

1 Copy-scAT: Deconvoluting single-cell chromatin accessibility of genetic subclones in cancer

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ABSTRACT

17 Single-cell epigenomic assays have tremendous potential to illuminate mechanisms of transcriptional control
18 in functionally diverse cancer cell populations. However, application of these techniques to clinical tumor
19 specimens has been hampered by the current inability to distinguish malignant from non-malignant cells,
20 which potently confounds data analysis and interpretation. Here we describe Copy-scAT, an R package that
21 uses single-cell epigenomic data to infer copy number variants (CNVs) that define cancer cells. Copy-scAT
22 enables studies of subclonal chromatin dynamics in complex tumors like glioblastoma. By deploying Copy-
23 scAT, we uncovered potent influences of genetics on chromatin accessibility profiles in individual subclones.
24 Consequently, some genetic subclones were predisposed to acquire stem-like or more differentiated
25 molecular phenotypes, reminiscent of developmental paradigms. Copy-scAT is ideal for studies of the
26 relationships between genetics and epigenetics in malignancies with high levels of intratumoral heterogeneity
27 and to investigate how cancer cells interface with their microenvironment.

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30 INTRODUCTION

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32 Single-cell genomic technologies have made enormous contributions to the deconvolution of complex
33 cellular systems, including cancer (1). Single-cell RNA sequencing (scRNA-seq) in particular has been widely
34 employed to understand the implications of intratumoral transcriptional heterogeneity for tumor growth,
35 response to therapy and patient prognosis (2–6). This field has hugely benefited from an emerging ecosystem
36 of computational tools that have enabled complex analyses of scRNA data. Since copy number variants
37 (CNVs) mostly accrue in malignant cells and are rare in non-malignant tissues, computational platforms that
38 use scRNA data to call CNVs have resulted in improved understanding of the behavior of genetic subclones
39 in tumors (7–9).

40 On the other hand, the application of single-cell epigenomic techniques, including the assay for transposase
41 accessible chromatin (scATAC) (10, 11), to study cancer has been slowed by computational bottlenecks. For
42 instance, unlike scRNA-seq, currently no dedicated tool exists to call CNVs using scATAC data. This
43 technical gap has prevented scATAC studies of clinical tumor specimens, which often are surgical resections

44 that include both malignant and non-malignant cells. Inability to deconvolute these cell populations after the
45 generation of scATAC datasets would confound downstream analyses and interpretation of this data type.

46 In this report, we describe Copy-scAT (Copy number inference using scATAC-seq data), a new
47 computational tool that uses scATAC datasets to call CNVs at the single-cell level. Using scATAC datasets
48 from adult glioblastoma (aGBM), pediatric GBM (pGBM) and multiple myeloma (MM), we demonstrate the
49 effectiveness of Copy-scAT in calling (A) focal amplifications, (B) segmental gains and losses and (C)
50 chromosome arm-level gains and losses. At the most basic level, Copy-scAT can therefore discriminate
51 between malignant and non-malignant cells in scATAC datasets based on the presence or absence,
52 respectively, of CNVs. This distinction is fundamental to ensure that downstream analyses include only the
53 appropriate tumor or microenvironment cell populations. At a more sophisticated level, we show that
54 implementation of Copy-scAT allows investigations of the relationship between genetic and epigenetic
55 principles governing the behavior of individual subclones. In this regard, we show that each genetic subclone
56 has characteristic accessible chromatin profiles, indicating that genetics imparts information that determines
57 key epigenetic features. Strong influence of genetics on chromatin states is demonstrated by the
58 predisposition of genetic subclones to have stem-like or more differentiated molecular profiles in GBM.

59

60 RESULTS

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62 Design and implementation of Copy-scAT

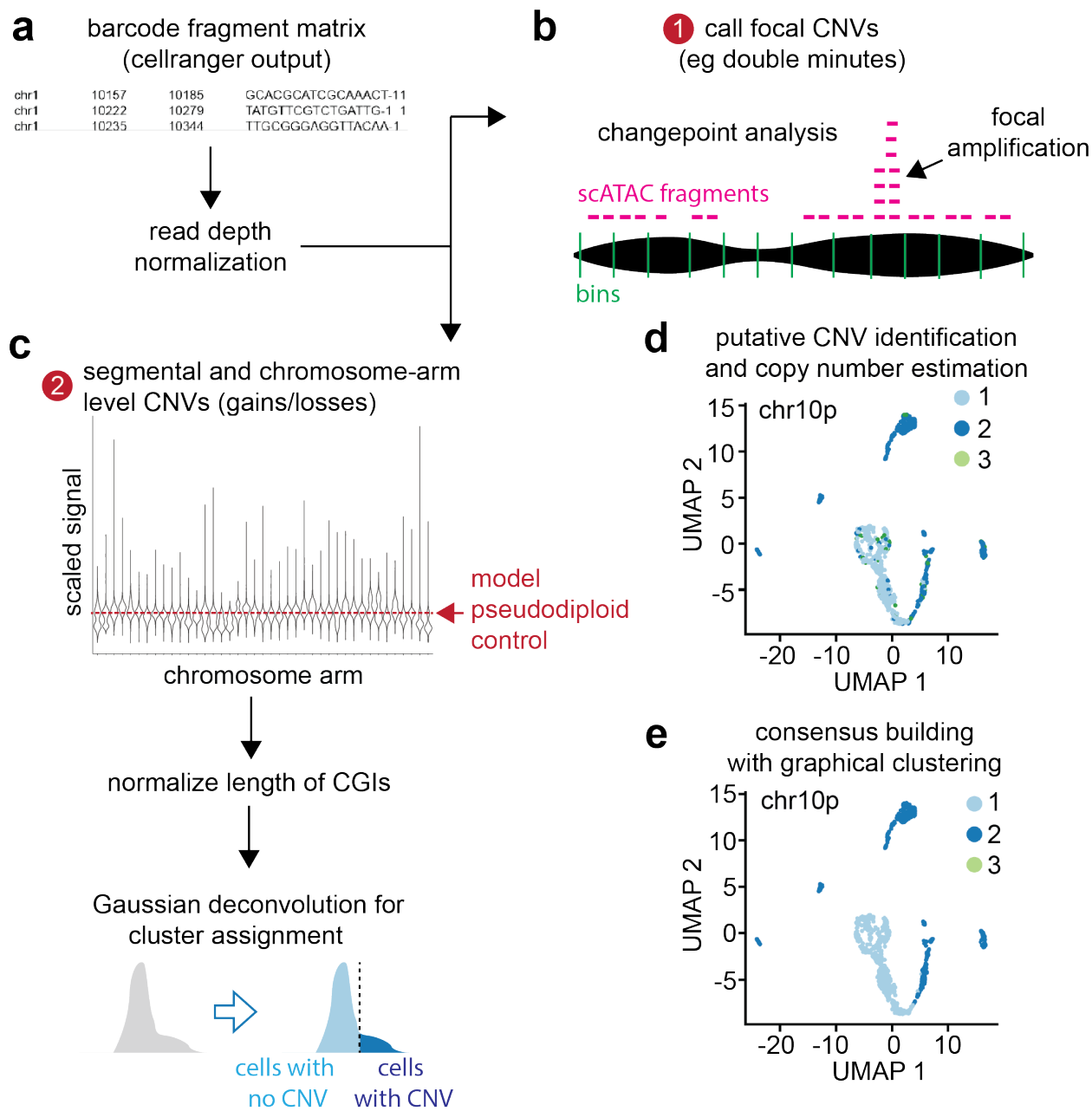
63 We designed Copy-scAT, an R package that uses scATAC-seq information to infer copy number alterations.
64 Copy-scAT uses fragment files generated by cellranger-atac (10xGenomics) as input to generate chromatin
65 accessibility pileups, keeping only barcodes with a minimum number of fragments (defaulting to 5,000
66 fragments). It then generates a pileup of total coverage (number of reads \times read lengths) over bins of
67 determined length (1 million bp as default) (**Fig. 1a**). Binned read counts then undergo linear normalization
68 over the total signal in each cell to account for differences in read depth, and chromosomal bins which
69 consist predominantly of zeros (at least 80% zero values) are discarded from further analysis. All parameters,
70 including reference genome, bin size, and minimum length cut-off are user-customizable. Copy-scAT then
71 implements different algorithms to detect focal amplifications and larger-scale copy number variation.

72 To call focal amplifications (**Fig. 1b**), Copy-scAT generates a linear scaled profile of density over normalized
73 1 Mbp bins along each chromosome on a single-cell basis, centering on the median and scaling using the
74 range. Copy-scAT then uses changepoint analysis (12) (see Methods) to identify segments of abnormally high
75 signal (Z score > 5) along each chromosome in each single cell. These calls are then pooled together to
76 generate consensus regions of amplification, in order to identify putative double minutes and
77 extrachromosomal amplifications. Each cell is scored as positive or negative for each amplified genomic
78 region.

79 Segmental losses are called in a similar fashion, by calculating a quantile for each bin on a chromosome,
80 running changepoint analysis to identify regions with abnormally low average signal, and then using Gaussian
81 decomposition of total signal in that region to identify distinct clusters of cells.

82 For larger copy number alterations, Copy-scAT pools the bins further at the chromosome arm level using a
83 trimmed mean, while normalizing the data on the basis of length of CpG islands contained in each bin (**Fig.**
84 **1c**). Data is then scaled for each chromosome arm, compared to a pseudodiploid control (expected signal
85 distribution for a diploid genotype) that is modeled for each sample, and cluster assignments are generated
86 using Gaussian decomposition. Cluster assignments are then normalized to get an estimate of copy number

87 for each cell (**Fig. 1d**). These assignments can be optionally combined with clustering information to
 88 generate consensus genotypes for each cluster of cells and further filter false positives (**Fig. 1e**) For full
 89 details regarding the execution of Copy-scAT, see Methods. A step-by-step tutorial for Copy-scAT is
 90 available on GitHub (see Methods).



91

92 **Fig. 1. Copy-scAT workflow.**

93 (a) Copy-scAT accepts barcode fragment matrices generated by cellranger (10xGenomics) as input.

94 (b) Large peaks in normalized coverage matrices can be used to infer focal CNVs.

95 (c) Normalized matrices can be used to infer segmental and chromosome-arm level CNVs.

96 (d) Example of chromosome-arm level CNV (chromosome 10p loss) called by Copy-scAT

97 (e) Consensus clustering is used to finalize cell assignment.

