

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

by

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

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Abstract

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Chapter 1

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

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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 textapprox 12% of mCPRC 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 (Figure 1.1a), ranking in the 95th percentile for 492 of 497 prostate tumours profiled in TCGA (Figure A.1a). 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 (Figure A.1b). 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 (Figure A.2a). 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 (Figure A.2b). 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 (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 prostate cancer cell proliferation (permutation test, $p = 1 \cdot 10^{-6}$, see Methods) (Figure A.3a). Growth assays in LNCaP and VCaP cells following FOXA1 knockdown using two independent siRNAs (Figure 1.1d, Figure A.3b) 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 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

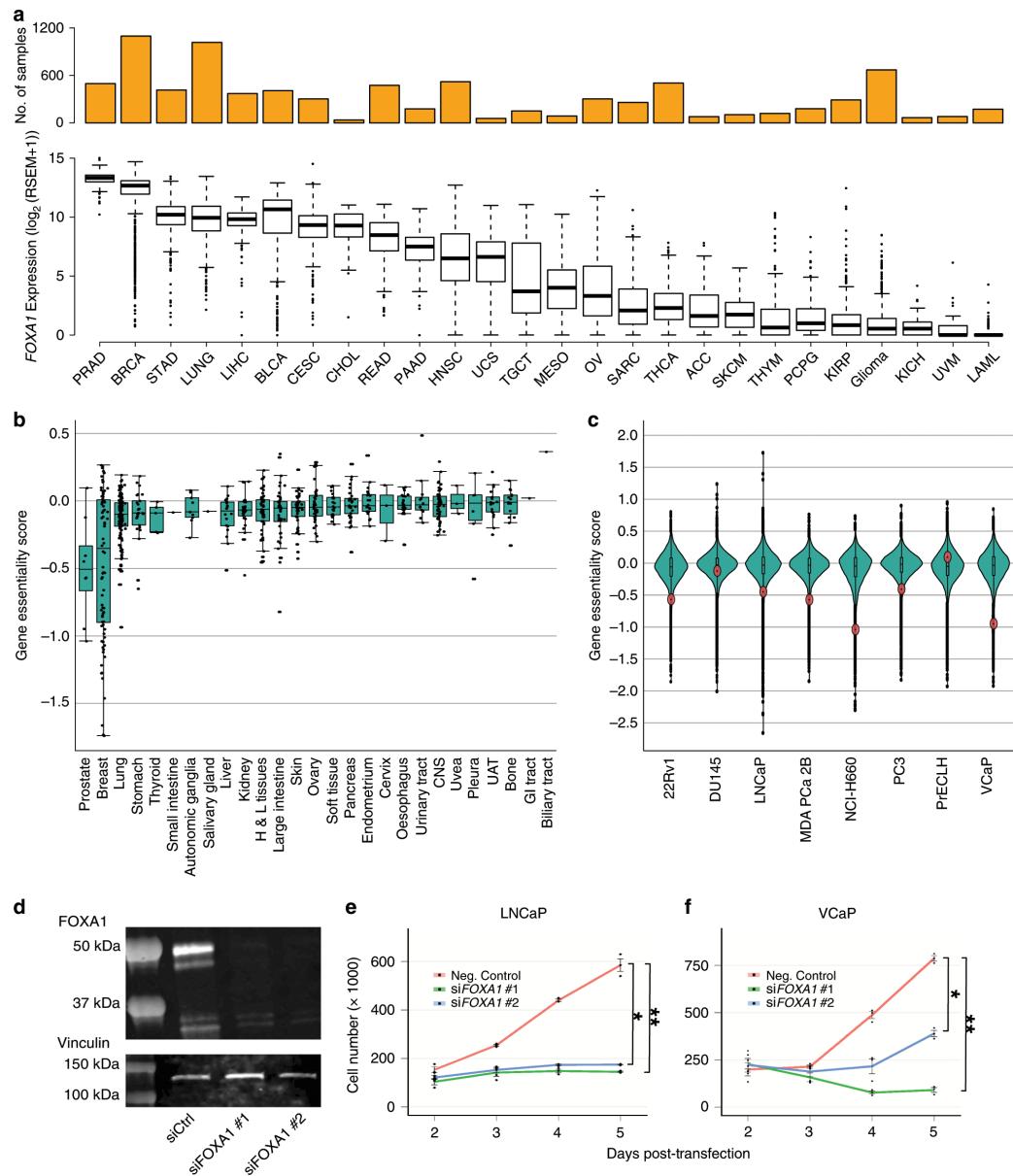


Figure 1.1: FOXA1 is highly expressed in prostate cancer and essential for prostate cancer cell proliferation.. **a.** The mRNA expression of FOXA1 across tumour types ($n = 26$) from RNA-seq data of TCGA. **b.** FOXA1 essentiality mediated through RNAi across various cell lines ($n = 707$) from DEPMAP. 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, * $p < 0.05$, ** $p < 0.01$.

interactions orchestrated by various factors including ZNF143, YY1, CTCF and the cohesin complex [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 ± 40 kbp adjusting for resolution) (Figure 1.2a). 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 1.2a). 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 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 with available ChIP-seq data for histone modifications and TFs conducted in LNCaP, 22Rv1, VCaP prostate cancer cell lines and primary prostate tumours (Figure 1.2b) [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 1.2b) [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 1.2b). We observe that these 6 CREs can be bound by multiple TFs in prostate cancer cells, including FOXA1, AR and HOXB13 (Figure 1.2b, Figure A.4). 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 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 prostate cancer. The Hi-C and C3D predictions suggest that they regulate FOXA1 expression.

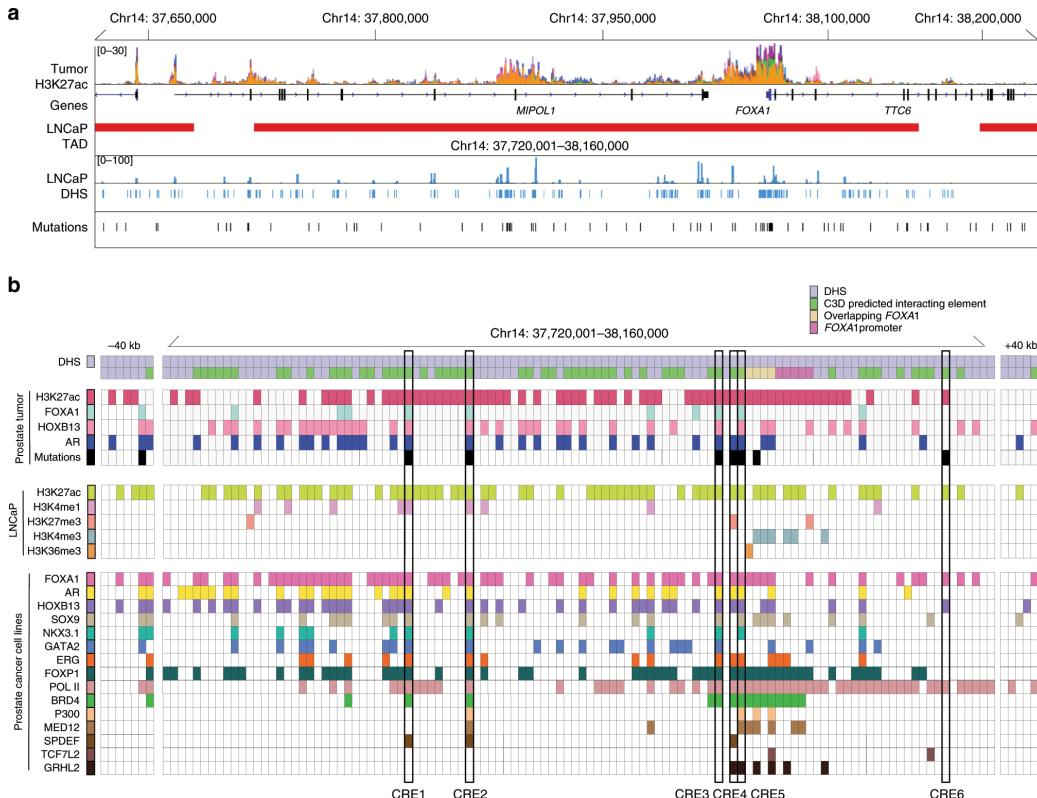


Figure 1.2: Epigenetic annotation of 14q21.1 locus and identification of *FOXA1* CREs..

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*. Mutations indicate SNVs identified in 200 primary prostate tumours. **b.** Functional annotation of putative *FOXA1* CREs using transcription factor and histone modification ChIP-seq conducted in primary tumours and prostate cancer 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.

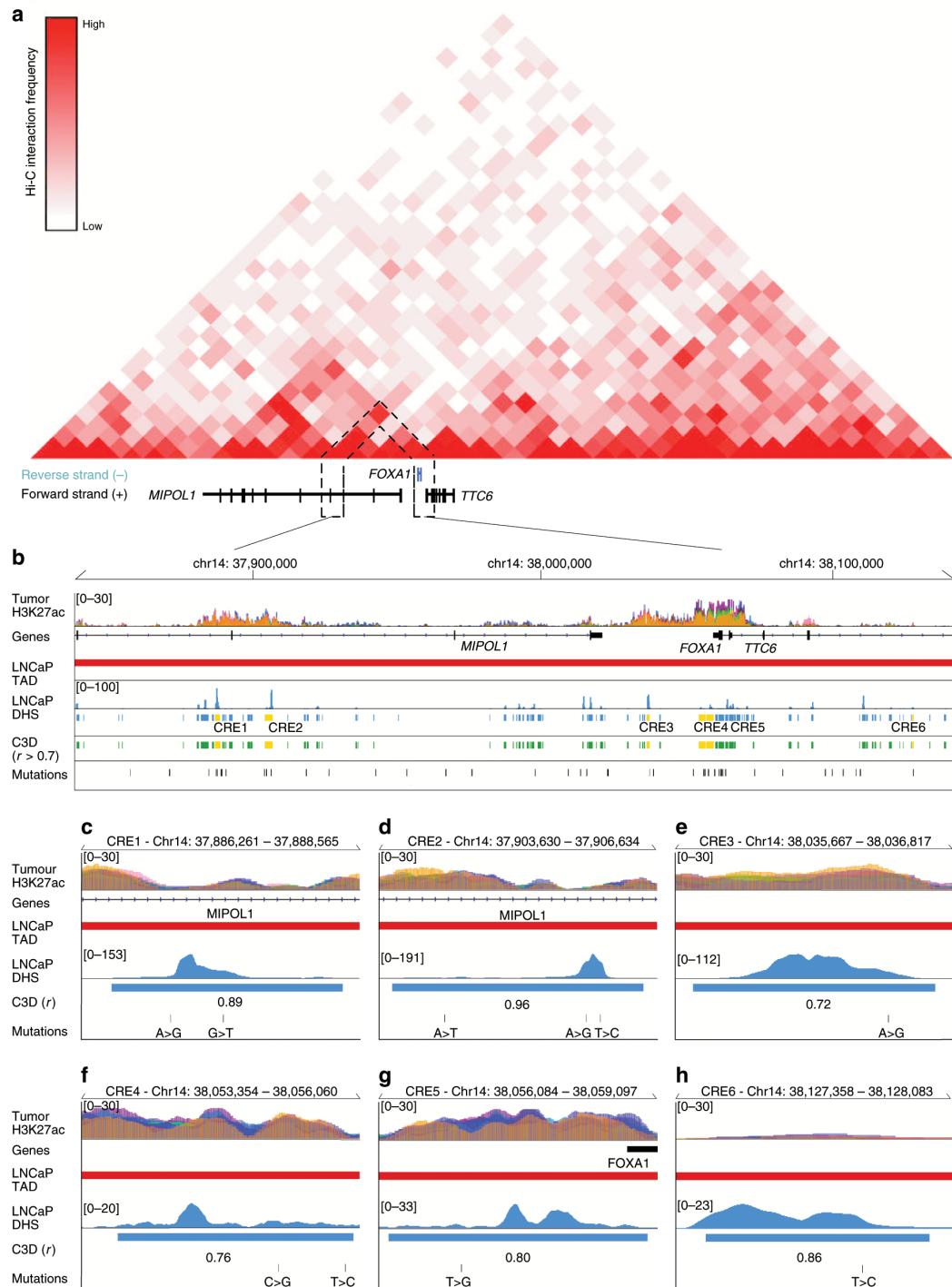


Figure 1.3: Putative CREs predicted to interact with *FOXA1* promoter. **a.** Hi-C conducted in LNCaP cells indicating physical interactions between putative *FOXA1* CREs and the *FOXA1* promoter. Hi-C resolution is 40 kbp. **b.** The six putative *FOXA1* CREs are coloured in yellow. **c-h.** Zoom-in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

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 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 (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 (Δ CRE1
textapprox 29.3 ± 8.3%, Δ CRE2
textapprox 40.1 ± 11.8%, Δ CRE 3
textapprox 30.6 ± 9.1%, Δ CRE4
textapprox 23.6 ± 8.2%, Δ CRE5
textapprox 25.3 ± 6.6%, Δ CRE6
textapprox 24.5 ± 10.2% and Δ FOXA1 (exon 1 and intron 1)
textapprox 87.4 ± 8.8% reduction relative to basal levels) (Figure 1.4c, Figure A.5a-f). In contrast, deletion of several negative control regions within the same TAD did not significantly reduce FOXA1 mRNA level (Figure 1.4c, Figure A.5g-i). Similar results were observed in 22Rv1 prostate cancer 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 $r = 0.49, p < 0.005$) (Figure A.6a) and agreeing trends for each individual CRE (Figure A.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 1.4e-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
textapprox 24.6 ± 6.2%, iCRE2
textapprox 42.2 ± 10.8%, iCRE3
textapprox 25.3 ± 9.2%, iCRE4

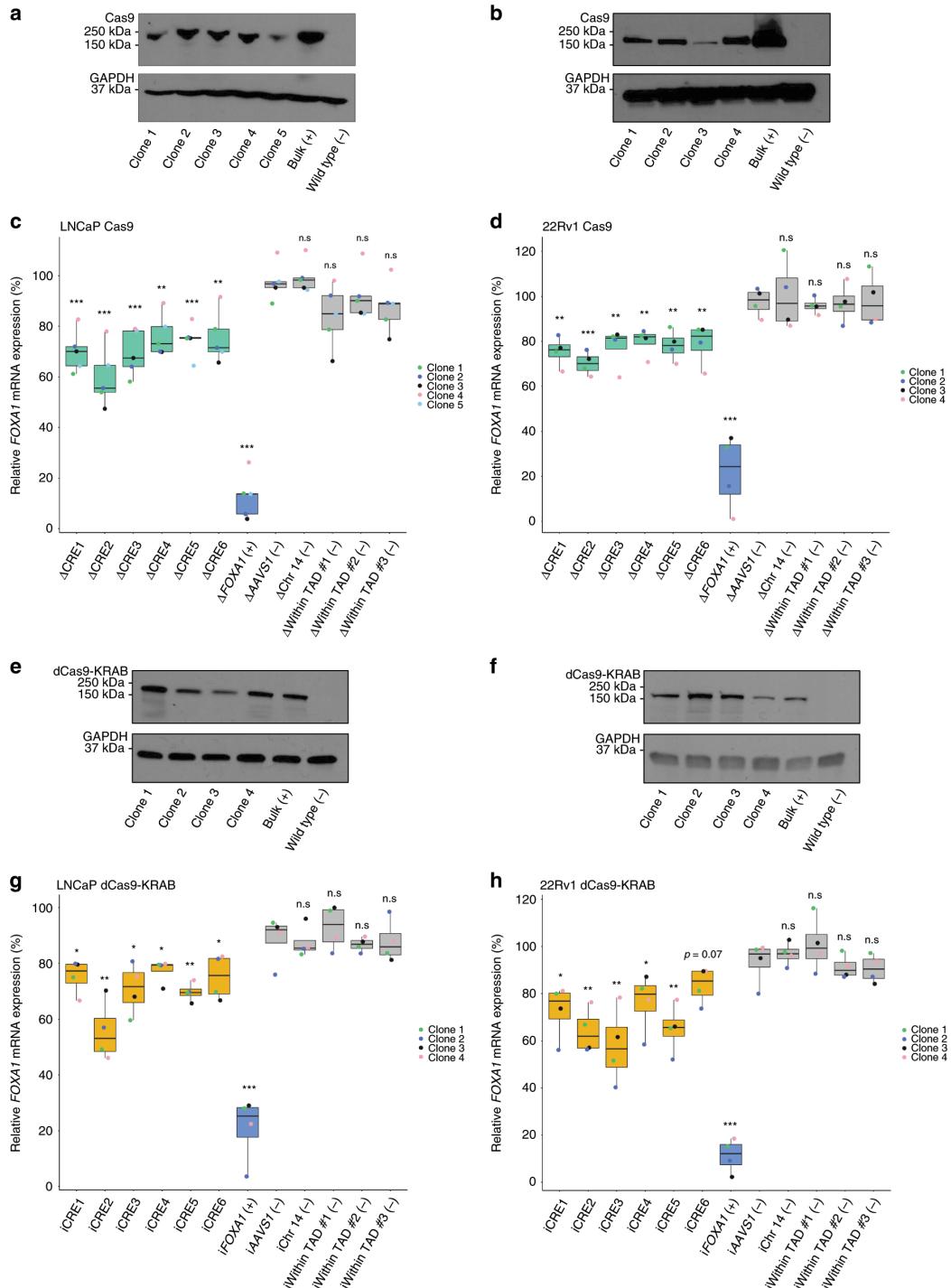


Figure 1.4: Functional dissection of putative *FOXA1* CREs. (Continued on the following page)

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, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

textapprox $23.3 \pm 4.3\%$, iCRE5

textapprox $30.2 \pm 3.4\%$ and iCRE6

textapprox $23.1 \pm 8.1\%$ reduction). Similarly, gRNAs targeting the dCas9-KRAB fusion protein to *FOXA1* decreased its expression (iFOXA1

textapprox $81.6 \pm 11.8\%$ 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

textapprox $38.4 \pm 6.4\%$ and

textapprox $48.4 \pm 9\%$, 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, $p < 0.05$, Figure A.7a).

