Fate mapping of human glioblastoma reveals an invariant stem cell hierarchy


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Human glioblastomas (GBMs) harbour a subpopulation of glioblastoma stem cells (GSCs) that drive tumourigenesis. However, the origin of intra-tumoural functional heterogeneity between GBM cells remains poorly understood. Here we study the clonal evolution of barcoded GBM cells in an unbiased way following serial xenotransplantation to define their individual fate behaviours. Independent of an evolving mutational signature, we show that the growth of GBM clones in vivo is consistent with a remarkably neutral process involving a conserved proliferative hierarchy rooted in GSCs. In this model, slow-cycling stem-like cells give rise to a more rapidly cycling progenitor population with extensive self-maintenance capacity, that in turn generates non-proliferative cells. We also identify rare “outlier” clones that deviate from these dynamics, and further show that chemotherapy facilitates the expansion of pre-existing drug-resistant GSCs. Finally, we show that functionally distinct GSCs can be separately targeted using epigenetic compounds, suggesting new avenues for GBM targeted therapy.
Introduction

Glioblastoma (GBM) is the most common and malignant form of adult brain tumor\(^1\). Central to our understanding of GBM biology is the idea that tumour initiation, maintenance, and regrowth following treatment are seeded by glioblastoma stem cells (GSCs)\(^2,3\). Evidence for a proliferative hierarchy in GBM has been derived from xenotransplantation of specific GBM subsets defined by surface marker expression\(^2\), genetic lineage tracing in mouse models\(^3\) and more recently, single-cell RNA-sequencing\(^4,5\). In parallel, GBMs exhibit substantial intra-tumoural genomic heterogeneity\(^6,7\) that could theoretically be based in GSCs with variations in growth potential, treatment responsiveness, or invasiveness\(^8-10\). However, recent evidence from other systems demonstrate that the intrinsic growth dynamics of a functionally homogeneous population of stem cells is already sufficient to create a wide range of clonal growth behaviours\(^11-14\). Therefore, it is yet unclear whether the heterogeneity of human GBM clones is primarily derived from their genomic heterogeneity, or the stochastic outcome of their hierarchical mode of growth.

DNA barcoding is a methodology that enables the proliferative capacity of individual cells to be resolved within polyclonal populations, with diverse applications in stem cell and cancer biology. Recent investigations with this strategy have already provided crucial insights into the lineage potential of normal stem cells\(^15\), the proliferative heterogeneity of their transformed counterparts\(^16\), as well as mechanisms of cancer drug resistance\(^17\) and metastasis\(^18\). Importantly, characterizations of population dynamics in a quantitative and unbiased way can be used to inform a mathematical framework to explain complex behaviours\(^13,17\). Here, we perform DNA barcoding of primary GBM cells in order to investigate the quantitative behaviours of GSC clones, creating a general, minimal model of GBM growth in which a high degree of intra-
tumoural functional complexity can be derived from a homogeneous population of stem-like cells.

**Lineage tracing of human GBM cells**

Lineage tracing assays based on genetic mouse models have demonstrated that quiescent stem-like cells promote brain tumour recurrence following chemotherapy\(^3,19\). However, it remains unclear how these cells contribute to tumour growth in genetically heterogeneous human GBM\(^6,7,20,21\). To identify potential differences in tumour clone-initiating potential, tolerance to chemotherapy and invasion capacity, we made use of a lentiviral barcoding strategy to trace the output of individual cells *in vivo* (Fig. 1a)\(^{15,16,22}\). Freshly dissociated cells from primary (GBM-719, -729, -735, -743, and -754) and recurrent (GBM-742) GBMs were transduced with a library of biologically neutral barcodes prior to their transplantation into the brains of NOD/SCID/IL-2γ\(^{-}\) (NSG) mice within 24 hours of isolation, a time window below the doubling time of GSCs (Extended Data Fig. 1a-c). For each tumour sample, spiked-in controls were included to estimate relative clone sizes from barcode read counts (Extended Data Fig. 1d-f). Given the high library diversity (~2×10\(^5\)) and limiting transduction efficiency across experiments (<38%), the majority of labelled cells were expected to carry unique barcodes (Extended Data Fig. 1g-h and Supplementary Theory 1).

Exome and RNA sequencing of primary tumours identified mutations in common GBM-associated genes (*TP53, EGFR, PDGFRA*) and signatures of the Classical and Proneural transcriptional subgroups (Extended Data Fig. 2a-b)\(^{20}\). Histologically, xenografts resemble human GBM and have abundant expression of the neural precursor marker nestin (Fig. 1b and
Extended Data Fig. 3a–b). Consistent with the significant inter-patient heterogeneity of human GBM\textsuperscript{20,21}, tumours generated from different primary samples differed in proliferative activity, apoptosis rates, growth rates and response to temozolomide (TMZ) chemotherapy (Extended Data Fig. 3c–d). In the following, we focused first on GBM-719 for which the largest xenograft data set was available, using xenografts from other GBMs to test for consistency in their properties.

Growth of GBM cells \textit{in vivo} was concomitant with expansion in both the injected (ipsilateral) and non-injected (contralateral) hemispheres (Fig. 1c and Extended Data Fig. 4a–b). For GBM-719, 1,532 clones (derived from \~3\% of barcoded cells) expanded above the detection threshold, with 475 present in both hemispheres. The sizes of these “surviving” clones were broadly distributed, with the majority remaining small (Fig. 2a). A further, smaller reduction in clone number was observed upon serial passaging, with a fraction becoming apparent only in the second passage, indicating that some clonogenic cells did not reach the detection threshold within the first passage (Fig. 1d). These observations suggest that the primary GBM population contained only a subset of cells with continuous tumour-maintaining activity (GSCs). However, the abundance of surviving clones and broad size distributions demonstrate that tumour growth does not rely on the activity of a few tumour-initiating cells (Fig. 2a and Extended Data Fig. 4e)\textsuperscript{4,5}.

**GBM clones are uniformly invasive**

We next sought to define the invasive capacity of barcoded GBM clones by comparing clonal composition between the ipsilateral and contralateral hemispheres, the latter representing expansion of invasive cells (Extended Data Fig. 4a–b). In all experiments, the sizes of clones in
both hemispheres were either highly correlated from the first passage on, or became highly correlated soon thereafter (Extended Data Fig. 4c), indicating that clonal behaviour in the contralateral side reflected their behaviour in the ipsilateral side. We then asked whether clones that were exclusively found in the contralateral side have a higher invasive capacity. However, xenografts derived from re-injecting contralaterally-harvested cells were primarily composed of clones that had been present in both hemispheres in the previous passage (Extended Data Fig. 4d). It follows that self-renewal and invasion capacity are coincident properties of the same labelled clones within each human GBM. Spatial separation of genetically distinct clones may therefore represent transient variations in local dispersal, which become amplified over time\textsuperscript{6,10,23,24}.

Neutral hierarchical growth dynamics

A consistent feature of clone sizes across all passages and between hemispheres was their broad distribution (Fig. 2a and Extended Data Fig. 4e). Such functional heterogeneity could derive from engrained “fitness” advantages of some tumour-initiating cells over others, resulting from heritable genetic or epigenetic alterations\textsuperscript{8}. Alternatively, variation in clonal output could result from “neutral” processes, reflecting the chance outcome of cell fate decisions obtained within an equipotent tumour-initiating population\textsuperscript{11,12}. To discriminate between these possibilities, we looked for evidence of equipotency in the distribution of relative clone size. Remarkably, the distributions were found to be consistent with a negative binomial dependence — as evidenced by the exponential form of the first incomplete moment (Fig. 2b, Extended Data Figs. 5-6 and Supplementary Theory 2). Some xenografts also showed a minority (<4%) of large clones that lay outside this distribution (Fig. 2b and Extended Data Fig. 4g, red arrowhead), a
feature returned to below. With clone size distributions across all 6 patient tumour samples largely characterized by just one parameter (the constant of the exponential), these observations suggest that GBM intra-tumoural heterogeneity derives primarily from the growth characteristics of a single equipotent cell population rather than an engrained differential fitness of subclones, an unexpected finding given the inter- and intra-patient genomic diversity of GBM and the ongoing genomic evolution observed in xenografts (Extended Data Fig. 5-6 and Supplementary Theory 3).

How could a negative binomial clone size distribution arise? Such behaviour is common in population dynamics and is typically associated with processes involving the sporadic creation of “individuals” —cells in this case— that, when born, undergo a stochastic process, selecting with equal probability between duplication (birth) or loss (death) and supported by a slow influx from another compartment (immigration) – a “critical birth-death process with immigration” (Supplementary Theory 3)\(^{25}\). In the tumour context, this behaviour translates to a proliferative hierarchy in which a slow-cycling stem cell-like population undergoes serial rounds of invariant asymmetric cell division, giving rise to a self-sustaining, rapidly-dividing progenitor population that generates short-lived non-proliferative progeny (Fig. 2c and Supplementary Theory 4).

But, is a mode of strictly invariant asymmetric cell division plausible? Since most barcoded clones survive dilution through serial passaging (Fig. 1d), individual clones at the end of the previous passage are likely to host a multiplicity of stem-like cells. Cell division must therefore also lead to symmetric fate outcomes so that their numbers can accumulate in individual clones. However, so long as asymmetric fate outcomes predominate, the resulting clone size distributions do not depart significantly from the observed negative binomial form (Supplementary Theory 4).
Based on a quantitative analysis of clone size, we propose that human GBM growth in xenografts is defined by a minimal model involving a defined GSC hierarchy (Fig. 2c and Supplementary Theory 4). To challenge the model and define the minimal set of parameters governing GBM growth, we used stochastic simulations to compare the predicted clonal dynamics with experimental findings (Fig. 2d-h and Supplementary Theory 5). In assessing the viability of the model, we constrained the simulation using a range of biologically plausible parameters based on the overall expansion of xenografts along with the proportion of actively dividing and apoptotic cells (Extended Data Fig. 3d and Supplementary Theory 5). Over the determined range of parameters, simulations revealed an approximately negative binomial clone size distribution across all serial passages (Fig. 2e), consistent with experiment. Using the unique barcoding of clones, we assessed correlations of clone size and survival likelihoods across serial passages. Remarkably, the minimal model captured the range of data to a high level of accuracy (Fig. 2f-h, Extended Data Fig. 4f and Supplementary Theory 6). Quantitative analysis of clone size distributions for GBM-742 and GBM-754, in addition to independent analysis of mutational data derived from GBM-719 xenografts, also provided strong evidence in favour of the same paradigm (Extended Data Fig. 6d-i, Supplementary Theory 6-7).

Two divergent GSC phenotypes

Building on the findings above, we next sought to define the effect of TMZ chemotherapy on clonal dynamics. Analysis of the TMZ-treated xenografts clearly distinguished two divergent behaviours: A majority of clones were sensitive to TMZ treatment and present at low abundances (“Group A” in Fig. 3a,b), while a minority were present at frequencies almost an order of magnitude greater, consistent with treatment resistance (“Group B” in Fig. 3a,b).
Comparison of the TMZ-treated secondary xenografts with the untreated primary xenograft indicated that the sizes of sensitive clones were largely uncorrelated across serial passages, whereas the sizes of the resistant clones appeared to be positively correlated (Fig. 3a).

Interestingly, the further coincidence of distinct resistant clones in drug-treated replicate xenografts (Figs. 3c,d) suggests that the resistance phenotype can be pre-existing within the parental population.

Based on this classification, we analysed the clone size distribution within each group separately. Sensitive clones maintained an approximate negative binomial dependence (Extended Data Fig. 5a) suggesting that, in sharp contrast with the mouse model\textsuperscript{3}, TMZ-treatment leaves the proliferative hierarchy of the majority of tumour cells unperturbed. In contrast, resistant clones could not be captured by the same dynamics (Extended Data Fig. 5a, red arrowheads).

