

Longitudinal molecular trajectories of diffuse glioma in adults

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1 LONGITUDINAL MOLECULAR TRAJECTORIES OF DIFFUSE GLIOMA IN ADULTS

2

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109

110 **ABSTRACT**

111 The evolutionary processes that drive universal therapeutic resistance in adult patients with
112 diffuse glioma remain unclear^{1,2}. Here, we analyzed temporally separated DNA sequencing data
113 and matched clinical annotation from 222 patients with glioma. Through mutational and copy
114 number analyses across the three major subtypes of diffuse glioma, we observed that driver
115 genes detected at initial disease were retained at recurrence, while there was little evidence of
116 recurrence-specific gene alterations. Treatment with alkylating-agents resulted in a
117 hypermutator phenotype at different rates across glioma subtypes, and hypermutation was not
118 associated with differences in survival. Acquired aneuploidy was frequently detected in recurrent
119 gliomas characterized by presence of an IDH mutation but without 1p/19q codeletion and further
120 converged with acquired cell cycle alterations and poor outcomes. We show that the clonal
121 architecture of each tumor remains similar over time and that absence of clonal selection was
122 associated with increased survival. Finally, we did not observe differences in immunoediting
123 levels between initial and recurrent glioma. Our results collectively argue that the strongest
124 selective pressures occur early during glioma development and that current therapies shape this
125 evolution in a largely stochastic manner.

126

127 **INTRODUCTION**

128 Diffuse glioma is the most common malignant brain tumor in adults and invariably relapse
129 despite treatment with surgery, radiotherapy, and chemotherapy. The molecular landscape of
130 glioma at diagnosis has been extensively characterized³⁻⁹. While these efforts have led to the
131 identification of driver genes and clinically relevant subtypes^{10,11}, it is unknown how the glioma
132 genetic landscape evolves over time and in response to therapy.

133 Intratumoral heterogeneity is a well-recognized characteristic of gliomas and results from
134 selective pressures such as a limited availability of nutrients, clonal competition, and
135 treatment¹²⁻¹⁵. Tumors are thought to circumvent these growth bottlenecks via dynamic
136 competition of subclones resulting in the most favorable environment for tumor sustenance¹.
137 Recent studies have suggested that stochastic changes in clone frequency (i.e. neutral
138 evolution) and immunogenic surveillance may further contribute to the observed intratumoral
139 heterogeneity^{16,17}. An understanding of evolutionary dynamics at multiple time points is needed
140 to develop strategies aimed at delaying or preventing the onset of tumor progression.

141 To investigate clonal dynamics over time and in response to therapeutic pressures, we
142 established the Glioma Longitudinal Analysis (GLASS) Consortium. GLASS is a community-
143 driven effort that seeks to overcome the logistical challenges in constructing adequately
144 powered longitudinal genomic glioma datasets by pooling datasets from patients treated at
145 institutions worldwide¹⁸. We have analyzed longitudinal profiles across the three molecular
146 glioma subtypes to identify the molecular processes active at initial and recurrent time points.
147 These analyses identified few common features of glioma evolution across subtypes, and
148 instead pointed toward highly variable and patient-specific trajectories of genomic alterations.

149 **RESULTS**

150 **GLASS cohort**

151 We pooled existing and newly generated longitudinal DNA sequencing datasets from 288
152 patients treated at 35 hospitals (Supplementary Table 1, Extended Data Fig. 1). After applying
153 quality filters, tumor samples from 222 patients with high-quality data in at least two time points
154 were classified according to molecular markers into three major glioma subtypes: 1. IDH-mutant
155 and chromosome 1p/19q co-deleted (IDHmutant-codel; n = 25) 2. IDH-mutant without
156 chromosome 1p/19q codeletion (IDHmutant-noncodel; n = 63) and 3. IDH wild type (IDHwt; n =
157 134), in alignment with the World Health Organization classification of Central Nervous System
158 tumors^{10,11}. For each patient we selected two time-separated tumor samples, henceforth initial
159 and recurrence, for further analysis.

160 **Mutational burdens and processes over time**

161 We first evaluated temporal changes in mutational burden and processes to understand general
162 patterns of glioma evolution. Mutation burdens in initial tumors were comparable with previously
163 reported rates^{6,7,19}. 2.20 mutations (single-nucleotide variants and small insertions/deletions)
164 per Megabase (Mutations/Mb) for IDHmutant-codels; 2.52 Mutations/Mb for IDHmutant-
165 noncodels; and 2.85 Mutations/Mb for IDHwt glioma (Fig. 1a; Extended Data Fig. 2a). Excluding
166 DNA hypermutation cases (> 10 Mutations/Mb, n = 35), the mutation burden increased after
167 recurrence in 70% of the cohort (Extended Data Fig. 2a). To study changes during tumor
168 progression, we separated mutations into three fractions: initial only, recurrence only, or shared.
169 Interestingly, private fraction but not shared fraction mutation burdens were comparable
170 between subtypes (Extended Data Fig. 2b). Patient age at diagnosis was significantly
171 associated with the shared mutational burden and to a lesser extent the mutation burden private
172 to the initial tumor (Extended Data Fig. 2c). On average, tumors with longer time to recurrence
173 had slightly higher mutation burdens (Extended Data Fig. 2d).

174 These fraction-specific differences in mutation burden suggested that the activity of
175 distinct mutational processes may also be time-dependent. We therefore classified mutations in
176 each fraction according to the Catalogue of Somatic Mutations in Cancer (COSMIC) signature
177 database²⁰. As expected, signature activity was closely related to subtype and fraction (Fig. 1b,
178 Extended Data Fig. 3a). Signature 1 (aging) was nearly always the dominant signature amongst
179 shared mutations in IDHwt tumors, whereas the shared fraction in IDHmut-noncodel and
180 IDHmut-codel tumors - tumor subtypes associated with a younger age of diagnosis - additionally
181 showed a strong presence of signature 16 (unknown etiology). Signatures 3 (double strand
182 break repair) and 15 (mismatch repair) along with signature 8 (unknown etiology) were mostly
183 confined to the private fractions, suggesting that these processes were of lesser importance to
184 tumor maintenance than those associated with aging.

185 Treatment of glioma includes alkylating agents that can induce post-treatment
186 hypermutation²¹⁻²³. We observed enrichment of the associated signature 11 in recurrent tumors
187 with a mutational load exceeding 10 Mutations/Mb and treated with alkylating agents (Fig. 1a,
188 Extended Data Fig. 3b). Treatment-associated hypermutation occurred most frequently among
189 IDHmutant-noncodels (47%), followed by IDHmutant-codels (25%), and IDHwt gliomas (16%)
190 (Fig. 1c). The difference in the proportion of hypermutation events was significantly different
191 between the three glioma subtypes (Fisher's exact-test $P = 2.0e-03$), suggesting that IDHmutant
192 noncodels are most sensitive to developing a hypermutator phenotype²⁴.

193 Treatment-induced hypermutation has been associated with disease progression²³. We
194 did not find overall survival differences between alkylating agent-treated hypermutators and
195 alkylating agent-treated non-hypermutators independent of age, subtype, and *MGMT*
196 methylation status (Fig. 1d, Supplementary Table 2a-b). In order to further assess the
197 pathogenicity of acquired mutations, we studied their clonality²⁵. Newly acquired clonal
198 mutations have penetrated most of the tumor (i.e., a selective sweep) between initial and
199 recurrence and mark clonal expansion²⁶. Conversely, acquired subclonal mutations are less
200 prevalent, and therefore less likely to drive disease progression. Previous reports have
201 suggested that alkylating agent-associated mutations hypermutation are frequently clonal²⁷. We
202 found that in 48% of hypermutated tumors a majority of the recurrence-only mutations were
203 clonal, potentially reflecting cases where a selective sweep occurred (Extended Data Fig. 4a).
204 However, IDHmut-noncodeI hypermutators with predominantly clonal mutations did not show
205 differences in survival compared with those harboring predominantly subclonal mutations (log-
206 rank test $P = 0.38$, Extended Data Fig. 4b). Alkylating agents such as temozolomide prolong
207 survival of adult patients with glioma^{28,29}. Our results show that treatment-induced
208 hypermutation is common across subtypes and does not associate with a reduced overall
209 survival supporting the noted benefit of alkylating agent therapy.

