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

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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.

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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). The AR remains an important driver of resistance and progression in ADT-RPCa via a number of potential mechanisms. 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 carcincogenic 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 represents a sub-set of the potential AR binding sites, suggesting there is considerable reprogramming 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, after ADT failure to augment current chemotherapies, or as a novel therapy following ADT-RPCa.

2. Keywords

prostate cancer, therapy resistance, enhancers, eRNA, transcription

3. Accomplishments

- What were the major goals of this project?

The overall 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. This will also serve as the experimental basis for advanced genomic training. I hypothesized that (i) eRNAs contribute to the normal functioning of the AR, and (ii) in ADT-RPCa the expression and genomic binding of eRNA is altered such that aberrantly activated

AR-bound enhancers induce eRNAs that lead to the aberrant recruitment and/or activity of coregulators. I further hypothesize (iii) 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 therefore:

(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?

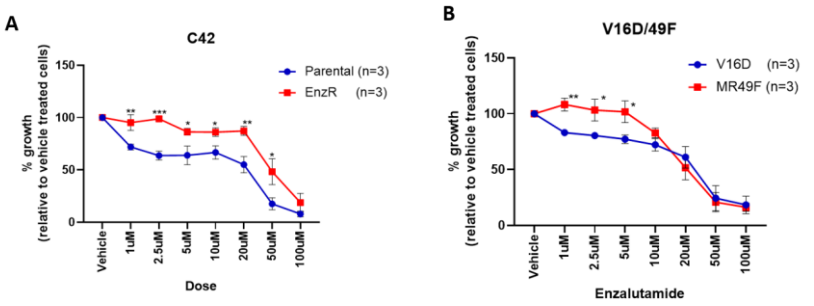
Work during this reporting period has primarily focussed on completing **Specific Aim 1:** To identify novel eRNA expression at aberrantly activated AR-bound enhancers in therapy resistant PCa.

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)**

Subtask 1 involved using two isogenic pairs of enzalutamide-responsive and enzalutamide-resistant cell lines ($C42^{Parental}$ and C42EnzR, V16D and $MR49F^{EnzR}$). Enzalutamide resistance was validated using growth assays for both cell line pairs (Figure 1) prior to setting up GRO-seq experiments.



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Cell Line	C42Control	C42EnzR	V16D	MR49FEnzR
IC50 (uM)	12.69	49.18	24.15	36.74

Figure 1: Validation of differences in enzalutamide resistance between isogenic pairs of cell lines (A) $C42^{Parental}$ and $C42^{EnzR}$ (B) V16D and $MR49F^{EnzR}$ (C) IC50 values for enzalutamide for all cell lines used in this study.

All cell lines were serum starved for 72h followed by treating with vehicle or 1nM DHT for 2 hours prior to harvesting for GRO-seq. GRO-seq experiments were performed as described in Barbieri et al 2020 [12]. Following sequencing, files were quality checked using FASTQC [13], trimmed using Trimmomatatic [14] and aligned to the human transcriptome (hg38) using HISAT2 [15]. Homer v4.11 [16] was used for de novo transcript detection, and differential expression analysis of genes (Table 1) as well as de novo transcripts (Table 2). Three biological replicates were sequenced for each cell line and condition, however due to high levels of sequencing duplication in one set of $C42^{EnzR}$, 3 replicates were analysed for each treatment condition in the $C42^{Parental}$ cells and 2 replicates for the $C42^{EnzR}$ cells.

Comparison	Total DE genes	Upregulated	Downregulated
$C42^{Parental}$ Veh vs DHT	103	98	5
$C42^{EnzR}$ Veh vs DHT	4	4	0
$C42^{Parental}$ Veh vs $C42^{EnzR}$ Veh	749	369	380
$C42^{Parental}$DHT vs $C42^{EnzR}$ DHT	1053	533	520
V16D Veh vs DHT	627	485	142
$MR49F^{EnzR}$ Veh vs DHT	612	385	227
V16D Veh vs $MR49F^{EnzR}$ Veh	6108	3016	3092
V16D DHT vs $MR49F^{EnzR}$ DHT	6237	3434	2803

Table 1: Differential expression of genes across cell line models and treatments. Differentially expressed (DE) genes are defined as those with abs(log2FC) <0.5 and FDR 0.1.