On the other hand, deletion of CREs each significantly reduced TTC6 mRNA expression relative to its basal levels (Δ CRE1

textapprox $52.9\% \pm 6.4\%$, Δ CRE2

textapprox $66 \pm 11.3\%$, Δ CRE3

textapprox $55.5 \pm 12.8\%$, 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, $p < 0.05$, Figure A.7b), in agreement with the fact that TTC6 shares its

promoter with FOXA1 as both genes are transcribed on opposing strands (Figure A.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; Δ CRE1
textapprox 190%, Δ CRE2
textapprox 162.8%, Δ CRE3
textapprox 147.5%, Δ CRE4
textapprox 133.3%, Δ CRE5
textapprox 137.3%, Δ CRE6
textapprox 120.8%, Δ FOXA1
textapprox 266.7%), ACPP (down-regulation; Δ CRE1
textapprox 73.5%, Δ CRE2
textapprox 62.5%, Δ CRE3
textapprox 69.6%, Δ CRE4
textapprox 75.6%, Δ CRE5
textapprox 70.9%, Δ CRE6
textapprox 74.6%, Δ FOXA1
textapprox 52.2%) and GRIN3A expression (up-regulation; Δ CRE1
textapprox 138.2%, Δ CRE2
textapprox 168.8%, Δ CRE3
textapprox 144.6%, Δ CRE4
textapprox 132.1%, Δ CRE5
textapprox 131.4%, Δ CRE6
textapprox 127%, Δ FOXA1
textapprox 228%) (Student's t-test, $p < 0.05$, Figure A.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: (Δ CRE1
textapprox $29.3 \pm 7.3\%$, Δ CRE2
textapprox $36 \pm 11.8\%$, Δ CRE3
textapprox $30.6 \pm 12.7\%$, Δ CRE4
textapprox $24.5 \pm 6.1\%$, Δ CRE5
textapprox $23.7 \pm 13.2\%$, Δ CRE6
textapprox $26.8 \pm 14.2\%$ and Δ *FOXA1*
textapprox $96.2 \pm 1.4\%$ reduction (Student's t-test, $p < 0.05$, Figure 1.5a, Figure A.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 Δ CRE1 + Δ CRE2, Δ CRE1 + Δ CRE4, and Δ CRE2 + Δ CRE4 resulted in a significant
textapprox $48.5 \pm 4.5\%$,
textapprox $50.4 \pm 2.9\%$ and
textapprox $45.2 \pm 5.5\%$ reduction in *FOXA1* mRNA expression, respectively (Student's t-test, $p < 0.05$, Figure 1.5b, Figure A.9a-f) a fold reduction greater than single CRE deletions (Student's t-test, Figure A.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 1.1b-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*

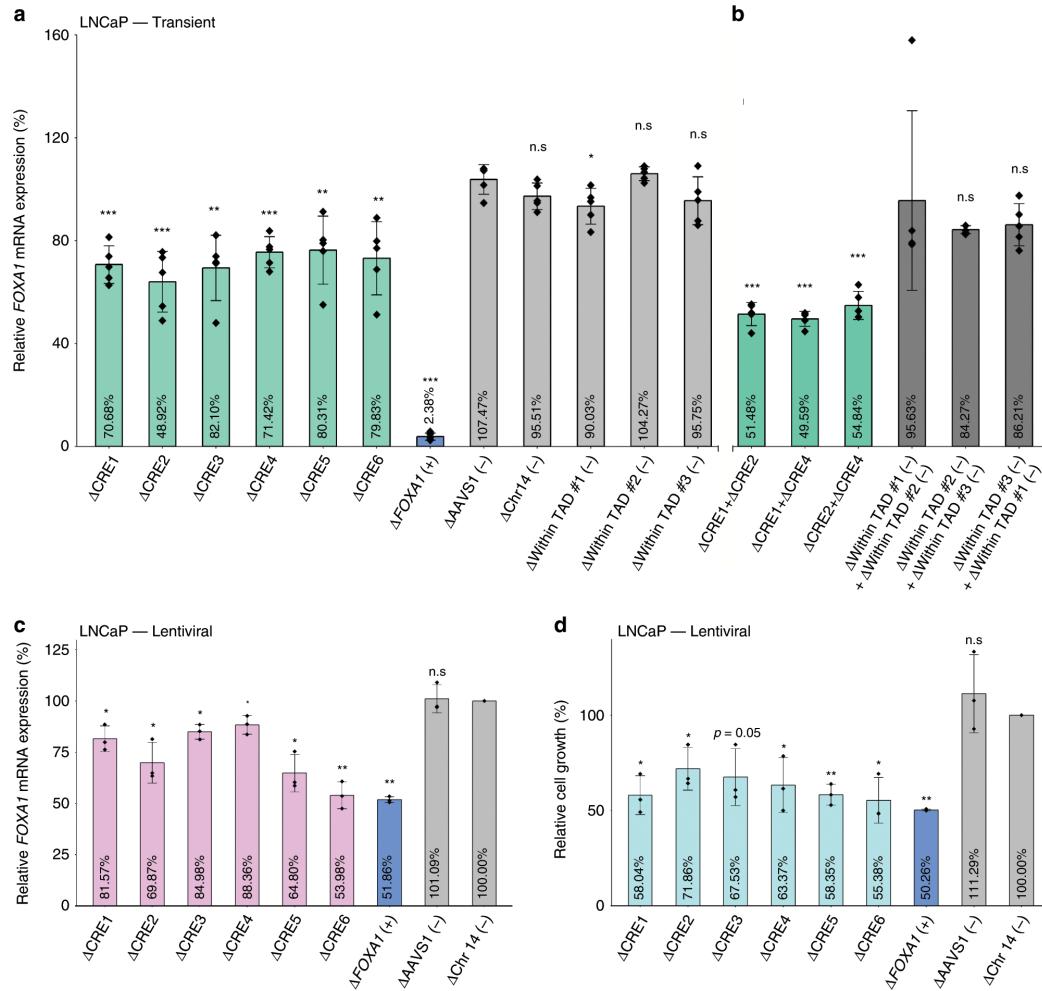


Figure 1.5: ***FOXA1* CREs collaborate to regulate its expression and are critical for prostate cancer cell proliferation.** **a.** *FOXA1* mRNA expression normalized to housekeeping *TBP* mRNA expression upon transient transfection-based CRISPR/Cas9-mediated deletion of CRE1, CRE2, CRE4, and sequential deletion combinations ($n = 5$ independent experiments). **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, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

mRNA expression (Δ CRE1

textapprox 18%, Δ CRE2

textapprox 30%, Δ CRE3

textapprox 15%, Δ CRE4

textapprox 12%, Δ CRE5

textapprox 35%, Δ CRE6

textapprox 46% and Δ FOXA1 (exon 1 and intron 1)

textapprox 48% reduction (Student's t-test, $p < 0.05$, Figure 1.5c, Figure A.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 (Δ CRE1

textapprox 42%, Δ CRE2

textapprox 28%, Δ CRE3

textapprox 33%, Δ CRE4

textapprox 27%, Δ CRE5

textapprox 42%, Δ CRE6

textapprox 44% and Δ FOXA1 (exon 1 and intron 1)

textapprox 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 1.6a). 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 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 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 1.6b, d-h). These results indicate that these SNVs can alter the transactivation potential of FOXA1 plexus CREs in prostate cancer cells.

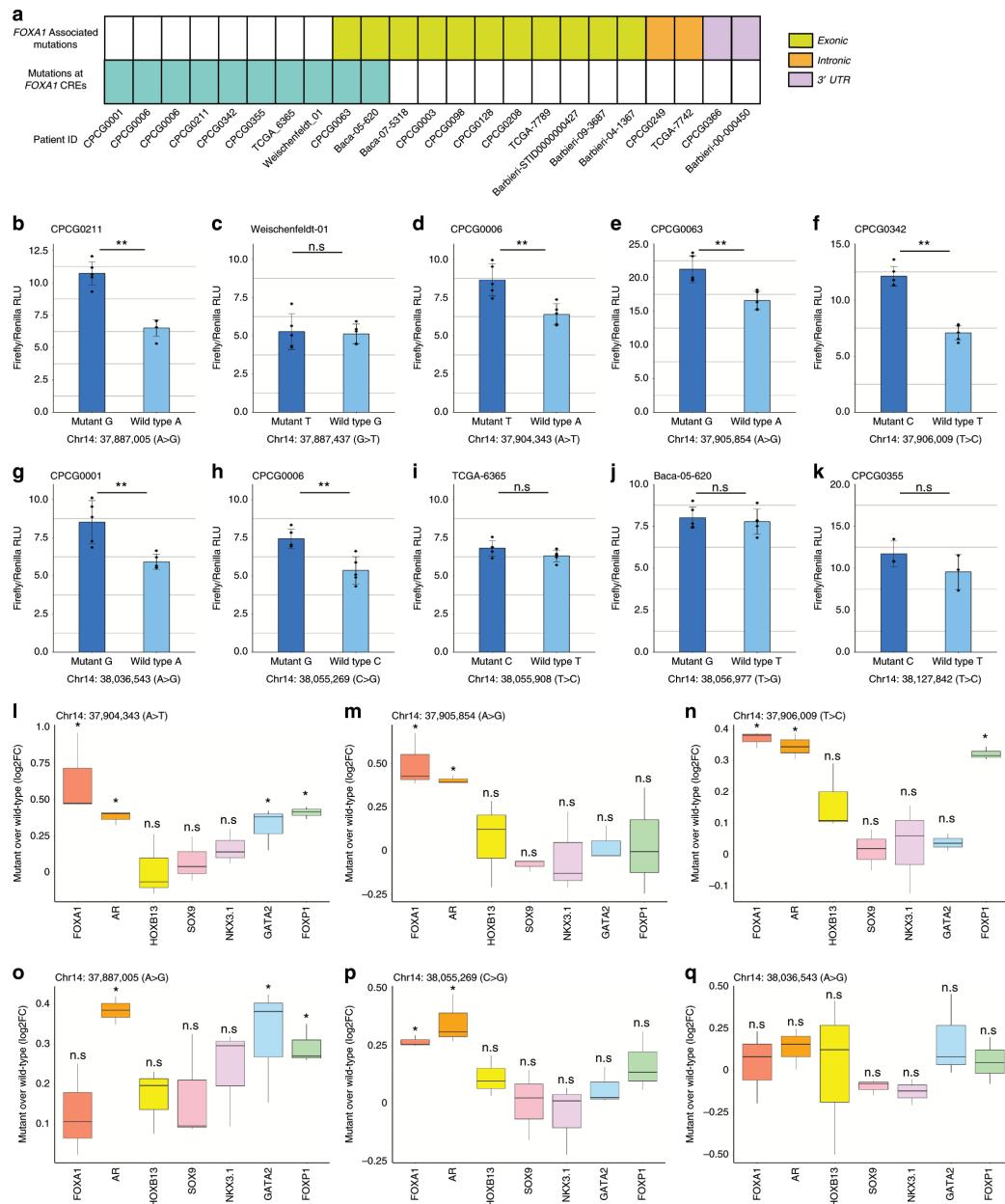


Figure 1.6: A subset of noncoding SNVs mapping to the *FOXA1* CREs are gain-of-function. **a.** Matrix showcasing the patients from the CPC-GENE dataset that harbour SNVs at the *FOXA1* CREs, exons, introns, and the 3' UTR of *FOXA1*. **b-k.** Luciferase assays are conducted in LNCaP cells. Bar plot showcases the mean firefly luciferase activity normalized by *renilla* luciferase activity. 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 prostate cancer 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, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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 1.6l-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 textapprox 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% CO₂. 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) [63] 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 [64]. 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 μ 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>) [37]. Predicted interacting DNase I Hypersensitivity Sites (DHS) with a Pearson’s correlation above 0.7 [65] 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/>) [66].

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 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. 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 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 $^{\circ}$ 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/>) [67] and the Zhang lab CRISPR Design tools (<http://crispr.mit.edu/>) [68]. 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 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 Nu-

cleofection (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 nucleic acid 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/>) [67] and the Zhang lab CRISPR Design tools (<http://crispr.mit.edu/>) [68]. 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 \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 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 guide RNA-RNP complex (10 μ g of Alt-R \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 [69]. 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 µg/mL, ThermoFisher) and blasticidin (7 µg/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 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 μ g per allele, per 1 million cells) using Lipofectamine 2000 (ThermoFisher Scientific), per manufacturer's instructions. Next day, each antibody (FOXA1 5 μ g, Abcam, ab23738; AR 5 μ g, Abcam, ab1083241; HOXB13 5 μ g, Abcam, ab201682; SOX9 5 μ g, Abcam, ab3697; GATA2 5 μ g, Abcam, ab22849; FOXP1 5 μ g, Abcam, ab16645; NKX3.1 10 μ 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 μ 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.

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 tu-

mors (<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.

Chapter 2

Cis-regulatory Element Hijacking by Structural Variants Overshadows Higher-Order Topological Changes in Prostate Cancer

S.Z., J.R.H., and M.L. conceptualized the study. J.R.H. and S.Z. co-led the study with equal contributions and can be interchangeably listed as first author. S.Z. designed and conducted all the experiments with help from C.A. G.G., and K.K. J.R.H. implemented all the computational and statistical approaches and analyses. R.H.-W. pre-processed the RNA-seq data from the primary tumours. Figures were designed by S.Z. and J.R.H. The manuscript was written by S.Z., J.H., and M.L with assistance from all authors. T.v.d.K., M.F., P.C.B., R.G.B., and M.L supervised the study. M.L oversaw the study.

2.1 Abstract

Prostate cancer is a heterogeneous disease whose progression is linked to genome instability. However the impact of this instability on the three-dimensional chromatin organization and how this

drives progression is unclear. Using primary benign and tumour tissue, we find a high concordance in the higher-order three-dimensional genome organization across normal and prostate cancer cells. This concordance argues for constraints to the topology of prostate tumour genomes. Nonetheless, we identify changes to focal chromatin interactions and show how structural variants can induce these changes to guide *cis*-regulatory element hijacking. Such events result in opposing differential expression on genes found at antipodes of rearrangements. Collectively, our results argue that *cis*-regulatory element hijacking from structural variant-induced altered focal chromatin interactions overshadows higher-order topological changes in the development of primary prostate cancer.

2.2 Introduction

The human genome is organized into hubs of chromatin interactions within the nucleus, setting its three-dimensional topology [70]. Two classes of higher-order topology, topologically associating domains (TADs) and compartments, define clusters of contacts between DNA elements that are linearly distant from each other, such as *cis*-regulatory elements (CREs) and their target gene promoters [71, 72]. Insulating these hubs to prevent ectopic interactions are TAD boundaries, maintained by CCCTC-binding Factor (CTCF) and the cohesin complex [73]. Disruption of TAD boundaries through genetic or epigenetic variants can activate oncogenes, as observed in medulloblastoma [74], acute myeloid leukemia [75], gliomas [76], and salivary gland acinic cell carcinoma [77]. However, recent studies depleting CTCF or the cohesin complex produced little effect on gene expression despite global changes to the three-dimensional chromatin organization [78–80]. In contrast, CRE hijacking caused by genetic alterations can result in large changes to gene expression, despite having little impact on the higher-order chromatin organization [48, 74]. These contrasting observations raise questions about the interplay between components of the genetic architecture, namely, how genetic alterations, chromatin states, and the three-dimensional genome cooperate to misregulate genes in disease. Understanding the roles that chromatin organization and *cis*-regulatory interactions play in gene regulation is crucial for understanding how their disruption can promote oncogenesis.

The roles of noncoding mutations targeting CREs in cancer are becoming increasingly clear [48, 81, 82]. Mutations to the TERT promoter, for example, lead to its over-expression and telomere elongation in multiple cancer types [45, 83, 84]. Similarly, mutations targeting CREs of the ESR1 and FOXA1 oncogenes in breast and prostate cancers, respectively, lead to their sustained over-expression [9, 32, 85], which is associated with resistance to hormonal therapies [86–89]. Point mutations have the potential to alter three-dimensional chromatin organization, albeit indirectly,

by modifying transcription factor or CTCF binding sites [90, 91]. Structural variants (SVs), on the other hand, are large rearrangements of chromatin that can directly impact its structure [92, 93]. This can establish novel CRE interactions from separate TADs or chromosomes, as has been observed in leukemia [94] and multiple developmental diseases [95, 96]. But how prevalent and to what extent these rearrangements affect the surrounding chromatin remains largely unstudied in primary tumours [82, 93, 97]. Hence, to understand gene misregulation in cancer, it is critical to understand how SVs impact three-dimensional chromatin organization and CRE interactions in primary tumours.

SVs play an important role in prostate cancer (PCa), both for oncogenesis and progression. An estimated 97% of primary tumours contain SVs [6, 82], and translocations and duplications of CREs for oncogenes such as AR [55], ERG [98], FOXA1 [9, 62] and MYC [9] are highly recurrent. While coding mutations of FOXA1 are found in

textapprox 10% of metastatic castration-resistant PCa patients, SVs that target FOXA1 CREs are found in over 25% of metastatic prostate tumours [9]. In addition to oncogenic activation, SVs in prostate tumours disrupt and inactivate key tumour suppressor genes including PTEN, BRCA2, CDK12, and TP53 [5, 62]. Furthermore, over 90% of prostate tumours contain complex SVs, including chromothripsis and chromoplexy events 38, making it a prime model to study the effects of SVs. However, despite large-scale tumour sequencing efforts, investigating the impact of SVs on three-dimensional prostate genome remains difficult, owing to constraints from chromatin conformation capture (i.e. Hi-C) assays. In this work, we build on recent technological advances in Hi-C protocols to investigate the three-dimensional chromatin organization of the prostate from primary benign and tumour tissues. Using patient-matched whole genome sequencing (WGS), RNA sequencing (RNA-seq), and chromatin immunoprecipitation (ChIP-seq) data, we show that SVs in PCa repeatedly hijacking CREs to disrupt the expression of multiple genes with minimal impact to higher-order three-dimensional chromatin organization.

