

CHROMATIN ARCHITECTURE ABERRATIONS CONTRIBUTE TO PROSTATE CANCER
ONCOGENESIS AND ACUTE LYMPHOBLASTIC LEUKEMIA RELAPSE

by

James Hawley

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Graduate Department of Medical Biophysics
University of Toronto

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Abstract

Cancer results from multiple aberrations at the molecular level that enable specific biological hallmarks, including sustained proliferation and genome instability. These aberrations can be found within the chromatin architecture of cancer cells, including the genome, the set of molecular modifications to the genome, and the three-dimensional organization of the chromatin fiber. The majority of genetic variants target non-coding regions of the genome and many genes affected by genetic and epigenetic variants have important roles in chromatin remodelling and maintenance. Thus, understanding the origins and trajectories of cancer progression requires investigating the targets of these aberrations and how they impact different components of the chromatin architecture.

First, I investigated the impact of non-coding single nucleotide variants that converge on the set of *cis*-regulatory elements for the *FOXA1* transcription factor in primary prostate tumours. We found that deletion and repression of these *cis*-regulatory elements significantly decreases *FOXA1* expression and prostate cancer cell growth by altering the potential of transcription factors to bind at these loci. These results identify *cis*-regulatory elements that control *FOXA1* expression in primary prostate cancer as potential targets for therapeutic intervention.

Secondly, I used chromatin conformation capture of 12 primary prostate cancer tumours and 5 benign prostate tissues to characterize the three-dimensional genome organization. We found that large-scale organization, including topologically associated domains and compartments, is largely stable over oncogenesis but that small-scale focal chromatin interactions change between benign and tumour tissue. We also investigated the impact of structural variants on chromatin organization and identify novel enhancer hijacking events. These results indicate that enhancer hijacking of prostate cancer oncogenes may be a more common driver of disease than previously recognized. Then, I developed a statistical framework for differential gene expression analysis to address the impact of non-recurrent structural variants in our primary prostate tumour cohort. This method improves on conventional gene expression fold change estimates in these unbalanced experimental designs.

Finally, I investigated the genetic and epigenetic changes that underlie B-cell acute lymphoblastic

leukemia relapse. I found recurrent loss of DNA methylation in patient-matched relapse samples that indicate a more stem-like chromatin state. Together, my work investigates the relationship between multiple components of the chromatin architecture, and how aberrations to this architecture connects oncogenesis, disease progression, and relapse.

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List of Research Works

Full Manuscripts

Published

- Seneviratne, A. K. *et al.* The Mitochondrial Transacylase, Tafazzin, Regulates for AML Stemness by Modulating Intracellular Levels of Phospholipids. *Cell Stem Cell* **24**, 621–636. doi:[10.1016/j.stem.2019.02.020](https://doi.org/10.1016/j.stem.2019.02.020) (Apr. 2019)
- Singh, R. P. *et al.* Disrupting Mitochondrial Copper Distribution Inhibits Leukemic Stem Cell Self-Renewal. *Cell Stem Cell* **26**, 926–937. doi:[10.1016/j.stem.2020.04.010](https://doi.org/10.1016/j.stem.2020.04.010) (June 2020)
- Zhou, S. *et al.* Noncoding Mutations Target Cis -Regulatory Elements of the FOXA1 Plexus in Prostate Cancer. *Nature Communications* **11**, 441. doi:[10.1038/s41467-020-14318-9](https://doi.org/10.1038/s41467-020-14318-9) (Jan. 2020)

In Preparation or Submitted

- Hawley, J. R. *et al.* *Reorganization of the Three-Dimensional Genome Pinpoint Non-Coding Drivers of Primary Prostate Tumours* Jan. 2021. doi:[10.1101/2021.01.05.425333](https://doi.org/10.1101/2021.01.05.425333)
- Ghamrasni, S. E. *et al.* *Non-Coding Mutations Reveal Cancer Driver Cistromes in Luminal Breast Cancer* May 2021. doi:[10.1101/2021.05.29.446210](https://doi.org/10.1101/2021.05.29.446210)

Selected talks and first author poster presentations

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- “Assessing epigenetic heterogeneity through DNA methylation entropy in cancer cell lines”. James Lepock Memorial Symposium. Toronto, Ontario, 2018.

List of Abbreviations

3C chromatin conformation capture

ALL acute lymphoblastic leukemia

AML acute myeloid leukemia

ANOVA Analysis of Variance

AR androgen receptor

ATAC-seq assay for transposase-accessible chromatin sequencing

B-ALL B-cell acute lymphoblastic leukemia

BAM binary alignment map

bp basepair

cDNA complementary DNA

ChIP-seq chromatin immunoprecipitation sequencing

CLL chronic lymphocytic leukemia

CML chronic myeloid leukemia

CMP common myeloid progenitor

CNV copy number variant

CPC-GENE Canadian Prostate Cancer Genome Network

CpG CG dinucleotide

crRNA CRISPR RNA

CRE *cis*-regulatory element

CRISPR clustered regularly interspaced short palindromic repeat

CTCF CCCTC-binding factor

CUT&RUN cleavage under targets and release using nuclease

DEPMAP Cancer Dependency Map

DHS DNase I hypersensitive sites

DLBCL diffuse large B-cell lymphoma

DMR differentially methylated region

DNA deoxyribonucleic acid

DNAme DNA methylation

DNase-seq DNase I hypersensitive sequencing

dRI disease relapse-initiating

Dx diagnosis

EarlyProB early progenitor B cell

FDR false discovery rate

FN false negative

FP false positive

FOX forkhead box

GLM generalized linear model

GMP granulocyte-macrophage progenitor

GO gene ontology

gRNA guide RNA

HSC hematopoietic stem cell

HSPC hematopoietic stem and progenitor cell

IDH isocitrate dehydrogenase

IID independent and identically distributed

ISUP International Society of Urological Pathology

JS James-Stein

KMT histone lysine methyltransferase

KO knockout

LDA limiting dilution assay

LMPP lymphoid-primed multi-potent progenitor

MeCapSeq DNA methylation capture sequencing

MEP megakaryocyte-erythrocyte progenitor

MNase-seq micrococcal nuclease sequencing

MSE mean square error

mCRPC metastatic castration-resistant prostate cancer

MDS myelodisplastic syndrome

MLP monocyte-lymphoid progenitor

MPP multi-potent progenitor

MRPA massively-parallel reporter assay

NSG NOD scid gamma

OLS ordinary least squares

mRNA messenger RNA

PCa prostate cancer

PDX patient-derived xenograft

PreProB pre-progenitor B cell

ProB progenitor B cell

PSA prostate-specific antigen

Rel relapse

RLU relative luciferase unit

RNA ribonucleic acid

RNAi RNA interference

RNA-seq RNA sequencing

shRNA small hairpin RNA

siRNA small interfering RNA

SNV single nucleotide variant

SRA Sequence Read Archive

SNF similarity network fusion

STR short tandem repeat

SV structural variant

T2E *TMPRSS2-ERG*

TAD topologically associated domain

TCGA The Cancer Genome Atlas

TET ten-eleven translocation

TSS transcription start site

TN true negative

TNM tumour node metastasis

TP true positive

TF transcription factor

tracrRNA trans-activating CRISPR RNA

UTR untranslated region

WES whole exome sequencing

WGBS whole genome bisulfite sequencing

WGS whole genome sequencing

WT wild-type

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. *et al.* Noncoding Mutations Target Cis -Regulatory Elements of the FOXA1 Plexus in Prostate Cancer. *Nature Communications* **11**, 441. doi:[10.1038/s41467-020-14318-9](https://doi.org/10.1038/s41467-020-14318-9) (Jan. 2020)