98

99 Copy-scAT effectively calls CNVs in diverse malignancies

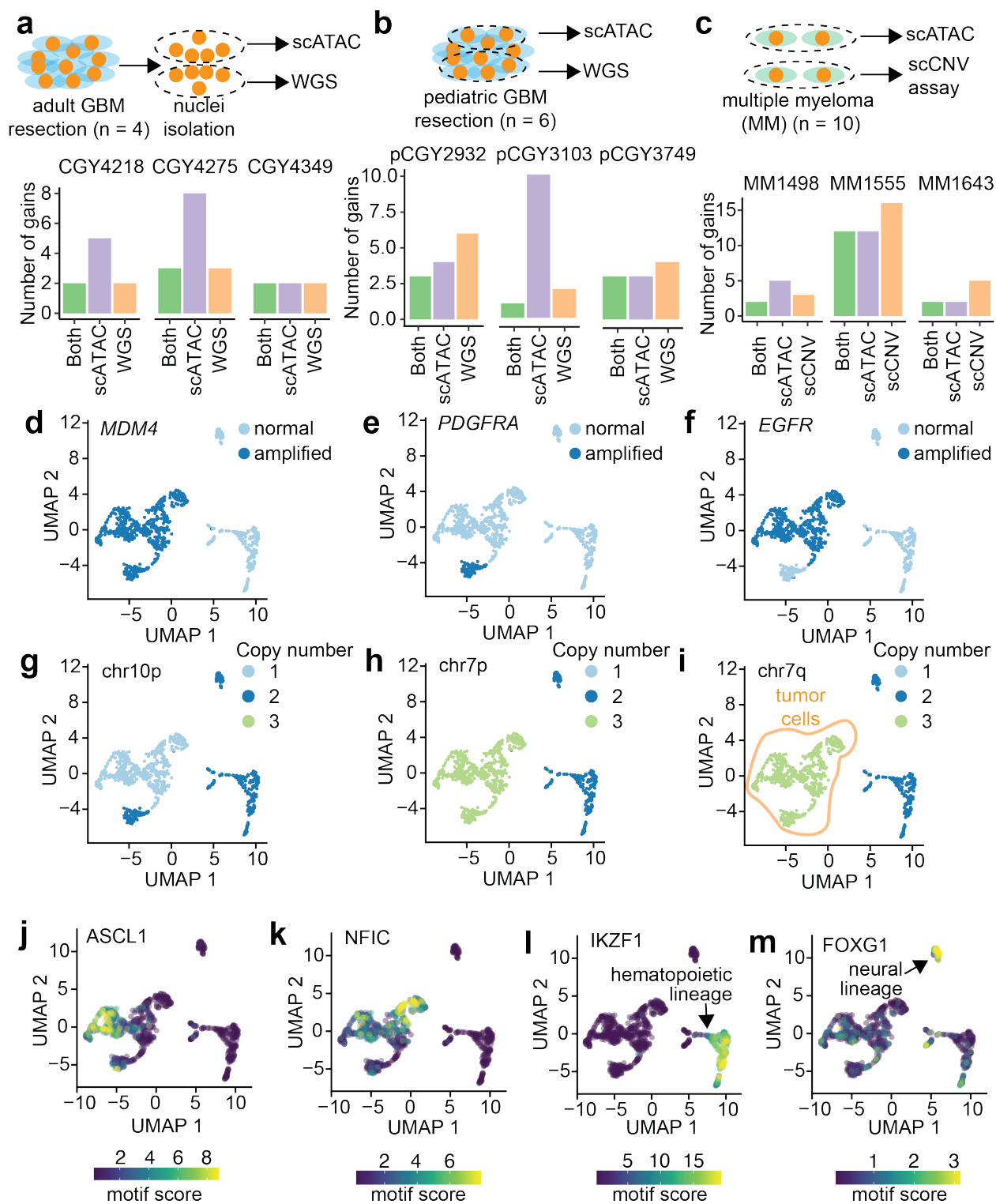
100 We have tested the ability of Copy-scAT to use scATAC data to call CNVs with three different approaches
101 and with different tumor types. First, we benchmarked Copy-scAT against CNV calls made with whole-
102 genome sequencing (WGS) data for adult GBM (aGBM) surgical resections (n = 4 samples, 3,647 cells). This
103 approach consisted in isolating nuclei from flash-frozen aGBM samples, mixing nuclei in suspension, and
104 then using these nuclei for either scATAC or WGS library construction (**Fig. 2a**). This was meant to ensure
105 similar representation of genetic subclones, which are usually regionally contiguous in this solid tumor, in
106 both scATAC and WGS libraries. Second, we benchmarked Copy-scAT against CNV calls made using
107 pediatric GBM (pGBM) surgical resections (n = 6 patient-matched diagnostic-relapse samples, 33,695 cells).
108 In this case, scATAC and WGS libraries were generated from separate geographical regions of the same
109 tumor (**Fig. 2b**). Third, we benchmarked Copy-scAT against CNV calls made with the single-cell CNV
110 (scCNV) assay (10xGenomics) using multiple myeloma (MM) clinical samples (n = 10 samples, 31,266 cells).
111 Overall, we observed that Copy-scAT correctly inferred all or most of the CNVs that were called with WGS
112 (**Figs. 2a,b; Figs. S1, S2**) or scCNV data (**Fig. 2c; Fig S3**). In total, we profiled 51,571 cells from 20
113 malignancies from 17 patients, and were able to infer CNV status for a total of 39,486 cells (**Table S1**). On
114 average, we were able to call CNVs for 78.09% of cells in each sample (range: 29.16 – 91.22%) (**Table S1**).
115 For chromosome-arm level CNV gains, sensitivity ranged from 0.51 for MM to 1.0 for aGBM and specificity
116 ranged from 0.93 to 0.94 (**Table S2**). For chromosome arm-level losses, sensitivity ranged from 0.67 to 0.79
117 and specificity from 0.89 to 0.95. The sensitivity and specificity of focal amplifications were very high
118 (>0.975, **Table S2**). The variation observed may reflect technical differences between the strategies used for
119 benchmarking. As expected, the calls of Copy-scAT for aGBM were the most accurate, likely because
120 scATAC and WGS datasets were generated by relatively homogeneous starting material, as described above.
121 Because of its design, it is also possible that Copy-scAT is more sensitive at inferring CNVs that occur in
122 relatively rare subclones compared to WGS, potentially explaining (in addition to true false positives) why
123 the number of CNVs inferred by our new tool is sometime higher than inferences made with WGS.
124

125 scATAC data can be used to distinguish malignant from non-malignant cells

126 Tumor cells often harbor CNVs, and we reasoned that the use of Copy-scAT should enable the use of
127 scATAC data to infer CNVs and therefore to distinguish between malignant and non-malignant cells. To
128 test this hypothesis, we overlaid CNVs called by Copy-scAT onto scATAC datasets displayed in uniform
129 manifold approximation and projections (UMAP) plots. This exercise led to the identification of cells that
130 were clearly positive for multiple CNVs and others that appeared to have a normal genome. As an illustrative
131 example, we found that the aGBM sample CGY4349 was composed of discrete cell populations that
132 harbored focal amplifications at the *MDM4* (**Fig. 2d**), *PDGFRA* (**Fig. 2e**) and *EGFR* (**Fig. 2f**) loci, as well
133 as chromosome 10p deletion (**Fig. 2g**) and chromosome 7 gain (**Fig. 2h,i**). Copy-scAT results suggest
134 specific lineage relationships between subclones. For instance, chromosome 7 amplifications are clonal in
135 this sample (**Fig. 2h,i**), whereas the chromosome 10 deletion is subclonal (**Fig. 2g**). In addition, our
136 computational tool predicts that *PDGFRA* (**Fig. 2e**) and *EGFR* (**Fig. 2f**) focal amplifications are mutually
137 exclusive, a phenomenon that has been reported in aGBM (13).
138

139 Altogether, these results illustrate one specific population of cells (shaded green in **Fig. 2i**) that harbors
140 several CNVs and are therefore putative cancer cells. At the same time, we also identified cells (labeled in
141 dark blue in **Fig. 2i**) that did not appear to have any CNVs and are therefore likely to be cells from the tumor
142 microenvironment. Equivalent results were obtained for pGBM (**Fig. S4**) and MM samples (**Fig. S5**). Since
143 the latter appear as multiple scATAC clusters, it is possible that our strategy detects multiple distinct non-
144 neoplastic cell clusters. Differential motif analysis with ChromVAR confirmed high scores for neural
145 progenitor cell-associated motifs like NFIC and ASCL1 in CNV⁺ cells (**Fig. 2j,k**), while the putative non-
146 neoplastic clusters showed increased occupancy at transcription factor motifs associated with hematopoietic
147 lineages, such as IKZF1 (**Fig. 2l**). Another CNV⁻ cluster showed enrichment of FOXG1 binding motifs in
148 accessible chromatin, in keeping with a non-neoplastic neural cell identity (**Fig. 2**). Using this approach, it

149 was possible to discriminate between malignant and cells from the tumor microenvironment in all tumor
 150 samples analyzed (**Extended Figs. S6-S8**). Copy-scAT therefore effectively uses scATAC data to infer
 151 CNVs, which can then be used to distinguish malignant from non-malignant cells and to infer lineage
 152 relationships between genetic subclones that coexist in a tumor.
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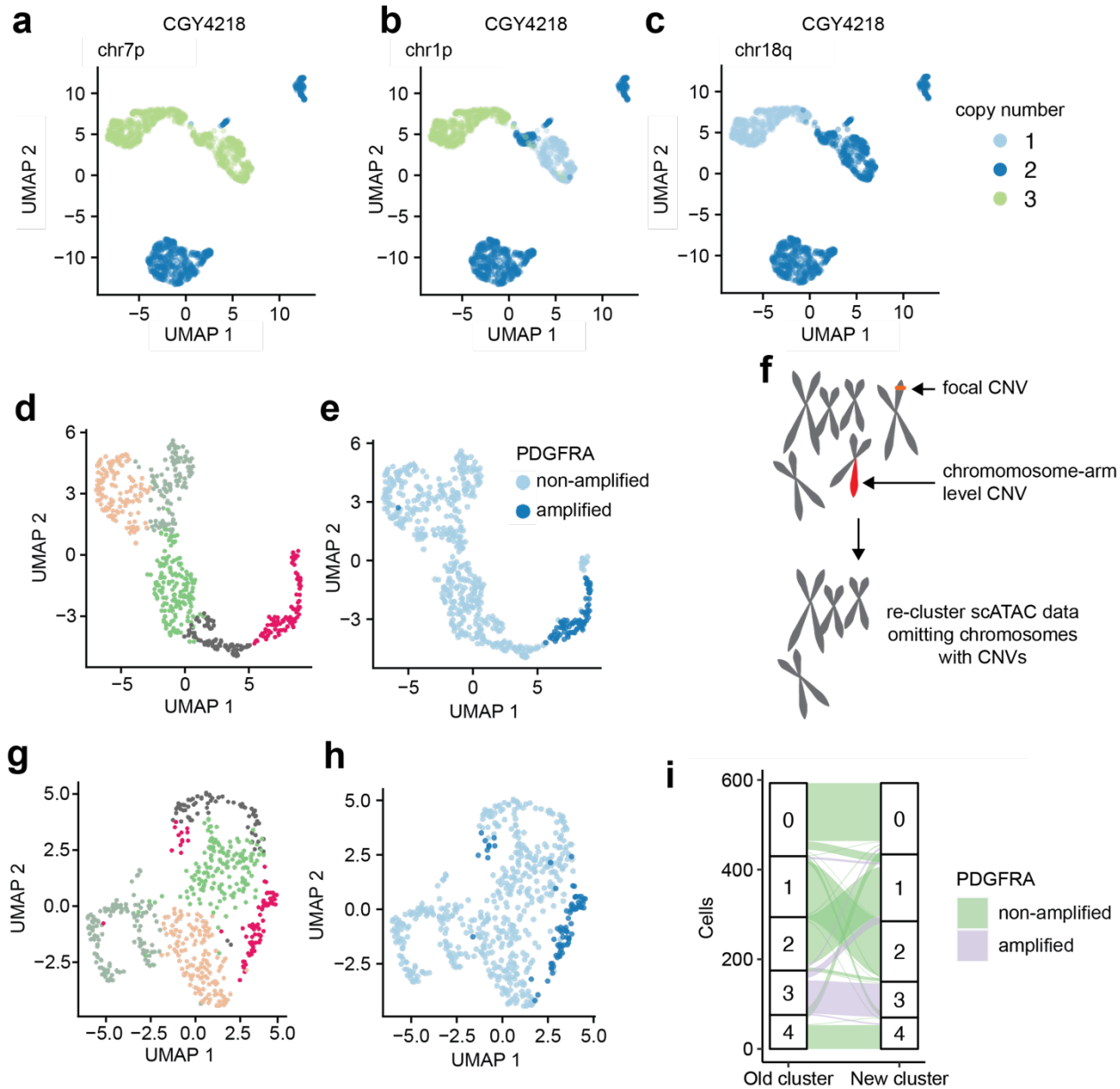
Fig. 2. Benchmarking of Copy-scAT with three methods involving clinical samples from three distinct malignancies.

- 158 (a) Banked frozen aGBM samples were used for both scATAC and WGS. Nuclei were isolated from the
159 samples, mixed, and used for both scATAC and WGS. Number of chromosome-arm level gains detected in
160 adult GBM samples identified using both methods, versus total numbers of gains detected by scATAC or
161 WGS.
- 162 (b) Surgical pGBM resections were split, and one section was used for scATAC and the other for WGS.
163 Number of chromosome-arm level gains detected in adult GBM samples identified using both methods,
164 versus total numbers of gains detected by scATAC or WGS.
- 165 (c) Multiple myeloma samples were profiled by both scATAC and the single-cell CNV assay. Number of
166 chromosome-arm level gains detected in adult GBM samples identified using both methods, versus total
167 numbers of gains detected by scATAC or scCNV assay.
- 168 (d) *MDM4* amplification in an adult GBM sample (CGY4349). Amplified cells are coloured dark blue, and
169 normal cells in pale blue.
- 170 (e) *PDGFRA* amplification in an adult GBM sample (CGY4349). Amplified cells are coloured dark blue,
171 and normal cells in pale blue.
- 172 (f) *EGFR* amplification in an adult GBM sample (CGY4349). Amplified cells are coloured dark blue, and
173 normal cells in pale blue.
- 174 (g) Chromosome 10p loss in an adult GBM sample.
- 175 (h) Chromosome 7P gain in an adult GBM sample.
- 176 (i) Chromosome 7Q gain in an adult GBM sample.
- 177 (j) ChromVAR activity score for *ASCL1*.
- 178 (k) ChromVAR activity score for *NFIC*.
- 179 (l) ChromVAR activity score for *IKZF1*.
- 180 (m) ChromVAR activity score for *FOXG1*.

