

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

by

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A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy

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Chapter 1

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

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

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

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

1.1 Abstract

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

1.2 Introduction

PCa is the second most commonly diagnosed cancer among men with an estimated 1.3 million new cases worldwide in 2018 [1]. Although most men diagnosed with primary PCa are treated with curative intent through surgery or radiation therapy, treatments fail in 30% of patients within 10 years [2] resulting in a metastatic disease [3]. Patients with metastatic disease are typically treated with anti-androgen therapies, the staple of aggressive PCa 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 PCa development and find novel strategies to treat patients. Sequencing efforts identified coding somatic SNVs mapping to *FOXA1* in up to 9% [5–10] and 13% [9–11] of primary and metastatic PCa patients, respectively. These coding somatic SNVs target the Forkhead and transactivation domains of *FOXA1* [12], altering its pioneering functions to promote PCa development [10, 13]. Outside of coding SNVs, whole genome sequencing (WGS) also identified somatic SNVs and indels in the 3' untranslated region (UTR) and C-terminus of *FOXA1* in textapprox 12% of mCRPC patients [14]. In addition to SNVs, the *FOXA1* locus is a target of structural rearrangements in both primary and metastatic PCa tumours, inclusive of duplications, amplifications, and translocations [9, 10]. Taken together, *FOXA1* is recurrently mutated taking into account both its coding and flanking noncoding sequences across various stages of PCa development.

FOXA1 serves as a pioneer TF that can bind to heterochromatin, promoting its remodelling to increase accessibility for the recruitment of other TFs [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 PCa, FOXA1 is known to pioneer and reprogram the binding of *AR* alongside HOXB13 [18]. Independent from its role in *AR* signalling, FOXA1 also regulates the expression of genes involved in cell cycle regulation in PCa [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 PCa development.

Despite the oncogenic roles of FOXA1, therapeutic avenues to inhibit its activity in PCa are lacking. In the breast cancer setting for instance, the use of cyclin-dependent kinases inhibitors have been suggested based on their ability to block FOXA1 activity on chromatin [28]. As such, understanding the governance of FOXA1 messenger RNA (mRNA) expression offers an alternative strategy to find modulators of its activity. Gene expression relies on the interplay between distal CREs, such as enhancers and anchors of chromatin interaction, and their target gene promoter(s) [29]. These elements can lie tens to hundreds of kilobases (kbps) 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 (3C)-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 PCa genome and delineate the FOXA1 regulatory plexus.

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

1.3 Results

1.3.1 *FOXA1* is essential for prostate cancer proliferation

We interrogated *FOXA1* expression levels across cancer types. We find that *FOXA1* mRNA is consistently the most abundant in prostate tumours compared to 25 other cancer types across patients (Figure 1.1a), ranking in the 95th percentile for 492 of 497 prostate tumours profiled in The Cancer Genome Atlas (TCGA) (??a). Using the same dataset we also find that *FOXA1* is the most highly expressed out of 41 other forkhead box (FOX) factors in prostate tumours (??b). We next analyzed expression data from Cancer Dependency Map (DEPMAP) and observed *FOXA1* to be most highly expressed in PCa cell lines compared to cell lines of other cancer types (??a). Amongst the eight PCa cell lines in the dataset (22Rv1, DU145, LNCaP, MDA-PCa-2B, NCI-H660, PrECLH, PC3, and VCaP), *FOXA1* mRNA abundance is above the 90th percentile in all but one cell line (PrECLH) compared to the > 56,000 protein coding and non-protein coding genes profiled (??b). These new results gained from the TCGA and DEPMAP validate previous understanding that *FOXA1* is one of the highest expressed genes in PCa [33].

Following up on *FOXA1* mRNA expression levels, we interrogated the essentiality of *FOXA1* for PCa cell growth. RNAi-mediated essentiality screens compiled in DEPMAP show that *FOXA1* lies in the 94th percentile across 6 of the 8 available PCa cell lines: 22Rv1, LNCaP, MDA PCa 2B, NCI-H660, PC3, and VCaP cells (Figure 1.1b-c). The median RNAi-mediated essentiality score for all prostate cell lines is significantly lower than all other cell lines, suggesting that *FOXA1* is especially essential for PCa cell proliferation (permutation test, $p = 1 \cdot 10^{-6}$, see Methods) (??a). Growth assays in LNCaP and VCaP cells following *FOXA1* knockdown using two independent siRNAs (Figure 1.1d, ??b) show significant growth inhibition in LNCaP (siRNA #1: 4-fold, siRNA #2: 3.35-fold) and VCaP (siRNA #1: 8.7-fold, siRNA #2: 2-fold) cells five days post-transfection (Mann-Whitney U Test, $p < 0.05$; Figure 1.1e-f). In accordance with previous reports, our results using essentiality datasets followed by knockdown validation reveals that *FOXA1* is oncogenic and essential for PCa cell proliferation.

1.3.2 Identifying putative *FOXA1* CREs

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

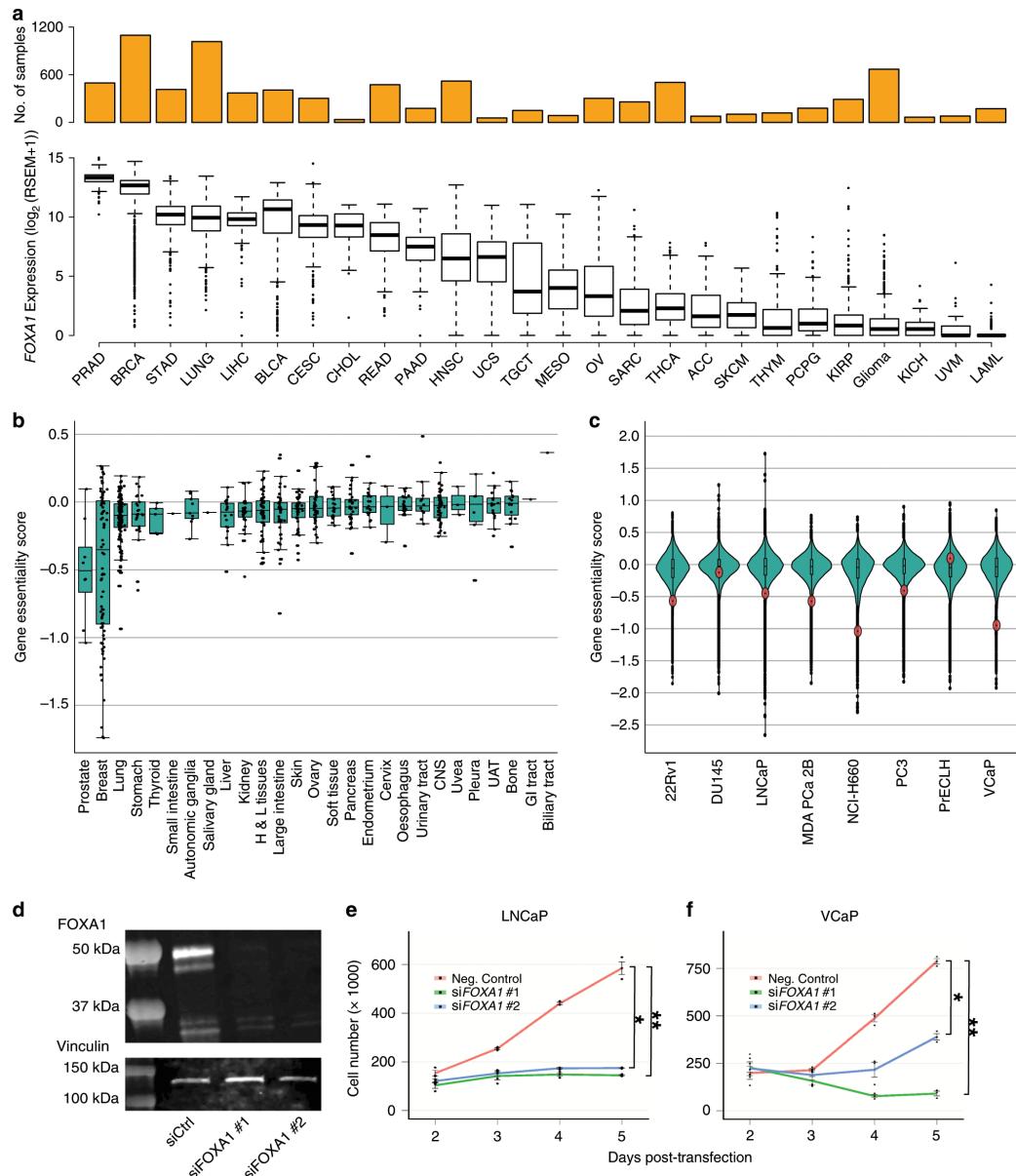


Figure 1.1: *FOXA1* is highly expressed in PCa and essential for PCa cell proliferation..

a. The mRNA expression of *FOXA1* across tumour types ($n = 26$) from RNA-seq data of TCGA.

b. *FOXA1* essentiality mediated through RNAi across various cell lines ($n = 707$) from DEPMAP. Gene essentiality scores are normalized z -scores. Higher scores indicate less essential, and lower scores indicate more essential for cell proliferation. x -axis indicate tissue of origin for each cell line tested. Each dot indicates one cell line.

c. Gene essentiality mediated through RNAi across PCa cell lines ($n = 8$) from DEPMAP. Each dot indicates one gene, red indicates *FOXA1*.

d. Representative Western blot against *FOXA1* in LNCaP cells 5 days post-transfection of non-targeting siRNA and two independent siRNA targeting *FOXA1*.

e. Cell proliferation assay conducted in LNCaP cells upon siRNA-mediated knockdown of *FOXA1* across 5 days.

f. Cell proliferation assay conducted in VCaP cells upon siRNA-mediated knockdown of *FOXA1* across 5 days. Error bars indicate \pm s.d. $n = 3$ independent experiments. Mann-Whitney U test, * $p < 0.05$, ** $p < 0.01$.