However, with an additional acquired resistance to apoptosis, we found that the original model parameters were sufficient to explain the scale of the observed behaviours of resistant clones (compare Fig. 3a to Fig. 3e and Fig. 3b to Fig. 3f, Supplementary Theory 6.5). Importantly, large outlier clones can be detected even in untreated tumours across different GBM cases (Extended Data Figs. 5-6). Taken together, these results demonstrate that a minority of clones in pre- and post-treatment tumours conform to perturbed growth dynamics, and may constitute a key driver in the clonal evolution of human GBM. We define these outliers as “Group B” clones, and the majority that behave according to the negative binomial distribution as “Group A”.

Epigenetic targeting of distinct GSCs

We next questioned whether the Group B phenotype exposes new therapeutic vulnerabilities. Primary GSC cultures\textsuperscript{26} established from xenografts maintained a mixture of
clones seen in primary, secondary and tertiary passages (Extended Data Fig. 7a-b). Moreover, both cultures and xenografts derived from the same parental TMZ-treated xenograft were relatively concordant in their relative clonal abundances (Extended Data Fig. 7c), suggesting that GSC cultures can recapitulate their growth behaviour \textit{in vivo}. Strikingly, Group A clones from the GBM-754 primary xenograft-derived culture model \cite{1}, for which the most data was available, maintained a negative binomial distribution after an approximate 7-fold expansion \textit{in vitro}, consistent with maintenance of the proliferative hierarchy under culture conditions \cite{754, 8a-b}. This included the correlations of outlier clones between replicates (Extended Data Fig. 7f), corroborating the previously observed presence of Group B clones in untreated xenografts (Fig. 2b). Most cultures derived from other xenografts also adhered to a negative binomial distribution once the largest outliers were removed (Extended Data Fig. 8a-b).

We next combined \textit{in vitro} drug selection of the (1)\textsubscript{754} culture with barcode sequencing to determine whether resistance arises proportionately from each clone type (Extended Data Fig. 9a,b). GSC cultures analyzed by assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq) identified a shared epigenetic state, leading us to focus on epigenetic targets (Extended Data Fig. 2d). Cells subjected to drug selection were allowed to repopulate to a similar density as control, in order to model tumour regrowth following therapy (Extended Data Fig. 9b). The drug treatments induced a range of changes to clonal dominance patterns (Extended Data Fig. 9c). However, the same negative binomial distribution was maintained in most cases, indicating that the underlying dynamics of Group A clones are largely unperturbed (Extended Data Fig. 10a,b). Intriguingly, a Menin-Mixed Lineage Leukemia (MLL) interaction inhibitor (MI-2-2)\textsuperscript{27-29} was selective against Group B clones, as repopulation following selection derived primarily from Group A clones (Fig. 3g, Extended Data Fig. 9d). By
the same logic, and consistent with the requirement for Enhancer of zeste homolog 2 (EZH2) in GSC maintenance\(^{30}\), we found that an EZH2 inhibitor (UNC1999) was instead selective against Group A clones (Fig. 3g, Extended Data Fig. 9d). MI-2-2 is growth inhibitory in a polyclonal context, consistent with its specificity for the highly proliferative clone type (Extended Data Fig. 9e). Targeting both clone types by combining MI-2-2 with an EZH2 inhibitor (UNC1999 or GSK343) was uniquely sufficient to eradicate self-renewal (Fig. 3h, Extended Data Fig. 9f-h). Consistent with TMZ-induced selection for Group B clones in GBM-719, MI-2-2 treatment of TMZ-transformed cells eradicated self-renewal and reduced tumour growth \textit{in vivo} (Fig. 3i-j, Extended Data Fig. 9i). Efficacy of the UNC1999/MI-2-2 combination was mirrored in 4 additional models (G523, G549, G564, G566) even when single drug treatments did not affect self-renewal, and in GBM-851 primary cells (Extended Data Fig. 9j-n). While Menin-MLL inhibition is especially effective in targeting paediatric glioma that carry histone 3 variant H3.3 mutations\(^{27}\), these findings warrant further pre-clinical studies of MI-2-2 in advanced, post-treatment adult GBM.

\textbf{Discussion}

Efforts to define the identity and behaviour of tumour-maintaining cells in human GBM have focused on genetic intra-tumoural heterogeneity\(^{9,31}\). Yet the majority of subclonal mutations in cancer may be biologically neutral\(^{14,32}\). At first sight, the emergence of clonal heterogeneity suggests that the evolving mutational landscape may confer a range of fitness advantages on GSCs. However, quantitative analysis of clone sizes indicates that clonal heterogeneity can be explained by robust features of a conserved proliferative hierarchy. In this model, heterogeneity in clonal expansion does not derive from genetic diversity but, in common with other cancer
models\textsuperscript{11,12}, emerges as the predictable outcome of chance fate decisions made by GSCs and their progeny. Given the correlation of human GBM cell transcriptomes with those of normal outer radial glial cells and intermediate progenitors\textsuperscript{33}, these results suggest that the initiation of human GBM may be associated with the aberrant reactivation of a surprisingly normal developmental program.

While the majority of GSC clones adhere to neutral, hierarchical growth dynamics (Group A), we identified a minority subset that showed a different growth characteristic (Group B). It is currently unknown whether Group B clones share common molecular features between different patient tumours. Intriguingly, however, these dominant clones are sensitive to an epigenetic drug (MI-2-2) previously shown to be effective in H3.3 mutant paediatric glioblastoma\textsuperscript{27}. Together with the fact that adult GSCs can converge into an epigenetic state reminiscent of paediatric GBM due to selective downregulation of H3.3 expression\textsuperscript{29}, it is tempting to speculate that Group B clones in adult GBM may share additional epigenetic features of H3.3 mutant paediatric GBM cells and H3.3-low adult GSCs\textsuperscript{29}. Alternatively, Group B clones may arise from Group A clones after a gradual accumulation of genetic mutations that alters their mode of growth\textsuperscript{7}. Future studies should target the origin and functional properties of these clones, and assess whether they contribute disproportionately to GBM malignancy.
References


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Author Contributions


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Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to P.B.D. (peter.dirks@sickkids.ca) and B.D.S. (bds10@cam.ac.uk).
Figure Legends

Figure 1 | Serial transplantation scheme and characterization of barcoded glioblastoma xenografts.

a, General transplantation scheme for barcoded xenografts derived from primary GBM tumour cells (GBM-719). b, Staining of a secondary GBM-719 xenograft with the indicated markers, scale bar = 100 μm. c, Tumour growth quantified as the estimated fold-change in cell number between injection and harvesting for different ipsilateral derived GBM-719 xenografts. Lines indicate serial transplantation trajectories. d, Proportional Venn diagrams depicting the number of barcoded clones unique to each passage or shared between passages for the indicated experiment.

Figure 2 | Clonal dynamics of GBM is consistent with a conserved proliferative hierarchy.

a, Clone size distributions of xenografts derived from GBM-719 cells across different passages. For the primary passage, distributions for the ipsilateral (blue) and contralateral sides (red) are shown. For the secondary and tertiary passages, distributions for the ipsilateral side from different replicate experiments are shown (shades of blue). b, First incomplete moment of the corresponding clone size distributions shown in panel (a), displayed on a logarithmic scale (Supplementary Theory 2). Dashed lines show exponentials as a guide for the eye. The red arrowhead indicate deviations from exponential behaviour due to a small number (<4%) of outlier clones. c, A minimal model of tumour growth based on a three-component hierarchy involving transitions from a slow-cycling stem-like compartment (S) to a more rapidly cycling progenitor population (P) to a non-dividing compartment (D). Following S cell divisions, a
fraction, $\varepsilon$, result in symmetric fate outcome while the remainder lead to asymmetric fate. With equal probability, P cells divide symmetrically or give rise to D cells which, in turn, rapidly undergo apoptosis. d, Representative clone size trajectories computed for the model shown in (c). Different curves correspond to different clones across three serial passages, along with the average over all trajectories, with the S cell division rate of 0.15/day, the P cell division rate of 1/day, the D cell apoptosis rate of 0.5/day and $\varepsilon = 15\%$ (for details, see Supplementary Theory 5). e, First incomplete moment of the clone size distribution across passages derived from $2 \times 10^6$ simulated clone trajectories. The shaded areas show the regions within which 95% of the respective curves fall for repeated simulations with $5 \times 10^4$ clones each. For each passage, the first incomplete moment follows an approximate exponential size dependence. Parameters as in panel (d). f, Clone size correlation for different passages in the model (distributions) and from representative xenografts derived from GBM-719 cells (data points). Distributions show model results within the biologically plausible parameter range (see Supplementary Theory, Table S2). See Supplementary Theory, Figure S3 for other patients. g, Fraction of initially injected clones growing above half of the characteristic clone frequency $n_0/2$ for the same datasets as in (f) (see Supplementary Theory 6.3). See Supplementary Theory, Figure S2 for other patients. h, Simulated examples of clone size correlations across successive serial passages. Parameters are as in panel (d).

Figure 3 | Chemotherapy reveals clonal transformations in GBM.

a, Correlation of clone sizes for the primary, untreated xenograft with secondary xenografts treated with TMZ (light and dark dots indicate two replicate secondary xenografts). Light dataset – Group A: 1255 data points, Group B: 15 data points; dark dataset – Group A: 1228 data points, Group B: 10 data points. b, Correlation of clone sizes for a secondary TMZ-treated xenograft
(light dots in panel (a)) with tertiary TMZ-treated xenografts, light and dark dots indicate two replicate tertiary xenografts. Light dataset – Group A: 95 data points, Group B: 15 data points; dark dataset – Group A: 117 data points, Group B: 15 data points. c, Correlation of the two replicate secondary xenografts shown in (a) with Spearman’s rho indicated. d, Correlation of the two replicate tertiary xenografts shown in (b) with Spearman’s rho indicated. e-f, Correlation of clone sizes obtained from simulations with a subset of clones being resistant to cell death (blue dots) and the remaining clones following unperturbed dynamics (green dots) for a primary and secondary passage (e) and a secondary and tertiary passage (f) (see Supplementary Theory 6.5).

The S cell division rate is set at 0.1/day, the P cell division rate is 1.5/day, \( \varepsilon = 10\% \), and the apoptosis rate is set at 0.7/day with a 0.5% chance of each clone to show resistance to apoptosis (see Supplementary Theory, Table S3). g, Selectivity of UNC1999 and MI-2-2 for group A and B clones respectively, representative of 2 technical replicate experiments. Shown are relative clone sizes after DMSO treatment, or regrowth following selection with indicate compounds. The indicated values are clone sizes for groups A (black) and B (blue), lines connect the same barcoded clone under different conditions. h, Reduction of self-renewal ability upon treatment with epigenetic compounds alone and in combination as assessed by limiting dilution analysis (LDA), representative of 3 independent experiments (MI-nc: inactive control for MI-2-2, M: MI-2-2, C: CI-994, G: GSK591, U: UNC1999). \( P = 0.0663 \) for DMSO vs. CI-994, 0.132 for DMSO vs. GSK591, 0.216 for DMSO vs. UNC1999, 5.74\( \times 10^{-13} \) for DMSO vs. MI-2-2, 4.11\( \times 10^{-18} \) for MI-nc vs. M, 1 for M vs. M+C, 0.432 for M vs. M+G, 8.53\( \times 10^{-8} \) for M vs. M+U. i, MI-2-2 abrogates self-renewal in TMZ-transformed GBM-719 population, representative of 3 independent experiments. \( P = 3.73\times 10^{-3} \) for DMSO vs. UNC1999, 1.16\( \times 10^{-27} \) for DMSO vs MI-2-2, 1.61\( \times 10^{-16} \) for UNC1999 vs MI-2-2. All LDA results are representative of 3 independent
experiments with the remaining experiments presented in Extended Data Fig. 9. Analysis of all LDA results was performed using ELDA software\textsuperscript{34}, error bars represent 95% confidence interval (ns $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$). MI-2-2 inhibits tumour growth in subcutaneous xenografts derived from TMZ-transformed GBM-719 cells, n = 9 tumours per group, two-sided unpaired t-test. The horizontal line indicates the mean tumour weight of each experimental group.
**Methods**

No statistical methods were used to predetermine sample size. For animal studies, all animals were included for the analysis. Animals from separate litters were randomly and evenly divided between experimental groups to control for animal age. The investigators were not blinded to group allocation during the experiments and outcome assessment.