The number of differentially expressed genes across cell lines and treatment are listed in Table 1. In the C42 pair of cell lines, treatment with DHT in $C42^{Parental}$ results in differential expression of 103 genes which includes classical AR targets such as NKX3.1, ZBTB16, TMPRSS2, KLK2 and FKBP5. The $C42^{EnzR}$ line however only shows significant upregulation of four genes upon DHT stimulation, confirming that it is not responsive to androgens and thus AR independent. Compared to the $C42^{Parental}$, the $C42^{EnzR}$ line has upregulated expression of the nuclear receptor, glucocorticoid receptor (GR), which has previously been shown to compensate for AR signalling in androgen independent cell lines [17]. In contrast, the V16D, $MR49F^{EnzR}$ pair are more responsive to androgen stimulation. Treatment with DHT resulted in

627 and 612 genes being upregulated in V16D and MR49F^{EnzR}, respectively. As with the C42^{Parental} line, differentially expressed genes include classical AR targets such as ZBTB16, PMEPA1, FKBP5, NKX3.1 and TMPRSS2. Gene expression differences between V16D and MR49F cells in our GRO-seq data is consistent with previously published sequencing data in these cell lines, such as upregulation of MYC, CEBPA and downregulation of TIMP3, KLK3, in the MR49F^{EnzR} cells versus the V16D cells [18].

For identifying potential eRNAs in the C42$^{Parental/EnzR}$ and V16D/MR49F^{EnzR} cell lines, the findPeaks program using groseq mode was used in Homerv4.11 [16]. Transcripts identified by findPeaks were further filtered using the findDistalPeaks program with intergenic and no TSS settings. This filters peaks to only those more than 3kb away from known TSSs and more than10kb downstream of known genes. Peaks were further filtered by removing those that intersected with rRNA, tRNAs and known UCSC genes using bedTools [19]. Finally, transcripts were filtered by size, removing all transcripts longer than 5kb since eRNAs are generally between 150bp-5kb in size [20]. eRNAs identified in this dataset have a median length of 575 bp, and the majority are within 1000bp in length.

A total of 9212 eRNAs were identified across the C42 cell lines and 37401 eRNAs in the V16D/MR49F^{EnzR} cell lines. Of these, 4576 eRNAs are common between both pairs of cell lines. The Cistrome Data Browser toolkit [21] was used to predict factors that have significant overlaps with eRNA expressing regions in prostate cancer cell lines based on existing ChIP-seq datasets (Figure 2A). For eRNAs identified in both sets of cell lines, binding of AR and RNA polymerase II subunit A (POL2RA) (which is the polymerase that transcribes eRNAs) are significantly enriched. Additionally, several other factors common between the two cell line pairs are known to be commonly enriched at enhancers, such as BRD4, CHD4 and MED1 [22, 23].

Differential expression of de novo transcripts was determined using the DESEQ2 wrapper in Homer v4.11. The number of differentially expressed transcripts across cell lines and treatments are listed in in Table 2. In the C42 cell lines, differential expression was only observed between cell lines but not on treating each cell line with DHT. In comparison, in the V16D and MR49F cells differential expression was observed between cell lines and within each cell line on treatment with DHT (Figure 2B,C). Overall, the V16D/MR49F pair appears to be more responsive to androgen stimulation compared to the C42$^{Parental/EnzR}$ pair in terms of both gene and eRNA expression. This may reflect differences in levels of androgen independence and means by which resistance has emerged. Candidate eRNAs found to be differentially expressed in the C42 cell lines from sequencing data were validated using qPCR (Figure 2D); validation is ongoing for candidate eRNAs identified in the V16D, MR49F cell lines.

Comparison	Total DE de novo transcripts/potential eRNA	Upregulated	Downregulated
C42^{Parental} Veh vs DHT	0	0	0
C42^{EnzR} Veh vs DHT	0	0	0
C42^{Parental} EtOH vs C42^{EnzR} EtOH	34	10	24

$C42^{Parental}$ DHT vs $C42^{EnzR}$ DHT	101	34	77
V16D Veh vs DHT	137	56	81
$MR49F^{EnzR}$ Veh vs DHT	122	27	95
V16D EtOh vs $MR49F^{EnzR}$ EtOH	3852	1822	2030
V16D DHT vs $MR49F^{EnzR}$ DHT	3154	1767	1387

Table 2: Differential expression of eRNAs across cell line models and treatments. Differentially expressed (DE) eRNAs are defined as those with abs(log2FC) <0.5 and FDR 0.1.