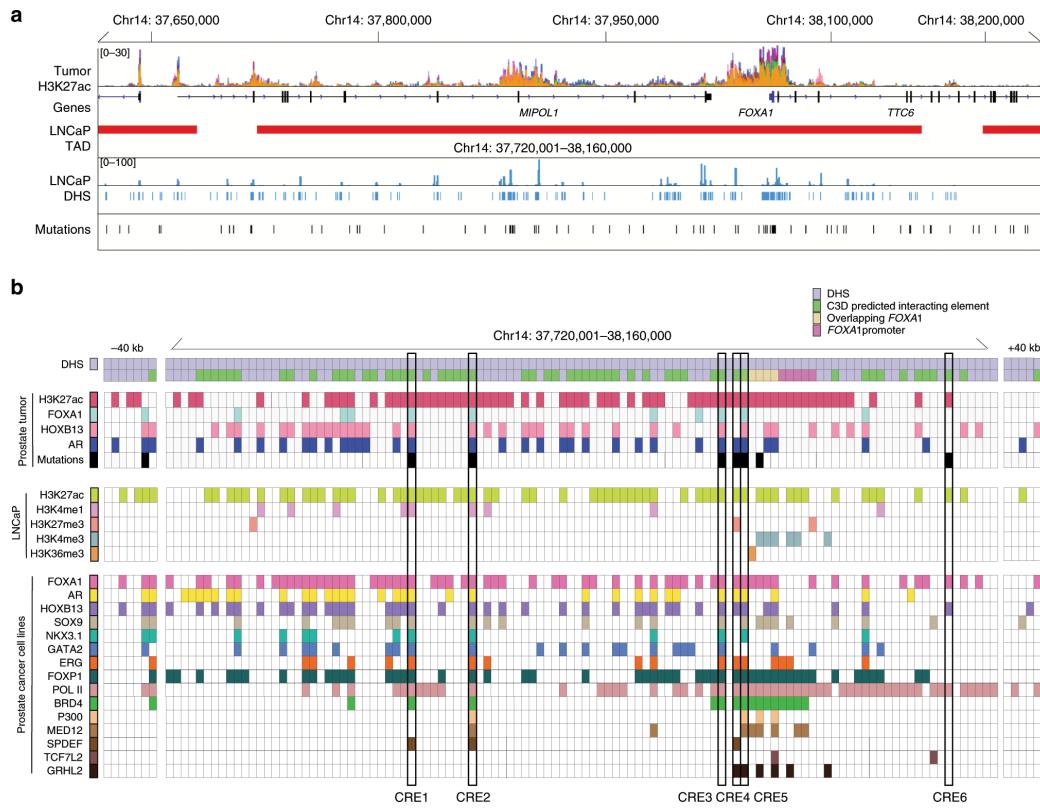


Figure 1.2: Epigenetic annotation of 14q21.1 locus and identification of *FOXA1* CREs.

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

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

[34–36]. Motivated by the oncogenic role of *FOXA1* in PCa, we investigated its regulatory plexus controlling its expression. According to chromatin contact frequency maps generated from Hi-C assays performed in LNCaP PCa cells, *FOXA1* lies in a 440 kbp topologically associated domain (TAD) (chr14: 37720002-38160000 \pm 40 kbp adjusting for resolution) (Figure 1.2a). By overlaying DNase-seq data from LNCaP PCa cells, there are a total of 123 putative CREs reported as DNase I hypersensitive sites (DHSs) that populate this TAD (Figure 1.2a). We next inferred the regulatory plexus of *FOXA1* using the C3D method [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 PCa 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 PCa 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 SNVs (10 total SNVs called from 9 tumours) (Figure 1.2b). We observe that these 6 CREs can be bound by multiple TFs in PCa cells, including *FOXA1*, AR and *HOXB13* (Figure 1.2b, ??). The Hi-C data from the LNCaP PCa cells corroborates the C3D predictions as demonstrated by the elevated contact frequency between the region harboring the *FOXA1* promoter and where the 6 CREs are located, compared to other loci in the same TAD (Figure 1.3a). The 6 CREs lie in intergenic or intronic regions (Figure 1.3b-h). Together, histone modifications, TF binding sites and noncoding SNVs support that these 6 putative CREs are active in primary PCa. The Hi-C and C3D predictions suggest that they regulate *FOXA1* expression.

1.3.4 Disruption of CREs reduces *FOXA1* mRNA expression

We next assessed the role of CREs toward *FOXA1* expression using LNCaP and 22Rv1 clones stably expressing the wild-type (WT) Cas9 protein (Figure 1.4a-b). Guide RNA (gRNA) 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 $\sim 29.3 \pm 8.3\%$, Δ CRE2 $\sim 40.1 \pm 11.8\%$, Δ CRE3 $\sim 30.6 \pm 9.1\%$, Δ CRE4 $\sim 23.6 \pm 8.2\%$, Δ CRE5 $\sim 25.3 \pm 6.6\%$, Δ CRE6 $\sim 24.5 \pm 10.2\%$ and Δ *FOXA1* (exon 1 and intron 1) $\sim 87.4 \pm 8.8\%$ reduction relative to basal levels) (Figure 1.4c, ??a-f). In contrast, deletion of several negative control regions within the same TAD did not significantly reduce *FOXA1* mRNA level (Figure 1.4c, ??g-i). Similar results were observed in 22Rv1

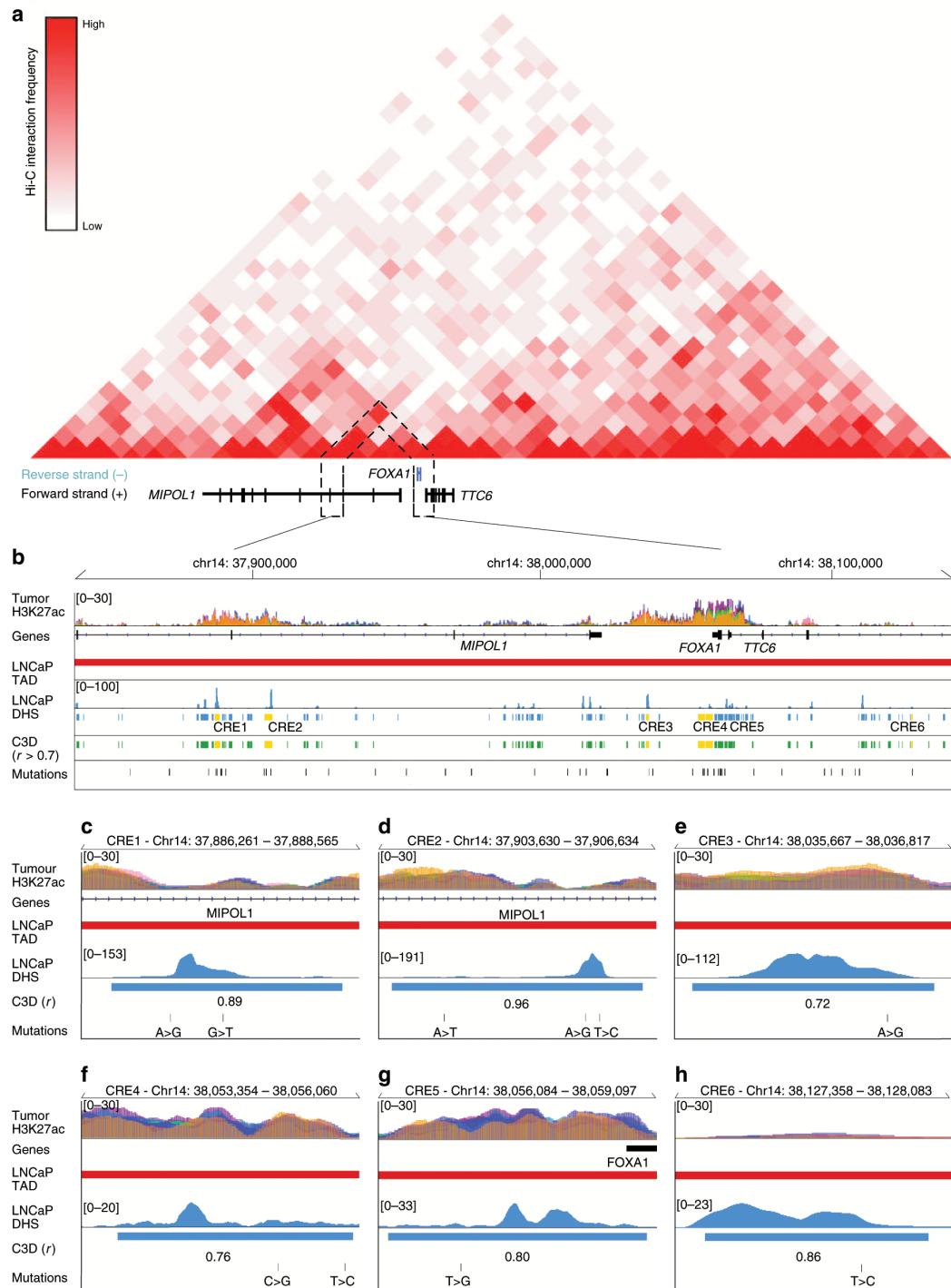


Figure 1.3: Putative CREs predicted to interact with *FOXA1* promoter. **a.** Hi-C conducted in LNCaP cells indicating physical interactions between putative *FOXA1* CREs and the *FOXA1* promoter. Hi-C resolution is 40 kbp. **b.** The six putative *FOXA1* CREs are coloured in yellow. **c-h.** Zoom-in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

PCa cells (Figure 1.4d). As each clone expressed Cas9 protein at different levels, there may be a difference between genome editing efficiencies between the clones. We compared the CRISPR/Cas9 on-target genome editing efficiency across the five LNCaP cell line-derived clones with the relative *FOXA1* mRNA levels, and indeed observe a significant inverse correlation across all CREs (Pearson's correlation $r = 0.49, p < 0.005$) (??a) and agreeing trends for each individual CRE (??b).

