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1 Glioma progression is shaped by genetic evolution and microenvironment interactions

2
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76

77 **Summary**

78 To interrogate the factors driving therapy resistance in diffuse glioma, we collected and analyzed
79 RNA and/or DNA sequencing data from temporally separated tumor pairs of 304 adult patients
80 with IDH-wild-type or IDH-mutant glioma. Tumors recurred in distinct manners that were
81 dependent on IDH mutation status and attributable to changes in histological feature composition,
82 somatic alterations, and microenvironment interactions. Hypermutation and acquired *CDKN2A*
83 deletions associated with an increase in proliferating stem-like malignant cells at recurrence in
84 both glioma subtypes, reflecting active tumor growth. IDH-wild-type tumors were more invasive
85 at recurrence, and their malignant cells exhibited increased expression of neuronal signaling
86 programs that reflected a possible role for neuronal interactions in promoting glioma progression.
87 Mesenchymal transition was associated with the presence of a specific myeloid cell state defined
88 by unique ligand-receptor interactions with malignant cells. Collectively, our results uncover
89 recurrence-associated changes that could be targetable to shape disease progression following
90 initial diagnosis.

91

92 **Keywords:** Glioma, glioblastoma, genomics, treatment resistance, microenvironment, single-cell

93

94 **Introduction**

95 Diffuse gliomas in adults are aggressive primary tumors of the central nervous system that are
96 characterized by a poor prognosis and the development of resistance to a treatment regimen that
97 typically includes surgery, alkylating chemotherapy, and radiotherapy (Stupp et al., 2005; Wen et
98 al., 2020). Genomic profiling of diffuse glioma has identified genetic drivers of disease progression
99 and led to the definition of clinically relevant subtypes based on the presence of somatic mutations
100 in the isocitrate dehydrogenase (IDH) genes and co-deletion of chromosome arms 1p and 19q
101 (Cancer Genome Atlas Research et al., 2015; Ceccarelli et al., 2016; Eckel-Passow et al., 2015;
102 Louis et al., 2016; Weller et al., 2015; Yan et al., 2009). Transcriptional profiling of whole tumors

103 and single cells has revealed that the gene expression programs in malignant glioma cells are
104 influenced by underlying somatic alterations and interactions with the tumor microenvironment.
105 Additionally, malignant cells exhibit high plasticity that enables them to respond dynamically to
106 diverse challenges (Johnson et al., 2021; Neftel et al., 2019; Patel et al., 2014; Phillips et al.,
107 2006; Venteicher et al., 2017; Verhaak et al., 2010; Wang et al., 2017). Studies of changes relating
108 to therapy using bulk genomics approaches have revealed mesenchymal transitions and both
109 branching and linear evolutionary patterns (Barthel et al., 2019; Kim et al., 2015a; Kim et al.,
110 2015b; Kocakavuk et al., 2021; Korber et al., 2019; Wang et al., 2016; Wang et al., 2017).
111 However, the extent to which individual malignant glioma and microenvironmental cells interact
112 and evolve over time to facilitate therapy resistance remains poorly understood.

113

114 To identify the drivers of treatment resistance in glioma, we established the Glioma Longitudinal
115 Analysis Consortium (GLASS) (Bakas et al., 2020; Barthel et al., 2019; Consortium, 2018). In our
116 initial effort, we assembled a set of longitudinal whole-exome and whole-genome sequencing data
117 from 222 patients to define the clonal dynamics that allow each glioma subtype to escape therapy.
118 In the current study, we build upon these analyses by integrating this genomic dataset with
119 overlapping and complementary longitudinal transcriptomic data. We apply single-cell-based
120 deconvolution approaches to these data to infer a tumor's physical structure and identify the cell
121 state interactions across IDH-wild-type and IDH-mutant glioma. Collectively, we find that gliomas
122 exhibit several common transcriptional and compositional changes at recurrence that represent
123 promising therapeutic targets for delaying disease progression.

124

125 **Results**

126 **Overview of the GLASS Cohort**

127 We expanded the GLASS cohort with an emphasis on collecting orthogonal RNA sequencing
128 profiles to include data from a total of 381 patients treated across 37 hospitals (**Table S1**). After

129 applying genomic and clinical quality control filters, the resulting dataset included genomic data
130 from a total of 304 patients, with 168 having RNA sequencing data available for at least two time
131 points, 256 having DNA sequencing data available for at least two time points, and 115 having
132 overlapping RNA and DNA available for at least two time points. The cohort of 168 tumors used
133 for RNA sequencing analyses comprised each of the three major glioma subtypes, with 128 IDH
134 wild-type (IDH-wild-type), 31 IDH mutant 1p/19q intact (IDH-mutant-noncode1), and 9 IDH mutant
135 1p/19q co-deleted (IDH-mutant-code1) glioma pairs (**Figure 1A**; **Table S2**). Given the limited
136 number of IDH-mutant-code1 cases, we grouped the IDH-mutant categories, unless specified
137 otherwise. To facilitate further investigation and discovery of the drivers of treatment resistance
138 in glioma, we have made this resource publicly available (<https://www.synapse.org/glass>).

139

140 **Transcriptional activity and cellular composition in glioma are variable over time**

141 To obtain a baseline understanding of transcriptional evolution in glioma, we assessed the
142 representation of the classical, mesenchymal, and proneural transcriptional subtypes in each
143 sample. IDH-wild-type tumors exhibited primarily classical and mesenchymal characteristics
144 compared to IDH-mutant tumors, which were largely proneural (**Figure 1A**). Longitudinally, the
145 dominant subtype in IDH-wild-type tumors switched in 49% of patients, with classical to
146 mesenchymal being the most common transition. IDH-mutant tumors were more stable, with 78%
147 of tumors remaining proneural at both time points (**Figure 1B**). *Classical IDH-wild-type and IDH-*
148 *mutant tumors switched subtype 52% of the time, while the mesenchymal and proneural subtypes*
149 *switched 38% of the time. This resulted in an overall reduction of classical tumors at recurrence,*
150 suggesting that the tumor cells underlying the classical subtype have higher plasticity than other
151 subtypes.

152

153 To understand the cellular phenotypes underlying the transcriptional dynamics over time, we
154 deconvoluted the GLASS gene expression dataset using CIBERSORTx (Newman et al., 2019)

155 integrated with reference cell state signatures derived from our previously established collection
156 of 55,284 single-cell transcriptomes from 11 adult patients spanning glioma subtypes and time
157 points (Johnson et al., 2021) (**Table S3, Table S4**). Unsupervised analyses of the single-cell data
158 had previously identified 12 cell states that represented the glial, stromal, immune, and malignant
159 compartments commonly present in glioma. The malignant population expressed a shared set of
160 markers (e.g., SOX2) and was split across three pan-glioma cell states, differentiated-like, stem-
161 like, and proliferating stem-like, that together capture the gradient between development, lineage
162 commitment, and proliferative status that has been observed across numerous glioma single-cell
163 studies (Bhaduri et al., 2020; Castellan et al., 2021; Couturier et al., 2020; Garofano et al., 2021;
164 Neftel et al., 2019; Richards et al., 2021; Tirosh et al., 2016; Venteicher et al., 2017; Wang et al.,
165 2019; Yuan et al., 2018). Specifically, the differentiated-like state encompassed malignant cells
166 exhibiting oligodendrocyte-like, astrocyte-like (EGFR+), and mesenchymal-like (CD44+)
167 processes, while the stem-like states could be segregated by cell cycle activity (Ki67+) and
168 resembled undifferentiated and progenitor-like malignant cells (OLIG2+) (Neftel et al., 2019;
169 Venteicher et al., 2017). To validate this approach, we applied the method to bulk glioma RNAseq
170 profiles that had ground truth cellular proportions determined by 1) synthetic mixing of single-cell
171 profiles, 2) single-cell RNAseq, and 3) histo-cytometry of whole-slide multiplex
172 immunofluorescence stains (**Figures S1A-C**). Together, these orthogonal analyses supported the
173 validity of the CIBERSORTx deconvolution approach in glioma.

174

175 When applying our deconvolution approach to the GLASS dataset, we observed variations in
176 cellular composition across each subtype consistent with prior literature (Neftel et al., 2019; Wang
177 et al., 2017). In both initial and recurrent tumors, the classical and mesenchymal subtypes were
178 dominated by differentiated-like malignant cells, with mesenchymal samples also having high
179 levels of stromal and immune cells. The proneural subtype, in contrast, contained high levels of
180 proliferating stem-like and stem-like malignant cells (**Figure 1C and S1D**). We observed

181 consistent associations in the TCGA glioma cohort. (Figure S1E). Longitudinally, we found that
182 IDH-wild-type tumors had significantly higher levels of oligodendrocytes and significantly lower
183 levels of differentiated-like malignant cells at recurrence ($P = 5e-6$ and $4e-3$, paired t-test). These
184 changes remained significant even after accounting for differences in the surgical resection extent
185 at each time point, suggesting a greater admixture of malignant cells and oligodendrocytes
186 (Figure S1F). We observed similar changes in cellular composition when using an independently
187 published integrative model of cell state classification that has been established for IDH-wild-type
188 glioma (Neftel et al., 2019), including a significant decrease at recurrence in the astrocyte-like
189 malignant cell state that is dominant in classical IDH-wild-type tumors ($P = 7e-3$, paired t-test;
190 Figure S1G). Recurrent IDH-mutant tumors exhibited significantly higher levels of proliferating
191 stem-like malignant cells and significantly lower levels of differentiated-like malignant cells ($P =$
192 $1e-3$ and $2e-6$, paired t-test; Figure 1C). Stratifying this group by 1p/19q co-deletion status
193 revealed that the increase in proliferating stem-like cells was only significant in IDH-mutant-
194 noncodels, while IDH-mutant-codels exhibited a significant increase in stem-like cells ($P = 0.04$,
195 paired t-test; Figure S1H). Overall, the differences IDH-wild-type and IDH-mutant tumors
196 exhibited over time suggested that distinct factors influence recurrence in each subtype.

197

198 **Histological features underlie subtype switching and cell state changes at recurrence**

199 Intratumoral heterogeneity is a hallmark of glioma and is abundant in hematoxylin and eosin-
200 stained (H&E) tissue slides, where features such as microvascular proliferation and necrosis are
201 used for diagnosis and grading by pathologists (Hambardzumyan and Bergers, 2015; Kristensen
202 et al., 2019). The Ivy Glioblastoma Atlas Project (Ivy GAP) has defined and microdissected five
203 “anatomic” features on the basis of reference histology, including two features found at the tumor
204 periphery (leading edge and infiltrating tumor), and three features found in the tumor core (cellular
205 tumor, pseudopalisading cells around necrosis, and microvascular proliferation) (Puchalski et al.,
206 2018). They have shown that each of these features has a distinct transcriptional profile,

207 suggesting that changes in a tumor's cell state composition at recurrence reflect changes in a
208 tumor's underlying physical structure. To obtain a better understanding of the cell states found in
209 these features, we assessed the cellular composition of each feature through transcriptional
210 deconvolution and multiplex immunofluorescence (Figures 2A, S2A). Each feature exhibited a
211 distinct cell state composition profile. Leading-edge features, which have been shown to exhibit
212 expression patterns associated with the proneural subtype and neural tissue (Gill et al., 2014; Jin
213 et al., 2017; Puchalski et al., 2018), were rich in oligodendrocytes and stem-like malignant cells.
214 Pseudopalisading cells around necrosis features, which are areas of hypoxia, exhibited the
215 highest levels of differentiated-like malignant cells. Conversely, microvascular proliferation
216 features were enriched in proliferating stem-like malignant cells, supporting the role of oxygen in
217 influencing cell state. Finally, the cellular tumor feature exhibited sample-specific variation, with
218 high levels of differentiated-like malignant cells in IDH-wild-type samples and high levels of stem-
219 like cells in IDH-mutant samples. Overall, each cell state's distribution was more significantly
220 associated with the histological feature than the patient from which it was derived (two-way
221 ANOVA; Figure S2B) (Puchalski et al., 2018).

