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TITLE: Functionally Characterizing the Noncoding Genome of Metastatic Prostate Cancer to Identify Therapeutic Vulnerabilities

PRINCIPAL INVESTIGATOR: Matthew Freedman

CONTRACTING ORGANIZATION: Dana-Farber Cancer Institute, Boston, MA

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13. SUPPLEMENTARY NOTES

14. ABSTRACT Prostate cancer (PCa) is dependent on the androgen receptor (AR) at all stages of the disease. Given this critical role, inhibiting AR function is the standard-of-care to treat advanced PCa. However, while treatment is initially effective, resistance to treatment inevitably occurs resulting in lethal metastatic castrate-resistant PCa (mCRPC). Importantly, the vast majority of mCRPC tumors still remain fully dependent on AR signalling. Yet when the cancer adapts to low circulating androgens the AR program is dramatically altered. We recently reported that during progression to castration resistance, AR binding sites (ARBS) on the DNA are heavily reprogrammed and gain a set of ~17,000 new binding sites. These gained metastatic AR binding sites (mARBS) reactivate a latent prostate developmental program. Notably, the massive change in the AR cistrome -the universe of all AR binding sites in the genome- is highly recurrent across patients, suggesting that this common mechanism that represents a universal feature of castration resistance in mCRPC tumors. Intriguingly, we observed that these gained ARBS are highly mutated and may potentially alter AR-mediated transcription to drive tumour growth in mCRPC. Yet while we can readily identify these gained mARBS and their mutations, the specific subset of sites that are critical to the metastatic process remains unknown. In addition, it remains elusive which biological pathways are selectively driving metastatic outgrowth in this setting. Our published work provides a clear foundation for studying the mCRPC-specific AR cistrome. We strongly believe that our results will reveal important insights into mechanisms of progressive metastatic PCa and will provide new avenues of how treatment can be improved for these patients. We propose that a subset of metastatic ARBS are responsible for driving the growth of CRPC and a fraction of these sites possess gain-of-function somatic mutations.

15. SUBJECT TERMS
None listed.

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TABLE OF CONTENTS

1. INTRODUCTION:

Prostate cancer (PCa) is dependent on the androgen receptor (AR) at all stages of the disease. Standard of care treatment for advanced PCa focuses on inhibition of androgen signaling. Despite initial efficacy, resistance inevitably occurs. Notably, this state, termed metastatic castration resistant prostate cancer (mCRPC), remains dependent on AR signaling. Our groups have identified a set of 17,000 gained AR binding sites that characterize mCRPC. Our proposal aims to functionally characterize these important sites through a variety of approaches.

2. KEYWORDS:

Metastatic castration resistant prostate cancer (mCRPC), cistrome, androgen receptor binding sites (ARBS), STARR-seq, epigenomics, CRISPR, HiChIP

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The major goals of the proposal are as follows:

Specific Aim 1: Characterize the AR cistrome to reveal functionally active regulatory elements.
This major tasks underlying this aim included STARR-seq. The milestones for STARR-seq were estimated to be completed in Year 1.

- The STARR-seq work is 75% completed. We have generated suitable STARRseq libraries and tested all gained mARBS sites for enhancer activity using a genome-wide STARRseq in our proposed model of primary PCa.
- The HiChIP work is 75% completed.

Specific Aim 2: Functionally test gained metastatic AR enhancers to drive castration resistance and metastatic outgrowth
The major tasks in this aim are a CRISPR dropout screen (to be completed within two years) and to perform in vivo validation of top-scoring enhancers (to be completed within four months after the initial screen).
Specific Aim 3: Identify somatic mutation that alter AR enhancer activity and characterize their function. The major tasks in this aim are to generate an AR mutated library and to validate hits. The milestones for this work are projected to be completed in years 2-3 of the proposal.

What was accomplished under these goals?

Specific aim 1: Test universe of 17,655 gained metastatic AR binding sites using STARR-seq
Subtask 1: Design Agilent DNA capture array, capture pooled DNA
Status: completed

- **Detailed description:** Due to recent technical innovations we chose to expand the scope of the experiments and implemented a genome-wide STARRseq library. This unpublished library has excellent ARBS coverage (>35x) and provides unbiased dataset that can be adopted to interrogate AR enhancer activity. Importantly, when compared to the smaller capture-based STARRseq activity we observed excellent correlation of AR enhancer activity (Figure 1)