Complementary to our findings using the WT CRISPR/Cas9 system, we next generated four LNCaP and four 22Rv1 cell line-derived dCas9-KRAB fusion protein expressing clones (Figure 1.4e-f). Transient transfection of the same gRNAs used in the WT Cas9 experiments, targeting the six *FOXA1* plexus CREs into our dCas9-KRAB LNCaP clones significantly decreased *FOXA1* expression relative to basal levels (iCRE1 $\sim 24.6 \pm 6.2\%$, iCRE2 $\sim 42.2 \pm 10.8\%$, iCRE3 $\sim 25.3 \pm 9.2\%$, iCRE4 $\sim 23.3 \pm 4.3\%$, iCRE5 $\sim 30.2 \pm 3.4\%$ and iCRE6 $\sim 23.1 \pm 8.1\%$ reduction). Similarly, gRNAs targeting the dCas9-KRAB fusion protein to *FOXA1* decreased its expression (i*FOXA1* $\sim 81.6 \pm 11.8\%$ reduction; Student's *t*-test, $p < 0.05$, Figure 1.4g). Analogous results were also observed in our four clonal 22Rv1 dCas9-KRAB cell lines (Student's *t*-test, $p < 0.05$, Figure 1.4h). Collectively, our results suggest that the six CREs control *FOXA1* expression.

We further assessed the regulatory activity of the six *FOXA1* plexus CREs by testing the consequent mRNA expression on other genes within the same TAD, namely *MIPOL1* and *MIPOL1*. Δ CRE1 and Δ CRE2 significantly reduced *MIPOL1* mRNA expression by $\sim 38.4 \pm 6.4\%$ and $\sim 48.4 \pm 9\%$, respectively relative to basal levels, whereas deletion of the other four CREs did not result in any significant *MIPOL1* expression changes (Student's *t*-test, $p < 0.05$, ??a). On the other hand, deletion of CREs each significantly reduced *MIPOL1* mRNA expression relative to its basal levels (Δ CRE1 $\sim 52.9\% \pm 6.4\%$, Δ CRE2 $\sim 66 \pm 11.3\%$, Δ CRE3 $\sim 55.5 \pm 12.8\%$, Δ CRE4 $44.9 \pm 10.6\%$, Δ CRE5 $43.1 \pm 11.9\%$ and Δ CRE6 $52.2 \pm 7.3\%$ reduction (Student's *t*-test, $p < 0.05$, ??b), in agreement with the fact that *MIPOL1* shares its promoter with *FOXA1* as both genes are transcribed on opposing strands (??c).

Reduction in *FOXA1* mRNA expression resulting from the deletion of *FOXA1* plexus CREs may also impact gene expression downstream of *FOXA1*, we assessed the mRNA expression of several *FOXA1* target genes, namely *SNAI2*, *ACPP*, and *GRIN3A*. Deletion of CREs resulted in significant change in *SNAI2* (up-regulation; Δ CRE1 $\sim 190\%$, Δ CRE2 $\sim 162.8\%$, Δ CRE3 $\sim 147.5\%$, Δ CRE4 $\sim 133.3\%$, Δ CRE5 $\sim 137.3\%$, Δ CRE6 $\sim 120.8\%$, Δ *FOXA1* $\sim 266.7\%$), *ACPP* (down-regulation; Δ CRE1 $\sim 73.5\%$, Δ CRE2 $\sim 62.5\%$, Δ CRE3 $\sim 69.6\%$, Δ CRE4 $\sim 75.6\%$, Δ CRE5 $\sim 70.9\%$, Δ CRE6 $\sim 74.6\%$, Δ *FOXA1* $\sim 52.2\%$) and *GRIN3A* expression (up-regulation; Δ CRE1 $\sim 138.2\%$, Δ CRE2 $\sim 168.8\%$, Δ CRE3 $\sim 144.6\%$, Δ CRE4 $\sim 132.1\%$, Δ CRE5 $\sim 131.4\%$, Δ CRE6 $\sim 127\%$, Δ *FOXA1*

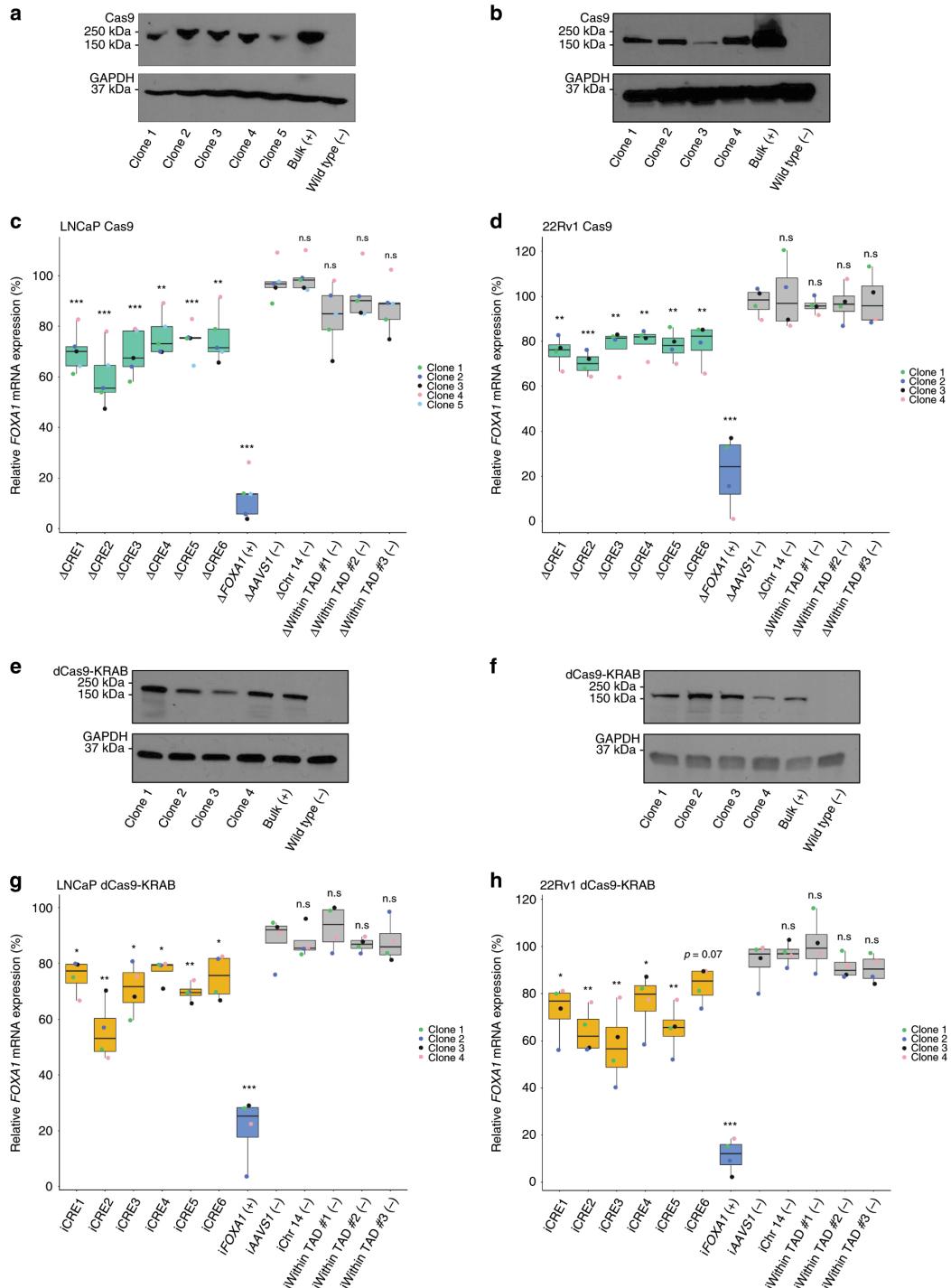


Figure 1.4: Functional dissection of putative *FOXA1* CREs. (Continued on the following page)

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

$\sim 228\%$) (Student's *t*-test, $p < 0.05$, ??d-f). Collectively, our results support the restriction of most *FOXA1* plexus CREs towards *FOXA1* and its target genes.

1.3.5 *FOXA1* CREs collaborate to regulate its expression

Expanding on the idea that multiple CREs can converge to regulate the expression of a single target gene [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 PCa 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 PCa cell clones stably expressing WT Cas9 and dCas9-KRAB, individual CRE deletion resulted in a significant reduction in *FOXA1* mRNA expression: (Δ CRE1 $\sim 29.3 \pm 7.3\%$, Δ CRE2 $\sim 36 \pm 11.8\%$, Δ CRE3 $\sim 30.6 \pm 12.7\%$, Δ CRE4 $\sim 24.5 \pm 6.1\%$, Δ CRE5 $\sim 23.7 \pm 13.2\%$, Δ CRE6 $\sim 26.8 \pm 14.2\%$ and Δ *FOXA1* $\sim 96.2 \pm 1.4\%$ reduction (Student's *t*-test, $p < 0.05$, Figure 1.5a, ??a-f). Next for combinatorial deletions, we prioritized the CREs that harbor more than 1 SNV (i.e CRE1, CRE2, CRE4), and transiently transfected RNP complexes that target both CREs in various combinations (i.e CRE1 + CRE2, CRE1 + CRE4, CRE2 + CRE4), and assessed *FOXA1* mRNA expression. Compared to negative control regions, the combinatorial deletion of Δ CRE1 + Δ CRE2, Δ CRE1 + Δ CRE4, and Δ CRE2 + Δ CRE4 resulted in a

significant $\sim 48.5 \pm 4.5\%$, $\sim 50.4 \pm 2.9\%$ and $\sim 45.2 \pm 5.5\%$ reduction in *FOXA1* mRNA expression, respectively (Student's *t*-test, $p < 0.05$, Figure 1.5b, ??a-f) a fold reduction greater than single CRE deletions (Student's *t*-test, ??, $p < 0.05$). These results together demonstrate that these CREs collaboratively contribute to the establishment and regulation of *FOXA1* expression in PCa.