210 **Selective pressures during glioma evolution**

211 Environmental and treatment-induced pressures may drive changes in clonal architecture at
212 recurrence. To evaluate selection over time we clustered copy number changes and mutations
213 based on their cancer cell fraction (CCF). CCF values represent the fraction of cancer cells
214 harboring a given alteration and reflect the relative timing of events, since alterations that are
215 present in a subset of cancer cells likely occurred later than events present in all cancer cells
216 (Fig. 2a). Most tumors (84%) demonstrated a mutational cluster with $CCF > 50\%$ that persisted
217 from the initial tumor into recurrence, likely reflecting the tumor trunk and harboring the tumor-
218 initiating driver mutations (Fig. 2b, Extended Data Fig. 5a)³⁰. To determine changes in clonal
219 dominance over time we ranked clusters within each sample by their CCF and found similarities
220 in clonal architecture throughout the course of disease (Kendall rank correlation, $\tau = 0.20$, $P =$
221 $3.76E-24$, Fig. 2b, Extended Data Fig. 5b-d). These results suggested that the clonal structure
222 at initial disease mostly persisted into recurrence.

223 To deepen our assessment of selective pressures, we evaluated selection in initial and
224 recurrent tumors by determining the normalized ratio between non-synonymous and
225 synonymous mutations (dNdScv). Higher ratios (> 1) suggest positive selection, and ratios less
226 than one suggest negative selection. We found evidence for positive selection at both time

227 points despite differences between subtypes (Fig. 2c). Separating mutations into mutational
228 fractions demonstrated that shared but not private mutations showed positive dN/dS ratios in all
229 three glioma subtypes indicating that only shared mutations (including truncal mutations) are
230 likely subject to positive selection (Fig. 2c). The dN/dS ratio of initial-only mutations showed that
231 these are neither positively nor negatively selected for, while recurrence-only mutations were
232 subject to negative selection in IDHwt.

233 To verify the reduced selective pressure in the private mutations we used an orthogonal
234 method to test for evidence of selection (neutralitytestr)³¹. The method uses variant allele
235 frequency distributions and estimated mutation rates to detect whether profiles significantly
236 deviate from a model of neutral evolution (i.e. as depicted by a linear relationship in Fig. 2d). In
237 accordance with dNdScv results, private mutations demonstrated dynamics consistent with
238 neutral evolution (Fig. 2d). Shared subclonal mutations deviated from linearity and were
239 consistent with selection both in non-hypermotators and hypermutators (Fig. 2d, Extended Data
240 Fig. 6a-b), providing additional evidence that the strongest selective forces occur early in
241 gliomagenesis.

242 Cohort-level analysis of selection masks the heterogeneity that exists in individual
243 evolutionary trajectories. To determine the selective effects at each tumor time point we used a
244 Bayesian framework (SubClonalSelection) which simultaneously provides sample-specific
245 probabilities for both selection and neutrality while modeling sources of noise in sequencing
246 data. The classification of a sample as “selection” or “neutral” is determined by whichever model
247 has the greater probability. Classification as “neutral” reflects the accumulation of random
248 mutations that are not subject to selection. Given the stringent algorithm requirements, 183
249 patients were included in this analysis with at least one time point, and 104 patients with both
250 time points (16 IDHmutant-codels, 29 IDHmutant-noncodels, 59 IDHwt, Supplementary Table
251 3). Neutral to neutral was the most common evolutionary trajectory across all three subtypes
252 (52%), and IDHwt tumors displayed the highest observed selection at any time point with
253 selection detected in 64% of tumors (Fisher’s exact test $P = 0.01$, Fig. 2e, Supplementary Table
254 3). IDHwt gliomas with evidence for selection at recurrence had a shorter overall survival than
255 IDHwt gliomas classified as neutral at recurrence ($P = 2.7E-02$; log-rank statistic, Fig. 2f),
256 suggesting that subclonal competition associates with more aggressive tumor behavior. To
257 address the limitations of smaller sample sizes in the IDH-mutant subtypes, we performed a
258 Cox proportional hazards model including age at first diagnosis, all three glioma subtypes, and
259 mode of selection at recurrence. This analysis revealed that selection at recurrence was
260 significantly associated with shorter survival across subtypes (HR = 1.53 95% CI 1.00-2.41, $P =$

261 4.8E-02, Supplementary Table 4). We next investigated whether radiation and chemotherapy
262 imposed a selective effect, by comparing the evolutionary status at recurrence with treatment
263 and other clinical variables. We did not observe significant associations between subclonal
264 selection and radiation therapy or chemotherapy (Fisher's exact-test $P > 0.05$, Supplementary
265 Table 5), suggesting that standard therapeutic approaches for glioma have limited impact on the
266 subclonal tumor architecture. While high-depth sequencing datasets may be required to detect
267 subtle selective effects²⁶, our analyses raise the possibility that the survival benefit derived from
268 standard chemoradiation results from tumor cell elimination where treatment sensitivity of
269 individual cells is not determined by genetic factors.

270 **Driver alteration frequencies across time**

271 We evaluated how stability, acquisition, and loss of mutation and copy number drivers⁶ over
272 time impact glioma evolution. We used dNdScv to nominate 12 candidate mutation driver genes
273 at both time points ($Q < 0.05$, Fig. 3a, Extended Data Fig. 7a) and determined significant copy
274 number alterations that recapitulated previously identified drivers (Extended Data Fig. 7b).
275 Mutations in *IDH1* and co-occurring 1p/19q chromosome-arm loss have been suggested as
276 glioma-initiating events¹, which was corroborated by the observation that these events were
277 never lost or acquired during the surgical interval (Fig. 3a, Extended Data Fig. 8a). Similarly, we
278 observed that *TERT* promoter mutations were almost always shared in the IDHmutant-codel
279 and IDHwt, though many samples lacked sufficient coverage in this GC-rich region.
280 Chromosome 7 gains and chromosome 10 losses were present in a large majority of IDHwt
281 initial tumors and persisted into recurrence.

282 Shifts in the fraction of cancer cells harboring an event may also indicate a time
283 dependency of drivers. We determined changes in cellular prevalence of shared driver events
284 by ordering events in each sample by their CCF (Extended Data Fig. 9). *ATRX* mutations in
285 IDHmutant-noncodel initial tumors demonstrated lower CCFs than *TP53* ($P = 0.03$) and *IDH1* (P
286 $= 0.10$) mutations, suggesting *IDH1* and *TP53* mutations precede *ATRX* inactivation¹. There
287 was no difference in CCF between *IDH1* and *TP53* amongst initial gliomas ($P = 0.98$), however,
288 *IDH1* mutations demonstrated significantly lower CCFs compared with *TP53* ($P = 0.0018$) in
289 recurrent gliomas. We did not observe any CCF differences among driver mutations detected in
290 IDHwt tumors at either time point. Chromosome 10 deletion CCFs were higher compared to
291 chromosome 7 amplifications ($P = 0.0036$) implying that chromosome 10 deletions arise earlier
292 ³². Similarly, there was no difference in CCF between *CDKN2A* deletion and *EGFR* amplification
293 ($P = 0.70$). *EGFR* and chromosomal arm events significantly differed (i.e. 10p del vs *EGFR*
294 amp, $P = 0.0019$) but not *CDKN2A* deletion and chromosomal events (i.e. 10p del vs *CDKN2A*

295 del, $P = 0.33$). The consistently high CCF for *EGFR* amplifications could indicate that these
296 events precede even some larger chromosomal aberrations, while not excluding the possibility
297 that high levels of extrachromosomal *EGFR*³³ artificially inflate CCF.

