of interest?**

Nothing to report

- **What do you plan to do during the next reporting period to accomplish the goals?**

During the next reporting period, I plan to complete outstanding tasks from Specific Aim 1 & Specific Aim 2.

4. Impact

- **What was the impact on the development of the principal discipline(s) of the project?**

This study has generated eRNA expression data for therapy resistant prostate cancer cell lines. This project will ultimately provide new insights into the role of eRNAs in enhancer functioning and the potential utility of eRNAs as therapeutic targets in ADT-RPCa.

- **What was the impact on other disciplines?**

Results from this study will contribute to a better understanding of the role of eRNA function in pathobiology of prostate cancer but can have an impact on other hormone driven malignancies as well.

5. CHANGES/PROBLEMS

- **Changes in approach and reasons for change**

Nothing to report

- **Actual or anticipated problems or delays and actions or plans to resolve them**

We have been awarded a 9-month no cost extension for completion of outstanding tasks. Delays in completion of work within the original timeframe were due to delays in finalising contracts and issues with getting the ChIA-PET protocol to work, as reported in annual report for year 1. We have also spent additional time spent identifying appropriate candidate eRNAs for downstream analysis during this reporting period.

- **Changes that had a significant impact on expenditures**

Nothing to report

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agent**

Nothing to report

6. PRODUCTS

- **Publications, conference papers, and presentations:**

Results were disseminated as oral presentations at the British Association for Cancer Research's Trailblazers in Cancer Research Conference (22nd-23rd June 2023) and the Endocrine Society of Australia's Annual Scientific Meeting (25th -28th Nov 2023). This work was also presented as a poster at the Gordon Research Seminar and Conference on Hormone Dependant Cancers (5th -11th Aug 2023), as an invited talk at Flinders University, Australia (4th Dec 2023), as a seminar at the Departmental seminar series (9th May 2023) at the Department of Surgery and Cancer, Imperial College London and as as a Work in Progress seminar for the Division of Cancer, Imperial College London (10th Oct 2023).

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name	Rayzel Fernandes
Role	Principal Investigator
Researcher ID	0000-0002-8834-3221
Nearest Person Months worked	24
Contributions to project	Designed and performed experiments, analysed data.
Funding Support	N/A

Name	Nuria Mascaro Fortuno
Role	Graduate student
Researcher ID	-
Nearest Person Months worked	2
Contributions to project	Performed experiments and analysed data.
Funding Support	N/A

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

- Organization name: Melbourne Urological Research Alliance (MURAL), Monash University
- Location of Organization: Melbourne, Australia
- Partner's contribution to the project:
  Other: Provided PDX material for eRNA expression experiments and PDX transcriptomic data

8. **SPECIAL REPORTING REQUIREMENTS**
   Nothing to report

9. **APPENDICES:**

N/A

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