2.3 Results

2.3.1 Three-dimensional chromatin organization is stable over oncogenesis

Chromatin conformation capture technologies enable the measurement of three-dimensional chromatin organization. These assays, however, are often limited to cell lines, animal models and liquid

tumours due to the amount of input required [99]. Here, we optimized and conducted low-input Hi-C [100] on 10 μm thick cryosections from 12 primary prostate tumours and 5 primary benign prostate sections (see Methods, Figure 2.1a, Supplementary Figure 1a). The 12 tumours were selected from the Canadian Prostate Cancer Genome Network (CPC-GENE) cohort previously assessed for whole-genome sequencing [6], RNA-seq [101], and H3K27ac ChIP-seq [38, 59] (Supplementary Table 1). All 12 of these PCa patients previously underwent radical prostatectomies and 6 of our 12 samples (50%) harbour the TMPRSS2-ERG genetic fusion (T2E) found in approximately half of the primary PCa patients [6]. The total percent of genome altered ranges from 0.99%-18.78% (Supplementary Table 1) [6]. The 12 tumour samples were histopathologically assessed to have $\geq 70\%$ cellularity while the cellularity was $\geq 60\%$ for our group of 5 normal prostate samples. Upon Hi-C sequencing, we reached an average of $9.90 \cdot 10^8$ read pairs per sample (range $5.84 \cdot 10^8 - 1.49 \cdot 10^9$ read pairs) with minimal duplication rates (range 10.6% - 20.8%) (Supplementary Table 2). Pre-processing resulted in an average of $6.23 \cdot 10^8$ (96.13%) valid read pairs per sample (range $3.95 \cdot 10^8 - 9.01 \cdot 10^8$, or 82.42% - 99.22%; Supplementary Table 2). Hence, we produced a high depth, high quality Hi-C library on 17 primary prostate tissue slices.

To characterize the higher-order organization of the primary prostate genome, we first identified TADs. Across the 17 primary tissue samples, we observed an average of 2,305 TADs with a median size of 560 kbp (Supplementary Tables 3-4). However, when considering all hierarchical levels of TAD organization, we did not observe significant differences in the number of TADs identified across length scales (Figure 2.1b), nor in the persistence of their boundaries (Figure 2.1c). This suggests few, if any, differences in three-dimensional chromatin organization at the TAD level between benign and tumour tissue. Notably, we observed differences in organization around essential genes for PCa between primary tissue and previously profiled cell lines. For example, chromatin around the AR gene that was previously found enriched in the 22Rv1 compared to RWPE1 prostate cell lines [52] were not recapitulated in either benign or tumour primary samples (Figure 2.1d, Supplementary Figure 1d). Moreover, when compared to other Hi-C datasets, the primary prostate samples clustered separately from cell lines (Supplementary Figure 1b), despite similar enrichment of CTCF binding sites near TAD boundaries (Supplementary Figure 1c). These results suggest that TADs are constrained over oncogenesis and that cell line models may not harbour disease-relevant three-dimensional chromatin organization.

We next investigated compartmentalization changes, the second class of higher-order three-dimensional chromatin organization. Recurrent changes to segments nearly the size of chromosome arms showed differential compartmentalization in multiple tumour samples compared to benign

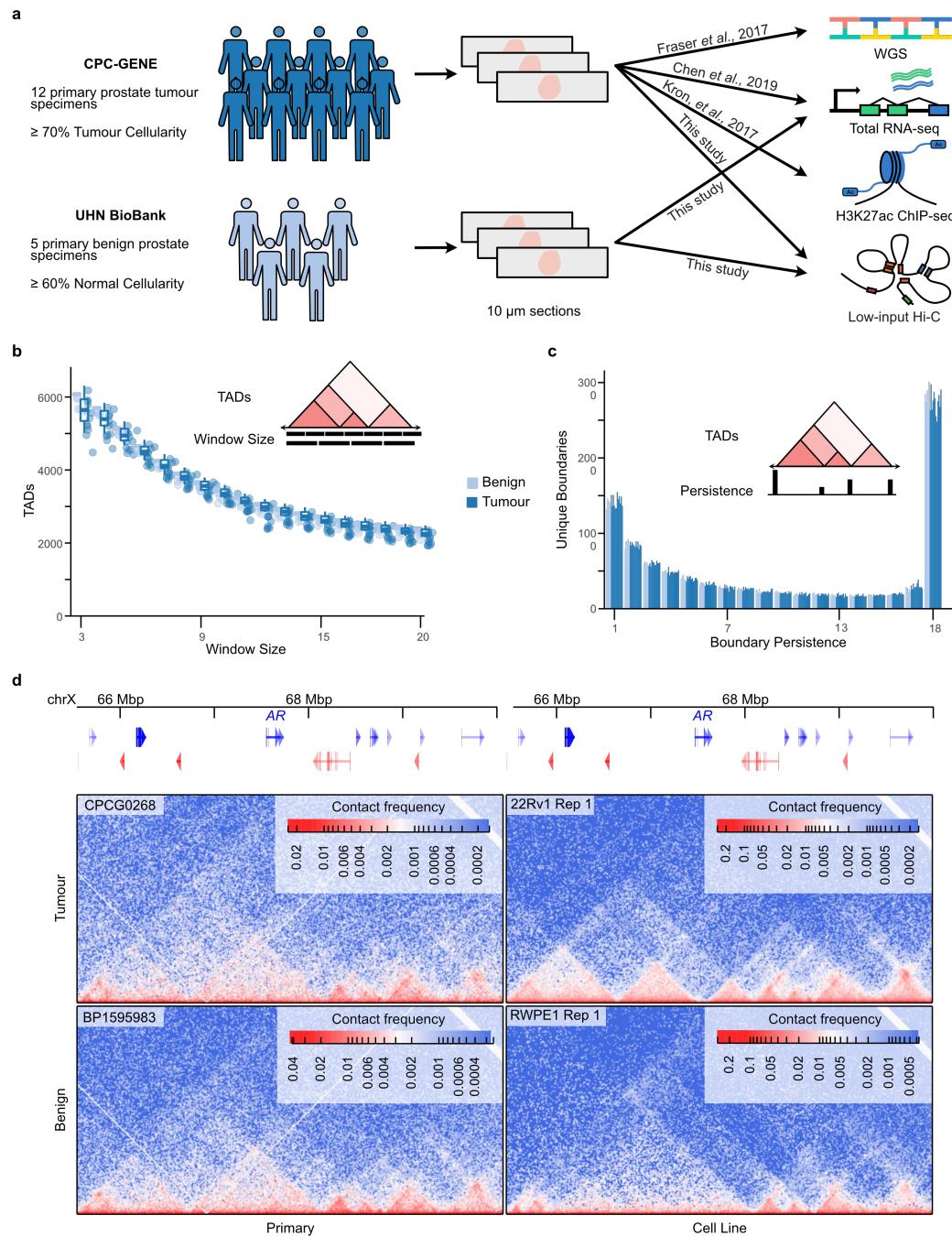


Figure 2.1: Topologically associated domains are stable over prostate oncogenesis. **a.** The sample collection and data usage of primary prostate samples in this study. 10 μm sections from 6 tumours previously identified as T2E+ and 6 T2E- were used for Hi-C sequencing. 5 additional 10 μm sections were collected from benign prostate specimens in the UHN BioBank. **b-c.** A comparison of the number of TADs detected at multiple window sizes (**b**) and boundary persistence (**c**) in each patient sample, with inset schematics. **d.** Contact matrices around the *AR* gene in primary samples and cell lines. Hi-C data for 22Rv1 and RWPE1 cell lines obtained from Rhie *et al.*, 2019.

samples, such as compartment B-to-A transitions on 19q and A-to-B transitions on chromosome Y (Supplementary Figure 2a-c). Only two genes on chromosome 19 were differentially expressed between the 8 tumours with benign-like compartmentalization and the other 4 (Supplementary Figure 2d). Similarly, no genes on chromosome Y were differentially expressed between the 4 tumours with benign-like compartmentalization and the remaining samples (Supplementary Figure 2e). Both arms on chromosome 3 show differential mean compartmentalization, but this appears to be driven by one tumour sample and one benign sample for each arm and is not recurrent (Supplementary Figure 2f). Collectively, these results suggest that phenotypic differences between benign and tumour tissues do not stem from differences in higher-order three-dimensional chromatin organization alone.

2.3.2 Focal chromatin interactions shift over oncogenesis

Changes to focal chromatin interactions have been observed in the absence of higher-order chromatin changes [102, 103], and we hypothesized that this may be the case in PCa. We detected chromatin interactions, identifying a median of 4,395 interactions per sample (range 1,286 - 6,993; Supplementary Figure 3a, Supplementary Table 5). Among these detected interactions, we identified known contacts in PCa such as those between two distal CREs on chromosome 14 and the FOXA1 promoter [85] (Supplementary Figure 3b), and CREs upstream of MYC on chromosome 8 that are frequently duplicated in metastatic disease [62] (Supplementary Table 5). 16,474 unique chromatin interactions were identified in at least one sample (Figure 2.2a), reaching an estimated textapprox 80% saturation of detection (Supplementary Figure 3c). Restricting our analysis to the 8,486 interactions present in at least two samples (51.5% of all interactions) yielded 1,405 tumour- and 273 benign-specific interactions, suggesting focal changes in three-dimensional chromatin organization occur over oncogenesis. Aggregate peak analysis revealed Hi-C contact enrichment at all detected interactions in all samples (Figure 2.2b-c), demonstrating that tumors- and benign-specific interactions are not binary. Rather, the contacts at “tumour-specific” loci are more enriched than those at “benign-specific” loci in tumour samples (Figure 2.2b). Similarly, the contacts at “benign-specific” loci are more enriched than those at “tumour-specific” loci in benign samples (Figure 2.2c). Together, these results suggest that more focal changes to chromatin interactions are present in prostate oncogenesis despite the stable higher-order organization.

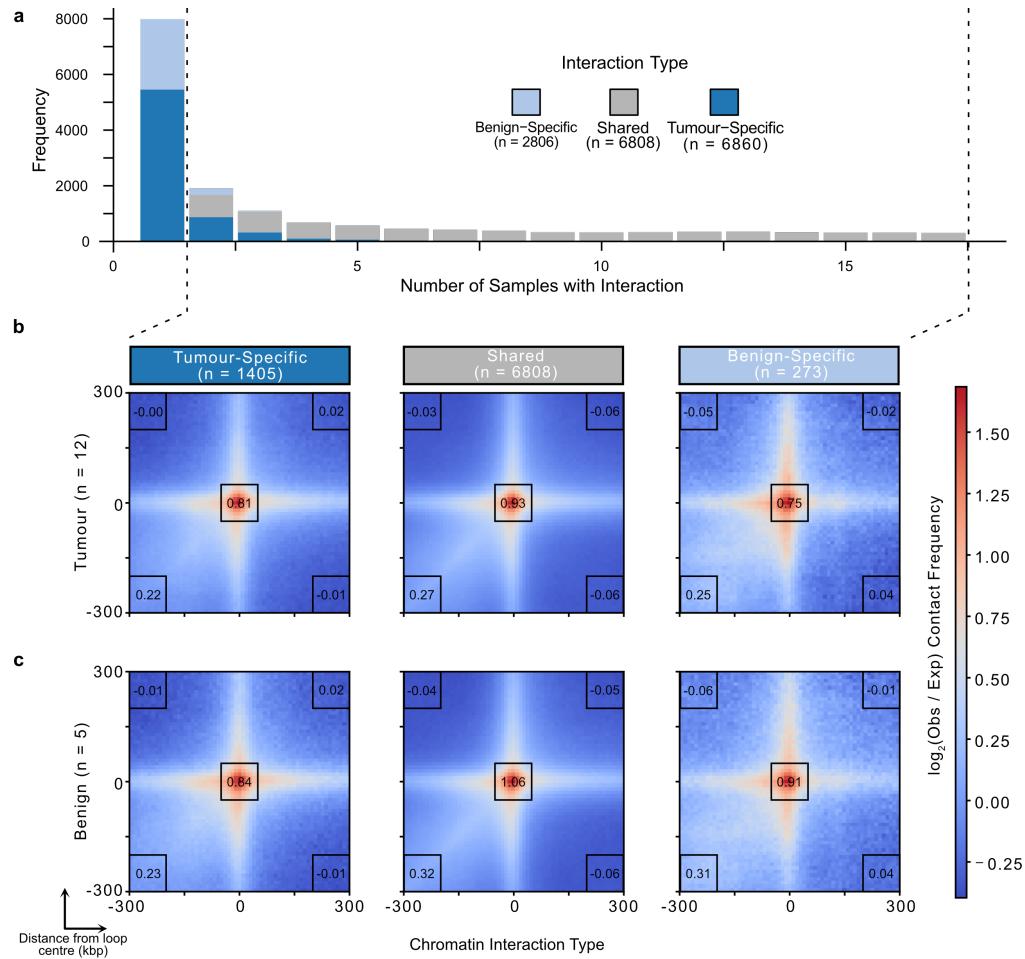


Figure 2.2: Focal chromatin interactions display subtle differences between benign and tumour tissue. **a.** Stacked bar plots of the number of samples that chromatin interactions were identified in. **b-c.** Aggregate peak analysis of tumour (**b**) or benign (**c**) contacts in tumour-specific, benign-specific, and shared interactions identified in two or more samples. Regions plotted are ± 300 kbp around the centre of each identified interaction. Inset numbers are the mean $\log_2(\text{obs}/\text{exp})$ contact frequencies within the 100 kbp \times 100 kbp black boxes.

2.3.3 Cataloguing structural variants from Hi-C data

In prostate tumours, SVs populate the genome to aid disease onset and progression [6, 62]. Advances in computational methods now enable the identification of SVs from Hi-C datasets [92, 104]. Applying an SV caller to our primary prostate tumour Hi-C dataset [92], we detected a total of 317 unique breakpoints with a median of 15 unique breakpoints per tumour (range 3-95; Figure 2.3a; Supplementary Table 6). As an example, we found evidence of the TMPRSS2-ERG (T2E) genetic fusion spanning the 21q22.2-3 locus in 6/12 (50%) patients (CPCG0258, CPCG0324, CPCG0331, CPCG0336, CPCG0342, and CPCG0366) (Figure 2.3b), in accordance with previous whole-genome sequencing (WGS) findings [6]. Combining unique breakpoint pairs into rearrangement events yielded 7.5 total events on average per patient (range 1 - 36, Supplementary Figure 4a-b). We also identified more inter-chromosomal breakpoint pairs with the Hi-C data in 11 of 12 tumours (Figure 2.3b), including a novel translocation event that encompasses the deleted region between TMPRSS2 and ERG into chromosome 14. Few loci contained SV breakpoints recurrent between patients (Supplementary Figure 4c). These numbers are smaller than previously reported from matched WGS data [6]; however, the median distance between breakpoints on the same chromosome was much larger at 31.6 Mbp for Hi-C-identified breakpoints, compared to 1.47 Mbp from WGS-identified breakpoints (Figure 2.3c). This is consistent with the inherent nature and resolution of the Hi-C method to detect larger, inter-chromosomal events [92]. No SVs were detected in the 5 primary benign prostate tissue samples from Hi-C data. While this does not rule out the presence of small rearrangements undetectable by Hi-C limited by its resolution, the absence of large and inter-chromosomal SVs further supports a difference in genome stability between benign and tumour tissues [6, 59, 105, 106]. Collectively, Hi-C defines a valid method to interrogate for the presence of SV in tumour samples, compatible with the detection of intra- and inter-chromosomal interactions otherwise missed in WGS analyses.

Among SVs detected in primary prostate tumours, we identified both simple and complex chains of breakpoints. While simple SVs correspond to fusion between two distal DNA sequences, complex chains are evidence of chromothripsis and chromoplexy [106]. These genomic aberrations affecting multiple regions of the genome are known to occur in both primary and metastatic PCa [6, 82, 106]. The chains can be pictured as paths connecting breakpoints in the contact matrix (Supplementary Figure 4d). 8 of the 12 (66.7%) tumour samples contained these chains, including one patient (CPCG0331) harbouring 11 complex events and three patients (CPCG0246, CPCG0345, and CPCG0365) each harbouring a single complex event. We observed a median of 1 complex event