298 Longitudinal changes in CCF values provide additional insights into evolutionary
299 dynamics. For instance, the CCF value may increase when a driver event is linked to clonal
300 expansion, or conversely, decrease when a clone is outcompeted. Most individual drivers did
301 not demonstrate significant consistent CCF changes between the initial tumor and recurrence
302 (Extended Data Fig. 10a). A notable exception was the *TP53* mutation CCF that increased over
303 time ($P = 0.037$) in IDHmut-noncodels, but not IDHwt gliomas ($P = 0.13$, Extended Data Fig.
304 10b). We did not observe any differences in *IDH1* CCF over time among IDHmut-noncode
305 tumors, possibly because the general trend of these tumors to increase in CCF is counteracted
306 by the biological loss of relevance of mutant *IDH1* over time (Extended Data Fig. 10c). Indeed, a
307 gross comparison of all shared mutation CCFs revealed an increase in recurrent IDHmut-
308 noncode
309 l tumors ($P < 0.0001$), which may reflect increased clonality and a reduction in
310 intratumoral heterogeneity (Extended Data Fig. 10d). In contrast, shared CCFs decreased in
311 IDHwt tumors, potentially indicating a general increase in intratumoral heterogeneity at
312 recurrence ($P < 0.0001$, Extended Data Fig. 10d). We confirmed that IDHmutant-noncode
313 l CCF
314 increases and IDHwt decreases were not biased by patients with high mutation burden through
315 the classification of patient-specific shared mutation CCF change (Extended Data Fig. 10e).

314 We next investigated whether specific somatic alterations were acquired or lost over
315 time. Gene-specific enrichment of many recurrence-only mutations was found in hypermutated
316 tumors, but there was no enrichment for somatic gene alterations in non-hypermutators
317 suggesting that glioma recurrence is not directed by particular sets of mutations (Extended Data
318 Fig. 8b). Within subtypes we detected an enrichment in *CDKN2A* homozygous deletions (Fig.
319 3a, Extended Data Fig. 8a) in recurrent IDHmutant-noncodels, which was corroborated by
320 additional cell cycle gene alterations (focal gain of *CCND2*, *CDK4*, *CDK6*, and mutation or
321 homozygous loss of *RB1*). Mutations in cell cycle checkpoint control genes are associated with
322 genomic instability³⁴. Therefore, we analyzed aneuploidy levels by determining the proportion of
323 the genome that had undergone aneuploidy events (Extended Data Fig. 11a-b). We observed
324 that IDHmutant-noncode
325 l tumors had a higher level of aneuploidy at recurrence (Wilcoxon rank
326 sum test $P = 1.4E-06$ total aneuploidy, $p = 8.6E-03$ arm-level aneuploidy, Extended Data Fig.
327 11c-d) with tumors carrying acquired cell cycle gene alterations displaying the largest increases
328 in aneuploidy ($P = 7.6E-06$; Wilcoxon rank sum test, Fig. 3b). We reasoned that *CDKN2A*
deletions may precede aneuploidy. Homozygous *CDKN2A* deletions had significantly higher

329 CCFs compared to average CNV CCF across the genome (as a surrogate for aneuploidy
330 related copy number changes), suggesting that *CDKN2A* loss occurred prior to aneuploidy (Fig.
331 3c). These alterations may hasten disease progression as patients with either cell cycle
332 alterations or the largest increases in aneuploidy at recurrence demonstrated significantly
333 shorter survival than patients without these alterations (log-rank test $P < 0.0001$, Fig. 3d). Taken
334 together, the persistence of drivers over time and the paucity of consistent change imply that
335 therapy does not result in selection of specific sets of molecular changes.

336 **Immunoediting activity in glioma**

337 We next investigated how the immune microenvironment affects evolutionary trajectories. The
338 immune system may prune tumor cells carrying immunogenic (neo-)antigens, resulting in the
339 selection of subclones capable of evading the immune response. Evidence of this
340 immunoediting process has been shown in several cancer types, including glioma³⁵⁻³⁸, and
341 suggests active immunosurveillance that may be therapeutically exploited³⁹. We
342 computationally predicted neoantigen-causing mutations⁴⁰. As expected, the neoantigen load
343 across the GLASS cohort was strongly correlated with exonic mutation burden (Spearman's
344 $Rho = 0.89$), with 42% of nonsynonymous exonic mutations giving rise to neoantigens on
345 average. This fraction did not significantly differ by glioma subtype or between initial and
346 recurrent tumors ($P > 0.05$, Wilcoxon rank-sum test; Fig. 4a). The most common neoantigen
347 arose from the clonal R132H mutation in *IDH1* and was present in 22 out of 88 *IDH*-mutant
348 initial and recurrent tumors. Beyond mutations in *IDH1*, no mutations gave rise to a neoantigen
349 found in more than three tumors at a given timepoint (Supplementary Table 6). Across the
350 dataset, neoantigens and non-immunogenic mutations exhibited similar changes in cancer cell
351 fractions between initial and recurrent tumors indicating a lack of neoantigen-specific selection
352 processes over time (Extended Data Fig. 12a).

353 We then examined the extent to which immunoediting occurred by comparing each
354 sample's observed neoantigen rate to an expected rate that was empirically derived from our
355 dataset. The output of this approach is a normally distributed set of ratios centered at 1.
356 Samples with an observed-to-expected neoantigen ratio < 1 exhibit evidence of neoantigen
357 depletion relative to the rest of the dataset, and thus are more likely to have been
358 immunoedited. We found that none of the three glioma subtypes harbored observed-to-
359 expected ratios that significantly differed from 1 ($P > 0.05$, one sample t-test), though *IDHwt*
360 tumors exhibited significantly lower scores compared to *IDHmut-noncodels* (t-test, $P = 0.04$; Fig.
361 4b). We additionally did not observe an association between the observed-to-expected ratio and
362 survival when adjusting for subtype and age (Wald test, $P > 0.05$), nor was there a difference

363 between samples with neutral evolution dynamics compared to those exhibiting evidence of
364 subclonal selection. When comparing samples longitudinally, we found that the observed-to-
365 expected neoantigen ratio was strongly correlated between initial and recurrent tumors of each
366 patient (Pearson's $R = 0.73$, $P = 5E-38$), suggesting that the neoantigen depletion level in the
367 recurrence reflects that of the initial tumor (Fig. 4c).

368 Immunoediting is most likely to take place in the tumors with high cytolytic activity and
369 low levels of immunosuppressive activity³⁸. Hypermutators, which have high neoantigen loads,
370 have previously been associated with highly cytolytic microenvironments³⁷. However, we did
371 not observe any differences in the observed-to-expected neoantigen ratio between
372 hypermutated recurrent tumors and their initial counterparts, nor did we observe differences
373 between hypermutated and non-hypermutated recurrent tumors, indicating that immunoediting
374 activity is not related to the total number of mutations in a sample (Wilcoxon rank-sum test $P >$
375 0.05 ; Extended Data Fig. 12b). To more directly determine whether there were immunologic
376 factors associated with neoantigen depletion, we analyzed CIBERSORT immune cell fractions
377 from a subset of samples that had undergone expression profiling in a previous study ($n = 84$
378 from 42 tumor pairs)^{37,41}. Initial tumors with an observed-to-expected neoantigen ratio >1
379 exhibited significantly higher levels of CD4+ T cells than those with a ratio < 1 , while recurrent
380 tumors with a ratio > 1 exhibited significantly higher levels of macrophages, neutrophils, and
381 significantly lower levels of plasma cells relative to those with ratio < 1 ($P < 0.05$, Wilcoxon rank-
382 sum test; Extended Data Fig. 12c).

383 While we did not detect many factors associated with the observed-to-expected
384 neoantigen ratio, we did observe that the ratio was significantly associated with the total number
385 of unique HLA loci in a patient (Spearman's $Rho = 0.28$, $P = 2E-9$), reflecting similar findings in
386 lung cancer⁴². This may bias analyses comparing the ratio across patients. To determine
387 whether immunoediting varies over time in a patient-agnostic manner, we compared the
388 observed-to-expected neoantigen ratio derived from a sample's clonal mutations, which likely
389 arose earlier in tumor evolution, to that derived from their subclonal mutations, which likely
390 arose later. We did not observe a significant difference in the observed-to-expected neoantigen
391 ratio of each patient's clonal and subclonal neoantigens, regardless of glioma subtype or
392 whether the sample was an initial tumor or recurrence ($P > 0.05$, paired t-test; Fig. 4d).
393 Together, these analyses suggest that neoantigens in glioma are not exposed to differing levels
394 of selective pressure throughout their development.