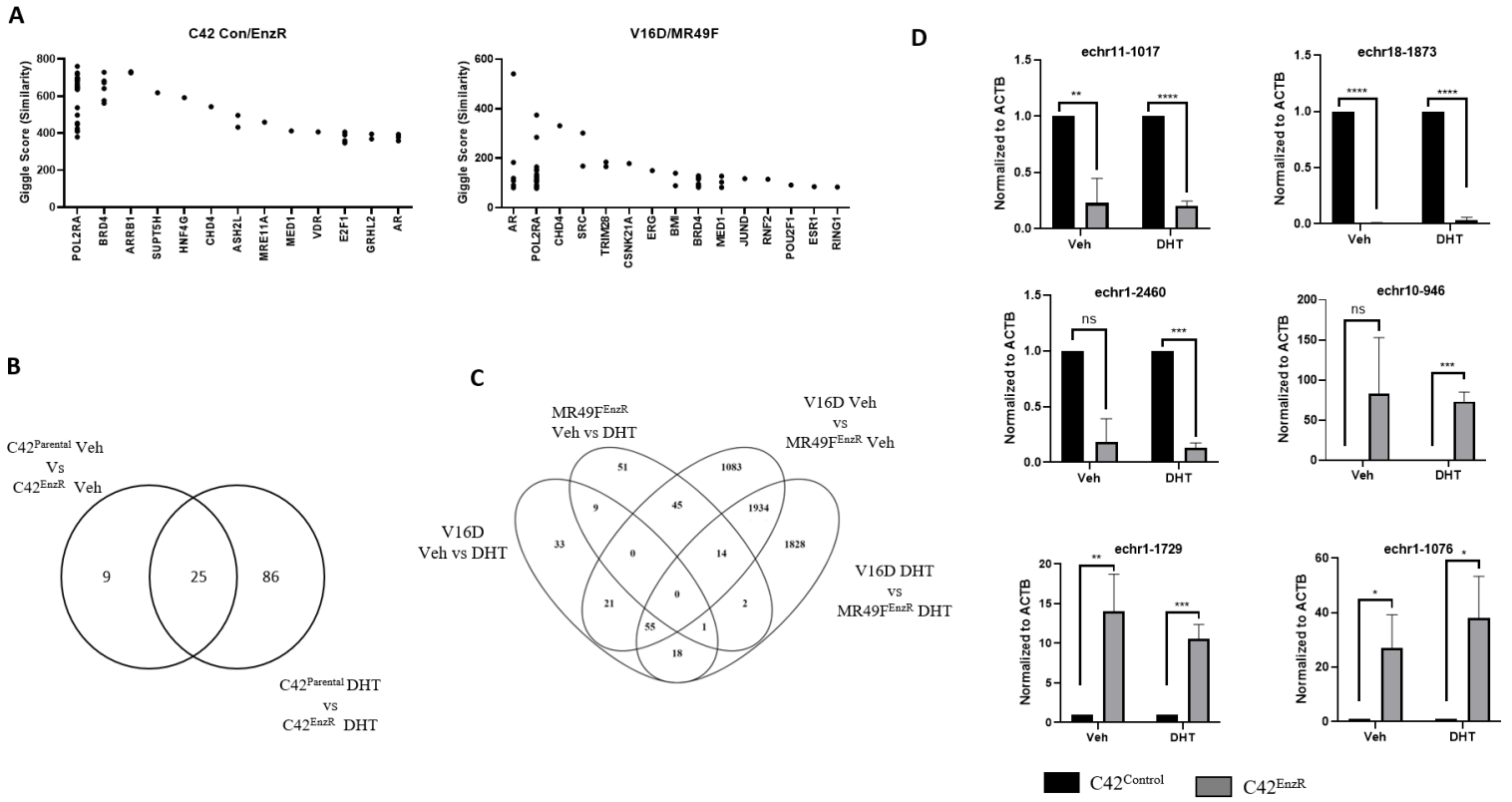

Figure 2: Identification and validation of eRNAs in enzalutamide resistant prostate cancer cell lines. (A) Giggle analysis of eRNA expressing regions for overlap with factors identified in published ChIP-seq datasets (B) Overlap between eRNAs differentially expressed in $C42^{Parental}$ vs $C42^{EnzR}$ cell treated with vehicle and $C42^{Parental}$ vs $C42^{EnzR}$ treated with DHT (C) Overlap between eRNAs differentially expressed in V16D vs MR49FEnzR cell treated with vehicle, V16D vs MR49FEnzR treated with DHT, V16D treated with vehicle vs DHT and MR49FEnzR treated with vehicle vs DHT. (D) Validation of differentially expressed eRNA in the C42 pair of cell lines.