**Processing of patient samples**

GBM tumour samples were obtained from consenting patients, and all procedures are approved by the Research Ethics Boards at The Hospital for Sick Children (Toronto, Canada), St. Michael’s Hospital (Toronto, Canada) and Toronto Western Hospital (Toronto, Canada). Following surgical resection, tumour specimens were immediately subjected to mechanical and enzymatic dissociation in artificial cerebrospinal fluid (aCSF) containing trypsin, hyaluronidase, and kynurenic acid at 37°C. GSC culture models were established as previously described\(^{(26)}\), and matched to primary GBM tumour tissue by microsatellite genotyping (The Centre for Applied Genomics, Hospital for Sick Children). GSC cultures were also randomly and intermittently tested for mycoplasma contamination by PCR. For barcoding experiments, primary single-cell suspensions were subjected to magnetic bead depletion to remove cells expressing human CD31 and CD45 markers (130-091-935, 130-045-801, Miltenyi Biotech), thereby excluding endothelial and hematopoietic lineages prior to lentiviral barcoding.

**Exome sequencing**
For the primary tumour samples, DNA was extracted from flash frozen primary tumour pieces using an AllPrep DNA/RNA Mini Kit (80204, Qiagen). Genomic DNA libraries from which exons are captured were constructed according to British Columbia Cancer Agency Genome Sciences Centre plate-based and paired-end library protocols on a Microlab NIMBUS liquid handling robot (Hamilton, USA). Briefly, 1 µg of high molecular weight genomic DNA was sonicated (Covaris LE220) in 62.5 µL volume to 250-350 bp. Sonicated DNA was purified with PCRClean DX magnetic beads (Aline Biosciences). The DNA fragments were end-repaired, phosphorylated and bead purified in preparation for A-tailing using a custom NEB Paired-End Sample Prep Premix Kit (New England Biolabs). Illumina sequencing adapters were ligated overnight at 16°C and adapter ligated products bead purified and enriched with 6 cycles of PCR using primers containing a hexamer index that enables library pooling. 200 ng for each of 6 different libraries were pooled prior to whole exome capture using Agilent SureSelect All Exon V6+UTR probes. The pooled libraries were hybridized to the RNA probes at 65°C for 24 hours. Following hybridization, streptavidin-coated magnetic beads (Dynal, MyOne) were used for exome capture. Post-capture material was purified on MinElute columns (Qiagen) followed by post-capture enrichment with 6 cycles of PCR using primers that maintain the library-specific indices. The pooled libraries were sequenced on Illumina Hiseq 2500 using V4 sequencing chemistry at PE125 following Illumina recommendations (Canada’s Michael Smith Genome Sciences Centre, BC Cancer Agency).

For the GBM-719 xenograft samples, GFP positive barcoded cells were isolated by FACS (MoFlo Astrios, Beckman Coulter) from in vitro expanded cells (p3 TMZ TMZ) or directly from dissociated tumours (all remaining samples) and subjected to DNA extraction using a PrepGEM
DNA extraction kit (PTI0050, ZyGEM) prior to whole-genome amplification using a REPLI-g Mini kit (150023, Qiagen). 200 ng of DNA per sample was used to generate cDNA libraries following Agilent SureSelect XT target enrichment kit as per protocol. 750 ng from each cDNA library was then hybridized for 24 hours using the All Exon V5 capture baits from Agilent. Captured, enriched libraries were size validated using the Agilent Bioanalyzer DNA high sensitivity chip and library concentration was validated by qPCR (Kapa Technologies). All libraries were normalized to 10 nM and diluted to 2 nM before being denatured with 0.1N NaOH. Denatured library pools were diluted for a final time down to 14 pM of pooled libraries and loaded onto Illumina cBot for cluster generation. The clustered flow cell was sequenced paired-end 100 cycles using an Illumina HiSeq 2000 (Princess Margaret Genomics Centre, University Health Network).

For the germline reference sample, DNA was extracted from the patient’s whole blood using a DNeasy Blood & Tissue kit (69504, Qiagen). The library was prepared using Agilent SureSelect Human Exome Library Preparation V4 kit for paired end sequencing on a HiSeq 2500 platform. In brief, 750 ng of genomic DNA was fragmented to 200-bp on average using a Covaris LE220 instrument. Sheared DNA was end-repaired and the 3’ ends adenylated prior to ligation of adapters with overhang-T. Genomic library was amplified by PCR using 10 cycles and hybridized with biotinylated probes that target exonic regions; the enriched exome libraries were amplified by an additional 8 cycles of PCR. Exome libraries were validated on a Bioanalyzer 2100 DNA High Sensitivity chip (Agilent Technologies) for size and by qPCR using the Kapa Library Quantification Illumina/ABI Prism Kit protocol (KAPA Biosystems) for quantities. Exome libraries were pooled and sequenced with the TruSeq SBS sequencing chemistry using a
Exome sequencing analysis of primary tumours

For the primary tumour samples, Fastq files were aligned to the human reference genome hg38 with BWA (0.7.9a, -M option)\textsuperscript{35}. The BAM files were further processed using MarkDuplicates (Picard Tools 2.6.0), indel realignment (GATK 3.6 RealignerTargetCreator and IndelRealigner) and BaseRecalibration (GATK 3.6 BaseRecalibrator and PrintReads)\textsuperscript{36}. Samtools 1.3.1 mpileup (-B, -q10 -d10000000 options)\textsuperscript{37} was run on the processed BAM files to generate the input to Varscan. Varscan (2.4.2), mpileup2cns was applied to call snp and indels in each sample (--p-value 0.01 --min-var-freq 0.03, other default parameters)\textsuperscript{38}. The calls were annotated with Annovar (20160201, using refGene genes)\textsuperscript{39}. To identify the important somatic variants, the calls were further filtered to include only the following annotated events: nonsynonymous\_SNV, stopgain, stoploss and frameshift\_deletion. In addition, calls were removed if they were in the dbSNP database\textsuperscript{40} as part of the snp147Common file downloaded from the UCSC server which contains uniquely mapped variants that appear in at least 1% of the population or are 100% non-reference. Therefore, the flagged SNPs (uniquely mapped variants, excluding Common SNPs, that have been flagged by dbSNP as "clinically associated") were not removed. In addition, calls were further filtered out if they had an AF>0.001 in ExAC (exac03, ExAC\_ALL)\textsuperscript{41} or 1000 Genome Project (1000g2015aug\_all)\textsuperscript{42}. Subclonal mutations with variant allele frequency < 0.2 were excluded.

Exome sequencing analysis of xenografts
For the GBM-719 xenograft samples, read pairs were aligned to the hg19 reference sequence using the Burrows-Wheeler Aligner (v0.7.12)\textsuperscript{35}, and samples were demultiplexed using Picard tools (v1.140). Data were then sorted and duplicate marked using Picard and SAMtools\textsuperscript{37}. Local realignment around insertions or deletions (indels) and base-quality score recalibration was performed using the Genome Analysis toolkit (v3.4-46)\textsuperscript{36}. QualiMap (v2.1)\textsuperscript{43} was used to evaluate resulting sequencing alignment data. To correct for coverage discrepancies between Agilent V4 (germline reference sample) and V5 (xenograft samples) capture baits, an intersection of common regions was performed using bedtools (v2.26.0)\textsuperscript{44}. Common regions with 0X coverage in the blood or greater than 500X coverage in either reference or xenografts were removed from subsequent analysis.

The MuTect (v.1.15) algorithm\textsuperscript{45} was used for somatic variant calling and false-positive filtering. Resulting variants were annotated using Oncotator (v.2.8.0)\textsuperscript{46}, including common database variants (ClinVar\textsuperscript{47}, 1000 Genomes (phase 1 variant set)\textsuperscript{48}, dbSNP (build 138)\textsuperscript{40}, COSMIC (v71)\textsuperscript{49}). Germline variants found in the 1000 Genomes Project, dbSNP build 138 were excluded. Cellularity, ploidy and allele-specific copy number was estimated from normal-xenograft pairs using the Sequenza algorithm (v2.1.2)\textsuperscript{50}. Cutoffs of log 2 copy number ratios between -0.35 and +0.3 were set to assign genome losses and gains, respectively.

**RNA sequencing**

RNA was extracted from the same flash frozen primary tumour pieces as for exome sequencing using a Qiagen AllPrep DNA/RNA Mini Kit (80204, Qiagen). Qualities of total RNA samples were determined using an Agilent Bioanalyzer RNA Nanochip or Caliper RNA assay and arrayed into a 96-well plate (Thermo Fisher Scientific). Polyadenylated (PolyA+) RNA was
purified using the NEBNext Poly(A) mRNA Magnetic Isolation Module (E7490L, NEB) from 500 ng total RNA normalized in 35 µL for DNase I-treatment (1 Unit, Invitrogen). DNase-treated RNA was purified using RNA MagClean DX beads (Aline Biosciences, USA) on a Microlab NIMBUS liquid handler (Hamilton Robotics, USA). Messenger RNA selection was performed using NEBNext Oligod(T)25 beads (NEB) with incubation at 65°C for 5 minutes followed by snap-chilling at 4°C to denature RNA and facilitate binding of poly(A) mRNA to the beads. mRNA was eluted in 36 µL of Tris Buffer.

First-strand cDNA was synthesized from the purified polyadenylated messenger RNA using the Maxima H Minus First Strand cDNA Synthesis kit (Thermo-Fisher, USA) and random hexamer primers at a concentration of 5 µM along with a final concentration of 1 µg/uL Actinomycin D, followed by PCR Clean DX bead purification on a Microlab NIMBUS robot (Hamilton Robotics, USA). The second strand cDNA was synthesized following the NEBNext Ultra Directional Second Strand cDNA Synthesis protocol (NEB) that incorporates dUTP in the dNTP mix, allowing the second strand to be digested using USER™ enzyme (NEB) in the post-adapter ligation reaction and thus achieving strand specificity.

cDNA was fragmented by Covaris LE220 sonication for 55 seconds at a “Duty cycle” of 20% and “Intensity” of 5 to achieve 200-250 bp average fragment lengths. The paired-end sequencing library was prepared following the BC Cancer Agency Genome Sciences Centre strand-specific, plate-based library construction protocol on a Microlab NIMBUS robot (Hamilton Robotics, USA). Briefly, the sheared cDNA was subject to end-repair and phosphorylation in a single reaction using an enzyme premix (NEB) containing T4 DNA polymerase, Klenow DNA
Polymerase and T4 polynucleotide kinase, incubated at 20°C for 30 minutes. Repaired cDNA was purified in 96-well format using PCR Clean DX beads (Aline Biosciences, USA), and 3’ A-tailed (adenylation) using Klenow fragment (3’ to 5’ exo minus) and incubation at 37°C for 30 minutes prior to enzyme heat inactivation. Illumina PE adapters were ligated at 20°C for 15 minutes. The adapter-ligated products were purified using PCR Clean DX beads, then digested with USER™ enzyme (1U/µL, NEB) at 37°C for 15 minutes followed immediately by 13 cycles of indexed PCR using Phusion DNA Polymerase (Thermo Fisher Scientific Inc. USA) and Illumina’s PE primer set. PCR parameters: 98°C for 1 minute followed by 13 cycles of 98°C 15 seconds, 65°C 30 seconds and 72°C 30 seconds, and then 72°C 5 minutes. The PCR products were purified and size-selected using a 1:1 PCR Clean DX beads-to-sample ratio (twice), and the eluted DNA quality was assessed with Caliper LabChip GX for DNA samples using the High Sensitivity Assay (PerkinElmer, Inc. USA) and quantified using a Quant-iT dsDNA High Sensitivity Assay Kit on a Qubit fluorometer (Invitrogen) prior to library pooling and size-corrected final molar concentration calculation for Illumina HiSeq 2500 sequencing with paired-end 75 base reads (Canada’s Michael Smith Genome Sciences Centre, BC Cancer Agency).