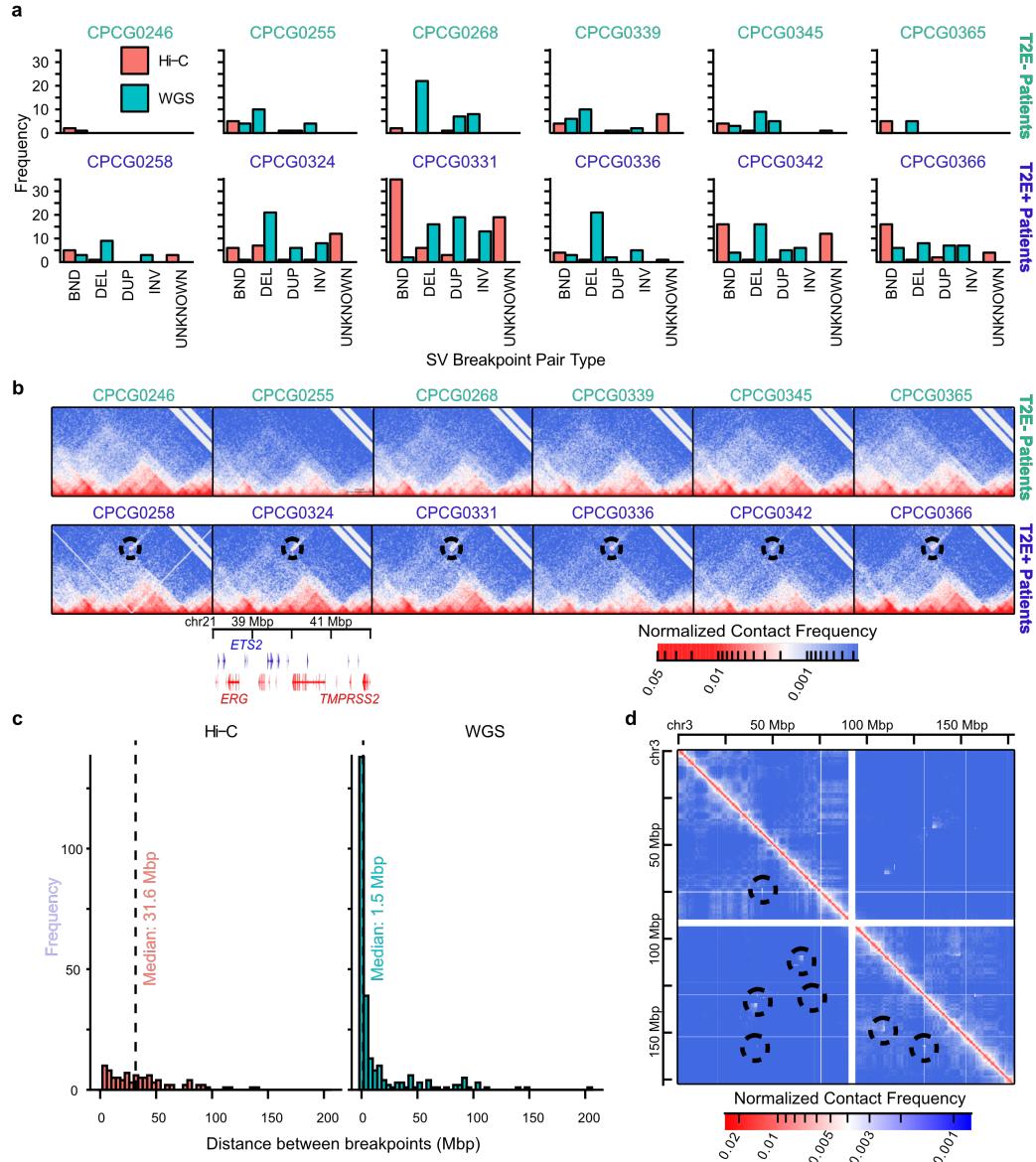


Figure 2.3: SVs are identified in primary tissue through chromatin conformation capture. **a.** Bar plot of SV breakpoint pairs identified by Hi-C and WGS on matched samples. BND = inter-chromosomal translocation, DEL = deletion, DUP = duplication, INV = inversion, UNKNOWN = breakpoint pair of unknown type. **b.** Hi-C contact matrices of the chr21:37-42 Mbp locus harbouring the *TMPPRSS2* and *ERG* genes. Circles indicate increased contact between *TMPPRSS2* and *ERG* in the T2E+ tumours. **c.** Histogram showing the distance between breakpoints on the same chromosome detected by Hi-C (left) versus WGS (right). **d.** An example of a complex set of rearrangements spanning both arms of chromosome 3 in a single patient.

per patient (range 0-11) consisting of a median of 3 breakpoints (range 3-7) spanning a median of 2 chromosomes per event (range 1-4, Supplementary Table 7, Supplementary Figure 4b). Patient CPCG0331 had 11 complex events, including a 6-breakpoint event spanning 3 chromosomes (Supplementary Figure 4b). A highly rearranged chromosome 3 was also found in the same patient (Figure 2.3d). The most common type of complex event involved 3 breakpoints and spanned 2 chromosomes, occurring 9 times across 5 of the 8 patients with complex events. In summary, using Hi-C, we detected both simple and complex SVs in primary prostate tumours not previously identified using WGS-based methods. We were able to identify known observations, such as a highly mutated region on chromosome 3 and subtype-specific differences in abundance, as well as find novel inter-chromosomal events not previously reported.

2.3.4 SVs alter gene expression independently of intra-TAD contacts

Using combined WGS called SVs with those from Hi-C data, we next systematically examined the impact of SVs on TAD structure. This led us to look at the intra-TAD and inter-TAD interactions around each breakpoint. We observed that only 18 of the 260 (6.9%) TADs containing SV breakpoints were associated with decreased intra-TAD or increased inter-TAD interactions (Figure 2.4a). 12 of 18 (66.7%) occurrences were within T2E+ tumours. We found no evidence that simple versus complex SVs were a factor in determining whether a TAD was altered (Pearson's χ^2 test, $\chi^2 = 0.0166$, $p = 0.8974$, $df = 1$). Similarly, the type of SV (deletion, inversion, duplication, or translocation) was not predictive of whether the TAD would be altered (Pearson's χ^2 test, $\chi^2 = 4.7756$, $p = 0.3111$, $df = 4$). Overall, we find that SVs are associated with higher-order topological changes in a small percentage of cases and that the presence of an SV breakpoint is not predictive alone of an altered TAD.

Despite the evidence that SVs rarely impact higher-order chromatin topology, we evaluated whether SVs affected the expression of genes within the TADs surrounding the breakpoint using patient-matched RNA-seq data [101]. We found that 23 of 260 breakpoints (8.8%) are associated with significant changes to local gene expression (Figure 2.4b). Complex events can have opposite effects at each breakpoint. For example, while the T2E fusion across all tumours leads to over-expression of ERG and under-expression of TMPRSS2 [6, 38], the deleted locus between these two genes was inserted into chromosome 14 as part of a complex translocation event in one patient (Figure 2.4c-f). This inserted fragment positions ERG towards the 5' end of the RALGAPA1 gene and TMPRSS2 towards the 3' end (Figure 2.4c) resulting in a significant drop in intra-TAD contacts

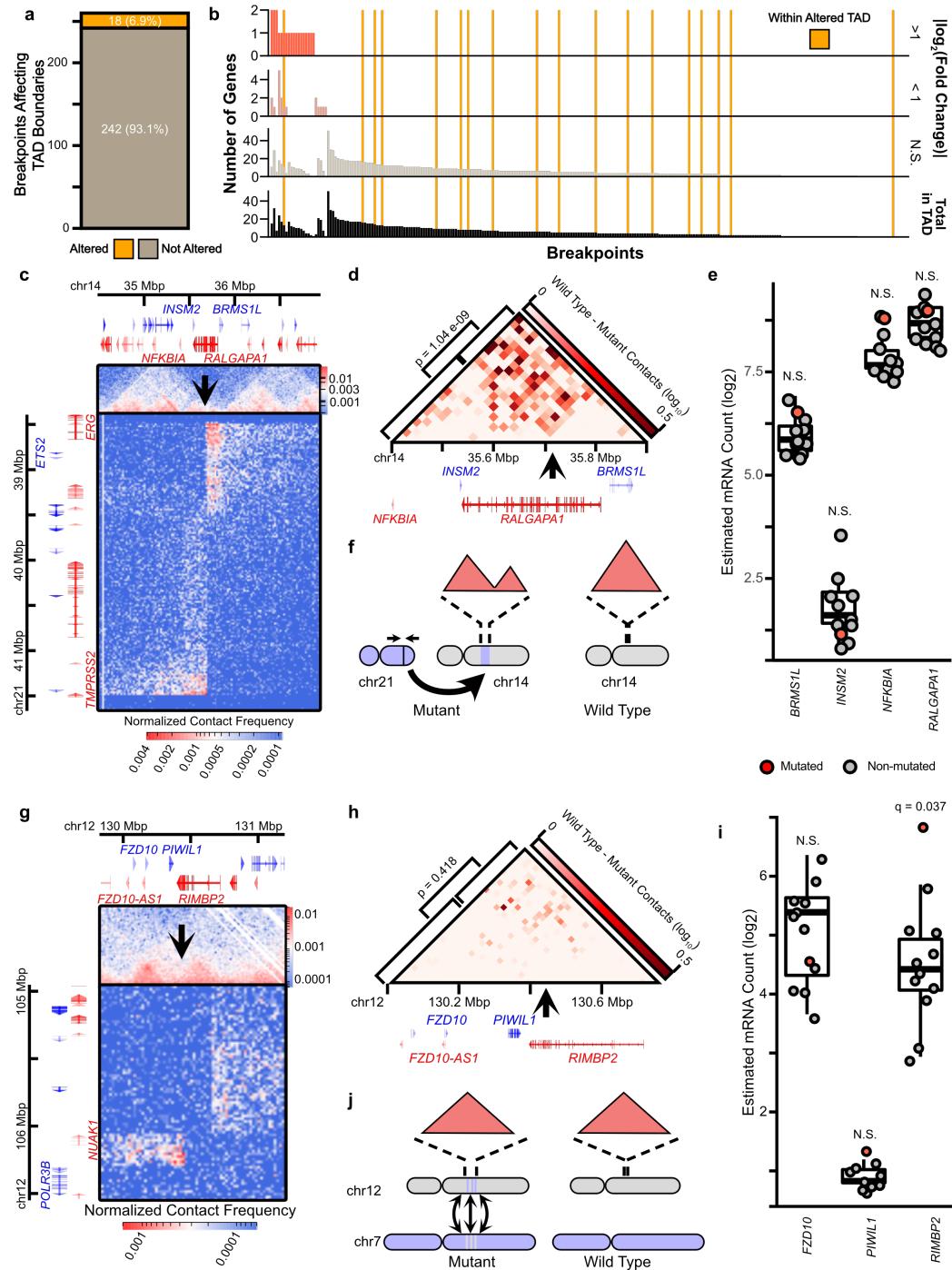


Figure 2.4: SVs can alter TADs or gene expression around breakpoints, but rarely alters both. (Continued on the following page)

Figure 2.4: **a.** A count of the number of SV breakpoints associated with altered TAD boundaries. **b.** Bar plot showing the number of genes differentially expressed around SV breakpoints. **c-f.** An example of an SV that alters intra-TAD contacts without significantly affecting gene expression of the nearby genes. **c.** The contact matrix showing a translocation of the *TMPRSS2-ERG* locus into chromosome 14 in the *RALGAPA1* gene. **d.** The differential contact matrix between the tumour containing this translocation and another tumour without it. **e.** Gene expression scatterplot and boxplot of genes within the affected TAD for each sample. **f.** A schematic representation of the translocation. **g-j.** An example of an SV that does not alter intra-TAD contacts but does alter the expression of the nearby genes. **g.** The contact matrix showing a complex rearrangement around the *RIMBP2* gene. **h.** The differential contact matrix between the tumour containing this rearrangement and another tumour without it. **e.** Gene expression scatterplot and boxplot of genes within the affected TAD for each sample. **j.** A schematic representation of the rearrangement. Boxplots highlight the first, second, and third quartiles of expression in the tumours without the example SV. Red dots represent the tumour with the example SV, grey dots represent the tumours without.

at the *RALGAPA1* locus on chromosome 14 (two-sample unpaired *t*-test, $t = 6.38$, $p = 1.04 \cdot 10^{-9}$; Figure 2.4d). Despite the significant topological change on chromosome 14, no significant changes to expression was detectable across genes within the same TAD on chromosome 14 (Figure 2.4e). Conversely, TAD alterations are not required changes to gene expression. As part of a complex SV involving the *RIMBP2* gene (Figure 2.4g-j), both ends of the gene contain breakpoints (Figure 2.4g). This rearrangement is not associated with changes to intra-TAD contacts (two-sample unpaired *t*-test, $t = 0.8101$, $p = 0.4183$; Figure 2.4h). However, *RIMBP2* is over-expressed in this patient (Figure 2.4i). More generally, only a single breakpoint was observed with both TAD contact and gene expression changes, although we did not find evidence to suggest these are dependent events (Pearson's χ^2 test, $\chi^2 = 6.31 \cdot 10^3$, $p = 0.9367$, $df = 1$). For TADs where at least one gene was differentially expressed, 19 (83%) of them had at least one gene with doubled or halved expression. Notably, we found that inter-chromosomal translocations are associated with altering the expression of genes nearby their breakpoints compared to intra-chromosomal breakpoints (Pearson's χ^2 test, $\chi^2 = 7.0088$, $p = 0.00811$, $df = 1$; Supplementary Figure 5). Taken together, these results suggest that while SVs can alter contacts within TADs, this is neither necessary nor sufficient to alter gene expression.

2.3.5 SVs alter focal chromatin interactions to hijack CREs and alter antipode gene expression

Mutations in prostate cancer have previously been found to converge on active CREs [59]. To assess if SVs function in a similar fashion, we investigated the convergence of SV breakpoints in active CREs. We find that SV breakpoints are enriched in the catalogue of CREs captured by

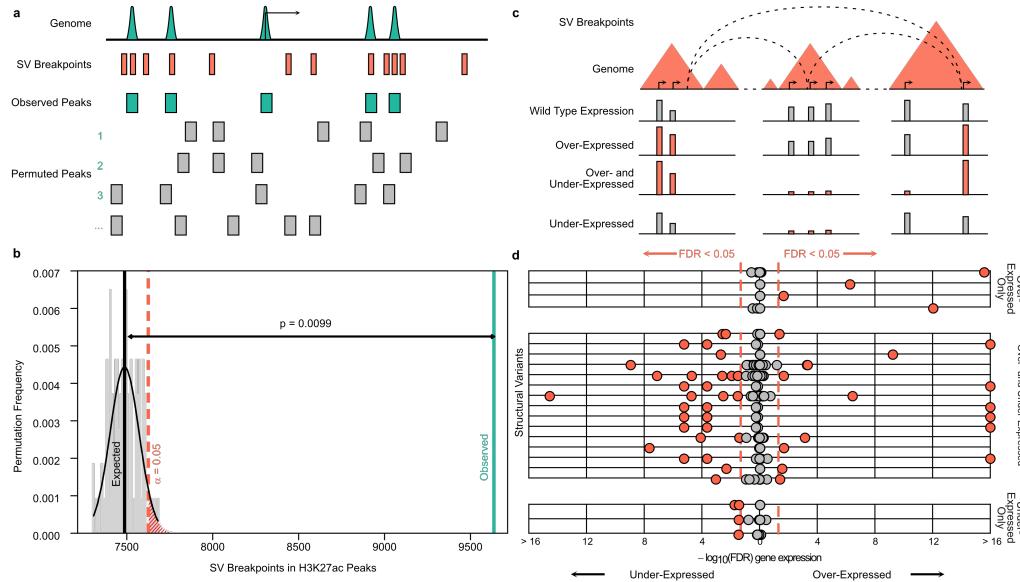


Figure 2.5: SV breakpoints are enriched in active CREs and repeatedly alter the expression of multiple genes. **a.** Schematic of permutation testing for the overlap between SV breakpoints in all CPC-GENE prostate tumours and the catalogue of active CREs in the 12 tumour samples in this study. **b.** Histogram of permutation test results in grey. The vertical black and green bars refer to the expected and observed overlap of SV breakpoints and CREs, respectively. P-value is obtained from the permutation test, $n = 100$. **c.** Schematic of how the expression of genes within TADs containing SV breakpoints are compared between mutant and wild type tumours are compared. **d.** Scatterplot of FDR values obtained from differential gene expression analysis as outlined in **c**. Red dots are differentially expressed genes ($FDR \leq 0.05$), grey dots are genes not differentially expressed between the mutant and wild type tumours.

H3K27ac ChIP-seq from our 12 primary prostate tumours compared to the rest of the genome (one-sided permutation z -test, $z = 25.591$, $p = 0.0099$, $n = 100$; Figure 2.5a-b). This is similar to the enrichment of point mutations in CREs active in prostate cancer [59], suggesting that SVs which alter gene expression may do so by recurrently targeting CREs. Since individual CREs can regulate multiple genes [107], we suspected that SVs that do alter gene expression may predominantly affect multiple genes at the same time, instead of single genes. In agreement, when considering all SVs associated with altered gene expression near a breakpoint we find 16 of the 22 (72.7%) SVs are associated with altered expression of multiple genes (Figure 2.5c-d). Notably, 15 of these 16 SVs (93.8%) are associated with both over- and under-expression of genes, instead of genes all being either over-expressed or under-expressed (Figure 2.5d). 12 of these 15 (80%) SVs are associated with expression changes at SV antipodes, opposite ends of a breakpoint pair (Supplementary Figure 6). The recurrent targeting of active CREs, combined with the opposite gene expression changes at SV antipodes, suggests that SVs may repeatedly alter expression by CRE hijacking.

The fusion of *PMEPA1* and *ZNF516* is an example of CRE hijacking resulting in opposite

differential gene expression. Specifically, the fusion results in the *PMEPA1* promoter being hijacked to the 5' end of the *ZNF516* gene. This is concomitant with the over-expression of *ZNF516* and under-expression of *PMEPA1* (Figure 2.6a-c). In addition to hijacking the *PMEPA1* promoter to the *ZNF516* gene, this fusion also coincides with gains in H3K27ac over the *ZNF516* gene body and of H3K27ac histone hypo-acetylation over the 3' end of *PMEPA1*'s gene body. This mirrors the creation of a Cluster Of Regulatory Elements (COREs) reported for the T2E fusion, reflective of new CREs enabling ERG over-expression and the concomitant under-expression of TMPRSS2 (Supplementary Figure 7) [38, 108, 109]. CRE hijacking is also observed with inter-chromosomal rearrangements such as seen at the SV connecting chromosomes 7 and 19, creating 2 fusion products (termed C2B and B2C; Figure 2.6d). This SV separates the 3' end of BRAF from its promoter and upstream enhancers on chromosome 7 (C2B; Figure 2.6d), fusing it to the 3' end of CYP4F11 (Figure 2.6e). Focal chromatin interactions between BRAF and multiple active CREs are only observed in the fusion on chromosome 19 (Figure 2.6e). Using matched RNA-seq data, we observe an estimated 5 fold increase in expression for the 3' exons of BRAF in the mutated tumour compared to others (fold-change = 4.976, FDR = 0.0181; Figure 2.6f). Collectively, over-expression of the oncogenes, such as ERG and BRAF, and suppression of the tumour suppressor *PMEPA1* demonstrates the disease-relevant effects of CRE hijacking mediated by SVs in primary prostate cancer resulting in changes to focal chromatin interactions, and that these effects overshadow the effect on higher-order topology in primary prostate cancer.

