

GENOMIC AND EPIGENOMIC ABERRATIONS OF GENE REGULATION IN PROSTATE  
CANCER AND LEUKEMIA

by

James Hawley

A thesis submitted in conformity with the requirements  
for the degree of Doctor of Philosophy  
Graduate Department of Medical Biophysics  
University of Toronto

© Copyright 2021 by James Hawley

Genomic and epigenomic aberrations of gene regulation in prostate cancer and leukemia

James Hawley

Doctor of Philosophy

Graduate Department of Medical Biophysics

University of Toronto

2021

## **Abstract**

Abstract text goes here. Maximum 350 words for doctoral or 150 words for master's thesis excluding title. Do not include graphs, charts, tables, or illustrations in the abstract. Uses style "Abstract text" (double spaced).

## Acknowledgments

Use Body Text or Normal style for text in this section.

# Contents

|          |   |          |
|----------|---|----------|
| <b>1</b> | <b>Noncoding mutations target the <i>FOXA1</i> plexus</b>                     | <b>1</b> |
| 1.1      | Abstract . . . . .  | 2        |
| 1.2      | Introduction . . . . .  | 2        |
| 1.3      | Results . . . . .   | 4        |
| 1.3.1    | <i>FOXA1</i> is essential for prostate cancer proliferation . . . . .         | 4        |
| 1.3.2    | Identifying putative <i>FOXA1</i> CREs . . . . .                              | 4        |
| 1.3.3    | Putative <i>FOXA1</i> CREs harbour TF binding sites and SNVs . . . . .        | 6        |
| 1.3.4    | Disruption of CREs reduces <i>FOXA1</i> mRNA expression . . . . .             | 7        |
| 1.3.5    | <i>FOXA1</i> CREs collaborate to regulate its expression . . . . .            | 8        |
| 1.3.6    | Disruption of <i>FOXA1</i> CREs reduces prostate cancer cell growth . . . . . | 9        |
| 1.3.7    | SNVs mapping to <i>FOXA1</i> CREs can alter their activity . . . . .          | 9        |
| 1.3.8    | SNVs mapping to <i>FOXA1</i> CREs can modulate the binding of TFs . . . . .   | 10       |
| 1.4      | Discussion . . . . .  | 10       |
| 1.5      | Methods . . . . .   | 12       |
| 1.5.1    | Cell Culture . . . . .  | 12       |
| 1.5.2    | Prostate tumours and cancer cell lines expression . . . . .                   | 12       |
| 1.5.3    | Prostate cancer cell line gene essentiality . . . . .                         | 13       |
| 1.5.4    | siRNA knockdown and cell proliferation assay . . . . .                        | 13       |
| 1.5.5    | Identifying putative FOXA1 CREs . . . . .                                     | 13       |
| 1.5.6    | Hi-C and TADs in LNCaP cells . . . . .  | 14       |
| 1.5.7    | Clonal wild-type Cas9 and dCas9-KRAB mediated validation . . . . .            | 14       |
| 1.5.8    | Transient Cas9-mediated disruption of CREs . . . . .                          | 15       |
| 1.5.9    | RT-PCR assessment of gene expression upon deletion of CREs . . . . .          | 15       |
| 1.5.10   | Confirmation of Cas9-mediated deletion of CREs . . . . .                      | 16       |
| 1.5.11   | Cell proliferation upon deletion of FOXA1 CREs . . . . .                      | 16       |
| 1.5.12   | Luciferase reporter assays . . . . .  | 16       |

|   |           |
|---|-----------|
| 1.5.13 Allele-specific ChIP-qPCR . . . . .      | 17        |
| <b>References</b>                               | <b>18</b> |
| <b>Appendices</b>                               | <b>24</b> |
| .1 Supplemental Figures for Chapter 2 . . . . . | 24        |

## List of Tables

## List of Figures

|  |   |
|--|---|
| 1.1 FOXA1 is highly expressed in prostate cancer and essential for prostate cancer cell proliferation. . . . . | 5 |
|--|---|

## List of Appendices

# Chapter 1

## Noncoding mutations target *cis*-regulatory elements of the *FOXA1* plexus in prostate cancer

This chapter is a version of the paper published in *Nature Communications* as follows:

Zhou, S., **Hawley, J. R.**, Soares, F., Grillo, G., Teng, M., Tonekaboni, S. A. M., Hua, J. T., Kron, K. J., Mazrooei, P., Ahmed, M., Arlidge, C., Yun, H. Y., Livingstone, J., Huang, V., Yamaguchi, T. N., Espiritu, S. M. G., Zhu, Y., Severson, T. M., Murison, A., Cameron, S., Zwart, W., van der Kwast, T., Pugh, T. J., Fraser, M., Boutros, P. C., Bristow, R. G., He, H. H., and Lupien, M. Noncoding mutations target *cis*-regulatory elements of the *FOXA1* plexus in prostate cancer. **Nature Communications**, 2020;11:1–13.

Contributions per the manuscript: S.Z. and M.L. conceptualized the study. S.Z. designed and conducted most of the experiments with help from F.S., G.G., M.T., K.J.K., J.T.H., C.A., H.Y.Y., Y.Z. and S.C. J.R.H. implemented most of the computational analyses and statistical approaches with help from S.A.M., P.M., M.A., A.M., V.H., T.N.Y., S.M.G.E., T.M.S. and J.L. under the supervision of W.Z., T.v.d.K., T.J.P., M.F., P.C.B., R.G.B., H.H.H., or M.L. Figures were designed by S.Z. with assistance from J.R.H. and S.A.M. The manuscript was written by S.Z., J.R.H. and M.L. with assistance from all authors. M.L. oversaw the study.

## 1.1 Abstract

Prostate cancer is the second most commonly diagnosed malignancy among men worldwide. Recurrently mutated in primary and metastatic prostate tumours, *FOXA1* encodes a pioneer transcription factor involved in disease onset and progression through both androgen receptor-dependent and androgen receptor-independent mechanisms. Despite its oncogenic properties however, the regulation of *FOXA1* expression remains unknown. Here, we identify a set of six *cis*-regulatory elements in the *FOXA1* regulatory plexus harboring somatic single-nucleotide variants in primary prostate tumours. We find that deletion and repression of these *cis*-regulatory elements significantly decreases *FOXA1* expression and prostate cancer cell growth. Six of the ten single-nucleotide variants mapping to *FOXA1* regulatory plexus significantly alter the transactivation potential of *cis*-regulatory elements by modulating the binding of transcription factors. Collectively, our results identify *cis*-regulatory elements within the *FOXA1* plexus mutated in primary prostate tumours as potential targets for therapeutic intervention.

## 1.2 Introduction

Prostate cancer is the second most commonly diagnosed cancer among men with an estimated 1.3 million new cases worldwide in 2018 [1]. Although most men diagnosed with primary prostate cancer are treated with curative intent through surgery or radiation therapy, treatments fail in 30% of patients within 10 years [2] resulting in a metastatic disease [3]. Patients with metastatic disease are typically treated with anti-androgen therapies, the staple of aggressive prostate cancer treatment [4]. Despite the efficacy of these therapies, recurrence ultimately develops into lethal metastatic castration resistant prostate cancer (mCRPC) [4]. As such, there remains a need to improve our biological understanding of prostate cancer development and find novel strategies to treat patients. Sequencing efforts identified coding somatic single-nucleotide variants (SNVs) mapping to *FOXA1* in up to 9% [5–10] and 13% [9–11] of primary and metastatic castration resistant prostate cancer (mCRPC) patients, respectively. These coding somatic SNVs target the Forkhead and transactivation domains of *FOXA1* [12], altering its pioneering functions to promote prostate cancer development [10, 13]. Outside of coding SNVs, whole genome sequencing also identified somatic SNVs and indels in the 3' UTR and C-terminus of *FOXA1* in 12% of mCRPC patients [14]. In addition to SNVs, the *FOXA1* locus is a target of structural rearrangements in both primary and metastatic prostate cancer tumours, inclusive of duplications, amplifications, and translocations [9, 10]. Taken together, *FOXA1*

is recurrently mutated taking into account both its coding and flanking noncoding sequences across various stages of prostate cancer development.

FOXA1 serves as a pioneer transcription factor (TF) that can bind to heterochromatin, promoting its remodelling to increase accessibility for the recruitment of other TFs [15]. FOXA1 binds to chromatin at cell-type specific genomic coordinates facilitated by the presence of mono- and dimethylated lysine 4 of histone H3 (H3K4me1 and H3K4me2) histone modifications [16, 17]. In prostate cancer, FOXA1 is known to pioneer and reprogram the binding of the Androgen Receptor (AR) alongside HOXB13 [18]. Independent from its role in AR signalling, FOXA1 also regulates the expression of genes involved in cell cycle regulation in prostate cancer [19, 20]. For instance, FOXA1 co-localizes with CREB1 to regulate the transcription of genes involved in cell cycle processes, nuclear division and mitosis in mCRPC [19–25]. FOXA1 has also been shown to promote feed-forward mechanisms to drive disease progression [26, 27]. Hence, FOXA1 contributes to AR-dependent and AR-independent processes favouring prostate cancer development.