222
223 Given the strong association between histological features and cellular composition, we examined
224 how the representation of these features varied over time by deconvoluting the GLASS dataset
225 with the available feature-specific gene signatures developed as part of Ivy GAP. To assess the
226 performance of this deconvolution, we compared the resulting proportions to pathologist
227 estimates of related features in a subset of samples with matched H&E slides (Table S5). This
228 revealed that the method was successfully able to distinguish between periphery- and tumor core-
229 associated features regardless of time point, (Figure S2C) and identified expected correlations
230 between the pseudopalisading cells around necrosis feature and pathologist estimates of the slide
231 area occupied by necrosis (Figure S2D). However, in recurrent samples, transcriptional
232 deconvolution of some tumor core-associated features was influenced by the presence of

233 recurrence-specific histological features not profiled by Ivy GAP (**Figure S2E**). Within the GLASS
234 dataset, deconvolution captured differences in each bulk transcriptional subtype's anatomy that
235 were consistent with their underlying cell state composition (**Figure 2B**). It also revealed that IDH-
236 wild-type tumors had significantly higher leading-edge content at recurrence ($P = 4e-5$, paired t-
237 test; **Figure 2B**), which was consistent with the increase in oligodendrocytes we had previously
238 observed (**Figure 1C**). In most cases this increase was independent of whether a tumor
239 underwent a transcriptional subtype transition, suggesting it was a general feature at recurrence
240 (**Figure 2C**). At the cell state level, we found that changes in the abundance of differentiated-like
241 malignant cells positively associated with increased cellular tumor features in IDH-wild-type
242 tumors, increased leading edge features in IDH-mutant tumors, and increased pseudopalisading
243 cells around necrosis features in both subtypes. Changes in stem-like malignant cells positively
244 associated with changes in leading-edge features in IDH-wild-type tumors and cellular tumor
245 features in IDH-mutant tumors. Finally, in both subtypes, changes in proliferating stem-like and
246 immune cells positively associated with changes in microvascular proliferation (**Figure 2D**).

247

248 Given these correlations, we hypothesized that subtype switches in IDH-wild-type tumors were
249 attributable to changes in histological feature composition over time. To test this, we recalculated
250 our malignant cell fractions by adjusting for the presence of non-malignant cells, as well as
251 leading-edge content which may vary by surgery. While most subtype switches associated with
252 changes in at least one malignant cell fraction pre-adjustment, the strongest difference observed
253 post-adjustment was a decrease in stem-like cells in tumors undergoing a proneural-to-
254 mesenchymal transition ($P = 3e-4$, paired t-test; **Figures S2F, S2G**). This association remained
255 significant even after adjusting for the remaining non-cellular tumor features, suggesting that
256 tumors undergoing this switch exhibit a loss of stem-like cells independent of histological feature
257 composition (**Figures 2E, S2F**). Collectively, these results indicate that while most subtype
258 switches in IDH-wild-type tumors are related to changes in a tumor's underlying physical structure

259 and microenvironment, the changes observed in the proneural-to-mesenchymal transition may
260 result from tumor-wide changes that reflect malignant cell-intrinsic processes at recurrence.

261

262 **Acquired somatic alterations at recurrence associate with changes in cellular composition**

263 While most tumors exhibited changes in cell state and associated histological feature
264 composition, the factors underlying these changes remained unclear. Somatic genetic alterations
265 have been shown to be associated with the cell state distribution of IDH-wild-type and IDH-mutant
266 glioma (Johnson et al., 2021; Neftel et al., 2019; Tirosh et al., 2016; Verhaak et al., 2010). We
267 thus hypothesized that changes in cellular composition resulted from genetic changes at
268 recurrence. To test this, we began by comparing how each malignant cell state differed across
269 samples that acquired or lost driver mutations (Ceccarelli et al., 2016) at recurrence. Within IDH-
270 mutant tumors, this identified acquired deletions of the cell cycle regulator *CDKN2A* and acquired
271 amplifications of the cell cycle regulator *CCND2* as genetic events that together associated with
272 an increase in proliferating stem-like cells ($P = 3e-3$, paired t-test, $n = 6$; **Figure 3A**). [Whole slide](#)
273 [multiplex immunofluorescence scans of a recurrent IDH-mutant tumor with an acquired *CDKN2A*](#)
274 [deletion and its matched initial tumor confirmed this association, with the recurrence exhibiting a](#)
275 [significantly higher number of SOX2+/Ki67+ cells \(\$P < 1e-5\$, Fisher's exact test; **Figure 3B**\)](#). We
276 did not observe this association in IDH-wild-type tumors, which typically harbor *CDKN2A* deletions
277 at initial presentation (**Figure S3A**).

278

279 We next examined how malignant cell states associated with treatment-induced hypermutation.
280 Approximately 20% of gliomas recur with a hypermutated phenotype following treatment with
281 alkylating agents, a standard-of-care chemotherapy (Barthel et al., 2019; Touat et al., 2020). This
282 phenotype has been associated with disease progression and distant recurrence (Yu et al., 2021).
283 We found that in both IDH-wild-type and IDH-mutant glioma, hypermutation also associated with
284 an increase in proliferating stem-like malignant cells ($n = 13$ and 7 , respectively; **Figure 3C**).

285 Multiplex immunofluorescence scans of an IDH-wild-type tumor pair with temozolomide-induced
286 hypermutation confirmed this association, with the recurrence having significantly higher number
287 of SOX2+/Ki67+ cells ($P < 1e-5$, Fisher's exact test; **Figure 3D**). In IDH-mutant tumors,
288 hypermutation largely occurred independent of acquired copy number changes in *CDKN2A* and
289 *CCND2*, suggesting that there are multiple genetic routes to increasing proliferating stem-like
290 malignant cells at recurrence (**Figure 3E**). Notably, neither of these alterations associated with
291 changes in microvascular proliferation, suggesting that increases in proliferating stem-like
292 malignant cells were a result of genetics and not microenvironmental interactions (**Figure S3B**).
293 Survival analyses revealed that that increases in proliferating stem-like malignant cells in IDH-
294 mutant tumors were significantly associated with reduced overall survival ($P = 0.02$, log-rank test;
295 **Figure 3F**), and remained so after adjusting for age, grade, and 1p/19q co-deletion status ($P =$
296 0.02 , Wald test; **Figure S3C**). Collectively, these results indicate that genetic evolution at
297 recurrence can alter malignant glioma cells toward a more proliferative phenotype that associates
298 with poor prognosis.

299

300 In addition to malignant cells, genetic alterations have been associated with changes in the
301 microenvironmental composition of tumors (Wellenstein and de Visser, 2018). We thus repeated
302 our longitudinal analyses with non-malignant cells, examining how each cell state differed in tumor
303 pairs that acquired or lost selected driver mutations at recurrence. In IDH-wild-type tumors, non-
304 hypermutated recurrences that acquired *NF1* mutations all underwent a mesenchymal transition
305 and exhibited a significant increase in granulocytes ($P = 0.03$, paired t-test, $n = 3$; **Figure S3D**).
306 Granulocytes have previously been associated with tumor necrosis, a feature that is prominent in
307 mesenchymal glioblastoma (Yee et al., 2020). There were additionally several copy number
308 alterations, including loss of *EGFR* or *PDGFRA* amplifications, that were associated with
309 increased non-malignant cell content ($P < 0.05$, paired t-test, $n = 11$ and $n = 4$, respectively), and
310 a transition to the mesenchymal subtype ($P = 0.05$, Fisher's exact test; **Figures S3E and S3F**).

311 We did not observe any significant changes in the fractions of non-malignant cells when
312 comparing hypermutated recurrences with their corresponding non-hypermutated initial tumors,
313 including T cells (**Figure S3G**). These results together indicate that while genetic evolution is a
314 major driver of changes in malignant cell state composition, it has less of an effect on a tumor's
315 microenvironment.

316

317 **IDH-wild-type malignant cells exhibit an increase in neuronal signaling gene expression** 318 **programs at recurrence**

319 While a subset of tumors demonstrated a genetic-associated increase in proliferating stem cell
320 content at recurrence, the remaining IDH-wild-type and IDH-mutant tumors did not exhibit a
321 shared longitudinal trajectory in their malignant cell composition. We hypothesized that the
322 expression programs of individual cell states may change following treatment in more subtle ways
323 that do not manifest as a ubiquitous shift in cellular composition. To test whether these changes
324 were taking place, we utilized our pan-glioma single-cell RNAseq dataset (Johnson et al., 2021)
325 as a reference to deconvolute GLASS bulk gene expression profiles into their component
326 differentiated-like, stem-like, proliferating stem-like, and myeloid gene expression profiles (**Figure**
327 **S4A**). To validate these profiles, we compared them to those derived from fluorescence-activated
328 cell sorting (FACS)-purified glioma-specific CD45⁻ and myeloid populations. This revealed strong
329 concordance between the corresponding profiles of each cell state (**Figures S4B and S4C**). To
330 ensure the profiles were capturing established biology, we employed differential expression and
331 gene ontology (GO) enrichment analyses to assess how each malignant cell profile differed
332 across each transcriptional subtype in IDH-wild-type glioma samples from TCGA. This revealed
333 subtle differences between the cell state-specific expression profiles, such as high levels of
334 immune-related functions in differentiated-like malignant cells from mesenchymal tumors, that
335 together were reflective of unique microenvironment interactions that were characteristic of each
336 subtype (**Figure S4D**).

337

338 To determine how the expression programs in each malignant cell state vary longitudinally, we
339 compared the cell state-specific gene expression profiles between the initial and recurrent tumor
340 for each pair receiving temozolomide and/or radiotherapy. In IDH-wild-type tumors, we found that
341 10.0% of the 7,511 genes that could be inferred in stem-like cells were significantly differentially
342 expressed at recurrence (false discovery rate (FDR) < 0.05, Wilcoxon signed-rank test). This
343 number was 7.6% of the 11,641 differentiated-like state genes and 6.1% of the 6,019 proliferating
344 stem-like state genes (**Figure 4A; Table S6**). Based on these results, we defined recurrence-
345 specific signatures as the genes that were significantly up-regulated at recurrence in each cell
346 state. Within our pan-glioma single-cell dataset, we confirmed the recurrence-specific nature for
347 each of these signatures by comparing their expression between malignant cells from unmatched
348 recurrent and initial tumors (**Figure S4E**). To understand the functions these cell states up-
349 regulate at recurrence, we then performed GO enrichment analysis on each signature. This
350 revealed that the stem-like signature was significantly enriched in terms relating to neuronal
351 signaling, while the differentiated-like and proliferating stem-like signatures exhibited similar, but
352 weaker, associations (**Figures 4B and S4F**).

353

354 Given our previous results that showed increased levels of oligodendrocytes and leading edge
355 content in recurrent IDH-wild-type tumors, we hypothesized that neuronal signaling in stem-like
356 malignant cells may be found in infiltrative regions of the tumor characterized by high tumor-
357 neuron interactions. To test this hypothesis, we examined how the stem-like malignant cell
358 recurrence signature associated with histological feature content in the GLASS cohort. This
359 revealed a positive association between stem-like malignant cell-specific expression of the
360 recurrence signature and leading edge content (**Figure 4C**). **Notably, we observed this result at**
361 **both time points, suggesting that neuronal signaling in stem-like malignant cells may be driven**
362 **more by tumor-neuron interactions than malignant cell-intrinsic changes specific to recurrence.**

363 We next utilized an independent single-cell RNAseq dataset to compare the recurrence-specific
364 signature between malignant cells collected from the invasive rim, where there are higher levels
365 of neurons, to those collected from the tumor core (Yu et al., 2020). This analysis revealed
366 significantly higher signature expression at the invasive rim, further supporting the association
367 between neuronal signaling and tumor-neuron interactions (**Figure 4D**). Finally, we performed
368 multiplex immunofluorescence to examine how malignant cell expression of neuronal markers
369 differed between pathologist-annotated histological features in recurrent glioma (**Figure S4G**).
370 Within the infiltrating tumor region, we found neurons (NeuN+) and a high number of malignant
371 cells (SOX2+) staining positively for SNAP25, a neuronal marker that was part of our stem-like
372 malignant cell recurrence signature. In contrast, there were few neurons and no SNAP25+ cells
373 in the cellular tumor region (**Figure 4E**). Collectively, these results suggest that increased normal
374 cell content at recurrence associates with higher signaling between malignant cells and
375 neighboring neural cells. Neuron-to-glioma synapses have been implicated in increased tumor
376 growth and invasion, and collectively our results support a model of greater tumor invasion into
377 the normal brain at recurrence that is likely facilitated by an increase in neuronal interactions
378 (Venkataramani et al., 2019; Venkatesh et al., 2015; Venkatesh et al., 2019; Venkatesh et al.,
379 2017).

