

1 **BRG1 promotes transcriptional patterns that are permissive to proliferation in cancer cells**

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4 **Authors:**

5 Katherine A. Giles^{1,2,3}, Cathryn M. Gould¹, Joanna Achinger-Kawecka^{1,4}, Scott G. Page², Georgia

6 Kafer², Phuc-Loi Luu^{1,4}, Anthony J. Cesare², Susan J. Clark^{1,4*}, Phillipa C. Taberlay^{3*}

7 *These authors contributed equally

8

9 **Affiliations:**

10 ¹Epigenetics Research Laboratory, Genomics & Epigenetics Theme, Garvan Institute of Medical
11 Research, Sydney, NSW, 2010, Australia

12 ²Genome Integrity Unit, Children's Medical Research Institute, University of Sydney, Westmead,
13 NSW, 2145, Australia

14 ³Tasmanian School of Medicine, College of Health and Medicine, University of Tasmania, Hobart,
15 TAS, 7000, Australia

16 ⁴St Vincent's Clinical School, UNSW Sydney, Sydney, NSW, 2000, Australia

17

18 **Correspondence:** Phillipa C. Taberlay (phillippa.taberlay@utas.edu.au)

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27 **ABSTRACT**

28 **Background:** BRG1 (encoded by *SMARCA4*) is a catalytic component of the SWI/SNF chromatin
29 remodelling complex, with key roles in modulating DNA accessibility. Dysregulation of BRG1 is
30 observed, but functionally uncharacterised, in a wide range of malignancies. We have probed the
31 functions of BRG1 on a background of prostate cancer to investigate how BRG1 controls gene
32 expression programs and cancer cell behaviour.

33 **Results:** Our investigation of *SMARCA4* revealed that BRG1 is universally overexpressed in 486
34 tumours from The Cancer Genome Atlas prostate cohort, as well as in a complementary panel of
35 21 prostate cell lines. Next, we utilised a temporal model of BRG1 depletion to investigate the
36 molecular effects on global transcription programs. Unexpectedly, depleting BRG1 had no impact
37 on alternative splicing and conferred only modest effect on global expression. However, of the
38 transcriptional changes that occurred, most manifested as down-regulated expression. Deeper
39 examination found the common thread linking down-regulated genes was involvement in
40 proliferation, including several known to increase prostate cancer proliferation (*KLK2*, *PCAT1* and
41 *VAV3*). Interestingly, the promoters of genes driving proliferation were bound by BRG1 as well
42 as the oncogenic transcription factors, AR and FOXA1. We also noted that BRG1 depletion
43 repressed genes involved in cell cycle progression and DNA replication but intriguingly, these
44 pathways operated independently of AR and FOXA1. In agreement with transcriptional changes,
45 depleting BRG1 conferred G1 arrest.

46 **Conclusions:** Our data have revealed that BRG1 has capacity to drive oncogenesis by coordinating
47 oncogenic pathways dependent on BRG1 for proliferation, cell cycle progression and DNA
48 replication.

49

50 **Keywords:** BRG1, *SMARCA4*, chromatin remodelling, cancer, gene expression, cell cycle,
51 transcription, DNA replication

52

53 **BACKGROUND**

54

55 Nucleosomes serve as a physical backbone for chromatin organization on a global scale and at
56 local gene regulatory elements. Nucleosomes therefore govern both genome-wide stability and
57 local DNA accessibility (1). Nucleosome positioning by ATP-dependent chromatin remodelers
58 plays a critical role in regulating DNA accessibility and allows genes to be expressed at the
59 appropriate place and time (1). Genomic profiling has demonstrated that dynamic regulation of
60 DNA accessibility occurs primarily at DNA regulatory elements, which are cell type specific, and
61 that DNA accessibility changes reflect concomitant transcriptional patterns. (2, 3). It is essential
62 for chromatin to be relaxed at active gene promoters to create an ordered nucleosome disassembly,
63 which permits binding of RNA pol II and the general transcription machinery (4, 5). In agreement,
64 ChIP-seq data show that transcription factors are concentrated on accessible DNA, with the highest
65 levels of bound transcription factors correlating with the most accessible genomic regions (6).
66 Conversely, chromatin condensation resulting in reduced DNA accessibility is necessary for
67 transcriptional repression (7). Disruption to the DNA accessibility landscape is a feature of cancer
68 (2, 8, 9). This was recently emphasized in genomic sequencing data from multiple cancers and
69 cancer subtypes, which revealed associations between the accessible chromatin organization and
70 mutation load (8). Moreover, studies of aged human and yeast cells demonstrated that nucleosome
71 loss compromises genome stability, gene regulation and transcription (10, 11).

72

73 Genes encoding ATP-dependent chromatin remodelers are themselves frequently mutated and
74 often atypically expressed in cancer (5, 12-16). Notably, the SWI/SNF chromatin remodelling
75 complex is mutated or transcriptionally deregulated in ~20% of cancers; a mutation frequency
76 approaching that of *TP53* (~26%) (12, 14, 17). The SWI/SNF complex is often described as a
77 tumour suppressor because it is required by the Retinoblastoma protein (Rb) family for regulation
78 of normal cell growth (18, 19). Disruptions of multiple SWI/SNF subunits are reported in human

79 tumours and cell lines (13-15, 20-37), often accompanied by a loss of heterozygosity consistent
80 with the inactivation of a tumour suppressor (13, 34). The specific SWI/SNF mutations observed
81 in tumours and the cancers associated with altered SWI/SNF function have been extensively
82 reviewed (12-15, 26, 31, 34, 38). However, the mechanism and functional consequences of
83 SWI/SNF dysregulation are still being defined.

84

85 Brahma-related gene 1 (BRG1) is one of the two mutually exclusive ATPases within the SWI/SNF
86 complex. Interestingly, *SMARCA4*, the gene encoding BRG1, has been observed in both down-
87 and up- regulated states in cancer, indicative of the diverse and complex BRG1 functions.
88 *SMARCA4* mRNA was seen to be down regulated in bladder, colon, non-triple negative breast
89 cancers, head and neck, oesophageal, melanoma, pancreatic, lung and ovarian cancers, and
90 *SMARCA4* mutation rates in these cancers have been reported between 4-13% (12-14, 22, 24, 30,
91 39-41). In contrast, *SMARCA4* has been reported as over expressed in cancers of the prostate, triple
92 negative breast cancers and some leukaemias (12, 22, 24, 30, 42, 43). In *SMARCA4* over
93 expressing cancers, no significant recurrent mutations have been reported (42, 44-46). The
94 importance of BRG1 in cancer is further evidenced through studies of synthetic lethality, where
95 BRG1 was observed to have a synthetic lethal relationship with the alternative SWI/SNF ATPase
96 Braham (BRM), and Aurora A kinase in lung cancer, and PTEN in prostate cancer (43, 47, 48).