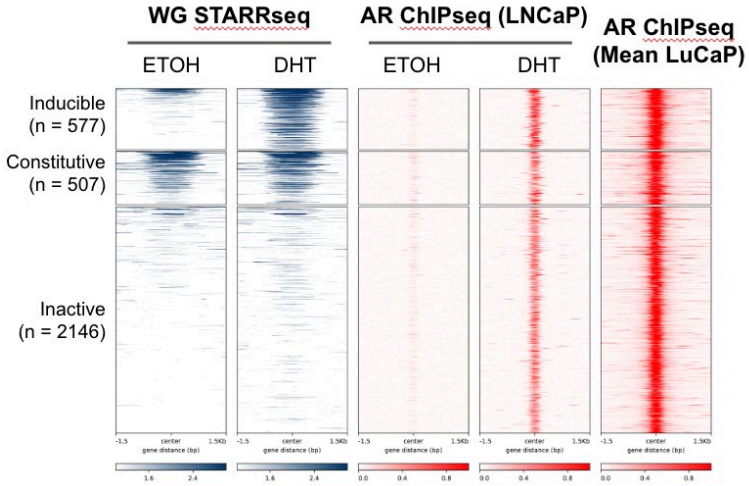


Figure 1: Comparison of primary PCa ARBS from whole-genomc STARRseq to capture-based annotations.

Subtask 2: Clone into STARR-seq plasmid
Status: completed

- **Detailed description:** Cloning was completed in the whole genome library preparation (see Subtask 1)

Subtask 2: Test for AR-driven enhancer activity in cell lines and analyze data
Status: Ongoing

- **Detailed description** With this library we have tested all gained metastatic AR binding sites (ARBS) for enhancer activity in our proposed model of primary PCa (LNCaP). In line with our hypothesis, we observed minimal AR-driven enhancer activity in these gained sites (Figure 2). Of the >17,000 gained ARBS tested only 473 (~2%) had AR-driven enhancer activity. Given the reproducibility of our results we are currently testing for AR enhancers within cellular models of mCRPC.

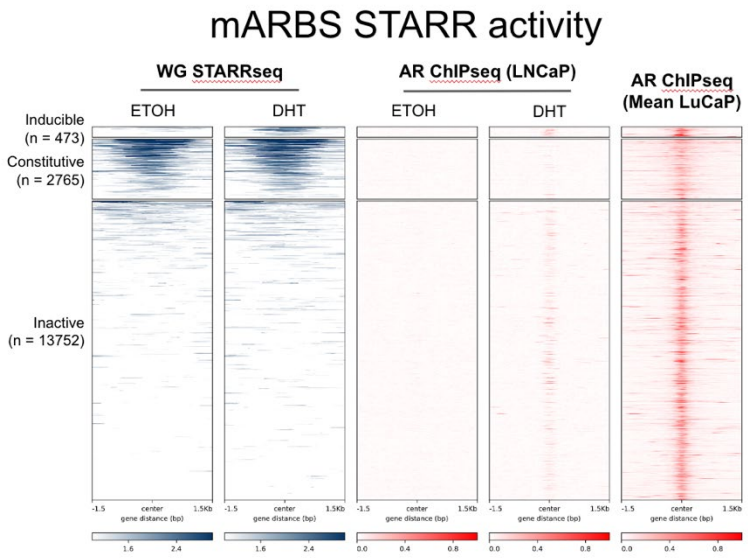


Figure 2: AR enhancer activity across all gained mARBS in a model of primary PCa (LNCaP).

Specific aim 2,

Subtask 1: design guide RNAs (gRNAs) across 17,655 enhancers
Status: completed

- **Detailed description**: The design of optimal CRISPR guide RNAs is a yield of continuous development. To maximize on-target efficiency and limit off-target effects of the sgRNAs, we made use of a newly developed tools to enhance CRISPR efficiency (PMID: 34050182) and decrease off-target effects (PMID: 30367669). Based on these criteria, we designed optimized sgRNAs targeting each of the 17655 metastasis-specific ARBS, with 4 sgRNAs for each site. In addition, 600 controls were designed 10kb upstream (n=300) or downstream (n=300) of the ARBS, as well as 100 non-targeting controls from the Brunello library. As positive controls, promoter-targeting sgRNAs were included targeting essential genes (PLK1, AR, FOXA1, HOXB13). CRISPRi performance was assessed by targeting the enhancer of NKX3.1, after which cut&tag for H3K9ac and H3K27ac was performed along with ATAC-seq to determine chromatin accessibility (see Figure 3). Clear increase of H3K9me3 signal was observed at the targeted enhancer element, with a decreased H3K27ac signal both at the KNX3.1 enhancer and promoter regions. Based on this, we conclude that the CRISPRi targeting was effective, without regional suppression or indirect promoter targeting.

