

1 **JARID2 facilitates transcriptional reprogramming in glioblastoma in response to standard**
2 **treatment**

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21 **ABSTRACT**

22 **Background:** Glioblastoma (GBM) is a fatal and incurable brain cancer with a dismal prognosis. In
23 order to impact on this disease, we need to understand how infiltrating, non resectable tumour
24 cells resist chemoradiation and facilitate disease recurrence. To this end, we generated or
25 acquired bulk tumour RNA sequencing data from 45 paired primary and locally recurrent GBM
26 tumours (split into original and validation cohorts) from patients that received standard treatment.
27 We also generated DNA methylation profiles for 9 pairs and sequenced RNA from single cells
28 isolated from a patient derived GBM spheroid model at different timepoints following *in vitro*
29 chemoradiation.

30 **Results:** We have identified a set of genes with Jumonji and AT-Rich Interacting Domain 2
31 (JARID2) binding sites in their promoters that are universally dysregulated in post-standard
32 treatment recurrent GBMs compared to the primary tumour. The direction of dysregulation is
33 patient-dependent and not associated with differential promoter DNA methylation. Our *in vitro*
34 experiments suggest that this dysregulation occurs dynamically following treatment as opposed to
35 resulting from selection of cells with specific expression profiles.

36 **Conclusion:** JARID2 is an accessory protein to a chromatin remodeling complex, responsible for
37 histone modifications observed during cell state transitions in both normal brain and GBM. We
38 propose that JARID2 facilitates GBM recurrence following treatment by indirect transcriptional
39 reprogramming of surviving cells in whichever manner is needed to reproduce the phenotypic
40 heterogeneity required for tumour regrowth *in vivo*. The mechanism of this reprogramming may
41 present a therapeutic vulnerability for more effective treatment of GBM.

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43 **Keywords:** Glioblastoma; High grade glioma; Recurrence; RNAseq; Cell plasticity; Chromatin
44 remodelling; Longitudinal; Treatment resistance; Tumour adaption; Transcriptional reprogramming

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46

47 **BACKGROUND**

48 Glioblastoma (GBM) is arguably one of the most challenging cancers to treat and is associated
49 with very poor prognosis. This is in part because GBM cells infiltrate the surrounding normal brain
50 making complete surgical removal impossible and, despite subsequent chemoradiation, the
51 remaining cells resist treatment and facilitate tumour regrowth in almost 100% of cases. If we ever
52 hope to more effectively treat GBM we must understand how and why unresected cells resist
53 treatment and form a recurrent tumour. To this end, we and others have focused our attention on
54 molecular profiling of paired primary and recurrent GBM tumours to specifically identify features
55 which are expanded post-treatment and may offer insight into the properties of cells which survive,
56 or the mechanisms that enable their continued proliferation^[1-5]. As part of the Glioma Longitudinal
57 AnalySiS consortium, we have analysed the genomes of more than 200 paired gliomas and
58 determined that there is no clear evidence for therapy-driven selection of cells bearing specific
59 resistance-conferring mutations (manuscript under review) in agreement with the work of Körber et
60 al.^[3, 6]. We have, therefore, focused our continued efforts herein on transcriptional features and the
61 possibility of therapy-driven selection of GBM cell populations defined by expression profiles.
62 Transcriptional heterogeneity is evident in GBM: expression profiles align with
63 neurodevelopmental-like hierarchies that span genomic subclones and are functionally distinct,
64 including with respect to treatment sensitivity *in vitro*^[7, 8]. We generated or acquired RNAseq data
65 from paired primary and recurrent GBMs from a cohort of 23 patients (our original cohort) that
66 underwent standard treatment (debulking surgery followed by chemoradiation with the alkylating
67 drug temozolomide) and had a local recurrence. We then acquired data from an additional 22 such
68 patients (our validation cohort). Our analyses of these data, and of DNA methylation profiles from 9
69 pairs, consistently highlight the likely role of a Polycomb Repressive Complex 2 (PRC2) accessory
70 protein called JARID2 (Jumonji and AT-Rich Interacting Domain 2) in the transcriptional changes
71 observed after treatment in GBM, via histone modifications and chromatin remodeling. However,
72 the direction of fold change is not consistent across patients. We then performed single cell
73 RNAseq on a patient derived GBM model at different time points following administration of
74 clinically relevant doses of chemoradiation *in vitro* and found that JARID2 associated
75 transcriptional changes occur dynamically after treatment as opposed to resulting from selection of

76 cells with a specific expression profile. JARID2's interaction with PRC2 is fundamental to
77 specifying neurodevelopmental cell lineages in response to environmental cues, and PRC2 has
78 been shown to be necessary for determining GBM cell phenotypes based on tumour
79 microenvironmental pressures, though the role of JARID2 in this has never been investigated^[9-12].
80 We propose that GBM recurrence results from JARID2-associated transcriptional reprogramming,
81 via PRC2, of unresected cells in whichever direction enables recapitulation of the transcriptional
82 heterogeneity needed for continued tumour growth *in vivo*^[13]. Therapeutic targeting of the
83 mechanism of such reprogramming may constitute a more effective treatment strategy than
84 targeting of the cell types that lie either side of the interconversions.