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 **(Incomplete, ongoing)**

There have been significant delays with optimizing the ChIA-PET protocol [24] proposed for this project. Issues included insufficient digestion, ligation and enrichment during various steps of the protocol. After discussion with colleagues and collaborators, it was decided to use an alternative approach. The Hi-ChIP protocol [25] which is a variation on ChIA-PET and can also be used to identify enhancer-promoter interactions was tested, however this was also unsuccessful.

ChIP-seq was listed as a contingency approach in the project proposal in case of problems with generating ChIA-PET data; I plan also to use 4C-ChIP-seq. While this won't give genome-wide information, it will provide 3D promoter-enhancer information for specific eRNAs and enhancers of interest.

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

Subtask 1: Integrate the eRNA and ChIA-PET data sets **(Ongoing ~75% complete)**

GEO Identifier	Description	Publication
GSE136128	AR/FOXA1/ H3K27Ac ChIP-seq in C42 Con/EnzR cells	[26]
GSE54946	AR ChIA-PET in VCaP cells	[27]
GSE121020	RNA polII ChIA-PET in prostate cell lines	[28]

Table 3: Publicly available datasets integrated with GRO-seq data from this study

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 (listed in Table 3) have been used to identify eRNA transcribing regions that are potentially bound by AR and that interact with other genomic loci. ChIP-seq data is available for AR in $C42^{Control}$ and $C42^{EnzR}$ cells from a study published by He at al in 2021 [26], although there are differences in EnzR resistance and gene expression between cell lines in the two studies - the C42 EnzR line in He et al was maintained in 20uM enzalutamide whereas the one used in our study was grown at 100uM.

On comparing differentially expressed eRNAs in the C42 cell line pair in our study with AR binding sites (ARBS) across both the parental and EnzR cell lines from He et al [26], 30 eRNAs were found to be associated with ARBS (Figure 3A). echr18-1873 and echr18-514, for example, are both upregulated in $C42^{Parental}$ cells and associated with binding of more AR, the pioneer factor FOXA1 and the H3K27Ac active histone mark (Fig 3E). eRNA expressing regions were also compared with publicly available AR ChIA-PET data from the VCaP cell

line [27] and RNA polII ChIA-PET data from prostate cancer cell lines [28] (Figure 3 B, F). Figure 3F shows AR and RNA polII interactions at the site of expression of echr18-1873 and echr18-514. No interactions are observed at these loci in the non-malignant RWPE cell line and the AR negative DU145 cell line. The majority of overlaps with ChIA-PET datasets were found with eRNAs that are lost in the EnzR line. This lack of overlaps with eRNAs upregulated in $C42^{EnzR}$ cells may potentially be attributed to the fact that neither VCaPs nor LNCaPs are enzalutamide resistant and therefore may not exhibit interactions specific to resistant cells.

Due to unavailability of AR ChIP-seq data for V16D, MR49F cell line pair, differentially expressed eRNA transcribing regions from the V16D, MR49F pair were compared to ARBS in the $C42^{Parental,\ EnzR}$ lines from He et al [26]. Overall, 219 out of the 5002 differentially expressed eRNA are associated with ARBS (Figure 3C). Additionally, 159 eRNAs are associated with AR interaction in the VCaP cell line (Figure 3D).

Currently, work is ongoing to integrate eRNA expression data from this study with additional ChIP-seq cell line datasets and patient datasets.