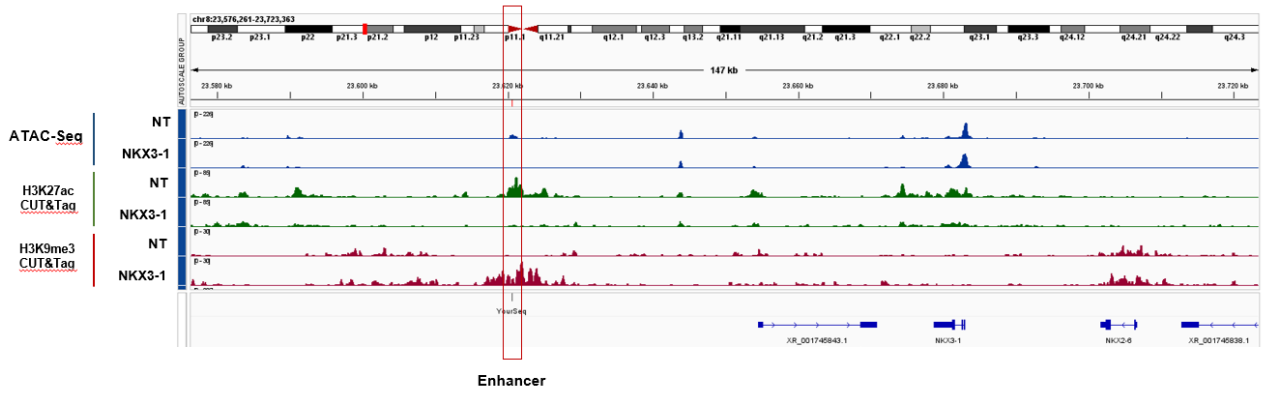


Figure 3: perturbation of the NKX3.1 enhancer by CRISPRi.

Subtask 2: Perform H3K27 ChIP seq on two hormone-sensitive (LNCaP, LAPC4) and three hormone-resistant cell lines (LNCaP-abl, 22Rv1, and VCaP)

Status: completed

Detailed description: Using wet lab and computational pipelines optimized by our teams (PMID: 32690948) to perform high-quality ChIP-seq analyses, H3K27ac ChIP-seq was successfully performed on two hormone-sensitive (LNCaP, LAPC4) and three hormone-resistant cell lines (LNCaP-abl, 22Rv1, and VCaP). For each cell line and each ChIP-seq condition, 3 biological replicates were generated, to facilitate statistical analyses downstream.

Subtask 3: Conduct pooled CRISPR/Cas9 screen targeting mARBS.

Status: ongoing

Detailed description: having designed the CRISPR library, we are currently in the process of library construction, after which the lentiviral library will be transduced into the above-mentioned cell lines, as originally planned.

Major Task 2: Perform HiChIP

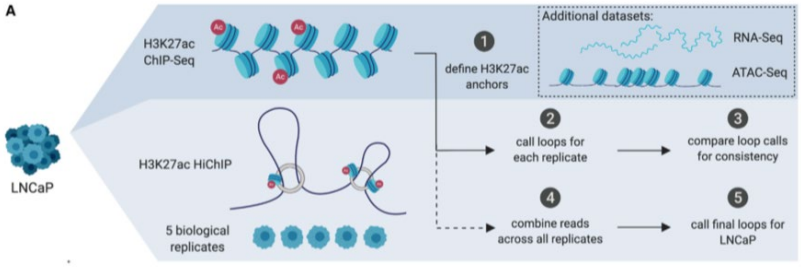
Status: Ongoing

Subtasks 1 and 2: Perform chromosome conformation capture and ChIP
Detailed description: We completed H3K27ac and H3K4me3 HiChIP. AR HiChIP is underway and we aim to complete it in the next reporting period (SA1/MT2). The HiChIP assay is labor-intensive requiring a significant amount of time. We were delayed in the AR HiChIP due to COVID-related restrictions at our institution.

- Briefly, for each HiChIP experiment, we cross-linked 10 million cells and digested with the MboI restriction enzyme. Biotinylated dATP is incorporated into the digested sample and then ligated. Immunoprecipitation is then performed with the appropriate antibody (H3K27ac, H3K4me3, and AR). Samples are decrosslinked,

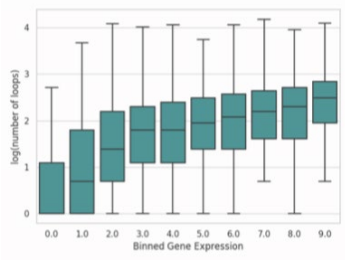
pulled down with streptavidin beads and treated with transposase to create libraries. These libraries are then sent for sequencing.