181 182 183 184 **Subclonal genetics shapes chromatin accessibility profiles in aGBM**

185 We noticed that in most tumors we analyzed, cells harboring a given CNV had a tendency to cluster together
186 (**Fig. 2d-i**). Individual clusters were in fact defined by the presence of specific CNVs (**Fig. 3a-c**). This was
187 an unexpected observation, because it is widely assumed that clustering of scATAC data reflects the global
188 patterns of chromatin accessibility. One possible explanation for this observation could be that chromosomal
189 regions affected by a CNV display imbalances in the fragment depth distribution of scATAC datasets, and
190 that these patterns have a dominant effect on cluster assignment. Most scATAC-seq workflows rely on some
191 variant of term-frequency inverse document frequency (TF-IDF) normalization rather than feature scaling,
192 and this may amplify the effects of CNV-driven DNA content imbalances. For instance, it is possible that
193 focal amplifications of the *PDGFRA* locus result in increased frequency of transposition events that are
194 mapped to this site. A dominant effect of chromatin accessibility at this amplified locus could result in
195 *PDGFRA*-amplified cells clustering together in UMAP representations of scATAC data (**Fig 3d,e**). Indeed
196 we found that compared to a random selection of peaks, the chromosomes which carried CNVs had
197 significantly different numbers of peaks ranked as highly variant than chromosomes that did not have CNVs,
198 leading to a markedly uneven distribution of top peaks ($p < 2.2E-16$; Chi-squared test; **Fig. S9a**) This was
199 not seen in non-neoplastic cells, which had relatively even top fragment distribution patterns ($p = 0.05472$,
200 Chi-squared test; **Fig. S9b**). To test this hypothesis, we used Copy-scAT to call CNVs in our tumor samples,
201 then removed all peaks mapping to chromosomes predicted to harbor CNVs, and finally re-clustered all cells
202 in each sample (**Fig. 3f**). We found that although removing chromosomes with CNVs from our analyses
203 changed the overall cluster structure of a sample (**Fig. 3g**), *PDGFRA*-amplified cells still clustered close to
204 each other (**Fig. 3h**). In fact, our results indicate that clustering after CNV removal is more granular but
205 overall very stable (**Fig. 3i**). In this case, *PDGFRA*-amplified cells localized to a single cluster before
206 removing chromosomes affected by CNVs. Following removal of CNV⁺ chromosomes and re-clustering,
207 most *PDGFRA*-amplified cells still clustered together, with only a few cells merging into a cluster that

208 included both amplified and non-amplified cells. Comparing the most variable peaks after chromosome
 209 CNV removal showed a distribution closer to normal, supporting the marked effect of the CNVs on the
 210 identification of variant peaks ($p = 2.418E-8$; **Fig. S9c**). Contrary to current views of cancer epigenomics,
 211 these data indicate that genetic subclones may have characteristic patterns of chromatin accessibility, and
 212 that a cell's genetic background has significant influence on its likelihood of attaining specific epigenetic
 213 states.
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215
 216 **Fig. 3. Subclonal genetics influences clustering of scATAC-seq data.**

217 (a-c) CNVs in adult GBM CGY4218 segregate within specific scATAC clusters.

218 (d, e) *PDGFRA*-amplified cells cluster together in adult GBM CGY4349.

219 (f) Diagram summarizing our strategy to remove CNVs from clustering of scATAC data. All chromosomes
 220 or regions with putative CNVs were removed from downstream analyses, and cells were re-clustered.

221 (g) Reclustering of (d) following removal of chromosomes and regions affected by CNVs in CGY 4349.

222 (h) Distribution of *PDGFRA*-amplified cells following re-clustering.

223 (i) Cluster assignments of cells in CGY4349 (aGBM specimen) before and after removal of CNV-containing
224 regions (purple: *PDGFR4*-amplified cells).

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227 Genetic events predispose subclones to the acquisition of developmental chromatin states

228 We further explored the notion that CNVs may shape chromatin accessibility profiles and its possible
229 implications for cell fate determination. As an illustrative example, we focused on an aGBM sample
230 (CGY4218) where CNVs at chromosome 1p characterized three genetic subclones, as determined with
231 Copy-scAT: (i) A subclone with two copies of chromosome 1p; (ii) a subclone with loss of 1p; (iii) a subclone
232 with gain of 1p (**Fig. 4a**).

233

234 We were interested in determining whether the major genetic subclones in this tumor had similar cycling
235 properties. Unlike scRNA-seq, we found it is not possible to use scATAC profiles at cell cycle genes to
236 determine whether a cell is proliferating. We reasoned that cells that are actively going through cell division
237 have to replicate their DNA. Given that cancer cells have numerous CNVs on autosomes and could lead to
238 noisy data, we decided to use Copy-scAT to identify cells that have doubled the number of their X
239 chromosomes and defined them as actively cycling cells. To validate this approach, we determined the
240 number of cells with double the number of expected X chromosomes – ie putative cycling cells – in
241 previously published scATAC datasets for mouse brain and peripheral blood mononuclear cells (PBMCs).
242 We hypothesized that we should be able to identify cycling cells in fetal mouse brain, but not in PBMCs. In
243 fact, we detected numerous cycling cells (with twice the expected number of X chromosomes) in brain tissue
244 but not in PBMCs (**Fig. S10**). This method detected putative cycling cells in our datasets (**Fig. 4b**). We used
245 scATAC data to arrange cells from this tumor along pseudotime with the package STREAM (14) (**Fig. 4c**)
246 and then superimposed cell cycle status determined with our X chromosome doubling method (**Fig. 4d**).
247 The results show that cells along branch 2, which is strongly enriched for cells with chromosome 1p gains,
248 are also the most proliferative (**Fig. 4e**), with over 25% of the cells actively going through replication ($P =$
249 7.776×10^{-14} ; Chi-square test). On the other hand, ~5% of cells along branch 1 and ~15% of cells along
250 branch 3 were cycling. These data therefore indicate functional differences between cells with gain or loss of
251 chromosome 1p.

252

253 We then used ChromVAR(15) and STREAM-ATAC to calculate scores for transcription factor (TF) binding
254 motifs that are associated with neurodevelopmental processes. This analysis revealed that motifs bound by
255 TFs that are associated with stem-like phenotypes, including OLIG2 and HOXA2, are enriched in accessible
256 chromatin regions in cells that have one copy of chromosome 1p (**Fig. 4f**). Motifs bound by TFs associated
257 with progenitor (**Fig. 4g**) and differentiated states (**Fig. 4h**) were enriched in the branch with more cells
258 showing gain of chromosome 1p. This was associated with a significant shift in the overall distribution of
259 enrichment of these motifs in cells along the different branches of the trajectory (**Fig. 4i-k**). A distribution
260 of genetic subclones along developmental chromatin accessibility states was observed in other tumor samples
261 we studied (**Fig. S11-S13**). Overall, the data support the notion that tumor cells sample a discrete number
262 of chromatin states, but their transition probabilities differ based on genotype. Consequently, chromatin
263 states associated with each genetic subclone manifest as different functional properties, here demonstrated
264 at the level of cell proliferation and stemness profiles.

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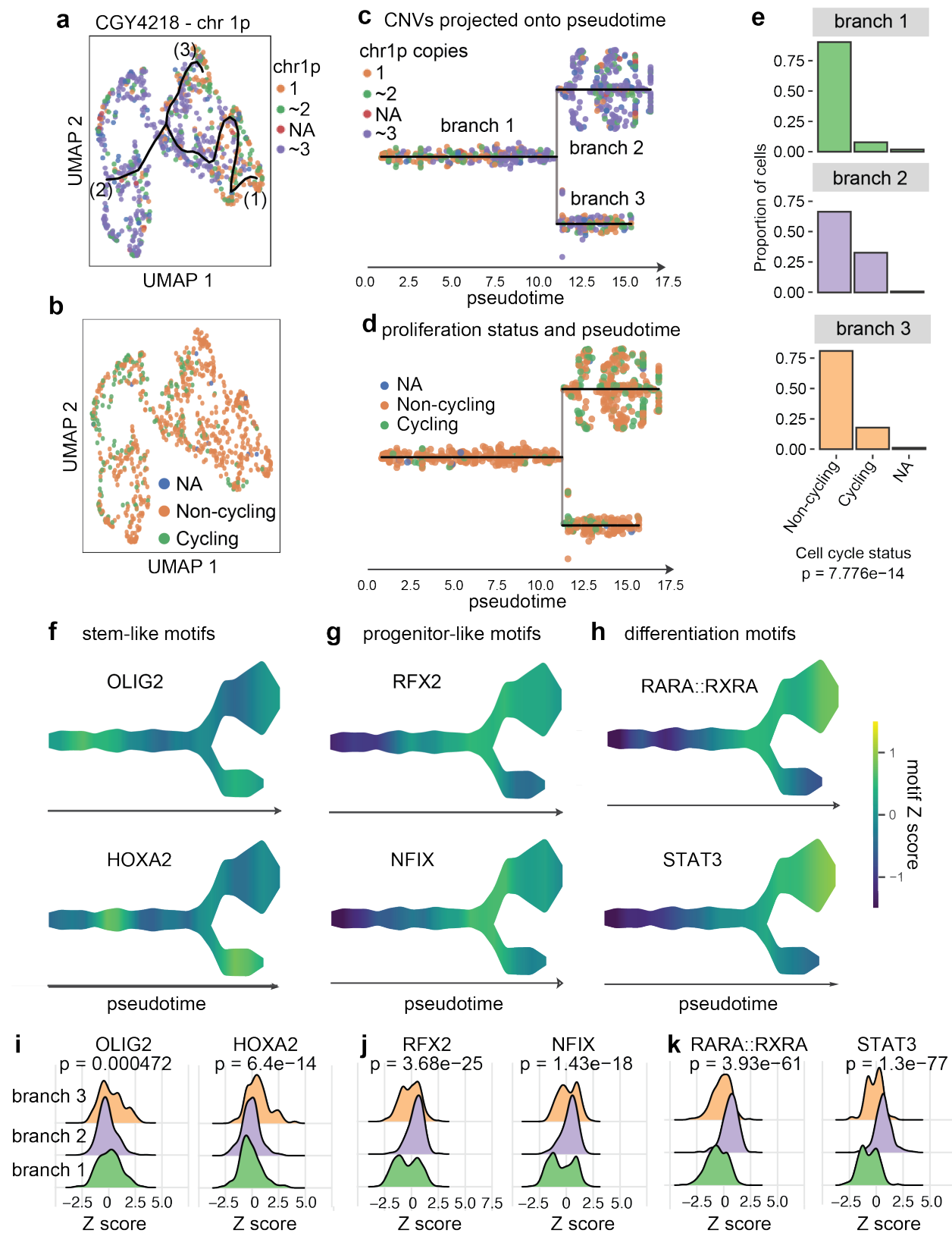


Figure 4. Subclonal genetic alterations predispose cells to adopt developmental chromatin states.

(a) Cells were clustered based on scATAC ChromVAR motif scores, then shaded based on the presence of 1, 2 or 3 copies of chromosome 1P.

(b) Cells were shaded based on their predicted cycling properties.

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- 272 **(a)** Data shown in **(a)** projected onto pseudotime. The resulting three branches are populated preferentially
273 by cells with gain or loss of chromosome 1P respectively.
- 274 **(d)** Proliferation status as shown in **(b)**, overlaid onto pseudotime.
- 275 **(e)** Branches enriched for 1P gain show greater proportions of proliferative cells (statistics: Chi-squared test).
- 276 **(f)** Scaled chromatin accessibility at binding motifs for OLIG2 and HOXA2, two TFs associated with
277 stemness.
- 278 **(g)** Scaled chromatin accessibility at binding motifs for RFX2 and NFIX, two TFs associated with
279 progenitor-like phenotypes.
- 280 **(h)** Scaled chromatin accessibility at binding motifs for RARA::RXRA and STAT3, two TFs associated with
281 differentiated phenotypes.
- 282 **(i)** Enrichment plot for motif Z scores for OLIG2 and HOXA2.
- 283 **(j)** Enrichment plot for motif Z scores for RFX and NFIX.
- 284 **(k)** Enrichment plot for motif Z scores for RARA::RXRA and STAT3. P values calculated by Kruskal-Wallis
285 test.
- 286

287 **DISCUSSION**

288 Here we describe Copy-scAT, the first computational tool dedicated to inferring CNVs using scATAC data.
289 Copy-scAT resolves a computational bottleneck that has restricted the application of single-cell epigenomic
290 techniques to the study of clinical tumor samples, which are often mixtures of malignant and non-malignant
291 cells. The presence of non-malignant cells can severely confound the analyses of these samples and
292 downstream data interpretation. Cell admixture is a particular problem for scATAC data because of the
293 inherent sparsity of these datasets and because they do not provide direct information on the expression
294 status of cell lineage markers that could be used to solve cellular identities. Because most tumor types harbor
295 CNVs, Copy-scAT provides a simple way of solving this problem.