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

PCa is the second most commonly diagnosed malignancy among men worldwide. Recurrently mutated in primary and metastatic prostate tumours, *FOXA1* encodes a pioneer transcription factor (TF) involved in disease onset and progression through both androgen receptor (*AR*)-dependent and

AR-independent mechanisms. Despite its oncogenic properties however, the regulation of *FOXA1* expression remains unknown. Here, we identify a set of six CREs in the *FOXA1* regulatory plexus harboring somatic SNVs in primary prostate tumours. We find that deletion and repression of these CREs significantly decreases *FOXA1* expression and PCa cell growth. Six of the ten SNVs mapping to *FOXA1* regulatory plexus significantly alter the transactivation potential of CREs by modulating the binding of TFs. Collectively, our results identify CREs within the *FOXA1* plexus mutated in primary prostate tumours as potential targets for therapeutic intervention.

1.2 Introduction

PCa is the second most commonly diagnosed cancer among men with an estimated 1.3 million new cases worldwide in 2018 [6]. Although most men diagnosed with primary PCa are treated with curative intent through surgery or radiation therapy, treatments fail in 30% of patients within 10 years [7] resulting in a metastatic disease [8]. Patients with metastatic disease are typically treated with anti-androgen therapies, the staple of aggressive PCa treatment [9]. Despite the efficacy of these therapies, recurrence ultimately develops into lethal metastatic castration-resistant prostate cancer (mCRPC) [9]. As such, there remains a need to improve our biological understanding of PCa development and find novel strategies to treat patients. Sequencing efforts identified coding somatic SNVs mapping to *FOXA1* in up to 9% [10–15] and 13% [14–16] of primary and metastatic PCa patients, respectively. These coding somatic SNVs target the Forkhead and transactivation domains of *FOXA1* [17], altering its pioneering functions to promote PCa development [15, 18]. Outside of coding SNVs, whole genome sequencing (WGS) also identified somatic SNVs and indels in the 3' untranslated region (UTR) and C-terminus of *FOXA1* in ~12% of mCRPC patients [19]. In addition to SNVs, the *FOXA1* locus is a target of structural rearrangements in both primary and metastatic PCa tumours, inclusive of duplications, amplifications, and translocations [14, 15]. Taken together, *FOXA1* is recurrently mutated taking into account both its coding and flanking noncoding sequences across various stages of PCa development.

FOXA1 serves as a pioneer TF that can bind to heterochromatin, promoting its remodelling to increase accessibility for the recruitment of other TFs [20]. *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 [21, 22]. In PCa, *FOXA1* is known to pioneer and reprogram the binding of *AR* alongside HOXB13 [23]. Independent from its role in *AR* signalling, *FOXA1* also regulates the expression of genes involved in cell cycle regulation in PCa [24],

[25]. For instance, FOXA1 co-localizes with CREB1 to regulate the transcription of genes involved in cell cycle processes, nuclear division and mitosis in mCRPC [24–30]. FOXA1 has also been shown to promote feed-forward mechanisms to drive disease progression [31, 32]. Hence, FOXA1 contributes to *AR*-dependent and *AR*-independent processes favouring PCa development.

Despite the oncogenic roles of FOXA1, therapeutic avenues to inhibit its activity in PCa 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 [33]. As such, understanding the governance of *FOXA1* messenger RNA (mRNA) expression offers an alternative strategy to find modulators of its activity. Gene expression relies on the interplay between distal CREs, such as enhancers and anchors of chromatin interaction, and their target gene promoter(s) [34]. These elements can lie tens to hundreds of kilobases away from each other on the linear genome but physically engage in close proximity with each other in the three-dimensional space [35]. By measuring contact frequencies between loci through the use of chromatin conformation capture (3C)-based technologies, it enables the identification of regulatory plexuses corresponding to sets of CREs in contact with each other [36, 37]. By leveraging these technologies, we can begin to understand the three-dimensional organization of the PCa genome and delineate the *FOXA1* regulatory plexus.

Here, we integrate epigenetics and genetics from PCa 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 PCa 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 (Figure 1.1a), ranking in the 95th percentile for 492 of 497 prostate tumours profiled in The Cancer Genome Atlas (TCGA) (??a). 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 (??b). We next analyzed expression data from Cancer Dependency Map (DEPMAP) and observed *FOXA1* to be most highly expressed in PCa cell lines compared to cell lines of other cancer types (??a).

Amongst the eight PCa 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 (??b). These new results gained from the TCGA and DEPMAP validate previous understanding that *FOXA1* is one of the highest expressed genes in PCa [38].

Following up on *FOXA1* mRNA expression levels, we interrogated the essentiality of *FOXA1* for PCa cell growth. RNAi-mediated essentiality screens compiled in DEPMAP show that *FOXA1* lies in the 94th percentile across 6 of the 8 available PCa cell lines: 22Rv1, LNCaP, MDA PCa 2B, NCI-H660, PC3, and VCaP cells (Figure 1.1b-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 PCa cell proliferation (permutation test, $p = 1 \times 10^{-6}$, see ??; ??a). Growth assays in LNCaP and VCaP cells following *FOXA1* knockdown using two independent siRNAs (Figure 1.1d, ??b) 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, $p < 0.05$; Figure 1.1e-f). In accordance with previous reports, our results using essentiality datasets followed by knockdown validation reveals that *FOXA1* is oncogenic and essential for PCa 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 [36, 37]. Regulatory plexuses stem from chromatin interactions orchestrated by various factors including ZNF143, YY1, CTCF and the cohesin complex [39–41]. Motivated by the oncogenic role of *FOXA1* in PCa, we investigated its regulatory plexus controlling its expression. According to chromatin contact frequency maps generated from Hi-C assays performed in LNCaP PCa cells, *FOXA1* lies in a 440 kbp topologically associated domain (TAD) (chr14: 37720001-38160000 ± 40 kbp adjusting for resolution; Figure 1.2a). By overlaying DNase-seq data from LNCaP PCa cells, there are a total of 123 putative CREs reported as DNase I hypersensitive sites (DHSs) that populate this TAD (Figure 1.2a). We next inferred the regulatory plexus of *FOXA1* using the C3D method [42]. C3D aggregates and draws correlation of DHS signal intensities between the cell line of choice and the DHS signal across all systems in a collection of cell lines and tissues [42]. Anchoring our analysis to the *FOXA1* promoter and using accessible chromatin regions defined in LNCaP PCa cells identified 55 putative CREs to the *FOXA1* regulatory plexus