395 **DISCUSSION**

396 We reconstructed the evolutionary trajectories of 222 patients with glioma to better understand
397 treatment failures and tumor progression. The longitudinal molecular profiles revealed common
398 features such as acquired hypermutation and aneuploidy, but highlighted the individualistic
399 paths of post-treatment glioma evolution. Our results provide evidence that current standard of
400 care therapies do not frequently coerce glioma down predictable paths. Instead, an unexpected
401 number of gliomas appeared to stochastically evolve following early driver events. We expect
402 that continuing to profile patient tumors over time using comprehensive sequencing approaches
403 will identify additional common evolutionary paths. Our results here highlight the exciting
404 prospects of several ongoing efforts that may inform new glioma therapies.

405 The observation that treatment-induced hypermutation occurred across subtypes, but
406 did not confer a detrimental effect on patient survival leaves the clinical significance of glioma
407 hypermutation uncertain^{21-24,27}. Future analyses that consider the number of therapy cycles and
408 *MGMT* DNA methylation status will help to elucidate factors that predispose tumors to
409 hypermutation and identify therapies that effectively exploit this phenotype's vulnerabilities (e.g.,
410 high mutation burden). Acquired cell cycle alterations and aneuploidy in recurrent IDHmut-
411 noncodel gliomas also provide a rationale to target these more aggressive phenotypes with
412 CDK inhibitors⁴³ or with compounds that disrupt microtubule dynamics⁴⁴. Finally, our analyses
413 revealed that immunoediting activity does not vary in glioma over time, though we did observe
414 variation between individual patients. Additional molecular and immunological data are needed
415 to fully understand the impact this variability has on glioma evolution and to devise therapies
416 directed at a glioma's immunogenicity¹⁷. To this end, we found that clonal neoantigens arising
417 from the *IDH1* R132H mutation persisted from the initial tumor into the recurrence, justifying
418 neoantigen vaccine approaches as treatments for initial and recurrent glioma^{45,46}.

419 Collectively, these findings help shape our perspective on what constitutes an optimal
420 treatment, and what approaches would result in the greatest removal or killing of glioma cells
421 possible. Genomic characterization efforts such as TCGA have greatly increased our
422 understanding of glioma biology, but were limited to a single snapshot in evolutionary time. The
423 GLASS resource provides a framework to study the patterns of glioma evolution and treatment
424 response.

425

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463 **CONFLICTS OF INTEREST**

464 R.G.W.V. declares equity in Boundless Bio, Inc. M.K. receives research grants from BMS and
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489 A.D.M., F.S.V., and R.G.W.V. wrote the manuscript. K.D.A. and J.F.D. took charge in
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493 the results and commented on the manuscript and Supplementary Information.

494

495 **Methods**

496 **Data reporting** No statistical methods were used to predetermine sample size.

497 **DNA sequencing and data collection** The GLASS dataset consists of both unpublished and
498 published sequencing data as outlined in Supplementary Table 1. Among the cohort were
499 exomes from 436 glioma samples (200 patients), whole-genome from 165 glioma samples (78
500 patients), with overlapping exome/whole-genome data on 78 glioma samples (38 patients). A
501 matching germline sequence was available for all patients. The dataset includes 257 sets of at
502 least two time-separated tumor samples, seventeen standalone recurrences, and 19 patients
503 with at least two geographically distinct tumor portions. More specifically, the dataset includes
504 exome or whole-genome sequencing data on 211 primary gliomas, 234 first recurrences, 32
505 second recurrences, 11 third recurrences and one fourth recurrence (Supplementary Table 7).

506 Newly generated whole genome sequencing data for the Chinese University of Hong Kong
507 (HK), Northern Sydney Cancer Centre (NS) and MD Anderson Cancer Center (MD) cohorts
508 were subjected to 150 base paired-end sequencing. The HK samples were sequenced using a
509 HiSeqX while the NS and MD cohorts were sequenced using a NovaSeq according to Illumina's
510 protocols. Whole exome capture was performed using the following platforms as reported in
511 previous publications. Agilent SureSelect Human All Exon 50Mb capture kit was used for
512 patients SF-0001- SF-0021, Agilent SureSelect Human All Exon V4 capture kit was used for
513 patients SF-0024 – SF-0029 in the UC San Francisco cohort. Agilent SureSelect Human All
514 Exon v4 or v5 was used to capture samples in the Kyoto University cohort. Samsung Medical
515 Center cohort reported using Agilent SureSelect kit for patients SM-R056 – SM-R071, SM-
516 R075, SM-R076, SM-R095- SM-R114 while Illumina TruSeq Exome-capture kit was used for
517 patient SM-R072. Exome capture was performed using Agilent SureSelect Human All Exon 50
518 Mb in The Cancer Genome Atlas (TCGA)-GBM cohort and Agilent SureSelect Human All Exon
519 v2.0, 44Mb kit in the TCGA-LGG cohort. Columbia University cases were captured using Agilent
520 V3 50M kit, sequencing 90bp PE for samples R009-TP, R009R1, R011TP, R011R1, R014TP,
521 R014R1, R017-R1, R018-R1, R019-R1. Mapping files of initial tumor and normal samples of
522 patients R017 – R019 were obtained from TCGA through CG-hub. All other samples were
523 captured using Agilent SureSelect XT Human All Exon v4 Kit, PE, 80M reads, 150X on target
524 coverage. Samples in the Henry Ford Hospital cohort were multiplexed and sequenced using
525 Illumina HiSeq 2000 by the Sequencing and Microarray Facility at an average target exome
526 coverage of 100x using 76-bp paired-end reads. Samples in the HK cohort were subjected to 75
527 base paired-end sequencing for HK-0001 – HK-0004 as performed NextSeq in high output
528 mode. In the Leeds Cohort (LU) SureSelectXT V5 kit (PE100) was used to construct exome
529 libraries. Illumina TruSeq Exome capture kit was used for samples at the Medical University of
530 Vienna – CeMM.

531 **GLASS identifiers** A GLASS barcode system was created, based on TCGA barcode design, in
532 an effort to de-identify patient information and provide an organized framework for the different
533 pieces of the dataset.

534 GLASS barcodes are composed of 24 characters. The first four characters specify the
535 project (either GLSS or TCGA). All datasets submitted to the GLASS consortium, published and
536 unpublished, were given the GLSS project ID. Samples that were part of the TCGA cohorts
537 (TCGA GBM and TCGA LGG) were given a TCGA designation. The next two characters
538 designate the center where the samples were either acquired or sequenced (Supplementary

539 Table 7). This is followed by the four-character center specific patient identification that was kept
540 as close as possible to the patient identification provided by the collaborators to allow a
541 simplified trace back process. Patient data is divided by a relative sample type, such as initial
542 tumor (TP), recurrent tumor (R1), normal tissue (NB, NM, etc), or metastatic tumor sample (M1).
543 If there was more than one recurrence the relative number was specified following “R”. Some
544 patients had surgeries for which a biospecimen was unavailable. Thus, a surgical number was
545 also provided to indicate temporal ordering (Supplementary Table 8). To include spatially
546 separated samples the portion designation was added, which is followed by one character
547 specifying the type of analyte, either DNA (D) or RNA (R). As there is variation in the
548 sequencing analysis, a three-character designation represents either whole genome (WGS) or
549 whole exome sequencing (WXS). The last part of the GLASS barcode is a six-character
550 designation unique to each barcode that was randomly generated.

551
552 **Computational pipelines** All pipelines were developed using snakemake 5.2.2 ⁴⁷. Unless
553 otherwise stated, all tools mentioned are part of the GATK 4 suite ⁴⁸. All data was collected at a
554 central location (The Jackson Laboratory) and was analyzed using homogenous pipelines
555 capable of processing both raw fastq files as well as re-process previously analyzed bam files.