1.3.6 Disruption of *FOXA1* CREs reduces PCa cell growth

As *FOXA1* is essential for PCa growth (Figure 1.1b-e), we next sought to assess the importance of the six *FOXA1* plexus CREs towards PCa cell growth. We adapted a lentiviral-based approach that expressed both the Cas9 protein and two gRNA that target each CRE for deletion (See Methods). Upon lentiviral transduction with subsequent selection, we separated LNCaP PCa cells for RNA, DNA and for cell proliferation. We first tested the system by measuring *FOXA1* mRNA expression, and independently observed significant reductions of *FOXA1* mRNA expression (Δ CRE1 $\sim 18\%$, Δ CRE2 $\sim 30\%$, Δ CRE3 $\sim 15\%$, Δ CRE4 $\sim 12\%$, Δ CRE5 $\sim 35\%$, Δ CRE6 $\sim 46\%$ and Δ *FOXA1* (exon 1 and intron 1) $\sim 48\%$ reduction (Student's *t*-test, $p < 0.05$, Figure 1.5c, ??a-f). We then seeded these cells at equal density. Six days post-seeding, we harvested the cells and observed a significant reduction in cell growth upon deleting any of the six *FOXA1* plexus CREs (Δ CRE1 $\sim 42\%$, Δ CRE2 $\sim 28\%$, Δ CRE3 $\sim 33\%$, Δ CRE4 $\sim 27\%$, Δ CRE5 $\sim 42\%$, Δ CRE6 $\sim 44\%$ and Δ *FOXA1* (exon 1 and intron 1) $\sim 50\%$ reduction (Student's *t*-test, $p < 0.05$, Fig 5d). These results suggest that the six *FOXA1* plexus contribute to PCa etiology, in agreement with their ability to regulate *FOXA1* expression and the essentiality of this gene in PCa cell growth.

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

SNVs can alter the transactivation potential of CREs [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 WT allele of each CRE (Figure 1.6b-k). We found that the variant alleles of 6 of the 10 SNVs displayed significantly greater luciferase reporter activity when compared to the WT alleles (Mann-Whitney U test, $p < 0.05$). Specifically, we observed the following fold-changes: chr14:37,887,005 A > G (1.65-fold), chr14:37,904,343 A > T (1.35-fold), chr14:37,905,854 A > G (1.28-fold), chr14:37,906,009 T > C (1.71-fold), chr14:38,036,543 A > G (1.44-fold), chr14:38,055,269 C > G (1.39-fold) (Figure 1.6b, d-h). These results indicate that these SNVs can alter the transactivation potential of *FOXA1* plexus CREs in PCa cells.

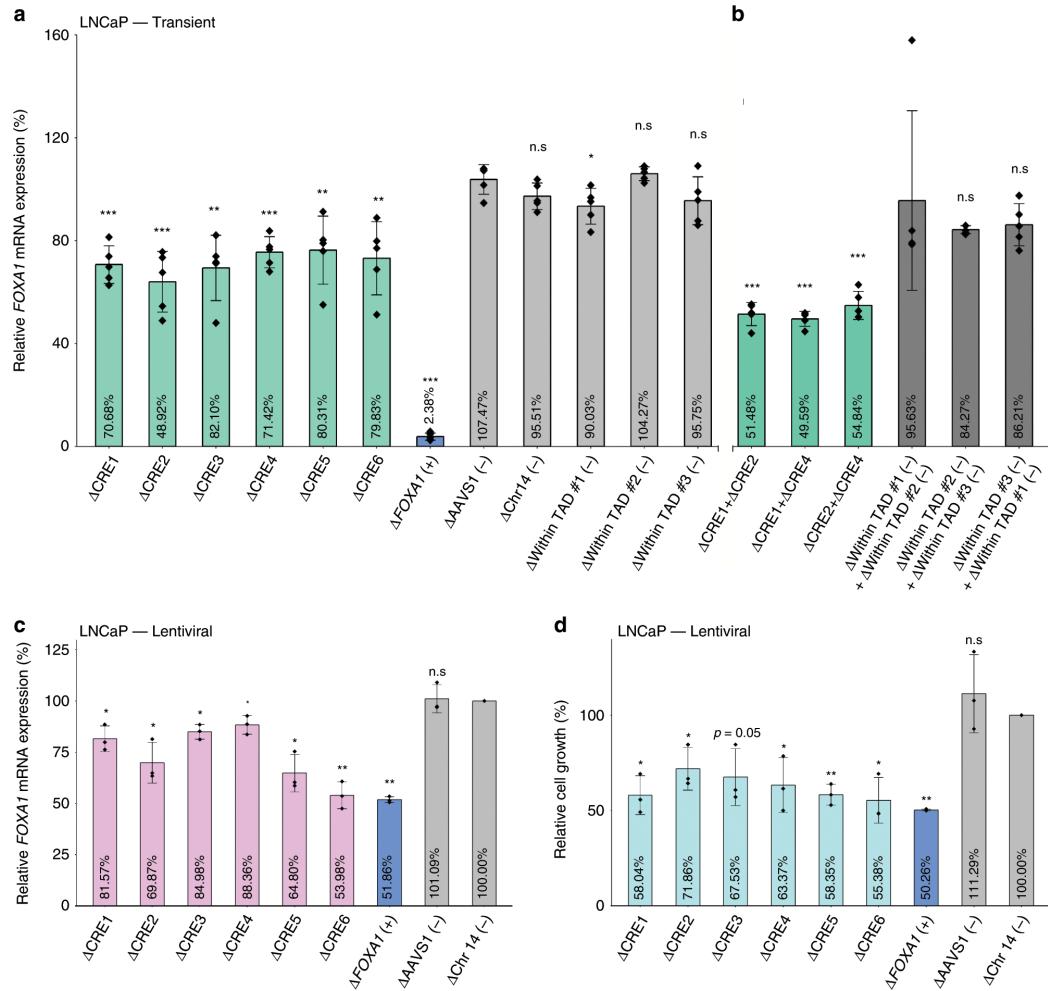


Figure 1.5: *FOXA1* CREs collaborate to regulate its expression and are critical for PCa cell proliferation.. **a.** *FOXA1* mRNA expression normalized to housekeeping *TBP* mRNA expression upon transient transfection-based CRISPR/Cas9-mediated deletion of CRE1, CRE2, CRE4, and sequential deletion combinations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