97

98 Examination of multiple prostate cancer cohorts has demonstrated elevated *SMARCA4* expression
99 or increased BRG1 protein levels. Clinical studies of primary prostate tumours reported an overall
100 increase in BRG1 protein by immunohistochemistry (42, 44-46). Moreover, increased *SMARCA4*
101 gene expression has been reported in tumours from The Cancer Genome Atlas (TCGA) prostate
102 cancer cohort (49, 50). While it is established that BRG1 is commonly up regulated in prostate
103 cancer, the full range of molecular pathways impacted by dysregulated BRG1 levels and the
104 contribution of these molecular changes to the atypical phenotype of prostate cancer cells remains

105 unclear.

106

107 BRG1 has known roles in regulating DNA for temporal gene expression at both promoters and
108 enhancer gene regulatory elements (4, 51-56). Moreover, BRG1 maintains the epigenetic
109 landscape of a cell at these gene regulatory elements. Specifically, BRG1 has been directly linked
110 to transcriptional output through its recognition of H3K14ac (57-59). In the absence of H3K14ac,
111 BRG1 is still present at promoters and histones are disassembled from the chromatin; however,
112 transcription is reduced (60). At enhancers, BRG1 depletion greatly reduces H3K27ac and subtlety
113 reduces H3K4me1, which is correlated with a decrease in chromatin accessibility (53). BRG1 is
114 also known to mediate inter-chromosomal looping interactions between specific loci such as the
115 *MYC* enhancer and promoter, the alpha-globulin genes, the *IgH* locus, and the class II major
116 histocompatibility complex gene locus (24, 61-64). On a global scale, BRG1 binding has been
117 found at DNA-loop anchors (56) and topological associated domain (TAD) boundaries where it
118 increases their stability (65). Together, this demonstrates an important role for BRG1 in
119 maintaining chromatin architecture at both local and global levels for transcription regulation.

120

121 Here we dissected the molecular role of BRG1 on the transcriptome in prostate cancer. We
122 confirmed that *SMARCA4* is over-expressed in prostate cancer irrespective of severity or cancer
123 subtype and identified *SMARCA4* was also over expressed in a panel of prostate cancer cell lines.
124 Depletion of BRG1 in LNCaP prostate cancer cells resulted in a modest effect on global gene
125 transcription with most changes resulting in down-regulated gene expression. Within the cohort
126 of down-regulated genes in BRG1 depleted cells we identified gene clusters defined by their co-
127 occupancy or independence from transcription factors AR and FOXA1, both of which are known
128 BRG1 co-activators (66-68). Our data revealed that BRG1, AR and FOXA1 co-regulate known
129 prostate cancer genes *KLK2*, *PCAT1* and *VAV3*. Gene ontology analysis further revealed that genes
130 regulated by BRG1 independent of AR and FOXA1 include factors regulating cell cycle and

131 proliferation processes including DNA replication. In agreement, depleting BRG1 promoted G1
132 arrest resulting in reduced cell proliferation. Cumulatively the data indicate BRG1 promotes
133 expression of cellular proliferation factors and cancer-associated genes in prostate cancer cells.

134

135 **RESULTS**

136

137 ***SMARCA4* is over expressed in prostate cancer irrespective of tumour grade or subtype**

138 We first examined the expression of *SMARCA4* in the TCGA (50) prostate normal and cancer
139 cohort. The 486 tumour samples were subset into the seven TCGA categorised molecular subtypes
140 of prostate cancer (50). These included those with fusion genes involving *ERG* (46%), *ETV1* (8%),
141 *ETV4* (4%) and *FLI1* (1%), or those with mutations in *SPOP* (11%), *FOXA1* (3%) or *IDH1* (1%)
142 (50). The remaining samples were grouped as ‘other’ (26%). Each subtype exhibited a statistically
143 significant increase in *SMARCA4* expression ($p<0.05$) with the exception of the ‘FLI1’ subtype
144 ($p=0.5899$) and ‘other’ ($p=0.1899$), which both demonstrated a non-significant increase in
145 *SMARCA4* expression (Figure 1A). Previous work examining *SMARCA4* expression in the TCGA
146 prostate cancer cohort demonstrated that it is also up-regulated irrespective of Gleason score (49).
147 Therefore, we conclude that at the mRNA level, *SMARCA4* is universally over-expressed in
148 prostate cancer, regardless of clinical grade or molecular subtype.

149

150 ***SMARCA4* is over expressed in prostate cancer and transformed prostate cell lines**

151 We next examined both BRG1 protein and *SMARCA4* gene expression levels in normal prostate
152 epithelial cells (PrEC) and compared to LNCaP (lymph node metastasis), an androgen-dependent
153 prostate cancer cell line, as well as PC3 (bone metastasis), an androgen-independent prostate
154 cancer cell line. We found that *SMARCA4* gene expression was increased ~9 fold in LNCaP cells
155 and ~6 fold in PC3 compared to PrEC ($p<0.001$; Figure 1B). Further, the BRG1 protein level was
156 increased ~20 and ~24 fold, respectively, in each of the prostate cancer cell lines compared to

157 PrEC (Figure 1C). We compared this to published RNA-seq data of several normal, cancer and
158 transformed prostate cell lines (69). The mean expression of *SMARCA4* was significantly
159 increased in both the cancer cell lines and the transformed cell lines compared to the normal cells
160 ($p=0.0148$ and $p=0.0353$ respectively; Figure 1D). The exception was DU145 cells that has a
161 known frameshift mutation in *SMARCA4*, resulting in reduced expression (36). This data show
162 that common prostate cancer cell lines reflect the same pattern of increased BRG1 protein that is
163 observed in prostate tumours compared to normal prostate samples and therefore provides an
164 appropriate model system to explore the functional consequences of BRG1 dysregulation on the
165 transcriptome.

166

167 **BRG1 is required for the maintenance of active gene expression**

168 Our previous work has shown that BRG1 occupancy is enriched at active promoter and enhancer
169 gene regulatory elements in LNCaP cells (56). We therefore hypothesised that BRG1 would play
170 an important role in maintaining the transcriptional profile of these cells. To assess this, we
171 depleted the level of BRG1 protein using two independent siRNAs targeting *SMARCA4* (si-
172 *SMARCA4*-1 and si-*SMARCA4*-2) and performed RNA-seq at 72 and 144 hours post transfection
173 (Figure 2A). Initial assessment of our RNA-seq data confirmed successful depletion of the
174 *SMARCA4* transcript (~80%) at both time points (Figure 2B). Additionally, we confirmed
175 substantial depletion of BRG1 levels reduced to ~40% of the non-targeting control at 72 hours,
176 and to ~20% of the non-targeting control at 144 hours post-transfection (Figure 2C). We note there
177 were no significant changes detected in the gene expression of any other SWI/SNF subunit proteins
178 (Supplementary Figure 1A). Further quality assessment of the RNA-seq data through a principal
179 component analysis demonstrated that the samples separated by time-point on the first dimension,
180 accounting for 43.39 % of the sample variance (Supplementary Figure 1B). We performed a
181 differential gene expression analysis and identified 169 down-regulated genes and 24 up-regulated
182 genes ($\log_{2}FC > 1.5$, $FDR < 0.05$) at 72 hours post BRG1 depletion (Figure 2D). This increased to

183 800 down-regulated genes and 174 up-regulated genes by 144 hours post-transfection (Figure 2E).
184 This suggests that the primary role of BRG1 in LNCaP cells is to maintain active gene expression
185 of a subset of genes.