556 **Alignment and pre-processing** Data pre-processing was conducted in accordance to the
557 GATK Best Practices using GATK 4.0.10.1. Briefly, aligned BAM files were separated by read
558 group, sanitized and stripped of alignments and attributes using ‘RevertSam’, giving one
559 unaligned BAM (uBAM) file per readgroup. Uniform readgroups were assigned to uBAM files
560 using ‘AddOrReplaceReadgroups’. Similarly, unaligned fastq files were assigned uniformly
561 designated readgroup attributes and converted to uBAM format using ‘FastqToSam’. uBAM files
562 underwent quality control using ‘FastQC 0.11.7’. Sequencing adapters were marked using
563 ‘MarkIlluminaAdapters’. uBAM files were finally reverted to interleaved fastq format using
564 ‘SamToFastq’, aligned to the b37 genome ('human_g1k_v37_decoy') using 'BWA MEM 0.7.17',
565 attributes were restored using 'MergeBamAlignment'. 'MarkDuplicates' was then used to merge
566 aligned BAM files from multiple readgroups and to mark PCR and optical duplicates across
567 identical sequencing libraries. Lastly, base recalibration was performed using 'BaseRecalibrator'
568 followed by 'ApplyBQSR'. Coverage statistics were gathered using 'CollectWgsMetrics'.
569 Alignment QC was performed running 'ValidateSamFile' on the final BAM file and QC results
570 were inspected using 'MultiQC 1.6a0' ⁴⁹. A haplotype database for fingerprinting was generated
571 using a modified version of the code on https://github.com/naumanjaved/fingerprint_maps. The
572 tool ‘CrosscheckFingerprints’ was used to confirm that all readgroups within a sample belong to
573 the same individual, and that all samples from one individual match. Any mismatches were
574 marked and excluded from further analysis.

575 **Variant detection** Variant detection was performed in accordance to the GATK Best practices
576 using GATK 4.1.0.0. Germline variants were called from control samples using Mutect2 in
577 artifact detection mode and pooled into a cohort-wide panel of normals. Somatic variants were
578 subsequently called in individual tumor samples (single-sample mode) and in entire patients
579 using GATK 4.1 Mutect2 in multi-sample mode. Mutect2 was given matched control samples,
580 the aforementioned panel of normals and the gnomAD germline resource as additional controls.
581 Cross-sample contamination was evaluated using ‘GetPileupSummaries’ and

582 'CalculateContamination' run for both tumor and matching control samples. Read orientation
583 artifacts were evaluated using 'CollectF1R2Counts' and 'LearnReadOrientationModel'. Somatic
584 likelihood, read orientation, sequence context, germline and contamination filters were applied
585 using 'FilterMutectCalls'.

586 **Variant post-processing** BCFTools 1.9 was used to normalize, sort and index variants⁵⁰. A
587 consensus VCF was generated from all variants in the cohort, removing any duplicate variants.
588 The consensus VCF file was annotated using GATK 4.1 Funcotator and the v1.6.20190124s
589 annotation data source. Allele frequencies (AFs) from multi-sample Mutect2 were used to
590 compare AFs between related samples. Multi-sample Mutect2 calls and filters mutations across
591 a patient as a whole and does not determine mutation calls in a single samples. Single-sample
592 mutation calls were overlaid on the multi-sample calls to infer whether variants were called in
593 individual samples. Single-sample called variants that were not present in the multi-sample
594 callset were discarded.

595 **Mutational burden** Mutational burden was calculated as the number of mutations per
596 megabase (Mb) sequenced. A minimum coverage threshold of 15x was required for each base.
597 DNA hypermutation was defined for recurrent tumors with greater than 10 mutations per Mb
598 sequenced as these values were considered outliers (1.5 times the interquartile range above
599 the upper quartile). Notably, there were a few initial gliomas that demonstrated a mutational
600 frequency above 10 mutations per Mb. However, the "hypermutation" classification was
601 restricted to only patients with this level at recurrence since these likely reflect different
602 evolutionary paths.

603 **Mutational signatures** The relative contributions of the COSMIC mutational signatures were
604 determined from a patient's initial-only, recurrence-only, and shared mutations by solving the
605 non-negative-least squares (NNLS) problem for each set of mutations using the 30 signatures
606 from version 2 (March 2015). Six signatures were dominantly enriched in at least 3% of the
607 fractions and we resolved the NNLS using the reduced six-signature model to increase
608 accuracy and reduce noise.

609 **Copy number segmentation** Copy number identification was performed according to the
610 GATK Best Practices and is outlined briefly here. The pipeline differs slightly for whole genomes
611 and whole exomes. For genomes, the genome was segmented into 10kb bins using
612 'PreprocessIntervals'. For exomes, overlapping regions between several commonly used
613 capture kits (Broad Human Exome b37, Nextera Rapid Capture, TruSeq Exome, SeqCap EZ
614 Exome V3, Agilent SureSelect V4, Agilent SureSelect V7) were identified using 'bedtools
615 multiIntersectBed'. The tool 'PreprocessIntervals' was used to apply 1kb padding and to merge
616 overlapping intervals. In parallel, 'SelectVariants' was used to subset the gnomAD resource of
617 germline variants to variants with a population AF greater than 5%. Next, 'CollectReadcounts'
618 was used to count reads in the bins generated by 'PreprocessIntervals' separately for
619 autosomes and allosomes. In parallel, 'CollectAllelicCounts' was used to count reference and
620 alternate reads at gnomAD variant sites with a population AF greater than 5%. The cohort was
621 subsequently split into batches determined by sequencing center and
622 'CreateReadCountPanelOfNormals' was used to create a panel of normal (PON) for each batch.
623 PONs were created separately for allosomes and autosomes, and allosomes were separated
624 further by sex. To further improve the panel of normals, GC content annotation of each interval

625 as determined by 'AnnotateIntervals' were given. Next, 'DenoiseReadCounts' was used to
626 denoise the binned readcounts output by 'CollectReadCounts', given a PON determined by
627 batch, chromosomes (allosomes or autosomes) and sex. Denoised copy ratios were plotted and
628 inspected for quality concerns using 'PlotDenoisedCopyRatios'. The tool 'ModelSegments' is an
629 implementation of a gaussian-kernel binary-segmentation algorithm and was used to merge
630 contiguous segments and assign copy and allelic ratios. The results of this segmentation were
631 plotted using 'PlotModeledSegments' and inspected for quality concerns.

632 **Copy number calling** A copy number caller loosely based on GATK 'CallCopyRatioSegments'
633 (which in turn is based off of ReCapSeg) and GISTIC was implemented to call both arm-level
634 and high-level copy number changes, respectively^{51,52}.

635 Segments (from 'ModelSegments') with a non-log₂ copy ratio between 0.9 and 1.1 were
636 determined to be neutral. These segments were then weighted by length and a weighted mean
637 and standard deviation (sd) non-log₂ copy ratio (once-filtered) were determined again. Outlier
638 segments are removed and once again a weighted mean and sd non-log₂ copy ratio (twice-
639 filtered) were determined. Segments with a non-log₂ copy ratio between 0.9 and 1.1 and
640 segments within two standard deviations of the twice-filtered mean were determined to be
641 neutral, and segments outside of these boundaries were determined to have a low-level
642 amplification or deletion, depending on the direction.

643 The weighted mean and sd of the non-log₂ copy ratio (once-filtered) was then
644 determined individually for each chromosome arm. Outlier segments were removed and the
645 weighted mean and sd of the non-log₂ copy ratio (twice-filtered) was determined again. In order
646 to determine a high-level amplification and deletion threshold, the most highly amplified and
647 deleted chromosome arms were selected, respectively. The twice-filtered mean plus (high level
648 amplification) or minus (high level deletion) two times the sd of the selected arms were used as
649 high-level thresholds.