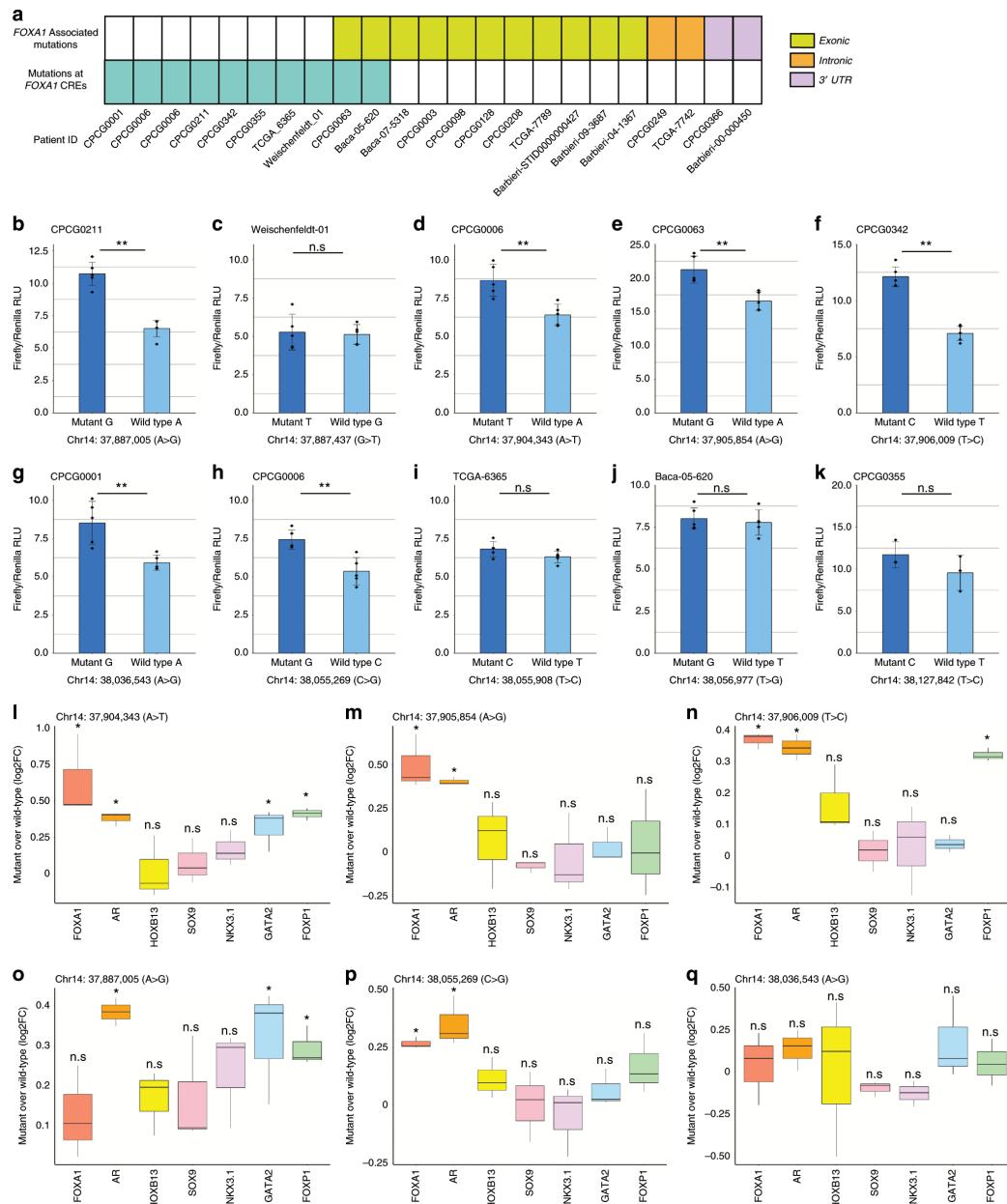


Figure 1.6: A subset of noncoding SNVs mapping to the *FOXA1* CREs are gain-of-function. **a.** Matrix showcasing the patients from the CPC-GENE dataset that harbour SNVs at the *FOXA1* CREs, exons, introns, and the 3' UTR of *FOXA1*. **b-k.** Luciferase assays are conducted in LNCaP cells. Bar plot 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 PCa cells. Data is presented as \log_2 fold-change of variant sequence upon comparison to WT sequence ($n = 3$ independent experiments per ChIP). Hypothesis testing done with Student's *t*-test, n.s. not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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 PCa 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 1.6l-q). Compared to the WT sequence, chr14:37,887,005 A > G significantly increased *AR* binding (1.31-fold increase), *GATA2* binding (1.25-fold increase) and *FOXP1* binding (1.23-fold increase); chr14:37,904,343 A > T significant increased *AR* binding (1.30-fold increase), *GATA2* (1.25-fold increase) and *FOXP1* (1.33-fold increase); chr14:37,905,854 A > G significantly increased *FOXA1* binding (1.41-fold increase) and *AR* binding (1.33-fold increase); chr14:37,906,009 T > C significantly increased the binding of *FOXA1* (1.29-fold increase), *AR* (1.31-fold increase), *HOXB13* (1.13-fold increase) and *FOXP1* (1.25-fold increase); and chr14:38,055,269 C > G significantly increased *FOXA1* binding (1.20-fold increase). Notably all six SNVs increased the binding of the TFs known to bind at these CREs. In contrast, none of the SNVs significantly decreased the binding of these TFs. Our observations suggest that gain-of-function SNVs populate the *FOXA1* plexus CREs.

1.4 Discussion

Modern technologies and understanding of the epigenome allow the possibility of probing CREs involved in regulating genes implicated in disease. Despite *FOXA1* being recurrently mutated [5–8, 11] and playing potent oncogenic roles in PCa 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 PCa.

We used the DHS profiled in PCa cells to identify putative *FOXA1* CREs through annotating these regions with five different histone modifications, TF binding sites and noncoding SNVs profiled in PCa cells and primary prostate tumours. Our efforts identified and validated a set of six active CREs involved in *FOXA1* regulation, agreeing with a recent report where a subset of our CREs map to loci suggested to be in contact with the *FOXA1* promoter [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 PCa 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 PCa [59]. Our findings complement recent reports of SNVs found in the noncoding space of *FOXA1* that could affect its expression [14, 61]. The *FOXA1* plexus CREs we identified here are also reported to be target of structural variants in both the primary and metastatic settings [9, 62], including tandem duplication in ~14% (14/101) mCRPC tumours over CRE2 [62], amplification, duplication and translocation over CRE3, CRE4, and CRE5 [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 PCa etiology. As a whole, our findings in conjunction with recent reports suggest that CREs involved in the transcriptional regulation of *FOXA1* may be hijacked in prostate tumours through various types of genetic alterations.

Despite initial treatment responses from treating aggressive primary and metastatic PCa through castration to suppress AR signalling [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 PCa, 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 downregulate *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 PCa TFs. The study supports the importance of

considering CREs not only as lone occurrences but as a team that works together to regulate their target genes, particularly when considering the impact of genetic alterations. As such, our work builds a bridge between the understanding of FOXA1 transcriptional regulation and new routes to FOXA1 inhibition. Aligning with recent reports [9, 10, 13], our findings support the oncogenic nature of FOXA1 in PCa. Gaining insight on the cis-regulatory plexuses of important genes such as FOXA1 in PCa may provide new avenues to inhibit other drivers across various cancer types to halt disease progression.

1.5 Methods

1.5.1 Cell Culture

LNCaP and 22Rv1 cells were cultured in RPMI medium, and VCaP cells were cultured in DMEM medium, both supplemented with 10% FBS, and 1% penicillin-streptomycin at 37 °C in a humidified incubator with 5% CO₂. These PCa cells originated from ATCC. 293FT cells were purchased from ThermoFisherScientific (Cat No. R70007) maintained in complete DMEM medium (DMEM with 10% FBS (080150, Wisent), L-glutamine (25030-081, ThermoFisher) and non-essential amino acids (11140-050, ThermoFisher) supplemented with 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 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 PCa cell lines and others, essentiality scores for FOXA1 were collected from all available cell lines ($n = 707$). To perform a permutation test, the median of 8 randomly selected

cell lines was calculated one million times to generate a background distribution of essentiality scores across all cell types available. The median essentiality score from the 8 PCa cell lines was calculated and its percentile within the background distribution is reported.

1.5.4 siRNA knockdown and cell proliferation assay

300,000 LNCaP cells (Day 0) were reverse transfected with siRNA (siFOXA1 using Lipofectamine®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 µg of lysate was subjected to SDS-PAGE. Upon completion of SDS-PAGE, protein was transferred onto PVDF membrane (Bio-Rad, Cat No. 1704156). The membrane was blocked with 5% non-fat milk for one hour at room temperature with shaking. After blocking, anti-FOXA1 (Abcam Cat No. 23737) in 2.5% non-fat milk was added, and was incubated at 4 °C overnight. Next day, the blot was washed and incubated with IRDye®800CW Goat Anti-Rabbit IgG secondary antibody (LI-COR, Cat No. 925-32211) at room temperature for 1 hour. The blot was then washed and assessed with the Odyssey®CLX imaging system (LI-COR).

1.5.5 Identifying putative FOXA1 CREs

Putative FOXA1 CREs were identified through the use of 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 LentidCas9-KRAB-blast plasmid (Addgene #89567, a gift from Gary Hon) and collected 72 hrs post transfection. LNCaP and 22Rv1 cells were then transduced for 24-48 hours with equal amounts of virus followed by selection with media containing blasticidin (7.5 μ g/mL for LNCaP cells, 6 μ g/mL for 22Rv1 cells). Upon selection, clones were derived by serial dilution with subsequent single cell seeding into 96-well plates containing selection media. dCas9-KRAB protein expression for each clone was then assessed through Western blotting (1 $^{\circ}$ 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 Nucleofection (SF Solution EN120 - 4D Nucleofector, Lonza). Cells were then harvested 24 hours post-transfection for RNA and DNA for RT-PCR and confirmation of deletion, respectively.

1.5.8 Transient Cas9-mediated disruption of CREs

Deletion of elements through this method were achieved through the transfection of Cas9 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 ®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 ®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 tumors (<https://dcc.iegc.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, TADs and compartments, define clusters of contacts between DNA elements that are linearly distant from each other, such as 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 PCas, 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 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 3C (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

3C 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 μ m thick

cryosections from 12 primary prostate tumours and 5 primary benign prostate sections (see Methods, Figure 2.1a, ??a). 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). Moreover, when compared to other Hi-C datasets, the primary prostate samples clustered separately from cell lines (??b), despite similar enrichment of CTCF binding sites near TAD boundaries (??c). 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 samples, such as compartment B-to-A transitions on 19q and A-to-B transitions on chromosome Y (??a-c). Only two genes on chromosome 19 were differentially expressed between the 8 tumours with benign-like compartmentalization and the other 4 (??d). Similarly, no genes on chromosome

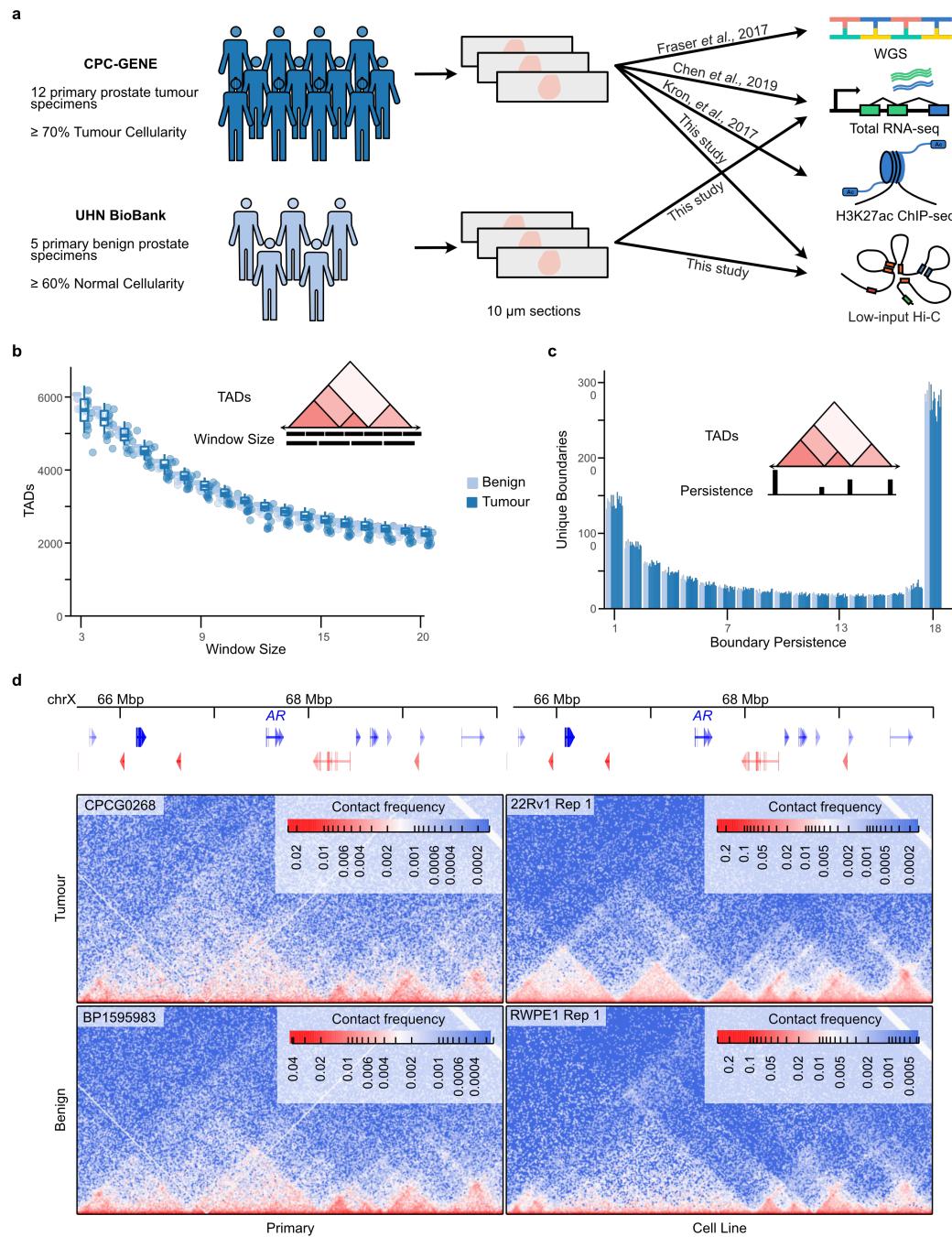


Figure 2.1: Topologically associated domains are stable over prostate oncogenesis. **a.** The sample collection and data usage of primary prostate samples in this study. 10 μm sections from 6 tumours previously identified as T2E+ and 6 T2E- were used for Hi-C sequencing. 5 additional 10 μm sections were 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.