RNA sequencing analysis

Fastq files were aligned with STAR (2.4.2a) on the hg38 human reference genome. FPKM values were computed with the DESeq2 fpkm function using the raw read count per gene (ReadsPerGene.out.tab file from STAR output), with size factor normalization and gene length derived from the hg38 GTF files used for the alignment. Subgroup classification was done using the simple GBM classifier. This 32-gene classifier permits greater accuracy of GBM subgroup classification when using RNA-seq data instead of gene expression microarrays, as was performed in the original subgrouping study. One of the 32 genes was not quantified in the
analysis so the classifier was run using 31 genes.

Assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq)
The open chromatin profiles of 11 GSC lines were defined using ATAC-seq as described previously and the prepared libraries were sequenced with 50 bp single end reads. Reads were mapped to hg19 using bowtie2 and peaks of open chromatin were called with MACS2. The correlation between samples was calculated as the Pearson correlation of the quantile-normalized signal across the peak catalogue. Here, the peak catalogue corresponds to all peak regions identified across the sample cohort, and the signal refers to the fold enrichment of the signal per million reads in a sample over a modelled local background. The chronic lymphocytic leukaemia (CLL) data used in this comparison was taken from a published dataset, and the raw signal was normalized together with the GSC cohort.

MGMT promoter methylation assay
Primary tumour DNA was subjected to bisulfite conversion using the EZ DNA Methylation-Gold Kit (D5005, Zymo Research), and MGMT promoter methylation status was assessed using a two-step PCR protocol as previously described. PCR products, including water control, were visualized by electrophoresis on a 2% agarose gel along with a 100 base pair ladder (NEB).

Lentiviral barcoding
The lentiviral barcode library has been described previously. For viral transduction, primary GBM cells were seeded per plate onto 10-cm cell culture dishes that are coated with
poly-L-ornithine (PLO, Sigma) and laminin (Sigma). The culture media consisted of serum-free Neurocult NS-A Basal (Stemcell Technologies) media, supplemented with 2 mmol/L L-gutamine, N2 and B27 supplements, 75 µg bovine serum albumin, 10 ng/mL recombinant human EGF (rhEGF), 10 ng/mL basic fibroblast growth factor (bFGF), and 2 µg/mL heparin (Sigma). Primary cells were incubated for approximately 12 hours at 37°C with lentivirus at an appropriate concentration to minimize multiple integration events. The concentration of lentivirus used was previously determined by titrating the library with a human fetal derived neural stem cell culture (HF7450), and assessing GFP positivity by flow cytometry (LSR II, BD Biosciences) 48 hours post-transduction. Barcoded cells were washed 5 times with PBS to remove remaining lentivirus, and immediately harvested by accutase (Sigma) treatment for orthotopic injection. A separate cell aliquot was cultured for 48 hours to allow for GFP expression, and transduction efficiency was determined by flow cytometry (LSR II, BD Biosciences).

Mouse xenografts

All mouse procedures were approved by The Hospital for Sick Children’s Animal Care Committee. For intracranial injections, animals were first anesthetized with isoflurane and given Ketoprofen as an analgesic. Tumour cells were then suspended in a 2 µl volume of PBS and injected in the forebrains of female NOD/SCID/IL-2γc (NSG) mice of age 1-3 months with a Hamilton syringe and stereotactic device. The coordinates for orthotropic injections are 4 mm anterior of lambda, 2 mm to the right of the midline, and 3 mm deep. For secondary and tertiary xenografts, 25 mg/kg TMZ (Sigma) solubilized in Cremophor or vehicle controls were administered by gastric gavage for 5 consecutive days, 10 days post-injection. Mice were
sacrificed for further processing once neurological symptoms are observed, or at experiment endpoint (6 months). Survival analysis was performed using GraphPad Prism 5 software.

**Processing of xenografts**

Forebrains were obtained from animals displaying neurological symptoms, and the two hemispheres (ipsilateral and contralateral) were dissected for processing separately. Each hemisphere was dissociated to single-cell suspensions as described in the “processing of patient samples” section. Cells were subsequently subjected to magnetic bead depletion to remove contaminating mouse cells (130-104-694, Miltenyi Biotech) prior to serial transplantation. Serial xenografts were always established without any intermediate culturing step. Either the ipsilateral or contralateral fraction from a single mouse was used to establish serial xenografts.

Approximately 15% of xenograft cells were used without magnetic bead depletion for PCR amplification, library preparation and deep amplicon sequencing of barcodes. One xenograft per experimental group was set aside for histological analysis. Splinkerette PCR according to a previously published protocol was performed in order to identify unique barcode vector integration sites from xenografts.

**Histopathology and immunohistochemistry**

Mouse brains were fixed in 4% paraformaldehyde (PFA), washed in 70% ethanol and paraffin embedded. 6 μm coronal sections were generated for further analysis. Haematoxylin and Eosin staining was carried out according to manufacturer’s instructions (MHS32-1L, Sigma-Aldrich and 6766009, Thermo Scientific). Antibodies for immunohistochemistry include anti-Nestin (MAB5326, Millipore used at 1:500), anti-Ki-67 (M7240, Dako used at 1:500) and anti-Cleaved
Caspase-3 (9661, Cell Signaling used at 1:500). A secondary anti-Mouse HRP antibody (A9044, Sigma 1:500) was used for detection using 3,3’-diaminobenzidine (DAB), Alkaline Phosphatase (AP) and Mouse on Mouse (M.O.M) detection kits (Vector Laboratories). Images were acquired using a 3DHistech Pannoramic 250 Flash II Slide Scanner and processed using Pannoramic Viewer software (3DHISTECH). Automatic detection and quantification of Ki-67 and Cleaved Caspase-3 staining was performed on six representative images per sample, using TMARKER software.

Barcode sequencing

Spiked-in controls were generated using a human fetal derived neural stem cell line (HF7450) using the previously described protocol, and combined into single wells of a 96-well plate. For the GBM-719 experiment and the first sequencing run, the cell numbers used as spiked-in controls were 10, 100, 250, 500, and 5000. For all subsequent in vivo experiments and the second sequencing run, the cell numbers used were 10, 100, and 5000. For all in vitro experiments and the third sequencing run, the cell numbers used were 10, 100, 500, and 5000. Separate spiked-in control only wells containing barcode sequences derived from 25,000 and 100,000 cells were also included in the GBM-719 experiment, to test accuracy of extrapolation for larger clones. The same was done in the third sequencing run for in vitro experiments, using a control of 50,000 cells. Xenograft samples were combined with spiked-in controls and subjected to DNA extraction using a PrepGEM DNA extraction kit (PTI0050, ZyGEM) followed by ethanol precipitation and deep amplicon sequencing as described previously. Briefly, a two-step PCR protocol was used to generate barcode amplicons with fault-tolerant sample indices, and equimolar samples were pooled and loaded onto a single lane of a flow cell for paired-end
Barcode data analysis

Barcode sequences were extracted from raw data files with custom scripts, and those with a minimum base quality of 20 that matched the flanking regions (with up to 3 mismatches) surrounding the barcode sequence were kept. A merging of highly similar barcodes was performed in order to limit the number of false positive barcode sequences that may arise from sequencing errors. Specifically, a list of read counts corresponding to all unique barcode sequences was generated, and read counts corresponding to sequences with up to three mismatches were combined into the most abundant sequence. Barcode sequence logograms were generated using the R package ggseqlogo (https://github.com/omarwagh/ggseqlogo). Spiked-in controls were retrieved for defining noise thresholds and clone size estimation as described previously. We defined fractional read value (FRV) as the read count for a particular barcode sequence divided by the sum read counts of all spiked-in controls in the sample. A relationship was generated between FRVs and control cell number for spiked-in controls across all samples. A Cook’s distance of 4/n was used to define outlier controls and the relationship was generated again with those outliers removed to estimate clone sizes. This step was performed to ensure that outlier controls do not influence the estimation of relative clone sizes in the majority of samples within a particular sequencing run. FRV thresholds were determined from spiked-in controls in order to maximize the difference between the true positive rate (TPR) and false positive rate (FPR), and only clones with FRVs greater than the threshold were kept. The total cell number for each sample was estimated by summing up estimated cell numbers for each clone in the sample.
that are above detection threshold. Relative clone sizes were then determined by dividing the cell numbers for each clone by the total cell number calculated for each sample. Proportional Venn diagrams for barcode sequences were generated with eulerAPE v3 software\textsuperscript{62}.

**Generation of xenograft-derived cultures**

Dissociated primary GBM xenografts were cultured as described in the “lentiviral barcoding” section after depletion of contaminating mouse cells (130-104-694, Miltenyi Biotech). All short-term cultures were subjected to 2 to 3 passages prior to barcode sequencing. Short-term cultures were not subjected to mycoplasma testing or microsatellite genotyping, although in all cases the identified barcode sequences of cultures matched those of the corresponding xenograft series.

**Cell culture assays**

For proliferation assays, GSCs were propagated for 11 days in triplicate under previously described conditions\textsuperscript{26}. Viable cells were counted on days 0, 2, 4, 7, 9, and 11 with a Countess Automated Cell Counter (Thermo Fischer Scientific), excluding apoptotic cells that stained positive for trypan blue (Thermo Fischer Scientific). Doubling times were calculated during exponential growth phase (between days 4 and 11) using the formula $t = \frac{\log_{10} 2 \times \log_{10}(N_{11}/N_{17})}{\log_{10}(2)}$, where $N_{17}$ and $N_{11}$ are the number of cells on days 4 and 11 respectively and t is the elapsed time in hours. For dosage response assays, GSCs were cultured with drug for 5 days with 6 technical replicates per dose, without any media changes. Cell viability relative to DMSO control was then assessed by AlamarBlue assay (Thermo Fisher Scientific) using a Gemini EM Fluorescence Microplate Reader (Molecular Devices).
Drug screening

Primary drug screens were carried out in 96-well format on passage 2-3 cultures that were grown under previously described conditions\textsuperscript{26}. An Incucyte Zoom live-cell analysis system (Essen Bioscience) was used to quantify confluency according to manufacturer’s instructions. In order to characterize drug responsiveness of barcoded clones, a second screen was performed where cells were seeded on 6-well plates, subjected to a single round of drug selection in duplicate, and harvested for barcode sequencing when the culture reached approximately the same confluency as DMSO controls (~90%). In this assay, culture media was refreshed every 3 days without drug. The concentrations of drugs used for screening were as follows: Rapamycin: 20 nM, Dasatinib: 125 nM, BIO; Daunorubicin: 1 \( \mu \)M, LGK-974; RO4929097; WP1066: 2 \( \mu \)M, Imatinib: 2.5 \( \mu \)M, Bromosporine; CI-994; GSK591; GSK-J4; GSK-LSD1; InSolution \( \gamma \)-Secretase Inhibitor X; IOX2; JQ-1; L-741,742; LAQ824; MI-2-2; MS023; OF-1; Olaparib; PFI-1; PNU96515E; SGC-CBP30; UNC1999: 5 \( \mu \)M, Erlotinib: 10 \( \mu \)M, TMZ: 50 \( \mu \)M. Once ~90% confluency is reached, all surviving cells were used for DNA extraction and barcode sequencing as described above.