296 It is important to note that Copy-scAT enables users to perform analyses on both malignant and non-
297 malignant cells from a tumor sample, because cell barcodes associated with both presence or absence of
298 CNVs can be selected for downstream analyses. Implementation of Copy-scAT will therefore be beneficial
299 to groups interested in defining the epigenomes of both tumor cells and their microenvironment. Because
300 chromatin accessibility datasets provide information on mechanisms of transcriptional regulation by distal
301 and proximal enhancer and super enhancer elements, Copy-scAT could be useful in clarifying epigenetic
302 mechanisms involved in immune suppression and T cell exhaustion, for instance. Copy-scAT also allows
303 scATAC studies of frozen banked cancer specimens (see Methods), because it requires no prior knowledge
304 of cell composition.

305 We show that the underlying CNV architecture plays a significant role in clustering of scATAC data, a
306 problem that is amplified by the use of TF-IDF algorithms for normalization. These effects are less
307 pronounced when clustering is based on motif activity scores (e.g. ChromVAR), likely as this incorporates
308 data from multiple chromosomes, thus dampening the effect of variation at any one specific locus. Further
309 studies are needed to identify the optimal way to address the effects of CNVs in downstream analyses, as
310 they may present a significant confounder and potentially mask significant biological relationships.

311 In this report, we provide evidence that Copy-scAT can be used to shed new light on how genetics and
312 epigenetics interface in cancer. We show that genetic subclones tend to have unique chromatin accessibility
313 landscapes that can promote or antagonize stem-like phenotypes. Consequently, we report that some genetic
314 subclones have greater proportions of stem-like cells, and others appear more differentiated. These results
315 offer a radically different view of functional hierarchies in GBM, where stem-like properties were thought to
316 be programmed by epigenetic factors, independently of genotype. These findings provide a simple
317 explanation for the observed intra-tumoral transcriptional heterogeneity in GBM ((5, 16)), by suggesting that
318 each genetic subclone achieves specific chromatin accessibility profiles, which in turn result in subclone-
319 specific transcriptional outcomes.

320 Copy-scAT will enable future studies of subclonal chromatin dynamics in complex tumor types and may be
321 an important tool in better understanding the functional relationships between subclones, their
322 microenvironment and therapy response.

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325 **MATERIALS AND METHODS**

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327 **Ethics and consent statement**

328 All samples were collected and used for research with appropriate informed consent and with approval by
329 the Health Research Ethics Board of Alberta.

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331 **scATAC-seq sample processing**

332 GBM samples were either frozen surgical resections (pediatric GBM) or cells dissociated from fresh surgical
333 specimens and cryopreserved (adult GBM). Samples were dissociated in a 1.5 mL microcentrifuge tube, using
334 a wide-bore P1000 pipette followed by a narrow bore P1000 pipette in nuclear resuspension buffer (10 mM
335 Tris-HCl; 10 mM NaCl; 3 mM MgCl₂; 0.1% IGEPAL, 0.1% Tween-20, 0.01% Digitonin, 1% BSA in PBS),
336 then vortexed briefly, chilled on ice for 10 minutes, then pipetted again, and spun at 4°C, 500 g for 5 minutes.
337 This step was repeated, and the sample was then resuspended in Tween wash buffer (10 mM Tris-HCl; 10
338 mM NaCl; 3 mM MgCl₂; 0.1% IGEPAL, 0.1% Tween-20; 1% BSA in PBS), then strained through a 35 µm
339 cell strainer FACS tube (Fisher Scientific 08-771-23) to remove debris. Nuclei were then quantified by trypan
340 blue on the Countess II (Invitrogen), spun down at 500 g at 4°C for 5 minutes, resuspended in the nuclear
341 isolation buffer (10X Genomics), and the rest of the scATAC was performed as per the 10X Genomics
342 protocol. MM samples were from bone marrow aspirates collected from patients; tumor cells were isolated
343 from mononuclear cell fractions through Ficoll gradients coupled with magnetic bead sorting of CD138⁺
344 cells. scATAC libraries were prepared from GBM and MM samples using a Chromium controller
345 (10xGenomics). Libraries were sequenced on NextSeq 500 or Novaseq 6000 instruments (Illumina) at the
346 Centre for Health Genomics and Informatics (CHGI; University of Calgary) using the recommended
347 settings.

348

349 **scATAC-seq initial data analysis**

350 The raw sequencing data was demultiplexed using cellranger-atac mkfastq (Cell Ranger ATAC, version 1.1.0,
351 10x Genomics). Single cell ATAC-seq reads were aligned to the hg38 reference genome (GRCh38, version
352 1.1.0, 10x Genomics) and quantified using cellranger-atac count function with default parameters (Cell
353 Ranger ATAC, version 1.1.0, 10x Genomics).

354

355 **Single-cell CNV analysis**

356 *Fragment pileup and normalization*

357 The fragment file was processed and signal was binned into bins of a preset size (default 1 Mb) across the
358 hg38 chromosomes to generate a genome-wide read-depth map. Only barcodes with a minimum of 5000
359 reads were retained, in order to remove spurious barcodes. This flattened barcode-fragment matrix pileup
360 was cleaned by removal of genomic intervals which were uninformative (greater than 80% zeros) and
361 barcodes with greater than a certain number of zero intervals. Cells passing this first filter were normalized
362 with counts-per-million normalization using *cpm* in the *edgeR* package (17).

363

364 *Chromosome arm CNV analysis*

365 The normalized barcode-fragment matrix was collapsed to the chromosome arm level, using chromosome
366 arm information from the UCSC (UCSC table: cytoBand), centromeres were removed, and signal in each
367 bin was normalized using the number of basepairs in CpG islands in the interval using the UCSC CpG islands
368 table (UCSC table: cpgIslandExtUnmasked). The signal was then summarized using a quantile-trimmed-
369 mean (between the 50th and 80th quantiles). Only chromosome arms with a minimum trimmed mean signal
370 were kept for analysis.

371 The chromosome arm signal matrix is mixed with a generated set proportion of pseudodiploid control cells,
372 defined using the mean of chromosome segment medians with a defined standard deviation. This cell-signal
373 matrix is then scaled across each chromosome arm and centered on the median signal of all chromosomes.
374 Each chromosome arm segment is then analyzed using Gaussian decomposition with Mclust (18). The
375 subsequent clusters are filtered based on Z scores and mixing proportions, and redundant clusters are
376 combined. These Z scores are then translated into estimated copy numbers for each segment for each

377 barcode. The barcode CNV assignments can be optionally used to assign consensus CNVs to clusters
378 generated in other software packages such as Loupe or Seurat/Signac.

379

380 *Detection of amplifications*

381 The normalized barcode-fragment matrix was scaled and mean-variance changepoint analysis using the
382 Changepoint package was performed for each cell and each chromosome to identify areas of abnormally
383 high signal (Z score greater than 5) (19). The consensus coordinates of each amplification region were
384 generated across all cells and only abnormalities affecting a minimum number of cells were kept for analysis.

385

386 *Detection of loss of heterozygosity*

387 The normalized barcode-fragment matrix was scaled as above. As overall coverage levels in these samples
388 are quite sparse, a chromosome-wide coverage profile was generated for the entire sample in bulk, using the
389 30% quantile as a cut-off, and then changepoint analysis was used to find inflection points. This was followed
390 by Gaussian decomposition of the values using Mclust to identify putative areas of loss or gain, thresholded
391 by a minimum difference in signal between the clusters identified by Mclust.

392

393 **scATAC trajectory analysis**

394 STREAM-ATAC and STREAM (20) were used to generate pseudotime trajectories based on motif
395 occupancy profiles generated using ChromVAR (21) with the JASPAR 2018 motif database as reference (22).
396 Dimensionality reduction was performed using the top 20 components and 50 neighbours, and an initial
397 elastic graph was generated on the 2D UMAP projection using 10 clusters, using the kmeans method with
398 $n_neighbours = 30$. An elastic principal graph was constructed using the parameters $epg_alpha = 0.02$,
399 $epg_mu = 0.05$, $epg_lambda = 0.02$ and $epg_trimmingradius = 1.2$, with branch extension using
400 ‘QuantDists’. Trees were rooted using the branch with highest motif activities for OLIG2 and ETV motifs
401 as root.

402

403 **Whole genome sequencing**

404 DNA was extracted from residual nuclei from the same samples and tissue fragments used for scATAC-seq
405 of adult GBM samples, using the Qiagen DNEasy Blood and Tissue DNA extraction kit (Qiagen # 69504).
406 Libraries were prepared using the NEBNext Ultra II DNA Library Prep Kit (#E7645) and sequenced on
407 the Novaseq 6000 (Illumina) at the CHGI (University of Calgary), in paired-end mode.

408

409 **Whole genome data processing**

410 Genome data was aligned to the hg38 assembly using bwa mem (bwa 0.7.17)(23). Samtools was used to
411 extract high-quality reads ($Q > 30$) and picard tools (Broad Institute) was used to remove duplicates (24).

412

413 **Whole genome SNV and CNV detection**

414 Gatk mutect2 (Broad Institute) was run on the filtered data to detect SNVs with low stringency using the
415 following settings: `--disable-read-filter MateOnSameContigOrNoMappedMateReadFilter`. CNVkit was subsequently
416 used to call copy number variants using the following parameters: `--filter cn -m clonal -purity 0.7` (25). Adjacent
417 segments were further combined and averaged using bedtools (26).

418 Data visualization and clustering

419 Data was visualized and UMAP plots were generated using Seurat 3.0.0 and Signac 1.0.0 (27) and Cell Loupe
420 version 4.0.0 (28).

421

422 Statistical analysis

423 Between-group differences in discrete values (e.g. chromosome peaks, branch assignments) were calculated
424 using the Chi-squared test. Differences in non-parametric distributions (motif accessibility in clusters) were
425 quantified using the Kruskal-Wallis test.

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427

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ACKNOWLEDGMENTS

535 **Funding:** A Canada Research Chair in Brain Cancer Epigenomics (tier 2) from the Government of Canada,
536 Project grants from the Canadian Institutes of Health Research (CIHR; PJT-156278, PJT-173475), a
537 Discovery grant from the Natural Sciences and Engineering Research Council (NSERC) and an Azrieli
538 Future Leader in Canadian Brain Research grant to MG; a Clinician Investigator Program fellowship from
539 Alberta Health Services and a fellowship from Alberta Innovates to AN; an Eyes High Scholarship from the
540 University of Calgary to DS; a Clark Smith postdoctoral fellowship and a CIHR postdoctoral fellowship to
541 MJ; a Canada Research Char in Precision Oncology (tier 2) and a CIHR grant (PJT-438802) to SM; an Alberta
542 Graduate Excellence Scholarship and Alberta Innovates scholarship to AG. This project has been made
543 possible by the Brain Canada Foundation through the Canada Brain Research Fund, with the financial
544 support of Health Canada and the Azrieli Foundation.

545

546 **Author contributions:** Conception and experimental design: AN, MG. Generation of datasets: AN, KE,
547 JC, PN, NB. Data acquisition and analysis: AN, DS, MJ, AG, SM, NB, MG. Data interpretation and creation
548 of new software: AN, MG. Manuscript preparation: All co-authors.

549

550 **Competing interests:** The authors declare no competing interests.

551

552 **Data and materials availability:** The Copy-scAT package and a sample tutorial are available on Github at
553 <http://github.com/spcdot/CopyscAT>. All datasets will be made available upon publication in a peer
554 reviewed journal.