380

381 We next compared how the expression profiles of each cell state differed between initial and
382 recurrent IDH-mutant tumors that received treatment. The resulting signatures were distinct from
383 those in IDH-wild-type tumors, with the largest proportion of differentially expressed genes found
384 in the differentiated-like state instead of the stem-like state (FDR < 0.05, Wilcoxon signed-rank
385 test; **Figure 4F, Table S5**). A GO enrichment analysis of the genes up-regulated at recurrence in
386 the differentiated-like cell state revealed a significant enrichment of terms related to the cell cycle
387 and mitosis (FDR < 0.05; **Figure 4G**), while the stem-like signature exhibited similar associations
388 at a relaxed significance threshold (FDR < 0.1; **Figure S4H**). These signatures were consistent

389 with those found in higher grade tumors, suggesting that the cell state-specific gene expression
390 changes were indicative of grade increases at recurrence. Accordingly, we observed that these
391 changes were strongest in the tumor pairs that recurred at a higher grade (**Figure S4I**).
392 Furthermore, when we compared signature expression in single cells of the same cell state, we
393 found that the signatures were differentially expressed in the cells derived from grade III versus
394 grade II tumors (**Figure S4J**). These results indicate that IDH-wild-type and IDH-mutant tumors
395 recur in distinct manners that may reflect their response to treatment.

396

397 **Mesenchymal malignant cell activity associates with a distinct myeloid cell phenotype**

398 The mesenchymal subtype of glioma has been associated with an accumulation of myeloid cells
399 as well as radiotherapy resistance and poor patient survival (Bhat et al., 2013; Kim et al., 2021;
400 Wang et al., 2017). Given the importance of this subtype on a patient's clinical trajectory, we
401 sought to understand the factors driving tumors toward this subtype over time. Within IDH-wild-
402 type tumors in the GLASS dataset, the mesenchymal subtype was the most common subtype at
403 recurrence. In agreement with previous findings, IDH-wild-type tumors with a mesenchymal
404 recurrence exhibited a significantly shorter surgical interval compared to those with non-
405 mesenchymal recurrences ($P = 0.03$, log-rank test; **Figure S5A**) (Wang et al., 2017). However,
406 this association was weaker in a multi-variate model (**Figure S5B**). Single-cell studies have
407 previously shown that samples of this subtype exhibit high levels of malignant cells that express
408 a distinct mesenchymal-like expression signature (Neftel et al., 2019). Analysis of the malignant
409 cell state-specific expression profiles in samples undergoing a mesenchymal transition revealed
410 that differentiated-like cells, but not stem-like cells, up-regulated this signature at recurrence
411 (**Figure S5C**).

412

413 Given the changes in cellular composition and malignant cell expression associated with a
414 mesenchymal transition, we hypothesized that this trajectory may be driven in part by unique

415 interactions between the tumor-infiltrating myeloid cells and malignant cells. To understand how
416 the myeloid compartment differed across each glioma subtype, we deconvoluted the myeloid-
417 specific gene expression profiles from a collection of diffuse glioma bulk RNAseq profiles ($n =$
418 687) from The Cancer Genome Atlas (TCGA). The myeloid compartment in IDH-wild-type tumors
419 was characterized by high expression of a previously defined blood-derived macrophage
420 signature (Muller et al., 2017), while myeloid cells in IDH-mutant-noncode1 tumors exhibited high
421 expression of a previously defined brain-resident microglia signature (**Figure 5A**). Stratifying this
422 cohort by transcriptional subtype revealed that the blood-derived macrophage signature followed
423 a stepwise increase with mesenchymal subtype representation, while microglial gene expression
424 was highest amongst tumors of the mixed subtype classification that is seen most frequently in
425 IDH-mutant-noncode1 glioma (**Figure S5D**). [Consistent with these results, principal component
426 analysis of tumor and normal brain myeloid cell expression profiles revealed that proneural tumors
427 most closely resembled those from normal brain tissue, while mesenchymal myeloid profiles were
428 more distinct \(Figure S5E\).](#) In IDH-wild-type tumors, blood-derived macrophage signature
429 expression was positively correlated with the abundance of microvascular proliferation and
430 pseudopalisading cells around necrosis features, while the microglia signature was most
431 positively correlated with leading-edge content. In IDH-mutant tumors, the blood-derived
432 macrophage signature negatively associated with leading-edge content, while the microglia
433 signature did not exhibit any clear associations (**Figure S5F**). Longitudinally, when holding
434 transcriptional subtype constant, we observed very few differentially expressed genes in the
435 myeloid cell profiles from matched initial and recurrent tumors in the GLASS cohort (**Figure S5G**).
436 However, the myeloid profiles in IDH-mutant tumors that increased grade at recurrence exhibited
437 a significant decrease in microglia signature expression, suggesting a shift in myeloid cell states
438 away from brain-resident microglia ($P = 4e-4$, [Wilcoxon signed-rank test](#); **Figure 5B**).
439

440 Macrophages are highly plastic and capable of changing their transcriptional programs in
441 response to different stimuli (Xue et al., 2014). We thus hypothesized that different glioma
442 transcriptional subtypes would exhibit distinct myeloid expression programs. To test this
443 hypothesis, we performed a differential expression analysis comparing the deconvoluted myeloid
444 cell expression profiles from each transcriptional subtype to those from normal brain tissue
445 (Figure S5H). This analysis revealed that myeloid cells from the classical and mesenchymal
446 subtypes exhibit an immunosuppressive phenotype, with each signature including several genes
447 from the blood-derived macrophage signature as well as the immune checkpoint genes,
448 *PDCD1LG2* and *IDO1*. In addition to this shared signature, myeloid cells from mesenchymal
449 glioma uniquely up-regulated another 300 genes not seen in the other subtypes, suggesting they
450 exhibit distinct biology. To better understand the processes taking place in this subtype, we
451 directly compared the myeloid gene expression profiles between mesenchymal and non-
452 mesenchymal IDH-wild-type tumors in TCGA. This analysis revealed a 186-gene signature that
453 was significantly upregulated in mesenchymal samples (FDR < 0.05, fold-change > 1.5; Figure
454 5C, Table S7) and enriched in chemokine signaling and lymphocyte chemotaxis functions (Figure
455 S5I). To validate this signature, we examined its expression in our scRNAseq dataset and found
456 that expression of this signature in a patient's myeloid cells strongly associated with the patient's
457 bulk RNAseq-derived mesenchymal glioma subtype score ($R = 0.89$, $P = 3e-3$; Figure S5J).
458 Longitudinally, IDH-wild-type tumors in the GLASS dataset undergoing a mesenchymal transition
459 exhibited myeloid-specific expression profiles with significantly higher expression of this signature
460 at recurrence ($P = 8e-8$, Wilcoxon signed-rank test; Figure 5D).

461
462 Overall, these analyses revealed a mesenchymal-specific myeloid cell state that was associated
463 with dynamic changes in malignant cell expression over time. We hypothesized that these cells
464 represent a subset of blood-derived macrophages that interact directly with mesenchymal
465 malignant cells. To determine where this myeloid cell state was located, we examined how the

466 expression of the mesenchymal myeloid signature varied across each of the Ivy GAP dataset's
467 histological feature samples. This revealed that the mesenchymal myeloid signature was
468 expressed most highly in the pseudopalisading cells around necrosis and microvascular
469 proliferation features that also harbor high levels of blood-derived macrophages (**Figure 5E**).

470 [Correlating the myeloid-specific expression of this signature with histological feature proportions](#)
471 [in TCGA revealed similar results \(Figure S5K\)](#). We next performed a ligand-receptor interaction
472 analysis to identify candidate ligand-receptor pairs that associate with mesenchymal-transitions
473 over time. To probe these interactions, we downloaded a set of 1,894 literature-supported ligand-
474 receptor pairs (Ramilowski et al., 2015) and identified all pairs that had one component expressed
475 in a tumor's deconvoluted myeloid profile and the other expressed in the differentiated-like
476 malignant cell profile. We then compared how the longitudinal change in expression of each
477 component associated with the change in each tumor pair's mesenchymal subtype score. This
478 identified 69 putative ligand-receptor pairs where each component exhibited a positive association
479 ($R > 0$, $FDR < 0.05$). Of these pairs, 35 also exhibited these associations in our single-cell dataset
480 (**Table S8**). Notably, this analysis revealed that expression of oncostatin M (*OSM*) and oncostatin
481 M receptor (*OSMR*) by myeloid cells and differentiated-like malignant cells, respectively, was one
482 of the strongest correlates of the mesenchymal subtype. This was consistent with studies showing
483 that this signaling associates with mesenchymal-like expression programs both *in vitro* and *in vivo*
484 (Hara et al., 2021; Junk et al., 2017). [To determine whether spatial convergence of OSM-](#)
485 [expressing myeloid cells \(CD14+\) and mesenchymal-like malignant cells \(CD44+/SOX2+\) takes](#)
486 [place in human tissue samples, we examined their distribution using multiplex](#)
487 [immunofluorescence. In mesenchymal IDH-wild-type glioma, we observed high OSM expression](#)
488 [in myeloid cells near blood vessels and mesenchymal malignant cells, while these expression](#)
489 [patterns were not observed in classical glioma \(Figure 5F\)](#). These analyses together identify a
490 candidate ligand-receptor interaction that can potentially be targeted to change a tumor's
491 trajectory following treatment.

492

493 **Antigen presentation is disrupted at recurrence in IDH-mutant-noncodeL glioma**

494 The interactions we identified between myeloid cells and mesenchymal malignant cells
495 demonstrated a role for the immune system in shaping glioma evolution. In addition to myeloid
496 cells, T cells have been implicated in driving the evolution of multiple cancer types through the
497 elimination of neoantigen-presenting tumor subclones (Grasso et al., 2018; McGranahan et al.,
498 2017; Rooney et al., 2015; Rosenthal et al., 2019; Zhang et al., 2018). While rare in glioma, these
499 cells have been shown to select for epigenetic changes and specific genetic alterations (Gangoso
500 et al., 2021; Kane et al., 2020) and converge with rare, recorded responses to checkpoint
501 inhibition (Cloughesy et al., 2019; Zhao et al., 2019). However, the extent to which T cell-mediated
502 selection interacts with standard-of-care treatment to influence glioma evolution at recurrence
503 remains unclear. We hypothesized that if T cell selection was taking place in glioma, we would
504 observe high rates of loss-of-heterozygosity (LOH) in the human leukocyte antigen (HLA) genes
505 that are central to the presentation of neoantigens. We thus called HLA LOH throughout the
506 GLASS cohort (**Figure 6A**). We observed that HLA LOH takes place in glioma, occurring in at
507 least one timepoint in 19% of patients. Within IDH-wild-type and IDH-mutant-codel tumors, HLA
508 LOH was found at similar rates between initial and recurrent tumors, with most affected pairs
509 exhibiting this alteration at both time points. This was not the case in IDH-mutant-noncodeL
510 tumors, where significantly more samples acquired HLA LOH at recurrence ($P = 0.04$, Fisher's
511 exact test). Given the increase in HLA LOH in recurrent IDH-mutant samples, we reasoned that
512 HLA LOH may be under positive selection at recurrence. To test this, we used a previously
513 established simulation approach (McGranahan et al., 2017) that determined whether focal losses
514 of the HLA genes occurred at a rate greater than expected by chance given a sample's overall
515 somatic copy number alteration (SCNA) burden. In both IDH-wild-type and IDH-mutant
516 recurrences we did not observe evidence of positive selection using this approach ($P > 0.05$).
517 Furthermore, we did not observe an association between HLA LOH status and T cell-mediated

518 selection metrics, including the fraction of infiltrating T cells in each tumor (**Figure 6B**), the rates
519 of neoantigen depletion (**Figure S6A**), and the number of neoantigens binding to the kept versus
520 lost alleles (**Figures S6B**).