650 Gene level copy number were called by intersecting the gene boundaries with the
651 segment intervals and by calculating the weighted non-log₂ copy ratio for that gene. The copy
652 number call for that gene was then determined by comparing the gene-level non-log₂ copy ratio
653 to the previously determined thresholds.

654 **dNdScv** The R package dNdScv⁵³ (<https://github.com/im3sanger/dndscv>) was run using the
655 default and recommended parameters for all mutations in initial tumor samples, recurrent tumor
656 samples, and for each mutational fraction (unique to initial, unique to recurrent and shared). All
657 analyses were conducted separately within the three main tumor subtypes.

658 **Aneuploidy calculation** The most reductive metric of aneuploidy was computed by taking the
659 size of all non-neutral segments divided by the size of all segments. The resulting aneuploidy
660 value indicates the proportion of the segmented genome that is non-diploid.

661 In parallel, an arm-level aneuploidy score modeled after a previously described method was
662 computed⁵⁴. Briefly, adjacent segments with identical arm-level calls (-1, 0 or 1) were merged
663 into a single segment with a single call. For each merged/reduced segment, the proportion of
664 the chromosome arm it spans was calculated. Segments spanning greater than 80% of the arm
665 length resulted in a call of either -1 (loss), 0 (neutral) or +1 (gain) to the entire arm, or NA if no
666 contiguous segment spanned at least 80% of the arm's length. For each sample the number of
667 arms with a non-neutral event was finally counted. The resulting aneuploidy score is a positive

668 integer with a minimum value of 0 (no chromosomal arm-level events detected) and a maximum
669 value of 39 (total number of autosomal chromosome arms excluding the short arms for
670 chromosomes 13, 14, 15, 21, and 22).

671 **Estimates of evolutionary pressures** Evolutionary pressures were evaluated both by variant
672 status and glioma subtype using the neutralitytestr algorithm as previously described (R-
673 package: neutralitytestr version: 0.0.2, <https://github.com/marcjwilliams1/neutralitytestr>)³¹.
674 Individual variant allele frequency vectors were merged at the level of glioma subtype by variant
675 status. Only mutations found in copy-neutral regions should be included in these analyses.
676 For all else, default parameters were used. Merged VAF distributions were deemed to be
677 selected when the neutral null hypothesis was rejected using several metrics. Tests for
678 neutrality required that both R^2 values < 0.98 and the area between the two curves of 1) merged
679 VAF data and 2) a normalized distribution expected under neutrality to be significantly different.

680 The SubclonalSelection algorithm was applied to GLASS mutation data to measure the
681 selection strength in individual tumor samples (Julia package: SubclonalSelection,
682 <https://github.com/marcjwilliams1/SubClonalSelection.jl>)¹⁶. Patients that had samples at both
683 timepoints with a TITAN-defined purity estimate ≥ 0.5 and ≥ 25 subclonal mutations in non-
684 diploid regions were included. Mean coverage across all mutations was used as the
685 “read_depth” input parameter and the model was run with the recommended 10^6 iterations and
686 1000 particles. Samples were classified as neutral or selected based on the model that had the
687 highest probability, in line with the prior applications to TCGA data¹⁶. Classification based on the
688 highest model probability yielded stable results there was not a significant change in proportions
689 when setting a higher classification probability threshold ($P > 0.05$, Pearson’s Chi-square test,
690 for both probability thresholds of 0.6 and 0.7). At all three probability thresholds (0.5, 0.6, and
691 0.7), Kaplan-Meier survival analyses between selection at recurrence and overall survival
692 continued to indicate that patients with IDHwt tumors that were selected had a worse overall
693 survival ($P = 0.03$ (n=81), $P = 0.01$ (n=66), $P = 0.01$ (n=56) respectively).

694 **Mutation clonality** Each patient’s clonal architecture was inferred using PyClone (version
695 0.13.1) by grouping SNVs into clonal clusters (<https://github.com/aroeth85/pyclone>)⁵⁵. The
696 patient-level input mutation matrix was reduced by limiting to sites with at least 30x coverage
697 across all samples. PyClone was subsequently ran using a binomial density model, connected
698 initiation, and 10000 iterations. Sample purities were provided for each patient and parental
699 copy number (minor and major allele counts) from TITAN were given. PyClone results were
700 post-processed using a burn-in of 1000, thin of 1, minimum cluster size of 2 and a maximum
701 number of clusters per patient of 12. Individual mutations were determined to be clonal if the
702 PyClone cancer cell fraction (CCF) values were ≥ 0.5 , subclonal for mutations with CCF ≥ 0.1
703 and CCF < 0.5 , mutations were considered non-clonal when CCF < 0.1 as previously described
704 ⁵⁶.

705 **CNV clonality** Allele specific copy number, tumor purity and ploidy estimates were derived
706 using a probabilistic model (TITAN, version 1.19.1) for both whole genome and whole exome
707 sequencing samples ⁵⁷. TITAN was supplied with the tumor denoised readcounts output by
708 GATK DenoiseReadCounts and the tumor allelic counts at loci found to be heterozygous in
709 control samples output by ModelSegments. An ‘alphaK’ (and ‘alphaKHigh’) parameter of 2500
710 and 10000 was used for exomes and genomes, respectively. The patient sex was provided in

711 order to improve fitting allosomes. For each tumor-control pair TITAN was ran assuming an
 712 initial ploidy of two or three, and assuming 1 to 3 clusters, resulting in a total of six possible
 713 solutions per tumor/control pair. To select the optimal solution, TITAN's internal selectSolution
 714 function was used with a threshold of 0.15 giving additional weight to diploid solutions.

715 **Timing analysis** The CCF values output by TITAN or PyClone were used for separately timing
 716 copy number changes or mutations. To time specific copy number changes in genes, the
 717 average CCF for that gene was calculated. When timing mutations in genes, the highest CCF
 718 amongst the non-synonymous mutations was taken.

719 **Neoantigen analyses** Neoantigens in this analysis were defined as all 8-11-mer peptides that
 720 arose from an exonic nonsynonymous SNV or indel and bound their respective patient's HLA
 721 class I molecules at a binding affinity score (IC50) that was ≤ 500 nM and better than or equal to
 722 the wild-type form of the peptide. Each patient's 4-digit HLA class I types were inferred using
 723 OptiType (version 1.3.1, <https://github.com/FRED-2/OptiType>) run on each patient's matched
 724 normal sample⁵⁸. VCF files for each tumor sample were annotated using Variant Effect Predictor
 725 (ensembl) with the Downstream and Wildtype plugins. Neoantigens from these VCFs were then
 726 called using pVACseq (version 4.0.10, <https://github.com/griffithlab/pVAC-Seq>)⁴⁰ run using
 727 netMHCpan (version 2.8, <http://www.cbs.dtu.dk/services/NetMHCpan-2.8/>)⁵⁹. For each
 728 pVACseq run, epitope length was set to 8, 9, 10, or 11, minimum binding affinity fold-change
 729 was set to 1, and downstream sequence length was set to full, with default parameters used for
 730 all other settings.

731 Downstream neoantigen analyses were performed using the pVACseq output linked to its
 732 respective mutation information. Neoantigen-causing mutations were defined as all mutations
 733 that gave rise to at least one neoantigen. The observed-to-expected neoantigen ratio was
 734 calculated using a previously developed approach that compares each tumor's observed
 735 neoantigen rate to an empirically derived expected rate that assumes no selection against
 736 neoantigen-causing mutations³⁸: From the gold set samples in the GLASS cohort ($n = 222$),
 737 define \bar{N}_s to be the expected number of nonsynonymous missense SNVs per synonymous SNV
 738 with trinucleotide context s . \bar{B}_s is then defined as the expected number of neoantigen-generating
 739 missense SNVs per nonsynonymous missense SNV with trinucleotide context s . For a given
 740 sample i , define Y_i as the sample's set of synonymous SNVs and $s(m)$ to be a synonymous
 741 SNV with trinucleotide context m . The expected number of nonsynonymous missense SNVs,
 742 N_{pred} , and neoantigen-causing mutations, B_{pred} , can then be calculated as follows:

743
$$N_{pred,i} = \sum_{m \in Y_i} \bar{N}_{s(m)}$$

744
$$B_{pred,i} = \sum_{m \in Y_i} \bar{N}_{s(m)} \bar{B}_{s(m)}$$

745 To obtain sample i 's final neoantigen depletion ratio R_i , the observed number of neoantigen-
 746 causing mutations in the sample, $B_{obs,i}$ is divided by the sample's observed number of
 747 nonsynonymous missense SNVs, $N_{obs,i}$, and then this ratio is divided by the ratio of $B_{pred,i}$ and
 748 $N_{pred,i}$. Thus:

749

$$R_i = \frac{B_{obs,i}/N_{obs,i}}{B_{pred,i}/N_{pred,i}}$$

750 For analyses examining clonal/subclonal neoantigen ratios, the observed and expected
751 numbers were calculated by subsetting a sample's SNVs by the respective criteria and then
752 recalculating the ratio as described above. To mitigate overfitting, all analyses presented here
753 utilized samples from patients with at least 3 neoantigen-causing mutations in their primary and
754 recurrent tumors.