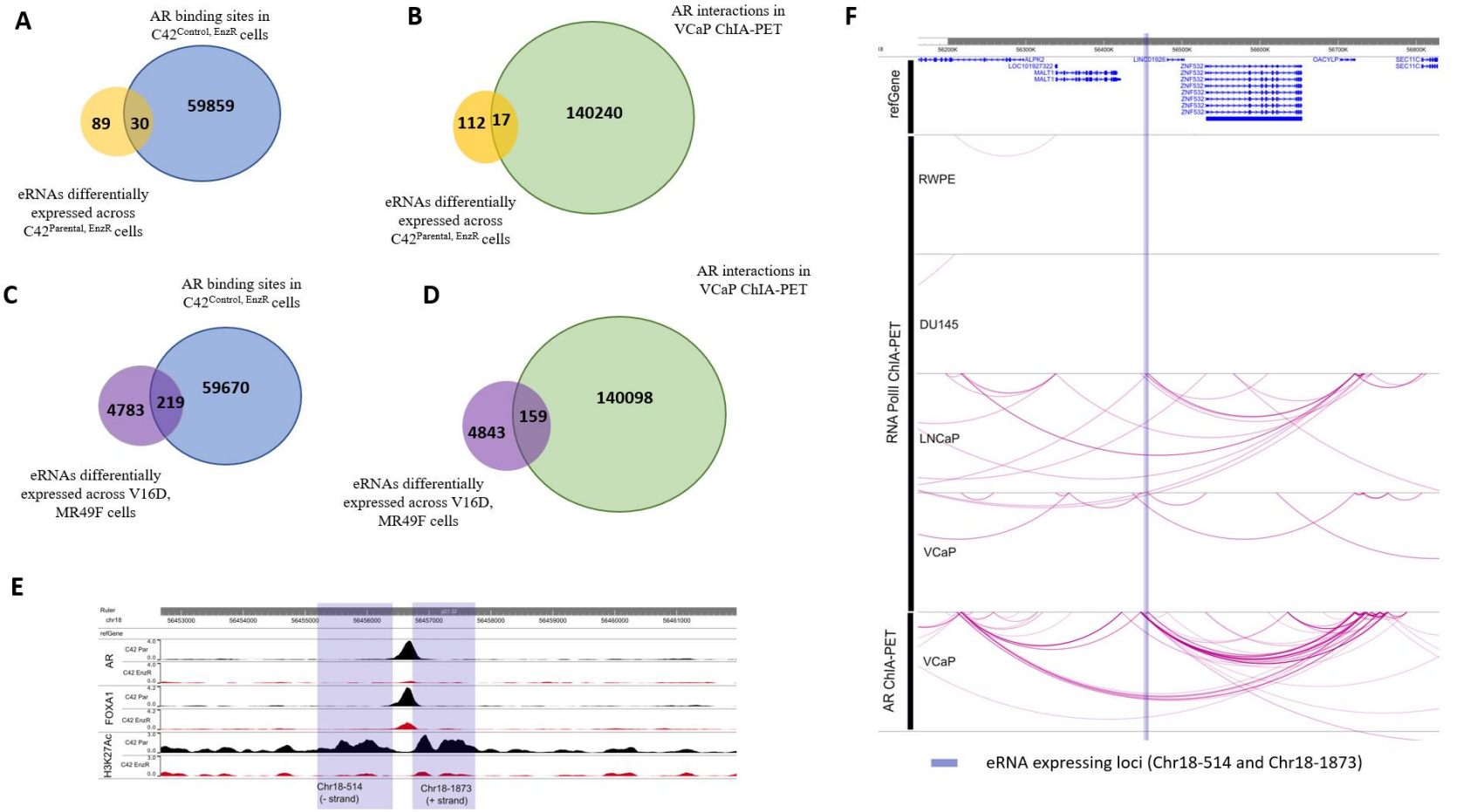

Figure 3: Integration of eRNAs and ChiP-seq, ChIA-PET datasets (A) Intersection of AR binding sites in [26] with differntial eRNA expressing loci in $C42^{Parental}$ vs $C42^{EnzR}$ cells (B) Intersection of AR interaction anchor points from ChIA-PET data [27] with differntial eRNA expressing loci in $C42^{Parental}$ vs $C42^{EnzR}$ cells (C) Intersection of AR binding sites in [26] with differntial eRNA expressing loci in V16D vs $MR49F^{EnzR}$ cells (D) Intersection of AR interaction anchor points from ChIA-PET data [27] with differntial eRNA expressing loci in V16D vs $MR49F^{EnzR}$ cells (E) AR, FOXA1 and HOXB13 binding at sites of expression of eRNA chr18-514 and chr18-1873 (F) eRNAs chr18-514 and chr18-1873 intersct with AR interaction anchor point in VCaP cells.