Despite the oncogenic roles of FOXA1, therapeutic avenues to inhibit its activity in prostate cancer are lacking. In the breast cancer setting for instance, the use of cyclin-dependent kinases inhibitors have been suggested based on their ability to block FOXA1 activity on chromatin [28]. As such, understanding the governance of FOXA1 mRNA expression offers an alternative strategy to find modulators of its activity. Gene expression relies on the interplay between distal cis-regulatory elements (CREs), such as enhancers and anchors of chromatin interaction, and their target gene promoter(s) [29]. These elements can lie tens to hundreds of kilobases (kbp) away from each other on the linear genome but physically engage in close proximity with each other in the three-dimensional space [30]. By measuring contact frequencies between loci through the use of chromatin conformation capture-based technologies, it enables the identification of regulatory plexuses corresponding to sets of CREs in contact with each other [31, 32]. By leveraging these technologies, we can begin to understand the three-dimensional organization of the prostate cancer genome and delineate the FOXA1 regulatory plexus.

Here, we integrate epigenetics and genetics from prostate cancer patients and model systems to delineate CREs establishing the regulatory plexus of FOXA1. We functionally validate a set of six mutated CREs that regulate FOXA1 mRNA expression. We further show that SNVs mapping to these CREs are capable of altering their transactivation potential, likely through modulating the binding of key prostate cancer TFs.



## 1.3 Results

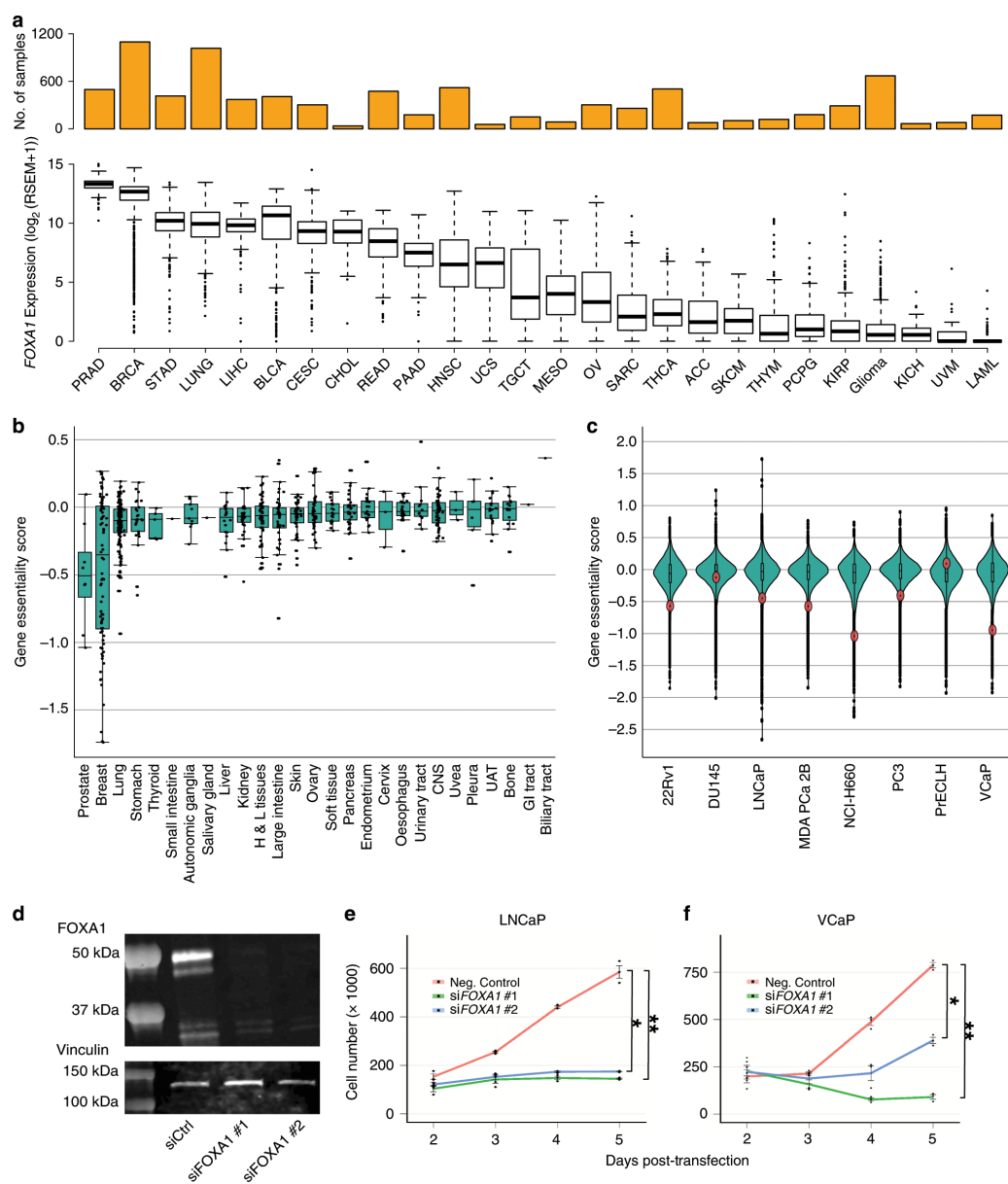
### 1.3.1 *FOXA1* is essential for prostate cancer proliferation

We interrogated FOXA1 expression levels across cancer types. We find that FOXA1 mRNA is consistently the most abundant in prostate tumours compared to 25 other cancer types across patients (fig1a), ranking in the 95th percentile for 492 of 497 prostate tumours profiled in TCGA (figs1a). Using the same dataset we also find that FOXA1 is the most highly expressed out of 41 other Forkhead Box (FOX) factors in prostate tumours (figs1b). We next analyzed expression data from DEPMAP and observed FOXA1 to be most highly expressed in prostate cancer cell lines compared to cell lines of other cancer types (figs2a). Amongst the eight prostate cancer cell lines in the dataset (22Rv1, DU145, LNCaP, MDA-PCa-2B, NCI-H660, PrECLH, PC3, and VCaP), FOXA1 mRNA abundance is above the 90th percentile in all but one cell line (PrECLH) compared to the > 56,000 protein coding and non-protein coding genes profiled (figs2b). These new results gained from the TCGA and DEPMAP validate previous understanding that FOXA1 is one of the highest expressed genes in prostate cancer [33].

Following up on FOXA1 mRNA expression levels, we interrogated the essentiality of FOXA1 for prostate cancer cell growth. RNAi-mediated essentiality screens compiled in DEPMAP show that FOXA1 lies in the 94th percentile across 6 of the 8 available prostate cancer cell lines: 22Rv1, LNCaP, MDA PCa 2B, NCI-H660, PC3, and VCaP cells (fig1b-c). The median RNAi-mediated essentiality score for all prostate cell lines is significantly lower than all other cell lines, suggesting that FOXA1 is especially essential for prostate cancer cell proliferation (permutation test,  $p = 1 \cdot 10^{-6}$ , see Methods) (figs3a). Growth assays in LNCaP and VCaP cells following FOXA1 knockdown using two independent siRNAs (fig1d, figs3b) show significant growth inhibition in LNCaP (siRNA #1: 4-fold, siRNA #2: 3.35-fold) and VCaP (siRNA #1: 8.7-fold, siRNA #2: 2-fold) cells five days post-transfection (Mann-Whitney U Test,  $p < 0.05$ ; fig1e-f). In accordance with previous reports, our results using essentiality datasets followed by knockdown validation reveals that FOXA1 is oncogenic and essential for prostate cancer cell proliferation.

### 1.3.2 Identifying putative *FOXA1* CREs

The interweaving of distal CREs with target gene promoters establishes regulatory plexuses with some to be ascribed to specific genes [31, 32]. Regulatory plexuses stem from chromatin interactions orchestrated by various factors including ZNF143, YY1, CTCF and the cohesin complex



**Figure 1.1: FOXA1 is highly expressed in prostate cancer and essential for prostate cancer cell proliferation..** **a.** The mRNA expression of FOXA1 across tumour types ( $n = 26$ ) from RNA-seq data of TCGA. **b.** FOXA1 essentiality mediated through RNAi across various cell lines ( $n = 707$ ) from DEPMAP. Gene essentiality scores are normalized Z-scores. Higher scores indicate less essential, and lower scores indicate more essential for cell proliferation. X-axis indicate tissue of origin for each cell line tested. Each dot indicates one cell line. **c.** Gene essentiality mediated through RNAi across prostate cancer cell lines ( $n = 8$ ) from DEPMAP. Each dot indicates one gene, red indicates FOXA1. **d.** Representative Western blot against FOXA1 in LNCaP cells 5 days post-transfection of non-targeting siRNA and two independent siRNA targeting FOXA1. **e.** Cell proliferation assay conducted in LNCaP cells upon siRNA-mediated knockdown of FOXA1 across 5 days. **f.** Cell proliferation assay conducted in VCaP cells upon siRNA-mediated knockdown of FOXA1 across 5 days. Error bars indicate  $\pm$  s.d.  $n = 3$  independent experiments. Mann-Whitney U test, \*  $p < 0.05$ , \*\*  $p < 0.01$ .