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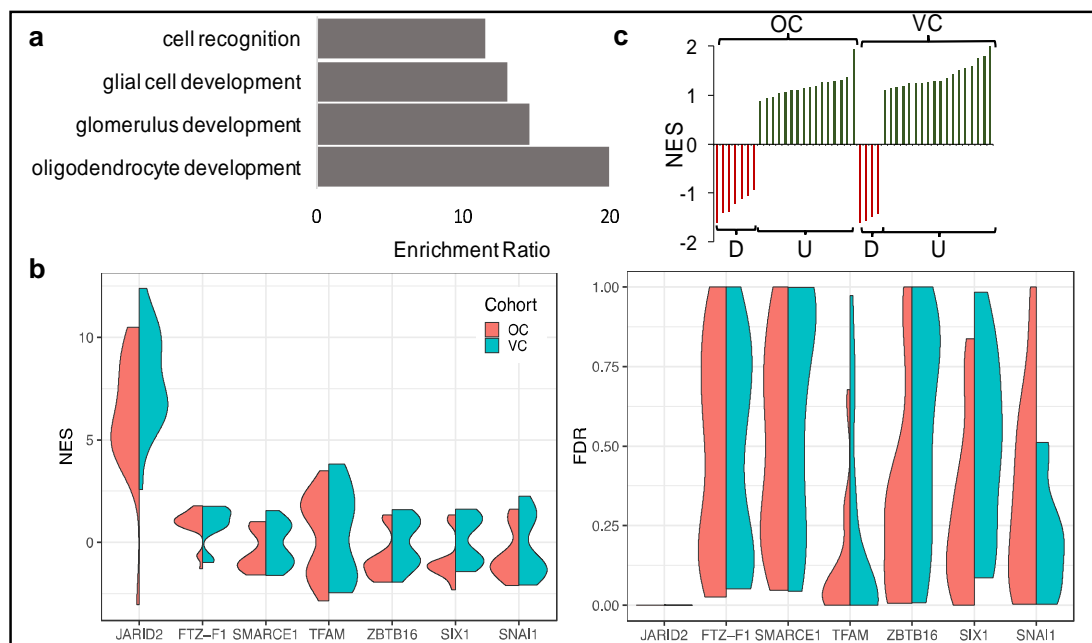
86 **RESULTS**

87 **Differential expression indicates a therapy-driven shift in neurodevelopmental genes**

88 Genes that were differentially expressed (DE) in the recurrent versus primary GBMs in our original
89 cohort (Supp.Table.1) were significantly enriched for those involved in cell development and
90 lineage determination, specifically in relation to neurodevelopment (Fig.1a and Supp.Table.2).
91 Brain cell fate is orchestrated by the combined actions of transcription factors (TFs) and chromatin
92 remodeling complexes, both of which have been implicated in establishing functionally
93 heterogeneous transcriptional hierarchies in gliomas^[14, 15]. We therefore reasoned that specific
94 DNA-binding factors may coordinately regulate the genes we observe to be altered after treatment,
95 potentially highlighting certain cell types that survive. Candidate master transcriptional regulators
96 can be identified from expression data via gene set enrichment analysis (GSEA). However, we
97 found that many neurodevelopmental TFs were missing from publicly available gene sets, so we
98 first developed a more comprehensive DNA-binding factor gene set using ChIPseq data from the
99 Gene Transcription Regulation Database (see Methods)^[16].

100 **Genes with JARID2 binding sites in their promoters (JBSgenes) are consistently and**
101 **significantly dysregulated in recurrent versus primary GBMs and stratify patients into two**
102 **response subtypes**

103 We performed per-patient GSEA, using our novel gene set, with genes pre-ranked by the
 104 magnitude of fold change in expression between the primary and recurrent tumour. Genes with a
 105 Jumonji and AT-Rich Interacting Domain 2 (JARID2) binding site in their promoters (JBSgenes)
 106 were the most significantly, consistently and highly enriched within the genes changed after
 107 therapy across patients: the normalized enrichment score (NES) for JARID2 was significant in all
 108 patients (FDR<0.05) and gave the highest score in 91% (n=21/23) (Fig.1b). To determine whether
 109 JBSgenes were consistently up-regulated or down-regulated after treatment, we repeated the
 110 analysis including the direction of fold change in the gene ranking. We found that the JBSgenes
 111 were altered in a consistent direction per patient but across patients the direction varied: in 30%
 112 (n=7/23) the JARID2 enrichment was being driven by down-regulation of JBSgenes (hereon
 113 referred to as D response subtype) and in the remaining 70% (n=16/23) it through up-regulation (U
 114 response subtype) (Fig.1c).