- We have performed extensive quality control analyses for H3K27ac HiChIP. Five biological replicates were performed. Please note that these analyses were performed ahead of schedule.
-



Overview of H3K27ac HiChIP experiment and analysis workflow

- We identified a total 126,280 loops using an FDR < 0.01 using FitHiChIP.
- Gene connectivity, defined as the number of loops per gene promoter is moderately correlated with gene expression activity as measured by RNA-seq (ρ=0.49; p value < 2.2e-16).



Gene connectivity and gene expression in LNCaP. We took the union of 17,690 genes with looping counts in LNCaP and 20,114 genes with expression counts in LNCaP, dropping all genes that do not have both looping and expression information. We binned the remaining 13,274 genes into deciles (1,327 genes per decile) **a.** X-axis is the binned gene expression (FPKM) of the LNCaP genes; Y-axis represents the number of loops.

What opportunities for training and professional development has the project provided?

- In Dr. Freedman's group, the project has provided the opportunity to increase training for Dr. Ji-Heui Seo, a research scientist in the laboratory. She has supervised and mentored Mr.

Matthew Davidsohn and Ms. Gita Lakshminarayanan, two research technicians in the laboratory for the HiChIP and the ChIP-seq work.

- Flora Huang (UBC, Canada)

Personal development:

Seminars/Meetings;
- Participating/presenting in group meetings (every week)
  - One-on-one mentoring session with Dr. Lack (every week)
  - Participating/presenting in Journal Clubs (every 2nd week)
  - Attending Vancouver Prostate Centre International Seminars (every week)

Emma Minnee (NKI, Amsterdam):

Personal development:

Conferences;
- Nov. 2021 - Oncode - CGC Annual Conference: Basic biology meets Cancer research (Amsterdam, the Netherlands)
- Nov. 2021 - 28th Annual Prostate Cancer Foundation (PCF) Scientific Retreat (online)
- Nov. 2021 – 13th Nuclear Receptor Network Meeting (NRRN) (Leiden, the Netherlands)
- Juli. 2022 - Oncode - CGC Annual Conference: Basic biology meets Cancer research 2022 (Amersfoort, the Netherlands)

Courses/Masterclasses;
- Jan. 2022 - Oncode Masterclass "Gene editing and its application 2.0" (Hubrecht Institute, Utrecht, The Netherlands)
- Jun. 2022 - FlowJo course (NKI, Amsterdam, the Netherlands)
- Jun. 2022 – 5 day Introduction to R course (NKI)

Seminars/Meetings;
- Participating/presenting in group meetings (every week)
  - One-on-one mentoring session with dr. Zwart (every week)
  - Participating/presenting in Journal Clubs (every 2nd week)
  - Attending NKI International Seminars (every week)
  - Attending NKI Research Club (twice a week)

How were the results disseminated to communities of interest?

The Zwart lab participates in a Patient Engagement Program, involving patients (including prostate cancer patient) in our group meetings, and interactions with researchers. In these events, the project and its implications have been extensively discussed.

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

- We will complete the AR HiChIP experiment in the LNCaP cell line
- Bioinformatic analyses will be performed to systematically identify enhancer-promoter interactions at our set of 17,655 metastatic AR binding sites that we previously identified.
- The plans have remained unchanged as the project is still on-track.
- Test mARBS for enhancer activity in models of mCRPC
- In the next, reporting period, we aim to have finalized the enhancer CRISPR screen, and identified those AR bound enhancers that are essential for tumor cell proliferation in the mCRPC setting. We aim to have hits validated independently and tested multiple cell line models of mCRPC.

4. **IMPACT: What was the impact on the development of the principal discipline(s) of the project?**

- The androgen receptor (AR) plays a critical role in prostate cancer initiation and progression. The AR is a protein called a transcription factor that works by turning on and off sets of genes. Our groups have defined the set of AR binding sites across the human genome in normal prostate tissue, localized prostate tumors, and metastatic prostate tumors. We identified a unique set of 17,000 AR binding sites in the metastatic state. This proposal seeks to functionally characterize these sites. A greater understanding of these sites and what programs they enact will set a foundation for

What was the impact on other disciplines?