[34–36]. Motivated by the oncogenic role of FOXA1 in prostate cancer, we investigated its regulatory plexus controlling its expression. According to chromatin contact frequency maps generated from Hi-C assays performed in LNCaP prostate cancer cells, FOXA1 lies in a 440 kbp TAD (chr14: 37720002-38160000  $\pm$  40 kbp adjusting for resolution) (Figure 3.2a). By overlaying DNase-seq data from LNCaP prostate cancer cells, there are a total of 123 putative CREs reported as DNase I Hypersensitive Sites (DHS) that populate this TAD (Figure 3.2a). We next inferred the regulatory plexus of FOXA1 using the Cross Cell-Type Correlation based on DNA accessibility (C3D) method [37]. C3D aggregates and draws correlation of DHS signal intensities between the cell line of choice and the DHS signal across all systems in the database [37]. Anchoring our analysis to the FOXA1 promoter and using accessible chromatin regions defined in LNCaP prostate cancer cells identified 55 putative CREs to the FOXA1 regulatory plexus ( $r > 0.7$ ) (Figure 3.2b).

### 1.3.3 Putative *FOXA1* CREs harbour TF binding sites and SNVs

To delineate the CREs that could be actively involved in the transcriptional regulation of FOXA1, we annotated the DHS with available ChIP-seq data for histone modifications and TFs conducted in LNCaP, 22Rv1, VCaP prostate cancer cell lines and primary prostate tumours (Figure 3.2b) [18, 38]. Close to 60% (33/55) of the putative FOXA1 plexus CREs are positively marked by H3K27ac profiled in primary prostate tumours [38], indicative of active CREs in tumours (Figure 3.2b) [39]. Next, considering that noncoding SNVs can target a set of CREs that converge on the same target gene in cancer [32], we overlapped the somatic SNVs called from the whole-genome sequencing across 200 primary prostate tumours to the 33 H3K27ac-marked DHS predicted to regulate FOXA1 [6, 40]. This analysis identified 6 out of the 33 DHS marked with H3K27ac (18.2%) harboring one or more SNV(s) (10 total SNVs called from 9 tumours) (Figure 3.2b). We observe that these 6 CREs can be bound by multiple TFs in prostate cancer cells, including FOXA1, AR and HOXB13 (Figure 3.2b, Appendix II Supplemental Figure 3.4a-f). The Hi-C data from the LNCaP prostate cancer cells corroborates the C3D predictions as demonstrated by the elevated contact frequency between the region harboring the FOXA1 promoter and where the 6 CREs are located, compared to other loci in the same TAD (Figure 3.3a). The 6 CREs lie in intergenic or intronic regions (Figure 3.3b-h). Together, histone modifications, TF binding sites and noncoding SNVs support that these 6 putative CREs are active in primary prostate cancer. The Hi-C and C3D predictions suggest that they regulate FOXA1 expression.

### 1.3.4 Disruption of CREs reduces *FOXA1* mRNA expression

We next assessed the role of CREs toward FOXA1 expression using LNCaP and 22Rv1 clones stably expressing the wild-type Cas9 protein (Figure 3.4a-b). Guide RNAs (gRNAs) designed against the FOXA1 gene (exon 1 and intron 1) served as positive controls while an outside-TAD region (i.e. termed Chr14 (-)), a region on a different chromosome (the human AAVS1 safe-harbor site at the PPP1R12C locus [38, 41]), and three regions within the TAD predicted to be excluded from the FOXA1 plexus served as negative controls. Individual deletion of the FOXA1 plexus CREs through transient transfection of gRNAs into the LNCaP cells (See Methods) led to significantly decreased FOXA1 mRNA expression ( $\Delta$  CRE1  $29.3 \pm 8.3\%$ ,  $\Delta$  CRE2  $40.1 \pm 11.8\%$ ,  $\Delta$  CRE 3  $30.6 \pm 9.1\%$ ,  $\Delta$  CRE4  $23.6 \pm 8.2\%$ ,  $\Delta$  CRE5  $25.3 \pm 6.6\%$ ,  $\Delta$  CRE6  $24.5 \pm 10.2\%$  and  $\Delta$  FOXA1 (exon 1 and intron 1)  $87.4 \pm 8.8\%$  reduction relative to basal levels) (Figure 3.4c, Appendix II Supplemental Figure 3.5a-f). In contrast, deletion of several negative control regions within the same TAD did not significantly reduce FOXA1 mRNA level (Figure 3.4c, Appendix II Supplemental Figure 3.5g-i). Similar results were observed in 22Rv1 prostate cancer cells (Figure 3.4d). As each clone expressed Cas9 protein at different levels, there may be a difference between genome editing efficiencies between the clones. We compared the CRISPR/Cas9 on-target genome editing efficiency across the five LNCaP cell line-derived clones with the relative FOXA1 mRNA levels, and indeed observe a significant inverse correlation across all CREs (Pearson's correlation  $r = 0.49, p < 0.005$ ) (Appendix II Supplemental Figure 3.6a) and agreeing trends for each individual CRE (Appendix II Supplemental Figure 3.6b).

Complementary to our findings using the wild-type CRISPR/Cas9 system, we next generated four LNCaP and four 22Rv1 cell line-derived dCas9-KRAB fusion protein expressing clones (Figure 3.4e-f). Transient transfection of the same gRNAs used in the wild-type Cas9 experiments, targeting the six FOXA1 plexus CREs into our dCas9-KRAB LNCaP clones significantly decreased FOXA1 expression relative to basal levels (iCRE1  $24.6 \pm 6.2\%$ , iCRE2  $42.2 \pm 10.8\%$ , iCRE3  $25.3 \pm 9.2\%$ , iCRE4  $23.3 \pm 4.3\%$ , iCRE5  $30.2 \pm 3.4\%$  and iCRE6  $23.1 \pm 8.1\%$  reduction). Similarly, gRNAs targeting the dCas9-KRAB fusion protein to FOXA1 decreased its expression (iFOXA1  $81.6 \pm 11.8\%$  reduction; Student's t-test,  $p < 0.05$ , Figure 3.4g). Analogous results were also observed in our four clonal 22Rv1 dCas9-KRAB cell lines (Student's t-test,  $p < 0.05$ , Figure 3.4h). Collectively, our results suggest that the six CREs control FOXA1 expression.

We further assessed the regulatory activity of the six FOXA1 plexus CREs by testing the consequent mRNA expression on other genes within the same TAD, namely MIPOL1 and TTC6.  $\Delta$

CRE1 and  $\Delta$  CRE2 significantly reduced MIPOL1 mRNA expression by  $38.4 \pm 6.4\%$  and  $48.4 \pm 9\%$ , respectively relative to basal levels, whereas deletion of the other four CREs did not result in any significant MIPOL1 expression changes (Student's t-test,  $p < 0.05$ , Appendix II Supplemental Figure 3.7a). On the other hand, deletion of CREs each significantly reduced TTC6 mRNA expression relative to its basal levels ( $\Delta$  CRE1  $52.9\% \pm 6.4\%$ ,  $\Delta$  CRE2  $66 \pm 11.3\%$ ,  $\Delta$  CRE3  $55.5 \pm 12.8\%$ ,  $\Delta$  CRE4  $44.9 \pm 10.6\%$ ,  $\Delta$  CRE5  $43.1 \pm 11.9\%$  and  $\Delta$  CRE6  $52.2 \pm 7.3\%$  reduction (Student's t-test,  $p < 0.05$ , Appendix II Supplemental Figure 3.7b), in agreement with the fact that TTC6 shares its promoter with FOXA1 as both genes are transcribed on opposing strands (Appendix II Supplemental Figure 3.7c).