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600 **SUPPLEMENTAL MATERIAL**

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Table S1. Summary of samples and cells profiled by Copy-scAT

Sample	Unique barcodes after pileup	Unique barcodes after filtering	Percent passing filters
CGY4218	1542	1335	86.58%
CGY4250	1371	947	69.07%
CGY4275	1004	609	60.66%
CGY4349	961	756	78.67%
pCGY2932	1203	802	66.67%
pCGY2937	1445	1200	83.04%
pCGY3103	2189	956	43.67%
pCGY3402	3162	2318	73.31%
pCGY3749	2774	2503	90.23%
pCGY4021	1963	1382	70.40%
MM1217	890	792	88.99%
MM1388	2538	2219	87.43%
MM1389	7438	6607	88.83%
MM1438	1774	1578	88.95%
MM1460	2048	1683	82.18%
MM1479	5135	4564	88.88%
MM1498	7408	2160	29.16%
MM1555	7220	6586	91.22%
MM1643	3141	2794	88.95%
MM1698	2844	2283	80.27%
Total cells profiled	58050	44074	76.86%

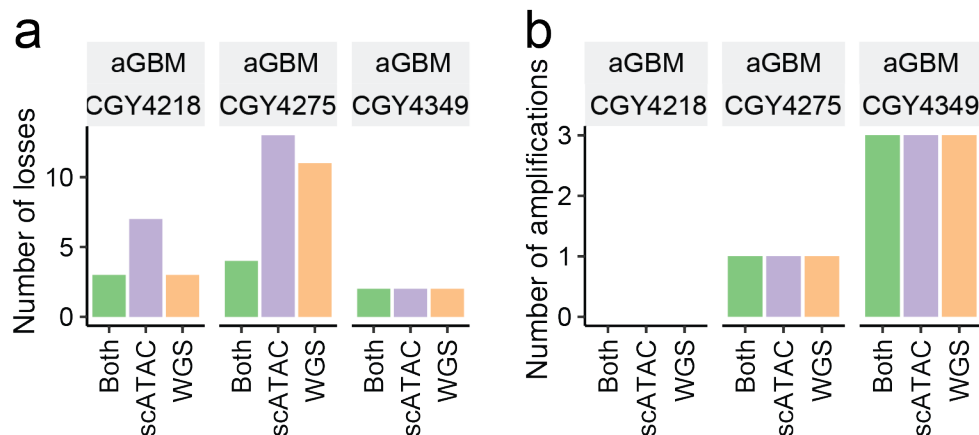
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623 **Table S2. Sensitivity and Specificity of Copy-scAT in aGBM, pGBM and MM samples**
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Samples	Gains		Losses		Amplifications	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
aGBM (n = 3)	1.0	0.94	0.79	0.89	1.0	1.0
pGBM (n= 6)	0.73	0.93	0.73	0.95	N/A	0.975
MM (n = 10)	0.51	0.94	0.67	0.89	N/A	N/A

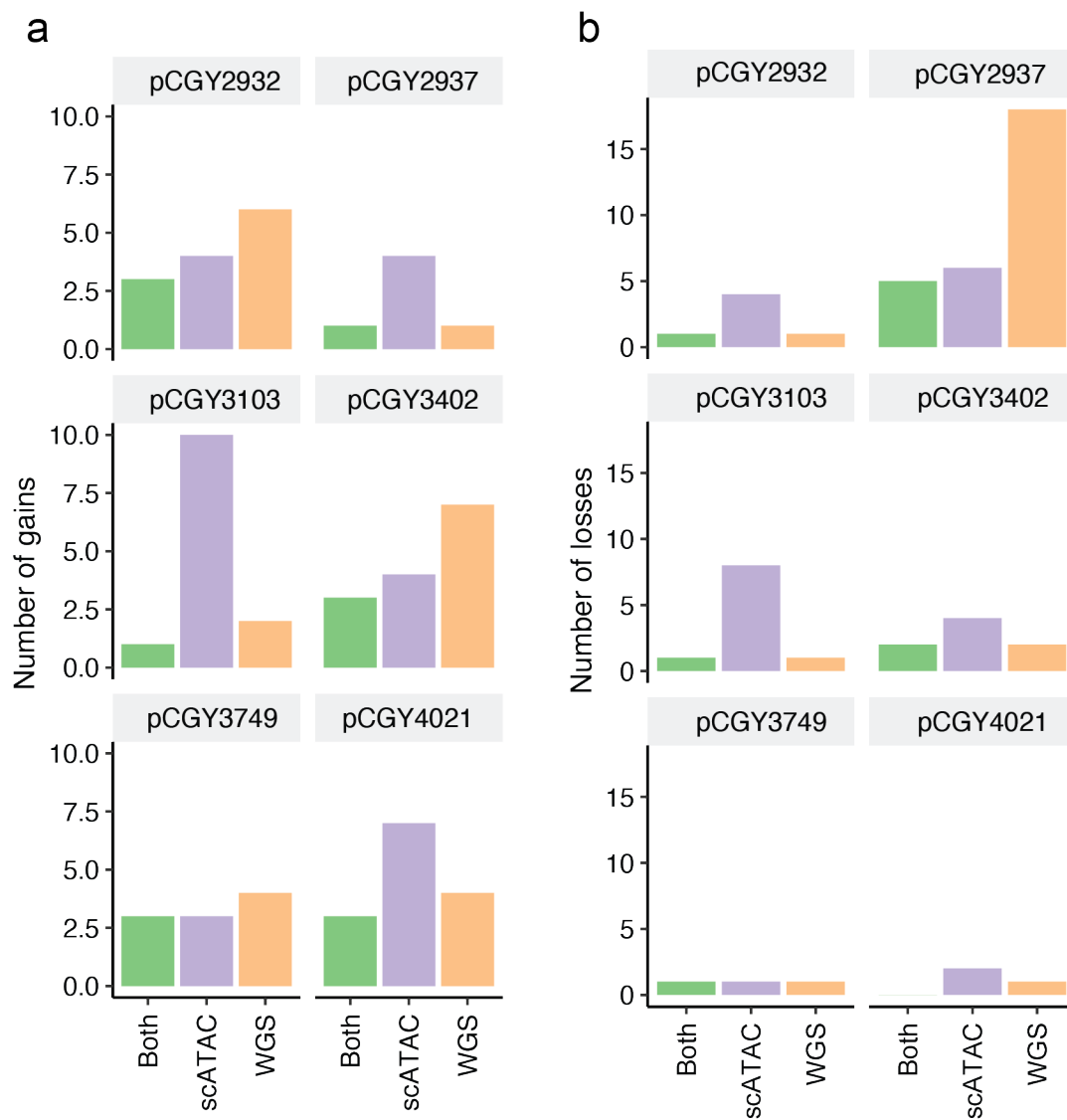
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668 **Fig. S1. Comparison of CNVs inferred by Copy-scAT and by WGS for adult GBM samples.**
669 **(A)** Comparison of chromosome arm level losses detected in three adult GBM samples by single cell
670 ATAC, WGS, or both methods.
671 **(B)** Comparison of focal amplifications detected in three adult GBM sample by scATAC, WGS, or both
672 methods.
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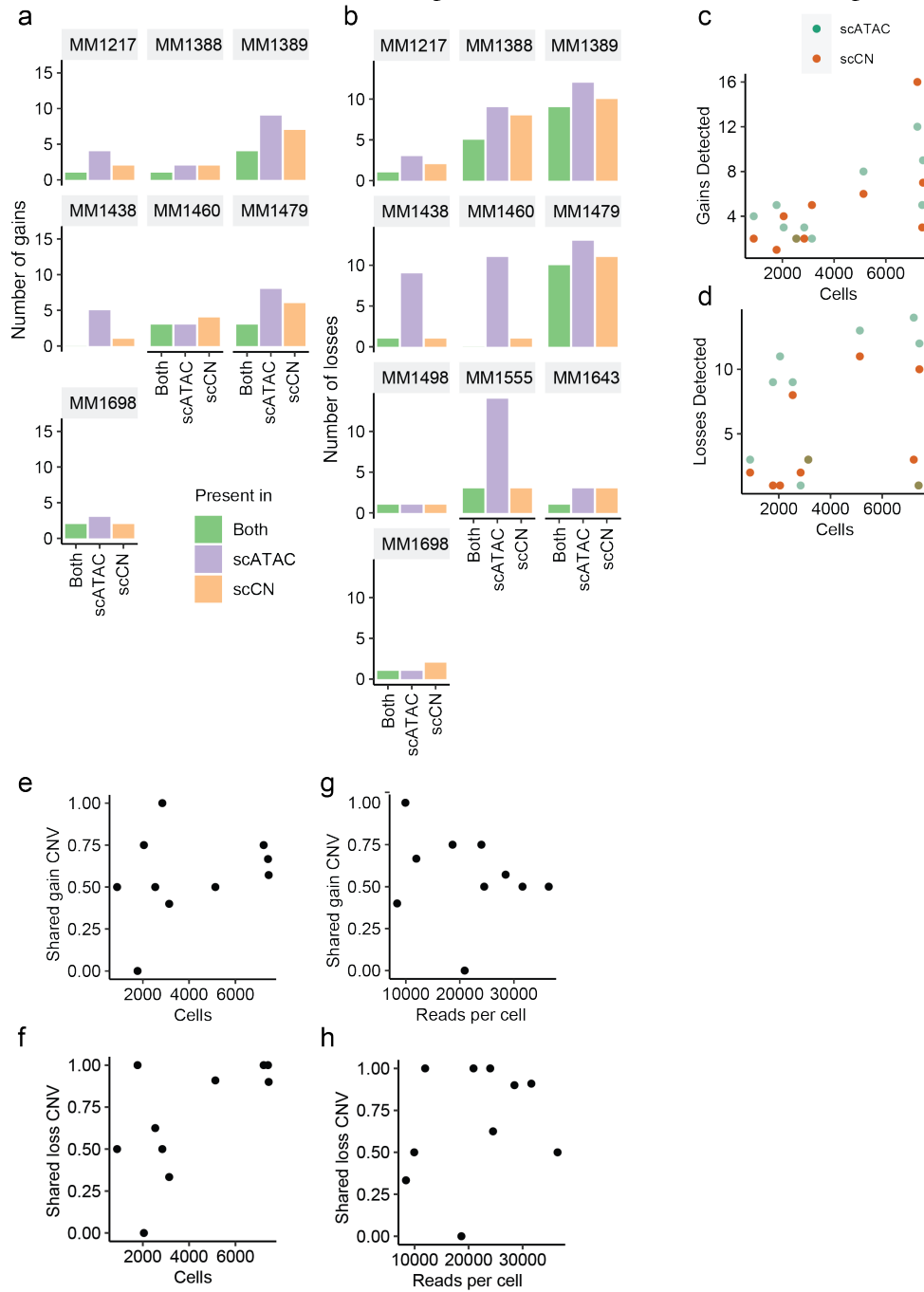
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707 **Fig. S2. Comparison of CNVs inferred by Copy-scAT or WGS in pediatric GBM samples.**
708 **(a) Gains detected in three pediatric GBM samples compared to linked-reads WGS.**
709 **(b) Losses detected in three pediatric GBM samples compared to linked-reads WGS.**
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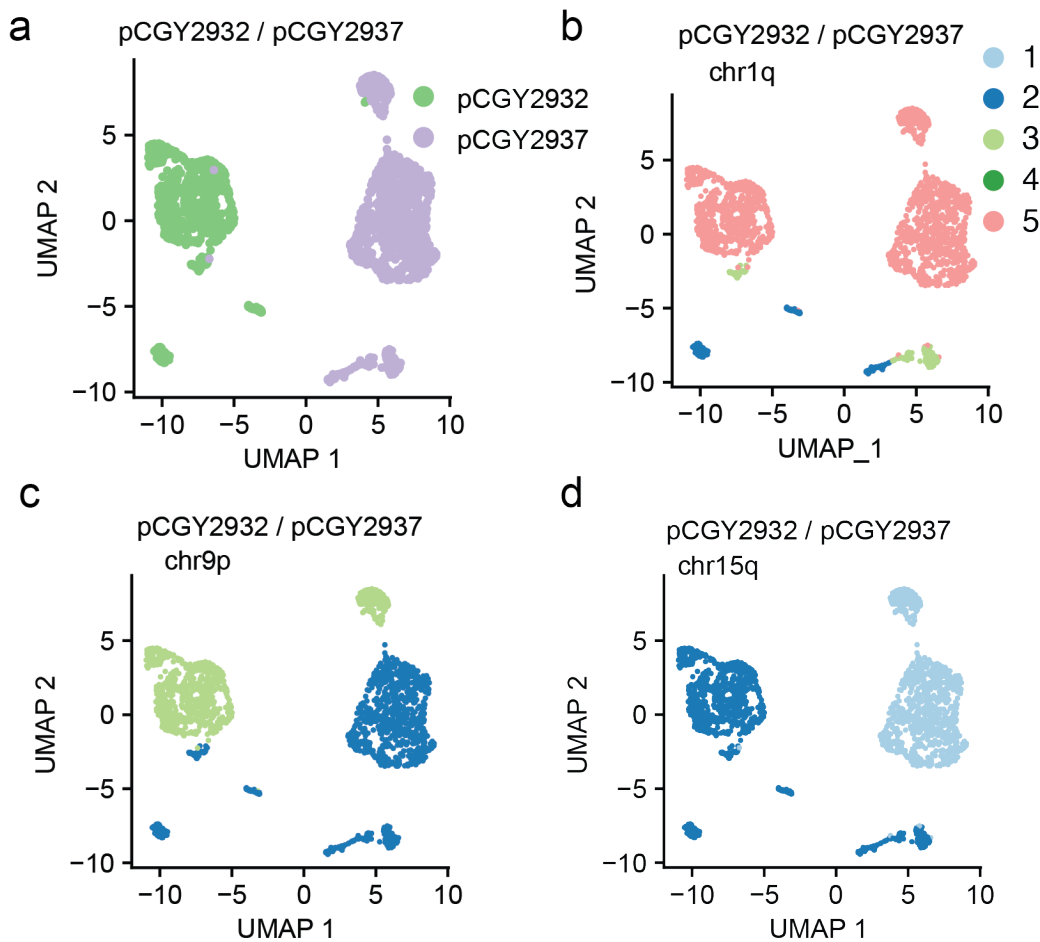
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727 **Fig. S3. Comparison of CNVs inferred by Copy-scAT or with the scCNV assay in multiple**
 728 **myeloma samples.**
 729 **(a)** Comparison of gains seen in additional myeloma samples versus 10x single-cell CNV sequencing. **(b)**
 730 Comparison of chromosome losses seen in additional myeloma samples versus 10x single-cell CNV
 731 sequencing. **(c,d)** Number of gains and losses detected by both methods compared to number of cells in
 732 scATAC-seq sample. **(e-f)** Number of shared gains or losses detected between the two methods, plotted
 733 versus the number of cells in the scATAC-seq experiment. **(g-h)** Number of shared gains or losses
 734 detected between the two methods, plotted versus the number of reads per cell in the scATAC.