Limiting dilution analysis (LDA)

Cells were plated onto Flat bottom 96 well plates (Sarstedt) in 100 \( \mu \)L of culture media, 6 replicates per cell dose. The culturing conditions are described previously\textsuperscript{26}, with the exception that culture plates were not coated with PLO and laminin to allow for sphere formation. For analysis of primary, uncultured GBM cells, two-fold dilutions from 4000 cells to 8 cells were used and scored after two weeks of culture. For analysis of established GSC cultures, two-fold dilutions from 2000 cells to 4 cells were used and scored after one to two weeks of culture. Drugs were added only once on the first day at either 1 \( \mu \)M or 5 \( \mu \)M as indicated for each
experiment, with 50 μL of fresh media added to each well after the first week. Investigators were
blinded to the label for each plate during data collection. Data were analyzed using ELDA
software\textsuperscript{34}.

\textbf{In vivo drug assay}

To test the effect of MI-2-2 treatment \textit{in vivo} on tumour growth, 200,000 (1,1\textsuperscript{T},1\textsuperscript{T})\textsubscript{179} cells were
transplanted subcutaneously into the flanks of NSG mice (6 mice per treatment group, total 12
mice) and allowed to grow for 1 week prior to drug treatment. Mice were then treated with either
20 mg/kg MI-2-2 (444825, Millipore) or vehicle control (15% DMSO, 25% PEG, 60% PBS) for
2 weeks by intraperitoneal injection. The treatment schedule was Monday, Wednesday, Friday of
each week for a total of 6 treatments. Mice were then monitored for tumour formation and
sacrificed once the control tumours reached endpoint for measurement (127 days between
injection and sacrifice). Flanks in which tumours were not visible were excluded from analysis.
Subcutaneous tumour size did not exceed the limit set by the experimental protocol with The
Hospital for Sick Children’s Animal Care Committee (17 mm in the longest dimension).

\textbf{Stochastic simulations}

A standard stochastic simulation algorithm\textsuperscript{63} was used to simulate realizations of the stochastic
process defined by the model shown in Fig. 2c and described fully in Supplementary Theory
section 5. Clone size distributions, clone size cross correlations and the ratio of surviving clones
were then calculated from 100,000 realizations of the system for each parameter set. To compare
the model with experiments, we simulated the system using 108 equidistant parameter sets
located in the region of biologically plausible parameters and compared the results to
experimental data points.

Data availability

ATAC-seq data have been deposited at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under the accession number GSE96088. WES and RNA-seq data have been deposited at the European Genome-phenome Archive (http://www.ebi.ac.uk/ega) under the accession number EGAS00001002424. All other data are available as Supplementary Data Tables, Source Data, or upon reasonable request from the corresponding authors (P.B.D. and B.D.S.).

Code availability

Code used throughout this study are available upon reasonable request from the corresponding authors (P.B.D. and B.D.S.).
Supplementary References


Extended Data Figure Legends

Extended data figure 1 | Barcode data processing.

a, Summary of GBM models used for barcoding experiments indicating TCGA subgroups as determined by RNA-Seq, self-renewing frequency as assessed by primary limiting dilution analysis (LDA), the number of primary xenografts successfully established and the cell dose used for primary xenografts (n.d: not done, n.s: no spheres). b, Proliferation kinetics of GSC cultures in vitro. Data are shown as mean ± sd of 3 technical replicates. c, Cell doubling times of GSCs grown in culture calculated using the data in (b). Data are shown as mean ± sd of 3 technical replicates, horizontal line marks 24 hours. d-f, Relationship between fractional read value (FRV) and input cell numbers in spiked-in controls for the three sequencing runs. The highly influential data points (Cook’s distance > 4/n) are grayed out and not used for regression analysis to estimate relative clone sizes. The black line is the line of best fit, and the grey box indicates sequencing noise threshold. g, Analysis of barcode sequence saturation across six in vivo experiments. h, Position weight matrices depicting the representation of variable nucleotides in the barcode library, the (1) ipsilateral sample, as well as the largest and smallest 100 clones in that sample. The height of nucleotides at each position represents its relative frequency, with the most frequently occurring nucleotide shown in the top position. i, Summary of unique barcode integration sites identified by splinkerette PCR.

Extended data figure 2 | Molecular characterization of GBMs and GBM xenografts.

a, Oncoprint plot of mutations identified in primary GBM tissue samples that are of the top 200 recurrently mutated genes in the provisional TCGA dataset. b, Multidimensional scaling plot for the 32-gene simple GBM classification method using RNA-Seq. Shown are the TCGA
samples with RNA-Seq data and 5 patient samples used in the current study. TCGA samples are labelled and coloured according to their original subgroup as determined from microarray expression analysis\textsuperscript{20}. c, Methylation-specific PCR assay for the MGMT promoter in 6 primary GBMs. L: ladder, -ve: water only control, U: unmethylated PCR product, M: methylated PCR product. Specific ladder marker sizes are shown in base pairs. d, Pairwise correlation of ATAC-Seq peak intensities across GSC culture models and compared with a chronic lymphocytic leukaemia (CLL) control\textsuperscript{57}. Black outline highlights correlations for GSC cultures derived from the GBMs used for the \textit{in vivo} barcoding study (G719, G729, G754). e, Summary of somatic mutations identified using exome sequencing from representative GBM-719 barcoded xenografts, grouped according to type. p2 Veh: passage 2; treated with vehicle, p2 TMZ: passage 2; treated with TMZ, p3 Veh Veh: passage 3; treated with vehicle at passages 2 and 3, p3 TMZ TMZ: passage 3; treated with TMZ at passages 2 and 3 and briefly expanded \textit{in vitro} prior to sequencing. f, Heat map representing relative copy number profiles from whole exome sequencing of GBM-719 xenograft samples. Segments of gains (red) or deletions (blue) are colour-coded based on log2 copy number ratios. Frequent loss of chromosome 10 is a common observation in GBM. g, Summary of patient characteristics for all tumour samples used throughout the study, and the experiment(s) that each sample is used for.

Extended data figure 3 | Functional characterization of GBMs and GBM xenografts.

a, H&E and human-specific nestin staining in primary glioblastoma specimens, scale bar = 100 µm. b, H&E and human-specific nestin staining for representative GBM xenografts, scale bar = 100 µm. c, Survival analysis of xenografts derived from the indicated GBM model and treatment conditions. All survival analyses were performed using a log-rank test (n = 4 mice per group
with the exception of the GBM-754 experiment, Vehicle – Vehicle group which contains 3
mice). d, Quantification of percentage proliferative activity in serial xenografts by Ki-67 staining
and percentage apoptosis by cleaved Caspase-3 staining, mean ± sd of 6 representative sections
from the same xenograft sample.

Extended data figure 4 | GSCs are able to invade contralaterally and have heterogeneous
clonal outputs.

a, Human-specific nestin staining in representative xenografts between ipsilateral and
contralateral hemispheres (scale bar = 1mm, Ipsi: ipsilateral hemisphere, Contra: contralateral
hemisphere). b, Comparison of cell numbers recovered from xenografts between the ipsilateral
and contralateral fractions, two-sided paired t-tests. Single data points are overlaid over the box
plot, the horizontal line represents the median, and the lower and upper hinges represent the 25th
and 75th quartiles respectively. The lower and upper whiskers extend from the hinge to the
lowest and highest values within 1.5 times the inter-quartile range (IQR). c, Plot of Pearson
correlation coefficients comparing relative clone sizes between two hemispheres, for the
indicated sample groups. The box-plots are displayed as with panel (b). d, Clonal composition
of tumours generated serially from contralateral fractions, grouped according to the geographical
distribution of each detected clone in the previous (primary) passage. e, Clone size distributions
for representative xenograft samples. All data shown are from ipsilateral hemispheres, not treated
with TMZ, and generated from ipsilateral-derived cells from the previous passage (in the case of
secondary and tertiary xenografts). Fits to a negative binomial distribution (curve) are included
for patients with rich data sets (GBM-719, GBM-742, and GBM-754), used for quantitative
analyses. Plot titles identify the respective sequence of serial passages by the nomenclature
introduced in the Supplementary Theory. f, Representative correlation of clone size between successive serial passages of GBM-719 untreated xenografts with Pearson’s r indicated. P1: primary passage, P2: secondary passage, P3: tertiary passage. g, Representative correlations of clone size between different secondary passage replicate experiments derived from the same primary xenograft as panel (f), with Pearson’s r indicated. The red arrowhead shows deviations from a linear correlation due to large outliers. R1: replicate 1, R2: replicate 2, R3: replicate 3.

Extended data figure 5 | First incomplete moment of clone size distributions for GBM-719, -729, and -735 xenografts.

a-c, First incomplete moments of the clone size distributions for all xenograft samples derived from patient tumours GBM-719 (a), GBM-729 (b), and GBM-735 (c). Samples are named according to the sequence of samples injected, V: vehicle treated, T: TMZ treated, C: generated from the contralateral fraction of the previous passage. For illustrative purposes, GBM-719 xenografts (a) that are TMZ-treated are marked with a red arrowhead where the distribution appears to deviate from the negative binomial. The indicated fit parameter n_o describe a characteristic clone size of the population (Supplementary Theory 2-3). Where Group B clones (large outliers) were removed to generate a more accurate fit, the number of clones removed is indicated and the re-calculated first incomplete moment distributions with outliers removed are plotted in grey. d, Schematic describing how a sequence of treatments resulting in a particular xenograft sample is incorporated into the sample nomenclatures.

Extended data figure 6 | First incomplete moment of clone size distributions for GBM-742, -743, and -754 xenografts and variant allele frequencies (VAFs) for GBM-719 xenografts.
a-c, First incomplete moments of the clone size distributions for all xenografts derived from the tumours GBM-742 (a), GBM-743 (b), and GBM-754 (c). Sample and plot annotations are as described for Extended data figure 5. d, Distribution of variant allele frequencies (VAFs) across GBM-719 xenograft samples. Mutations with a VAF of 0.5 likely corresponds to variants in the clonal population (found in all cells within the tumour), while less prevalent mutations correspond to subclonal populations defined by recent mutational events found only in a subset of cells. e, Comparison of VAF values for mutations in paired secondary and tertiary passages. f, First incomplete moments show a negative binomial distribution for VAF values below 0.5 across xenograft samples. The dashed line shows a fit to the exponential and the vertical line marks a VAF of 0.5. g, First incomplete moments for mutations that are newly detected in the tertiary vehicle- and TMZ-treated passage. h, Same as panel (f) after filtering out mutations that do not occur in diploid regions of the genome. i, Same as panel (g) after filtering out mutations that do not occur in diploid regions of the genome.

Extended data figure 7 | Barcode analysis of xenograft derived cultures.

a, Proportional Venn diagrams depicting the number of unique and shared barcoded clones as defined by the in vivo passages (primary, secondary, or tertiary), that are also detectable within the specified xenograft-derived cultures. b, Comparison of clone sizes between paired primary xenografts and primary xenograft-derived GSC cultures. c, Correlation of clone sizes between TMZ-treated GBM-719 xenografts, and cultures derived from these xenografts. A select cluster of clones that become outcompeted after secondary xenografts are outlined in blue, and Spearman’s rho coefficients are as indicated. d, First incomplete moments of the full clone size distributions for GBM-754 primary xenograft cultures at different times throughout culture.
expansion. e, First incomplete moments of the clone size distributions used in panel (d), with the 14 largest outlier clones removed from each sample. f, Pairwise clone size comparisons between replicate cultures in (d), with Spearman’s rho indicated.