Reduction in FOXA1 mRNA expression resulting from the deletion of FOXA1 plexus CREs may also impact gene expression downstream of FOXA1, we assessed the mRNA expression of several FOXA1 target genes, namely SNAI2, ACPP, and GRIN3A. Deletion of CREs resulted in significant change in SNAI2 (up-regulation;  $\Delta$  CRE1 190%,  $\Delta$  CRE2 162.8%,  $\Delta$  CRE3 147.5%,  $\Delta$  CRE4 133.3%,  $\Delta$  CRE5 137.3%,  $\Delta$  CRE6 120.8%,  $\Delta$  FOXA1 266.7%), ACPP (down-regulation;  $\Delta$  CRE1 73.5%,  $\Delta$  CRE2 62.5%,  $\Delta$  CRE3 69.6%,  $\Delta$  CRE4 75.6%,  $\Delta$  CRE5 70.9%,  $\Delta$  CRE6 74.6%,  $\Delta$  FOXA1 52.2%) and GRIN3A expression (up-regulation;  $\Delta$  CRE1 138.2%,  $\Delta$  CRE2 168.8%,  $\Delta$  CRE3 144.6%,  $\Delta$  CRE4 132.1%,  $\Delta$  CRE5 131.4%,  $\Delta$  CRE6 127%,  $\Delta$  FOXA1 228%) (Student's t-test,  $p < 0.05$ , Appendix II Supplemental Figure 3.7d-f). Collectively, our results support the restriction of most FOXA1 plexus CREs towards FOXA1 and its target genes.

### 1.3.5 *FOXA1* CREs collaborate to regulate its expression

Expanding on the idea that multiple CREs can converge to regulate the expression of a single target gene [31, 32, 42], we asked whether the CREs we identified collaboratively regulate FOXA1 mRNA expression. Here, we applied a transient approach that delivers Cas9 protein:gRNA as a ribonucleoprotein (RNP) complex formed prior to transfection that would avoid the heterogeneity of Cas9 protein expression across the prostate cancer cell clones (See Methods). We first validated this system through single CRE deletions, where we transiently transfected a set of gRNA targeting the CRE of interest. In accordance with data from our prostate cancer cell clones stably expressing wild-type Cas9 and dCas9-KRAB, individual CRE deletion resulted in a significant reduction in FOXA1 mRNA expression: ( $\Delta$  CRE1  $29.3 \pm 7.3\%$ ,  $\Delta$  CRE2  $36 \pm 11.8\%$ ,  $\Delta$  CRE3  $30.6 \pm 12.7\%$ ,  $\Delta$  CRE4  $24.5 \pm 6.1\%$ ,  $\Delta$  CRE5  $23.7 \pm 13.2\%$ ,  $\Delta$  CRE6  $26.8 \pm 14.2\%$  and  $\Delta$  FOXA1  $96.2 \pm 1.4\%$  reduction (Student's t-test,  $p < 0.05$ , Figure 3.5a, Appendix II Supplemental Figure

3.8a-f). Next for combinatorial deletions, we prioritized the CREs that harbor more than 1 SNV (i.e CRE1, CRE2, CRE4), and transiently transfected RNP complexes that target both CREs in various combinations (i.e CRE1 + CRE2, CRE1 + CRE4, CRE2 + CRE4), and assessed FOXA1 mRNA expression. Compared to negative control regions, the combinatorial deletion of  $\Delta$  CRE1 +  $\Delta$  CRE2,  $\Delta$  CRE1 +  $\Delta$  CRE4, and  $\Delta$  CRE2 +  $\Delta$  CRE4 resulted in a significant  $48.5 \pm 4.5\%$ ,  $50.4 \pm 2.9\%$  and  $45.2 \pm 5.5\%$  reduction in FOXA1 mRNA expression, respectively (Student's t-test,  $p < 0.05$ , Figure 3.5b, Appendix II Supplemental Figure 3.9a-f) a fold reduction greater than single CRE deletions (Student's t-test, Appendix II Supplemental Figure 10,  $p < 0.05$ ). These results together demonstrate that these CREs collaboratively contribute to the establishment and regulation of FOXA1 expression in prostate cancer.

### 1.3.6 Disruption of *FOXA1* CREs reduces prostate cancer cell growth

As FOXA1 is essential for prostate cancer growth (Figure 3.1b-e), we next sought to assess the importance of the six FOXA1 plexus CREs towards prostate cancer cell growth. We adapted a lentiviral-based approach that expressed both the Cas9 protein and two gRNA that target each CRE for deletion (See Methods). Upon lentiviral transduction with subsequent selection, we separated LNCaP prostate cancer cells for RNA, DNA and for cell proliferation. We first tested the system by measuring FOXA1 mRNA expression, and independently observed significant reductions of FOXA1 mRNA expression ( $\Delta$  CRE1 18%,  $\Delta$  CRE2 30%,  $\Delta$  CRE3 15%,  $\Delta$  CRE4 12%,  $\Delta$  CRE5 35%,  $\Delta$  CRE6 46% and  $\Delta$  FOXA1 (exon 1 and intron 1) 48% reduction (Student's t-test,  $p < 0.05$ , Figure 3.5c, Appendix II Supplemental Figure 11a-f). We then seeded these cells at equal density. Six days post-seeding, we harvested the cells and observed a significant reduction in cell growth upon deleting any of the six FOXA1 plexus CREs ( $\Delta$  CRE1 42%,  $\Delta$  CRE2 28%,  $\Delta$  CRE3 33%,  $\Delta$  CRE4 27%,  $\Delta$  CRE5 42%,  $\Delta$  CRE6 44% and  $\Delta$  FOXA1 (exon 1 and intron 1) 50% reduction (Student's t-test,  $p < 0.05$ , Fig 5d). These results suggest that the six FOXA1 plexus contribute to prostate cancer etiology, in agreement with their ability to regulate FOXA1 expression and the essentiality of this gene in prostate cancer cell growth.

### 1.3.7 SNVs mapping to *FOXA1* CREs can alter their activity

SNVs can alter the transactivation potential of CREs [32, 43–51]. In total, we found 10 SNVs called from 9 out of the 200 tumours that map to the six FOXA1 plexus CREs (Figure 3.6a). To assess the impact of these noncoding SNVs, we conducted luciferase assays comparing differential

reporter activity between the variant and the wild-type allele of each CRE (Figure 3.6b-k). We found that the variant alleles of 6 of the 10 SNVs displayed significantly greater luciferase reporter activity when compared to the wild-type alleles (Mann-Whitney U test,  $p < 0.05$ ). Specifically, we observed the following fold-changes: chr14:37,887,005 A > G (1.65-fold), chr14:37,904,343 A > T (1.35-fold), chr14:37,905,854 A > G (1.28-fold), chr14:37,906,009 T > C (1.71-fold), chr14:38,036,543 A > G (1.44-fold), chr14:38,055,269 C > G (1.39-fold) (Figure 3.6b, d-h). These results indicate that these SNVs can alter the transactivation potential of FOXA1 plexus CREs in prostate cancer cells.

### 1.3.8 SNVs mapping to *FOXA1* CREs can modulate the binding of TFs

We next assessed if the changes in transactivation potential induced by noncoding SNVs related to changes in TF binding to CREs by allele-specific ChIP-qPCR [32, 44, 51] in LNCaP prostate cancer cells. We observed differential binding of FOXA1, AR, HOXB13, GATA2 and FOXP1 for the chr14:37887005 (A > G) SNV found in CRE1; the chr14:37904343 (A > T), chr14:37905854 (A > G) and chr14:37906009 (T > C) SNVs found in CRE2; and the chr14:38055269 (C > G) SNV found in CRE4 (Student's t-test,  $p < 0.05$ , Figure 3.6l-p). In contrast, SOX9 and NKX3.1 binding was unaffected by these SNVs (Figure 3.6l-q). Compared to the wild-type sequence, chr14:37,887,005 A > G significantly increased AR binding (1.31-fold increase), GATA2 binding (1.25-fold increase) and FOXP1 binding (1.23-fold increase); chr14:37,904,343 A > T significant increased AR binding (1.30-fold increase), GATA2 (1.25-fold increase) and FOXP1 (1.33-fold increase); chr14:37,905,854 A > G significantly increased FOXA1 binding (1.41-fold increase) and AR binding (1.33-fold increase); chr14:37,906,009 T > C significantly increased the binding of FOXA1 (1.29-fold increase), AR (1.31-fold increase), HOXB13 (1.13-fold increase) and FOXP1 (1.25-fold increase); and chr14:38,055,269 C > G significantly increased FOXA1 binding (1.20-fold increase). Notably all six SNVs increased the binding of the TFs known to bind at these CREs. In contrast, none of the SNVs significantly decreased the binding of these TFs. Our observations suggest that gain-of-function SNVs populate the FOXA1 plexus CREs.

## 1.4 Discussion

Modern technologies and understanding of the epigenome allow the possibility of probing CRE(s) involved in regulating genes implicated in disease. Despite FOXA1 being recurrently mutated [5–8, 11] and playing potent oncogenic roles in prostate cancer etiology [9, 10, 13], the CREs involved in its transcriptional regulation are poorly understood. Understanding how FOXA1 is expressed can

provide a complementary strategy to antagonize FOXA1 in prostate cancer.