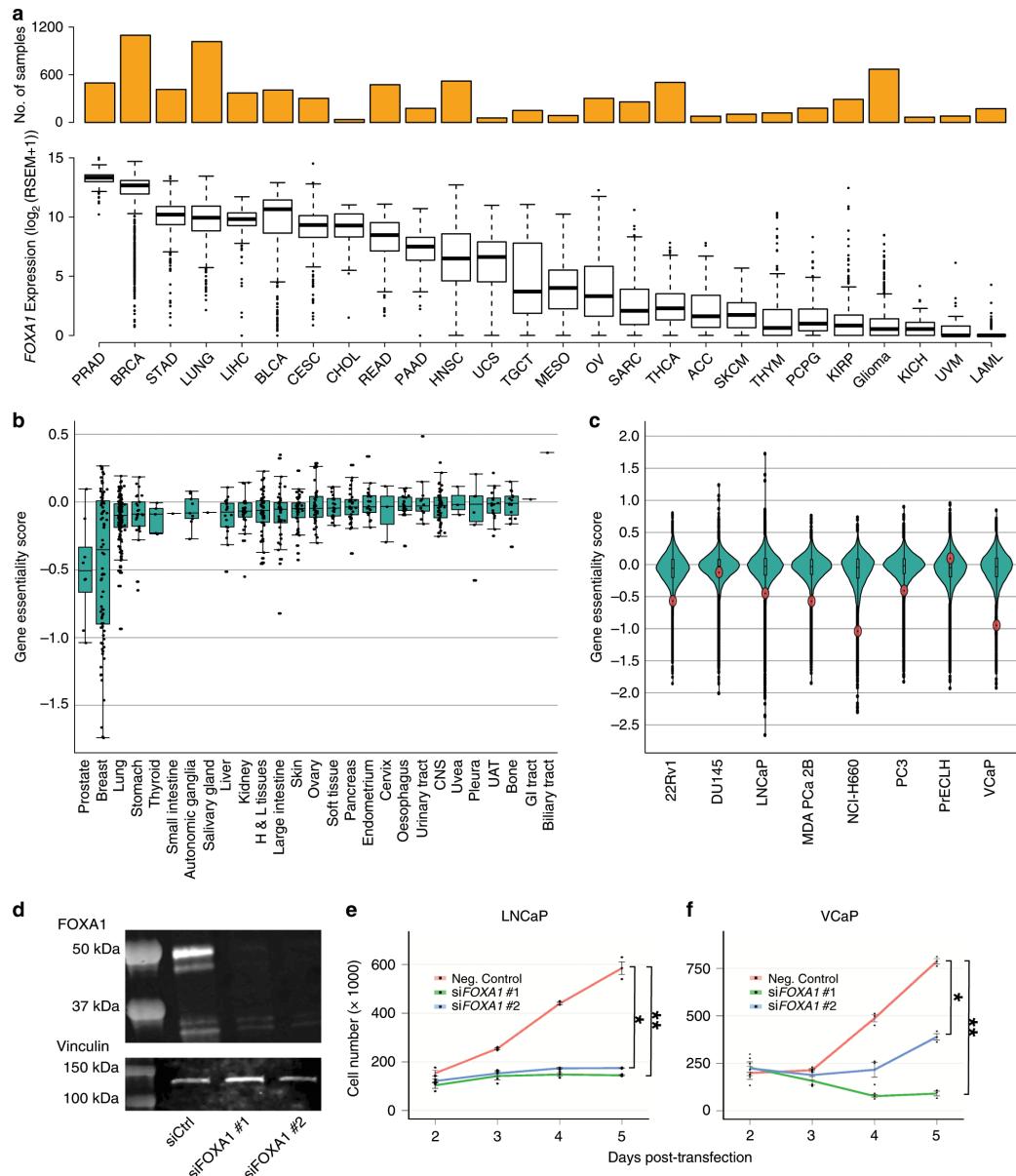


Figure 1.1: *FOXA1* is highly expressed in PCa and essential for PCa cell proliferation..

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 PCa 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, * $p < 0.05$, ** $p < 0.01$.

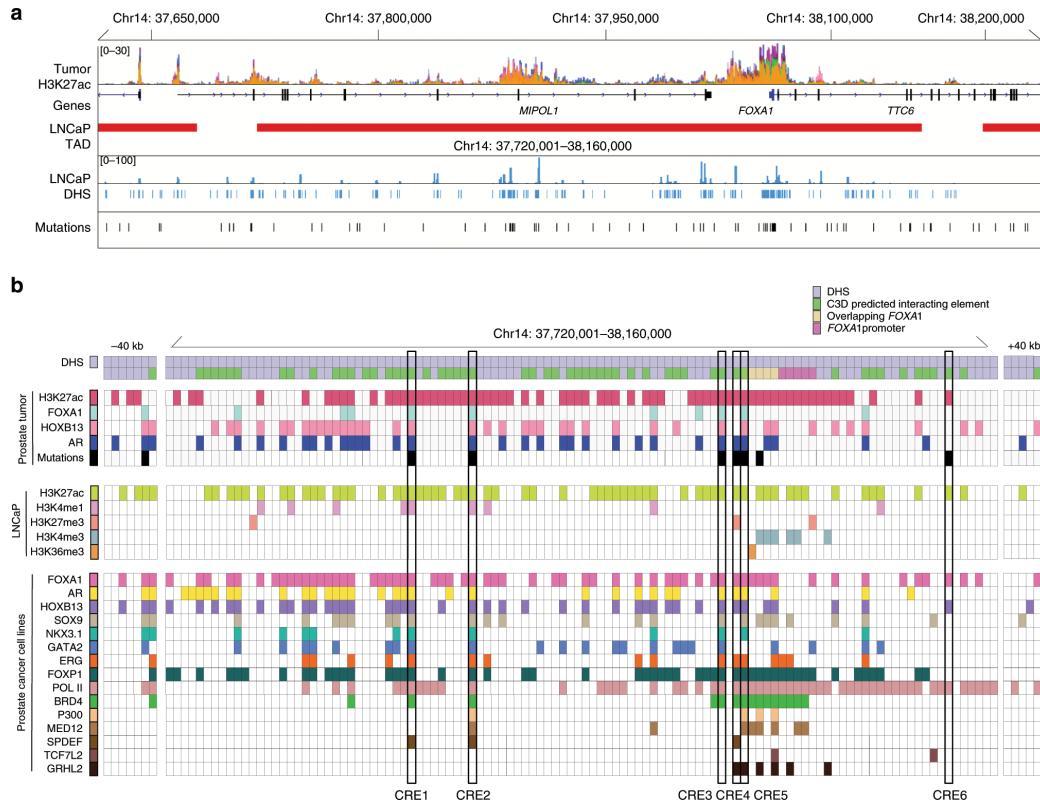


Figure 1.2: Epigenetic annotation of 14q21.1 locus and identification of *FOXA1* CREs.

a. Overview of *cis*-regulatory landscape surrounding *FOXA1* on the 14q21.1 locus. H3K27ac signal track is the ChIP-seq signal overlay of 19 primary prostate tumours. LNCaP Hi-C depicts the TAD structure around *FOXA1*. LNCaP signal values with peak calls below indicate the accessibility of the chromatin around *FOXA1*. Mutations indicate SNVs identified in 200 primary prostate tumours.

b. Functional annotation of putative *FOXA1* CREs using TF and histone modification ChIP-seq conducted in primary tumours and PCa cell lines. Annotated in the matrix are all DHS within the TAD and \pm 40 kbp resolution left and right of the TAD. Putative *FOXA1* CREs targeted by noncoding SNVs for downstream validation are boxed. Coordinates are 1-based in the hg19 reference genome.

($r > 0.7$; Figure 1.2b).