Y were differentially expressed between the 4 tumours with benign-like compartmentalization and the remaining samples (??e). 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 (??f). 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; ??a, 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] (??b), 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 (??c). 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.

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;

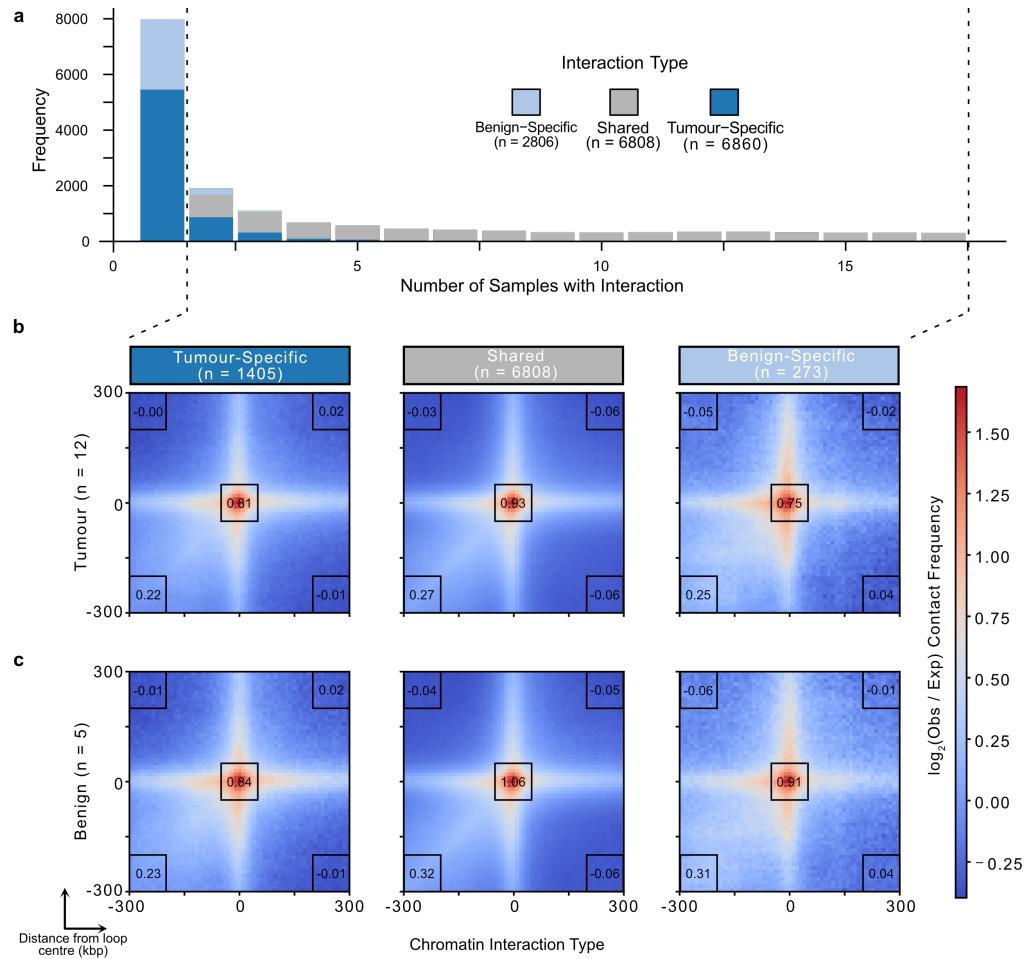


Figure 2.2: Focal chromatin interactions display subtle differences between benign and tumour tissue. **a.** Stacked bar plots of the number of samples that chromatin interactions were identified in. **b-c.** Aggregate peak analysis of tumour (**b**) or benign (**c**) contacts in tumour-specific, benign-specific, 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.

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, ??a-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 (??c). 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 (??d). 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 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, ??b). Patient CPCG0331 had 11 complex events, including a 6-breakpoint event spanning 3 chromosomes (??b). 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

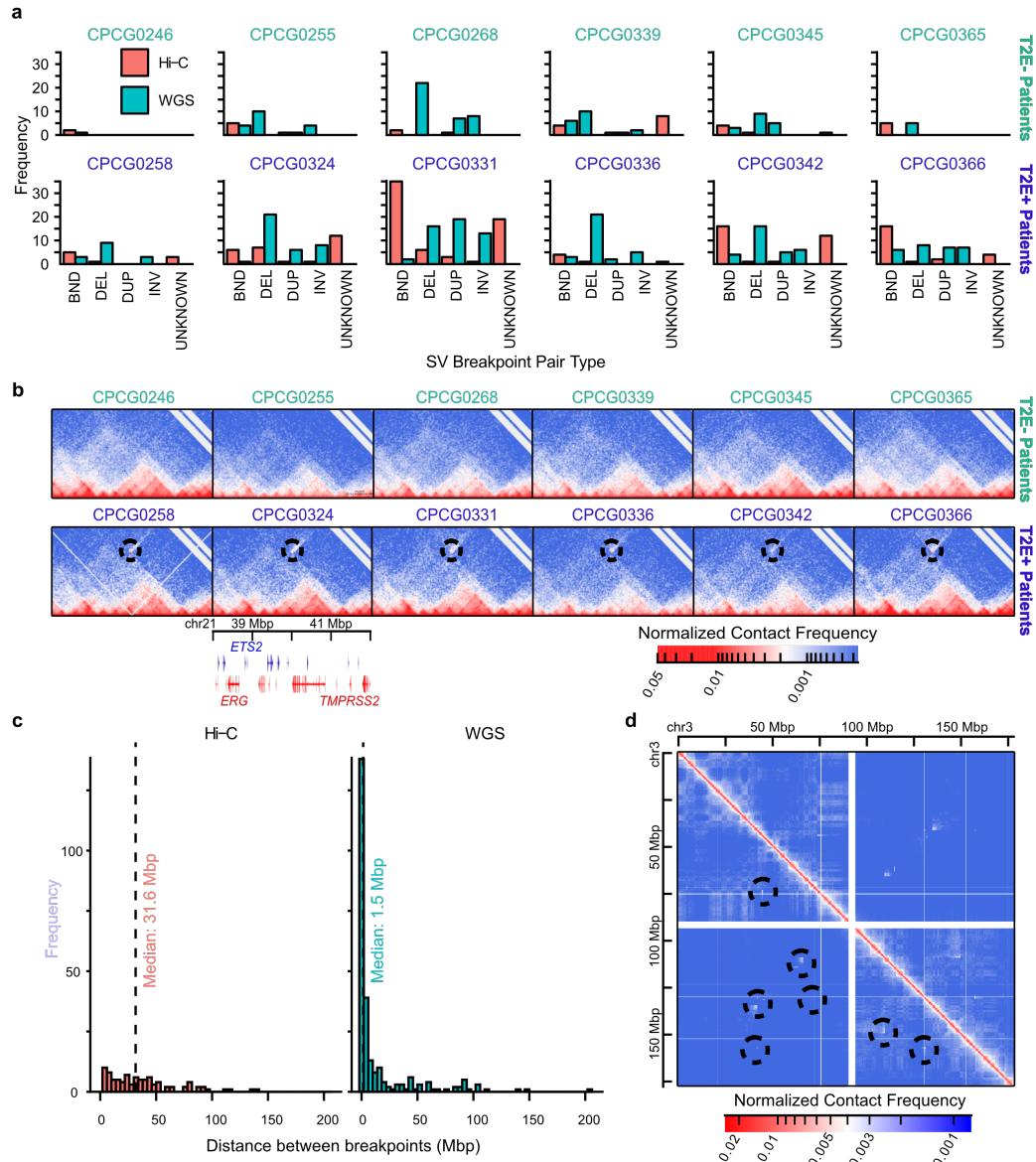


Figure 2.3: SVs are identified in primary tissue through chromatin conformation capture. **a.** Bar plot of SV breakpoint pairs identified by Hi-C and WGS on matched samples. BND = inter-chromosomal translocation, DEL = deletion, DUP = duplication, INV = inversion, UNKNOWN = breakpoint pair of unknown 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.