2.3.6 Discussion

Genetic alterations that subvert the higher-order chromatin organization to allow for aberrant focal interactions may be more common in cancer than previously recognized. In this work we demonstrated that CRE hijacking by SVs is often associated with opposing gene expression changes at SV antipodes, whereby genes on one flank of the breakpoint are up-regulated while genes on the other flank are repressed. Complex SVs, such as chromoplexy and chromothripsis, are found in numerous cancer types [82, 106], providing many opportunities for widespread effects on gene expression and CRE hijacking. This is in addition to many known cancer drivers that alter CRE interactions, including the AR and FOXA1 enhancer amplifications in primary and metastatic prostate tumours [9, 38, 55, 56, 62, 85]. More recent findings also fit this model, such as accumulation of extra-chromosomal circular DNA activating oncogenes that would otherwise be constrained by chromatin topology [110–113]. These insights stress the importance of investigating all ends of an SV

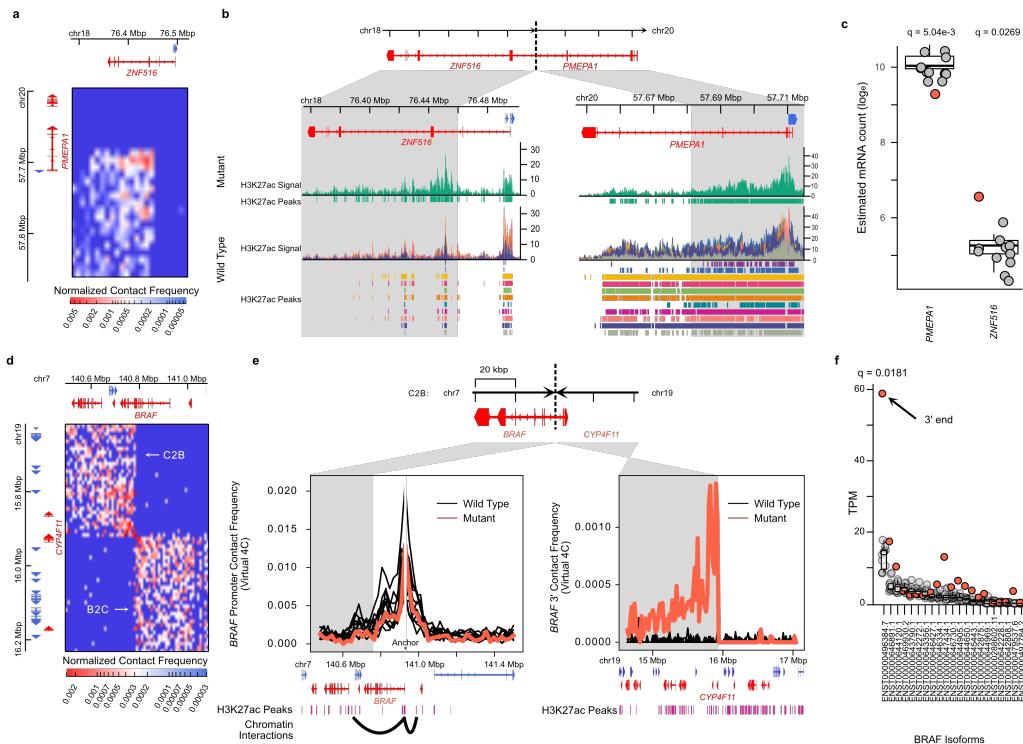


Figure 2.6: SVs altering gene expression by rewiring focal chromatin interactions. **a.** The contact matrix of the deletion between *PMEPA1* and *ZNF516*. **b.** Genome tracks of H3K27ac ChIP-seq signal around the *ZNF516* and *PMEPA1* genes with the rearrangement. Grey regions are loci brought into contact by the SV. **c.** Gene expression of *PMEPA1* and *ZNF516* in all tumour samples. Boxplots represent the first, second, and third quartiles of wild type patients (grey dots). Red dots are the gene expression for the mutated patient. **d.** The contact matrix of an inter-chromosomal break between chromosome 7 and chromosome 19. **e.** Contact frequencies of the *BRAF* promoter on chromosome 7 (left) and the 3' end of *BRAF* on chromosome 19 (right). SV-associated contacts between the 3' end of *BRAF* on chromosome 19 (right) are focally enriched at H3K27ac peaks downstream of *CYP4F11*. Bar plot of SVs categorized by how differentially expressed genes altered.

to assess the biological impact of these mutations on the cis-regulatory landscape as a whole, as opposed to focusing on CREs or SV breakpoints as single entities.

Changes to the three-dimensional genome reported in disease onset or development are often inferred from alterations in TAD boundaries [78, 93]. For instance, CTCF activity is targeted by somatic mutations that enrich at its binding sites in colorectal, esophageal, and liver cancers [91, 114]. Furthermore, gains in DNA methylation at CTCF binding sites are linked to altered TAD structures in gliomas [76]. In primary PCa 97% of differentially methylated regions genome-wide in primary PCa are losses of DNA methylation [115, 116], an epigenetic process previously shown to have limited impact on CTCF chromatin binding [117]. This suggests that aberrant CTCF binding at TAD boundaries is not a hallmark of prostate oncogenesis. Our observation of stable chromatin organization supports this model. Notably, stable TAD structures observed in these primary tissues contrast previous reports of chromatin organization in cell lines derived from prostate cells [52, 118], highlighting the necessity of low-input protocols and primary tissues [100]. Our findings further support recent reports of shared higher-order chromatin organization among phenotypically distinct cell types in model organisms [71, 93, 97, 119–121]. Taken together, this body of evidence suggests that large disruptions to TADs and compartments may constrain the transformation of normal to cancer cells or the divergent subtyping within prostate tumours. Instead, changes to focal chromatin interactions seem to reflect alterations in the genetic architecture leading to cancer development. Investigating these focal chromatin interactions may provide insights on the relationship between CREs, such as between enhancers and their target gene promoter [122, 123] to better understand the etiology of disease.

In conclusion, by bypassing technical limitations to characterize the three-dimensional genome organization across benign and tumour prostate tissue, our work reveals the predominant stable nature of genome topology across prostate oncogenesis. Instead, alterations to discrete chromatin interactions populate the PCa genome. These impact the function of CREs, such as we report for SV-mediated CRE hijacking events. Considering the contribution of SVs across human cancers [124], our collective work presents a framework inclusive of genetics, chromatin state, and three-dimensional genome organization to understand the genetic architecture across individual primary tumours.

2.4 Methods

2.4.1 Patient selection criteria

Patients were selected from the CPC-GENE cohort of Canadian men with indolent PCa, Gleason scores of 3+3, 3+4, and 4+3. All primary human material was obtained with informed consent with approval of our institutional research ethics board (UHN 11-0024). The intersection of previously published data for whole genome sequencing [6], RNA abundance [101], and H3K27ac ChIP-seq [38] led to 25 samples having data for all assays. 11 of these tested positive for ETS gene family fusions (T2E status), and 14 without. To accurately represent the presence of this subtype of PCa in the disease generally, and to ensure minimum read depths required to perform accurate analysis on chromatin conformation data, we selected approximately half of these remaining samples (6 T2E+ and 6 T2E-).

2.4.2 Patient Tumour *in situ* low-input Hi-C Sequencing

We followed the general *in situ* low input Hi-C (Low-C) protocol from Diaz *et al.*, 2018 [100] with our own re-optimization for solid tumour tissue sections. It is worth noting that throughout the protocol, the pellet would be hardly visible and would require careful pipetting. The specific modifications of the protocol are described below.

Tumour Tissue Preparation

Twelve cryopreserved-frozen PCa tumour tissue specimens were obtained from primary PCa patients as part of the Canadian PCa Genome Network (CPC-GENE) effort [6]. Informed consent was obtained from all patients with REB approval (UHN 11-0024). These tumour specimens were sectioned into 10 μ m sections. Sections before and after the sections used for Hi-C were stained with hematoxylin and eosin (H&E) and assessed pathologically for $\geq 70\%$ PCa cellularity. The percentage of infiltrating lymphocytes was also estimated by pathological assessment to be $\leq 3\%$. Stratification into T2E+ or T2E- was determined through either whole-genome sequencing detection of the rearrangement, immunohistochemistry, or mRNA expression microarray data [6].

Normal Tissue Preparation

Five snap-frozen prostate tumour-adjacent normal tissue specimens were obtained. Informed consent was obtained from all patients with REB approval (UHN 11-0024). Tissue specimens were

sectioned into 5, 10, and 20 μm sections. Sections used for Hi-C and RNA-seq were stained with H&E and assessed pathologically for $\geq 60\%$ prostate glandular cellularity.

Fixation and Lysis

One or two sections (consecutive; depending on surface area) for each patient were thawed and fixed by adding 300 μL of 1% formaldehyde in PBS directly onto the tissue sample, followed by a 10-minute incubation at room temperature (RT) (Supplementary Figure 1a). The formaldehyde was quenched by adding 20 μL of 2.5M glycine to the sample reaching a final concentration of 0.2M followed by 5 minutes of incubation at RT. The samples were then washed three times with 500 μL cold PBS and scraped off the microscope slide with a scalpel into 1.5 mL centrifuge tube containing 250 μL of ice-cold Low-C lysis buffer (10 mM Tris-Cl pH 8.0, 10 mM NaCl, 0.2% IGEPAL CA-630 (Sigma-Aldrich)) supplemented with protease inhibitor. The samples were then mixed thoroughly by gentle pipetting and left on ice for 20 minutes with intermittent mixing. Upon lysis, the samples were snap-frozen with liquid nitrogen and stored at -80 °C until processing the next day. As a note, stagger fixation times when processing multiple samples to prevent needless rush and chance of under/over-fixation.

Enzyme Digestion and Overhang Fill-In

The samples stored at -80 °C were thawed on ice and spun down at 300 \times g for 5 minutes at 4 °C. The samples were then re-suspended in 125 μL of ice-cold 10X NEB2 Buffer (New England Biolabs), and again spun down at 13,000 \times g for 5 minutes at 4 °C. The pellet was then re-suspended in 25 μL of 0.4% SDS and incubated at 65 °C for 10 minutes without agitation for permeabilization. To quench the SDS, 10% Triton X-100 in water (12.5 μL + 75 μL water) was then added to the samples and incubated at 37 °C for 45 minutes at 650 rpm. For enzymatic digestion, 35 μL of 10X NEB2.1 buffer (New England Biolabs) was added to each sample, follow by the addition of 50 U of MboI and 90 minutes incubation at 37 °C with gentle agitation (add 30 U first, incubate 45 minutes, followed by the addition of another 20 U and another 45 minutes of incubation). Upon digestion, the MboI enzyme was inactivated by incubating at 62 °C for 20 minutes. The overhangs generated by the MboI enzyme was then filled-in by adding a mix of dNTPs and DNA Polymerase I Klenow Fragment directly to each sample (10 μL of 0.4 mM biotin-14-dCTP, 0.5 μL of 10 mM dATP, 0.5 μL of 10 mM dGTP, 0.5 μL of 10 mM dTTP, 4 μL of 5U/ μL DNA Polymerase I Klenow Fragment). The samples were then mixed by gentle pipetting followed by incubation at 37 °C for 90 minutes with gentle agitation.

Proximity Ligation and De-crosslinking

Upon overhang fill-in, each sample was subject to proximity ligation through the addition of 328.5 μ L water, 60 μ L of 10X T4 DNA Ligase Buffer (ThermoFisher Scientific), 50 μ L of 10% Triton X-100, 6 μ L of 20 mg/mL BSA (New England Biolabs) and 3.5 μ L of 5 Weiss U/ μ L T4 DNA Ligase (ThermoFisher). The samples were mixed through gentle pipetting and incubated at RT (20-22 °C) with rotation for 4 hours. The samples were then spun down at 13,000 \times g for 5 minutes at RT and re-suspended in 250 μ L of Extraction Buffer (50 mM Tris-Cl pH 8.0, 50 mM NaCl, 1 mM EDTA, 1% SDS) upon removal of supernatant. Next, 10 μ L of 20 mg/mL Proteinase K (New England Biolabs) was added to each sample and incubated at 55 °C for 30 minutes at 1,000 rpm. Then 65 μ L of 5 M NaCl was added to each sample and incubated at 65 °C at 1,000 rpm overnight.

DNA Extraction

Phenol-chloroform extraction columns were spun down at 17,000 \times g for 1 minute at 4 °C to get gel down to the bottom of the tube. The samples incubated overnight were then added to the column. Next, an equal volume (~325 μ L) of phenol-chloroform-isoamyl alcohol mixture (25:24:1) (Sigma) was also added to the column. The column was then inverted for thorough mixing and spun down at 17,000 \times g for 5 minutes at 4 °C. The surface layer on top of the gel upon spinning contains the sample and is transferred to a clean 1.5 mL tube (~325 μ L). Each sample was mixed with 31.5 μ L of 3M sodium acetate, 2 μ L of GlycoBlue (ThermoFisher Scientific), and 504 μ L of 100% ethanol for DNA precipitation. The samples were inverted several times for mixing and incubated at -80 °C for 20 minutes, followed by a centrifuge spin at 17,000 \times g for 45 minutes at 4 °C. The supernatant was carefully discarded and the pellet was washed with 800 μ L of ice-cold 70% ethanol followed by a centrifuge spin at 17,000 \times g for 5 minutes at 4 °C. The supernatant was then discarded and the tube was air-dried until no traces of ethanol was left prior to dissolving the DNA pellet with 30 μ L of Elution Buffer (Qiagen PCR Clean-Up Kit). 1 μ L of RNase A (ThermoFisher Scientific) was added to each sample followed by incubation at 37 °C for 15 minutes. A mix of 5 μ L of 10X NEB2.1 buffer (New England Biolabs), 1.25 μ L of 1 mM dATP, 1.25 μ L of 1 mM dCTP, 1.25 μ L of 1 mM dGTP, 1 mM of dTTP, 0.5 μ L of 10 mg/mL BSA, 5 μ L of water, 3.5 μ L of 3 U/ μ L T4 DNA Polymerase (New England Biolabs) was added to each sample. The samples were mixed thoroughly by gentle pipetting, and then incubated at 20 °C for 4 hours.

Fragmentation and Biotin Pull-down

70 μ L of water was added to each sample bringing total volume up to 120 μ L, and the samples were transferred into Covaris sonication tubes. The samples were then sonicated using Covaris M220 sonicator to attain 300-700 bp fragments. For biotin pull-down using a magnetic rack, 30 μ L of Dynabeads MyOne Streptavidin C1 beads (Life Technologies) for each sample was washed once with 400 μ L of 1X B&W buffer + 0.1% Triton X-100. The beads were then re-suspended in 120 μ L of 2X B&W buffer and transferred to the 120 μ L of sample (1:1 ratio). The sample was then incubated with gentle rotation at RT for 20 minutes. The supernatant was discarded and the beads were re-suspended with 400 μ L of 1X B&W buffer + 0.1% Triton X-100 followed by a 2-minute incubation at 55 °C with mixing. The wash was repeated once more, then re-suspended in 400 μ L of 1X NEB2 buffer (New England Biolabs).

Library Preparation and Size Selection

The beads containing the Hi-C samples were separated on a magnetic rack to remove the supernatant. The beads were then re-suspended in a total volume of 10 μ L for library preparation using the SMARTer ThruPLEX DNA-seq library preparation kit (Takara Biosciences) per manufacturer's protocol with an adjustment on the last step, a PCR reaction for library amplification. Upon reaching that step, the reaction was carried out on a regular PCR for two cycles to amplify the Hi-C samples off the streptavidin beads. Next, the samples were transferred onto a new tube where 20X SYBR was added. The samples were then subject to real-time qPCR and pulled out from the qPCR machine mid-exponential phase. Ultimately, this is done to reduce PCR duplication rates, a huge limitation for low-input Hi-C protocols. The Hi-C libraries were then double size-selected for 300-700 bp using Ampure XP beads and sent for BioAnalyzer analysis prior to sequencing.

2.4.3 Hi-C Sequencing and Data Pre-processing

Sequencing

The Hi-C libraries for each tumour sample were sent for shallow paired-end 150 bp sequencing (~10-15 million reads per sample) on a NextSeq 500. Upon confirming library quality and low duplication rates (< 2%), samples were sent for deep paired-end 150 bp sequencing with the aim of 800 million raw read pairs per sample on NovaSeq 6000.

Sequence alignment and Hi-C artefact removal

Paired-end FASTQ files were pre-processed with HiCUP (v0.7.2) [125]. Reads were truncated at MboI ligation junction sites prior to alignment with `hicup_digester`. Each mate was independently aligned to the hg38 genome and were then paired and assigned to MboI restriction sites by `hicup_map`. `hicup_map` uses Bowtie2 (v2.3.4) [126] as the underlying aligner which has the following parameters: `--very-sensitive --no-unal --reorder`. Reads that reflect technical artefacts were filtered out with `hicup_filter`. Duplicate reads were removed with `hicup_deduplicator`.

Reads that came from different sequencing batches were then aggregated for each tumour sample at this stage using `sambamba merge` (v0.6.9) [127]. This resulted in an average of 1.12×10^9 read per tumour sample (Supplementary Table 2).

Contact matrix generation and balancing

Aggregated binary alignment map (BAM) files were converted to the pairs format using pairtools (v0.2.2) [128] and then the cooler format using the cooler package (v0.8.5) [129]. The pairs files were generated with the following command:

```
pairtools parse -c {genome} --assembly hg38 -o {output_pairs} {
    input_bam}
```

The cooler files were generated at an initial matrix resolution of 1000 bp with the following command:

```
cooler cload pairs --assembly hg38 -c1 2 -p1 3 -c2 4 -p2 5 {genome
}:1000 {input_pairs} {output_cooler}
```

The raw contact matrices stored in the cooler file format were balanced using cooler's implementation of the ICE algorithm [130] using the `cooler balance` command. Contact matrices at different resolutions were created with the `cooler zoomify` command.

2.4.4 Hi-C Data Analysis

TAD identification

Contact matrices were binned at a resolution of 40 kbp. To remove sequencing depth as a confounding factor, contact matrices for all samples were first downsampled to match the sequencing depth of the shallowest sample. For comparisons including cell lines, this was 120×10^6 contacts.

For comparisons only involving primary samples, this was 300×10^6 contacts. This was achieved with Cooltools (v0.3.2) [131] with the following command:

```
cooltools random-sample -c 120000000 {input}:::/resolutions/40000 {
    output}
```

TADs were identified using TopDom [132] on the downsampled, ICE-normalized contact matrices. To identify domains at multiple length scales, similar in concept to Artamus' gamma parameter [133], TopDom was run multiple times per sample, with the window size parameter set at values between 3 and 40, inclusive (corresponding to 120 kbp and 1.6 Mbp). The lower bound for the window size parameter allowed for the identification of domains multiple megabases in size at the upper end and domains < 100 kbp at the lower end without being dominated by false calls due to sparsity of the data. Despite TopDom being more resistant to confounding by sequencing depth than other TAD calling tools [134], biases in boundary persistence were evident between samples of different sequencing depth. Downsampling contact matrices to similar depths resolved these biases.