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 from LNCaP cells with available ChIP-seq data for histone modifications and TFs conducted in LNCaP, 22Rv1, VCaP PCa cell lines and primary prostate tumours (Figure 1.2b) [23, 43]. Close to 60% (33/55) of the putative *FOXA1* plexus CREs are marked by H3K27ac profiled in primary prostate tumours [43], indicative of active CREs in tumours (Figure 1.2b) [44]. Next, considering that noncoding SNVs can target a set of CREs that converge on the same target gene in cancer [37], we overlapped the somatic SNVs called from WGS across 200 primary prostate tumours

to the 33 H3K27ac-marked DHS predicted to regulate *FOXA1* [11, 45]. This analysis identified 6 out of the 33 DHS marked with H3K27ac (18.2%) harboring one or more SNVs (10 total SNVs called from 9 tumours; Figure 1.2b). We observe that these 6 CREs can be bound by multiple TFs in PCa cells, including *FOXA1*, AR and *HOXB13* (Figure 1.2b, ??). The Hi-C data from the LNCaP PCa 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 1.3a). The 6 CREs lie in intergenic or intronic regions (Figure 1.3b-h). Together, histone modifications, TF binding sites and noncoding SNVs support that these 6 putative CREs are active in primary PCa. 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 (WT) Cas9 protein (Figure 1.4a-b). Guide RNAs (gRNAs) designed against the *FOXA1* gene (exon 1 and intron 1) served as positive controls while an outside-TAD region (termed Chr14 (-)), a region on a different chromosome (the human *AAVS1* safe-harbor site at the *PPP1R12C* locus [43, 46]), 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 Section 1.5) led to significantly decreased *FOXA1* mRNA expression ($\Delta\text{CRE1} \sim 29.3 \pm 8.3\%$, $\Delta\text{CRE2} \sim 40.1 \pm 11.8\%$, $\Delta\text{CRE3} \sim 30.6 \pm 9.1\%$, $\Delta\text{CRE4} \sim 23.6 \pm 8.2\%$, $\Delta\text{CRE5} \sim 25.3 \pm 6.6\%$, $\Delta\text{CRE6} \sim 24.5 \pm 10.2\%$ and ΔFOXA1 (exon 1 and intron 1) $\sim 87.4 \pm 8.8\%$ reduction relative to basal levels; Figure 1.4c, ??a-f). In contrast, deletion of several negative control regions within the same TAD did not significantly reduce *FOXA1* mRNA level (Figure 1.4c, ??g-i). Similar results were observed in 22Rv1 PCa cells (Figure 1.4d). 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 $r = 0.49$, $p < 0.005$; ??a) and agreeing trends for each individual CRE (??b).

Complementary to our findings using the WT CRISPR/Cas9 system, we next generated four LNCaP and four 22Rv1 cell line-derived dCas9-KRAB fusion protein expressing clones (Figure 1.4e-f). Transient transfection of the same gRNAs used in the WT Cas9 experiments, targeting the six

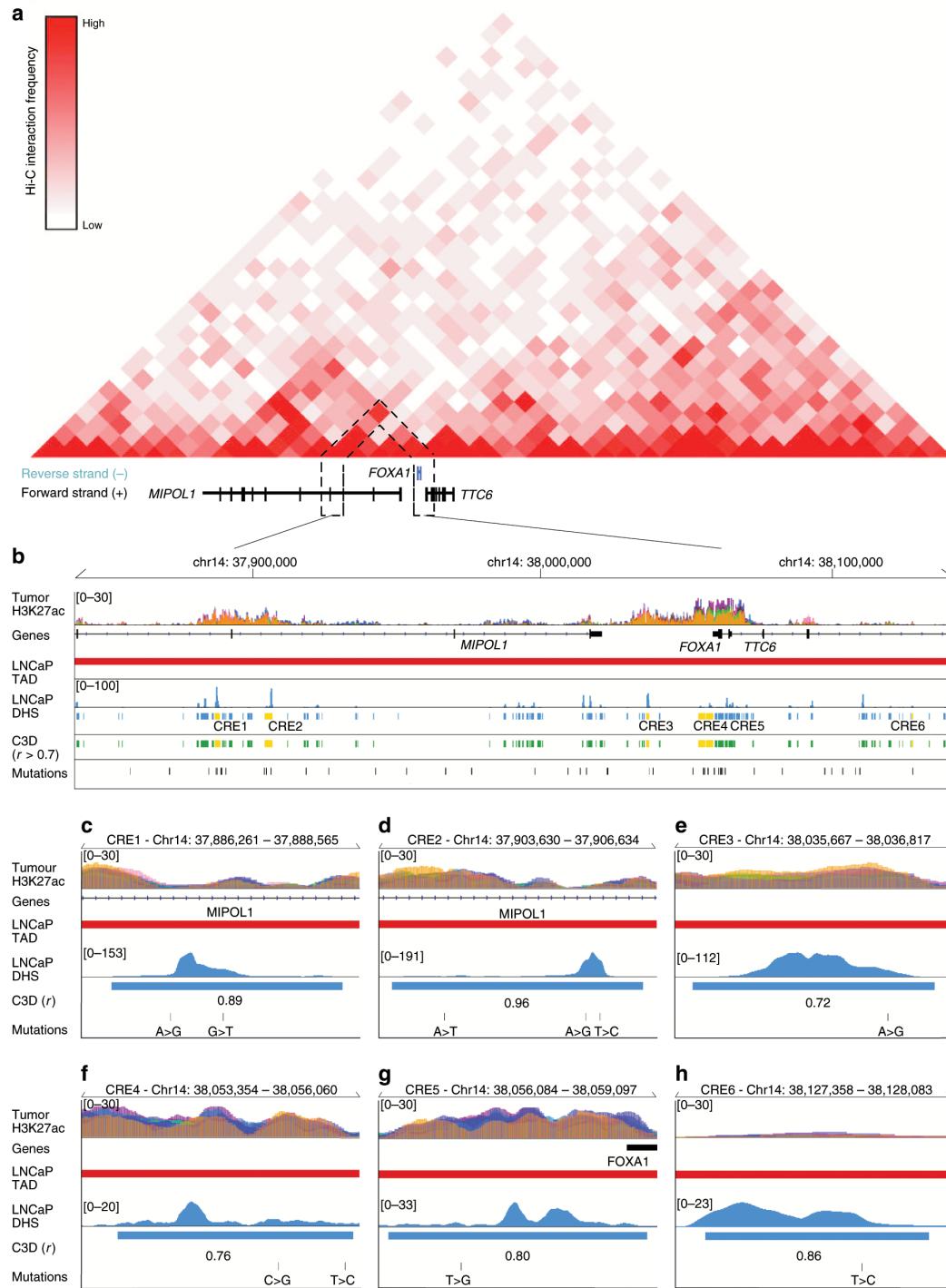


Figure 1.3: Putative CREs predicted to interact with *FOXA1* promoter. **a.** Hi-C contact matrix conducted in LNCaP cells indicating physical interactions between putative *FOXA1* CREs and the *FOXA1* promoter. Hi-C resolution is 40 kbp. **b.** Epigenome annotations around the *FOXA1* locus. The six putative *FOXA1* CREs are coloured in yellow. **c-h.** Zoom-in of each individual putative *FOXA1* CRE. C3D value is the Pearson correlation of DHS signal between LNCaP and the DHS reference matrix. Coordinates are 1-based in the hg19 reference genome.