Extended data figure 8 | First incomplete moment of clone size distributions for remaining GBM xenograft derived cultures.

a, Plots of first incomplete moment for cultures derived from the indicated GBM xenografts. b, Same as (a), with the indicated number of large outlier clones removed from the analysis.

Extended data figure 9 | Epigenetic drug screening of GBM-754 primary xenograft culture.

a, Primary drug screen of GBM-754 primary xenograft-derived culture, with growth assessed as culture density relative to DMSO control. Compounds highlighted in blue were used in subsequent experiments. b, Strategy to identify clonal differences in drug response. Cells are treated in duplicate with each compound, and allowed to repopulate to the same density as DMSO controls prior to barcode sequencing. c, Summary of results from drug repopulation experiments. The top plot shows the ratio between sum relative clone sizes of Group B and Group A, technical replicates are denoted as 1, 2, or 3. The horizontal line marks the mean Group B/Group A ratio for DMSO treated cultures. The bottom plot shows the number of reads obtained from each sample after repopulation, relative to DMSO. The horizontal line marks the mean number of reads for DMSO samples. d, Additional technical replicate experiments related to Fig. 3g, demonstrating selectivity of UNC1999 and MI-2-2 on Group A and B clones respectively. e, Dose response assays for the indicated GSC culture models upon UNC1999 and MI-2-2 treatment, mean ± sd of 6 technical replicates. f, Two additional independent experiments
related to Fig. 3h. $P$ values for the left and right replicates respectively are $6.95 \times 10^{-4}$; $0.148$ for DMSO vs. CI-994, $0.338$; $0.55$ for DMSO vs. GSK591, $3.31 \times 10^{-3}$; $0.0177$ for DMSO vs. UNC1999, $2.15 \times 10^{-11}$; $0.963$; $0.408$ for M vs. M + C, $0.338$; $0.55$ for DMSO vs. GSK591, $3.31 \times 10^{-3}$; $0.0177$ for DMSO vs. UNC1999, $2.15 \times 10^{-11}$; $1.59 \times 10^{-7}$ for DMSO vs. MI-2-2, $1.49 \times 10^{-10}$; $3.7 \times 10^{-12}$ for MI-nc vs. M, $0.963$; $0.408$ for M vs. M + U. 

$g$, Combined effect of GSK343 and MI-2-2 on self-renewal. $P=4.42 \times 10^{-6}$ for DMSO vs GSK343, $2.96 \times 10^{-12}$ for DMSO vs MI-2-2, $3.62 \times 10^{-6}$ for GSK343 vs M + G, $0.0125$ for MI-2-2 vs M + G.

$h$, Combined effect of UNC1999 and MI-2-2 on self-renewal when used at $1 \mu M$, representative of 3 independent experiments. $P=0.147$ for DMSO vs. UNC1999, $0.129$ for DMSO vs MI-2-2, $9.84 \times 10^{-4}$ for DMSO vs. M + U.

$i$, Two additional independent experiments related to Fig. 3i. $P$ values for the left and right replicates respectively are $4.59 \times 10^{-5}$; $4.81 \times 10^{-15}$ for DMSO vs. UNC1999, $3.28 \times 10^{-25}$; $1.13 \times 10^{-31}$ for DMSO vs MI-2-2, $1.86 \times 10^{-11}$; $3.61 \times 10^{-6}$ for UNC1999 vs MI-2-2. $j$-$m$, Combined effect of UNC1999 and MI-2-2 on self-renewal in the indicated GSC culture models. $P$ values for the G523, G549, G564, G566 experiments respectively are $1.9 \times 10^{-5}$; $1$; $0.758$; $0.799$ for DMSO vs UNC1999, $8.14 \times 10^{-18}$; $2.14 \times 10^{-4}$; $0.503$; $6.12 \times 10^{-4}$ for DMSO vs MI-2-2, $2.72 \times 10^{-12}$; $3.28 \times 10^{-30}$; $1.15 \times 10^{-21}$; $2.54 \times 10^{-8}$ for UNC1999 vs M + U, $7.69 \times 10^{-3}$; $1.26 \times 10^{-15}$; $2.61 \times 10^{-18}$; $8.82 \times 10^{-3}$ for MI-2-2 vs M + U.

$n$, Combined effect of UNC1999 and MI-2-2 on self-renewal of uncultured GBM-851 cells. $P=3.01 \times 10^{-3}$ for DMSO vs UNC1999, $1.36 \times 10^{-4}$ for DMSO vs MI-2-2, $3.11 \times 10^{-3}$ for UNC1999 vs M + U, $0.0276$ for MI-2-2 vs M + U. Analysis of LDA results was performed using ELDA software$^{34}$, error bars represent 95% confidence interval (ns $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

**Extended data figure 10** | First incomplete moment of the clone size distributions for drug-
treated GBM-754 primary xenograft cultures.

a, First incomplete moments of the full clone size distributions of GBM-754 primary xenograft cultures treated with different drugs. b, First incomplete moments of the clone size distributions used in panel (a), with 5 group B clones removed.
Harvesting a tumour following primary injection leads to tumour growth. Tumour harvesting at each passage (primary, secondary, tertiary) involves re-injection with or without TMZ and Veh. Imaging and sequencing at each passage show H&E, Nestin, Ki-67, and Cleaved Caspase-3 staining. Diagrams illustrate the fold change in cell number across passages for GBM-719 to GBM-754.
Supplemental Theory for
Fate mapping of human glioblastoma reveals an invariant stem cell hierarchy

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In this Supplementary Text, we describe the quantitative analysis of clonal data obtained from serial transplantation experiments of human glioblastoma (GBM) xenografts involving lentiviral barcoding. Our strategy is to analyse the features of barcode frequency distributions to infer the underlying cell fate dynamics giving rise to the heterogeneity of clonal behaviour observed in experiments. This heterogeneity could either be (i) a consequence of differential engrained or evolving fitness advantages of cells or (ii) reflecting stochastic fate choices of equipotent progenitor pools (Clayton et al., 2007; Blanpain and Simons, 2013). Here we show that the experimental data is consistent with the latter scenario and that the key features of barcode frequency distributions and correlations can be explained by a simple proliferative hierarchy with glioblastoma stem-like cells at the apex.

In Section 1, we address statistical properties of lentiviral barcoding and give estimates of the amount of uniquely labelled cells. In Section 2, we show that the experimentally obtained barcode frequencies follow a negative binomial distribution. This behaviour is characteristic of a specific class of proliferative hierarchies—in Section 3, we show how such a distribution generically arises. Based on these observations, we develop a minimal model of tumour growth in Section 4 and study its predictions on tumour expansion and composition. In Section 5, we use our model to develop a simulation of the serial transplantation experiments which permits a direct comparison of our model with experiments. In Section 6, we infer plausible parameter ranges for our model on biological grounds and compare the model results of our theory with experiments. In Section 7, we use the experimentally obtained data from exome deep sequencing to probe the mutational heterogeneity of the parent tumour and as an independent window on the clonal dynamics of GBM cells.

1 Statistical properties of lentiviral barcoding

Lentiviral barcoding relies on the random infection of cells. While it entails the possibility to uniquely identify clone lineages, the randomness of the barcoding procedure may lead to the same cell acquiring multiple barcodes or to the same barcode being present in more than one cell. Since this can affect the statistical properties of the derived barcode frequency distributions, we here give an estimate for the relative amount of multiply labelled cells and barcodes present in multiple cells.

For a library consisting of $N_B$ unique barcodes with a barcoding event occurring with probability $p_B$, the number $n_B$ of barcodes acquired by a single cell follows the binomial distribution $Q(n_B) = P_{\text{Binomial}}(n_B|N_B, p_B)$, where $P_{\text{Binomial}}(n|N, p) = \binom{N}{n} p^n (1 - p)^{N-n}$. For large $N_B$, the distribution $Q$ can be approximated by a
Supplemental Information

Table S1 Probabilities characterising the statistical properties of lentiviral barcoding with a library of \( N_B = 2 \times 10^5 \) barcodes (L. V. Nguyen, M. Makarem, \textit{et al.}, 2014).

<table>
<thead>
<tr>
<th>Patient</th>
<th>( N_C \times 10^4 )</th>
<th>( \eta )</th>
<th>( Q_0 )</th>
<th>( Q_1 )</th>
<th>( Q_{&gt;1} )</th>
<th>( R_0 )</th>
<th>( R_1 )</th>
<th>( R_{&gt;1} )</th>
<th>( Q )</th>
</tr>
</thead>
<tbody>
<tr>
<td>GBM719</td>
<td>12.5</td>
<td>37.8%</td>
<td>62.2%</td>
<td>29.5%</td>
<td>8.3%</td>
<td>74.3%</td>
<td>22.1%</td>
<td>3.6%</td>
<td>69.6%</td>
</tr>
<tr>
<td>GBM729</td>
<td>12.5</td>
<td>21.6%</td>
<td>78.4%</td>
<td>19.1%</td>
<td>2.5%</td>
<td>85.9%</td>
<td>13.1%</td>
<td>1.0%</td>
<td>84.4%</td>
</tr>
<tr>
<td>GBM735</td>
<td>3</td>
<td>37.6%</td>
<td>62.4%</td>
<td>29.4%</td>
<td>8.2%</td>
<td>93.2%</td>
<td>6.6%</td>
<td>0.2%</td>
<td>91.6%</td>
</tr>
<tr>
<td>GBM742</td>
<td>2.4</td>
<td>28.7%</td>
<td>71.3%</td>
<td>24.1%</td>
<td>4.6%</td>
<td>96.0%</td>
<td>3.9%</td>
<td>0.1%</td>
<td>95.3%</td>
</tr>
<tr>
<td>GBM743</td>
<td>8</td>
<td>17.3%</td>
<td>82.7%</td>
<td>15.7%</td>
<td>1.6%</td>
<td>92.7%</td>
<td>7.0%</td>
<td>0.3%</td>
<td>92.0%</td>
</tr>
<tr>
<td>GBM754</td>
<td>12.5</td>
<td>33.2%</td>
<td>66.8%</td>
<td>27.0%</td>
<td>6.2%</td>
<td>77.7%</td>
<td>19.6%</td>
<td>2.7%</td>
<td>74.1%</td>
</tr>
</tbody>
</table>

Poisson distribution,

\[
Q(n_B) \simeq \frac{v^n_B}{n_B!} e^{-v}. \tag{1}
\]

where \( v = p_B N_B \). Using Eq. (1), the relative amount of unlabelled cells, \( Q_0 = Q(0) \), the relative amount of cells labelled with one barcode, \( Q_1 = Q(1) \), and the relative amount of cells carrying more than one barcode, \( Q_{>1} = \sum_{n_B>1} Q(n_B) \), are obtained as

\[
Q_0 = e^{-v}, \quad Q_1 = v e^{-v}, \quad Q_{>1} = 1 - (1 + v) e^{-v}. \tag{2}
\]

The parameter \( v \) characterizing the distribution of barcodes can be obtained from the labelling efficiency \( \eta \), which denotes the relative amount of cells that bear at least one barcode, by requiring \( 1 - Q_0 = \eta \). This yields

\[
v = -\ln(1 - \eta). \tag{3}
\]

Conversely, we can ask how likely it is that the same barcode appears in multiple cells. Out of a total of \( N_C \) cells prepared for barcoding, the number \( n_C \) of cells acquiring the same barcode is distributed according to \( R(n_C) = P_{\text{Binomial}}(n_C|N_C, p_B) \). Again, for a large number of cells \( N_C \), this can be approximated by a Poisson distribution,

\[
R(n_C) \simeq \frac{k^{n_C}}{n_C!} e^{-k}, \tag{4}
\]

where \( k = p_B N_C = v N_C / N_B \). Analogous to Eqs. (2), we obtain the relative amount of barcodes that are present in no cell, \( R_0 = R(0) = e^{-k} \), the relative amount of barcodes present in exactly one cell, \( R_1 = R(1) = k e^{-k} \) and the relative amount of barcodes that have been acquired by more than one cell, \( R_{>1} = \sum_{n_C>1} R(n_C) = 1 - (1 + k) e^{-k} \).
Multiple barcoding of the same cell is unproblematic for the quantitative analysis of barcode frequency distributions—it generates copies of clones which are however subject to the same distribution of barcode frequencies. On the other hand, barcodes distributed to multiple cells lead to an effective merging of the sizes of derived clones and thus may alter the statistical properties of the barcode frequency distribution. Among the labelled cells, the relative amount of uniquely labelled cells, i.e., cells with a unique combination of one or more barcodes, is given by

\[
Q = \frac{1}{1 - Q_0} \sum_{n_B = 1}^{\infty} Q(n_B) \left[ (1 - p_B)^{N_C - 1} \right]^{n_B},
\]

which, for \( N_C \gg 1 \) and \( p_B \ll 1 \), can be approximated in terms of the probabilities \( Q_0 \) and \( R_0 \) as

\[
Q \approx \frac{1 - Q_0^{-R_0}}{1 - Q_0^{-1}}.
\]

Table S1 summarizes the respective probabilities for all xenografts used in this study; a large majority of labelled cells carries a unique combination of barcodes in all xenografts.