We used the DHS profiled in prostate cancer cells to identify putative FOXA1 CREs through annotating these regions with five different histone modifications, TF binding sites and noncoding SNVs profiled in prostate cancer cells and primary prostate tumours. Our efforts identified and validated a set of six active CREs involved in FOXA1 regulation, agreeing with a recent report where a subset of our CREs map to loci suggested to be in contact with the FOXA1 promoter [52]. The disruption of these six distal CREs each significantly reduced FOXA1 mRNA levels, similar to what has been demonstrated for ESR1 in luminal breast cancer [32], MLH1 in Lynch syndrome [53], MYC in lung adenocarcinoma and endometrial cancer [54], and AR in mCRPC [55, 56]. Through combinatorial deletion of two CREs, FOXA1 mRNA levels were further reduced in comparison with single CRE deletions, raising the possibility of CRE additivity [57]. The deletion of the FOXA1 plexus CREs also significantly reduced prostate cancer cell proliferation at levels comparable to what has been reported upon deletion of the amplified CRE upstream of the AR gene in mCRPC [55], suggestive of onco-CREs as reported in lung [54] and prostate [55] cancer.

More than 90% of SNVs found in cancer map to the noncoding genome [58, 59] with a portion of these SNVs mapping to CREs altering their transactivation potential [32, 44–46] and/or downstream target gene expression [48, 58, 60]. We extended this concept with SNVs identified from primary prostate tumours mapping to FOXA1 plexus CREs. We observed that a subset of these SNVs can alter transactivation potential by modulating the binding of specific TFs whose cistromes are preferentially burdened by SNVs in primary prostate cancer [59]. Our findings complement recent reports of SNVs found in the noncoding space of FOXA1 that could affect its expression [14, 61]. The FOXA1 plexus CREs we identified here are also reported to be target of structural variants in both the primary and metastatic settings [9, 62], including tandem duplication in 14% (14/101) mCRPC tumours over CRE2 [62], amplification, duplication and translocation over CRE3, 4, 5 [9]. Notably, the translocation and duplication defining the FOXMIND enhancer driving FOXA1 expression reported in primary and metastatic settings harbors the CRE3 element we characterized [9]. Collectively, these studies combined with our discoveries reveal the fundamental contribution of the FOXA1 plexus in prostate cancer etiology. As a whole, our findings in conjunction with recent reports suggest that CREs involved in the transcriptional regulation of FOXA1 may be hijacked in prostate tumours through various types of genetic alterations.

Despite initial treatment responses from treating aggressive primary and metastatic prostate cancer through castration to suppress AR signalling [4], resistance ensues as 80% of mCRPC tumours harbor either AR gene amplification, amplification of a CRE upstream of AR, or activating AR



coding mutations [11, 55, 62]. Given the AR-dependent [15, 18] and AR-independent [25] oncogenic activity of FOXA1 in prostate cancer, its inhibition is an appealing alternative therapeutic strategy. Our dissection of the FOXA1 cis-regulatory landscape complement recent findings through revealing loci that are important for the regulation of FOXA1. Theoretically, direct targeting of the CREs regulating FOXA1 would down-regulate FOXA1 levels and could therefore serve as a valid alternative to antagonize its function.

Taken together, we identified FOXA1 CREs targeted by SNVs that are capable of altering transactivation potential through the modulation of key prostate cancer TFs. The study supports the importance of considering CREs not only as lone occurrences but as a team that works together to regulate their target genes, particularly when considering the impact of genetic alterations. As such, our work builds a bridge between the understanding of FOXA1 transcriptional regulation and new routes to FOXA1 inhibition. Aligning with recent reports [9, 10, 13], our findings support the oncogenic nature of FOXA1 in prostate cancer. Gaining insight on the cis-regulatory plexuses of important genes such as FOXA1 in prostate cancer may provide new avenues to inhibit other drivers across various cancer types to halt disease progression.

## 1.5 Methods

### 1.5.1 Cell Culture

LNCaP and 22Rv1 cells were cultured in RPMI medium, and VCaP cells were cultured in DMEM medium, both supplemented with 10% FBS, and 1% penicillin-streptomycin at 37 °C in a humidified incubator with 5% CO<sub>2</sub>. These prostate cancer cells originated from ATCC. 293FT cells were purchased from ThermoFisherScientific (Cat No. R70007) maintained in complete DMEM medium (DMEM with 10% FBS (080150, Wisent), L-glutamine (25030-081, ThermoFisher) and non-essential amino acids (11140-050, ThermoFisher) supplemented with 50mg/mL Geneticin (4727894001, Sigma-Aldrich). The cells are regularly tested for Mycoplasma contamination. The authenticity of these cells was confirmed through Short Tandem Repeat profiling.

### 1.5.2 Prostate tumours and cancer cell lines expression

Cancer cell line mRNA abundance data were collected from the Cancer Dependency Map Project (DEPMAP; <https://depmap.org/portal/>; RNA-seq TPM values from 2018q4 version with all 5 non-cancer cell lines were removed) 325 projects. Prostate tumour mRNA abundance data was collected

from The Cancer Genome Atlas (TCGA) prostate cancer (TCGA-PRAD) project via the Xena Browser (<https://xenabrowser.net/>; dataset description: TCGA prostate adenocarcinoma (PRAD) gene expression by RNA-seq (polyA+ Illumina HiSeq; RSEM)).

### 1.5.3 Prostate cancer cell line gene essentiality

Essentiality scores were collected from the Cancer Dependency Map Project. To compare gene essentiality between prostate cancer cell lines and others, essentiality scores for FOXA1 were collected from all available cell lines ( $n = 707$ ). To perform a permutation test, the median of 8 randomly selected cell lines was calculated one million times to generate a background distribution of essentiality scores across all cell types available. The median essentiality score from the 8 prostate cancer cell lines was calculated and its percentile within the background distribution is reported.

### 1.5.4 siRNA knockdown and cell proliferation assay

300,000 LNCaP cells (Day 0) were reverse transfected with siRNA (siFOXA1 using Lipofectamine®RNAiMax reagent (ThermoFisher Scientific, Cat No. 13778150)). Cells were counted using Countess™ automated cell counter (Invitrogen). Whole cell lysates LNCaP cells after siRNA-mediated FOXA1 knockdown was collected at 96-hours post-transfection in RIPA buffer. Protein concentrations were determined through the bicinchoninic acid method (ThermoFisher Scientific, Cat No. 23225). Then 25  $\mu$ g of lysate was subjected to SDS-PAGE. Upon completion of SDS-PAGE, protein was transferred onto PVDF membrane (Bio-Rad, Cat No. 1704156). The membrane was blocked with 5% non-fat milk for one hour at room temperature with shaking. After blocking, anti-FOXA1 (Abcam Cat No. 23737) in 2.5% non-fat milk was added, and was incubated at 4 °C overnight. Next day, the blot was washed and incubated with IRDye®800CW Goat Anti-Rabbit IgG secondary antibody (LI-COR, Cat No. 925-32211) at room temperature for 1 hour. The blot was then washed and assessed with the Odyssey®CLX imaging system (LI-COR).

### 1.5.5 Identifying putative FOXA1 CREs

Putative FOXA1 CREs were identified through the use of Cross Cell-Type Correlation based on DNase I Hypersensitivity (C3D) (<https://github.com/tahmidmehdi/C3D>) 311. Predicted interacting DNase I Hypersensitivity Sites (DHS) with a Pearson's correlation above 0.7 133 were kept for downstream analysis.

### 1.5.6 Hi-C and TADs in LNCaP cells

Hi-C and TADs conducted and called, respectively, in LNCaP cells are publicly available off ENCODE portal (ENCSR346DCU). Visualization of the Hi-C dataset is available on the Hi-C Browser (<http://promoter.bx.psu.edu/hi-c/>) 326.

### 1.5.7 Clonal wild-type Cas9 and dCas9-KRAB mediated validation

Lentiviral particles were generated in 293FT cells (ThermoFisher) using the pMDG.2 and psPAX2 packaging plasmids (Addgene; #12259 and #12260, a gift from Didier Trono) alongside the Lenti-Cas9-2A-Blast plasmid (Addgene #73310, a gift from Jason Moffat) and collected 72 hrs post transfection. LNCaP and 22Rv1 cells were then transduced for 24-48 hours with equal amounts of virus followed by selection with media containing blasticidin (7.5  $\mu\text{g}/\text{mL}$  for LNCaP cells, 6  $\mu\text{g}/\text{mL}$  for 22Rv1 cells). Upon selection, clones were derived by serial dilution with subsequent single cell seeding into 96-well plates containing selection media. Cas9 protein expression for each clone was then assessed through Western blotting (1 $^{\circ}$  Ms-Cas9 (Cell Signalling Technology, Cat No. #14697) 1:1000, Ms-GAPDH 1:5000 (Santa Cruz Biotechnology, Cat No. #sc47724) in 5% non-fat milk; 2 $^{\circ}$  HRP-linked Anti-Mouse IgG (Cell Signalling Technology, Cat No. #7076S) 1:10 000 in 2.5% non-fat milk. The full unprocessed blot is in the Source Data File.