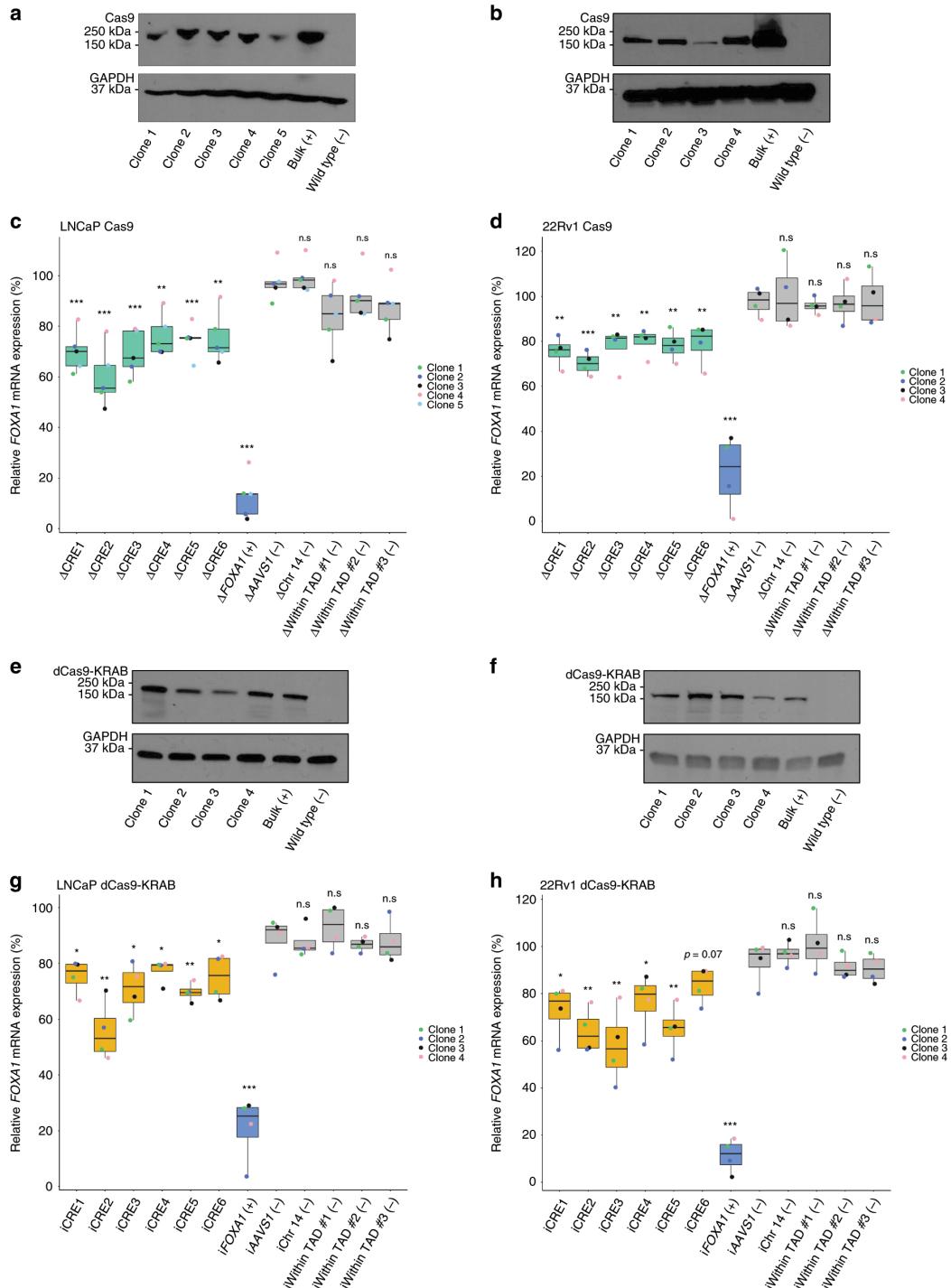


Figure 1.4: Functional dissection of putative *FOXA1* CREs. (Continued on the following page)

Figure 1.4: **a.** Representative western blot probed against Cas9 in LNCaP clones ($n = 5$ clones) derived to stably express Cas9 protein upon blasticidin selection. **b.** Representative western blot probed against Cas9 in 22Rv1 clones ($n = 4$ clones) derived to stably express Cas9 protein upon blasticidin selection. **c.** *FOXA1* mRNA expression normalized to housekeeping *TBP* mRNA expression upon CRISPR/Cas9-mediated deletion of each CRE using LNCaP clones ($n = 5$ independent experiments, each dot represents an independent clone). **d.** *FOXA1* mRNA expression normalized to housekeeping *TBP* mRNA expression upon CRISPR/Cas9-mediated deletion of each CRE using 22Rv1 clones ($n = 4$ independent experiments, each dot represents an independent clone). **e.** Representative western blot probed against Cas9 in LNCaP clones ($n = 4$ clones) derived to stably express the dCas9-KRAB fusion protein upon blasticidin selection. **f.** Representative western blot probed against Cas9 in 22Rv1 clones ($n = 4$ clones) derived to stably express dCas9-KRAB fusion protein upon blasticidin selection. **g.** *FOXA1* mRNA expression normalized to housekeeping *TBP* mRNA expression upon dCas9-KRAB-mediated repression of each CRE using LNCaP clones ($n = 4$ independent experiments, each dot represents an independent clone). **h.** *FOXA1* mRNA expression normalized to housekeeping *TBP* mRNA expression upon dCas9-KRAB-mediated repression of each CRE using 22Rv1 clones ($n = 4$ independent experiments, each dot represents an independent clone). *FOXA1* mRNA expression was normalized to basal *FOXA1* expression prior to statistical testing. Δ indicates CRISPR/Cas9-mediated deletion, i indicates dCas9-KRAB-mediated repression. Error bars indicate \pm s.d. Student's *t*-test, n.s. not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

FOXA1 plexus CREs into our dCas9-KRAB LNCaP clones significantly decreased *FOXA1* expression relative to basal levels (iCRE1 $\sim 24.6 \pm 6.2\%$, iCRE2 $\sim 42.2 \pm 10.8\%$, iCRE3 $\sim 25.3 \pm 9.2\%$, iCRE4 $\sim 23.3 \pm 4.3\%$, iCRE5 $\sim 30.2 \pm 3.4\%$ and iCRE6 $\sim 23.1 \pm 8.1\%$ reduction). Similarly, gRNAs targeting the dCas9-KRAB fusion protein to *FOXA1* decreased its expression (i*FOXA1* $\sim 81.6 \pm 11.8\%$ reduction; Student's *t*-test, $p < 0.05$, Figure 1.4g). Analogous results were also observed in our four clonal 22Rv1 dCas9-KRAB cell lines (Student's *t*-test, $p < 0.05$, Figure 1.4h). 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*. Δ CRE1 and Δ CRE2 significantly reduced *MIPOL1* mRNA expression by $\sim 38.4 \pm 6.4\%$ and $\sim 48.4 \pm 9\%$, 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, $p < 0.05$, ??a). On the other hand, deletion of CREs each significantly reduced *TTC6* mRNA expression relative to its basal levels (Δ CRE1 $\sim 52.9\% \pm 6.4\%$, Δ CRE2 $\sim 66 \pm 11.3\%$, Δ CRE3 $\sim 55.5 \pm 12.8\%$, Δ CRE4 $44.9 \pm 10.6\%$, Δ CRE5 $43.1 \pm 11.9\%$ and Δ CRE6 $52.2 \pm 7.3\%$ reduction (Student's *t*-test, $p < 0.05$, ??b), in agreement with the fact that *TTC6* shares its promoter with *FOXA1* as both genes are transcribed on opposing strands (??c).