## 2 Barcode frequencies follow a negative binomial distribution

To obtain a quantitative understanding of tumour growth, we analyse the distribution of barcode frequencies obtained from serial transplantation experiments. Here, we show that the distributions \( p(n) \) of barcode frequencies above the detection threshold for all passages and replicate experiments follow a negative binomial distribution,

\[
p(n) = \frac{1}{\mathcal{N}_0} \frac{e^{-n/n_0}}{n},
\]

where \( n_0 \) is a characteristic barcode frequency of the respective population and \( \mathcal{N}_0 \) is a normalisation constant. A robust method to detect negative binomial distributions is to obtain the first incomplete moment of the distribution \( p \), defined by

\[
\mu(n) = \frac{1}{\langle n \rangle} \sum_{n' = n}^{\infty} n' p(n')
\]

where \( \langle n \rangle = \sum_n n p(n) \) is the average barcode frequency. By definition, \( \mu(n) \) is the relative average barcode frequency of all barcode frequencies larger than \( n \). If the
barcode frequency distribution $p(n)$ has the negative binomial form Eq. (7), the first incomplete moment acquires an exponential dependence on the barcode frequency,

$$
\mu(n) = \frac{1}{N_1} e^{-n/n_0} ,
$$

(9)

where $N_1$ is another normalization constant. Since the first incomplete moment, together with the average barcode frequency $\langle n \rangle$, carries the same information as the original barcode frequency distribution\(^1\), an exponential dependence of $\mu$ is completely equivalent to a negative binomial barcode frequency distribution.

Fig. 2b in the main text and Extended Data Figs. 5-6 show all first incomplete moments of the experimental barcode frequency distributions. They clearly exhibit an exponential behaviour (linear on a logarithmic scale) over many decades of barcode frequencies, indicating negative binomial distributions across different patients, xenografts, passages, and replicate experiments. By definition of the first incomplete moment, data points with large barcode frequencies outside the negative binomial distribution show up as a strong deviation from the exponential behaviour (see, e.g., red arrowhead in Fig. 2b). This is caused by the barcode frequency entering as a multiplicative term in the definition Eq. (8). Importantly, this does not affect its ability to detect negative binomial distributions for small barcode frequencies.

### 3 Emergence of negative binomial distributions

What can the barcode frequency distribution tell us about the proliferative dynamics underlying tumour growth? A generic mechanism giving rise to a negative binomial distribution is a process long-known in population dynamics, termed ‘critical birth-death process with immigration’ (Bailey, 1990; Simons, 2016). Translated into the language of cell population dynamics, such a process can be realized by a population of cells that stochastically divide (‘birth’) and differentiate (‘death’) with equal probability (‘critical’), with a slow influx of cells from another cell compartment (‘immigration’) through differentiation. In the tumour context, such a process could naturally arise if there is (i) a slowly cycling glioblastoma stem cell (GSC) compartment at the apex of a proliferative hierarchy that sporadically gives rise to progenitor cells by asymmetric division and (ii) the resulting progenitor population undergoes division and differentiation that are balanced on the population level. Schematically, the dynamics of stem cells (S) and progenitors (P) can be expressed as

$$
\begin{align*}
S & \xrightarrow{\omega} S \quad \xrightarrow{\lambda} P \\
S & \xrightarrow{\text{prob. 1/2}} P \\
P & \xrightarrow{\text{prob. 1/2}} P \\
& \xrightarrow{\text{prob. 1/2}} P
\end{align*}
$$

(10)

\(^1\)The barcode frequency distribution can be retrieved from the first incomplete moment via the relation $p(n) = \langle n \rangle [\mu(n) - \mu(n - 1)]/n$. 

6
where $\lambda$ is the loss-and-replacement rate of the progenitors and $\omega$ is the asymmetric division rate of the stem cells, also called ‘immigration rate’ since it describes the rate at which cells enter the progenitor compartment. If the immigration rate $\omega$ is small compared to the loss-and-replacement rate $\lambda$, uniquely barcoded stem-like cells produce clones with a negative binomial barcode frequency distribution, Eq. (7). To show this, we describe the cell fate dynamics shown in scheme (10) as independent Poisson processes with rates $\omega$ and $\lambda$, respectively. Formally, the corresponding master equation that governs the dynamics of the probability $P = P(n, t)$ to find $n$ progenitor cells in a clone derived from a single uniquely labelled S-type cell,

$$\frac{\partial P}{\partial t} = \left\{ \omega (\hat{E}^{-} - 1) + \frac{\lambda}{2} (n-1) \hat{E}^{-} + \frac{\lambda}{2} (n+1) \hat{E}^{+} - \lambda n \right\} P,$$

(11)

where we have introduced the ladder operators $\hat{E}^\pm$, defined by $\hat{E}^{\pm} P(n, t) = P(n \pm 1, t)$. The first term in brackets describes the asymmetric division of a single S-type cell whereas the remaining three terms describe symmetric division and death of P-type cells. Note that asymmetric division of S-type cells leaves the number of S-type cells unchanged so that it is sufficient to only describe the number $n$ of P-type cells.

The master equation (11) describes the dynamics of S-type and P-type cells shown in the scheme (10) as independent Poisson processes. An analytical solution can readily be obtained by standard methods (Walczak et al., 2012). For initially no progenitor cells being labelled, $P(n, 0) = \delta_{n,0}$, the exact solution to the master equation (11) is given by the negative binomial distribution

$$P(n, t) = \frac{1}{n!} \frac{\Gamma(\xi + n)}{\Gamma(\xi)} \left( \frac{n_0(t)}{1 + n_0(t)} \right)^n \left( 1 - \frac{n_0(t)}{1 + n_0(t)} \right)^\xi,$$

(12)

where $n_0(t) = \lambda t/2$, the dimensionless parameter $\xi = 2\omega/\lambda$ is the ratio of immigration rate and progenitor loss-and-replacement rate, and $\Gamma(x) = \int_0^\infty u^{x-1} e^{-u} \text{d}u$.

$^2$The structure of the master equation (11) can be understood by considering, for instance, a reduced dynamics that only describes asymmetric divisions of the S-type cell. This amounts to setting $\lambda = 0$ in Eq. (11) which yields the reduced equation $\frac{\partial P}{\partial t} = \omega P(n-1, t) - \omega P(n, t)$, where we have used the definition of the ladder operator $\hat{E}^{-}$. This equation describes the rate of change of the probability $P(n, t)$ to find $n$ P-type cells. A state with $n$ P-type cells can only be reached if there are already $n-1$ P-type cells and an asymmetric division of an S-type cell occurs, giving rise to another P-type cell. The corresponding contribution $\omega P(n-1, t)$ to the rate of change $\frac{\partial P}{\partial t}$ is given by the probability $P(n-1, t)$ to find the system in the state $n-1$ multiplied by the rate $\omega$ of asymmetric divisions. Conversely, the state with $n$ P-type cells is left if another asymmetric division of the S-type cell occurs, raising the number of P-type cells to $n+1$. The analogous contribution $-\omega P(n, t)$ enters with a negative sign as it describes the process of leaving the state $n$. The other terms in the full master equation (11) follow the same logic. For more detailed reviews on general master equations and birth-death processes, we refer the reader to standard textbooks (Gardiner, 2009; Bailey, 1990).
is the Gamma function. On average, barcode frequencies grow linearly in time,
\[ \langle n(t) \rangle = 1 + \omega t, \]
where \( \langle n(t) \rangle = 1 + \sum_n n P(n, t) \) is the average barcode frequency with the first term accounting for the stem cell. For small immigration rates \( \omega \), the barcode frequency distribution of cell populations with at least one progenitor cell, given by \( P_*(n, t) = P(n, t)/(1 - P(0, t)) \), acquires the form Eq. (7),
\[ P_*(n, t) \approx \frac{1}{\mathcal{N}_0(t)} \frac{e^{-n/n_0(t)}}{n}, \]
where \( \mathcal{N}_0(t) = \ln n_0(t) \). For non-vanishing but small immigration rates \( \omega \), the resulting barcode frequency distribution is still well-approximated by Eq. (14). Thus, the dynamics (10) generically give rise to negative binomial barcode frequency distributions and hence are the starting point for our quantitative analysis.

Comparison with clone size distributions emerging from engrained proliferative heterogeneity

Could a negative binomial barcode frequency distribution also be caused by engrained proliferative heterogeneity instead of equipotency? To address this question, let us consider a large population of clones in which the cell of each clone \( i \) undergoes loss and replacement with clone-specific probabilities. For concreteness, we consider the following cell fate dynamics in which each cell undergoes loss and replacement with different probabilities,
\[ p_{\text{rob.1/2 + } \delta_i} \] and \[ p_{\text{rob.1/2 - } \delta_i}. \]

The parameter \( \delta_i \) determines whether cell \( i \) is primed for proliferation (\( \delta_i > 0 \)) or loss (\( \delta_i < 0 \)). The average size of a clone derived from cell \( i \) evolves according to \( \langle n_i(t) \rangle = e^{2\delta_i \lambda t} \) and on average, clones will thus either grow exponentially (\( \delta_i > 0 \)) or die out (\( \delta_i < 0 \)). In this picture, engrained proliferative heterogeneity is captured by a broad distribution of the \( \delta_i \), so that some clones expand faster than others while some clones die. For a clone with a given \( \delta_i \), the resulting surviving clone size distribution at large times is exponential (Bailey, 1990),
\[ p(n|\alpha_i) \approx \alpha_i e^{-\alpha_i n}. \]
with an exponent \( \alpha_i \) that depends on the proliferative potential and on time. Hence, a distribution in engrained proliferative advantages \( \delta_i \) entails a distribution in the
shape parameter $\alpha_i$ of the clone size probabilities for the different clones. As an example, let us consider the distribution of $\alpha_i$ at a fixed time $t = t_0$. For simplicity, we here consider a Gamma distribution\(^3\) for $\alpha$, which ensures that $\alpha > 0$,

$$\tilde{\rho}(\alpha) = \frac{\alpha^{m-1}e^{-m\alpha/\alpha_0}}{(\alpha_0/m)^m \Gamma(m)}.$$  

(17)

The clone size distribution resulting from this distribution of clone size scales is given by

$$p(n) = \int_0^\infty p(n|\alpha) \tilde{\rho}(\alpha) d\alpha = \frac{\alpha_0}{(\alpha_0 n/m + 1)^{m+1}},$$  

(18)

which asymptotically has the power law behaviour $n^{-(m+1)}$ and is therefore distinctly different from the negative binomial form $e^{-n/n_0}/n$. Which distribution of proliferative potentials would be needed to generate a negative binomial clone size distribution under these circumstances? In fact, a negative binomial form can only be obtained under very artificial conditions: the distribution for $\alpha$ would have to take the non-normalizable discontinuous form $\tilde{\rho}(\alpha) \propto \alpha^{-1} \Theta(\alpha - \alpha_0)$ where $\Theta$ is the Heaviside step function; in this case, the clone size distribution would sensitively depend on the position $\alpha_0$ of the step as it determines the characteristic scale of clone sizes, $p(n) = e^{-\alpha_0 n}/n$. While being simplistic, this minimal model of engrained proliferative heterogeneity illustrates that negative binomial clone size distributions do not generically arise from a mere loss and replacement of clones—rather, the cell fate dynamics have to display certain distinctive features, such as the minimal hierarchy of the type (10), which robustly leads to such clone size distributions.