- The algorithms and computational tools generated in this project will be rendered publicly available, to benefit researchers of other disciplines
- We anticipate this project to serve as blueprint for other researchers and may inspire others, when searching for the impact of non-coding mutations in cancer.

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

- Note that the pandemic affected (and still affects) our Institutional policies including how many people can be at the bench at one time. Therefore, we are operating at ~65% capacity. As can be seen by the data generated, we have done our best to overcome this limitation and will continue to generate and analyze data while adhering to Institute policy.

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

Nothing to report

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 agents

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

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

Journal publications.

1. Qiu X, Boufaied N, Hallal T, Feit A, de Polo A, Luoma AM, Alahmadi W, Larocque J, Zadra G, Xie Y, Gu S, Tang Q, Zhang Y, Syamala S, Seo JH, Bell C, O'Connor E, Liu Y, Schaeffer EM, Jeffrey Karnes R, Weinmann S, Davicioni E, Morrissey C, Cejas P, Ellis L, Loda M, Wucherpfennig KW, Pomerantz MM, Spratt DE, Corey E, Freedman ML, Shirley Liu X, Brown M, Long HW, Labbé DP. MYC drives aggressive prostate cancer by disrupting transcriptional pause release at androgen receptor targets. Nat Commun. 2022 May 13;13(1):2559. doi: 10.1038/s41467-022-30257-z. PMID: 35562350; PMCID: PMC9106722.; yes

2. Giambartolomei C, Seo JH, Schwarz T, Freund MK, Johnson RD, Spisak S, Baca SC, Gusev A, Mancuso N, Pasaniuc B, Freedman ML. H3K27ac HiChIP in prostate cell lines identifies risk genes for prostate cancer susceptibility. Am J Hum Genet. 2021 Dec 2;108(12):2284-2300. doi: 10.1016/j.ajhg.2021.11.007. Epub 2021 Nov 24. PMID: 34822763; PMCID: PMC8715276.; yes

- **Books or other non-periodical, one-time publications.**

None

- **Other publications, conference papers and presentations.**

None

- **Website(s) or other Internet site(s)**

None

- **Technologies or techniques**

None

- **Inventions, patent applications, and/or licenses**

None

- **Other Products**

None

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Matthew Freedman
Project role:	Principal Investigator
Researcher Identifier (ORCID ID)	0000-0002-0151-1238
Nearest person month worked:	1
Contribution to the Project:	Overall PI, responsible for project direction and lab supervision
Name:	Nathan Lack
Project role:	Sub Site PI
Researcher Identifier (ORCID ID)	0000-0001-7399-5844
Nearest person month worked:	2.4
Contribution to the Project:	Resposible for STARRseq and TOTEM mutagenesis
Name:	Wilbert Zwart
Project role:	Sub Site PI
Researcher Identifier (ORCID ID)	0000-0002-9823-7289
Nearest person month worked:	0.6
Contribution to the Project:	Responsible for ACURO approval, and experiments related to CRISPR-Cas9
Name:	Emma Minnee
Project role:	Graduate Student
Researcher Identifier (ORCID ID)	**0000-0003-1012-4800**
Nearest person month worked:	6.0
Contribution to the Project:	Ms. Minnee is performing experiments on CRISPRi perturbations in prostate cancer cells, designing the CRISPRi library, performs the screen and analyses the hits
Name:	Ji-Heui Seo
Project role:	Research Scientist
Nearest person month worked:	1.1
Contribution to the Project:	Dr. Seo designed, performed, and analyzed all of the HiChIP experiments
Name:	Sandor Spisak
Project role:	Instructor
Nearest person month worked:	1.8
Contribution to the Project:	Dr. Spisak assisted with experiments and data reporting.
Name:	Flora Huang
Project role:	Research Scientist
Nearest person month worked:	1
Contribution to the Project:	Dr. Huang designed, performed, and analyzed all of the STARRseq experiments

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

Freedman:
R01CA204954 ended 3/31/21
W81XWH21-1-0339 awarded 8/15/21
Movember PCF Challenge Award funded 10/1/21
Lack:
Prostate Cancer Canada ended 3/31/22
Turkish Scientific and Technological Research Council ended 8/31/21
Turkish Scientific and Technological Research Council awarded 10/1/21
Zwart:
N/A

What other organizations were involved as partners?

University of British Columbia – work is funded through a subagreement to support Nathan Lack's research

Netherlands Cancer Institute – work is funded through a subagreement to support Wilbert Zwart's research

These collaborations were included in the original application and responsibilities were outlined in the SOW.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

N/A

9. APPENDICES:

None