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; $\Delta\text{CRE1} \sim 190\%$, $\Delta\text{CRE2} \sim 162.8\%$, $\Delta\text{CRE3} \sim 147.5\%$, $\Delta\text{CRE4} \sim 133.3\%$, $\Delta\text{CRE5} \sim 137.3\%$, $\Delta\text{CRE6} \sim 120.8\%$, $\Delta\text{FOXA1} \sim 266.7\%$), *ACPP* (down-regulation; $\Delta\text{CRE1} \sim 73.5\%$, $\Delta\text{CRE2} \sim 62.5\%$, $\Delta\text{CRE3} \sim 69.6\%$, $\Delta\text{CRE4} \sim 75.6\%$, $\Delta\text{CRE5} \sim 70.9\%$, $\Delta\text{CRE6} \sim 74.6\%$, $\Delta\text{FOXA1} \sim 52.2\%$) and *GRIN3A* expression (up-regulation; $\Delta\text{CRE1} \sim 138.2\%$, $\Delta\text{CRE2} \sim 168.8\%$, $\Delta\text{CRE3} \sim 144.6\%$, $\Delta\text{CRE4} \sim 132.1\%$, $\Delta\text{CRE5} \sim 131.4\%$, $\Delta\text{CRE6} \sim 127\%$, $\Delta\text{FOXA1} \sim 228\%$; Student's *t*-test, $p < 0.05$, ??d-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 [36, 37, 47], 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 PCa cell clones (see Section 1.5). 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 PCa cell clones stably expressing WT Cas9 and dCas9-KRAB, individual CRE deletion resulted in a significant reduction in *FOXA1* mRNA expression: ($\Delta\text{CRE1} \sim 29.3 \pm 7.3\%$, $\Delta\text{CRE2} \sim 36 \pm 11.8\%$, $\Delta\text{CRE3} \sim 30.6 \pm 12.7\%$, $\Delta\text{CRE4} \sim 24.5 \pm 6.1\%$, $\Delta\text{CRE5} \sim 23.7 \pm 13.2\%$, $\Delta\text{CRE6} \sim 26.8 \pm 14.2\%$ and $\Delta\text{FOXA1} \sim 96.2 \pm 1.4\%$ reduction; Student's *t*-test, $p < 0.05$, Figure 1.5a, ??a-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 $\Delta\text{CRE1} + \Delta\text{CRE2}$, $\Delta\text{CRE1} + \Delta\text{CRE4}$, and $\Delta\text{CRE2} + \Delta\text{CRE4}$ resulted in a significant $\sim 48.5 \pm 4.5\%$, $\sim 50.4 \pm 2.9\%$ and $\sim 45.2 \pm 5.5\%$ reduction in *FOXA1* mRNA expression, respectively (Student's *t*-test, $p < 0.05$, Figure 1.5b, ??a-f) a fold reduction greater than single CRE deletions (Student's *t*-test, ??, $p < 0.05$). These results together demonstrate that these CREs collaboratively contribute to the establishment and regulation of *FOXA1* expression in PCa.

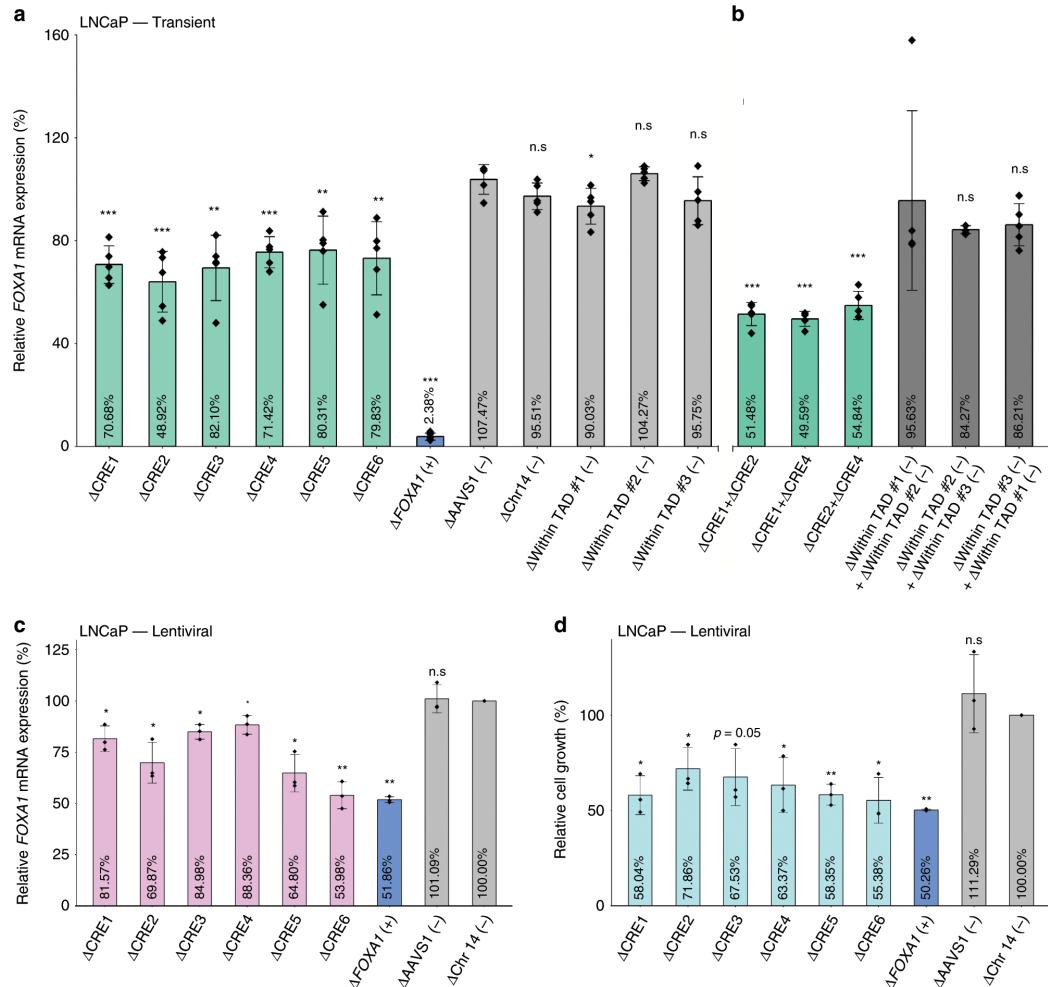


Figure 1.5: *FOXA1* CREs collaborate to regulate its expression and are critical for PCa cell proliferation. **a.** *FOXA1* mRNA expression normalized to housekeeping *TBP* mRNA expression upon transient transfection-based CRISPR/Cas9-mediated deletion of CRE1, CRE2, CRE4, and sequential deletion combinations ($n = 5$ independent experiments). Dots represent values from individual replicates. **b.** *FOXA1* mRNA expression normalized to housekeeping *TBP* mRNA expression upon bulk lentiviral-based CRISPR/Cas9-mediated deletion of each CRE in LNCaP cells ($n = 3$ independent experiments). **c.** Cell proliferation assay conducted after puromycin and blasticidin selection for LNCaP cells carrying deleted regions of interest. Data was based on cell counting 6 days after seeding post-selection ($n = 3$, representative of three independent experiments). *FOXA1* mRNA expression upon deletion was normalized to basal *FOXA1* expression prior to statistical testing. *FOXA1* mRNA expression was normalized to the basal LNCaP *FOXA1* expression prior to statistical testing. Δ indicates CRISPR/Cas9-mediated deletion. Error bars indicate \pm s.d. Student's *t*-test, n.s. not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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