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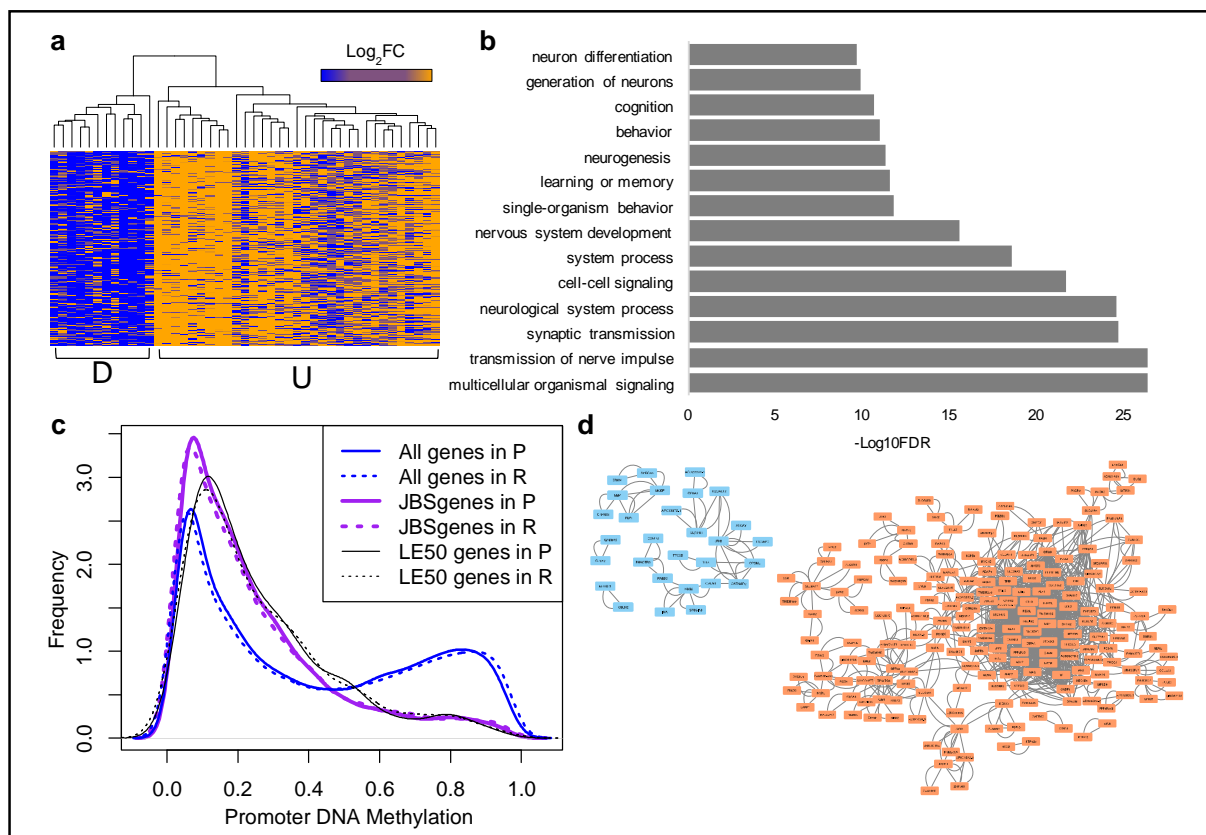
116 **Fig.1a)** Biological processes enriched in the genes differentially expressed between matched
 117 primary and recurrent GBMs (enrichment ratio and term count>10, $p<0.0005$); **b)** Per-patient
 118 normalised enrichment scores (NES, left plot) and false discovery rates (FDR, right plot) for the
 119 top-scoring promoter-binding factors associated with gene expression changes in recurrent vs
 120 primary GBMs, highlighting the significance of JARID2; **c)** The NES for JARID2 for each patient (x-
 121 axis) when direction of fold change is taken into account, shows that there are two response

122 subtypes based on whether genes are up (U) or down (D) regulated. OC: original cohort. VC:
 123 validation cohort.

124

125 We acquired data from an additional 22 paired primary and recurrent GBMs from patients who
 126 underwent standard treatment and who had a local recurrence (the validation cohort) which
 127 corroborated our findings (Figs.1b and c) with a similar ratio of D response subtype (n=4/22) and U
 128 response subtype (n=18/22) patients (Fisher's exact test, $p=0.52$)^[5].

129



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132 **Fig.2a)** Heatmap of fold change in expression after treatment per patient (columns) for the genes
 133 in the leading edge of the JARID2 GSEA results in more than 50% of patients across both cohorts
 134 (LE50 genes, rows). These same genes are upregulated in U response subtype patients as are
 135 downregulated in D response subtype patients; **b)** The biological processes (with <2000 terms)
 136 most enriched in LE50 genes; **c)** The distribution of average promoter DNA methylation for all
 137 genes, JARID2 binding site (JBS)genes and LE50 genes in primary (P) and recurrent (R) GBM

138 *samples; d) Networks of genes (nodes) for which expression is highly correlated (edges: $R > |0.9|$)*
139 *with LE70 genes in the primary (blue, top left) and recurrent (orange, right) GBM samples.*

140

141 **The same JBSgenes are dysregulated in each response subtype and their promoter DNA is**
142 **unmethylated in both primary and recurrent GBMs**

143 To investigate whether the JBSgenes driving the enrichment differed across individual patients or
144 between response subtypes, we quantified how often each gene was present in the leading edge
145 of the GSEA results across the 45 patients in the original and validation cohorts combined. 335
146 genes were observed in the leading edge of more than 50% of patients (denoted LE50 genes) and
147 43 genes in more than 70% (LE70 genes) (Supp.Table.3). The per-patient fold-changes of the
148 LE50 genes showed that these same JBSgenes drive the enrichment across patients irrespective
149 of response subtype i.e. the same genes are downregulated in D response patients as are
150 upregulated in U response patients (Fig.2a). The LE50 genes are enriched in functional
151 annotations associated with neurodevelopment and neuronal differentiation such as synaptic
152 plasticity and interneuronal communication (Fig.2b and Supp.Table.4). To investigate the DNA
153 methylation status of the promoters of these genes in primary and recurrent GBMs, in comparison
154 to other genes, we performed genome-wide methylation arrays on DNA from 9 pairs (see
155 Supp.Table.1). No gene promoters were differentially methylated between primary and recurrent
156 samples ($q > 0.45$ for all genes). However, the distribution of promoter methylation across all genes
157 was significantly different to that of the JBSgenes and the LE50 genes in isolation, revealing that
158 the DNA in the promoters of the latter two is unmethylated in both the primary and recurrent GBMs
159 (Fig.2c). This indicates that the change in expression of these genes that we observe post-
160 treatment is not driven by DNA methylation.

161 **LE70 JBSgenes are more coordinately expressed in recurrent GBM**

162 We hypothesised that JARID2 is involved in the tighter co-regulation of leading edge JBSgenes in
163 GBM tumours after treatment, independent of whether that results in an increase or decrease in
164 their expression. To investigate this, we identified all genes for which expression is highly
165 correlated ($R > |0.9|$) in the primary GBMs and recurrent GBMs separately. We then determined the
166 prevalence of the LE70 genes and their connectivity in these two correlation networks. We found

167 that the LE70 JBSgenes correlate with significantly more genes and with significantly more
168 connectivity in recurrent versus primary samples: 1% (29/2603) in the primary GBM network
169 compared to 7% (202/2855) in the recurrent GBM network (chi-squared; $p=0$ for both tests)
170 (Fig.2d). This implies that either cells in which these genes are co-regulated by JARID2 become
171 more prevalent post-treatment, or that their co-regulation by JARID2 occurs in response to
172 treatment. To inspect this further we designed an *in vitro* experiment to investigate the time course
173 of JBSgene dysregulation following treatment.