186
187 **BRG1 does not function in the regulation of alternative splicing**

188 The nucleosome barrier within genes is reported to contribute to alternative splicing, where there
189 is a higher conservation of nucleosomes at the splice sites of constitutive exons compared to
190 skipped exons (70-72). Since the contribution of BRG1 to alternative splicing regulation is
191 unknown, we investigated if alterations in alternative splicing may explain down regulation of
192 gene expression after BRG1 depletion in LNCaP cells. To do this we performed a multivariate
193 analysis of transcript splicing (MATS; (73-75)) of our RNA-seq datasets. After 72 hours of BRG1
194 depletion, MATS pairwise comparison detected a genome wide total of 13 and 11 skipped exons,
195 and 14 and 9 retained introns with si-SMARCA4-1 and si-SMARCA4-2 respectively
196 (Supplementary Figure 1C). At 144 hours post BRG1 knockdown this increased to 240 and 260
197 skipped exons, and 27 and 26 retained introns with si-SMARCA4-1 and si-SMARCA4-2,
198 respectively (Supplementary Figure 1D). Given the relatively large number of intron-exon
199 junctions within the total LNCaP transcriptome, we conclude BRG1 does not extensively
200 contribute to alternative splicing as the mechanism for predominant gene down-regulation.
201 However, we do note that at 144 hours post-knockdown the MATS analysis identified retention
202 of the first intron from the Kallikrein 3 gene, which encodes prostate specific antigen (PSA)
203 (Supplementary Figure 1E). This splice variant has previously been reported in LNCaP cells and
204 generates a unique protein from canonical PSA (76). While PSA has a well-known link to prostate
205 cancer, the function of its alternative splice variant remains unknown.

206
207 **BRG1 binding is associated with expression of prostate cancer associated genes**
208 We further examined our RNA-seq datasets to determine which genes showed a significant change

209 in expression at 72 hours that was maintained at 144 hours. Of the genes that were down-regulated
210 at the 72 hour time point, 126 genes (75 %) remained down-regulated at 144 hours. Similarly, of
211 the up-regulated genes, 16 (67 %) remained up-regulated at the extended time point (Figure 3A).
212 Within the down-regulated gene set we note a number of genes that have previously been
213 associated with increased proliferation in prostate cancer; these include kallikrein 2 (*KLK2*), long
214 non-coding RNA prostate cancer associated transcript 1 (*PCAT1*), Vav guanine nucleotide
215 exchange factor 3 (*VAV3*) (69, 77-84) (Figure 3B-D). We also examined the panel of prostate cell
216 lines (69) and confirmed that, on average there is elevated expression of these genes in both
217 prostate cancer cells and transformed prostate cell lines compared to normal prostate cells
218 (Supplementary Figure 2A). This suggests a role for BRG1 in maintaining the expression of genes
219 associated with prostate cancer proliferation.

220
221 We next sought to further explore commonalities in the genes with a significant change in
222 expression at both time points. We used ‘Enrichr’ (85, 86) to determine which existing ChIP-seq
223 datasets of transcription factors had enriched binding at the promoters of these genes. We
224 discovered that the most significantly enriched datasets were for the androgen receptor (AR) and
225 Forkhead box A1 (FOXA1) (Figure 3E), both of which are important for prostate cancer growth
226 (66, 67, 87-91). To investigate the potential coordinated function of these transcription factors with
227 BRG1, we compare the ChIP-seq signal of BRG1 (91), AR (87) and FOXA1 (87) at BRG1
228 genome-wide binding sites in LNCaP cells. We found the profiles separated into three clusters.
229 Cluster 1 sites displayed strong AR and FOXA1 binding, cluster 2 had moderate AR and strong
230 FOXA1, and cluster 3 had minimal to no signal for AR or FOXA1 (Figure 3F). We next examined
231 the key BRG1 regulated genes *KLK2*, *PCAT1* and *VAV3*, and found coordinated binding of all
232 three factors at the promoters of *KLK2* and *PCAT1*, and binding of BRG1 and FOXA1 upstream
233 of the internal 3-prime promoter of *VAV3* (Figure 3G). Furthermore, we showed that the expression
234 of AR or FOXA1 themselves was not regulated by BRG1 (Supplementary Figure 2B-C),

235 suggesting that the loss of BRG1 is enough to disrupt expression and regulation of *KLK2*, *PCAT1*
236 and *VAV3*.

237

238 **BRG1 binding is associated with the expression of DNA replication genes**

239 As the majority of significant gene changes occurred at 144 hours post-knockdown, we next
240 investigated potential gene regulatory networks. Gene ontology analysis with Enrichr (85, 86)
241 identified several significant (FDR < 0.05) GO terms pertaining to biological processes, cellular
242 component and molecular function that were all broadly related to the cell cycle (Figure 4A). As
243 BRG1 has previously been shown to interact with cell cycle master regulators, such as Rb and p53
244 (19, 92-94), we explored the relationship between the cell cycle and BRG1 further in our datasets.
245 We compiled a list of 250 genes related to cell cycle processes, curated from the cell cycle GO
246 terms, and of these examined the top 40 most significantly down-regulated genes in our dataset.
247 Of note among the list were several key genes involved in DNA replication initiation such as
248 *CDC6*, *CDT1* and *CDC45*, as well as the Minichromosome Maintenance (MCM) replicative
249 helicase components *MCM2* and *MCM5* (Figure 4B). To investigate if the effect on replication
250 initiation gene expression was more widespread, we reviewed the gene expression of the other
251 components in the MCM2-7 replicative helicase and the Origin Recognition Complex (ORC) and
252 found that several of these genes were also down-regulated (Figure 4C-D). We confirmed the
253 down regulation of MCM5, CDC6 and ORC6 via Western blot, along with cell cycle regulator
254 CHK1, which revealed almost undetectable expression by 144 hours post BRG1 knockdown
255 (Figure 4E-F).

256

257 We investigated whether AR and FOXA1 were also colocalised with BRG1 at DNA replication
258 genes. We examined the ChIP-seq binding profiles of AR, FOXA1 and BRG1 at the promoters of
259 91 DNA replication genes (determined from the DNA replication GO terms) that were expressed
260 in LNCaP cells. We found at promoters of these genes containing the active histone marks

261 H3K4me3 and H3K27ac, also displayed a weak BRG1 ChIP-seq signal, but were completely
262 absent of AR and FOXA1 ChIP-seq peaks (Supplementary Figure 3A), for example at the
263 promoters of *CDC45*, *ORC6* (Supplementary Figure 3B). Additionally, we also note this pattern
264 at a putative enhancer region within the *MCM2* gene (Supplementary Figure 3B). Our data
265 suggests that BRG1 binding is associated with the expression of DNA replication genes in prostate
266 cancer cells that is independent of AR and FOXA1.