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 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

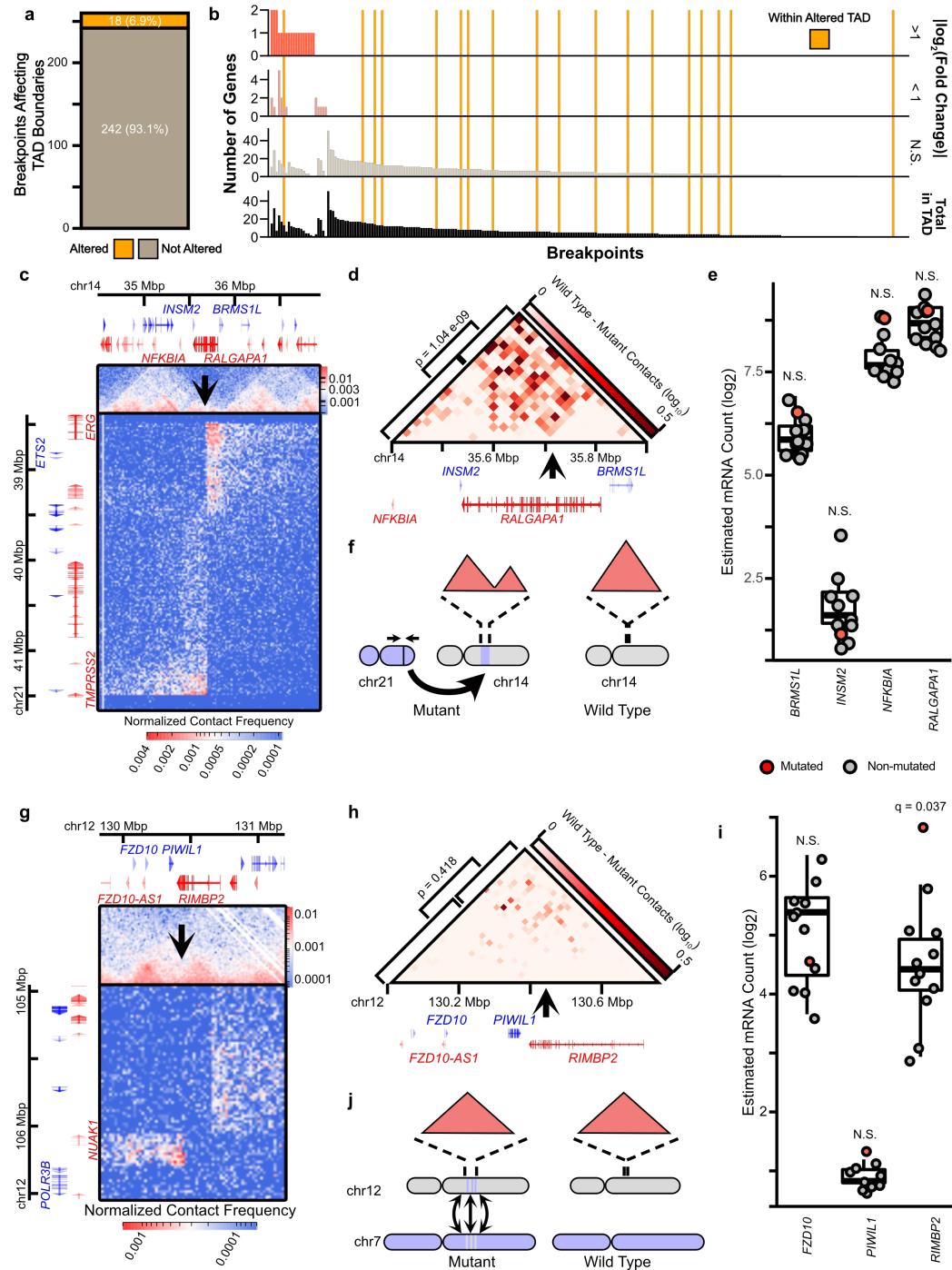


Figure 2.4: SVs can alter TADs or gene expression around breakpoints, but rarely alters both. (Continued on the following page)

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 *RALGAP41* 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. **i.** 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.

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, $\chi^2 = 7.0088$, $p = 0.00811$, $df = 1$; ??). 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 PCa 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 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 PCa [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).

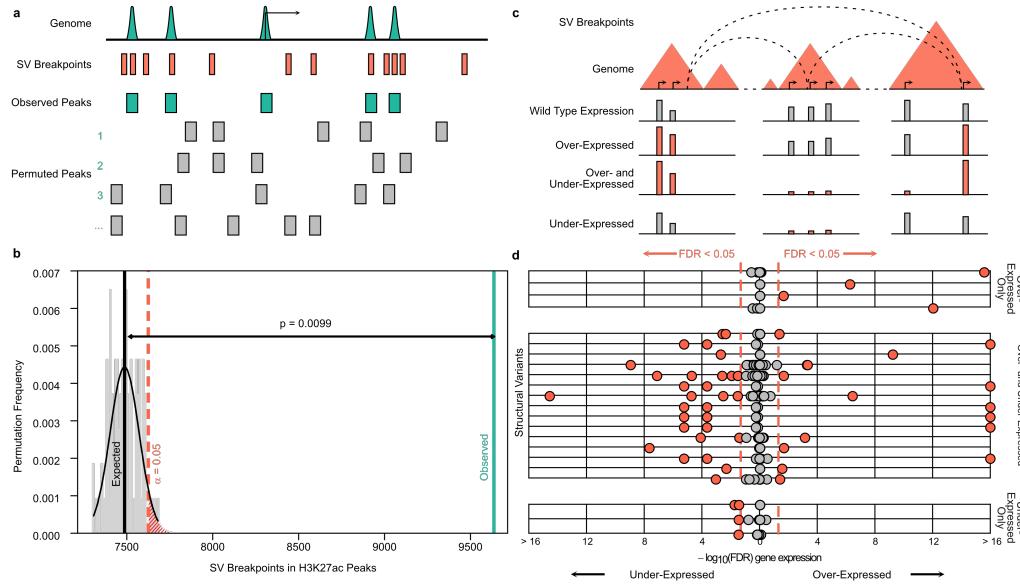


Figure 2.5: SV breakpoints are enriched in active CREs and repeatedly alter the expression of multiple genes. **a.** Schematic of permutation testing for the overlap between SV breakpoints in all CPC-GENE prostate tumours and the catalogue of active CREs in the 12 tumour samples in this study. **b.** 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 ($FDR < 0.05$), grey dots are genes not differentially expressed between the mutant and wild type tumours.

12 of these 15 (80%) SVs are associated with expression changes at SV antipodes, opposite ends of a breakpoint pair (??). 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 (??) [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;

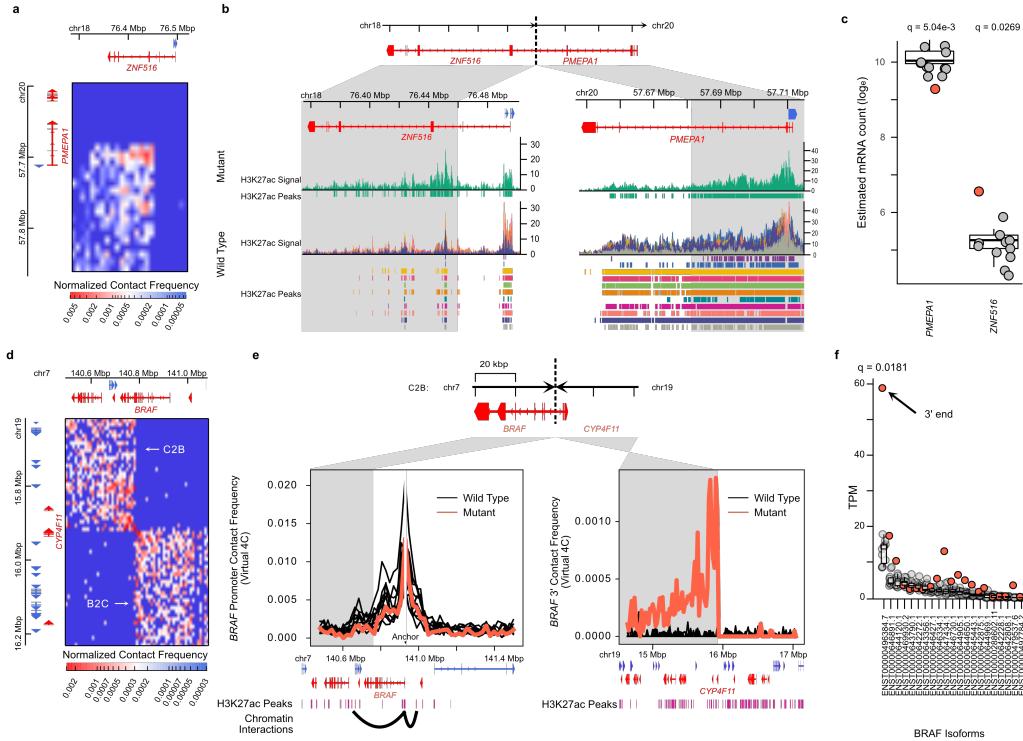


Figure 2.6: SVs altering gene expression by rewiring focal chromatin interactions. **a.** The contact matrix of the deletion between *PMEPA1* and *ZNF516*. **b.** Genome tracks of H3K27ac ChIP-seq signal around the *ZNF516* and *PMEPA1* genes with the rearrangement. Grey regions are loci brought into 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.

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 PCa resulting in changes to focal chromatin interactions, and that these effects overshadow the effect on higher-order topology in primary PCa.