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740 **Fig. S4. CNVs are detected in scATAC clusters with Copy-scAT in pediatric GBM samples.**
741 **(a)** Overview of cell assignments in two paired patient libraries.
742 **(b-d)** Representative WGS-confirmed alterations detected in pCGY2932 and pCGY2937.
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764 **Fig. S5. CNVs are identified by Copy-scAT in specific scATAC clusters in multiple myeloma**
765 **samples. (a) Gain of chromosome 11p restricted to neoplastic cell populations. (b) Similar pattern with**
766 **gain of chromosome 11q. (c) Similar pattern with loss of chr13q.**
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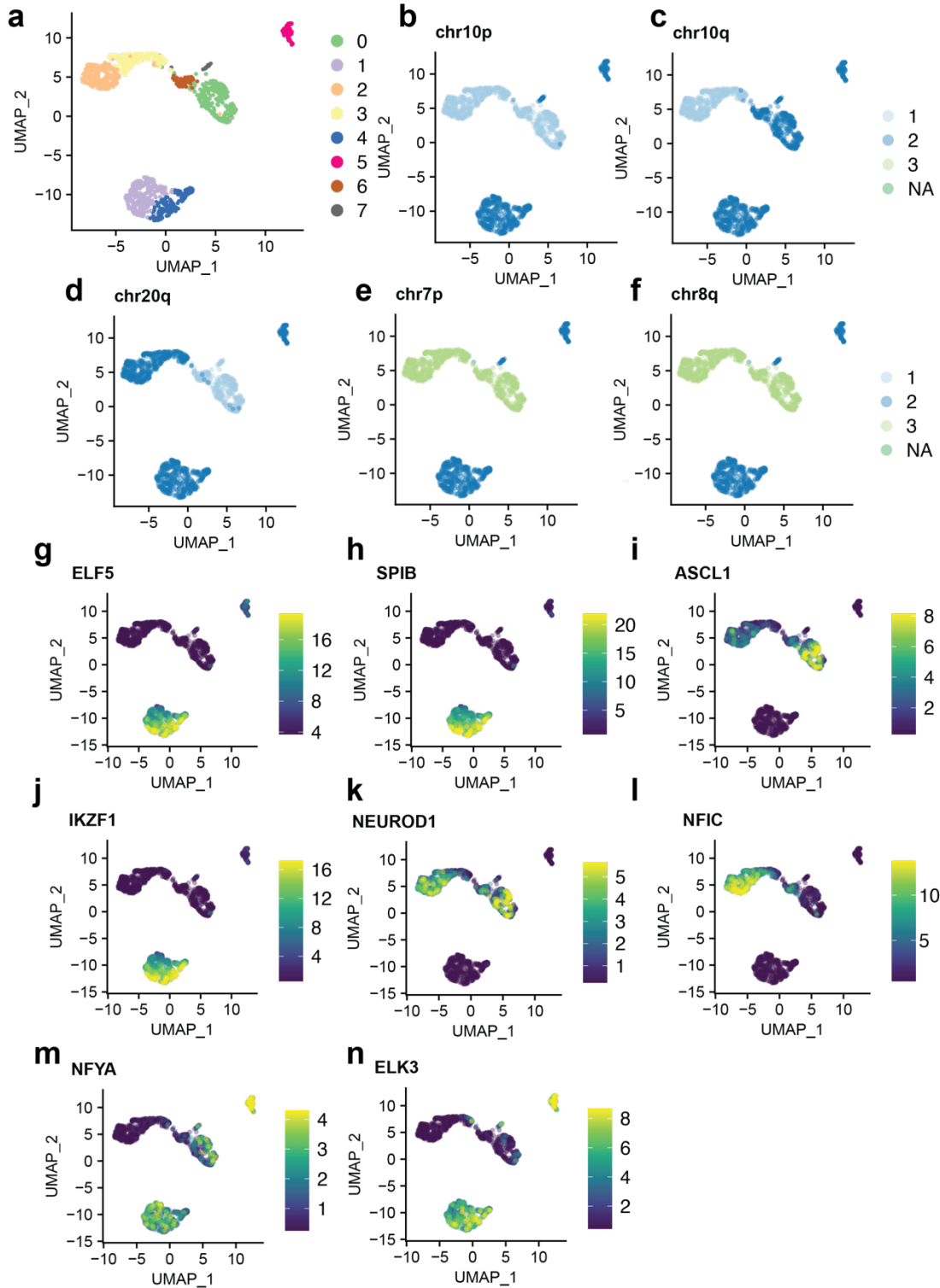
788 **Fig. S6. Additional chromosome copy number analyses for CGY4218.**

789 **(a)** Initial neighbourhood clustering results from Signac.

790 **(b-f)** Representative chromosome-level copy number alteration profiles for tumour and normal cells. **(g-n)**

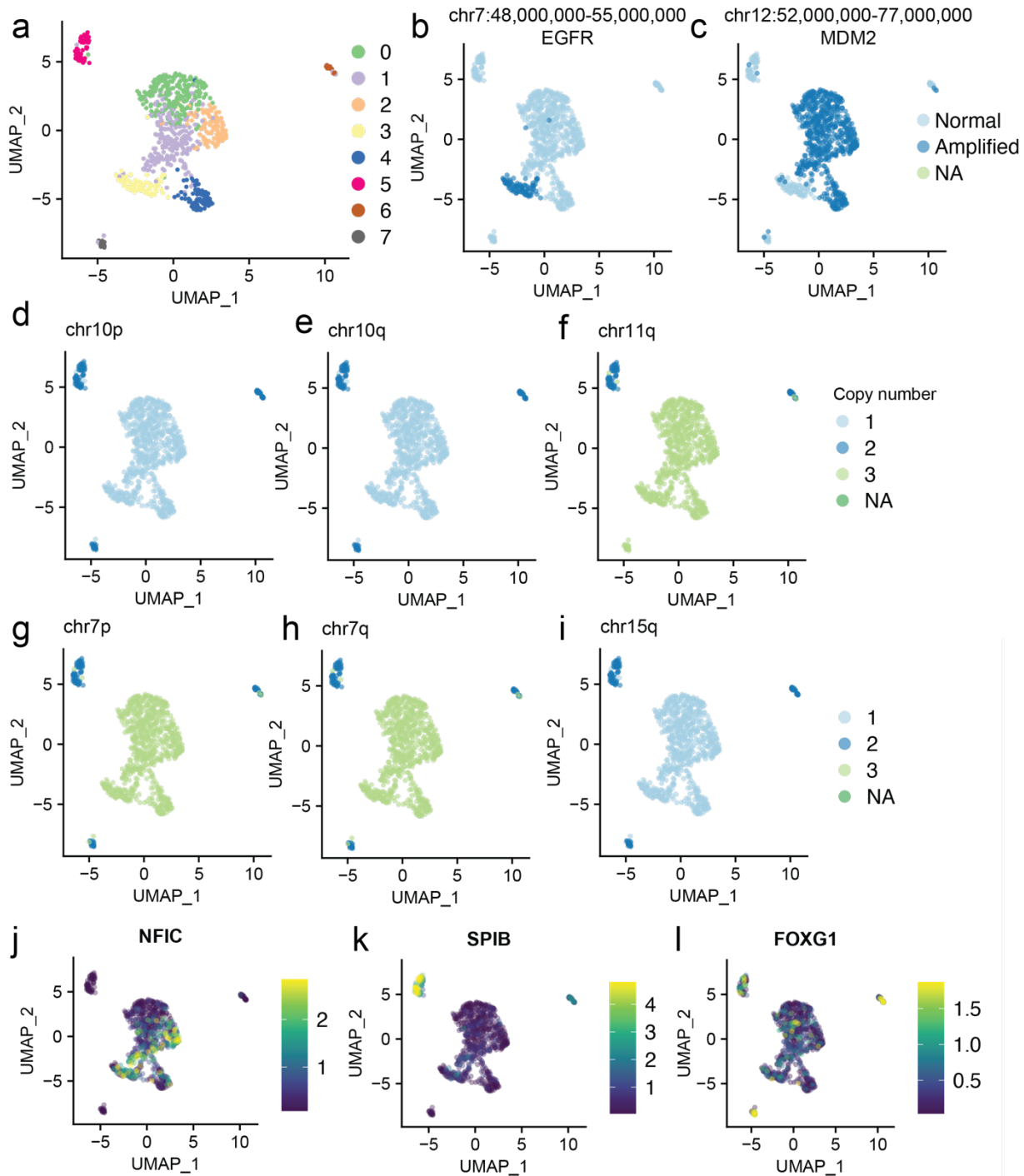
791 Representative motif scores from ChromVAR for different motifs, including **(g)** ELF5, **(h)** SPIB, **(i)**

792 **ASCL1**, **(j)** IKZF1, **(k)** NEUROD1, **(l)** NFIC, **(m)** NFYA, **(n)** ELK3.