174 **Single cell analysis indicates that JARID2 associated gene co-regulation is an adaptive** 175 **response to therapy**

176 We cultured two plates of spheroids directly from a freshly resected primary GBM, in serum-free
177 conditions. We treated one plate with physiologically relevant single doses of TMZ (30 μ M) and
178 radiation (2Gy). We captured and sequenced RNA from single cells from spheroids one week post-
179 treatment when there was a significant deviation in the untreated vs treated spheroid growth
180 curves, and three weeks post-treatment when growth of the treated spheroids appeared to have
181 recovered (Fig.3). Our bespoke GSEA revealed that JBSgenes were significantly enriched
182 amongst the genes altered in treated versus untreated spheroids three-weeks post-treatment
183 (FDR=0.18) but not one week post-treatment (FDR=0.65). Furthermore, the genes that were DE
184 ($p<0.05$) between treated and untreated cells included significantly more LE50 genes at the three-
185 week time point compared to the one week time-point (chi-squared, $p=0.007$). These results
186 suggest that the universal JBSgene dysregulation that we observe in recurrent tumours is not
187 caused by selection of a fixed transcriptional profile, but rather transcriptional reprogramming
188 following treatment.

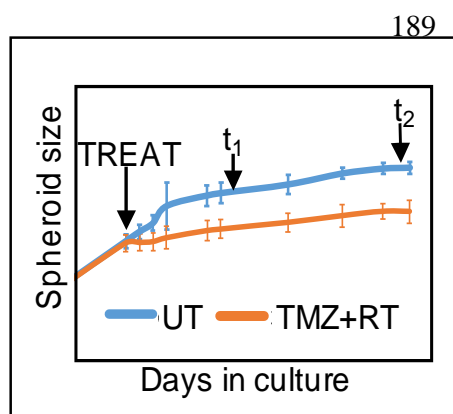


Fig.3. Growth curves for untreated (UT) and treated (TMZ+RT) patient-derived GBM spheroids. Time of treatment (TREAT) is indicated (arrow), in relation to single cell capture 1 week (t_1) and 3 weeks (t_2) post-treatment.

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193 **JARID2 is involved in cell plasticity and implicated in interconversions between cell states**

194 **in glioma**

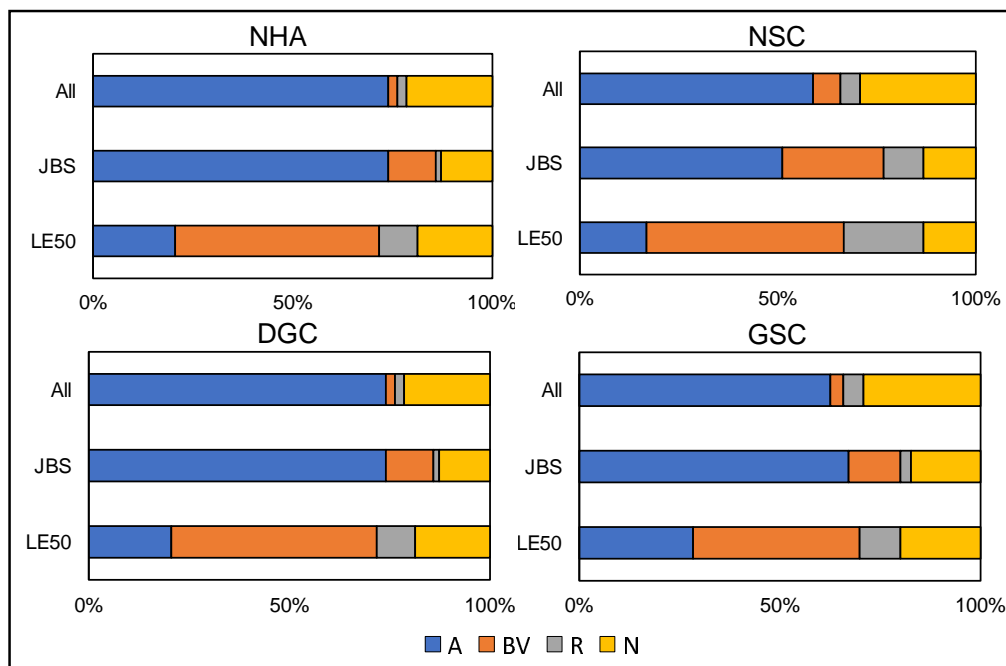
195 JARID2 is an accessory protein responsible for the genomic positioning of Polycomb Repressive

196 Complex 2 (PRC2)^[12]. PRC2 is a chromatin remodeller that is indispensable for lineage

197 determination during neurogenesis^[10]. It is responsible for the trimethylation of lysine 27 on histone

198 H3 (H3K27me3) which results in epigenetic silencing of the marked gene. PRC2 is directly

199 implicated



200

201 **Fig.4** Relative quantification of promoter status, with respect to specific histone marks, across all

202 genes compared to JBSgenes and LE50 genes in normal human astrocytes (NHA), neural stem

203 cells (NSC), differentiated glioma cells (DGC) and glioma stem cells (GSC). A=active=H3K4me3;

204 R=repressed=H3K27me3; BV=bivalent=H3K27me3+H3K4me3; and N=neither mark.

205

206 in cell plasticity in GBM by studies showing that its catalytic subunit is required to enable

207 conversions between stem-like and differentiated cell types^[11]. It is also indirectly implicated by the

208 fact that the prevalence and location of H3K27me3 significantly differs between normal brain and

209 glioma cells, and between GBM cells with different phenotypes^[17]. These differences occur most
 210 frequently at bivalent promoters: those harbouring both the repressive H3K27me3 and an
 211 activating mark (H3K4me3) causing the gene to be silenced but primed for activation upon PRC2
 212 disassociation and H3K27 demethylation. Developmental gene promoters are commonly bivalent
 213 in embryonic stem cells to enable subsequent rapid activation of specific lineage determination
 214 genes once cell fate is resolved, further highlighting PRC2's role in cell-type transitions^[18].