755 **Immune cell analyses** CIBERSORT relative immune cell fraction data used in downstream
756 neoantigen analyses were downloaded from a previous publication³⁷.

757 **Statistical methods** All data analyses were conducted in R 3.4.2, Python 2.7.15, PostgreSQL
758 10.5, and Julia 0.7. All survival analyses including Kaplan-Meier plots and Cox proportional
759 hazards models were conducted using the R packages survival and survminer.

760 **Data availability** All deidentified, non-protected access somatic variant profiles and clinical data
761 are accessible via Synapse (<http://synapse.org/glass>). Raw data of the various sequencing
762 datasets can be obtained per the overview provided in the Supplement.

763 **Code availability** All custom scripts and pipelines are available on the project's github page
764 (<https://github.com/TheJacksonLaboratory/GLASS>).

765

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907

908 Figure Legends

909 **Fig. 1 | Temporal changes in glioma mutational burden and processes.** **a.** Each column
910 represents a single patient ($n = 222$) at two separate timepoints grouped by glioma subtype and
911 ordered left-to-right by decreasing mutation frequency at recurrence. Top, mutation frequency
912 differences between initial and recurrent tumors. Blue dotted line indicates increased mutation
913 frequency while a red dotted line indicates decreased mutational frequency. Stacked bar plot
914 reflects the proportion of total mutations shared (mustard), private to initial (magenta), or private
915 to recurrence (blue). Clinical information including hypermutation status, therapy, and grade
916 changes. **b.** Stacked bar plot ($n=219$) indicating the dominant mutational signature among initial,
917 recurrent and shared mutation fractions stratified by glioma subtype. **c.** The proportion of glioma
918 recurrences with alkylating agent-related hypermutation, grouped by glioma subtype. Fisher's
919 exact test was used to compare proportions between subtypes. **d.** Kaplan-Meier curve depicting
920 overall survival in hypermutant (red) versus non-hypermutant (blue) alkylating agent treated
921 patients amongst IDHwt (left, $n = 99$) and IDHmut-noncodel (right, $n = 32$) tumors. Log-rank test
922 P-values are shown.

923 **Fig. 2 | Quantifying selective pressures during glioma evolution.** **a.** Schematic depiction of
924 cancer cell fraction (CCF) values during tumor evolution indicating clonality and associated
925 relative timing. **b.** Comparison of PyClone clusters ranked by CCF in matched initial and recurrent
926 tumors. **c. Left:** dN/dS ratio for all variants (i.e. global) in initial and recurrent tumors for each
927 subtype. Hypermutators were not included ($n = 187$). Dots represent the global dN/dS ratio with

928 associated Wald confidence intervals. *Right*: global dN/dS ratios for variant fractions per subtype.
929 **d.** Cumulative distribution of subclonal mutations by their inverse variant allele frequency.
930 Mutations were separated by timepoint, variant fraction, and glioma subtype. Deviation from a
931 linear relationship, significant Kolmogorov-Smirnov P-values and R^2 below 0.98 indicate
932 selection. **e.** Sankey plot indicating the breakdown of SubClonalSelection evolutionary modes by
933 subtype and therapy ($n = 104$). The sizes of the bands reflect sample sizes and band colors
934 highlight the glioma subtype. Gray coloring reflects instances when treatment information was not
935 available. **f.** Kaplan-Meier curve showing survival differences between IDHwt recurrent tumors
936 demonstrating selection ($n = 39$) compared with neutrally evolving tumors ($n = 44$). Log-rank P-
937 value is indicated.

938 **Fig. 3 | Patterns of glioma driver frequencies over time.** **a.** Driver dynamics for SNVs
939 nominated by the dNdScv and CNVs nominated by GISTIC ($n = 222$). Each column represents a
940 single patient at two separate time points stratified by subtype and ordered left-to-right by the
941 number of driver alterations. The degree of aneuploidy difference (recurrence – initial) offers a
942 summary metric for increases (> 0) or decreases (< 0) in aneuploidy at recurrence. Variants are
943 marked and different shapes indicate whether a variant was shared or private. The variant type
944 is depicted by its color. Stacked bar plots accompanying each gene/arm provide cohort-level
945 proportions for whether the alteration was shared, lost, or acquired. **b.** Aneuploidy comparison in
946 matching initial and recurrent IDHmut-noncode1 tumors. **c.** Within-sample CCF comparison of
947 *CDKN2A* homozygous deletion (homdel) to genome-wide CCF as a proxy for aneuploidy. A
948 relative higher CCF indicates temporal precedence. Wilcoxon signed-rank test P-value is
949 indicated. **d.** Kaplan-Meier curve comparing survival in IDHmut-noncode1 tumors with an alteration
950 in the cell cycle, acquired aneuploidy, or both (shades of red) versus unaltered IDHmut-noncode1
951 tumors (blue). Log-rank P-value is shown.

952 **Fig. 4 | Neoantigen selection during tumor progression.** **a.** Mean proportion of coding
953 mutations giving rise to neoantigens (neoantigens/nonsynonymous) stratified by glioma subtype
954 and timepoint ($n = 222$). Error bars represent standard deviation. **b.** Boxplot depicting the
955 distribution of observed to expected neoantigen ratios in the GLASS cohort stratified by glioma
956 subtype. P-value was calculated using the Wilcoxon rank-sum test. Each box spans quartiles,
957 with the lines representing the median ratio for each group. Whiskers represent absolute range,
958 excluding outliers. **c.** Scatterplot depicting the association between the observed-to-expected
959 neoantigen ratio in a patient's initial versus recurrent tumor. Each point represents a single
960 patient. R represents Pearson correlation coefficient. Panels b and c only include samples with at
961 least 3 neoantigens in the initial and recurrent tumors ($n = 131, 63, \text{ and } 24$ for IDHwt, IDHmut-
962 noncode1, and IDHmut-code1, respectively). **d.** Ladder plot depicting the difference in observed-
963 to-expected neoantigen ratio between a tumor's clonal and subclonal neoantigens. Each set of
964 points connected by a line represents one tumor. Tumors are stratified by whether they were a
965 patient's initial or recurrent tumor. Lines are colored by each patient's glioma subtype. Panel d
966 only includes samples with at least 3 clonal neoantigens and at least 3 subclonal neoantigens in
967 both the initial and recurrent tumors ($n = 35, 20 \text{ and } 9$ for IDHwt, IDHmut-noncode1, and IDHmut-
968 code1, respectively). P-value was calculated using a paired two-sided t-test. Colors in each panel
969 represent the glioma subtype and are denoted at the bottom of the figure.

970 **Extended Data Fig. 1 | Sample Selection.** **a.** Quality control workflow steps identifying all
971 GLASS samples available as a resource and the identification of the highest quality set of patient
972 pairs ($n = 222$) used for the presented mutational and copy number analyses. **b.** Additional
973 available datasets.