Given the stochasticity of Hi-C sequencing, boundaries called at one window size may not correspond to the exact same location at a different window size. To attempt to resolve these different boundary calls and leverage power from multiple window sizes, boundaries for a given patient were considered at all window sizes. Boundaries within one bin (40 kbp) of each other and called at different window sizes were marked as conflicting calls. If only two boundaries were in conflict and all the window sizes where the first boundary was called are smaller than the window sizes where the second boundary was called, the second boundary was selected since larger smoothing windows are less sensitive to small differences in contact counts. If only two boundaries were in conflict but there is no proper ordering of the window sizes, the boundary that was identified most often between the two was selected. If three boundaries are in conflict, the middle boundary was selected. If four or more boundaries were in conflict, the boundary that was identified most often was selected.

To determine the maximum window size for TAD calls, TAD calls were compared across window sizes for the same patient using the BPscore metric [135]. TAD calls are identical when the BPscore is 0, and divergent when 1. The cut-off window size for a single patient was determined when the difference between TAD calls at consecutive window sizes was < 0.005 , twice in a row. The maximum window size was determined by the maximum window size cut-off across all samples in a comparison. For comparisons involving only primary samples, the maximum window size was determined to be $w = 20 \times 40$ kbp. For comparisons involving cell lines, this was $w = 32 \times 40$ kbp.

The persistence of a TAD boundary was calculated as the number of window sizes where this

region was identified as a boundary.

Sample clustering by TADs

Using the TAD calls at the window size $w = 32 \times 40$ kbp, the similarity between samples was calculated with BPscore. The resulting matrix, containing the similarity between any two samples, was used as the distance matrix for unsupervised hierarchical clustering with `ward.D2` linkage.

Compartment identification

Contact matrices were binned at a resolution of 40 kbp, similarly to TAD identification. To remove sequencing depth as a confounding factor, contact matrices for all samples were first down-sampled to match the sequencing depth of the shallowest sample. Contact matrix eigenvectors were calculated with Cooltools. To standardize the sign of each eigenvector, the GC content of the reference genome, binned at 40 kbp, was used as a phasing reference track. This reference track was calculated with the `frac_gc` function from the Bioframe Python package (v0.0.12) [136]. The first eigenvector was used to identify compartments with the following command:

```
cooltools call-compartments --bigwig --reference-track gc-content-
phase.bedGraph -o {output} {input}
```

Identification of significant chromatin interactions

Chromatin interactions were identified in all 17 primary samples with Mustache (v1.0.2) [137]. Using the Cooler files from above, Mustache was run on the ICE-normalized 10 kbp contact matrix for each chromosome with the following command:

```
mustache -f {input} -r 10000 -ch {chromosome} -p 8 -o {output}
```

Interaction calls on each chromosome were merged for each sample to create a single table of interaction calls across the entire genome.

To account for variances in detection across samples and to identify similarly called interactions across samples, interaction anchors were aggregated across all samples to form a consensus set. Interaction anchors were merged if they overlapped by at least 1 bp. Interaction anchors for each sample were then mapped to the consensus set of anchors, and these new anchors were used in all subsequent analyses.

Chromatin interaction saturation analysis

To estimate the detection of all chromatin interactions across all samples, a nonlinear regression on an asymptotic model was performed. This is similar in method to peak saturation analysis used to assess peaks detected in ChIP-seq experiments from a collection of samples [38]. Bootstrapping the number of unique interactions detected in a random selection of n samples was calculated for n ranging from 1 to 17. 100 iterations of the bootstrapping process were performed. An exponential model was fit against the mean number of unique interactions detected in n samples using the `nls` and `SSaymp` functions from the stats R package (v3.6.3). The model was fit to the following equation:

$$\mu = \alpha + (R_0 - \alpha) \exp(-kn) \quad (2.1)$$

where μ is the mean number of chromatin interactions for a given number of samples, n , α is the asymptotic limit of the total number of mean detected interactions, R_0 is the response for $n = 0$, and k is the rate constant. The estimated fit was used to predict the number of samples required to reach 50%, 90%, 95%, and 99% saturation of the asymptote (Supplementary Figure 3c).

Structural variant breakpoint pair detection

Structural variant annotation and graph construction

Determination of structural variant breakpoints altering TAD boundaries

Virtual 4C

2.4.5 Patient Tumour Tissue H3K27ac ChIP-seq

Sequence alignment

Peak calling

Differential acetylation analysis

Structural variant breakpoint enrichment

2.4.6 Primary Tissue RNA Data Analysis

Tumour sample RNA sequencing

RNA sequencing data pre-processing

Differential gene expression analysis

Appendix A

Supplementary Material for Chapter 2

Table A.1: SNVs within the FOXA1 TAD.

Chromosome	Coordinate	SNV
chr14	37 695 514	c > G
chr14	37 700 981	a > G
chr14	37 703 714	a > T
chr14	37 715 593	C > T
chr14	37 715 710	T > C
chr14	37 730 447	A > G
chr14	37 755 665	g > T
chr14	37 762 423	A > G
chr14	37 781 081	c > A
chr14	37 785 616	c > T
chr14	37 787 646	A > C
chr14	37 803 915	a > G
chr14	37 829 761	g > A
chr14	37 857 322	G > C
chr14	37 870 712	g > A
chr14	37 884 260	T > C

(Continued)

Chromosome	Coordinate	SNV
chr14	37 887 005	A > G
chr14	37 887 437	G > T
chr14	37 888 682	a > G
chr14	37 888 869	g > T
chr14	37 890 288	G > A
chr14	37 903 468	t > G
chr14	37 904 343	A > T
chr14	37 905 854	A > G
chr14	37 906 009	T > C
chr14	37 918 105	G > A
chr14	37 925 787	A > G
chr14	37 941 995	g > A
chr14	37 952 831	c > T
chr14	37 966 152	a > T
chr14	37 975 348	g > T
chr14	37 997 221	C > G
chr14	38 008 477	a > G
chr14	38 012 766	g > A
chr14	38 015 049	C > G
chr14	38 022 140	c > T
chr14	38 022 335	a > T
chr14	38 036 543	A > G
chr14	38 037 962	G > C
chr14	38 051 839	G > T
chr14	38 055 269	C > G
chr14	38 055 908	T > C
chr14	38 056 977	T > G
chr14	38 059 153	G > A
chr14	38 060 558	C > T
chr14	38 061 208	G > C
chr14	38 061 229	A > C

(Continued)

Chromosome	Coordinate	SNV
chr14	38 061 231	A > C
chr14	38 061 231	A > G
chr14	38 061 231	A > T
chr14	38 061 250	G > A
chr14	38 061 294	G > A
chr14	38 061 313	C > T
chr14	38 061 317	G > T
chr14	38 061 340	A > C
chr14	38 061 528	c > T
chr14	38 061 975	G > T
chr14	38 062 840	C > G
chr14	38 072 955	C > A
chr14	38 082 628	c > T
chr14	38 097 288	g > A
chr14	38 099 831	g > T
chr14	38 103 657	g > A
chr14	38 107 989	t > G
chr14	38 109 595	T > C
chr14	38 127 842	t > C
chr14	38 145 684	a > G
chr14	38 151 460	a > C
chr14	38 152 352	T > G
chr14	38 161 158	a > G
chr14	38 165 472	A > T
chr14	38 165 665	A > G
chr14	38 178 575	t > A
chr14	38 193 969	T > C
chr14	38 197 633	a > C

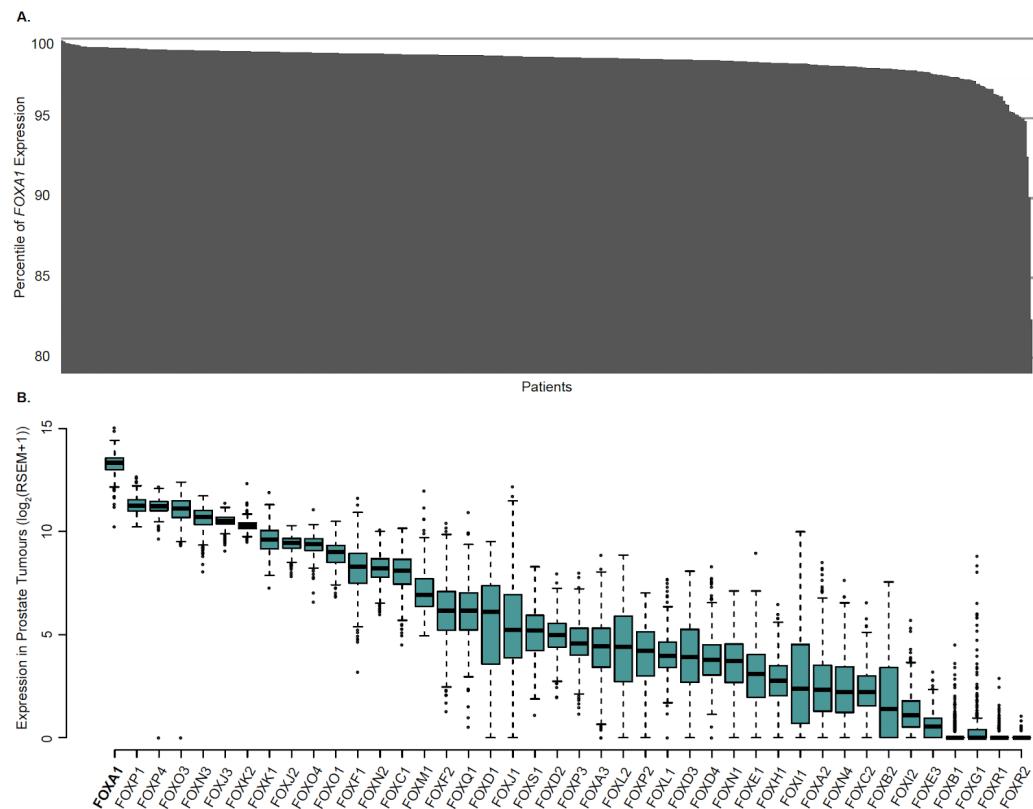


Figure A.1: ***FOXA1* mRNA expression in prostate tumours.** **a.** The ranking of *FOXA1* mRNA expression across 497 primary prostate tumours profiled in TCGA. **b.** mRNA expression of all genes coding for FOX transcription factors across 497 primary prostate tumours profiled in TCGA.

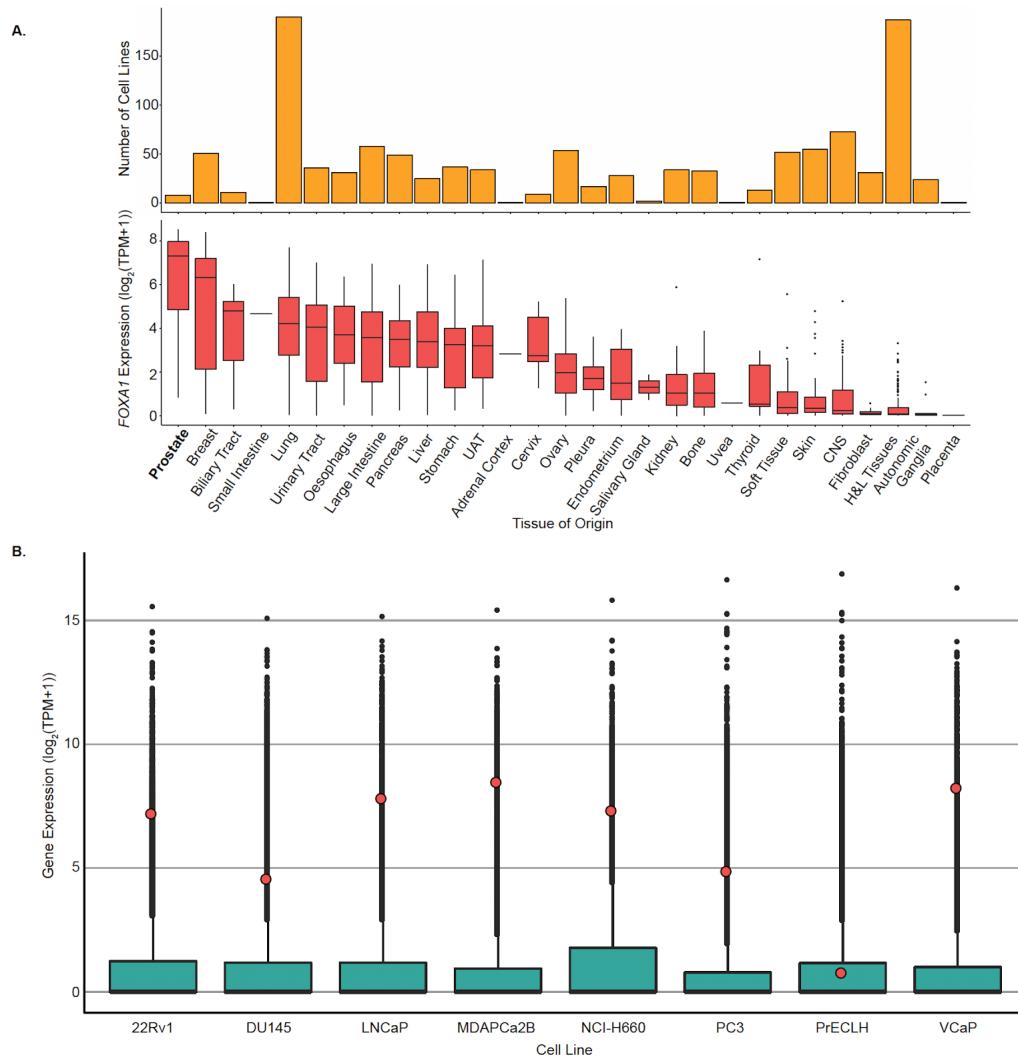


Figure A.2: ***FOXA1* mRNA expression across prostate cancer cell lines.** **a.** *FOXA1* mRNA expression across all cancer cell lines from DEPMAP, profiled by RNA-seq (see Methods). UAT = Upper Aerodigestive Tract, CNS = Central Nervous System, H&L Tissues = Hematopoietic and Lymphoid Tissues. **b.** *FOXA1* mRNA expression across eight prostate cancer cell lines from DEPMAP, profiled by RNA-seq (see Methods). Red dots indicate *FOXA1*.

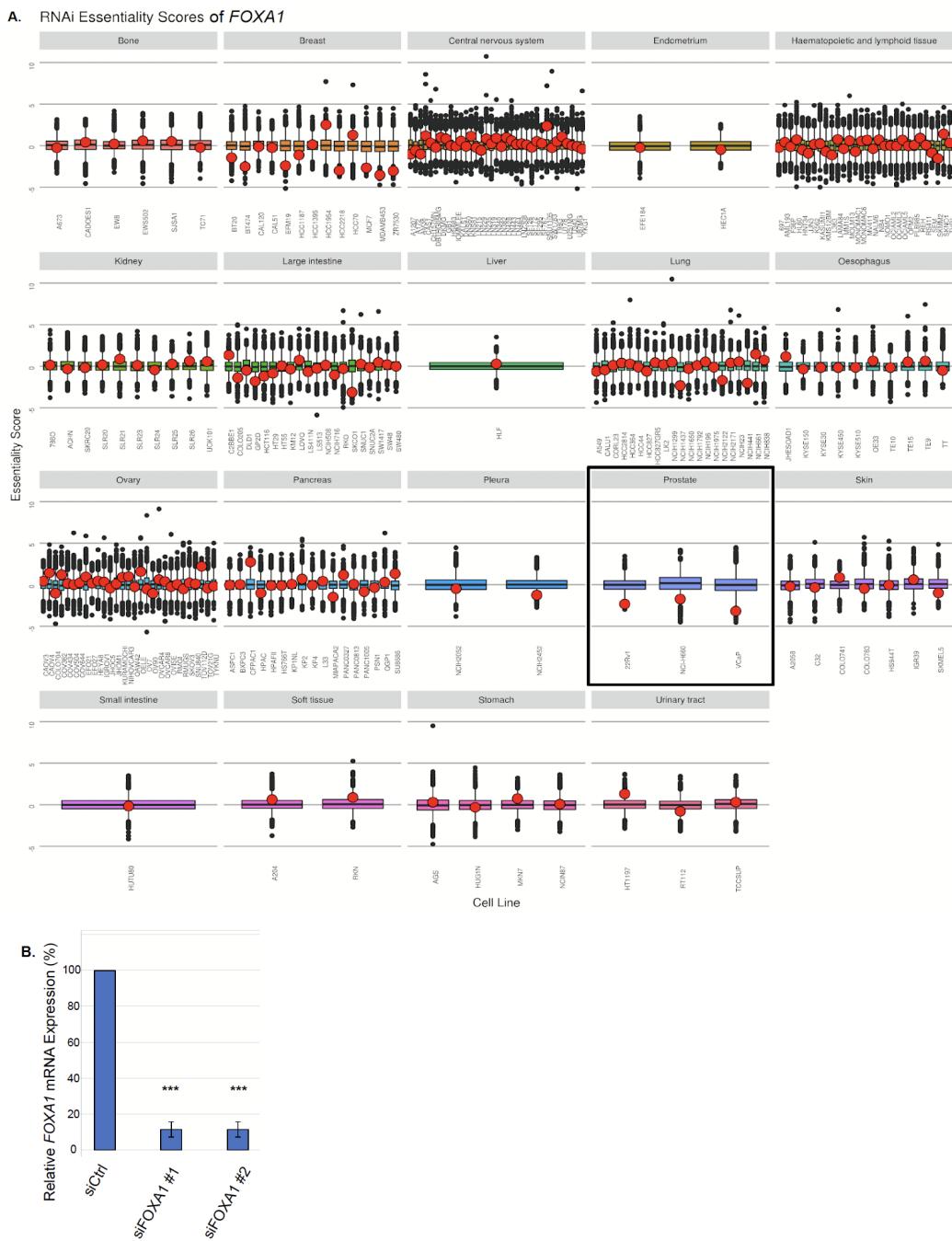


Figure A.3: Essentiality of *FOXA1* across cancer cell lines of various cancer types. **a.** Gene essentiality screen mediated through shRNA/RNAi across various cancer cell lines ($n = 707$). Higher score indicates less essential, and lower score indicates more essential for cell proliferation. Red dot indicates *FOXA1*. **b.** *FOXA1* mRNA expression normalized to housekeeping TBP mRNA expression upon siRNA-mediated knockdown, five days post-transfection ($n = 3$ independent experiments). Error bars indicate \pm s.d., Student's t-test, *** $p < 0.001$.