267

268 **BRG1 depletion arrests cells in G1**

269 Given BRG1 regulates several genes involved in proliferation and replication, we next asked if
270 BRG1 depletion would alter cell cycle progression in LNCaP cells. We investigated this utilising
271 the same siRNA-mediated approach to target BRG1 by depleting *SMARCA4* and conducted flow
272 cytometry cell cycle analysis at 72 and 144 hours post knockdown. We detected an increase of
273 cells in G1 at 72 hours, which was enhanced by 144 hours. Specifically, at 144 hours post BRG1
274 depletion there was ~20% increase of cells in G1 and equivalent loss of cells in S phase (Figure
275 4G-H). These data suggest that a loss of BRG1 reduces proliferation through mediating a G1 arrest.

276

277 **DISCUSSION**

278

279 Here we examined the involvement of the SWI/SNF chromatin remodeller BRG1 and its
280 associated encoding gene *SMARCA4* in prostate cancer transcriptional deregulation. We found that
281 over expression of *SMARCA4* commonly occurs in both the TCGA prostate cancer cohort,
282 irrespective of tumour subtype, and in a panel of prostate cancer cell lines. We also found that
283 knockdown of the *SMARCA4* gene, and consequently the BRG1 protein, results in down-
284 regulation of pro-proliferative transcriptional pathways. These included genes already known to
285 promote prostate cancer proliferation, as well as cell cycle and DNA replication genes. Reduction
286 of gene expression in these pathways was concomitant with G1 arrest. Taken together, our results

287 provide new insights into BRG1's contribution to transcriptional patterns relating to proliferation
288 in prostate cancer.

289
290 We have demonstrated that *SMARCA4* mRNA over expression is a universal feature of prostate
291 cancer. Clinical datasets have shown BRG1 protein levels are over-expressed in prostate cancer,
292 in the absence of consistent significant deleterious genetic mutations evident in *SMARCA4* (42,
293 44-46). Using the large prostate cancer cohort from TCGA (50) we found that *SMARCA4* was
294 significantly over-expressed. Consistent with this, *SMARCA4* expression was increased in a panel
295 of both prostate cancer and transformed cell lines. These data emphasise that the overall increased
296 expression of *SMARCA4* is a characteristic of prostate cancer, irrespective of subtype.

297
298 BRG1 depletion followed by RNA-seq revealed multiple transcriptomic alterations that were
299 regulated by BRG1 and related to proliferation. BRG1 depletion primarily resulted in the down-
300 regulation of BRG1's target genes, indicating the main role of BRG1 is to promote active gene
301 expression. Within the down-regulated genes were genes associated with increased proliferation
302 in prostate cancer including *KLK2*, *PCAT-1* and *VAV3*. *KLK2* is a known activator of PSA, which
303 is an important biomarker of prostate cancer, and associated with decreased apoptosis (77, 84).
304 *PCAT-1* promotes proliferation through the oncprotein Myc (69, 81), while *VAV3* regulates AR
305 activity to stimulate growth in prostate cancer (78-80, 82). Both *PCAT-1* and *VAV3* are correlated
306 with disease progression. Through an analysis of gene ontologies, we also found several cell cycle
307 gene pathways were downregulated with BRG1 depletion. This included numerous genes involved
308 in DNA replication, which were among the most significantly down regulated genes following
309 BRG1 depletion. BRG1 is known to have a role in driving self-renewal and malignancy in B-cell
310 acute lymphoblastic and acute myeloid leukaemias, cancers which also have over expressed BRG1
311 (22, 24). Specifically, these leukaemias require high levels of BRG1 for de-condensation of the
312 cell specific *MYC* enhancer. In these cancers, a loss of BRG1 causes a reduction of enhancer-

313 promoter interactions, reduced transcription factor occupancy and DNA looping which in turn
314 reduces *MYC* expression (24). This implies that the overexpression of BRG1 contributes to driving
315 oncogenic transcriptional programs which influence the proliferation capacity of cancer cells.

316
317 Our data revealed that BRG1 co-occupied the promoters of proliferation associated genes (*KLK2*,
318 *PCAT-1* and *VAV3*) along with AR and FOXA1, and that these genes were down-regulated across
319 our experimental time course. Co-regulation of transcription by AR and FOXA1 in prostate cancer
320 is associated with reprogrammed binding of AR and oncogenic patterns of gene expression that
321 are essential for AR-driven proliferation (95, 96). Additionally, there is a high overlap of these
322 reprogrammed AR binding sites between LNCaP cells and primary prostate tumour tissue (96).

323 Here we have shown BRG1 gene regulation overlaps with these transcription factors at gene
324 promoters, which is concomitant with expression of prostate cancer associated genes. However, it
325 is noteworthy that BRG1 depletion also altered the expression of DNA replication genes through
326 a mechanism that appears independent of AR and FOXA1. This data suggests that BRG1 has
327 additional roles in other gene regulatory networks, which may indirectly influence cell
328 proliferation. As BRG1 is known to interact with cell cycle regulators in other cancers, it is
329 possible that genes co-regulated by BRG1, AR and FOXA1 are important in a prostate cancer
330 context, while regulation of cell cycle and DNA replication genes may be a general feature of
331 BRG1 over expression in cancer.

332

333 **CONCLUSIONS**

334
335 In summary, our data identifies fundamental role for BRG1 in maintaining active transcription for
336 proliferation of prostate cancer cells. We find that BRG1 promotes gene expression in prostate
337 cancer models with varying degrees of dependence on AR and FOXA1. BRG1 is required to drive
338 the expression of numerous prostate cancer specific genes in an AR/FOXA1 dependant manner,

339 but also works independently to drive the expression of pro-proliferative and DNA replication
340 genes. These results provide important functional information regarding the role of BRG1
341 controlling proliferation in prostate cancer cells.

342

343 **METHODS**

344

345 ***Cell Culture and siRNA Transfection***

346 Normal Prostate Epithelial Cells (PrEC) cells (Cambrex Bio Science, #CC-2555) were cultured in
347 PrEBM (Clonetics, #CC-3165) according to the manufacturer's protocol. Briefly, PrEC cells were
348 seeded at 2,500 cells per cm² and medium was replaced every two days. Cells were passaged at
349 approximately 80 % confluence. To passage a T75 flask, PrEC cells were rinsed in 6 mls Hanks
350 Balanced Salt Solution (Thermo Fisher Scientific, #14025076) then detached with 2 ml pre-
351 warmed 0.025 % Trypsin-EDTA and incubated at room temperature for 5 minutes. Trypsin was
352 inactivated with 12 mls of Trypsin-Neutralizing Solution (Clonetics, #CC-5002) and cells were
353 centrifuged at 300 x g for 5 minutes. The supernatant was aspirated, and the cell pellet was re-
354 suspended in PrEBM. The number of cells was determined on the Countess automated counter
355 and were re-seeded at the appropriate density based on experimental needs. Cells were discarded
356 after ~16 population doublings.