974 **Extended Data Fig. 2 | Mutation burden by time point and subtype.** **a.** Boxplots and paired
975 lines depicting coverage adjusted mutation frequencies in initial and matched recurrent samples
976 across three subtypes. Wilcoxon signed-rank test P-values and sample sizes are indicated. **b.**
977 Bee swarm plot depicting coverage adjusted mutation frequencies in fractions by subtype.
978 Dashed line indicates the mean. One-way ANOVA P-values comparing three subtypes are
979 indicated. **c.** Scatter plot showing the relationship between age at diagnosis and coverage
980 adjusted mutation burdens by subtype and fraction. Linear model P-values are indicated and were
981 adjusted by subtype. **d.** Similar to the analysis presented in **c**, but showing the relationship
982 between time to recurrence and coverage adjusted mutation burdens.

983 **Extended Data Fig. 3 | Mutational signatures by fraction and subtype.** **a.** Correlation plot
984 showing the Pearson's chi-squared (X^2) residuals for each signature by fraction and subtype. A
985 X^2 was performed for each subtype and P-values are indicated. Positive residuals (blue) indicate
986 a positive correlation, whereas negative residuals (red) indicate an anticorrelation. The point size
987 reflects the contribution to X^2 estimate. **b.** The same ordered of patients as Fig. 1a along with
988 relevant clinical information is provided alongside the fraction-specific mutational signatures.
989 PyClone mutational clusters are also presented.

990 **Extended Data Fig. 4 | Hypermutator clonality.** **a.** Bar plots represent counts of recurrence-
991 only mutations per hypermutator tumor that were known to receive alkylating agent therapy and
992 were successfully run through the PyClone algorithm. Colors indicate mutation clonality and color
993 intensity indicates whether the mutations resulted in coding changes. **b.** Kaplan-Meier curve
994 comparing alkylating agent-treated patients with IDHmut-noncodel hypermutator tumors that were
995 predominantly clonal ($n = 8$), predominantly subclonal ($n = 7$), versus IDHmut-noncodel non-
996 hypermutators known to be treated with alkylating agents and had available PyClone data ($n =$
997 17). Log-rank P-value is shown.

998 **Extended Data Fig. 5 | Clonal structure evolution over time.** **a.** The minimum cancer cell
999 fraction of the most persistent (shared between initial and recurrence) PyClone cluster. **b.**
1000 Comparison of PyClone clusters ranked by CCF in matched initial and recurrent tumors, as Fig.
1001 2b but separated by subtype. **c-d.** Examples of cluster CCF dynamics over time in three separate
1002 samples, including **(c)** two multi-timepoint samples **(d)** and one multi-sector sample. These
1003 additional data are available in the GLASS resource, but only two time-separated samples were
1004 used throughout the manuscript to ensure clarity.

1005 **Extended Data Fig. 6 | Variant allele fraction distribution (a)** Non-hypermutator variant allele
1006 fraction distributions for copy neutral variants in coding regions ($n = 181$ patients). Variants are
1007 separated by subtype, fraction, and also whether the variant was non-synonymous or
1008 synonymous mutation in a coding region. R^2 goodness-of-fit measure and associated P-values
1009 are shown for both mutation types. Note that this data considers only the coding portion of genome
1010 while Fig. 2d presents both coding and non-coding. **(b)** The cumulative distribution of the

1011 subclonal mutations in copy-neutral regions for hypermutators (n = 31 patients). For each variant
1012 fraction and subtype, the R² goodness-of-fit measure and P-values are shown.

1013 **Extended Data Fig. 7 | Driver gene nomination.** **a.** Local (gene-wise) dNdScv estimates by
1014 subtype (rows) and fraction (columns). Genes are sorted by Q-value and P-value. The Q-value is
1015 shown in color, whereas the P-value is indicated in light gray. The Q-value threshold of 0.05 is
1016 indicated by a horizontal red line. **b.** GISTIC significant amplification (red) and deletion (blue) plots
1017 in initial (left) and recurrent tumors (right). Chromosomal locations are ordered on the y-axis, Q-
1018 values are shown on the x-axis, and selected drivers are indicated by their chromosomal location
1019 on the right.

1020 **Extended Data Fig. 8 | Driver acquisition over time** **a.** Tabulated numbers of SNV (top) and
1021 CNV (bottom) driver events that were shared, initial-only, or recurrence-only. P-values were
1022 obtained using a two-sided Fisher test comparing the initial-only fraction to the recurrence-only
1023 fraction testing for acquisition. **b.** One-sided Fisher test comparing the initial-only fraction to the
1024 recurrence-only fraction amongst previously implicated glioma drivers testing for driver
1025 acquisition. P-values were adjusted for multiple testing using the FDR (x-axis). Hypermutators
1026 (red) and non-hypermutators (black) were separately analyzed.

1027 **Extended Data Fig. 9 | Intra-tumor CCF comparison.** Ladder plots comparing the CCF of co-
1028 occurring drivers in single tumor samples. The color of the lines and points indicates whether the
1029 sample shown is an initial (brown) or recurrent (green) tumor. Two-sided Wilcoxon rank-sum test
1030 P-values are shown for all initial samples, all recurrent samples, as well as all samples (black).

1031 **Extended Data Fig. 10 | Between time point intra-patient CCF comparison.** **a.** Driver-gene
1032 CCF comparison between initial and matched recurrences. Lines are colored by variant
1033 classification. Two-sided Wilcoxon rank-sum test P-values are shown. **b.** *TP53* CCF by subtype,
1034 otherwise as in **(a)**. **c.** *IDH1* CCF by subtype, otherwise as in **(a)**. **d.** Ladder plot visualizing CCF
1035 change across all SNVs between initial and recurrent tumors, separated by subtype. Wilcoxon
1036 rank-sum test was used to test for differences between time points. **e.** Initial and recurrent
1037 mutations in each patient were compared using a Wilcoxon rank-sum test. Bar plot with counts of
1038 patients in each subtype are shown. Patients lacking significant change are shown in yellow,
1039 those with a significant increase or decrease are shown in dark and light blue, respectively.

1040

1041 **Extended Data Fig. 11 | Aneuploidy calculation** **a.** Heatmap displaying the chromosomal arm-
1042 level events (x-axis) with patients represented in each row. Patients are placed in the same order
1043 for both the initial (left) and recurrence (right). White space was inserted as a break between the
1044 three subtypes. **b.** Distribution of total aneuploidy difference. Acquired aneuploidy determination
1045 (upper-quartile) indicated with a red line. **c.** Comparison of aneuploidy score between initial and
1046 recurrent tumors separated by subtype **d.** As **(c)**, comparing aneuploidy value.

1047 **Extended Data Fig. 12 | Neoantigen evolution and cellular analysis** **a.** Bar plots representing
1048 the number of shared mutations that give rise to neoantigens (top row, “immunogenic”) and those
1049 that do not give rise to neoantigens (bottom row, “non-immunogenic”) stratified by longitudinal

1050 clonality (“(clonality in initial)-(clonality in recurrence)”) and further separated by subtype.
1051 Percentage of longitudinal clonality per subtype and mutation immunogenicity are presented
1052 above the respective bars. **b.** *Left:* Ladder plot depicting the difference in observed-to-expected
1053 neoantigen ratio between the initial and recurrent tumors of patients with hypermutated tumors at
1054 recurrence. Each set of points connected by a line represents one tumor ($n = 70$). *Right:* Boxplot
1055 depicting the distribution of observed to expected neoantigen ratios in recurrent tumors stratified
1056 by hypermutator status ($n = 35$ and 183 for hypermutators and non-hypermutators, respectively).
1057 Each box spans quartiles, with the lines representing the median ratio for each group. Whiskers
1058 represent absolute range, excluding outliers. P-values for panel b were calculated using a paired
1059 and unpaired two-sided t-test, respectively. **c.** Stacked bar plots depicting the average relative
1060 fraction of 11 CIBERSORT cell types in the neoantigen depleted (< 1) and non-depleted (> 1)
1061 initial and recurrent tumor subgroups. Asterisks to the right of each plot indicate a significant
1062 difference ($P < 0.05$, Wilcoxon rank-sum test) between the depleted and non-depleted groups for
1063 the noted cell type at that time.

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