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 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 μm 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 µL water, 60 µL of 10X T4 DNA Ligase Buffer (ThermoFisher Scientific), 50 µL of 10% Triton X-100, 6 µL of 20 mg/mL BSA (New England Biolabs) and 3.5 µL of 5 Weiss U/µL 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 × g for 5 minutes at RT and re-suspended in 250 µL of Extraction Buffer (50 mM Tris-Cl pH 8.0, 50 mM NaCl, 1 mM EDTA, 1% SDS) upon removal of supernatant. Next, 10 µL 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 µL 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 × 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 µL) 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 × 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 µL). Each sample was mixed with 31.5 µL of 3M sodium acetate, 2 µL of GlycoBlue (ThermoFisher Scientific), and 504 µL 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 × g for 45 minutes at 4 °C. The supernatant was carefully discarded and the pellet was washed with 800 µL of ice-cold 70% ethanol followed by

a centrifuge spin at 17,000 × 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 µL of Elution Buffer (Qiagen PCR Clean-Up Kit). 1 µL of RNase A (ThermoFisher Scientific) was added to each sample followed by incubation at 37 °C for 15 minutes. A mix of 5 µL of 10X NEB2.1 buffer (New England Biolabs), 1.25 µL of 1 mM dATP, 1.25 µL of 1 mM dCTP, 1.25 µL of 1 mM dGTP, 1 mM of dTTP, 0.5 µL of 10 mg/mL BSA, 5 µL of water, 3.5 µL of 3 U/µL 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 µL of water was added to each sample bringing total volume up to 120 µL, 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 µL of Dynabeads MyOne Streptavidin C1 beads (Life Technologies) for each sample was washed once with 400 µL of 1X B&W buffer + 0.1% Triton X-100. The beads were then re-suspended in 120 µL of 2X B&W buffer and transferred to the 120 µL 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 µL 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 µL 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 µL 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 `SSasymp` functions from the stats R package (v3.6.3). The model was fit to the following equation:

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

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

Breakpoint pairs for each patient were called on the merged BAM files using `hic_breakfinder` (commit 30a0dcc6d01859797d7c263df7335fd2f52df7b8) [92]. Pre-calculated expected observation files for the hg38 genome were downloaded from the git repository on July 24, 2019, as per the instructions. Breakpoints were explicitly called with the following command:

```
hic_breakfinder --bam-file {BAM} --exp-file-inter inter_expect_1Mb.  
hg38.txt --exp-file-intra intra_expect_100kb.hg38.txt --name {  
Sample_ID} --min-1kb.
```

For the T2E fusion, only one patient had the deletion identified by `hic_breakfinder` with default parameters (CPCG0336). Difficulties identifying SVs with `hic_breakfinder` have been previously noted [104]. After adjusting the detection threshold, we were able to identify the fusion in other

samples. To ensure the T2E+ tumours were effectively stratified for future analyses, the fusion was annotated using the same coordinates for the other T2E+ samples. No other additions to breakpoint calls were made. Certain breakpoints that appeared to be artefacts were removed, as described below.

Structural variant annotation and graph construction

The contact matrix spanning 5 Mbp upstream and downstream around the breakpoint pairs were plotted and annotated according to previously published heuristics (Supplementary Figure 4 for [92]). Breakpoint pairs that were nearby other breakpoints or did not match the heuristics in this figure were labelled as UNKNOWN. These annotations were matched against the annotations identified from the previously published whole genome sequencing structural variants [6]. Breakpoint pairs matching the following criteria were considered as detection artefacts and were ignored.

1. At least one breakpoint was > 1 Mbp
2. At least one breakpoint was surrounded by empty regions of the contact matrix
3. At least one breakpoint corresponded to a TAD or compartment boundary shared across all samples that lacked a distinct sharp edge that is indicative of a chromosomal rearrangement

To identify unique breakpoints that were identified in multiple breakpoint pairs, breakpoints that were within 50 kbp of each other were considered as possibly redundant calls. This distance was considered as the resolution of the non-artefactual calls is 100 kbp. Plotting the contact matrix 5 Mbp around the breakpoint, breakpoints calls were considered the same breakpoint if the sharp edge of each breakpoint was equal to within 5 kbp. Similar in concept to the ChainFinder algorithm [106], we consider each breakpoint as a node in a graph. Nodes are connected if they are detected as a pair of breakpoints by `hic_breakfinder`. Simple structural variants are connected components in the breakpoint graph containing only two nodes, and complex variants those with greater than two nodes. A visual representation of these graphs can be found in ??b. Graphs are displayed with a spring-force layout, adjusted using the Kamada Kawai optimization [138] from the NetworkX Python package (v2.4) [139].

Determination of structural variant breakpoints altering intra-TADs contacts

Patients are assigned into one of two groups using hierarchical clustering (complete linkage) with the matrix of pairwise BPscore [135] values as a distance matrix. If the clustering equals the

mutated samples from the non-mutated samples (i.e. the clustering matches the mutation status in this locus), then the local topology was classified as **altered** because of the SV.

Virtual 4C

Two parts of the BRAF gene were used as anchors for virtual 4C data: the promoter region (1500 bp upstream, 500 bp downstream of the TSS) and the entire gene downstream of the breakpoint. Contact frequencies from the ICE-normalized, 20 kbp contact matrices were extracted, with the rows as the bins containing the anchor and the columns as the target regions (the x-axes in Figure 2.6e). The row means were calculated to produce a single vector where each element is the average normalized contact frequency between the anchor of interest and the distal 20 kbp bin. These vectors were plotted as lines in Figure 2.6e.

2.4.5 Patient Tumour Tissue H3K27ac ChIP-seq

ChIP-seq against H3K27ac was previously published for these matching samples in [38]. Sequencing data was processed similarly to the previous publication of this data [38]; however, the hg38 reference genome was used instead of hg19.

Sequence alignment

FASTQ files from single-end sequencing were aligned to the hg38 genome using Bowtie2 (v2.3.4) with the following command:

```
bowtie2 -x {genome} -U {input} 2> {output_report} | samtools view -u
> {output_bam}
```

For FASTQ files from paired-end sequencing, only the first mate was considered and reads were aligned with the following command:

```
bowtie2 -x {genome} -U {input} -3 50 2> {output_report} | samtools
view -u > {output_bam}
```

This ensured that all H3K27ac ChIP-seq data had the same format (single-end) and length (52 bp) before alignment to mitigate possible differences in downstream analyses due to different sequencing methods. Duplicate reads were removed with sambamba (v0.6.9) via **sambamba markdup -r** and were then sorted by position using **sambamba sort**.

Peak calling

Peak calling was performed using MACS2 (v2.1.2) [140] with the following command:

```
macs2 callpeak -g hs -f BAM -q 0.005 -B -n {output_prefix} -t {  
    seq_chip} -c {seq_input}
```

ENCODE hg38 blacklist regions were then removed from the narrow peaks [141]. Peaks calls are in Supplementary Table 8.

Differential acetylation analysis

Unique peak calls and de-duplicated pull-down and control BAM files from tumour samples were loaded into R with the DiffBind package (v2.14.0) [142] using DESeq2 (v1.26.0) 84 as the differential analysis model. 3 of the 12 samples had low quality peak calls compared to the other 9 and were not considered when calculating differential acetylation (CPCG0268, CPCG0255, and CPCG0336). We considered each unique breakpoint one at a time in the remaining 9 samples. Samples were grouped by their mutation status (i.e. a design matrix where the mutation status is the only covariate) and DiffBind’s differential binding analysis method was performed to identify all differentially acetylated regions between the two groups. Acetylation peaks outside of the TADs overlapping the breakpoint were filtered out. Multiple test correction with the Benjamini-Hochberg FDR method [143] was performed on all peaks after all breakpoints were considered, due to similar group stratifications depending on the breakpoint under consideration.

Structural variant breakpoint enrichment

Structural variant breakpoint coordinates from WGS data from the CPC-GENE cohort were obtained from the International Cancer Genome Consortium (structural somatic mutations from the PRAD-CA dataset, release 28). Breakpoint coordinates were lifted over to hg38 coordinates using the liftOver function from the rtracklayer R package (v1.46.0) [144]. Permutation tests were performed with the regioneR R package (v1.18.0) [145], selecting randomized regions from the hg38 genome, excluding the ENCODE blacklist regions [141] and masked loci. 100 permutations were calculated and a one-sided permutation z -test was used to calculate statistical significance.

2.4.6 Primary Tissue RNA Data Analysis

Tumour sample RNA sequencing

Total RNA was extracted for the CPC-GENE tumour samples as previously described [101]. Briefly, total RNA was extracted with mirVana miRNA Isolation Kit (Life Technologies) according to the manufacturer’s instructions. RNA samples were sent to BGI Americas where it underwent QC and DNase treatment. For each sample, 200 ng of total RNA was used to construct a TruSeq strand-specific library with the Ribo Zero protocol (Illumina, Cat. #RS-122-2203). The libraries were sequenced on a HiSeq 2000 to a minimal target of 180 million, 2×100 bp paired-end reads.

RNA sequencing data pre-processing

RNA sequencing FASTQ files were pseudo-aligned to the hg38 genome using Kallisto (v0.46.1) [146] with the following command:

```
kallisto quant --bootstrap-samples 100 --pseudobam --threads 8 --
index /path/to/GRCh38.idx --output-dir {output_dir} {input_R1.
fastq.gz} {input_R2.fastq.gz}
```

Differential gene expression analysis

To assess whether SVs were associated with local gene expression changes, we considered each unique breakpoint one at a time. For each breakpoint, we compared the gene expression between the mutated and non-mutated tumour samples using Sleuth (v0.30.0) [147, 148] with a linear model where the mutation status was the only covariate. To reduce the chance of falsely identifying genes as differentially expressed, only genes located within the TADs (window size $w = 20$) containing breakpoints were considered. Fold-change estimates of each transcript were assessed for significance using a Wald test. Transcript-level p-values are combined to create gene-level p-values using the Lancaster aggregation method provided by the Sleuth package [148]. Correcting for multiple tests was then performed with the Benjamini-Hochberg FDR correction for all genes that were potentially altered in the mutated sample(s).

Glossary

3C chromatin conformation capture

AR androgen receptor

ChIP-seq chromatin immunoprecipitation sequencing

CPC-GENE Canadian Prostate Cancer Genome Network

CRE *cis*-regulatory element

DEPMAP Cancer Dependency Map

DHS DNase I hypersensitive sites

FOX forkhead box

gRNA guide RNA

kbp kilobase

mCRPC metastatic castration-resistant prostate cancer

mRNA messenger RNA

RNAi RNA interference

RNA-seq RNA sequencing

SNV single nucleotide variants

PCa prostate cancer

SV structural variant

TAD topologically associated domain

TCGA The Cancer Genome Atlas

TF transcription factor

UTR untranslated region

WGS whole genome sequencing

WT wild-type

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