357

358 PC3 cells (ATCC, #CRL-1435) were maintained in RPMI medium (Gibco, #11875-093) with 10
359 % FBS, 11 mls of 1 M HEPES (Gibco, #15630080) and Pen/Strep. LNCaP cells (ATCC, #CRL-
360 1740) were cultured using custom T-Medium from Gibco (DMEM low glucose (GIBCO cat#
361 31600-034), Kaighn's modified F-12 medium (F-12K; cat# 211227-014), insulin 500x bovine
362 pancreas (Sigma cat# I1882.10MG), T3 6.825 ng/ml Tri-iodothyronine (Sigma cat# T5516),
363 Transferrin 500x (Sigma cat# T5391), Biotin 500x (Sigma cat# B4639), Adenine 500x (Sigma
364 cat# A3259)). Both prostate cell lines were cultured under recommend conditions; 37°C with 5 %

365 CO₂. When the cells reached ~80 % confluence they were passaged or seeded as per experimental
366 requirements. For siRNA transfection LNCaP cells were seeded into 6-well plates at a density of
367 2.5 x 10⁵ cells per well or 10cm dishes at 1.5 x 10⁶ cells per dish. The cells were transfected with
368 either on target *SMARCA4* siRNA (Horizon, #J-010431-06-0005 or #J-010431-07-0005) or the
369 non-targeting control siRNA pool (Horizon, #D-001810-10-05) 24 hours after seeding the cells
370 using DharmaFECT 2 (Thermo Scientific, #T-2002-03) as per the manufacturer's instructions. To
371 maintain the knockdown over a 6-day period, at 72 hours post transfection the cells were harvested,
372 split at a ratio of 1:2 into two new wells, and reverse-transfected with siRNA. The cells were then
373 incubated for a further 72 hours before collection.

374

375 ***Quantitative Real-Time PCR (qRT-PCR)***

376 RNA was extracted with TRIzol reagent (Thermo Scientific, #15596026), according to the
377 manufacturer's protocol. Extracted RNA was re-suspended in 30 ul of nuclease-free water and
378 quantified on the NanoDrop spectrophotometer (Thermo Scientific). cDNA synthesis was carried
379 out with 500 ng of RNA using the SensiFAST cDNA Synthesis Kit (Bioline, #BIO-65054)
380 according to the manufacturer's instructions.

381

382 qRT-PCR was carried out on the CFX384 Touch Real-Time PCR Detection System (Bio-Rad). A
383 master mix was made for each qRT-PCR target containing 5 ul of KAPA Universal SYBR Fast
384 PCR mix (KAPA Biosystems, #KK4602), 0.6ul of 5 uM forward primer, 0.6 ul of 5 uM reverse
385 primer and 1.8 ul of nuclease free water per reaction. Reactions conditions were 95°C for 3
386 minutes, followed by 45x cycles of 95 °C for 3 seconds and 60 °C for 30 seconds, then a melt
387 curve analysis (65 °C to 95 °C, increasing at a rate of 0.5 °C every 5secs). Primers to detect
388 *SMARCA4* were CAGAACGCACAGACCTTCAA (forward) and
389 TCACTCTCGCCTTCACT (reverse) and for detection of 18S
390 GGGACTTAATCAACGCAAGC (forward) and GCAATTATTCCCCATGAACG (reverse).

391 Relative gene expression was calculated using ddCt and normalised to *18S*. A significant change
392 in gene expression of *SMARCA4* between PrEC, LNCaP and PC3 cells was determined by one-
393 way ANOVA and corrected with Tukey's test for multiple comparisons.

394

395 ***Western Blot***

396 Whole cell lysates were collected with lysis buffer (50 mM HEPES, 150 mM NaCl, 10% Glycerol,
397 1 % Triton-X-100, 1.5 mM MgCl₂, 1 mM EGTA, 10 mM Pyrophosphate, 100 mM NaF, Roche
398 protease inhibitor cocktail 1x), and protein level quantified using the Pierce BCA Assay Kit
399 (Thermo Scientific, #23227) according to the manufacturer's instructions. Sample reducing agent
400 (Thermo Scientific, NP0004), loading buffer (Thermo Scientific, NP0007) and 10 ug protein were
401 combined with water to a final volume of 25 ul. Protein samples were heated at 90 °C for 5 minutes
402 then allowed to cool to room temperature. Protein samples were loaded on a NuPage Novex Bis-
403 Tris 4-12 % gel (Thermo Scientific, NP0321BOX) and electrophoresed at 100V for 1.5 hours in a
404 1x MOPS buffer (50 mM MOPS (Biochemicals Astral Scientific, #BIOMB03600, 50 mM
405 Tris base, 0.1% SDS, 1 mM EDTA [pH 7.7]). Proteins were transferred to a polyvinylidene
406 fluoride membrane (Bio-Rad, #1620177) at 30 volts for 1 hour using 1x transfer buffer (25 mM
407 Tris base, 192 mM Glycine [pH 8.3]) with 10 % methanol (Sigma-Aldrich, #322415). Membranes
408 were blocked for 1 hour with 5 % skim milk in TBS-T (20 mM Tris, 150 mM NaCl, 0.1% Tween
409 20 [pH 7.6]) at 4°C. Primary antibodies used were BRG1 (Santa Cruz, sc-10768X), GAPDH
410 (Ambion, AM4300), CHK1 (CST, 2360S), ORC6 (CST, 4737S), CDC6 (CST, 3387S) and MCM5
411 (abcam, ab17967). Primary antibodies were incubated on samples overnight at 4 °C with rotation.
412 The membrane was then washed three times for 10mins each in TBS-T with rotation. Secondary
413 antibodies goat anti-mouse (Santa Cruz, sc-2005) and goat anti-rabbit (Santa Cruz, sc-2004) were
414 diluted in TBS-T containing 5 % skim milk and incubated at 4 °C with rotation for 1 hour. The
415 membrane was washed three times for 10 minutes in TBS-T. The membrane was then covered
416 with ECL solution (Perkin Elmer, #NEL104001EA), incubated for 1min at room temperature, and

417 visualized by X-ray film. Adjusted relative density calculations were processed through ImageJ
418 (97, 98).

419

420 ***Flow cytometric cell cycle analysis***

421 LNCaP cells were seeded at 1.5 x 10⁶ cells per 10 cm dish and transfected with siRNA as
422 described. At 72 and 144 hours post transfection the cells were treated with 10 uM EdU for 30
423 minutes. Remaining EdU was washed off the cells with PBS before harvesting cells, then 1 x 10⁶
424 cells were fixed in 70 % ethanol and frozen at -20 °C. Cells were then diluted 1 in 4 with PBS then
425 pelleted and re-suspended in 1 ml of PBS containing 1 % BSA (Sigma-Aldrich, #A2058). Cells
426 were again pelleted, re-suspended in 500 ul of click reaction mix (10uM carbocyfluorescine TEG-
427 azide, 10 mM Sodium L-ascorbate, and 2 mM Copper-II-sulphate diluted in PBS), and incubated
428 in the dark at room temperature for 30 minutes. Samples were then diluted with 5 mls of PBS
429 containing 1 % BSA and 0.1 % Tween-20. Cells were again pelleted, washed with PBS and then
430 resuspended in 500 ul of PBS containing 1% BSA, 0.1 mg/ml of RNase and 1 ug/ml of DAPI.
431 Samples were analysed on the Canto II (BD Biosciences). Forward and side scatter were used to
432 select a population of cells free of cell debris and doublets. Cells were analysed using B450 (FTIC
433 – EdU positive) and B510 (DAPI) lasers. 50,000 single cell events were recorded for each ample.
434 FlowJo software v10.5, was used to analyse the data. Data was collected in biological duplicate.