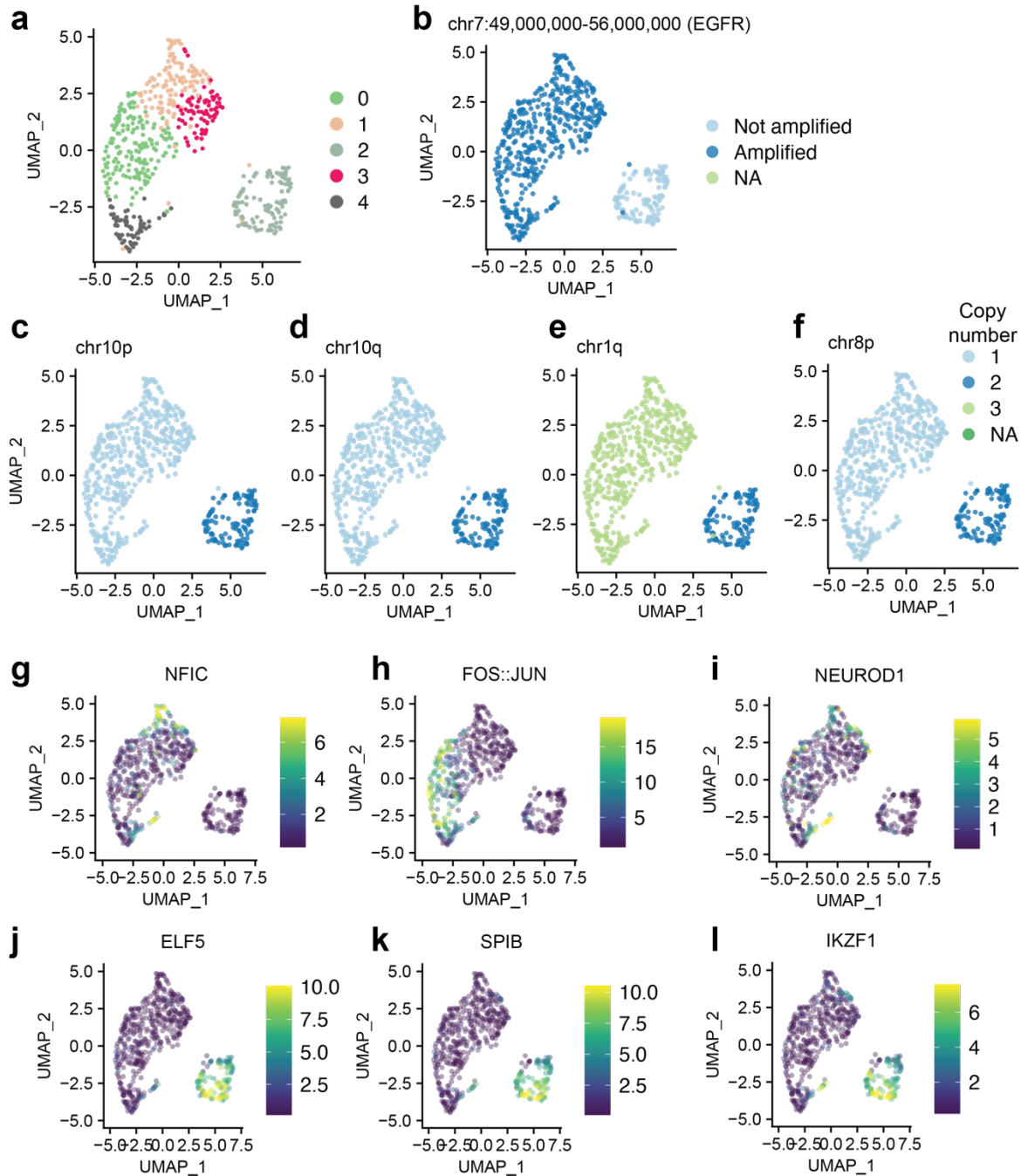
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797 **Fig. S7. Representative copy number information and distribution for aGBM sample CGY4250.**
798 **(a)** Neighbourhood clustering results from Signac.
799 **(b-c)** Distribution of amplifications in *EGFR* and *MDM2*.
800 **(d-i)** Representative chromosome-level copy number alteration profiles for tumour and normal cells. **(j-l)**
801 Representative motif scores from ChromVAR for different motifs, including **(j)** NFIC, **(k)** SPIB and **(l)**
802 FOXG1.



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808 **Fig. S8. Representative copy number information and distribution for aGBM sample CGY4275.**
809 **(a)** Neighbourhood clustering results from Signac.
810 **(b)** Distribution of amplifications in *EGFR*.
811 **(c-j)** Representative chromosome-level copy number alteration profiles for tumour and normal cells. **(g-l)**
812 Representative motif scores from ChromVAR for different motifs, including **(g)** NFIC, **(h)** FOS::JUN, **(i)**
813 NEUROD1, **(j)** ELF5, **(k)** SPIB, and **(l)** IKZF1.
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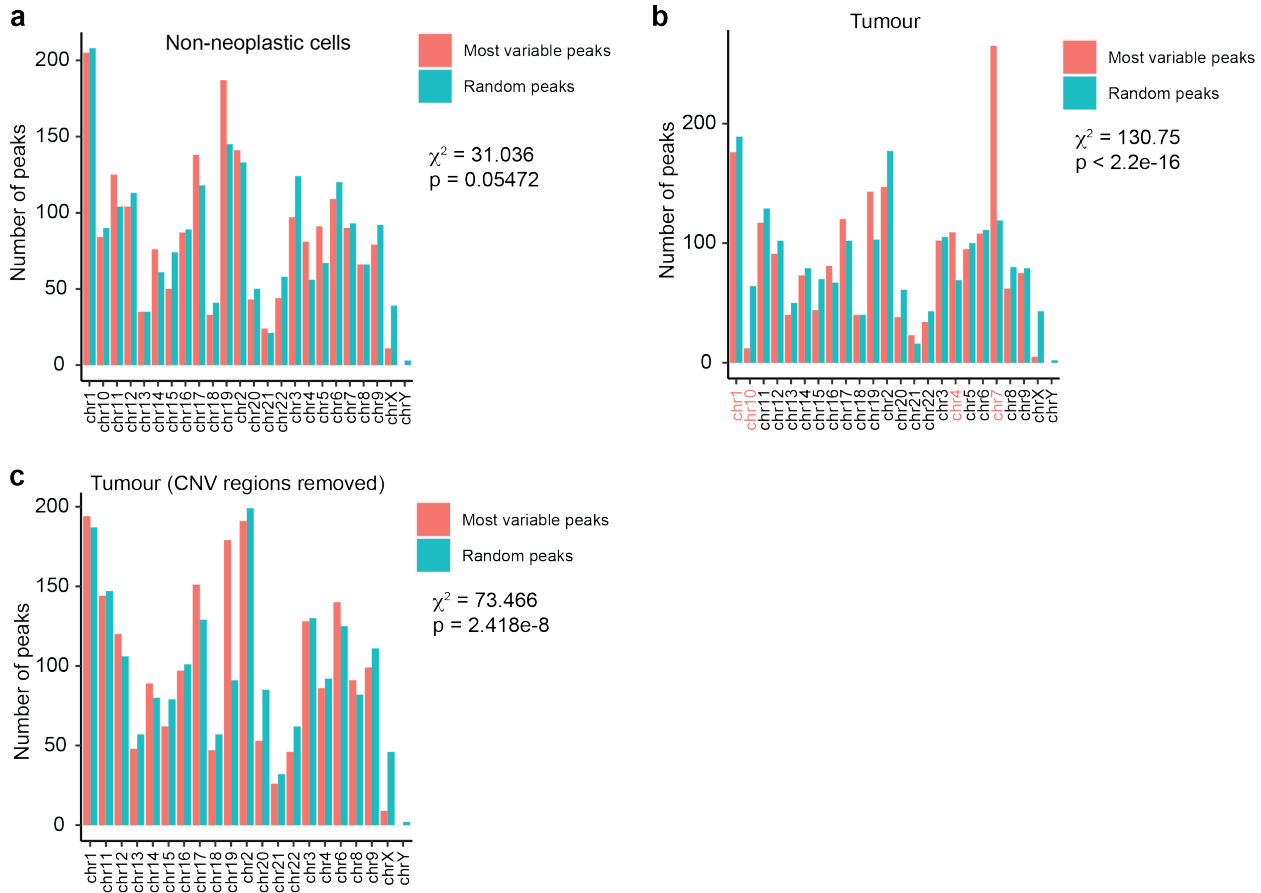
821 **Fig. S9. Effects of removing CNVs on variance in aGBM sample CGY4349.**

822 **(a)** Distribution of the top 2000 most variable peaks in the tumour cells after filtering out non-neoplastic
823 cells; p value from Chi-squared test.

824 **(b)** Distribution of top 2000 most variable peaks in non-neoplastic cells after filtering (P VALUE FROM
825 CHI-SQUARED TEST). Chromosomes with CNVs or amplification regions are highlighted in pink.

826 **(c)** Distribution of top 2000 most variable peaks in tumour cells after filtering of non-neoplastic cells and
827 removal of regions containing CNVs (P VALUE FROM CHI-SQUARED TEST).