521

522 Overall, our results suggested that HLA LOH in glioma was not selected for, contrasting it with
523 other cancer types (Grasso et al., 2018; McGranahan et al., 2017; Rosenthal et al., 2019; Zhang
524 et al., 2018). We hypothesized instead it was a passenger event, and thus would be more likely
525 to occur in samples with high SCNA burdens. In support of this, we found that while IDH-mutant-
526 noncodel tumors generally exhibit significantly higher SCNA burdens at recurrence (Barthel et al.,
527 2019), the tumors that acquired HLA LOH at recurrence exhibited significantly higher changes in
528 SCNA burden than those that did not ($P = 0.02$, Wilcoxon rank-sum test; **Figure 6C**). In IDH-wild-
529 type tumors, we did not observe these longitudinal associations. However, at both the initial and
530 recurrent time points IDH-wild-type tumors with HLA LOH exhibited significantly higher SCNA
531 burdens than those with both HLA alleles, supporting that HLA LOH is a passenger event in these
532 tumors as well ($P < 0.05$, Wilcoxon rank-sum test; **Figure S6C**). Taken together, these results
533 suggest that disruption of antigen presentation in glioma is likely a byproduct of genome-wide
534 copy number changes rather than being a result of selection by cytolytic T cells.

535

536 **Discussion**

537 To understand the factors driving the evolution and treatment resistance of diffuse glioma, we
538 integrated genomic and transcriptomic data from the initial and recurrent tumor pairs of 304
539 patients. By integrating this resource with data from single-cell RNAseq experiments, a
540 histological transcriptional atlas, multiplex immunofluorescence imaging, and a multitude of
541 external transcriptional datasets, we have comprehensively defined the longitudinal
542 transcriptional and compositional changes that gliomas sustain at recurrence. As a result of these
543 findings, we have grouped recurrent tumors into three “recurrence states”: neuronal,

544 mesenchymal, or proliferative, based on their shared cellular, genetic, and histological features
545 (Figure 7). These states associate with different clinical trajectories and are present in IDH-wild-
546 type and IDH-mutant tumors at different rates, with IDH-wild-type tumors exhibiting all three states
547 at recurrence and IDH-mutant tumors primarily exhibiting the proliferative state. Notably, these
548 states are not mutually exclusive, with some IDH-wild-type tumors simultaneously exhibiting
549 features associated with multiple states. Overall, this grouping offers a framework through which
550 to better understand progression in diffuse glioma and can help guide clinical decision-making for
551 recurrent disease.

552

553 In this study, we employed single-cell deconvolution approaches to enable high-resolution
554 quantification of the cellular composition of gliomas. Available cell state classification models have
555 been developed for diffuse glioma using single cells of a single glioma subtype (Castellan et al.,
556 2021; Garofano et al., 2021; Neftel et al., 2019; Richards et al., 2021; Venteicher et al., 2017). In
557 contrast, our reference matrix utilized cell states derived from a pan-glioma single-cell dataset
558 composed of initial and recurrent tumors of all major clinically relevant glioma subtypes, and thus
559 included malignant and normal cell states commonly found across diffuse glioma. The resulting
560 cellular proportions reflected true cell state levels in multiple benchmarking analyses, making this
561 an invaluable approach for comparing and contrasting the longitudinal changes taking place
562 across IDH-wild-type and IDH-mutant tumors. In the future this approach can continue to be
563 refined as the number of cells per tumor and patients profiled by scRNAseq increases and enables
564 even higher resolution estimates of glioma cell state composition and heterogeneity.

565

566 While transcriptional subtype switching has been reported to occur frequently in IDH-wild-type
567 glioma, the role these switches play in treatment resistance is unclear. Pathology-defined
568 histological features from Ivy GAP exhibit distinct transcriptional profiles that correspond to
569 different glioma transcriptional subtypes, suggesting that subtype switching may be more

570 reflective of changes in the tumor's histological feature composition at recurrence (Jin et al., 2017;
571 Puchalski et al., 2018). Ivy GAP comprises features defined from primary tumors, which we found
572 to be useful proxies to measure the biological changes at recurrence that underlie subtype
573 switching. Limitations of the Ivy GAP resource may include the absence of commonly observed
574 features, such as necrotic tissue and depopulated tumor, which may be more present following
575 radiation therapy. We showed that the proneural-to-mesenchymal transition is independent of
576 histological feature composition and reflects transcriptional changes in the cellular tumor.
577 Mesenchymal transitions have been shown to associate with several factors, including increased
578 myeloid cell infiltration, radiation-induced NF- κ B activation, altered tumor metabolism, and
579 hypoxia (Bhat et al., 2013; Garofano et al., 2021; Kim et al., 2021; Mao et al., 2013; Osuka et al.,
580 2021; Schmitt et al., 2021; Wang et al., 2017). Our results indicate that the proneural-to-
581 mesenchymal transition is likely influenced by tumor-wide changes, supporting the hypothesis
582 that this transition is involved in therapy resistance. Additional studies where multiple biopsies are
583 obtained from the same tumor over time may help to further elucidate the relationship between
584 histological feature composition and gene expression subtype.

585

586 Across IDH-wild-type and IDH-mutant glioma, we identified a sub-population of samples that
587 exhibited an increase in proliferating stem-like malignant cells at recurrence. Analysis of the
588 acquired somatic alterations in these tumors revealed that hypermutation was associated with
589 this change in both subtypes. This finding across both subtypes suggests that hypermutation may
590 represent a pan-glioma treatment resistance mechanism. Hypermutation did not associate with
591 patient survival in the GLASS dataset but has been found more frequently in distant recurrences
592 and linked to reduced survival following high-grade progression in low-grade IDH-mutant tumors
593 (Barthel et al., 2019; Touat et al., 2020; Yu et al., 2021). Given these findings, our data highlights
594 methods to predict treatment-induced hypermutation represent a previously unrecognized unmet
595 clinical need in the field. Integrating such methodologies into clinical care pathways would help to

596 identify patients that may benefit from therapies that complement chemotherapy and further target
597 cycling cells.

598

599 We did not identify any somatic alterations associated with changes in malignant cell composition
600 outside of hypermutation and copy number changes in cell cycle regulators. Despite this, we
601 found that malignant glioma cells in IDH-wild-type tumors exhibited a significant increase in the
602 expression of genes involved in neuronal signaling. This change coincided with an increase in
603 oligodendrocytes at recurrence that was independent of the extent of tumor resection, providing
604 a medium for increased interactions between malignant and normal cells in the brain. Additionally,
605 neuronal signaling was most significantly up-regulated within the malignant stem-like cells, which
606 are found at the highest levels at the leading edge of the tumor and frequently resemble
607 oligodendroglial precursor-like malignant cells involved in neuronal signaling (Venkatesh et al.,
608 2019). Increased neuronal signaling has previously been reported in malignant cells that have
609 infiltrated into the surrounding tissue in response to low oxygen content and our study extends
610 these observations to glioma progression (Darmanis et al., 2017). Collectively these findings
611 coupled with our results relating to proneural-to-mesenchymal transition support a model where
612 recurrent IDH-wild-type tumors, in response to changes in hypoxia or tumor metabolism at
613 recurrence, invade the surrounding peripheral tissue where they actively interact with neighboring
614 neuronal cells. Given the growing appreciation of the role neuron-glioma interactions play in
615 glioma invasion and progression, it will be critical to understand the extent to which these
616 interactions facilitate tumor regrowth and treatment resistance (Venkataramani et al., 2019;
617 Venkatesh et al., 2015; Venkatesh et al., 2019; Venkatesh et al., 2017).

618

619 In agreement with other studies, we found that the myeloid cell phenotype varied in relation to
620 tumor subtype and malignant cell state (Klemm et al., 2020; Muller et al., 2017; Ochocka et al.,
621 2021; Pombo Antunes et al., 2021; Venteicher et al., 2017). Notably, we found that this variation

622 was most apparent in mesenchymal tumors, where myeloid cells exhibited a distinct
623 transcriptional program. Ligand-receptor analyses revealed several candidate interactions
624 involved in driving malignant and myeloid cells toward this mesenchymal phenotype. Resolving
625 the directionality of these interactions, or determining whether additional factors mediate them,
626 will be an important step toward understanding the contribution myeloid cells make in
627 mesenchymal transformation. We did not observe any differences in T cell activity, nor did we
628 observe evidence of T cell-mediated selection, making glioma distinct from several other cancers
629 (Grasso et al., 2018; McGranahan et al., 2017; Rooney et al., 2015; Rosenthal et al., 2019; Zhang
630 et al., 2018). Despite this, we did observe that antigen presentation in IDH-mutant-noncode
631 tumors is frequently disrupted at recurrence and is associated with increases in SCNA burden.
632 [As data from studies of immunotherapy trials in glioma are published, it will be important to assess](#)
633 [whether antigen presentation loss and SCNA burden serve as biomarkers of response.](#) These
634 results may inform the design of T cell-based immunotherapies going forward, as standard-of-
635 care therapies may inadvertently disrupt malignant cells' ability to present neoantigens to T cells.
636

637 Therapy resistance remains a significant obstacle for patients with diffuse glioma and must be
638 overcome to improve patient survival and quality of life. Overall, our results reveal that gliomas
639 undergo changes in cell states that associate with changes in genetics and the microenvironment,
640 providing a baseline towards building predictive models of treatment response. Taking into
641 consideration the current histopathologic diagnostic criteria for gliomas and their longitudinal
642 follow-up, future efforts by the GLASS Consortium are now underway. These include expansion
643 of the cohort, integration of digitized tissue sections, and association with clinical and genomic
644 datasets with radiographic imaging data (Bakas et al., 2020). Computational imaging studies have
645 shown mounting evidence and promise in revealing imaging signatures associated with increased
646 invasion and proliferation for glioma patients harboring particular mutations (Bakas et al., 2017;
647 Binder et al., 2018; Fathi Kazerooni et al., 2020; Mang et al., 2020; Zwanenburg et al., 2020), and

648 given their use in clinical monitoring, are highly complementary to the longitudinal datasets
649 established here. Going forward, the transcriptional and compositional changes we have identified
650 can be integrated with these imaging-based results to more broadly assess the molecular and
651 microenvironmental heterogeneity of glioma and identify clinically targetable factors to aid in
652 shaping a patient's disease trajectory.

653

654 **Limitations of the Study**

655 In this study, we applied single-cell RNAseq-based deconvolution approaches to bulk RNAseq
656 glioma expression profiles to infer the cellular composition, cell state-specific transcriptional
657 activity, and cellular interactions within each tumor. While such deconvolution approaches have
658 been validated extensively, both during their own development and for this study, they are limited
659 in their ability to detect rare cellular subpopulations and can only attribute cell state-specific
660 expression activity to the cell states defined in their input single-cell signature matrix. Due to these
661 limitations, our analyses were mainly directed at understanding broad differences between
662 longitudinal samples and transcriptional subtypes where we were well-powered to make
663 comparisons. Furthermore, we focused our cell state-specific gene expression profile analyses
664 on malignant cells and myeloid cells, as these cells represent the most common cell states in
665 glioma and thus contribute the strongest expression signals to bulk RNAseq profiles. Going
666 forward, transcriptomic analyses of smaller glioma patient subpopulations or rare cell states will
667 require a combination of a larger cohort of samples and/or a higher resolution technology such
668 as single-cell RNAseq to make robust conclusions. Spatially resolved transcriptomic methods can
669 additionally be applied to infer cellular interactions and identify ligand-receptor signaling that drive
670 glioma cells towards different cell states. Collectively, these approaches can build upon the results
671 in this study to refine our understanding of how gliomas evolve following therapy and improve
672 patient treatment regimens.

673

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833

834 **Declaration of Interests**

835 R.G.W.V. is a co-founder of Boundless Bio and a consultant for Stellanova Therapeutics. M.K.
836 has received research funding from AbbVie and Bristol Myers Squibb, is on the advisory board

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848 advisory board member for Mundipharma Research Limited, Prelude Therapeutics, Kiyatec, Cure
849 Brain Cancer Foundation, Merck Sharp & Dohme Co., and Sapience Therapeutics; owns stock in
850 in Ziopharm Oncology and WuXi Biologics; and has a spouse employed by Ziopharm Oncology.
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855 bureau for AbbVie and IgynTA.

856

857 **Figure Legends**

858 **Figure 1. Diffuse glioma exhibits transcriptional and cellular heterogeneity across**
859 **samples, subtypes, and time.** (A) Overview of the GLASS dataset. Each column represents a
860 tumor pair, and their initial (I) and recurrent (R) samples are labelled. All tumor pairs with RNAseq
861 data at each time point are included. Pairs are arranged based on the representation of the
862 proneural and mesenchymal subtypes in their initial tumors. The first track indicates whether there

863 is whole exome or whole genome sequencing data available for that pair. The next three tracks
864 indicate the representation of each bulk subtype across each sample. The stacked bar plots
865 indicate the cell state composition of each sample based on the single cell-based deconvolution
866 method, CIBERSORTx. The bottom tracks indicate molecular and clinical information for each
867 tumor pair. (B) Sankey plot indicating whether the highest-scoring transcriptional subtype
868 changed at recurrence. Each color reflects the transcriptional subtype in the initial tumors.
869 Number in parentheses indicates number of samples of that subtype. Subtype abbreviations:
870 proneural (Pro.), classical (Class.) and mesenchymal (Mes.). (C) Left: The average cell state
871 composition of each bulk transcriptional subtype across all GLASS samples. Right: The average
872 cell state composition of initial and recurrent tumors stratified by IDH mutation status.
873 Abbreviations: IDH-wild-type (IDHwt) and IDH-mutant (IDHmut). Colors in (C) are identical to
874 those used in (A).