As *FOXA1* is essential for PCa growth (Figure 1.1b-e), we next sought to assess the importance of the six CREs in the *FOXA1* plexus towards PCa cell growth. We adapted a lentiviral-based approach that expressed both the Cas9 protein and two gRNA that target each CRE for deletion (see ??). Upon lentiviral transduction with subsequent selection, we separated LNCaP PCa 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 (Δ CRE1 ~18%, Δ CRE2 ~30%, Δ CRE3 ~15%, Δ CRE4 ~12%, Δ CRE5 ~35%, Δ CRE6 ~46% and Δ *FOXA1* (exon 1 and intron 1) ~48% reduction (Student's *t*-test, $p < 0.05$, Figure 1.5c, ??a-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 (Δ CRE1 ~42%, Δ CRE2 ~28%, Δ CRE3 ~33%, Δ CRE4 ~27%, Δ CRE5 ~42%, Δ CRE6 ~44% and Δ *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 PCa etiology, in agreement with their ability to regulate *FOXA1* expression and the essentiality of this gene in PCa cell growth.

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

SNVs can alter the transactivation potential of CREs [37, 48–56]. In total, we found 10 SNVs called from 9 out of the 200 tumours that map to the six *FOXA1* plexus CREs (Figure 1.6a). To assess the impact of these noncoding SNVs, we conducted luciferase assays comparing differential reporter activity between the variant and the WT allele of each CRE (Figure 1.6b-k). We found that the variant alleles of 6 of the 10 SNVs displayed significantly greater luciferase reporter activity when compared to the WT 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 1.6b, d-h). These results indicate that these SNVs can alter the transactivation potential of *FOXA1* plexus CREs in PCa 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 [37, 49, 56] in LNCaP PCa cells. We observed differential binding of FOXA1, AR, HOXB13, GATA2, and FOXP1 for the

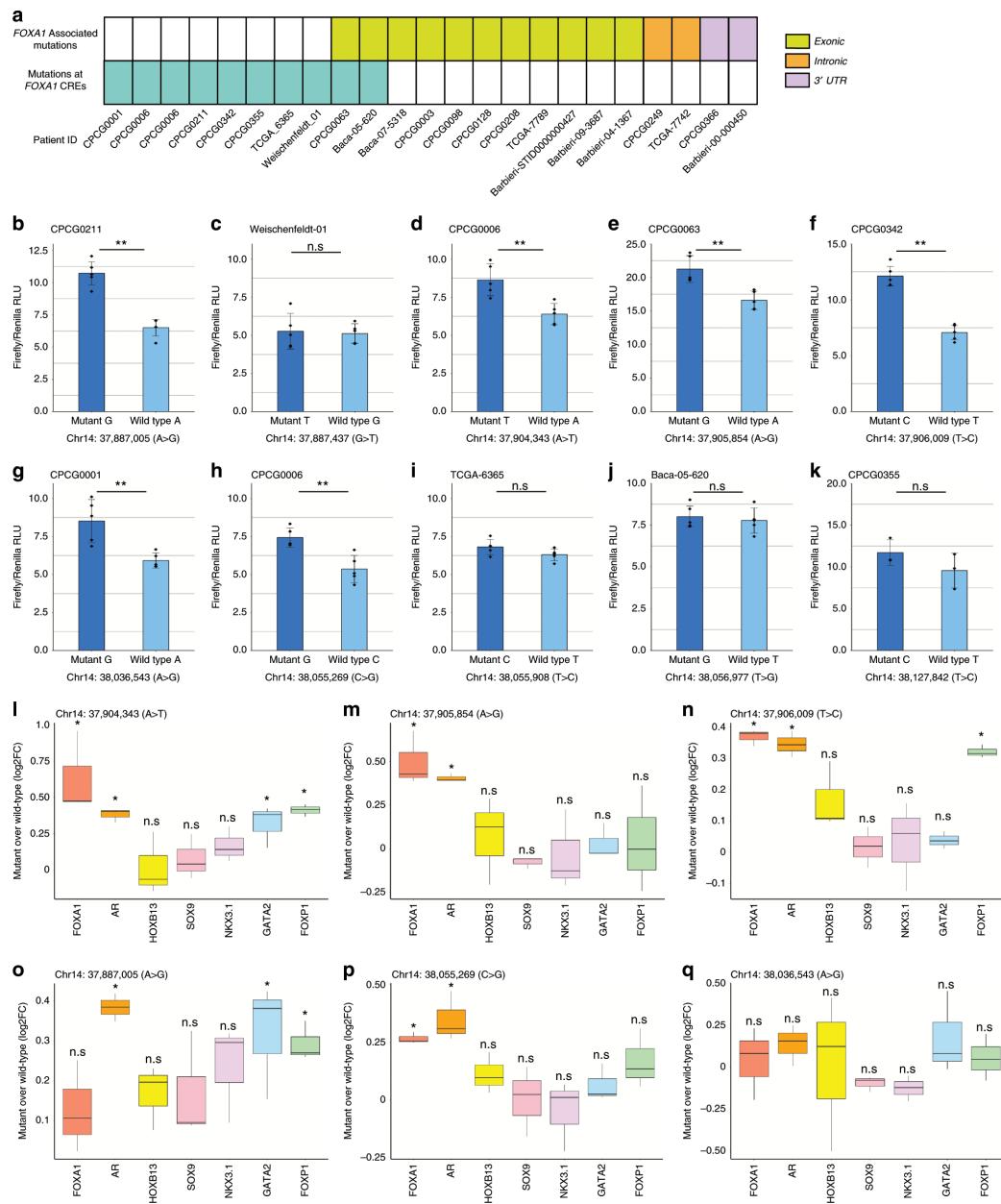


Figure 1.6: A subset of noncoding SNVs mapping to the *FOXA1* CREs are gain-of-function. **a.** Matrix showcasing the patients from the CPC-GENE dataset that harbour SNVs at the *FOXA1* CREs, exons, introns, and the 3' UTR of *FOXA1*. **b-k.** Luciferase assays are conducted in LNCaP cells. Bar plot showcases the mean firefly luciferase activity normalized by *Renilla* luciferase activity in RLUs. Error bars indicate \pm s.d. $n = 5$ independent experiments for all CREs except for chr14:38,127,842 T > C where $n = 3$. Each diamond represents an independent experiment. Hypothesis testing done with Mann-Whitney U test. **l-q.** Allele-specific ChIP-qPCR conducted on plasmids carrying the WT or variant sequence upon transient transfection in PCa cells. Data is presented as \log_2 fold-change of variant sequence upon comparison to WT sequence ($n = 3$ independent experiments per ChIP). Hypothesis testing done with Student's *t*-test, n.s. not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Coordinates are 1-based in the hg19 reference genome.

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 1.6l-p). In contrast, SOX9 and NKX3.1 binding was unaffected by these SNVs (Figure 1.6l-q). Compared to the WT 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 populate the *FOXA1* plexus CREs.