Lentiviral particles were generated in 293FT cells (ThermoFisher) using the pMDG.2 and psPAX2 packaging plasmids (Addgene; #12259 and #12260, a gift from Didier Trono) alongside the Lenti-dCas9-KRAB-blast plasmid (Addgene #89567, a gift from Gary Hon) and collected 72 hrs post transfection. LNCaP and 22Rv1 cells were then transduced for 24-48 hours with equal amounts of virus followed by selection with media containing blasticidin (7.5  $\mu\text{g}/\text{mL}$  for LNCaP cells, 6  $\mu\text{g}/\text{mL}$  for 22Rv1 cells). Upon selection, clones were derived by serial dilution with subsequent single cell seeding into 96-well plates containing selection media. dCas9-KRAB protein expression for each clone was then assessed through Western blotting (1 $^{\circ}$  Ms-Cas9 (Cell Signalling Technology, Cat No. #14697) 1:1000, Ms-GAPDH 1:5000 (Santa Cruz Biotechnology, Cat No. #sc47724) in 5% non-fat milk; 2 $^{\circ}$  HRP-linked Anti-Mouse IgG (Cell Signalling Technology, Cat No. #7076S) 1:10 000 in 2.5% non-fat milk. The full unprocessed blot is in the Source Data File.

For gRNA design, five to six unique crRNA molecules (Integrated DNA Technologies) were designed to tile across the region of interest using the CRISPR (<http://crispor.tefor.net/>) 327 and the Zhang lab CRISPR Design tools (<http://crispr.mit.edu/>) 328. See published manuscript for gRNA. Each CRISPR RNA (crRNA) and tracrRNA (Integrated DNA Technologies) were duplexed

according to company supplier protocol to a concentration of 50  $\mu$ M. Upon generation of the clones, six guides (crRNA-tracrRNA duplexes) for each region of interest were pooled into a single tube (1  $\mu$ L each guide, 6  $\mu$ L per reaction) (Integrated DNA Technologies). Lastly, 1  $\mu$ L (100  $\mu$ M) of electroporation enhancer (Integrated DNA Technologies) was added to the mix (7  $\mu$ L total) prior to transfection. The entire transfection reaction was transfected into 350 000 cells through Nucleofection (SF Solution EN120 - 4D Nucleofector, Lonza). Cells were then harvested 24 hours post-transfection for RNA and DNA for RT-PCR and confirmation of deletion, respectively.

### 1.5.8 Transient Cas9-mediated disruption of CREs

Deletion of elements through this method were achieved through the transfection of Cas9 nuclease protein complexed with the crRNA (Integrated DNA Technologies). Briefly, five to six unique crRNA molecules (Integrated DNA Technologies) were designed to tile across the region of interest using the CRISPR (<http://crispor.tefor.net/>) 327 and the Zhang lab CRISPR Design tools (<http://crispr.mit.edu/>) 328. Each crRNA and tracrRNA (Integrated DNA Technologies) were duplexed according to company supplier protocol to a concentration of 50  $\mu$ M. The six crRNA-tracrRNA duplexes were pooled into a single tube (6  $\mu$ L per reaction), prior to adding 1  $\mu$ L (5  $\mu$ g) of Alt-R  $\text{\textcircled{R}}$ S.p HiFi Cas9 Nuclease 3NLS (Integrated DNA Technologies). The reaction was incubated at room temperature for 10 minutes for ribonucleoprotein (RNP) complex formation. Lastly, 1  $\mu$ L (100  $\mu$ M) of electroporation enhancer (Integrated DNA Technologies) was added to the mix prior to transfection. The entire transfection reaction was transfected into 350 000 cells through Nucleofection (SF Solution EN120 - 4D Nucleofector, Lonza). Cells were then harvested 24 hours post-transfection for RNA and DNA for RT-PCR and confirmation of deletion, respectively. For double deletions, two sets of guide RNA-RNP complex (10  $\mu$ g of Alt-R  $\text{\textcircled{R}}$ S.p HiFi Cas9 Nuclease 3NLS) were transfected and harvested 24 hours post-transfection for RNA and DNA for RT-PCR and confirmation of deletion, respectively. To control for double deletions, two negative control regions within the TAD were also compounded. Due to size, see published manuscript for primers.

### 1.5.9 RT-PCR assessment of gene expression upon deletion of CREs

DNA and RNA were harvested with Qiagen AllPrep RNA/DNA Kit (Qiagen, Cat No. 80204). Next, cDNA was synthesized from 300 ng of RNA using SensiFast cDNA Synthesis kit (Bioline, Cat No. BIO-65054), and mRNA expression levels for various genes of interest were assessed. Due to size, see published manuscript for the primer sequences used for expression evaluation. Differential gene

expression was calculated upon normalization with TBP (housekeeping gene). Statistical significance was calculated using Student's t-test in R.

#### 1.5.10 Confirmation of Cas9-mediated deletion of CREs

Deletion of CREs were confirmed through PCR amplification of the intended region for deletion, followed by the T7 Endonuclease Assay (Integrated DNA Technology). Due to size, see published manuscript for primer sequences used for PCR amplification. PCR products were then loaded onto a 1% agarose gel for visualization. The agarose gel to assess the on-target genome editing efficiency was done through densitometry using ImageJ. The correlation between on-target genome editing efficiency and FOXA1 mRNA expression reduction was drawn through Pearson's correlation in R.

#### 1.5.11 Cell proliferation upon deletion of FOXA1 CREs

Pairs of gRNAs flanking the CREs of interest, FOXA1 promoter and control regions were designed using CRISPOR (<http://crispor.tefor.net/>) and Zhang lab CRISPR Design tool (<http://crispr.mit.edu/>) (due to size, see published manuscript). Each pair of gRNAs were cloned into the lentiCRISPRv2 (Addgene; a gift from Feng Zhang #52961) and the lentiCRISPRv2-Blast (Addgene; a gift from Feng Zhang #83480) plasmid as previously described 329. Lentiviral particles were generated in 293FT cells (ThermoFisher) using the pMDG.2 and psPAX2 packaging plasmids (Addgene; #12259 and #12260, a gift from Didier Trono), and collected 72 hrs post transfection. LNCaP cells were transduced for 24-48hrs with equal amounts of virus, followed by selection with media containing puromycin (3.5  $\mu\text{g}/\text{mL}$ , ThermoFisher) and blasticidin (7  $\mu\text{g}/\text{mL}$ , Wisent). Cells were harvested upon selection for RNA and DNA for RT-PCR and confirmation of DNA cleavage, respectively. For cell proliferation, cells were seeded at equal density per well (on a 96-well plate; Day 1) upon puromycin and blasticidin selection. Growth of the cells were monitored through cell counting using Countess <sup>TM</sup> automated cell counter (Invitrogen). Cell numbers were calculated as a percentage compared to negative control. Statistical significance was calculated using Student's t-test.

#### 1.5.12 Luciferase reporter assays

Each region of interest was ordered as gBlocks from Integrated DNA Technologies. The regions were cloned into the BamHI restriction enzyme digest site of the pGL3 promoter plasmid (Promega). On Day 0, 90 000 LNCaP cells were seeded in 24-well plates. Next day (Day 1), pGL3 plasmids harboring the wild-type and variant sequences were co-transfected with the pRL Renilla plasmid

(Promega) using Lipofectamine 2000. 48-hours later, the cells were harvested, and dual luciferase reporter assays were conducted (Promega). Notably, inserts of both forward and reverse directions were tested using this assay as enhancer elements are known to be direction-independent. Final luminescence readings are reported as firefly luciferase normalized to renilla luciferase activity. The assessment of each mutation was conducted in five biological replicates. Statistical significance was assessed by Mann-Whitney U test in R. See published manuscript for gBlock sequences.