435

436 ***RNA-seq Experiments***

437 Total RNA was extracted with TRIzol reagent, quantified on the Qubit and quality assessed with
438 the Bioanalyzer. An aliquot of 500 ng of total RNA was spiked with external controls ERCC RNA
439 spike-in Mix (Thermo Scientific, 4456740) and libraries constructed with the TruSeq Stranded
440 mRNA sample preparation kit (Illumina, 20020594) according to the manufacturer's protocol.
441 mRNA Libraries were quantified on Qubit and then stored at -20 °C. Library quality and fragment
442 size of RNA-seq libraries was assessed on the Bioanalyzer, then KAPA Library Quantification

443 (KAPA Biosystems, #KK4824) was performed according to the manufacturer's protocol. The
444 KAPA quantification results were used to dilute the libraries to 2 nM for sequencing. RNA-seq
445 samples were sequenced for 100 cycles of paired-end reads on the Illumina HiSeq 2500 platform,
446 with four samples multiplexed per lane of the high output run.

447

448 ***RNA-seq Data Analysis***

449 RNA-seq data was processed as described in Taberlay & Achinger-Kawecka *et al.* (9). Briefly,
450 read counts were normalized with ERCC spike in controls, mapped to hg19/GRCh37 using STAR
451 and counted into genes using the featureCounts (99) program. GENCODE v19 was used as a
452 reference transcriptome to determine the transcript per million read (TPM) value. Fold change was
453 calculated within each time point as the log2 ratio of normalized reads per gene using the *edgeR*
454 package in R. Genes with a fold change of ± 1.5 and FDR < 0.01 were considered significantly
455 different. Volcano plots of differential expression were created in R with *ggplots2* and heatmaps
456 with the *heatmap2* package with normalised row Z-score. PCA was performed in R using the
457 *edgeR* package with log counts per million (logCPMS) over GENCODE v19 annotated gene
458 coordinates and normalizing the read counts to library size. RNA-seq multivariate analysis of
459 transcript splicing (MATS) to calculate exon skipping and intron retention was performed with the
460 MATS python package v4.0.2 (73-75). Transcription factor and GO term enrichment was obtained
461 from Enrichr (<http://amp.pharm.mssm.edu/Enrichr/>) online gene list analysis tool (85, 86).

462

463 ***TCGA and prostate cell line expression analysis***

464 Pre-processed RNA-seq data from the TCGA prostate adenocarcinoma cohort was downloaded
465 (cancergenome.nih.gov) for both normal and tumour samples. The average of tumour (n = 486)
466 and normal (n = 52) samples was calculated to determine mean expression. Separation of tumours
467 by Gleason score and molecular subtype was performed in R using the associated clinical data to
468 subset the appropriate groups. Significance was calculated for tumour versus normal using an

469 unpaired T-test. For comparison between Gleason score or molecular subtype, significance was
470 calculated using one-way ANOVA with Dunnett's multiple comparison correction.

471
472 Expression data for prostate cell lines from Presner et al. (69) was downloaded from
473 http://www.betastasis.com/prostate_cancer/. Significance between normal, cancer and
474 transformed cell lines was calculated using one-way ANOVA with Dunnett's multiple comparison
475 correction.

476
477 ***ChIP-seq data***
478 The following LNCaP ChIP-seq data was obtained from GEO (ncbi.nlm.nih.gov/geo/); BRG1
479 accession GSE72690 (91), H3K4me3 and H3K27me3 accession GSE38685 (100), H3K27ac and
480 H3K4me1 accession GSE73785 (9). These data were processed through NGSane pipeline as
481 previously described (9, 100). Pre-processed bigwig files for FOXA1 and AR were obtained from
482 GEO accession GSE114274 (87). Genome browser images of ChIP-seq data were taken from IGV.
483 Heatmaps of ChIP-seq signal were created with *deeptools* (101).

484
485 **DECLARATIONS**

486
487 ***Ethics approval and consent to participate***
488 Not Applicable

489
490 ***Consent for publication***
491 Not Applicable

492
493 ***Availability of data and materials***

494 The BRG1 knockdown RNA-seq data generated for this study has been submitted to GEO,
495 accession number GSE150252. Reviewer access for the submitted data is available from;
496 <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE150252>

497

498 ***Competing interests***

499 The authors declare that they have no competing interests.

500

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508

509 ***Authors' contributions***

510 This study was initiated and deisgned by KAG, SJC and PCT. Experiments were performed by
511 KAG, SGP and GK. Analysis and interpretation of next-generation data was performed by KAG,
512 CMG, JAK and PL. Initial manuscript draft was written by KAG. Manuscript editing and
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520

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800

801 **FIGURE LEGENDS**

802

803 **Figure 1. SMARCA4 (BRG1) is over expressed in prostate cancer.** A) *SMARCA4* gene
804 expression (logPRKM) in TCGA data (tumours n= 486, normal = 52) with tumour samples
805 separated by molecular subtype defined by the TCGA. *SMARCA4* expression is increased across
806 all groups, with subtypes ERG, ETV1, ETV4, IDH1, SPOP, and FOXA1 all significantly up
807 regulated, one-way ANOVA Dunnett's multiple comparison correction $^{**}p<0.05$. B) *SMARCA4*
808 gene expression in prostate cell lines normalised to *I8S* and relative to PrEC (n = 2). Significance
809 determined by one-way ANOVA with Tukey's multiple comparison correction $^{***}p<0.001$. Bars
810 denote mean, and error bars are SD. C) Representative Western blot of BRG1 protein level in
811 prostate cell lines. Quantification above Western Blot by adjusted relative density normalized to
812 GAPDH and relative to PrEC. D) Expression of *SMARCA4* from RNA-seq in prostate cell lines
813 grouped as normal, cancer or transformed. The mean of each group was calculated, and a
814 significance was tested by one-way ANOVA Dunnett's multiple comparison correction,
815 $^{**}p<0.05$.

816

817 **Figure 2. Loss of BRG1 results in a down regulation of gene expression.** A) Schematic of
818 temporal BRG1 knockdown model used for RNA-seq. Samples were collected at 72hrs (si-NT
819 control, si-*SMARCA4*-1 and si-*SMARCA4*-2) and 144hrs (si-NT, si-*SMARCA4*-1 and si-
820 *SMARCA4*-2) post siRNA transfection in duplicate for each condition at each time point (n=2).
821 Cells were transfected with either control siRNA (si-NT) or *SMARCA4* siRNA. B) *SMARCA4*
822 gene expression in control and post BRG1 depletion in the RNA-seq data, shown as transcripts

823 per million reads (TPM). Control siRNA for 72 and 144 hours are shown collectively as si-NT.
824 *SMARCA4* expression is significantly down regulated at both time points, *** $p<0.0001$. Bars
825 denote mean, and error bars are SD. C) Representative Western blots of BRG1 and GAPDH
826 protein levels at 72 and 144 hours post transfection. Adjusted relative density for BRG1 is
827 calculated relative to GAPDH and normalized to the non-targeting control. Bars denote mean, and
828 error bars are SD. D-E) Volcano plots of differentially expressed genes at 72 hours and 144 hours
829 post knockdown. Significantly down regulated genes are blue and significantly up regulated genes
830 for 72 and 144 hours post knockdown are shown in orange and red respectively. *SMARCA4*
831 differential expression is highlighted in purple. Expression is shown as normalised log2 counts per
832 million reads.