215 To determine whether JBSgenes, and specifically the LE50 genes, are implicated in cell
 216 type switching we mined published data on histone marks (H3K27me3 and H3K4me3) and gene
 217 expression in different normal and GBM cell types^[17, 19]. Normal brain cell types were human
 218 neural stem cells (NSCs) and normal human astrocytes (NHAs). Different GBM 'cell types' pertains
 219 to the

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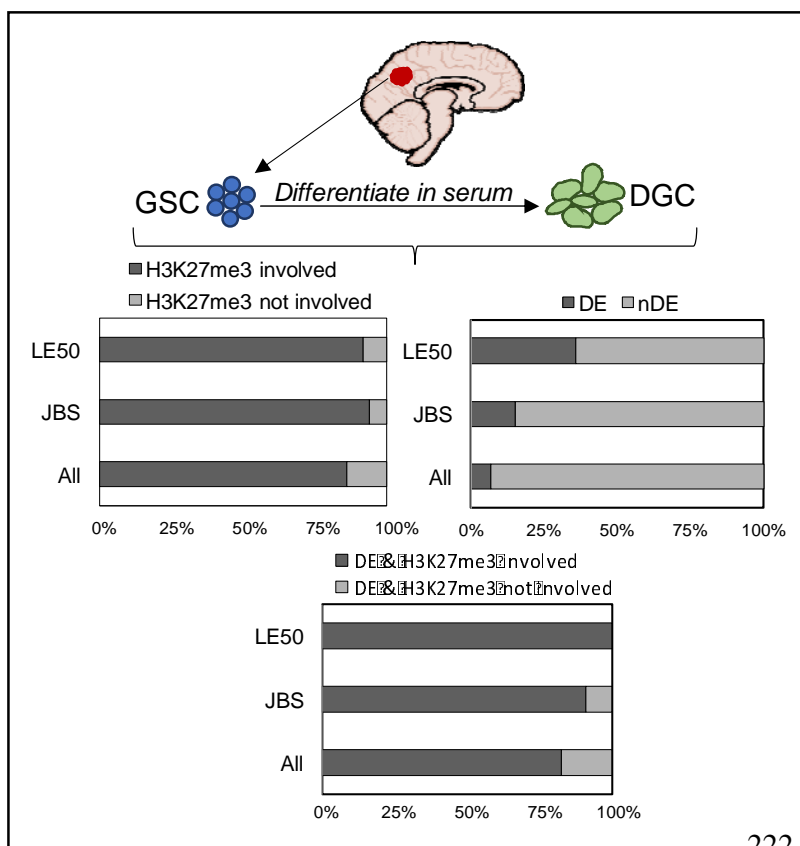


Fig.5. Glioma stem cell (GSC) cell lines can be derived from patient GBM tumours and then further cultured in serum to form differentiated glioma cells (DGC). The bar charts indicate the proportion of genes for which H3K27me3 is involved in any changes in promoter status (top left), or which were differentially expressed (DE, top right), or both (bottom), between matched GSC and DGC cell lines for all genes, JBSgenes or LE50 genes ability to derive phenotypically

223 distinct cell lines from the same patient GBM under different conditions: those which enrich for
 224 glioma stem cells (GSCs, which can be considered analogous to

225 NSCs in the normal brain) and those which enrich for differentiated glioma cells (DGCs, somewhat
226 analogous to NHAs)^[20]. We first quantified promoter status for each different cell types:
227 active=H3K4me3; repressed=H3K27me3; bivalent=H3K27me3+H3K4me3; and neither mark. In
228 support of the role of JARID2 as a PRC2 accessory protein, and increasing confidence that our
229 novel gene set has captured bona fide JBSgenes, we found that H3K27me3 was significantly
230 enriched at JBSgenes in all cell types investigated (Fig.4: middle compared to top barplot in each
231 panel; grey and orange shading pertains to promoters with the H3K27me3 mark), and that this was
232 particularly pronounced at bivalent promoters (Fig.4; orange shading). Moreover, the presence of
233 H3K27me3, again especially at bivalent promoters, was further significantly enriched within the
234 LE50 subset of JBSgenes (Fig.4 bottom compared to middle barplot in each panel). We then
235 characterized changes in promoter status between cells of different phenotype in the normal brain
236 (NPC vs NHA) and GBM (GSC vs DGC: Fig.5). We found, in agreement with the results from the
237 original publication, that changes involving H3K27me3 are more pronounced than any other. We
238 further found that this is significantly more evident in the promoters of JBSgenes: 85% of changes
239 between NPC and NHA involve H3K27me3 at JBSgene promoters compared to 76% at all gene
240 promoters (chi- squared, $p= 3.9 \times 10^{-8}$) and 93% of changes between GSC and DGC involve
241 H3K27me3 at JBSgene promoters compared to 78% at all gene promoters (Fig.5; chi-squared, $p=$
242 5.6×10^{-4}). This implicates JARID2 in chromatin remodeling of gene promoters that differ between
243 cell types in the both normal brain and in GBM.