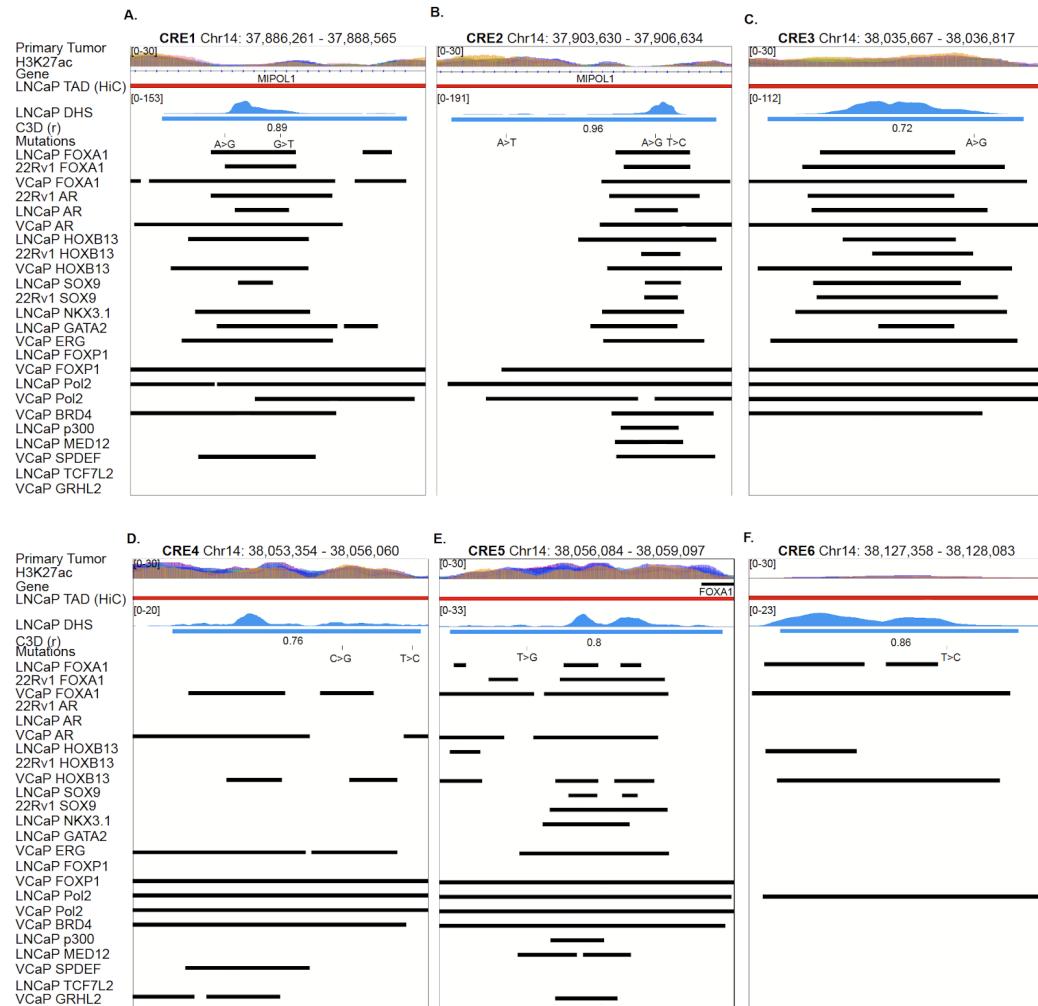


Figure A.4: Visualization of the functional annotation of the six *FOXA1* CREs. a-f. Visualization of Functional annotation of the six FOXA1 CREs using public and in-house ChIP-seq datasets.

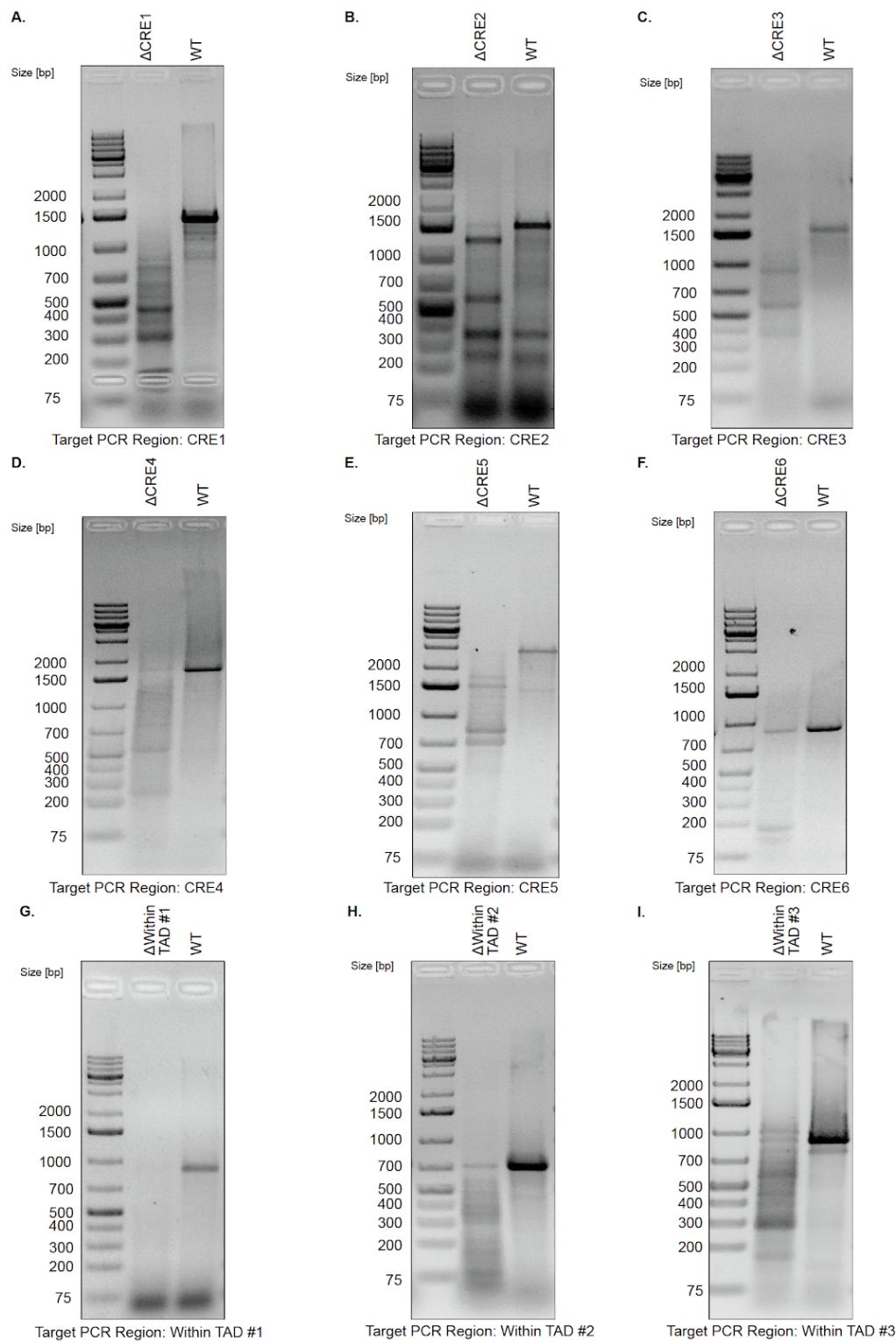


Figure A.5: Validation of clonal Cas-mediated deletions of CREs. a-f. Representative agarose gels from LNCaP clonal CRISPR/Cas9-mediated deletion products or wild-type (WT) product from PCR amplification of intended CRE, followed by T7 Endonuclease I assay.

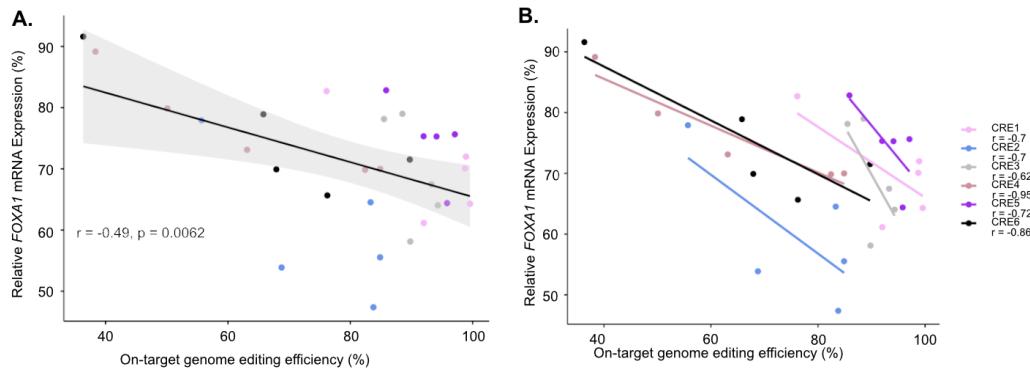


Figure A.6: Genome editing efficiency (%) is inversely correlated with *FOXA1* mRNA expression. **a.** Pearson's correlation to investigate the relationship between genome editing efficiency mediated by CRISPR/Cas9 and *FOXA1* mRNA expression in LNCaP cells. The Pearson's correlation here is across all of the CREs. **b.** Pearson's correlation based on each individual CRE, correlation between genome editing efficiency mediated by CRISPR/Cas9 and *FOXA1* mRNA expression in LNCaP cells.

Table A.2: Guide RNA for Clonal and Transient CRISPR/-Cas9 and dCas9-KRAB experiments.

Target	gRNA for Cas9	Location (hg19)
CRE1_1	GTGGAGGTTAATTGAAACC	chr14:37 886 718-37 886 737
CRE1_2	AACATGACCATAGACCTACT	chr14:37 886 860-37 886 879
CRE1_3	CCAATTCCATTCAATGGTCT	chr14:37 887 321-37 887 340
CRE1_4	ACCCATTTTCATCACCGTA	chr14:37 887 646-37 887 665
CRE1_5	TATTGAAATGTGGTCTTCTA	chr14:37 887 034-37 887 053
CRE1_6	CATAATTCCAGGTGACATAA	chr14:37 887 749-37 887 768
CRE2_1	TGACTATAGCTGGTGTTCAC	chr14:37 905 489-37 905 508
CRE2_2	CAGGTGAATAAGTCTACTC	chr14:37 905 215-37 905 234
CRE2_3	ATTTACTAATGCATGTGTCA	chr14:37 905 617-37 905 636
CRE2_4	AACTAACAGCAGGTTGCCT	chr14:37 904 850-37 904 869
CRE2_5	GCATTAAGCTTAATTGTGC	chr14:37 905 060-37 905 079
CRE2_6	CAGAACAGGATTCTGTGAC	chr14:37 905 425-37 905 444
CRE2_7	TGGCAGTACAGTCAGATATC	chr14:37 905 915-37 905 934
CRE2_8	CTTAGGTTGGAATAGTCAG	chr14:37 906 218-37 906 237
CRE3_1	CACTATTCTCGATCCAAAC	chr14:38 035 926-38 035 945
CRE3_2	GCGGTAAATACTAGAAATT	chr14:38 036 041-38 036 060

(Continued)

Target	gRNA for Cas9	Location (hg19)
CRE3_3	GTTTCTGGAACCTGTTAGT	chr14:38 036 102-38 036 121
CRE3_4	GAGTCGCCTTATCTCCACA	chr14:38 036 347-38 036 366
CRE3_5	GTCCTTAAGACTCCATATG	chr14:38 036 460-38 036 479
CRE3_6	CTTCTATCCCCTATCTAAA	chr14:38 036 572-38 036 591
CRE4_1	CCTTCGTGCGACACGTAGTT	chr14:38 053 998-38 054 017
CRE4_2	TGATGCTAATGCTCGGTCT	chr14:38 054 179-38 054 198
CRE4_3	TAACCCGGATCCTTAGCGGA	chr14:38 054 385-38 054 404
CRE4_4	CGAGGCCCTGCGCTACAATA	chr14:38 054 499-38 054 518
CRE4_5	CGCAGCGGTAGTTGGCGCCC	chr14:38 054 577-38 054 596
CRE4_6	AACTGACCCGGGATATGAGC	chr14:38 054 901-38 054 920
CRE5_1	CACAGGTGTTCGGAAGTAGA	chr14:38 056 803-38 056 822
CRE5_2	AAATGTTGCTCGGTAGCGT	chr14:38 057 009-38 057 028
CRE5_3	TCGCCAGCTCCGAAGACCA	chr14:38 057 208-38 057 227
CRE5_4	CCGCAGGAGCCGTAACGAGG	chr14:38 057 452-38 057 471
CRE5_5	CCCGCGCGCGTCCTAACGCG	chr14:38 057 639-38 057 658
CRE5_6	CTTCCGAGGTCTCAAATTAA	chr14:38 058 088-38 058 107
CRE6_1	AGTTTAAGCTTGTTGAACC	chr14:38 127 236-38 127 255
CRE6_2	GTTAGTCATTATGGGATC	chr14:38 127 547-38 127 566
CRE6_3	ACATTGGTCTGACCTTGC	chr14:38 127 651-38 127 670
CRE6_4	ACACACATGGATGTAGTGT	chr14:38 127 732-38 127 751
CRE6_5	TTCAGACATTTACTTACCC	chr14:38 127 948-38 127 967
CRE6_6	GTTGGAGCTAAATTACACAA	chr14:38 128 193-38 128 212
FOXA1 (+) 1	TCTTGTCGGCGGACAAAT	chr14:38 064 525-38 064 544
FOXA1 (+) 2	GAGTTCAATCCAGTATCGCC	chr14:38 066 997-38 067 016
FOXA1 (+) 3	CGCAGTACCTGAGCGGCACT	chr14:38 067 708-38 067 727
FOXA1 (+) 4	ACGTCTCGAATTAAACGGT	chr14:38 063 687-38 063 706
FOXA1 (+) 5	CCCGACTCTCGCAGCCGGAG	chr14:38 063 887-38 063 906
FOXA1 (+) 6	GTAGTAGCTGTTCCAGTCGC	chr14:38 064 121-38 064 140
AAVS1 (-) 1	ATTCCCAGGGCCGGTTAATG	chr19:55 627 186-55 627 205
AAVS1 (-) 2	GTCCCCCTCCACCCACAGTG	chr19:55 627 139-55 627 158
AAVS1 (-) 3	GGGGCCACTAGGGACAGGAT	chr19:55 627 120-55 627 139

(Continued)

Target	gRNA for Cas9	Location (hg19)
AAVS1 (-) 4	ACTAGGAAGGAGGGAGGCCTA	chr19:55 627 077-55 627 096
AAVS1 (-) 5	CGTGGGGTACCCCTAACGAACT	chr19:55 625 766-55 625 785
AAVS1 (-) 6	GATTCCCTCTCAGGTTACG	chr19:55 626 035-55 626 054
chr14 different TAD non-targeting (-) 1	AAACGTCACTAATGTTGGGG	chr14:30 211 476-30 211 495
chr14 different TAD non-targeting (-) 2	AAGAGCCCTCACCCCCATGA	chr14:30 212 122-30 212 141
chr14 different TAD non-targeting (-) 3	GCGAGTCCAAAATCTGCTAT	chr14:30 211 558-30 211 577
chr14 different TAD non-targeting (-) 4	GTATTATCTAGCTCTCAGT	chr14:30 212 024-30 212 043
chr14 different TAD non-targeting (-) 5	GCCGTACAGCATTACAGAAG	chr14:30 211 388-30 211 407
chr14 different TAD non-targeting (-) 6	GCATATCTCAGACACCTTCATG	chr14:30 212 137-30 212 156
Within_TAD_Neg1 1	CATATCAGCTGTAGTGTATG	chr14:37 812 350-37 812 369
Within_TAD_Neg1 2	TATTCCAAGTTATTGTAATG	chr14:37 812 510-37 812 529
Within_TAD_Neg1 3	TTCACATTGACACCAAAGTT	chr14:37 812 661-37 812 680
Within_TAD_Neg1 4	AGAGTTGTATCACATCCAAG	chr14:37 812 733-37 812 752
Within_TAD_Neg1 5	TAAAAAACTTAGACGATGTTA	chr14:37 812 833-37 812 852
Within_TAD_Neg1 6	GCTGGTATCATGAATCCTTA	chr14:37 813 115-37 813 134
Within_TAD_Neg2 1	AGCCACCCCACAGTTGTTAA	chr14:38 151 715-38 151 734
Within_TAD_Neg2 2	GCATGCTTGAAACTAACGCAT	chr14:38 151 841-38 151 860
Within_TAD_Neg2 3	TTTCCAACAGAGCGTGCATA	chr14:38 151 953-38 151 972
Within_TAD_Neg2 4	TACCGTTAAAGACACCCACT	chr14:38 152 153-38 152 172
Within_TAD_Neg2 5	CCATACGTTGGTCCTTGCCT	chr14:38 152 244-38 152 263
Within_TAD_Neg2 6	GAAGTGCAAGTTGCCACTT	chr14:38 152 312-38 152 331
Within_TAD_Neg3 1	CATTTAGCTAAGGACTTGC	chr14:37 875 337-37 875 356
Within_TAD_Neg3 2	GTATAGAAGAAATTCTACCC	chr14:37 875 447-37 875 466
Within_TAD_Neg3 3	GTGGGTTCAAGCATGTGTT	chr14:37 875 530-37 875 549
Within_TAD_Neg3 4	TTACGACTCAAGTGTAAAGG	chr14:37 875 669-37 875 688
Within_TAD_Neg3 5	GTACCAGAGAGCTTAGTGAA	chr14:37 875 775-37 875 794
Within_TAD_Neg3 6	TACCAAGCCAAGTGTCTTG	chr14:37 875 913-37 875 932