### 1.5.13 Allele-specific ChIP-qPCR

Briefly, pGL3 plasmids containing the wild-type sequence and the mutant sequence used in the luciferase reporter assay were transfected into 7 million cells (2  $\mu$ g per allele, per 1 million cells) using Lipofectamine 2000 (ThermoFisher Scientific), per manufacturer's instructions. Next day, each antibody (FOXA1 5  $\mu$ g, Abcam, ab23738; AR 5  $\mu$ g, Abcam, ab1083241; HOXB13 5  $\mu$ g, Abcam, ab201682; SOX9 5  $\mu$ g, Abcam, ab3697; GATA2 5  $\mu$ g, Abcam, ab22849; FOXP1 5  $\mu$ g, Abcam, ab16645; NKX3.1 10  $\mu$ L, Cell Signalling Technology, #83700) was conjugated with 10 uL of each Dynabeads A and G (Thermo Fisher Scientific) for each ChIP for 6 hours with rotation at 4 °C. When antibody-beads conjugates were ready for use, cells were lifted using trypsin and fixed by re-suspending with 300 uL of 1% formaldehyde in PBS for 10 minutes at room temperature. 2.5M Glycine was added to quench excess formaldehyde (final concentration 0.125 M). Cells were then washed with cold PBS and lysed using 300 uL of Modified RIPA buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA; 140 mM NaCl; 1% Triton X-100; 0.1% SDS; 0.1% sodium deoxycholate) supplemented with protease inhibitor. The lysate was subject to 25 cycles of sonication (30s ON 30s OFF) using Diagenode Bioruptor Pico (Diagenode). 15 uL of sonicated lysate was set aside as input with the rest used for chromatin pulldown through addition of antibody-beads conjugates and overnight incubation at 4 °C with rotation. Next day, the beads were washed once with Modified RIPA buffer, washed once with Modified RIPA buffer + 500 mM NaCl, once with LiCl buffer (10 mM TrisHCl, pH 8.0; 1 mM EDTA; 250 mM LiCl; 0.5% NP-40; 0.5% sodium deoxycholate) and twice with Tris-ETDA buffer (pH 8). After washes, beads and input were de-crosslinked by addition of 100  $\mu$ L De-crosslinking buffer and incubation at 65 °C for 6 hours. Samples were then purified and eluted. ChIP and input DNA were then used for allele-specific ChIP-qPCR using MAMA primers as described previously. Fold-change significance was calculated using Student's t-test in R.

All analyses were done using hg19 reference genome coordinates.

# References

1. Bray, F. *et al.* Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. en. *CA: A Cancer Journal for Clinicians* **68**, 394–424. ISSN: 00079235 (Nov. 2018).
2. Boorjian, S. A. *et al.* Long-Term Outcome After Radical Prostatectomy for Patients With Lymph Node Positive Prostate Cancer in the Prostate Specific Antigen Era. en. *Journal of Urology* **178**, 864–871. ISSN: 0022-5347, 1527-3792 (Sept. 2007).
3. Litwin, M. S. & Tan, H.-J. The Diagnosis and Treatment of Prostate Cancer: A Review. en. *JAMA* **317**, 2532. ISSN: 0098-7484 (June 2017).
4. Attard, G. *et al.* Prostate Cancer. en. *The Lancet* **387**, 70–82. ISSN: 01406736 (Jan. 2016).
5. Abeshouse, A. *et al.* The Molecular Taxonomy of Primary Prostate Cancer. en. *Cell* **163**, 1011–1025. ISSN: 00928674 (Nov. 2015).
6. Fraser, M. *et al.* Genomic Hallmarks of Localized, Non-Indolent Prostate Cancer. en. *Nature* **541**, 359–364. ISSN: 1476-4687 (Jan. 2017).
7. Barbieri, C. E. *et al.* Exome Sequencing Identifies Recurrent SPOP , FOXA1 and MED12 Mutations in Prostate Cancer. en. *Nature Genetics* **44**, 685–689. ISSN: 1546-1718 (June 2012).
8. Grasso, C. S. *et al.* The Mutational Landscape of Lethal Castration-Resistant Prostate Cancer. en. *Nature* **487**, 239–243. ISSN: 0028-0836, 1476-4687 (July 2012).
9. Parolia, A. *et al.* Distinct Structural Classes of Activating FOXA1 Alterations in Advanced Prostate Cancer. en. *Nature* **571**, 413–418. ISSN: 1476-4687 (July 2019).
10. Adams, E. J. *et al.* FOXA1 Mutations Alter Pioneering Activity, Differentiation and Prostate Cancer Phenotypes. en. *Nature* **571**, 408–412. ISSN: 0028-0836, 1476-4687 (July 2019).
11. Robinson, D. *et al.* Integrative Clinical Genomics of Advanced Prostate Cancer. en. *Cell* **161**, 1215–1228. ISSN: 00928674 (May 2015).

12. Robinson, J. L. L., Holmes, K. A. & Carroll, J. S. FOXA1 Mutations in Hormone-Dependent Cancers. *Frontiers in Oncology* **3**. ISSN: 2234-943X (2013).
13. Gao, S. *et al.* Forkhead Domain Mutations in FOXA1 Drive Prostate Cancer Progression. en. *Cell Research* **29**, 770–772. ISSN: 1001-0602, 1748-7838 (Sept. 2019).
14. Annala, M. *et al.* Frequent Mutation of the FOXA1 Untranslated Region in Prostate Cancer. en. *Communications Biology* **1**, 122. ISSN: 2399-3642 (Aug. 2018).
15. Yang, Y. A. & Yu, J. Current Perspectives on FOXA1 Regulation of Androgen Receptor Signaling and Prostate Cancer. en. *Genes & Diseases* **2**, 144–151. ISSN: 23523042 (June 2015).
16. Lupien, M. *et al.* FoxA1 Translates Epigenetic Signatures into Enhancer-Driven Lineage-Specific Transcription. *Cell* **132**, 958–970. ISSN: 0092-8674 (Mar. 2008).
17. Eeckhoutte, J. *et al.* Cell-Type Selective Chromatin Remodeling Defines the Active Subset of FOXA1-Bound Enhancers. en. *Genome Research* **19**, 372–380. ISSN: 1088-9051 (Dec. 2008).
18. Pomerantz, M. M. *et al.* The Androgen Receptor Cistrome Is Extensively Reprogrammed in Human Prostate Tumorigenesis. en. *Nature Genetics* **47**, 1346–1351. ISSN: 1061-4036, 1546-1718 (Nov. 2015).
19. Imamura, Y. *et al.* FOXA1 Promotes Tumor Progression in Prostate Cancer via the Insulin-Like Growth Factor Binding Protein 3 Pathway. en. *PLoS ONE* **7** (ed AgoulNIK, I.) e42456. ISSN: 1932-6203 (Aug. 2012).
20. Xu, Y., Chen, S.-Y., Ross, K. N. & Balk, S. P. Androgens Induce Prostate Cancer Cell Proliferation through Mammalian Target of Rapamycin Activation and Post-Transcriptional Increases in Cyclin D Proteins. en. *Cancer Research* **66**, 7783–7792. ISSN: 0008-5472, 1538-7445 (Aug. 2006).
21. Jin, H.-J., Zhao, J. C., Ogden, I., Bergan, R. C. & Yu, J. Androgen Receptor-Independent Function of FoxA1 in Prostate Cancer Metastasis. en. *Cancer Research* **73**, 3725–3736. ISSN: 0008-5472, 1538-7445 (June 2013).
22. Yang, Y. A. *et al.* FOXA1 Potentiates Lineage-Specific Enhancer Activation through Modulating TET1 Expression and Function. en. *Nucleic Acids Research* **44**, 8153–8164. ISSN: 0305-1048, 1362-4962 (Sept. 2016).
23. Zhang, G. *et al.* FOXA1 Defines Cancer Cell Specificity. en. *Science Advances* **2**, e1501473. ISSN: 2375-2548 (Mar. 2016).