244 To determine whether this remodeling associates with gene expression changes we
245 identified genes that are DE ($q < 0.2$) between GSC and DGC using RNAseq data from Patel et
246 al.^[19] from matched lines derived from three patient samples. As shown in Fig.5, we found that
247 significantly more JBSgenes are DE (15% JBSgenes vs 5% of non-JBSgenes; chi-squared, $p=0$)
248 and that, within the JBSgenes, significantly more LE50 genes are DE (36% of LE50 genes vs 15%
249 of remaining JBSgenes; chi-squared, $p=2.5 \times 10^{-5}$). We then overlaid the expression and histone
250 mark data and found, as also shown in Fig.5, that chromatin remodeling involving H3K27me3 is
251 more pronounced at DE JBSgenes (91% of changes at DE JBSgene promoters involve
252 H3K27me3) and DE LE50 genes (100% of changes) compared with all DE genes (where 82% of
253 promoter status changes involve H3K27me3). Together, these data suggest that chromatin

254 remodeling associated with JARID2 affects the expression of genes that distinguish different cell
255 types in the normal and human brain.

256

257 **DISCUSSION**

258 We found that the genes differentially expressed between pairs of primary and locally recurrent
259 GBM tumours post standard treatment were enriched for those involved in brain cell development
260 and lineage determination, suggesting that GBM cell types defined within neurodevelopmental-like
261 transcriptional hierarchies may be associated with treatment resistance and tumour regrowth in
262 patients. Brain and GBM cell specification results from a combination of the concerted action of
263 transcription factors (TFs) and differential genome accessibility imposed by a variety of chromatin
264 remodeling molecules and complexes. We, therefore, applied an unbiased approach to investigate
265 whether any such DNA-binding factors were repeatedly implicated across patients in the master
266 regulation of genes for which we observe altered expression in recurrent versus primary GBM
267 tumours. We found that genes with Jumonji and AT-Rich Interacting Domain 2 (JARID2) binding
268 site(s) in their promoter (JBSgenes) are consistently and significantly dysregulated in recurrent
269 GBM tumours, in both our original and validation cohorts. JARID2 is indirectly responsible for
270 eliciting the programmes of epigenetic gene silencing required for cell lineage determination during
271 neurodevelopment^[9]. It does so by docking Polycomb Repressive Complex 2 (PRC2) to specific
272 genomic loci where it trimethylates H3K27 to repress gene expression^[12, 21]. As well as normal
273 brain cell delineation, PRC2 and the H3K27me3 mark have been specifically implicated in cell
274 state transitions in glioblastoma^[11, 17, 22]. However, the involvement of JARID2 in this process, and
275 the genes in which expression is altered by this mechanism to dictate GBM cell phenotype have
276 not previously been elucidated. Our results highlight subsets of genes with JARID2 binding sites in
277 their promoters (JBSgenes) that are most commonly dysregulated (LE50 in more than 50% and
278 LE70 in more than 70% of patients) in GBM tumours after treatment. In support of the notion that
279 expression of these genes is regulated by JARID2-associated mechanisms in GBM, we have
280 shown that the DNA in their promoters is unmethylated in both primary and recurrent samples and
281 that their promoters are significantly more associated with the H3K27me3 mark than those of other
282 genes in GBM cell lines. The increasing importance of this coordinated regulation by JARID2 in

283 GBM after treatment is indicated by the larger and more connected networks of highly correlated
284 LE70 JBSgenes in recurrent versus primary samples.

285 Together these results could suggest that specific GBM cell types, defined by
286 transcriptional profiles resulting from JARID2-associated epigenetic programming, resist treatment
287 and expand during tumour recurrence. It is widely thought, for example, that glioma stem cells
288 specifically resist treatment and are responsible for GBM regrowth^[23, 24]. However, a confounding
289 result in relation to this interpretation is the fact that, whilst a specific subset of JBSgenes are
290 universally dysregulated in recurrent versus primary tumours, the direction of dysregulation is
291 inconsistent; the same genes are upregulated in the recurrence in ~70% of patients and down-
292 regulated in the remaining 30%. Furthermore, our *in vitro* work indicates that changes in
293 expression of JBSgenes occurs dynamically following treatment as opposed to resulting from an
294 increased signal from expansion of a fixed transcriptional profile. Our hypothesis is, therefore, that
295 JARID2-associated chromatin remodeling is not a treatment resistance mechanism per se, but a
296 mechanism by which GBM tumours recover from treatment to enable regrowth. In this way, the
297 different directions of gene dysregulation are owing to the need to recapitulate the GBM
298 transcriptional heterogeneity, required for tumour growth *in vivo*, from whichever cell types
299 survived in that particular patient^[13, 25, 26]. This is supported by our findings that the JBSgenes
300 dysregulated during treatment are a) significantly more likely to have bivalent promoters (i.e.
301 poised for activity relating to lineage decisions in response to environmental queues) and b)
302 significantly enriched amongst the genes differentially expressed between cells at either end of the
303 GBM transcriptional hierarchy (i.e. glioma stem cells and differentiated glioma cells). Recent
304 landmark findings also support our hypothesis, which posits that different GBM cell types are able
305 to resist treatment and that interconversions between cell types, as opposed to one-way transitions
306 down a differentiation pathway, are needed to enable tumour regrowth: i) differentiated GBM cells
307 form networks *in vivo* that enable them to better survive chemoradiation, negating the idea that
308 only stem-like cells are able to survive^[27]; ii) glioma stem-cells, needed for tumour regrowth owing
309 to their proliferation capabilities, can form via non-hierarchical conversion of differentiated cells in
310 GBM^[11, 14, 25].