833

834 **Figure 3. BRG1 regulates genes associated with prostate cancer.** A) Heatmap illustrating
835 RNA-seq differential gene expression data for up (n = 16) and down (n = 126) regulated genes
836 common to both time points after BRG1 depletion. Expression is represented as the normalised
837 row Z-score of TPM. B-D) *KLK2*, *VAV3* and *PCAT-1* gene expression from the RNA-seq datasets
838 shown as TPM. Bars denote mean, and error bars are SD. E) Gene set enrichment analysis using
839 ‘Enrichr’ of differentially expressed genes that are common to both time points, showing the
840 adjusted p-value (log 10, reversed x-axis) of significantly enriched transcription factor ChIP-seq
841 from ChEA curated data ($p<0.05$). F) Heatmap of BRG1, AR and FOXA1 ChIP-seq signal at
842 BRG1 binding sites in LNCaP cells, +/- 2.5 kb from the centre of the binding site. Data is clustered
843 into three groups by k-means. G) IGV images of the genes *KLK2*, *PCAT-1* and *VAV3*. Grey shaded
844 regions contain ChIP-seq signal peaks for BRG1, AR and FOXA1.

845

846 **Figure 4. BRG1 regulates genes involved the cell cycle.** A) Gene set enrichment analysis using
847 ‘Enrichr’ of down regulated genes at 144 hours post BRG1 knockdown. Enriched GO terms are
848 classified as biological processes, cellular component or molecular function. Adjusted p-value (log

849 10, reversed x-axis) of the 10 most significant GO terms are shown. B) Heatmap of gene
850 expression profiles from the top 40 differential cell cycle genes after BRG1 depletion. Expression
851 is shown as the normalised row Z-score of transcripts per million reads (TPM), with blue indicating
852 higher expression and red indicating lower expression. Genes involved in DNA replication
853 initiation are indicated in blue. C-D) Gene expression from RNA-seq (TPM), of the MCM2-7
854 helicase components (top) and the Origin of Replication complex (ORC) subunits (bottom). Error
855 bars denote mean and standard deviation. Bars denote mean, and error bars are SD. E)
856 Representative Western blot showing protein levels of replication initiation genes MCM5, CDC6
857 and ORC6, along with CHK1, after 72 and 144 hours post BRG1 depletion. Error bars demonstrate
858 SD. F) Quantification of Western blots demonstrating adjusted relative density to GAPDH (n =
859 2). G) Representative flow cytometry scatter of DAPI (x-axis) and EdU (y-axis) fluorescence
860 intensity at 72 and 144 hours post BRG1 knockdown. G1 cells are shown by boxed gate. H)
861 Percentage of cells in each phase of the cell cycle from flow cytometry data, error bars show
862 standard deviation (n = 2). Error bars show SD.

863
864 **Supplementary Figure 1.** A) SWI/SNF subunit gene expression (TPM) from RNA-seq data. All
865 subunits, except *SMARCA4* (shown in Figure 2A), are not significantly altered. Bars denote mean,
866 and error bars are SD. B) PCA plot characterising the trend in expression profiles between the
867 non-targeting control and after BRG1 knockdown. Each point on the plot represents an RNA-seq
868 sample. Samples are separated by principal components 1 and 2, which together explain 58.37 %
869 of the variance between the samples. C) Number of skipped exons at 72 hours and 144 hours after
870 BRG1 knockdown with si-*SMARCA4*-1 (black) and si-*SMARCA4*-2 (grey). D) Number of retained
871 introns at 72 hours and 144 hours post BRG1 depletion with si-*SMARCA4*-1 (black) and si-
872 *SMARCA4*-2 (grey). E) Sashimi plot of exons one and two of the *KLK3* gene in the non-targeting
873 and 144 hour knockdown RNA-seq data. Arcs represent the number of split reads across the exons.
874 Lower numbers represent increased retention of the first intron after BRG1 knockdown.

875

876 **Supplementary Figure 2.** A) Expression of *KLK2*, *PCAT-1* and *VAV3* in prostate cell lines
877 grouped as normal, cancer or transformed. B) *AR* and *FOXA1* gene expression from the RNA-seq
878 datasets shown as TPM. Bars denote mean, and error bars are SD.

879

880 **Supplementary Figure 3.** A) Heatmap of replication gene promoters, +/- 5kb from the
881 transcription start site. B) IGV images of the genes *CDC45*, *ORC6* and *MCM2*. Grey shaded
882 regions contain ChIP-seq signal peaks for BRG1 and active histone modifications.