875

876 **Figure 2. Histological features underlie changes in the cellular composition of diffuse**
877 **glioma over time.** (A) The cell state composition of each of the reference histology-defined Ivy
878 GAP histological features across 10 patients. Patient and IDH mutation status tracks are included
879 beneath the stacked bar plots. For the patient track, each colored block represents a unique
880 patient. (B) Left: The average histological feature composition of each bulk transcriptional subtype
881 across all GLASS samples. Right: The average histological feature composition of initial and
882 recurrent tumors stratified by IDH mutation status. Abbreviations: IDH-wild-type (IDHwt) and IDH-
883 mutant (IDHmut). (C) Heatmap depicting the changes in each histological feature between initial
884 and recurrent tumors undergoing the indicated subtype transition. The initial subtype is indicated
885 in the columns and the recurrent subtype is indicated in the rows. Colors represent the change in
886 fraction of the indicated features between initial and recurrent tumors, while * indicates a paired
887 t-test P -value < 0.05 . (D) Heatmap depicting the Pearson correlation coefficients measuring the
888 association between the change in a given histological feature and the change in a given cell

889 state when going from an initial tumor to recurrence. * indicates a significant correlation ($P < 0.05$).
890 (E) Left: Ladder plot depicting the change in the adjusted stem-like cell proportion between paired
891 initial and recurrent tumors undergoing a proneural-to-mesenchymal transition. Right: The
892 average adjusted proportions for malignant cells for the tumor pairs outlined on the left. Malignant
893 cell proportions were adjusted for the presence of non-malignant cells as well as non-cellular
894 tumor content.

895

896 **Figure 3. Hypermethylation and acquired cell cycle alterations associate with increased**
897 **proliferating stem-like malignant cells in IDH-wild-type and IDH-mutant glioma.** (A) Left:

898 Ladder plot depicting the change in the proliferating stem-like cell proportion between paired initial
899 and recurrent IDH-mutant tumors that acquired *CDNK2A* deletions or *CCND2* amplifications.

900 Right: Stacked bar plot depicting the average proportions of each cell state for the tumor pairs in
901 the ladder plots. (B) Left: Representative multiplex immunofluorescence images from a matched

902 initial and recurrent IDH-mutant tumor that acquired a *CDKN2A* deletion at recurrence. Scale bars
903 represent 50 μm . Right: Stacked bar plot depicting the proportion of SOX2+/Ki67+ cells among

904 all SOX2+ cells across the entire tissue section for each sample. Exact proportions are indicated
905 in the graph. (C) Top: Ladder plots depicting the change in the proliferating stem-like cell

906 proportion between paired initial and recurrent tumors that did and did not undergo hypermethylation.
907 Point colors indicate IDH mutation and 1p/19q co-deletion status. Paired t-test P -values are

908 indicated. Bottom: The average proportions of each cell state for the tumor pairs outlined above.

909 (D) Left: Representative multiplex immunofluorescence images from a matched initial and
910 recurrent IDH-wild-type tumor that was hypermethylated at recurrence. Scale bars represent 50 μm .

911 Right: Stacked bar plot depicting the proportion of SOX2+/Ki67+ cells among all SOX2+ cells
912 across the entire tissue section for each sample. Exact proportions are indicated in the graph. (E)

913 Top: The change in proliferating stem-like cell fraction between initial and recurrent tumors from
914 IDH-mutant tumor pairs. Each bar represents a tumor pair. Bottom: Molecular and clinical

915 information for each tumor pair. (F) Kaplan-Meier plot depicting the survival distributions of
916 patients that exhibited an increase or non-increase in proliferating stem-like cells at recurrence.
917 *P*-value was calculated using the log-rank test.

918

919 **Figure 4. Malignant cells exhibit increased neuronal signaling and cell cycle activation**

920 **programs in recurrent IDH-wild-type and IDH-mutant tumors.** (A) Heatmaps depicting the

921 average normalized \log_{10} expression level of genes that were differentially expressed between
922 malignant cell states from initial and recurrent IDH-wild-type tumors that received treatment.

923 Fractions on each plot's right indicate the number of differentially expressed genes (numerator)
924 out of the number of genes inferred for that cell state's profile using CIBERSORTx (denominator).

925 (B) Bar plot depicting the $-\log_{10}$ (adjusted *P*-value) from a GO enrichment analysis of the genes

926 significantly up-regulated at recurrence in stem-like malignant cell-specific gene expression
927 profiles from IDH-wild-type tumors. The top 15 GO terms are included. (C) Scatterplot depicting

928 the association between the leading edge fraction and the average expression of the stem-like
929 malignant cell recurrence signature for samples in the GLASS dataset. Pearson correlation

930 coefficients are indicated. (D) Violin plot depicting the average expression of the stem-like
931 malignant cell recurrence signature in malignant single-cells collected from the invasive rim and

932 tumor core of 9 grade IV gliomas (Yu et al. 2020). *P*-value was calculated using the Wilcoxon
933 rank-sum test. (E) Multiplex immunofluorescence images of the interface between the cellular

934 tumor (top right; CT) and infiltrating tumor (bottom right; IT) histological features in a recurrent
935 IDH-wild-type tumor. Histological features were defined by a neuropathologist using the H&E

936 image in **Figure S4G**. Scale bars represent the values indicated in the figure. (F) Heatmaps
937 depicting the average normalized \log_{10} expression level of genes that were differentially

938 expressed between malignant cell states from initial and recurrent IDH-mutant tumors that
939 received treatment. Fractions are as outlined in (A). (G) Bar plot depicting the $-\log_{10}$ (adjusted *P*-

940 value) from a GO enrichment analysis of the genes significantly up-regulated at recurrence in

941 differentiated-like malignant cell-specific gene expression profiles from IDH-mutant tumors. The
942 top 15 GO terms are included. In (B) and (G), dashed line corresponds to adjusted P -value <
943 0.05.

944

945 **Figure 5. Myeloid cells in diffuse glioma exhibit diverse phenotypes based on IDH mutation**

946 **status, transcriptional subtype, and recurrence status.** (A) Left: Uniform Manifold

947 Approximation and Projection (UMAP) dimensionality reduction plot of the CIBERSORTx-inferred

948 myeloid profiles from TCGA. Colors indicate bulk transcriptional subtype; shapes indicate IDH

949 and 1p/19q co-deletion status. Abbreviations: IDH-wild-type (IDHwt) and IDH-mutant (IDHmut).

950 When all three bulk transcriptional subtypes were significantly represented in a sample, the

951 'mixed' classification was used. Right: UMAP plot colored based on the relative mean expression

952 of macrophage and microglia signatures. (B) Box and ladder plots depicting the difference in the

953 mean expression of the indicated signatures between initial and recurrent IDH-mutant tumors

954 from GLASS that do and do not recur at higher grades. Point colors indicate 1p/19q co-deletion

955 status. *** indicates Wilcoxon signed-rank test P -value < $1e-3$. (C) Heatmap depicting the

956 normalized expression z-score of genes that were differentially expressed between myeloid cells

957 from mesenchymal and non-mesenchymal TCGA tumors. Rows indicate genes and columns

958 indicate samples. Top sidebar indicates the bulk mesenchymal score of each sample divided by

959 1,000. Right sidebar indicates the $-\log_{10}$ adjusted Wilcoxon rank-sum test P -value of the

960 association for each gene. Bottom sidebar indicates the transcriptional subtype of each sample

961 per panel (A). (D) Box and ladder plots depicting the difference in the mean expression of the

962 mesenchymal myeloid signature between initial and recurrent IDH-wild-type tumors undergoing

963 a mesenchymal transition in GLASS. **** indicates Wilcoxon signed-rank test $P < 1e-5$. (E)

964 Boxplot depicting the mean mesenchymal myeloid signature expression for CIBERSORTx-

965 inferred myeloid profiles from different histological features in the Ivy GAP dataset. Features in

966 this dataset include the leading edge (LE), infiltrating tumor (IT), cellular tumor (CT),

967 pseudopalisading cells around necrosis (PAN), and microvascular proliferation (MVP). (F)
968 Representative multiplex immunofluorescence images of myeloid cells near blood vessels from
969 classical (left) and mesenchymal (right) IDH-wild-type tumors. Scale bars represent 20 μ m.

970

971 **Figure 6. Loss of heterozygosity in HLA genes is associated with increased somatic copy**
972 **number alterations in IDH-mutant non-1p/19q co-deleted glioma.** (A) Left: Sankey plot
973 indicating whether a tumor pair acquires or loses HLA LOH at recurrence. Colored lines reflect
974 the IDH and 1p/19q co-deletion status of the tumor pair and indicate HLA LOH in the initial tumor.
975 Dark gray lines indicate acquired HLA LOH. Right: Stacked bar plot indicating the proportion of
976 samples for each glioma subtype that acquired HLA LOH at recurrence. * indicates Fisher's exact
977 test P -value < 0.05. (B) Violin plot depicting the difference in T cell proportion in samples with and
978 without HLA LOH. P -values were calculated using the t-test. (C) Left: Ladder plots depicting the
979 change in SCNA burden between paired initial and recurrent IDH-mutant-noncode1 tumors that
980 did and did not acquire HLA LOH. P -values were calculated using the Wilcoxon signed-rank test.
981 Right: Boxplot depicting the difference in the change in SCNA burden between IDH-mutant-
982 noncode1 tumor pairs that did and did not acquire HLA LOH. P -value was calculated using the
983 Wilcoxon rank-sum test. Abbreviations: IDH-wild-type (IDHwt) and IDH-mutant (IDHmut).

984

985 **Figure 7. Recurrent diffuse gliomas can be grouped into three recurrence states based on**
986 **their shared cellular, genetic, and histological features.** Analysis of the GLASS dataset
987 reveals that IDH-wild-type and IDH-mutant tumors can be grouped into three "recurrence states":
988 neuronal, mesenchymal, and proliferative. Each of these tumor states are associated with unique
989 cellular and histological features and molecular alterations with some also associating with poor
990 patient survival.

991

992 **Figure S1. Validation of deconvolution results and IDH-wild-type-specific cell state**
993 **profiles. Related to Figure 1.** (A) Scatterplots depicting the association between the true
994 proportion and the CIBERSORTx-inferred proportion for each cell state in gene expression
995 profiles from synthetic mixtures composed of different combinations of single cells. (B)
996 Scatterplots depicting the association between the proportion of each malignant cell state
997 determined from single-cell RNAseq and the non-malignant cell-adjusted malignant cell state
998 proportion inferred from CIBERSORTx applied to each sample's respective bulk tumor RNAseq
999 profile. (C) Scatterplots depicting the association between the proportion of each malignant cell
1000 state, as well as myeloid cells, as determined using whole slide multiplex immunofluorescence
1001 scans and histo-cytometry. In all plots, Pearson correlation coefficients are indicated. (D) The
1002 average cell state composition of each bulk transcriptional subtype across initial (left) and
1003 recurrent (right) GLASS samples. (E) The average cell state composition of each bulk
1004 transcriptional subtype across all TCGA samples. (F) Left: Stacked bar plot indicating the
1005 proportion of samples of IDH-wild-type tumors that underwent a gross total resection at each
1006 timepoint. Right: The average proportions of each cell state for tumors that underwent a subtotal
1007 resection at initial and a gross total resection at recurrence (Subtotal-gross total) and tumors that
1008 underwent a gross total resection at both time points (Gross total-gross total). (G) Left: The
1009 average Neftel et al. cell state composition of each bulk transcriptional subtype for all initial IDH-
1010 wild-type GLASS tumors. Right: The average Neftel et. al cell state composition of initial and
1011 recurrent IDH-wild-type tumors. (H) The average cell state composition of initial and recurrent
1012 IDH-mutant tumors stratified by 1p/19q co-deletion status. Colors for all panels are indicated at
1013 the bottom of the figure. Abbreviations: IDH-wild-type (IDHwt) and IDH-mutant (IDHmut).