Subtask2: Annotation of eRNA-stabilized AR-dependent loops to genes **(Ongoing ~75% complete)**

To generate eRNA-target gene links, eRNA coordinates were intersected with publicly available enhancer-gene annotations in prostate cell lines, that have been generated based on RNA polII ChIA-PET data [28] or using H3K27Ac ChIP-seq data and gene distance [29]. eRNA coordinates were also intersected with super-enhancer annotations [29].

Several genes associated with prostate cancer progression were found to be linked to enhancers associated with eRNAs identified in this study. For instance, eRNAs that are upregulated in the MR49F cells versus the V16D cells are expressed from enhancers associated with the MYC oncogene gene [28](Figure 4A). This region is also annotated as a super-enhancer for MYC in the PC-3 prostate cancer cell line and in cell lines representing other cancers [29]. Additionally, these regions are associated with AR binding in prostate cancer lines with ectopic expression of AR (Fig 4B). Other genes of interest associated with differentially expressed eRNAs include IRF2BPL, which was recently identified as an AR coregulator that is associated with poor outcomes in prostate cancer, the YY1 transcription factor, the p53 regulator MDM4, the transcriptional repressor RCOR1 and the long non-coding RNAs RBMX and SCHLAP1. Pathway analysis was performed for predicted gene targets (Fig 4C,D), which revealed that these genes are related to cell cycle, steroid receptor signalling and other signalling pathways.

Work is currently ongoing to determine how expression of target genes relates with clinical information in publicly available prostate cancer patient datasets.

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 ~15% complete**)

This subtask involves specific knockdown of identified eRNAs. I am currently testing custom siRNA against shortlisted candidate eRNAs to confirm knockdown; this will be followed by assays to determine effects on enzalutamide growth and target genes.