FIGURE 1

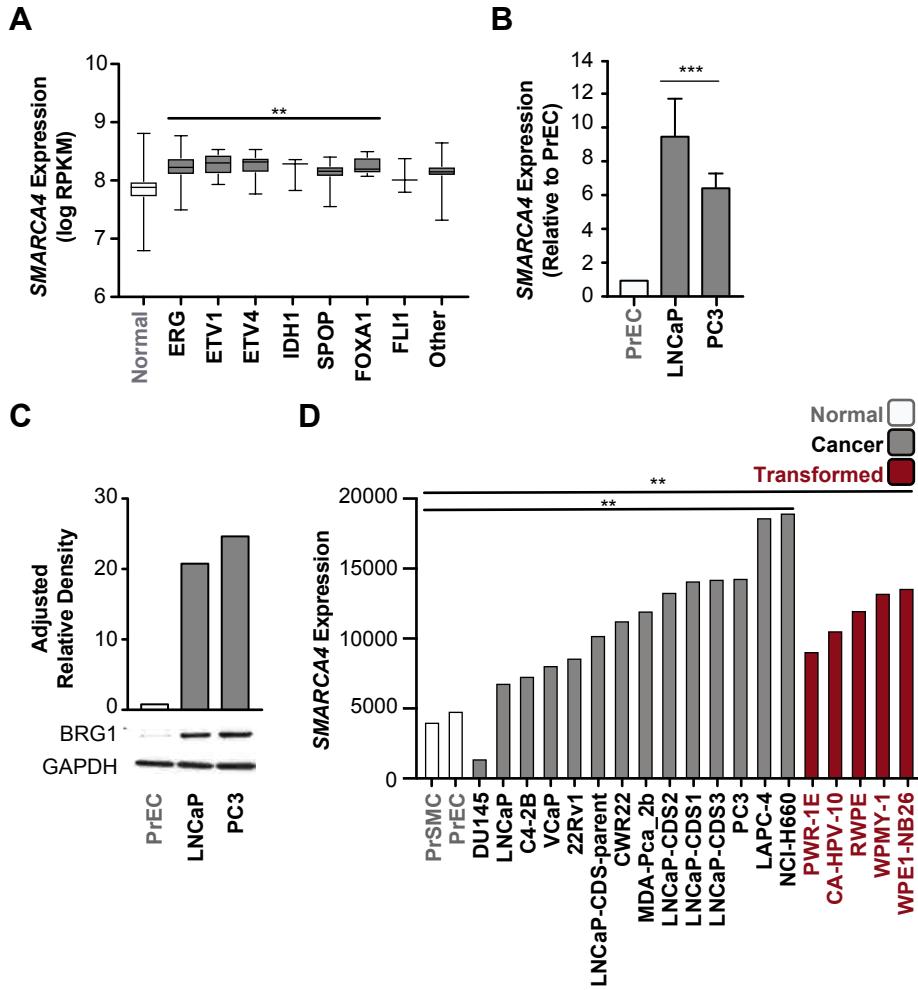


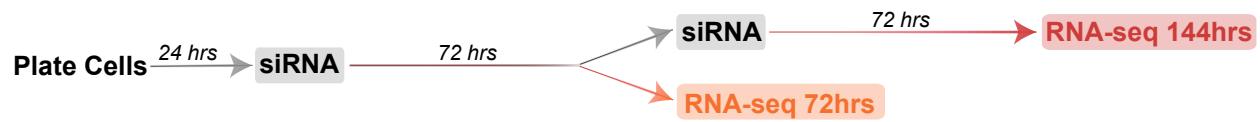
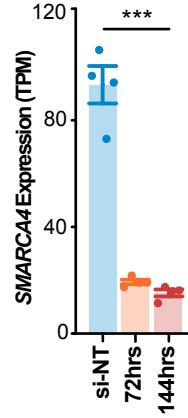
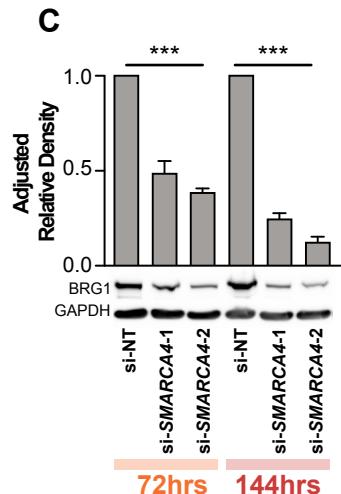
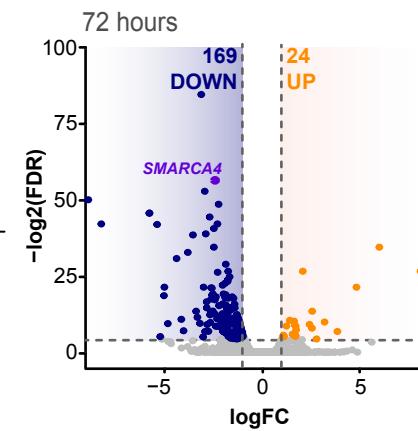
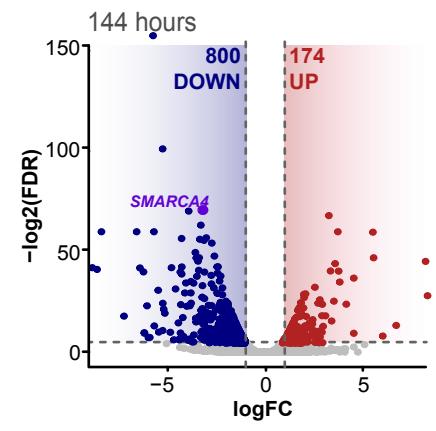
FIGURE 2**A****B****C****D****E**

FIGURE 3

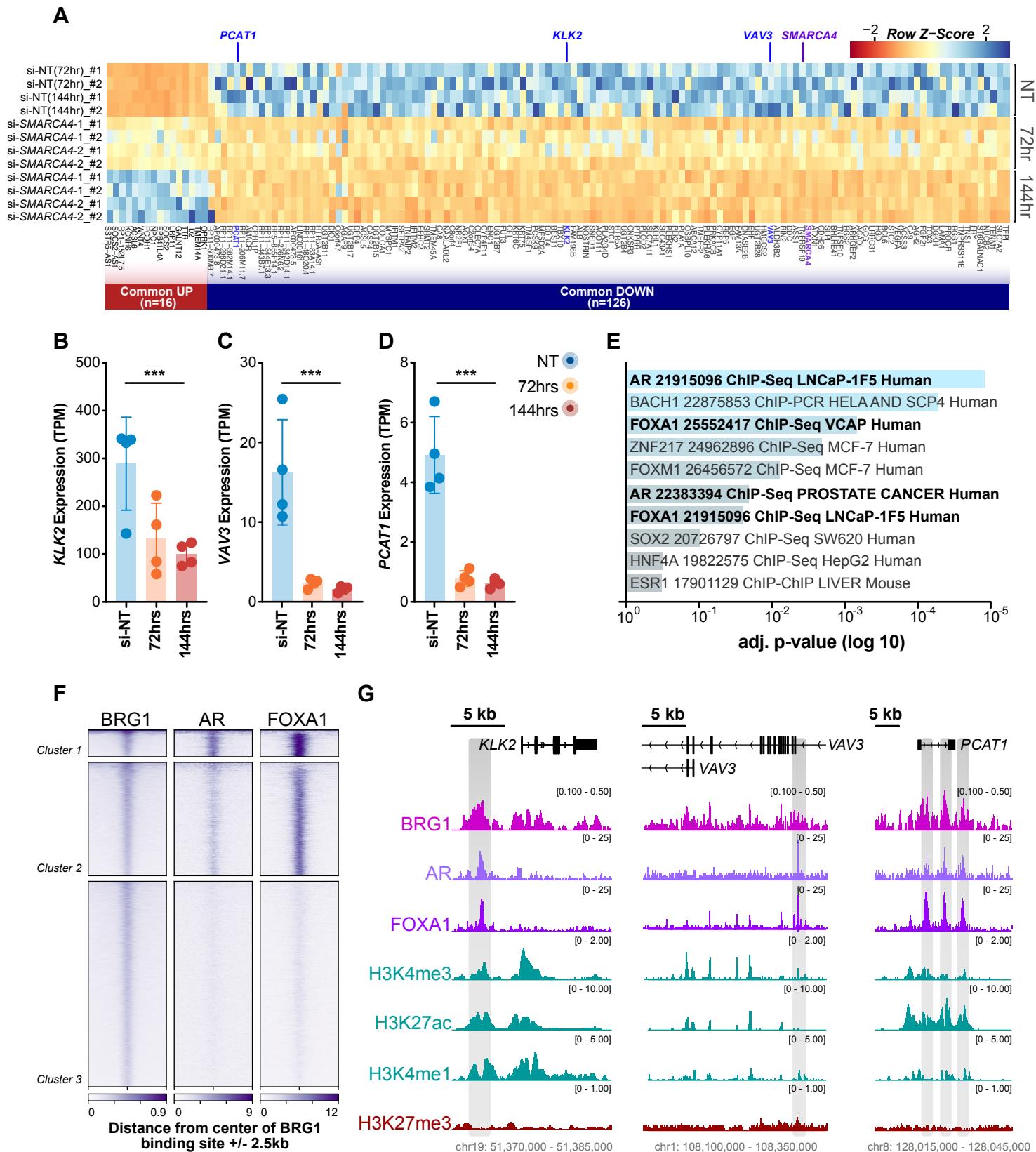


FIGURE 4