1014

1015 **Figure S2. Relationship between bulk subtype switching and cell state changes after**
1016 **adjusting for histological feature composition. Related to Figure 2.** (A) Representative H&E
1017 and multiplex immunofluorescence images for each Ivy GAP histological feature. Features were

1018 identified by a neuropathologist based on the H&E images on the left. The leading edge, infiltrating
1019 tumor, and cellular tumor features are from GLSS-LU-0B10 (primary), while the pseudopalisading
1020 cells around necrosis and microvascular proliferation features are from GLSS-LU-00B9 (primary).
1021 Scale bars represent 50 μm . (B) Bar plot depicting the $-\log_{10} P$ -value from a two-way ANOVA
1022 measuring whether the fractions of each cell state in a sample associate with the patient the
1023 sample was derived from (red bar) and the feature the sample represents (blue bar). Dashed line
1024 corresponds to $P = 0.05$. (C) Heatmap depicting the Pearson correlation coefficients measuring
1025 the association between pathologist and CIBERSORTx estimates of tumor core- and tumor
1026 periphery-related histological features. Evaluations were performed across 5 initial and 5
1027 recurrent samples (D) Scatterplots depicting the association between pathologist estimates of
1028 necrosis and CIBERSORTx estimates of the IvyGAP pseudopalisading cells around necrosis
1029 (PAN) feature in the GLASS and TCGA datasets. Shapes indicate initial and recurrence status.
1030 (E) Heatmap depicting the Pearson correlation coefficients measuring the association between
1031 pathologist estimates of recurrence-specific nucleated histological features and CIBERSORTx
1032 estimates of IvyGAP features. Evaluations were performed across 5 recurrent samples. In (C-E),
1033 pathologist estimates were based on the percent of the H&E slide area occupied by a given
1034 feature while CIBERSORTx estimates were based on RNAseq. (F) Heatmap depicting the
1035 changes in each malignant cell state between initial and recurrent tumors undergoing the
1036 indicated subtype transition. The initial subtype is indicated in the columns and the recurrent
1037 subtype is indicated in the rows. Each row of heatmaps reflects a different histological feature
1038 adjustment. Colors represent the change in fraction of the indicated features between initial and
1039 recurrent tumors, while * indicates a paired t-test P -value < 0.05 . (G) Left: Ladder plot depicting
1040 the change in the adjusted stem-like cell proportion between paired initial and recurrent tumors
1041 undergoing a proneural-to-mesenchymal transition. Right: The average adjusted proportions for
1042 malignant cells for the tumor pairs outlined on the left. Malignant cell proportions were adjusted
1043 for the presence of non-malignant cells and leading-edge content.

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Figure S3. Cell state composition changes associated with the acquisition and loss of somatic alterations. Related to Figure 3. (A) Left: Ladder plot depicting the change in the proliferating stem-like cell proportion between paired initial and recurrent IDH-wild-type tumors that acquired *CDNK2A* deletions or *CCND2* amplifications. Right: Stacked bar plot depicting the average proportions of each cell state for the tumor pairs in the ladder plots. (B) Ladder plots depicting the difference in microvascular proliferation fraction in IDH-mutant and IDH-wild-type tumors that underwent hypermutation at recurrence. (C) Forest plot depicting the results of a multivariable Cox proportional hazards model that included proliferating stem-like cell increase, age, initial grade, and 1p/19q co-deletion status as variables. Points represent the hazard ratio and lines represent the 95% confidence interval. *P*-values were calculated using the Wald test. (D) Left: Ladder plots depicting the change in granulocyte proportion in IDH-wild-type tumors that acquired mutations in *NF1* at recurrence. Right: The average proportions of each cell state for the tumor pairs in the ladder plots. (E) Non-malignant cell state differences in IDH-wild-type tumors that lost *EGFR* or *PDGFRA* amplifications at recurrence. Panel is split by alteration. Ladder plots depict the change in the non-malignant cell state proportion between paired initial and recurrent tumors while stacked bar plots depict the average proportions of each cell state for these tumors. (F) Sankey plot indicating whether the highest scoring transcriptional subtype changed at recurrence for the tumors depicted in (E). Each color reflects the transcriptional subtype in the initial tumors. Numbers in parentheses indicate number of samples. Subtype abbreviations: proneural (Pro.), classical (Class.) and mesenchymal (Mes.). (G) Ladder plots depicting the difference in T cell fraction in IDH-mutant and IDH-wild-type tumors that underwent hypermutation at recurrence. In all figures, *P*-values were calculated using a paired t-test unless otherwise noted. Abbreviations: IDH-wild-type (IDHwt) and IDH-mutant (IDHmut).

1069 **Figure S4. Validation and differential expression analysis of cell state-specific gene**
1070 **expression profiles. Related to Figure 4.** (A) Schema for single-cell RNAseq-based
1071 deconvolution of cell state-specific gene expression profiles. (B) Heatmap depicting the
1072 relationship between the CIBERSORTx-inferred gene expression profiles and gene expression
1073 profiles from analogous cell types from a FACS-purified ground truth dataset (Klemm et al.). In
1074 the CD45neg column in the Klemm et al. heatmap, which represents a composite gene
1075 expression profile from the non-immune cells purified from a collection of glioma tumors, gene
1076 expression patterns from all three malignant cell states can be observed. (C) Heatmap depicting
1077 the correlation coefficients between each CIBERSORTx-inferred cell state-specific gene
1078 expression profile and the gene expression profiles from the FACS-purified ground truth dataset.
1079 (D) Heatmap depicting the results from a GO enrichment analysis of the genes from the
1080 differentiated-like and stem-like-specific expression profiles that are significantly up-regulated in
1081 TCGA IDH-wild-type samples of the indicated transcriptional subtype versus samples from the
1082 remaining subtypes. The top two GO terms for each subtype are shown, including ties. Subtype
1083 abbreviations: proneural (Pro.), classical (Class.) and mesenchymal (Mes.). (E) Boxplot depicting
1084 the average signature expression in single cells of the indicated malignant cell states from
1085 unmatched initial and recurrent IDH-wild-type tumors. (F) Bar plot depicting the $-\log_{10}(\text{adjusted } P\text{-value})$
1086 from a GO enrichment analysis of the genes significantly up-regulated at recurrence in the
1087 differentiated-like and proliferating stem-like malignant cell-specific gene expression profiles from
1088 IDH-wild-type tumors. The top 5 GO terms for each cell state are included. Dashed line
1089 corresponds to adjusted $P\text{-value} < 0.05$. (G) H&E image used to define the histological features
1090 used for multiplex immunofluorescence staining in **Figure S4E**. Cellular tumor and infiltrating
1091 tumor features are highlighted in the indicated colors. (H) Bar plot depicting the $-\log_{10}(\text{adjusted } P\text{-value})$
1092 from a GO enrichment analysis of the genes significantly up-regulated at recurrence in the
1093 differentiated-like malignant cell-specific gene expression profiles from IDH-mutant tumors.
1094 Dashed line corresponds to adjusted $P\text{-value} < 0.1$. (I) Boxplot depicting the average signature

1095 expression in the analogous cell state-specific gene expression profiles for each IDH-mutant
1096 tumor pair in GLASS. Comparisons are stratified based on whether the tumor pair was grade
1097 stable or exhibited a grade increase at recurrence. (J) Boxplot depicting the average signature
1098 expression in single cells of the indicated malignant cell states from grade II and grade III. Across
1099 all panels, **** indicates Wilcoxon rank-sum test P -value $< 1e-5$, *** indicates Wilcoxon signed
1100 rank test P -value $< 1e-3$ and * indicates $P < 0.05$.

1101

1102 **Figure S5. Characterization of the mesenchymal myeloid signature. Related to Figure 5.**

1103 (A) Kaplan-Meier plot depicting the survival distributions of patients with tumors that were and
1104 were not mesenchymal at recurrence. P -value was calculated using the log-rank test. (B) Forest
1105 plot depicting the results of a multivariable Cox proportional hazards model that included recurrent
1106 tumor subtype, age, and initial grade as variables. Points represents the hazard ratio and lines
1107 represent the 95% confidence interval. P -values were calculated using the Wald test. (C) Box and
1108 ladder plots depicting the difference in the median-normalized mean expression of the Neftel et
1109 al. MES-like signature between initial (Init.) and recurrent (Rec.) IDH-wild-type tumors from
1110 GLASS undergoing a mesenchymal transition. Point colors indicate transcriptional subtype. P -
1111 values were calculated using the Wilcoxon signed-rank test. (D) Boxplots depicting the average
1112 macrophage and microglia gene expression signatures in CIBERSORTx-inferred myeloid-specific
1113 gene expression profiles from TCGA. Samples are stratified by IDH and 1p/19q co-deletion status
1114 (left) and bulk transcriptional subtype (right). **** indicates Wilcoxon rank-sum test P -value $< 1e$ -
1115 5. (E) Left: Principal component analysis plot of the CIBERSORTx-inferred myeloid profiles from
1116 TCGA and GTEx. Colors indicate bulk transcriptional subtype; shapes indicate tissue subtype.
1117 Right: Density plot depicting the distribution of principal component 1 (PC1) among each
1118 transcriptional subtype. (F) Bar plots depicting the Spearman correlation coefficients measuring
1119 the association between the myeloid-specific expression scores for the macrophage and microglia
1120 signatures versus the presence of the four Ivy GAP histological features in TCGA. The features

1121 measured were leading edge (LE), cellular tumor (CT), microvascular proliferation (MVP), and
1122 pseudopalisading cells around necrosis (PAN). (G) Heatmaps depicting the average normalized
1123 \log_{10} expression level of genes that were differentially expressed between myeloid cell states from
1124 initial and recurrent IDH-wild-type and IDH-mutant tumors in GLASS that did not undergo a
1125 subtype switch. Fractions on the right of each plot indicate the number of differentially expressed
1126 genes (numerator) out of the number of genes inferred for that cell state's profile in GLASS using
1127 CIBERSORTx (denominator). (H) Upset plot depicting the intersection of significantly up-
1128 regulated genes in the myeloid-specific gene expression profiles from each transcriptional
1129 subtype relative to normal brain cortex. Intersections between signatures are shown in the
1130 combination matrix. Number of genes uniquely found in each set are indicated above each bar.
1131 (I) Bar plot depicting the $-\log_{10}(\text{adjusted } P\text{-value})$ from a GO enrichment analysis for the genes in
1132 the mesenchymal myeloid signature. The GO term "chemokine-mediated signaling pathway" has
1133 been abbreviated to "chemokine-med. sign. pathway." (J) Scatterplot depicting the association
1134 between the mean mesenchymal myeloid signature expression in single myeloid cells and the
1135 mesenchymal subtype score calculated from bulk RNAseq for each patient. (K) Bar plots depicting
1136 the Spearman correlation coefficients measuring the association between the myeloid-specific
1137 expression scores for the mesenchymal myeloid signature versus the presence of the four Ivy
1138 GAP histological features in TCGA, as in (F). Abbreviations: IDH-wild-type (IDHwt) and IDH-
1139 mutant (IDHmut).

1140

1141 **Figure S6. Analysis of neoantigen-mediated T cell selection in glioma. Related to Figure 6.**

1142 (A) Scatterplots depicting the association between the T cell proportion and the neoantigen
1143 depletion rate in initial and recurrent GLASS samples. (B) Box and ladder plots depicting the
1144 difference in the number of neoantigens binding to the kept and lost allele. Points are colored
1145 based on whether the sample was an initial or recurrent tumor. P -values were calculated using
1146 the Wilcoxon signed-rank test. (C) Violin plots depicting the distribution of the somatic copy

1147 number alteration burden in initial and recurrent IDH-wild-type GLASS samples that did and did
1148 not exhibit HLA LOH. *P*-values were calculated using the Wilcoxon rank-sum test.

1149

1150 **Methods**

1151 **GLASS Datasets**

1152 Datasets added to GLASS came from both published and unpublished sources (**Table S1**).

1153 Collectively, the newly added data consisted of exomes from 83 glioma samples (40 patients) and
1154 RNA-sequencing data from 351 samples (184 patients).