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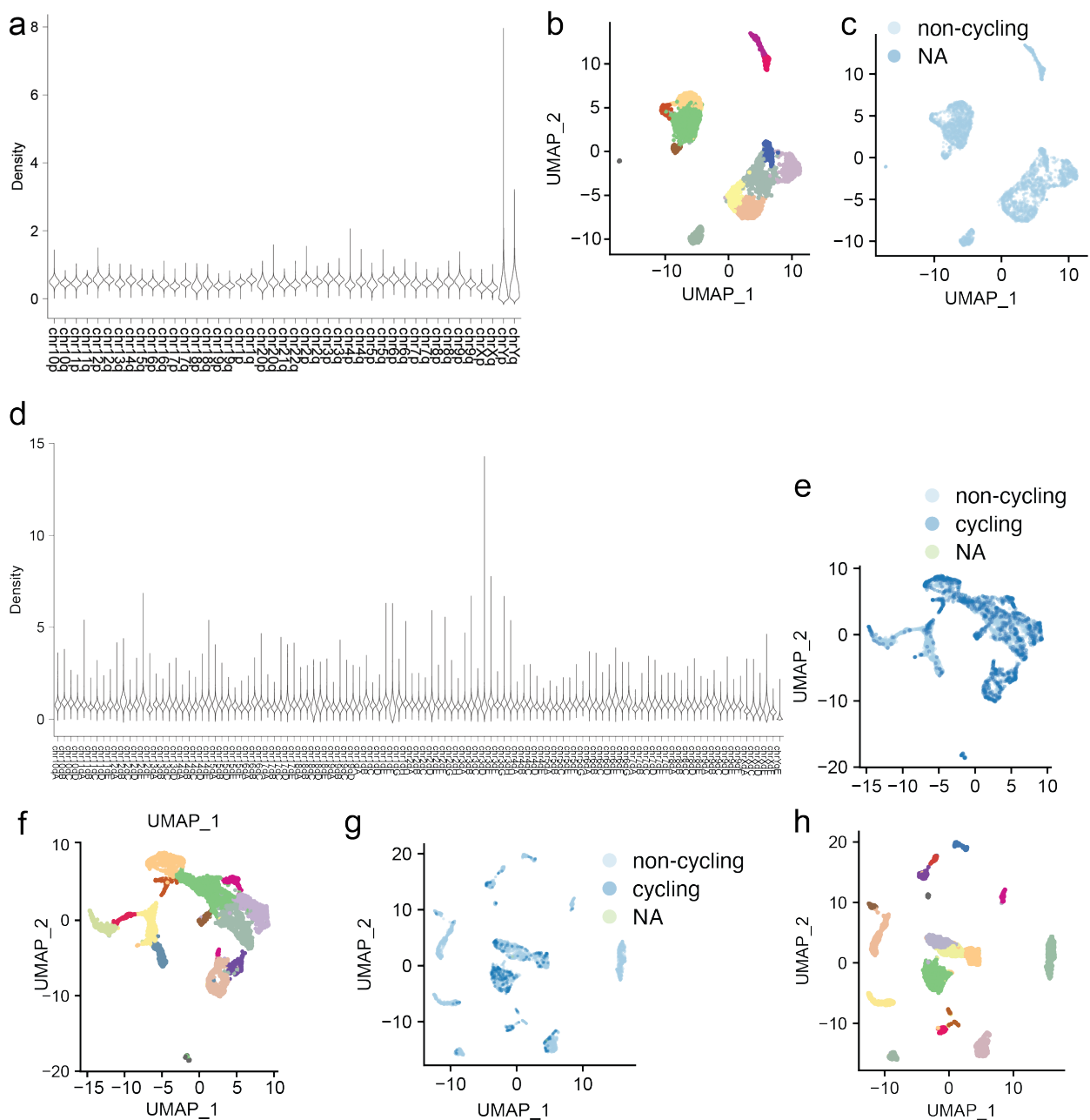
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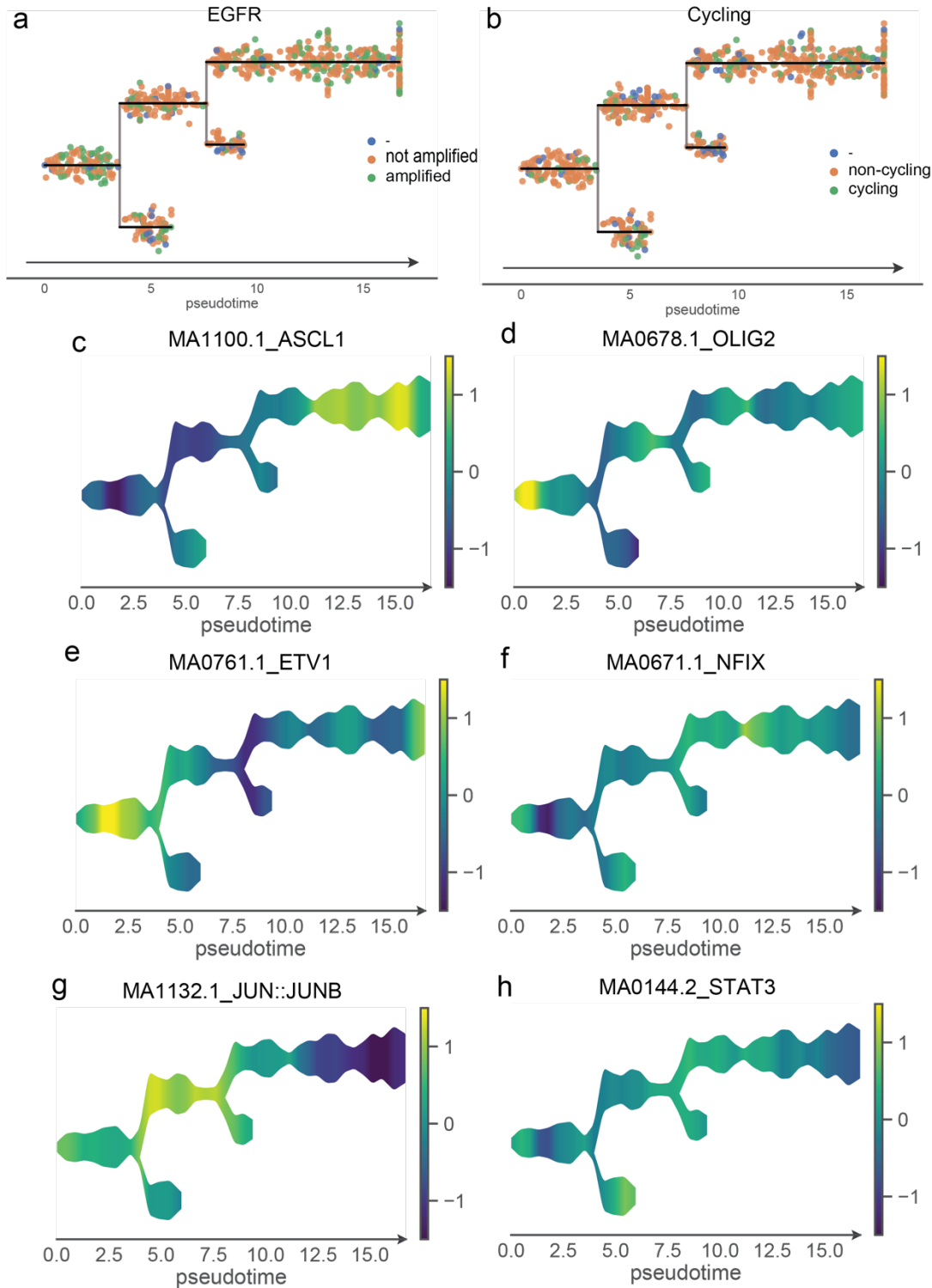
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847 **Fig. S10. Validation of Copy-scAT and identification of putative proliferative cells in non-**
848 **neoplastic datasets. (a)** Chromosome copy number distribution in a 10X dataset of 5000 human PBMCs.
849 **(b)** Seurat clusters for the 10X dataset of 5000 human PBMCs. **(c)** Estimate of cycle status for the 10X
850 dataset of 5000 human PBMCs. **(d)** Chromosome copy number distribution in a 10X dataset of mouse
851 embryonic brain at E18. **(e,f)** Predicted cycle status and cluster assignments in E18 mouse brain. **(g,h)**
852 Predicted cell cycle status and cluster profile in P50 mouse brain dataset from 10X.
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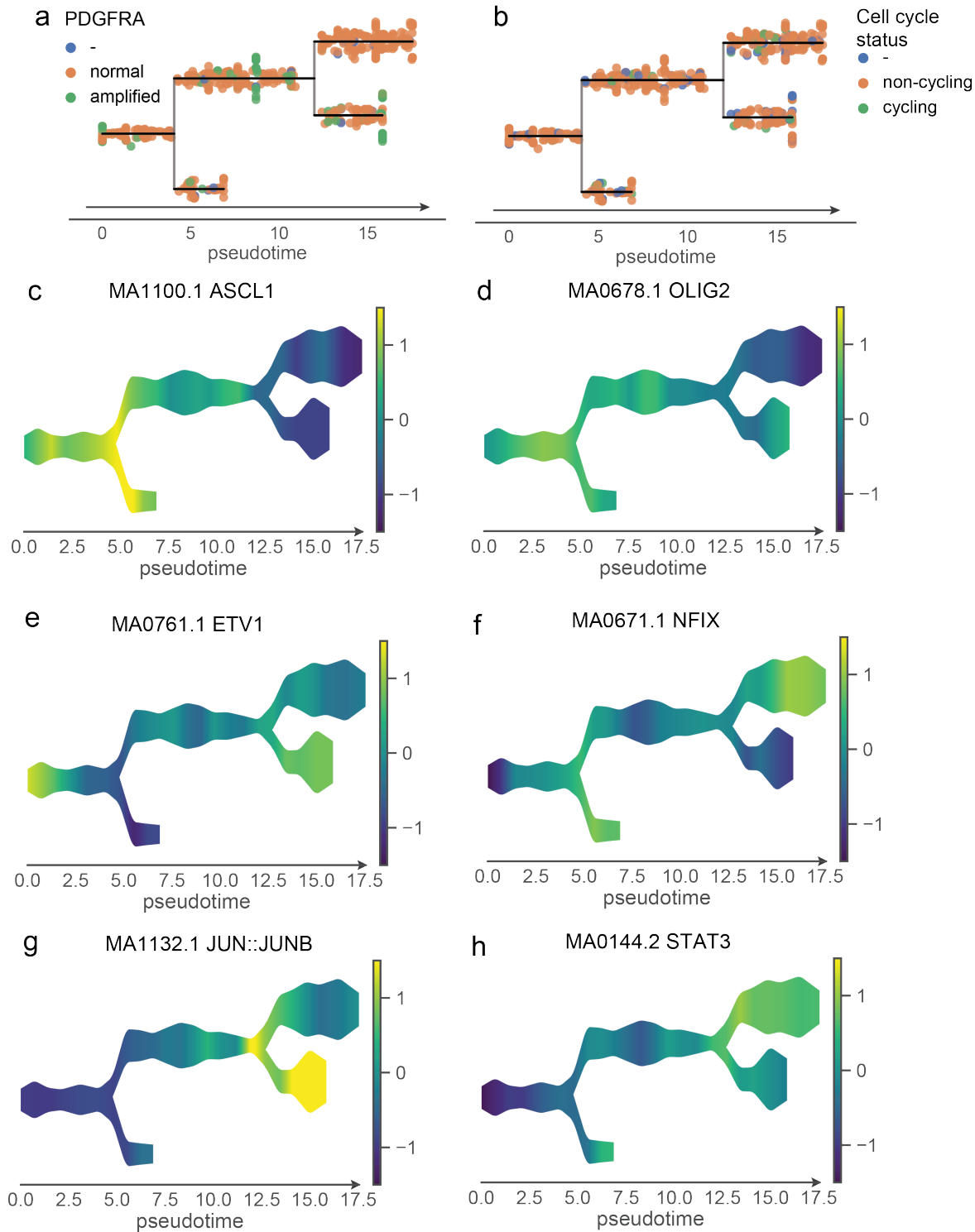
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861 **Fig. S11. Pseudotime trajectory analysis of aGBM sample CGY4250.** Distribution of EGFR
862 amplification **(a)** and cell cycle status **(b)** amongst branches. Distribution of ChromVAR motif scores in
863 branches for proneural motifs ASCL1 and OLIG2 **(c,d)**, ETV1 **(e)**, NFIX **(f)**, and mesenchymal motifs
864 JUN::JUNB **(g)** and STAT3 **(h)**.
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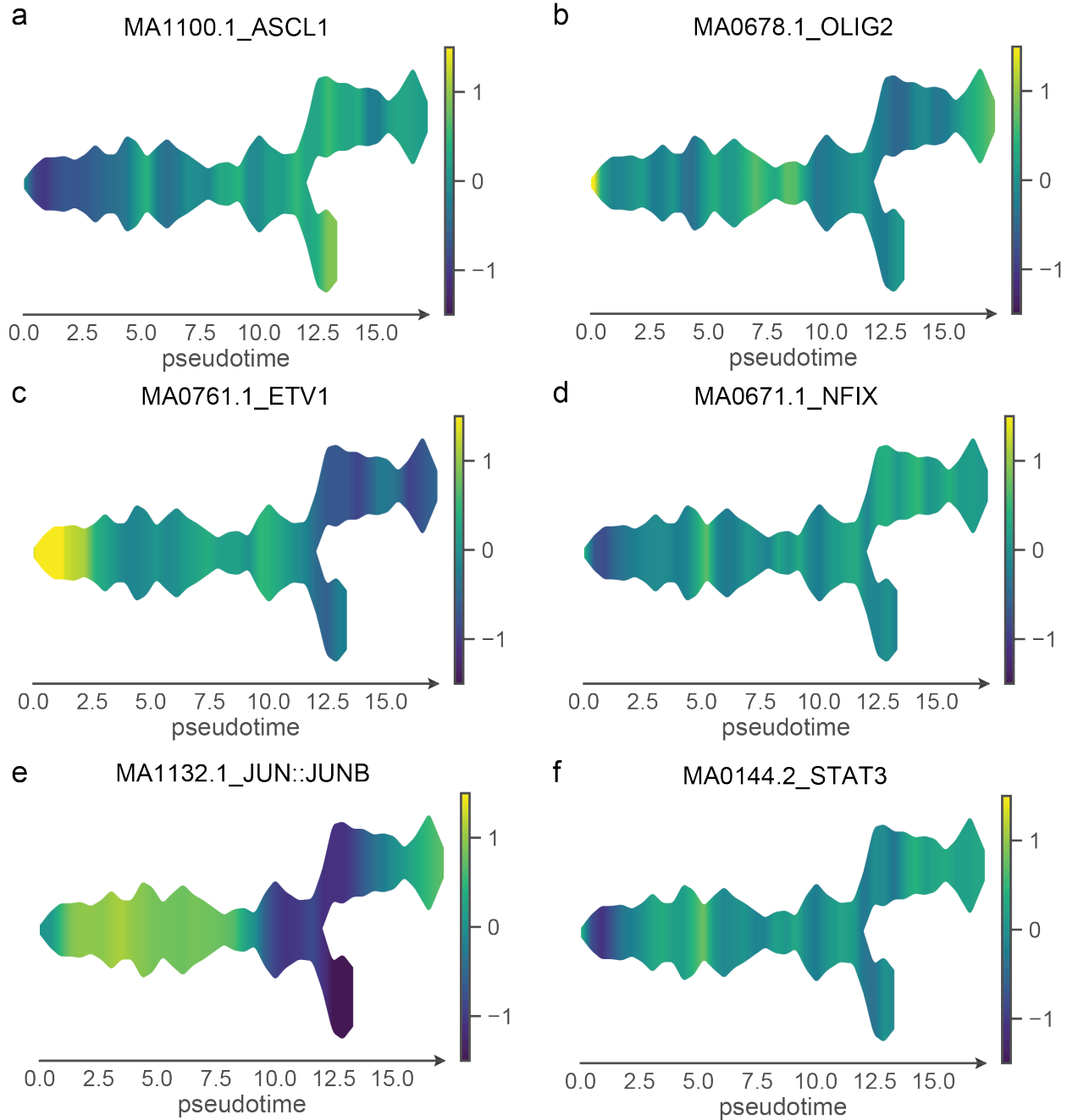
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870 **Fig. S12. Pseudotime trajectory analysis of aGBM sample CGY4349.** Distribution of PDGFRA
871 amplification (a) and cycling status (b) amongst branches. Distribution of ChromVAR motif scores in
872 branches for proneural motifs ASCL1 and OLIG2 (c,d), ETV1 (e), NFIX (f), and mesenchymal motifs
873 JUN::JUNB (g) and STAT3 (h).
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879 **Fig. S13. Pseudotime trajectory analysis of aGBM sample CGY4275.** Distribution of ChromVAR
880 motif scores in branches for proneural motifs ASCL1 and OLIG2 (a,b), ETV1 (c), NFIX (d), and
881 mesenchymal motifs JUN::JUNB (e) and STAT3 (f).
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