311 Our hypothesis represents a paradigm shift, also recently suggested by Dirkse et al.^[25], that
312 challenges the notion that effective treatment of GBM will be possible by therapeutically targeting
313 any one cell population, such as glioma stem cells. Instead, we propose that effective treatment
314 will only be possible by targeting the mechanisms of GBM cell plasticity resulting from
315 transcriptional reprogramming, which our results suggest are fundamentally linked with the role of
316 JARID2. The precise nature of this role, and its potential for therapeutic targeting, are the focus of
317 our ongoing work.

318

319 **CONCLUSION**

320 We have found that a subset of genes is universally dysregulated in patient GBMs following
321 standard treatment, likely because of epigenetic remodeling of their promoters via mechanisms
322 involving JARID2, as an adaptive response that facilitates tumour regrowth. The direction of this
323 adaptive response is, however, not constant across patients and may depend upon the cell state
324 transitions needed to recapitulate transcriptional heterogeneity in the recurrent tumour. This is the
325 first time that JARID2 has been implicated in GBM cell plasticity in association with tumour
326 recurrence, and highlights subsets of genes that may be involved in cell state transitions required
327 for adaption of GBM tumours to treatment.

328

329 **METHODS**

330 **Archival Samples and Profiling Data**

331 Four independent sources of paired patient GBM samples (surgical tissue from primary GBM and
332 subsequent recurrent samples) were used in this work. Samples were allocated to the original
333 cohort if they had undergone whole transcriptome RNA sequencing, and to the validation cohort if
334 they had undergone poly-A transcriptome sequencing. Clinical information and cohort assignment
335 are given in Supp.Table.1.

336 Stead Samples: 21 patients from four tissue banks (Leeds, Liverpool, Cambridge and Preston)
337 with tumour in paraffin blocks. Ethical approval was acquired (REC 13/SC/0509). RNA and DNA
338 was extracted from the same cells from neuropathologist annotated tumour regions (>60% cancer
339 cells) using appropriate Qiagen kits (Qiagen, Sussex, UK). Paired end strand-specific whole

340 transcriptome libraries were prepared for 16 pairs using the NEBNext Ultra Directional RNA Library
341 Prep Kit for Illumina (New England BioLabs, UK), following rRNA depletion with NEBNext rRNA
342 Depletion Kit or Ribo-Zero Gold. Libraries were sequenced on an Illumina HiSeq. DNA from 9 pairs
343 (4 of which also underwent RNAseq) was profiled using the Illumina Infinium Human Methylation
344 450K Bead Chip array.

345 Rabadan Samples: Nine patients from Wang et al.^[4] with transcriptome sequencing data (7 with
346 whole transcriptome data and 2 with poly-A transcriptome data) for paired tumours, downloadable
347 from the sequencing read archive (accession SRP074425).

348 Verhaak Samples: Four patients from Kim et al.^[1] with poly-A transcriptome sequencing alignment
349 data acquired, and converted to raw fastq format, following application to the European Genome-
350 Phenome Archive (accession EGAS00001001033).

351 Nam Samples: 16 patients from Kim et al.^[5] with poly-A transcriptome sequencing alignment data
352 acquired, and converted to raw fastq format, following application to the European Genome-
353 Phenome Archive (accession EGAD00001001424).

354 **Sequencing Data: Alignment, Differential Expression, Functional Enrichment and** 355 **Correlation Analysis**

356 RNAseq data was analysed as previously described except that reads were aligned to human
357 reference genome GRCh38, using the gencode.v27 genome annotation as a guide, using
358 STARv2.4.3a and functional enrichment analysis was done using WebGestalt^[28-30].

359 **Gene set enrichment analysis (GSEA)**

360 We developed a novel gene set file for use in GSEA using the Gene Transcription Regulation
361 Database (GTRD v18.01), which contained the genomic binding locations of 682 human DNA-
362 binding factors from 4236 chromatin immunoprecipitation sequencing (ChIPseq) experiments^[16]. A
363 gene was assigned to a DNA-binding factor's gene set if its promoter (transcription start site from
364 gencodev27 \pm 1kbp) contained a binding site for that factor in \geq 2 independent ChIPseq
365 experiments. We first performed pre-ranked GSEA, per patient, ordering genes by the magnitude
366 of fold change in expression $\log_2(\text{recurrent FPKM} + 0.01 / \text{primary FPKM} + 0.01)$ in classical mode.
367 To indicate directionality of dysregulation we then ranked genes by absolute fold changes ie. using
368 $\log_2(\text{recurrent FPKM} + 0.01 / \text{primary FPKM} + 0.01)$ and weighted by magnitude^[31].

369 **DNA methylation analysis**

370 The RnBeads package was used to import, quality check and preprocess IDAT files and then
371 perform pairwise differential methylation analysis. The combined and adjusted p-value
372 (comb.p.adj.fdr) in the promoter results file was used to determine significance. The average
373 methylation signal for each promoter (mean.mean) was extracted for both the primary and
374 recurrent samples and used to plot the distribution for different genes subsets in R using the
375 density function.