24. Augello, M. A., Hickey, T. E. & Knudsen, K. E. FOXA1: Master of Steroid Receptor Function in Cancer: FOXA1: Master of Steroid Receptor Function in Cancer. en. *The EMBO Journal* **30**, 3885–3894. ISSN: 02614189 (Oct. 2011).
25. Sunkel, B. *et al.* Integrative Analysis Identifies Targetable CREB1/FoxA1 Transcriptional Co-Regulation as a Predictor of Prostate Cancer Recurrence. en. *Nucleic Acids Research* **45**, 6993–6993. ISSN: 0305-1048, 1362-4962 (June 2017).
26. Ni, M. *et al.* Amplitude Modulation of Androgen Signaling by C-MYC. en. *Genes & Development* **27**, 734–748. ISSN: 0890-9369 (Apr. 2013).
27. Sasse, S. K. & Gerber, A. N. Feed-Forward Transcriptional Programming by Nuclear Receptors: Regulatory Principles and Therapeutic Implications. en. *Pharmacology & Therapeutics* **145**, 85–91. ISSN: 01637258 (Jan. 2015).
28. Wang, S., Singh, S., Katika, M., Lopez-Aviles, S. & Hurtado, A. High Throughput Chemical Screening Reveals Multiple Regulatory Proteins on FOXA1 in Breast Cancer Cell Lines. en. *International Journal of Molecular Sciences* **19**, 4123. ISSN: 1422-0067 (Dec. 2018).
29. Rowley, M. J. & Corces, V. G. Organizational Principles of 3D Genome Architecture. en. *Nature Reviews Genetics* **19**, 789–800. ISSN: 1471-0056, 1471-0064 (Dec. 2018).
30. Vernimmen, D. & Bickmore, W. A. The Hierarchy of Transcriptional Activation: From Enhancer to Promoter. en. *Trends in Genetics* **31**, 696–708. ISSN: 01689525 (Dec. 2015).
31. Sallari, R. C. *et al.* Convergence of Dispersed Regulatory Mutations Predicts Driver Genes in Prostate Cancer. *bioRxiv*, 38–38 (2016).
32. Bailey, S. D. *et al.* Noncoding Somatic and Inherited Single-Nucleotide Variants Converge to Promote ESR1 Expression in Breast Cancer. *Nature Genetics* **48**, 1260–1269 (2016).
33. Tsourlakis, M. C. *et al.* FOXA1 Expression Is a Strong Independent Predictor of Early PSA Recurrence in ERG Negative Prostate Cancers Treated by Radical Prostatectomy. en. *Carcinogenesis* **38**, 1180–1187. ISSN: 0143-3334, 1460-2180 (Dec. 2017).
34. Phillips, J. E. & Corces, V. G. CTCF: Master Weaver of the Genome. en. *Cell* **137**, 1194–1211. ISSN: 00928674 (June 2009).
35. Weintraub, A. S. *et al.* YY1 Is a Structural Regulator of Enhancer-Promoter Loops. en. *Cell* **171**, 1573–1588.e28. ISSN: 00928674 (Dec. 2017).
36. Bailey, S. D. *et al.* ZNF143 Provides Sequence Specificity to Secure Chromatin Interactions at Gene Promoters. en. *Nature Communications* **6**, 6186. ISSN: 2041-1723 (May 2015).

37. Mehdi, T., Bailey, S. D., Guilhamon, P. & Lupien, M. C3D: A Tool to Predict 3D Genomic Interactions between Cis-Regulatory Elements. en. *Bioinformatics* **35** (ed Kelso, J.) 877–879. ISSN: 1367-4803, 1460-2059 (Mar. 2019).
38. Kron, K. J. *et al.* TMPRSS2–ERG Fusion Co-opts Master Transcription Factors and Activates NOTCH Signaling in Primary Prostate Cancer. en. *Nature Genetics* **49**, 1336–1345. ISSN: 1546-1718 (Sept. 2017).
39. Creighton, M. P. *et al.* Histone H3K27ac Separates Active from Poised Enhancers and Predicts Developmental State. en. *Proceedings of the National Academy of Sciences* **107**, 21931–21936. ISSN: 0027-8424, 1091-6490 (Dec. 2010).
40. Espiritu, S. M. G. *et al.* The Evolutionary Landscape of Localized Prostate Cancers Drives Clinical Aggression. English. *Cell* **173**, 1003–1013.e15. ISSN: 0092-8674, 1097-4172 (May 2018).
41. DeKever, R. C. *et al.* Functional Genomics, Proteomics, and Regulatory DNA Analysis in Isogenic Settings Using Zinc Finger Nuclease-Driven Transgenesis into a Safe Harbor Locus in the Human Genome. en. *Genome Research* **20**, 1133–1142. ISSN: 1088-9051 (Aug. 2010).
42. Pennacchio, L. A., Bickmore, W., Dean, A., Nobrega, M. A. & Bejerano, G. Enhancers: Five Essential Questions. en. *Nature Reviews Genetics* **14**, 288–295. ISSN: 1471-0056, 1471-0064 (Apr. 2013).
43. Rheinbay, E. *et al.* Recurrent and Functional Regulatory Mutations in Breast Cancer. *Nature* **547**, 55–60. ISSN: 1476-4687 (Electronic) 0028-0836 (Linking) (June 2017).
44. Zhang, X., Cowper-Sal{middle dot}lari, R., Bailey, S. D., Moore, J. H. & Lupien, M. Integrative Functional Genomics Identifies an Enhancer Looping to the SOX9 Gene Disrupted by the 17q24.3 Prostate Cancer Risk Locus. en. *Genome Research* **22**, 1437–1446. ISSN: 1088-9051 (Aug. 2012).
45. Huang, F. W. *et al.* Highly Recurrent TERT Promoter Mutations in Human Melanoma. en. *Science* **339**, 957–959. ISSN: 0036-8075, 1095-9203 (Feb. 2013).
46. Horn, S. *et al.* TERT Promoter Mutations in Familial and Sporadic Melanoma. en. *Science* **339**, 959–961. ISSN: 0036-8075, 1095-9203 (Feb. 2013).
47. Fuxman Bass, J. I. *et al.* Human Gene-Centered Transcription Factor Networks for Enhancers and Disease Variants. en. *Cell* **161**, 661–673. ISSN: 00928674 (Apr. 2015).
48. Zhou, S., Treloar, A. E. & Lupien, M. Emergence of the Noncoding Cancer Genome: A Target of Genetic and Epigenetic Alterations. *Cancer Discovery* **6**, 1215–1229 (Nov. 2016).

49. Feigin, M. E. *et al.* Recurrent Noncoding Regulatory Mutations in Pancreatic Ductal Adenocarcinoma. en. *Nature Genetics* **49**, 825–833. ISSN: 1061-4036, 1546-1718 (June 2017).
50. Khurana, E. *et al.* Role of Non-Coding Sequence Variants in Cancer. en. *Nature Reviews Genetics* **17**, 93–108. ISSN: 1471-0056, 1471-0064 (Feb. 2016).
51. Cowper-Sal-lari, R. *et al.* Breast Cancer Risk-Associated SNPs Modulate the Affinity of Chromatin for FOXA1 and Alter Gene Expression. en. *Nature Genetics* **44**, 1191–1198. ISSN: 1061-4036, 1546-1718 (Nov. 2012).
52. Rhie, S. K. *et al.* A High-Resolution 3D Epigenomic Map Reveals Insights into the Creation of the Prostate Cancer Transcriptome. en. *Nature Communications* **10**, 1–12. ISSN: 2041-1723 (Sept. 2019).
53. Liu, Q. *et al.* Disruption of a -35 Kb Enhancer Impairs CTCF Binding and *MLH1* Expression in Colorectal Cells. en. *Clinical Cancer Research* **24**, 4602–4611. ISSN: 1078-0432, 1557-3265 (Sept. 2018).
54. Zhang, X. *et al.* Identification of Focally Amplified Lineage-Specific Super-Enhancers in Human Epithelial Cancers. en. *Nature Genetics* **48**, 176–182. ISSN: 1546-1718 (Feb. 2016).
55. Takeda, D. Y. *et al.* A Somatic Acquired Enhancer of the Androgen Receptor Is a Noncoding Driver in Advanced Prostate Cancer. English. *Cell* **174**, 422–432.e13. ISSN: 0092-8674, 1097-4172 (July 2018).
56. Viswanathan, S. R. *et al.* Structural Alterations Driving Castration-Resistant Prostate Cancer Revealed by Linked-Read Genome Sequencing. en. *Cell* **174**, 433–447.e19. ISSN: 00928674 (July 2018).
57. Osterwalder, M. *et al.* Enhancer Redundancy Provides Phenotypic Robustness in Mammalian Development. en. *Nature* **554**, 239–243. ISSN: 0028-0836, 1476-4687 (Feb. 2018).
58. Melton, C., Reuter, J. A., Spacek, D. V. & Snyder, M. Recurrent Somatic Mutations in Regulatory Regions of Human Cancer Genomes. en. *Nature Genetics* **47**, 710–716. ISSN: 1061-4036, 1546-1718 (July 2015).
59. Mazrooei, P. *et al.* Cistrome Partitioning Reveals Convergence of Somatic Mutations and Risk Variants on Master Transcription Regulators in Primary Prostate Tumors. English. *Cancer Cell* **36**, 674–689.e6. ISSN: 1535-6108, 1878-3686 (Dec. 2019).

60. Weinhold, N., Jacobsen, A., Schultz, N., Sander, C. & Lee, W. Genome-Wide Analysis of Noncoding Regulatory Mutations in Cancer. en. *Nature Genetics* **46**, 1160–1165. issn: 1061-4036, 1546-1718 (Nov. 2014).
61. CAMCAP Study Group *et al.* Sequencing of Prostate Cancers Identifies New Cancer Genes, Routes of Progression and Drug Targets. en. *Nature Genetics* **50**, 682–692. issn: 1061-4036, 1546-1718 (May 2018).
62. Quigley, D. A. *et al.* Genomic Hallmarks and Structural Variation in Metastatic Prostate Cancer. English. *Cell* **174**, 758–769.e9. issn: 0092-8674, 1097-4172 (July 2018).

# Appendices

## .1 Supplemental Figures for Chapter 2