1155

1156 Newly generated whole exome data and RNAseq data was collected for a cohort of frozen
1157 samples from Henry Ford Health System. From each sample, DNA and RNA was simultaneously
1158 extracted using the AllPrep DNA/RNA Mini Kit from Qiagen (#80204). Exon capture was then
1159 performed using the Agilent's SureSelect XT Low-Input Reagent Kit and the V6 + COSMIC
1160 capture library and the resulting reads were subjected to 150 base pair paired-end sequencing at
1161 the University of Southern California using an Illumina NovaSeq 6000. RNA from these tissues
1162 was processed and sequenced at Psomagen. New RNAseq data was also generated for cohorts
1163 coming from Case Western Reserve University, the Chinese University of Hong Kong, and MD
1164 Anderson Cancer Center. For Case Western Reserve University, RNA from frozen tissues was
1165 processed at Tempus (Chicago, IL) using the Tempus xO assay and then sequencing using an
1166 Illumina HiSeq 4000 platform. For the Chinese University of Hong Kong cohort, RNAseq libraries
1167 were prepared with the KAPA Stranded mRNAseq kit (Roche) per manufacturer's instructions
1168 and then sequenced at The Jackson Laboratory for Genomic Medicine using an Illumina
1169 HiSeq4000 platform generating paired end reads of 75 base pairs. For the MD Anderson cohort,
1170 purified double-stranded cDNA generated from 150 ng of FFPE sample-derived RNA was
1171 prepared using the NuGEN Ovation RNAseq System and subjected to paired-end sequencing
1172 using a HiSeq 2000 or HiSeq 2500 Sequencing System.

1173

1174 The remaining datasets were generated as described in their respective publications. For most of
1175 these cohorts, whole exome and/or whole genome sequencing data were downloaded and
1176 processed as described during creation of the initial GLASS dataset (Barthel et al., 2019).
1177 RNAseq fastq files from the Samsung Medical Center (SM) cohort were delivered via hard disk
1178 and are available to download from the European Genome-Phenome Archive (EGA) under
1179 accession numbers EGAS00001001041 and EGAS00001001880 (Kim et al., 2015b; Wang et al.,
1180 2016). RNAseq bam files for the original Henry Ford Health System (HF) and the University of
1181 California San Francisco (SF) cohorts were downloaded from EGA under accession numbers
1182 EGAS00001001033 and EGAS00001001255, respectively, and converted to fastq files for
1183 subsequent processing using bedtools (Kim et al., 2015a; Mazor et al., 2015). RNAseq fastq files
1184 for the University of Leeds (LU) cohort were downloaded from EGA under accession number
1185 EGAS00001003790 (Droop et al., 2018). For the first Columbia cohort (CU-R), which consisted
1186 of samples originally collected from the Istituto Neurologico C. Besta, RNAfastq files were
1187 delivered via hard disk and are available to download at the Sequencing Read Archive (SRA)
1188 under BioProject number PRJNA320312 (Wang et al., 2016). For the second Columbia cohort
1189 (CU-P), which featured samples that had been treated with immune checkpoint inhibitors, raw
1190 fastq reads for whole exome and RNAseq were obtained from SRA under BioProject number
1191 PRJNA482620 (Zhao et al., 2019). RNAseq fastq files from the Low Grade Glioma (LGG) and
1192 Glioblastoma Multiforme (GBM) projects in TCGA were obtained from the Genomic Data
1193 Commons legacy archive (<https://portal.gdc.cancer.gov/legacy-archive/>) (Brennan et al., 2013;
1194 Cancer Genome Atlas Research et al., 2015).

1195

1196 **Public Datasets**

1197 Processed RNAseq data from the TCGA glioma (GBMLGG) cohort was obtained from GDAC
1198 FireHose (RNAseqV2, RSEM). Normalized gene-level fragments per kilobase million (FPKM) for

1199 the Ivy Glioblastoma Atlas Project (Ivy GAP) dataset were obtained from the Ivy GAP website
1200 (<https://glioblastoma.alleninstitute.org/static/download.html>) (Puchalski et al., 2018). Processed
1201 single-cell data and associated metadata for a set of 28 IDH-wild-type glioblastomas processed
1202 using SmartSeq2 was obtained from the Broad Single Cell Portal (Study: Single cell RNA-seq of
1203 adult and pediatric glioblastoma;
1204 [https://singlecell.broadinstitute.org/single_cell/study/SCP393/single-cell-rna-seq-of-adult-and-](https://singlecell.broadinstitute.org/single_cell/study/SCP393/single-cell-rna-seq-of-adult-and-pediatric-glioblastoma)
1205 [pediatric-glioblastoma](https://singlecell.broadinstitute.org/single_cell/study/SCP393/single-cell-rna-seq-of-adult-and-pediatric-glioblastoma)) (Neftel et al., 2019). Raw count data and clinical annotation data from a
1206 set of glioma-derived cell populations purified using fluorescence activated cell sorting (FACS)
1207 was obtained from the Brain Tumor Immune Micro Environment (BrainTIME) portal and converted
1208 to counts per million (CPM) for downstream analysis (<https://joycelab.shinyapps.io/braintime/>)
1209 (Klemm et al., 2020).

1210

1211 **Whole exome and whole genome analysis**

1212 Whole exome and genome alignment, fingerprinting, variant detection, variant post-processing,
1213 mutation burden calculation, copy number segmentation, copy number calling, copy number-
1214 based purity, ploidy, HLA typing, and neoantigen calling were all performed using previously
1215 described pipelines that were developed during the initial GLASS data release (Barthel et al.,
1216 2019). Briefly, whole exome and whole genome reads were aligned to the b37 genome
1217 (human_g1k_v37_decoy) using BWA MEM 0.7.17 and pre-processed according to GATK Best
1218 Practices with GATK 4.0.10.1. Fingerprinting on the resulting files was performed using
1219 'CrosscheckFingerprints' to confirm all readgroups from a given sample and all samples from a
1220 given patient match, with all mismatches being labelled and dropped from downstream analysis.
1221 Somatic mutations were called using GATK4.1 MuTect2. Hypermutation was defined for all
1222 recurrent tumors that had more than 10 mutations per megabase sequenced, as described
1223 previously (Barthel et al., 2019). Copy number segmentation and calling was performed according
1224 to GATK Best Practices as previously described. Copy number-based tumor purity and ploidy

1225 were determined using TITAN (Ha et al., 2014). Four-digit HLA class I types were determined
1226 from the normal bams for each sample using OptiType v1.3.2 (Szolek et al., 2014). Neoantigens
1227 were called from each patient's somatic mutations and HLA types using pVACseq v4.0.10
1228 (Hundal et al., 2016). Neoantigen depletion was calculated as described previously (Barthel et
1229 al., 2019). Loss of heterozygosity (LOH) for each sample's HLA type was called from their
1230 respective matched tumor and normal bam files using LOHHLA run with default parameters and
1231 a coverage filter of 10 (<https://bitbucket.org/mcgranahanlab/lohhlal/>) (McGranahan et al., 2017).
1232 HLA LOH was called if the estimated copy number for an allele using binning and B-allele
1233 frequency was < 0.5 and the P -value for allelic imbalance was < 0.05 (paired t-test).

1234

1235 **RNA preprocessing**

1236 To ensure each RNAseq file matched to the DNA and RNAseq files from their respective sample
1237 and patient, RNAseq fastq files were aligned to the b37 genome using STARv2.7.5 and the
1238 resulting bams were then preprocessed using the same pipelines described for DNA sequencing
1239 (Barthel et al., 2019). Fingerprinting was then performed on each bam at the readgroup and
1240 patient levels using 'CrosscheckFingerprints.' For each patient-level comparison, each RNA bam
1241 was compared to all other RNA and DNA bams coming from the same patient. All mismatches
1242 were labelled and dropped from downstream analysis.

1243

1244 RNAseq fastq files were pre-processed with fastp v0.20.0. Transcripts per million (TPM) values
1245 were then calculated from each sample's set pre-processed files using kallisto v0.46.0 inputted
1246 with an index file built from the Ensemblv75 reference transcriptome. Strand-specific library
1247 preparation information was obtained for each sample from the source provider or using
1248 STARv2.7.5 quantMode set with the 'GeneCounts' parameter. The resulting TPM values for each
1249 sample were combined into a transcript expression matrix for downstream analysis. To create a

1250 gene expression matrix, transcript TPM values were collapsed and summed by their respective
1251 gene symbols.

1252

1253 **Quality control**

1254 For DNA samples to be included in longitudinal downstream analyses, two samples from a given
1255 patient had to pass a previously described quality control process based on fingerprinting,
1256 coverage, copy number variation, and clinical annotation criteria (Barthel et al., 2019). The
1257 resulting set of 243 whole exome or whole genome tumor pairs, known as the “gold set”, was
1258 used in all downstream DNA-only analyses. For RNA samples to be included in longitudinal
1259 downstream analyses, two samples from a given patient had to pass a patient-level fingerprinting
1260 filter that ensured that the RNA samples matched each other and the patient’s respective DNA
1261 samples if available, as well as a clinical annotation filter. The resulting set of 150 RNAseq pairs,
1262 known as the “RNA silver set”, was used in all downstream RNA-only analyses. Across the gold
1263 set and the RNA silver set, there were 101 tumor pairs that had DNA and RNA from the same
1264 sample at both timepoints. This overlapping set of pairs, known as the “platinum set”, was used
1265 in all downstream analyses that integrated DNA and RNA data.

1266

1267 **Bulk transcriptional subtype classification**

1268 Bulk transcriptional subtyping was performed on each GLASS or TCGA sample’s processed
1269 RNAseq profile using the “ssgsea.GBM.classification” R package (Wang et al., 2017). This
1270 method outputs an enrichment score quantifying the representation each of the three bulk glioma
1271 subtypes in a sample as well as a *P*-value indicating the significance of this representation. For
1272 each sample, the subtype with the lowest *P*-value was designated as that sample’s bulk
1273 transcriptional subtype. In cases where there were ties between subtypes, the subtype with the
1274 highest enrichment score was chosen.

1275

1276 **Joint single-cell and bulk RNAseq dataset**

1277 Single-cell and bulk RNA sequencing data were generated and processed as previously
1278 described (Johnson et al., 2021). Briefly, tumor surgical specimens were freshly collected,
1279 minced, and partitioned into single-cell and bulk fractions from the same tumor aliquot. The
1280 tissues aliquoted for single cell analyses were then mechanically and enzymatically dissociated
1281 using the Brain Tumor Dissociation Kit (P) according to the manufacturer's protocol (Miltenyi Cat.
1282 No. 130-095-942). FACS was performed to select for viable single cells (Propidium Iodide-,
1283 Calcein+ singlets) and enrich for tumor cells by limiting the proportion of non-tumor cells (e.g.,
1284 immune (CD45+) and endothelial (CD31+) cells). Sorted cells were then loaded on a 10X
1285 Chromium chip using the single-cell 3' mRNA kit according to the manufacturer's protocol (10X
1286 Genomics). A limitation of single-cell dissociation techniques is the exclusion of specific cell types,
1287 including neurons, that are found in glioma and surrounding tissue. Prior publications have
1288 estimated the neuronal content of central nervous system tumors to be less than 5% and therefore
1289 likely represent a minor non-malignant cell population in our dataset (Grabovska et al., 2020). The
1290 Cell Ranger pipeline (v3.0.2) was used to convert Illumina base call files to fastq files and align
1291 fastqs to hg19 10X reference genome (version 1.2.0) to be compatible with our bulk sequencing
1292 data. Data preprocessing and analysis was performed using the Scanpy package (1.3.7) (Wolf et
1293 al., 2018) with batch correction performed using BBKNN (Polanski et al., 2020). RNA was
1294 extracted for tissues with sufficient tissue and bulk RNAseq libraries were prepared with KAPA
1295 mRNA HyperPrep kit (Roche). Bulk RNA sequencing data was processed with the same pipeline
1296 as the GLASS samples.