376 **Patient-derived spheroids**

377 A patient presenting with a suspected GBM was consented for the use of their tissue in research
378 through the Leeds Multidisciplinary Research Tissue Bank (REC 15/YH/0080). GBM diagnosis was
379 confirmed intraoperatively by a neuropathologist who identified a tumour cell rich piece of tissue,
380 surplus to diagnosis, for transport to the laboratory in cold PBS for use in this work. Tissue was
381 washed in PBS and chopped in Accutase (Sigma-Aldrich, 500 μ L) before incubation at 37°C for 5
382 min. The sample was triturated and Neural Basal (NB) medium, consisting of Neurobasal Medium,
383 N2 and B27 supplements (ThermoFisher, 250mL, 1.25 and 2.5mL respectively), recombinant basic
384 fibroblast growth factor (bFGF), and epidermal growth factor (EGF) (R&D Systems 40ng/mL each),
385 was added to a total volume of 10 mL prior to spinning (1200 rpm at 5 min). The pellet was
386 resuspended in 5ml DNaseI then 1ml RBC lysis buffer (VWR International) with 1 min incubation at
387 room temp, addition of PBS to 10mL and further spinning following each resuspension. The pellet
388 was resuspended in 10mL PBS, filtered via a 70 μ m and 30 μ m strainer consecutively and counted.
389 Finally, cells were resuspended in NB-medium to a concentration of 2x10⁴cells/1mL with 200 μ L of
390 this cell suspension added into each well of an ultra-low-adherence plate and incubated at 37°C
391 5%CO₂. 100 μ L of medium was replaced per well every 3 days. Cells were imaged regularly on the
392 EVOS Cell Imaging System (ThermoFisher) until they reached approximately 300 μ m in diameter.
393 At this point TMZ (Sigma) was concentrated in 100 μ L of NB media and used in a media
394 replacement for one plate of cells to give a final dose, per well, of 30 μ M; one hour later the same
395 plate was irradiated with 2Gy.

396 **Single cell capture and sequencing**

397 Single cells were captured from treated spheroids 1 week and 3 weeks post-treatment, and cells

398 from untreated spheroids the following day, by extracting and combining 8 spheroids per time point
399 and dissociating them via a PBS wash, Accutase incubation, and further PBS washing (as above)
400 to a concentration of 2.5×10^5 cells/mL. Cells were diluted in C1 cell suspension reagent at a ratio
401 of 3:2, respectively. Single cells were captured on a medium (10–17 μ M) C1 Single-Cell Auto Prep
402 IFC for mRNAseq, lysed and underwent on-chip cDNA amplification via SMART Seq2 according to
403 the manufacturer's instructions using the Fluidigm C1 Single-Cell Auto Prep System and protocols
404 on Script Hub™ (Fluidigm)^[32]. cDNA was quantified using the High Sensitivity DNA Assay on the
405 Agilent 2100 Bioanalyser and paired end Nextera XT (Illumina) libraries were made and
406 sequenced, with multiplexing using indexes provided by Dr Iain Macaulay, on an Illumina HiSeq.
407 RNA sequencing data was processed and expression was quantified as per the bulk tissue. GSEA
408 analysis was performed twice using expression (in fragments per kilobase per million mapped)
409 datasets to identify differences between untreated and treated cells at the 1 week timepoint and
410 three week timepoint separately. Differentially expressed genes were identified using Seurat^[33].

411 **Cell type expression and histone modification status**

412 Data on gene promoter histone status in different normal and GBM cell types were extracted from
413 the supplementary material (Table S2) from Rheinbay et al.^[17]. This included data from three
414 patient GBM tumours that had been used to derive glioma stem cells (GSCs) which were
415 subsequently cultured in serum to produce differentiated glioma cells (DGC). We required that all
416 three samples had been assigned the same status to be included in our analyses. Raw
417 sequencing data was downloaded on a further three GSC and DGC pairs from Patel et al.^[19] via
418 the Gene Expression Omnibus (accession GSE57872). These data were processed and aligned,
419 and pairwise differential expression analysis was performed, exactly as for the paired primary and
420 recurrent RNAseq data. Quantification and assessment of the significance of overlap in genes with
421 different promoter states and/or differential expression with the JBSgenes or LE50 genes was
422 done in the R statistical package.

423

424 **LIST OF ABBREVIATIONS**

425 ChIP- Chromatin immunoprecipitation

426 DE – Differentially expressed

427 DGC – Differentiated glioma cell
428 GBM – Glioblastoma
429 GSC – Glioma stem cell
430 GSEA – Gene set enrichment analysis
431 H3K4me3 - Trimethylated histone 3 at lysine 4
432 H3K27me3 – Trimethylated histone 3 at lysine 27
433 JARID2 - Jumonji And AT-Rich Interaction Domain Containing 2
434 JBSgenes – JARID2 binding site genes
435 LE - Leading edge
436 NES – Normalized enrichment score
437 PRC2 – Polycomb Repressive Complex 2
438 RNAseq – RNA sequencing
439 TF – Transcription factor
440

441 **DECLARATIONS**

442 ***Ethics approval and consent to participate***

443 All patients whose data was used in this study consented to the use of their tissue in research. The
444 data generated for this study was used in accordance with ethical approval acquired from NHS
445 NRES Committee South Central - Oxford A (REC 13/SC/0509).

446 ***Consent for publication***

447 Not applicable

448 ***Availability of data and materials***

449 The RNAseq datasets analysed during the current study are available in the following repositories:
450 Rabadan samples are downloadable from the sequencing read archive (accession SRP074425);
451 Verhaak and Nam samples are available following application to the European Genome-Phenome
452 Archive (accession numbers EGAS00001001033 and EGAD00001001424, respectively). The
453 RNAseq and DNA methylation datasets generated during the current study are not publicly
454 available as data transfer agreements are required to ensure compliance with ethical approvals but
455 are available from the corresponding author on reasonable request.

456 ***Competing interests***

457 The authors declare that they have no competing interests

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464 manuscript.

465 ***Authors' contributions***

466 LFS devised the project. LFS and SS acquired funding. MDJ and AB sourced samples and
467 provided clinical annotation. NR and AFB processed samples following annotation and diagnostic
468 confirmation from AC and AI. LFS, GT, AD, MC and JW performed data analysis and LFS
469 interpreted it. NR performed experimental work with assistance from ABR. NR and CT captured
470 and sequenced single cells. LFS wrote the manuscript, which was reviewed and approved by all
471 authors.

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484

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