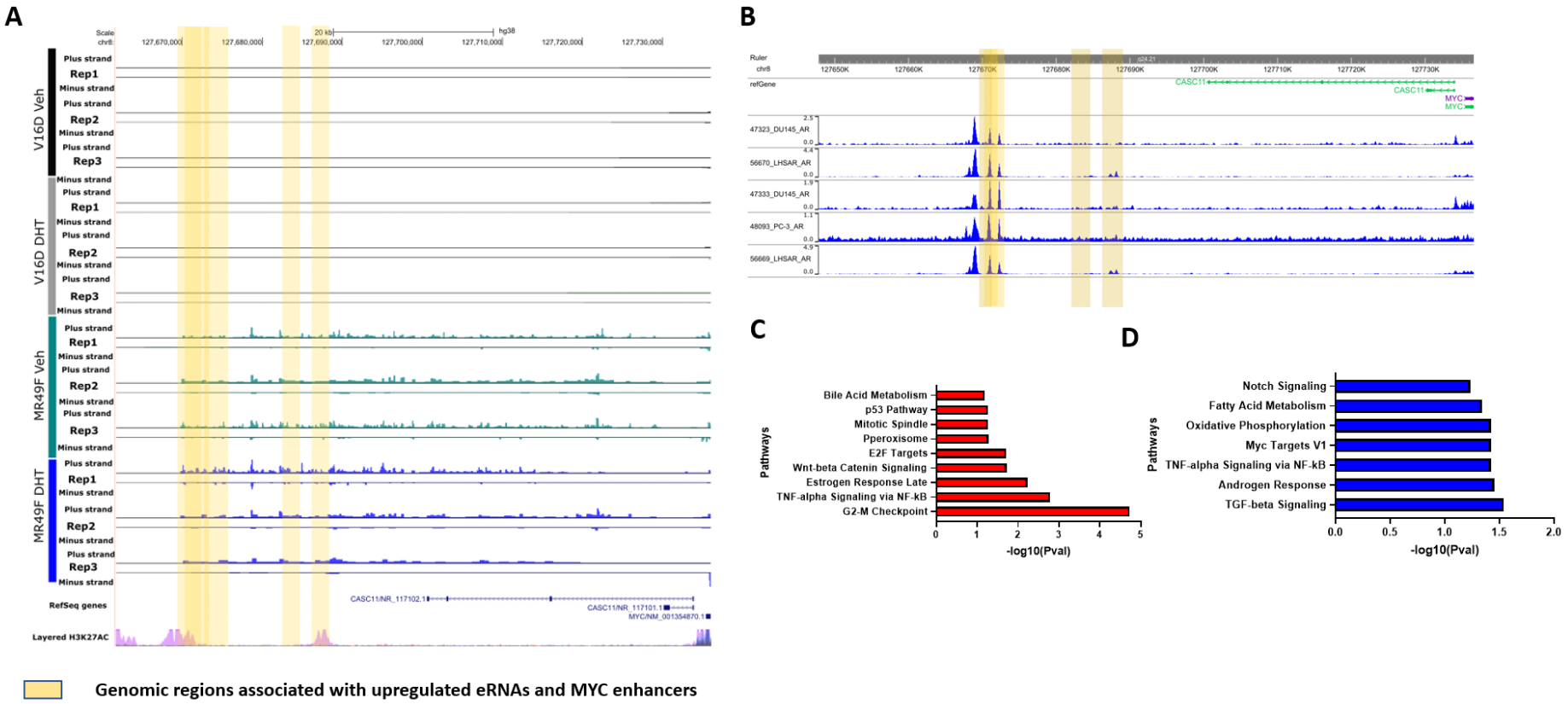

Figure 4: Annotation of eRNAs with target genes (A) eRNA expressing regions overlap with annotated enhancers for the MYC gene[28] (B) eRNA expressing regions overlap regions bound by AR in MYC enhancers (C) Pathways associated with genes linked to upregulated eRNAs (D) Pathways associated with genes linked to downregulated eRNA

- **Opportunities for training and development**

This project has provided training opportunities in both new experimental skills, e.g. GRO-seq, as well as bioinformatics skills including processing, analysing of sequencing data and integration with existing data. During the course of this reporting period, I have also exploited opportunities for professional development by attending workshops on science communication (Research Engagement and Impact; Social Media for Academics; Personal Pitching) conducted by Imperial College London's Postdoc and Fellows Development Centre.

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

Results were disseminated as a Work in Progress seminar (14th Dec 2022) for the Department of Surgery and Cancer, Imperial College London. I am currently submitting abstracts for conferences that will be taking place in mid-2023 (British Association for Cancer Research - Trailblazers in Cancer Research, Gordon Research Seminar and Conference).

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

During the next reporting period, I plan to complete Specific Aim 2: To test the functional implications of eRNA expression on coregulator functions, AR signalling and prostate cancer cell growth. This involves currently ongoing work to test the effect of eRNA knockdown on cell line growth in the presence of enzalutamide and other readouts such as invasion and migration. I will also be using the CRISPR Capture assay to identify coregulators associated with differentially expressed eRNAs, validate eRNA-coregulator interactions using tethering assays and finally, confirm eRNA functional effects in organoid models of prostate cancer. Additionally, I plan to perform ChIP-seq assay to complete Major Task 1, Subtask 2.

In terms of professional development and training, I plan to attend and present at the Gordon Research Conference on Hormone Dependant Cancers (6th -11th Aug 2023) , the associated Gordon Research Seminar (5th-6th Aug 2023), British Association for Cancer Research-Trailblazers in Cancer Research Conference (22nd-23rd June 2023) and the annual conference of the Society for Endocrinology (13th-15th Nov 2023).I will also participate in professional development workshops organized by Imperial College London's Postdoc and Fellows Development Centre.

4. Impact

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

This study has generated novel eRNA expression data for paired enzalutamide sensitive and resistant prostate cancer cell lines. The role of eRNAs in prostate cancer progression remains relatively unknown and this work adds to the limited number of eRNA expression datasets available for prostate cancer. Datasets generated in this study will be made available to collaborators and eventually the wider scientific community. Ultimately, this project will 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. This project will also have an impact on the understanding of the role of eRNAs in transcription in general, 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**

The ChIA-PET approach was part of Major Task 1, and after extensive optimisation and discussion it has not been possible to have this working sufficiently well. I therefore plan to use ChIP-seq, which was listed as an alternative in the project proposal for such an eventuality. Issues with ChiA-PET have delayed progress by approximately 2-3 months. I intend to apply for a no-cost extension at the end of the next reporting period to complete alternative ChIP-seq experiments.

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

No change in planned expenditure itself, but delays in finalising contracts resulted in delay in timing of spend, therefore (after consulting your Grants office) I plan to apply for a no cost extension towards the end of the current period to allow completion of the work.

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

Nothing to report

6. PRODUCTS

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name	Rayzel Fernandes
Role	Principal Investigator
Researcher ID	0000-0002-8834-3221
Nearest Person Months worked	12
Contributions to project	Designed and performed all experiments, 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?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report

9. APPENDICES:

N/A

10. References

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