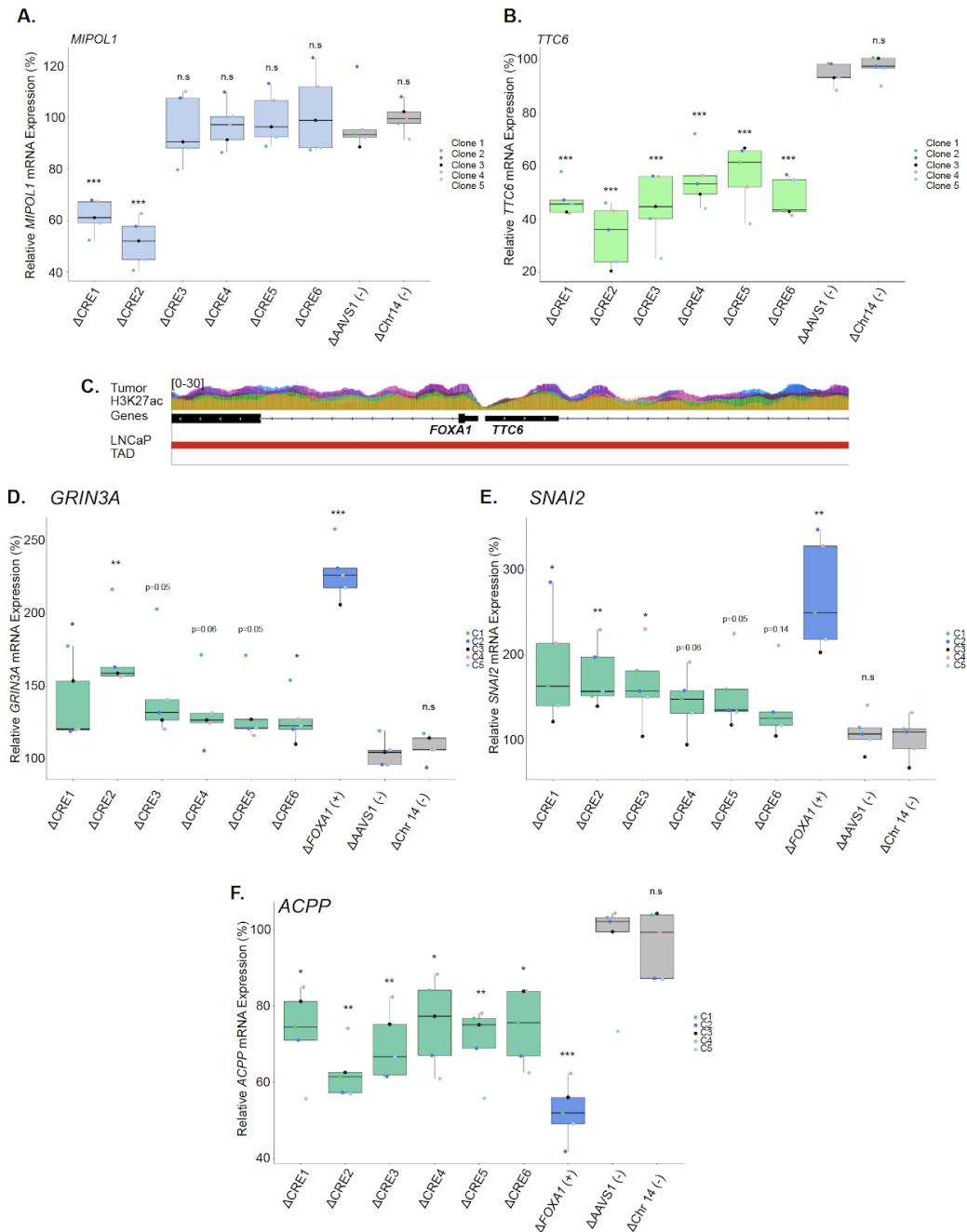


Figure A.7: Intra-TAD genes and *FOXA1* downstream genes are significantly changed upon deletion of CREs. a. *MIPO1* mRNA expression normalized to housekeeping gene *TBP* upon deletion of each region of interest. b. *TTC6* mRNA expression normalized to housekeeping gene *TBP* upon deletion of each CRE. c. Zoom-in view of the *FOXA1* and *TTC6* locus. d-f. mRNA expression of *GRIN3A*, *SNAI2* and *ACPP* normalized to housekeeping gene *TBP* upon deletion of each region of interest. Δ indicates CRISPR/Cas9-mediated deletion ($n = 5$ independent experiments, each dot represents an independent clone). Error bars indicate \pm s.d. Student's t-test, * $p < 0.05$, ** $p < 0.01$, * $p < 0.001$.**

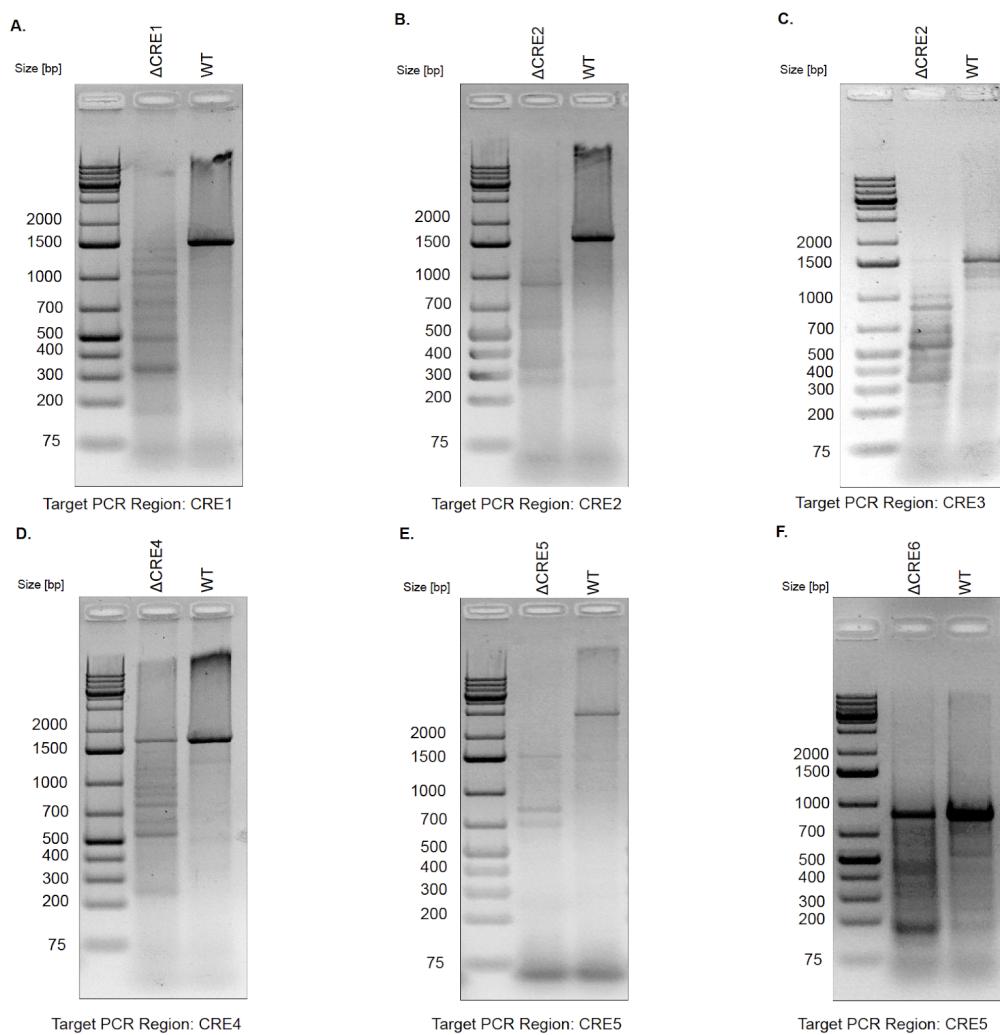


Figure A.8: Validation of transient Cas9-mediated single deletion of CREs. a-f. Agarose gel of transient transfection RNP-based Cas9-mediated deletion product from PCR amplification of intended CRE followed by T7 Endonuclease I assay.

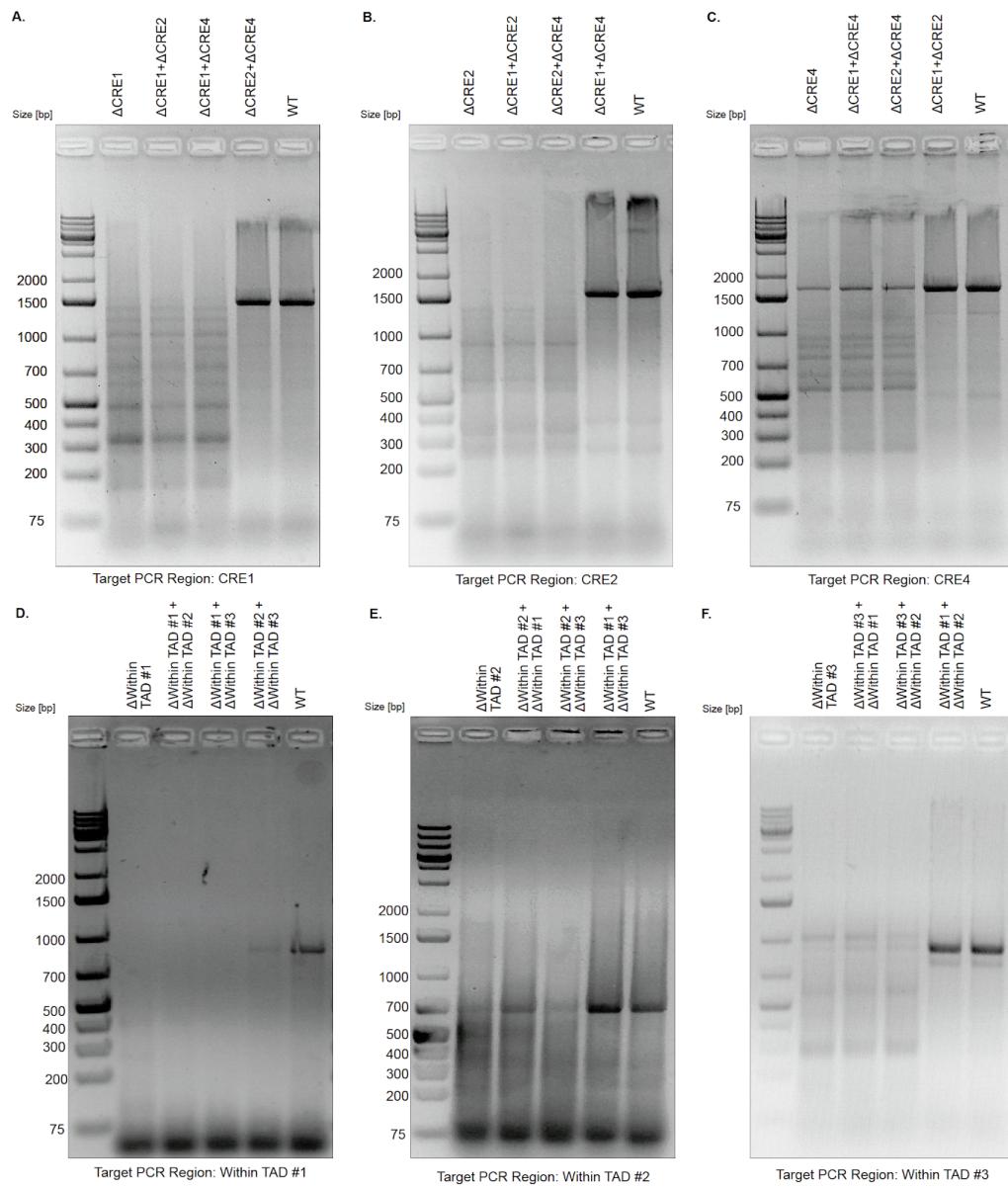


Figure A.9: Validation of transient Cas9-mediated double deletion of CREs. a-f. Agarose gel of transient transfection RNP-based Cas9-mediated deletion product from PCR amplification of intended CREs followed by T7 Endonuclease I assay.

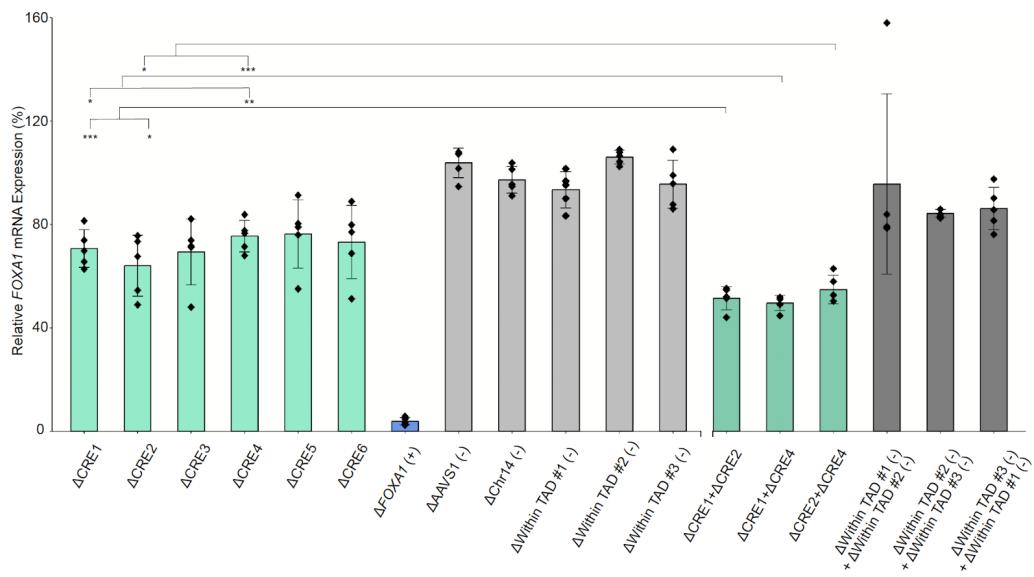


Figure A.10: Comparison of *FOXA1* mRNA expression upon double versus single deletion of CRE(s). *FOXA1* mRNA expression normalized to housekeeping gene *TBP* upon single or double deletion of target CREs. Δ indicates CRISPR/Cas9-mediated deletion ($n = 5$ independent experiments). Error bars indicate \pm s.d., Student's t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

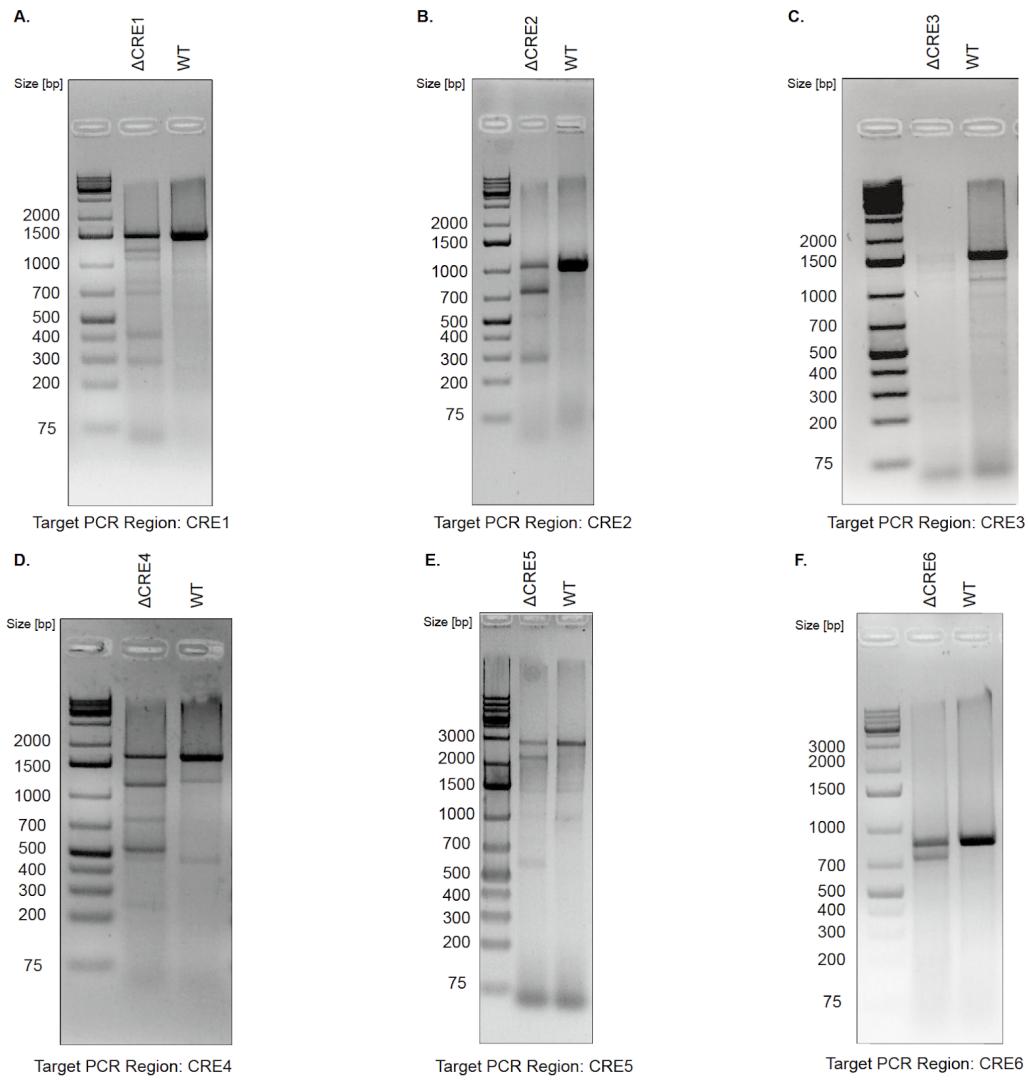


Figure A.11: Validation of Cas9-mediated deletion of CREs from lentiviral system expressing both Cas9 protein and gRNA for cell proliferation assays. a-f. Agarose gel of lentiviral-based (expression of Cas9 protein and two gRNA) Cas9-mediated deletion product from PCR amplification of intended CREs followed by T7 Endonuclease I assay.

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