1297

1298 **Deconvolution analyses**

1299 Cellular proportions and cell state-specific gene expression matrices were inferred from bulk
1300 RNAseq gene expression matrices using CIBERSORTx (Newman et al., 2019). Reference
1301 scRNAseq signature matrices were created from our internal 10x-derived scRNAseq dataset

1302 (Johnson et al., 2021) and a publicly available SmartSeq2-derived scRNAseq dataset (Neffel et
1303 al., 2019) using the ‘Create Signature Matrix’ module on the CIBERSORTx webserver
1304 (<https://cibersortx.stanford.edu/>) using default parameters and quantile normalization disabled.
1305 The Ivy GAP signature matrix was downloaded from a prior publication (Puchalski et al., 2018).
1306 The CIBERSORTx webserver currently recommends users input no more than 5,000 different
1307 single-cell profiles when creating their signature matrix (Steen et al., 2020). To meet this
1308 recommendation, our internal scRNAseq dataset, which is made up of 55,284 single cells, was
1309 randomly downsampled to 5,000 cells using the ‘sample’ command in R with the seed set to 11.
1310 The cells not included in signature matrix formation were then set aside for validation analyses.
1311
1312 Single-cell-derived cellular proportions and cell state-specific gene expression profiles were
1313 inferred from bulk RNAseq datasets using the CIBERSORTx High-Resolution docker container
1314 (<https://hub.docker.com/r/cibersortx/hires>) following CIBERSORTx instructions. For all runs, the
1315 bulk RNAseq dataset was input as the ‘mixture’ file and the respective signature matrix was input
1316 as the ‘sigmatrix’ file. For runs using our 10x-derived internal scRNAseq signatures, batch
1317 correction was done in ‘S-mode’ by setting the ‘rmbatchSmode’ parameter to TRUE, while for
1318 runs using SmartSeq2-derived scRNAseq signatures batch correction was done in ‘B-mode’ by
1319 setting the ‘rmbatchBmode’ parameter to TRUE. For each run, the inputted signature matrix’s
1320 respective CIBERSORTx-created “source gene expression profile” was input for batch correction.
1321 For all runs, the ‘subsetgenes’ parameter was set to a file containing the intersection of the gene
1322 symbols between the run’s respective source gene expression profile and the bulk RNAseq matrix
1323 that was being deconvoluted. For the run applying our internal scRNAseq dataset to the bulk
1324 GLASS RNAseq matrix, the ‘groundtruth’ parameter was set to a ground truth FACS-purified
1325 dataset that was generated as described below. Cellular proportions representing pre-created Ivy
1326 GAP signatures were inferred using the ‘Impute Cell Fractions’ module on the CIBERSORTx

1327 webserver set to relative mode with quantile normalization and batch correction disabled and 100
1328 permutations for significance analysis.

1329

1330 **Immunofluorescence staining and image acquisition**

1331 Tissue samples used in multiplex immunofluorescence microscopy were formalin-fixed, paraffin-
1332 embedded and sectioned to a thickness of 5 μ m unless otherwise stated. Tissue sections were
1333 heated at 58°C for 10 minutes, dewaxed in HistoClear (National Diagnostics) for 20 min and
1334 rehydrated in a graded series of alcohol (ethanol:deionized water 100:0, 90:10, 70:30, 50:50,
1335 0:100; 5 min each). Heat-induced epitope retrieval (95°C) was conducted in citrate buffer (pH 6)
1336 for 15 min using a BioSB TinoRetriever. After antigen retrieval, tissue sections were permeabilized
1337 with PBS 0.1% Triton-X100, washed with PBS and consecutively treated with Fc Receptor Block
1338 (Innovex bioscience) for 40 min + Background Buster (Innovex bioscience) for an additional 30
1339 min. The sections were then stained with primary antibodies, diluted in PBS + 5% BSA overnight
1340 at 4°C, and then washed and stained with the secondary antibodies at room temperature for 30
1341 minutes. Afterwards, tissues were washed and secondary antibodies were saturated with rabbit
1342 normal serum diluted at 1/20 in PBS for 15 minutes at room temperature. Tissues were then
1343 stained with directly conjugated antibody mix for 1 hour at room temperature and washed. Nuclei
1344 were counterstained with 4',6-diamidino-2-phenylindole (1 μ g/mL) or SytoxBlue 1/3000 for 2
1345 minutes. Tissues were then mounted in Fluoromount-G mounting media.

1346

1347 Images were acquired on a Leica SP8 confocal microscope equipped with an automated
1348 motorized stage. Spectral unmixing was achieved with combination of white light laser tuned laser
1349 line for each specific fluorophore, tunable detection window for each marker and sequential
1350 acquisition. Whole-slide scans were acquired with a dry 20x objective, while partial slide scans
1351 for OSM and SNAP25 panels were acquired with a 40x oil immersion objective. Tiles were stitched
1352 and max projected using Leica LAS X software.

1353

1354 **Histo-cytometry**

1355 Quantification of single-cell protein expression from immunofluorescence scans was performed
1356 using histo-cytometry as previously described (Gerner et al., 2012; Wang et al., 2018; Wu et al.,
1357 2018). Briefly, each whole slide tissue scan was segmented using Imaris software (version 9.0.2).
1358 Using the “spot” function in Imaris, images were segmented using individual cells with a nucleus
1359 equal or larger than 5 μm as a seeding point to extend each cells’ surface. The accuracy of the
1360 segmentation was manually verified for each sample and adjusted if needed. Finally, for each
1361 generated spot, x and y coordinates and mean intensity values for all channels were combined
1362 and exported into a csv file for further analysis in R.

1363

1364 **Validation of cell state proportions**

1365 Cell state proportions derived from our internal scRNAseq dataset were validated using three
1366 approaches. In the first approach, synthetic mixtures were made using the single-cell gene
1367 expression profiles that had been left out of signature creation. Each synthetic mixture
1368 represented the average expression profile of 5,000 single cells where the number of cells of one
1369 cell state were manually set and the remaining cells were randomly sampled. Each cell state had
1370 its level manually set in 11 mixtures, where it represented 0% of the cells in the first mixture and
1371 then increased in 10% increments until reaching 100% in the final mixture. In cases where there
1372 were fewer than 5,000 single cells of a given cell state, making 100% representation not possible,
1373 the preset proportion instead represented the percent of available cells of that cell state rather
1374 than the percent of cells in the mixture. Each synthetic mixture had its true proportions recorded
1375 and the resulting mixtures were input into CIBERSORTx for deconvolution. Comparisons of the
1376 true and inferred proportions were then performed through correlation analysis.

1377

1378 In the second approach, the cell state proportions inferred from bulk RNAseq data were compared
1379 to the cell state proportions quantified by scRNAseq for each sample in our internal scRNAseq
1380 dataset. Samples in the scRNAseq dataset were enriched for CD45⁺ cells via FACS and therefore
1381 precluded true cell state abundance when considering both malignant and non-malignant cells.
1382 To address this, comparisons were restricted to the relative proportions of each malignant cell
1383 state. Non-malignant cell proportions were removed, and malignant cells proportions were then
1384 renormalized so that the sum of each malignant cell state proportion in each sample added up to
1385 1.

1386

1387 In the third approach, cell state proportions inferred from bulk RNAseq data were compared to
1388 the cell state proportions quantified through multiplex immunofluorescence and histo-cytometry
1389 analyses performed on whole tissue scans for a subset of samples in the GLASS cohort. To
1390 determine the identity of each cell in a tissue scan, expression thresholds were set for each
1391 marker based on the marker's expression distribution across the dataset. For bimodal
1392 distributions the threshold was set to the local minima between the two maxima, while for normal
1393 distributions the threshold was set to the global maximum. Cells that were negative for all markers
1394 were excluded from further analysis. To facilitate comparisons between expression and
1395 immunofluorescence-based estimates, analyses were restricted only to the cell states identified
1396 in both platforms, and the resulting fractions were renormalized so that the sum of each proportion
1397 added up to 1.

1398

1399 **Annotation and validation of histological features**

1400 Digitized images of H&E slides were obtained for a subset of GLASS samples and stored centrally
1401 on the Digital Slide Archive (<https://styx.neurology.emory.edu/girder/>). In a subset of samples for
1402 which FFPE slides were available for multiplex immunofluorescence staining, representative
1403 histological features were digitally outlined by a board-certified neuropathologist.

1404

1405 Transcriptomic histological deconvolution was validated by comparing expression-based and
1406 neuropathologist-based estimates of feature abundance. To accomplish this, a team of six
1407 neuropathologists were instructed to estimate the proportion of the slide area occupied by
1408 different histological features for 10 GLASS samples (5 primary-recurrent tumor pairs) where the
1409 H&E slide was directly adjacent to the tumor region sent for RNA-sequencing. Neuropathologists
1410 were blinded to the type of glioma in each slide and did not have knowledge of the expression-
1411 based scores prior to scoring. To standardize feature evaluation across neuropathologists,
1412 common definitions for each feature were established. Definitions for features expected to be
1413 observed in primary and recurrent tumors were loosely based on those used by Ivy GAP, while
1414 recurrence-specific features were collaboratively defined by the neuropathologist team. During
1415 the evaluation process, each evaluator received a template with these feature definitions and was
1416 instructed to annotate the entire slide so that the total estimates for each sample summed to 100%
1417 (**Table S5**). Consensus pathology estimates for each slide were then calculated as the mean
1418 neuropathologist estimate of a given feature and were used for all downstream analyses. Results
1419 for the necrosis feature samples were additionally reproduced using publicly available
1420 neuropathologist estimates from TCGA H&E slides (Cooper et al., 2012).

1421

1422 **Validation of cell state gene expression profiles**

1423 Concordance between CIBERSORTx-inferred cell state-specific gene expression profiles and a
1424 ground truth set of FACS-purified gene expression profiles was assessed using the ‘groundtruth’
1425 parameter in CIBERSORTx. The ground truth dataset used in this step was generated from a
1426 previously released glioma dataset (Klemm et al., 2020) by collapsing all glioma-derived CD45⁺
1427 profiles into an average CD45⁺ profile and all glioma-derived macrophage/microglia profiles into
1428 an average myeloid cell profile. This dataset was input into CIBERSORTx using the ‘groundtruth’
1429 parameter during the run applying our internal scRNAseq signature matrix to the GLASS bulk

1430 RNAseq dataset. The resulting quality control files output during this run, primarily
1431 “SM_GEPs_HeatMap.txt”, were then used to perform correlation analyses assessing the
1432 similarity between the inferred malignant cell and myeloid profiles and the ground truth profiles.

1433

1434 **Analysis of cell state-specific gene expression profiles**

1435 To facilitate downstream analyses on each CIBERSORTx-inferred cell state-specific gene
1436 expression profile, each of the resulting expression matrices were log10-transformed and all
1437 genes that could not be imputed or had a variance of 0 across the dataset were removed. For
1438 each cell state-specific gene expression matrix, Wilcoxon signed-rank tests were used to
1439 determine the differentially expressed genes between initial and recurrent tumors and the
1440 resulting *P*-values were corrected for multiple testing using the Benjamini-Hochberg procedure.
1441 Signature scores in cell state-specific gene expression profiles and single-cell RNAseq profiles
1442 were defined as the average expression of the genes in the signature. In cases where the
1443 expression of some of the genes in the signature could not be determined, the intersection of the
1444 signature and the available genes was taken when calculating the signature score. For GO
1445 enrichment analyses on signatures derived from cell state-specific gene expression profiles, the
1446 background gene set only included the genes CIBERSORTx was able to impute for the cell state
1447 from which the signature was derived.

1448

1449 **Histological feature adjustment**

1450 For analyses examining how histological features influenced subtype switching, a tumor sample’s
1451 cell state composition profile was adjusted to remove cell states that could be attributed to a
1452 specific histological feature. To do this, the tumor sample’s proportion of a given histological
1453 feature was multiplied by the average proportion of each cell state from all samples of that feature
1454 in Ivy GAP. These numbers were then subtracted from their respective cell state’s proportion in
1455 the tumor sample and the resulting profile was then renormalized so that all proportions summed

1456 to 1. In cases where the new cell state proportion was less than 0, the value was set to 0 before
1457 renormalization.

1458

1459 **Statistical analysis**

1460 All data analyses were conducted in R 3.6.1 and PostgreSQL 10.6. GO enrichment analyses were
1461 performed using the “classic” algorithm in the R package “topGO” v2.38.1. When comparing
1462 variables between groups, t-tests were used for cell state proportions while non-parametric tests
1463 were used for all other variables (i.e., gene expression, signature score, neoantigen number).
1464 Clinical variables used throughout the study were defined as previously described in the
1465 Supplementary Information of the original GLASS study (Barthel et al., 2019).

1466

1467 **Code and data availability**

1468 All custom scripts, pipelines, and code used in figure creation will be made available at the time
1469 of publication on the project’s Github page. Processed data for the GLASS consortium is available
1470 on Synapse (<https://www.synapse.org/glass>).

1471

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1473

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