1.4 Discussion

Modern technologies and understanding of the epigenome allow the possibility of probing CREs involved in regulating genes implicated in disease. Despite *FOXA1* being recurrently mutated [10–13, 16] and playing potent oncogenic roles in PCa etiology [14, 15, 18], the CREs involved in its transcriptional regulation are poorly understood. Understanding how *FOXA1* is expressed can provide a complementary strategy to antagonize *FOXA1* in PCa.

We used the DHSs profiled in PCa cells to identify putative *FOXA1* CREs through annotating these regions with five different histone modifications, TF binding sites and noncoding SNVs profiled in PCa 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 [57]. 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 [37], *MLH1* in Lynch syndrome [58], *MYC* in lung adenocarcinoma and endometrial cancer [59], and *AR* in mCRPC [60, 61]. Through combinatorial deletion of two CREs, *FOXA1* mRNA levels were further reduced in comparison with single CRE deletions, raising the possibility of CRE additivity [62]. The deletion of the FOXA1 plexus CREs also significantly reduced PCa cell proliferation at levels comparable to what has been reported upon deletion of the

amplified CRE upstream of the *AR* gene in mCRPC [60], suggestive of onco-CREs as reported in lung [59] and prostate [60] cancer.

More than 90% of SNVs found in cancer map to the noncoding genome [63, 64] with a portion of these SNVs mapping to CREs altering their transactivation potential [37, 49–51] and/or downstream target gene expression [53, 63, 65]. 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 PCa [64]. Our findings complement recent reports of SNVs found in the noncoding locus of *FOXA1* that could affect its expression [19, 66]. The *FOXA1* plexus CREs we identified here are also reported to be target of structural variants (SVs) in both the primary and metastatic settings [14, 67], including tandem duplication in ~14% (14/101) mCRPC tumours over CRE2 [67], amplification, duplication and translocation over CRE3, CRE4, and CRE5 [14]. Notably, the translocation and duplication defining the FOXMIND enhancer driving *FOXA1* expression reported in primary and metastatic settings harbors the CRE3 element we characterized [14]. Collectively, these studies combined with our discoveries reveal the fundamental contribution of the *FOXA1* plexus in PCa 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 PCa through castration to suppress *AR* signalling [9], resistance ensues as 80% of mCRPC tumours harbor either *AR* gene amplification, amplification of a CRE upstream of *AR*, or activating *AR* coding mutations [16, 60, 67]. Given the *AR*-dependent [20, 23] and *AR*-independent [30] oncogenic activity of *FOXA1* in PCa, its inhibition is an appealing alternative therapeutic strategy. Our dissection of the *FOXA1* *cis*-regulatory landscape complements 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* 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 PCa 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 [14, 15, 18], our findings support the oncogenic nature of *FOXA1* in PCa. Gaining insight on the *cis*-regulatory plexuses of important genes such

as *FOXA1* in PCa 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% CO₂. These PCa 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 50 mg/mL Geneticin (4727894001, Sigma-Aldrich). The cells are regularly tested for *Mycoplasma* contamination. The authenticity of these cells was confirmed through short tandem repeat (STR) profiling.

1.5.2 Prostate tumours and cancer cell lines expression

Cancer cell line mRNA abundance data were collected from DEPMAP (<https://depmap.org/portal/>); RNA-seq TPM values from 2018q4 version with all 5 non-cancer cell lines were removed) [68] projects. Prostate tumour mRNA abundance data was collected from TCGA prostate cancer (TCGA-PRAD) project via the Xena Browser (<https://xenabrowser.net/>; dataset description: TCGA prostate adenocarcinoma gene expression by RNA-seq (polyA+ Illumina HiSeq; RSEM)).

1.5.3 Prostate cancer cell line gene essentiality

Essentiality scores were collected from the DEPMAP Project [69]. To compare gene essentiality between PCa cell lines and others, essentiality scores for *FOXA1* were collected from all available cell lines ($n = 707$). To perform a permutation test, the median expression 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 PCa 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 mRNAMax 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 µg 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 C3D method based on DNase I Hypersensitivity [42]. Predicted interacting DHS with a Pearson's correlation above 0.7 [70] 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 (experiment accession ENCSR346DCU; FASTQ file accessions: ENCFF726LGW, ENCFF550SKU, ENCFF950CQX, and ENCFF411TZJ; TADs file accession: ENCFF139JCA). Visualization of the Hi-C dataset is available on the Hi-C Browser [71].

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 µg/mL for LNCaP cells, 6 µg/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 (primary 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; secondary 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 LentidCas9-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 μ g/mL for LNCaP cells, 6 μ g/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 °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 °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 CRISPR RNA (crRNA) molecules (Integrated DNA Technologies) were designed to tile across the region of interest using the CRISPOR tool (<http://crispor.tefor.net/>) [72] and the Zhang lab CRISPR Design tools (<http://crispr.mit.edu/>) [73]. See published manuscript for gRNA sequences. Each crRNA and trans-activating CRISPR RNA (tracrRNA) (Integrated DNA Technologies) were duplexed according to company supplier protocol to a concentration of 50 μ M. Upon generation of the clones, six guides (crRNA-tracrRNA duplexes) for each region of interest were pooled into a single tube (1 μ L each guide, 6 μ L per reaction) (Integrated DNA Technologies). Lastly, 1 μ L (100 μ M) of electroporation enhancer (Integrated DNA Technologies) was added to the mix (7 μ 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/>) [72] and the Zhang lab CRISPR Design tools (<http://crispr.mit.edu/>) [73]. Each crRNA and tracrRNA (Integrated DNA Technologies) were duplexed according to company supplier protocol to a concentration of 50 μ M. The six crRNA-tracrRNA duplexes were pooled into a single tube (6 μ L per reaction), prior to adding 1 μ L (5 μ g) of Alt-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 μ L (100 μ 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 gRNA-RNP complex (10 μ g of Alt-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 by normalizing against *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 [74]. 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 μ g/mL, ThermoFisher) and blasticidin (7 μ g/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 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 WT and variant sequences were co-transfected with the pRL Renilla plasmid (Promega) using Lipofectamine 2000. 48 h 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 WT sequence and the mutant sequence used in the luciferase reporter assay were transfected into 7 million cells (2 μ g per allele, per 1 million cells) using Lipofectamine 2000 (ThermoFisher Scientific), per manufacturer's instructions. Next day, each an-

tibody (*FOXA1* 5 µg, Abcam, ab23738; *AR* 5 µg, Abcam, ab1083241; HOXB13 5 µg, Abcam, ab201682; SOX9 5 µg, Abcam, ab3697; GATA2 5 µg, Abcam, ab22849; FOXP1 5 µg, Abcam, ab16645; NKX3.1 10 µl, 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 µL 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.

1.6 Data availability

Genomic and Epigenomic data sets used to support this study can be found from the following accession codes: primary tumors—H3K27ac ChIP-seq (GSE96652), SNVs called from primary tumors (<https://dcc.icgc.org/projects/PRAD-CA>), *FOXA1*, *AR*, and HOXB13 ChIP-seq in primary prostate tumors is available under the following accession code: GSE137527 and EGAS00001003928, TF ChIP-seq data were from public databases of ReMap and ChIP-Atlas. All other relevant data supporting the key findings of this study are available within the article and its Supplementary Information files or from the corresponding author upon reasonable request.

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