4 Theoretical model of tumour growth

In Section 3, we have shown how a negative binomical barcode frequency distribution can arise from a single uniquely labelled stem cell at the apex of a critical birth-death process with immigration. However, there are several reasons why growth of glioblastoma as observed in serial transplantation experiments warrant a more comprehensive model: First, the model (10) only considers strictly asymetrically dividing stem cells, leading to linear growth of barcode frequencies on average. However, there is no reason to a priori rule out symmetric stem cell divisions, which potentially provide a considerable contribution to tumour growth. Second, in the model (10), loss of the stem cell leads to a remaining progenitor cell population that will not grow on average and will eventually die out (Clayton et al., 2007). In

\(^3\)The Gamma distribution as defined in Eq. (17) has mean $\alpha_0$ and variance $\alpha_0^2/m$; in the limit of large $m$, it is approximately equal to a normal distribution with the same mean and variance. For $m = 1$, the Gamma distribution reduces to an exponential distribution.
the serial transplantation experiments, only small fractions of a harvested tumour (~5%) are chosen for reinjection. If clones were indeed maintained by a single stem cell, it would thus be likely that the stem cell is lost upon reinjection, giving rise to a massive loss of barcodes across passages which is not observed in experiments. Third, the model (10) neglects the potential presence of a non-proliferating compartment undergoing apoptosis that may affect the tumour size and composition. This non-proliferating compartment may be the differentiating progeny of the progenitor population or a quiescent progenitor population.

Therefore, in this section, we now formulate a more comprehensive model of glioblastoma growth and study its predictions on tumour growth and composition. Our model makes falsifiable predictions and to compare it with experiments, we introduce a simulation procedure that combines the clonal dynamics with harvesting and reinjection scheme to mimic the experimental procedure (Section 5). Subsequently, we compare our model to experimental data and show that it captures the key features of clonal dynamics (Section 6).

4.1 Stochastic dynamics of cell division and differentiation

Our model of tumour growth describes the dynamics of three cell compartments: a stem-like cell compartment (S), a progenitor compartment (P), and a non-proliferating compartment that may account for differentiating progeny (D). In our model, stem-like cells divide symmetrically with a probability \( \varepsilon \) and asymmetrically with probability \( 1 - \varepsilon \). Progenitor cells either divide symmetrically or differentiate into their progeny, both with probability \( \frac{1}{2} \), so that division and differentiation are balanced on the population level. The differentiating compartment has a finite lifetime and constitutes the lowest level of the differentiation hierarchy in our model. Schematically, the model can be expressed as

\[
\begin{align*}
\text{S} & \xrightarrow{\omega} \text{S} \quad \xrightarrow{1 - \varepsilon} \text{S} \\
\text{S} & \xrightarrow{\varepsilon} \text{S} \quad \xrightarrow{\text{P}} \text{P} \\
\text{P} & \xrightarrow{1/2} \text{P} \\
\text{P} & \xrightarrow{1/2} \text{D} \\
\text{D} & \xrightarrow{\Gamma} \text{D}
\end{align*}
\]

where \( \omega \) and \( \lambda \) are the division rates of stem cells and progenitors, respectively, and \( \Gamma \) is the apoptosis rate of the differentiating progeny. Defining \( P(n^S, n^P, n^D, t) \) as the probability to find \( n^S \) stem cells, \( n^P \) progenitor cells, and \( n^D \) differentiated cells at time \( t \) within a clone, we write down a master equation governing the stochastic dynamics in the same spirit as in the previous section,

\[
\begin{align*}
\frac{\partial P}{\partial t} &= \left\{ \varepsilon \omega (n^S - 1) \hat{E}_S^- + (1 - \varepsilon) \omega n^S \hat{E}_P^- - \omega n^S + \frac{\lambda}{2} (n^P - 1) \hat{E}_P^- + \frac{\lambda}{2} n^P \hat{E}_P^+ \hat{E}_D^- - \lambda n^P + \Gamma (n^D + 1) \hat{E}_D^+ - \Gamma n^D \right\} P,
\end{align*}
\]

(20)
where we have again used ladder operators defined by \( \hat{E}_S^\pm P(n^S, n^P, n^D, t) = P(n^S \pm 1, n^P, n^D, t) \) and analogously for the other cell compartments P and D. Together with an initial condition \( P(n^S, n^P, n^D, 0) \) that characterizes the initially barcoded population, Eq. (20) permits to compute the clone composition and barcode frequency distribution of our model at any later time. The distribution \( p(n, t) \) of total barcode frequencies \( n = n^S + n^P + n^D \) is obtained from the joint distribution \( P \) by summing over all barcode frequency configurations that lead to a total size \( n \),

\[
p(n, t) = \sum_{n'=0}^{n} \sum_{n''=0}^{n-n'} P(n', n'', n - n' - n'', t) .
\]

While the full clonal dynamics of our model can only be explored by means of numerical simulations, several important insights about growth and composition of the tumour can be drawn from analytical arguments.

### 4.2 Composition of the tumour

Using the master equation (20), we can obtain insights into the composition of the tumour in our model, i.e., its relative content of stem cells, progenitors and differentiating progeny. The time evolution of the mean cell numbers is given by

\[
\begin{align*}
\langle n^S \rangle &= \epsilon \omega \langle n^S \rangle , \\
\langle n^P \rangle &= (1 - \epsilon) \omega \langle n^S \rangle , \\
\langle n^D \rangle &= \frac{1}{2} \lambda \langle n^P \rangle - \Gamma \langle n^D \rangle ,
\end{align*}
\]

where the dot denotes the time derivative. In particular, the evolving clone, while steadily growing, acquires a steady-state composition characterized by a constant relative amount of stem-like cells, progenitor cells, and differentiated cells: Defining the relative cell contents \( \phi^S = \langle n^S \rangle / \langle n \rangle \), \( \phi^P = \langle n^P \rangle / \langle n \rangle \), and \( \phi^D = \langle n^D \rangle / \langle n \rangle \) where \( \langle n \rangle = \langle n^S \rangle + \langle n^P \rangle + \langle n^D \rangle \) is the total barcode frequency, this stationary composition satisfies \( \dot{\phi}^S = \dot{\phi}^P = \dot{\phi}^D = 0 \) and is given by

\[
\begin{align*}
\phi^S &= \epsilon \Omega^{-1} , \\
\phi^P &= (1 - \epsilon) \Omega^{-1} , \\
\phi^D &= 1 - \Omega^{-1} ,
\end{align*}
\]

with \( \Omega \) being a dimensionless parameter given by

\[
\Omega = 1 + \frac{\lambda}{2} \left( \frac{1 - \epsilon}{\Gamma + \epsilon \omega} \right) .
\]

Eqs. (23a–c) show that the probability \( \epsilon \) for symmetric stem cell division determines the relative fraction of stem-like and progenitor cells while the composite parameter \( \Omega \) determines the relative fraction of the differentiating progeny and the remaining two compartments. Note that in general the ratio of averages does not correspond to the average of the ratio, \( \langle n^X \rangle / \langle n \rangle \neq \langle n^X / n \rangle \) for \( X = S, P, D \). However, simulations show that Eqs. (23a–c) are excellent approximations for the averages \( \langle n^X / n \rangle \) in the considered parameter ranges.
4.3 Tumour expansion

The average growth of a clone (and thus the tumour) can be determined from Eqs. (22) as well. Defining the fold-change in cell number compared to the initial barcode frequency, \( \gamma(t) = \frac{\langle n(t) \rangle}{\langle n(0) \rangle} \), we obtain

\[
\gamma(t) = e^{\epsilon \omega t},
\]

given that, from the outset, the tumour has the stationary composition given by Eqs. (23). Hence, the tumour expands exponentially with the growth speed given by the rate \( \epsilon \omega \) of symmetric stem cell divisions.

5 Simulation of transplantation experiments

To capture the dynamics of the serial transplantation experiments, we develop a simulation of the clonal dynamics involving the repeated procedure of injection, unperturbed growth, and harvesting of the tumour. To this end, we use a stochastic simulation algorithm to compute many realizations of the clonal dynamics (Gillespie, 1977). The simulation consists of (i) the injection of a single uniquely labelled cell, (ii) unperturbed clonal dynamics according to the process (19), and (iii) subsequent harvesting of cells for sequencing and reinjection. Key observables such as barcode frequency distributions, numbers of surviving barcodes, and clonal growth are then obtained by performing statistics over the computed realizations.

5.1 Primary injection

To mimic the experimental procedure in our simulation, we start the primary passage by injecting a single labelled S or P cell, each with a probability that reflects the steady-state fractions given in Eqs. (23a,b). Differentiating progeny (represented by the D compartment in our model) are unlikely to survive the process of serial transplantation. The corresponding initial condition for the probability \( P \) is thus given by

\[
P(n^S, n^P, n^D, 0) = \frac{\phi^S \delta_{n^s,1} \delta_{n^p,0} \delta_{n^d,0} + \phi^P \delta_{n^s,0} \delta_{n^p,1} \delta_{n^d,0}}{\phi^S + \phi^P}.
\]

5.2 Tumour growth

After the injection, the clone is subject to unperturbed growth according to Eq. (20) for the duration \( \tau_i \) of the corresponding passage \( i \).

5.3 Harvesting and reinjection

After each passage, the next passage \( i \) is initiated by reinjecting cells harvested from the previous passage \( i - 1 \). This amounts to setting a new initial condition for the
probability $P$ at the injection time $t_{i}^{\text{inj}}$, which coincides with the harvesting time $t_{i}^{\text{harv}} = \sum_{j=1}^{i-1} \tau_{j}$ of the previous passage, where $\tau_{i}$ is the passage duration of passage $i$. Again, assuming that it is unlikely for differentiating cells (D) to survive the process of serial transplantation, only stem-like cells (S) and progenitors (P) are reinjected, each such a cell with a probability $p_{i}^{\text{inj}}$. The probability $p_{i}^{\text{inj}}$ is determined by requiring that on average, the number $n_{i}^{\text{inj}}$ of injected cells matches the number in the corresponding experiment. The probability $p_{i}^{\text{inj}}$ can be calculated as follows. From Eqs. (22), the average growth of a clone can be calculated for any initial composition of the clone. If only S and P cells are injected, with cell numbers that reflect the stationary composition given by Eqs. (23a,b), the fold change $\gamma(t) = \langle n(t) \rangle / \langle n(0) \rangle$ in cell number is given by

$$
\gamma(t) = \Omega e^{\omega t} - (\Omega - 1)e^{-\Gamma t}.
$$

with $\Omega$ defined in Eq. (24). Hence, the total tumour size after passage $i$ is given by $n_{i}^{\text{inj}}\gamma(\tau)$ where $n_{i}^{\text{inj}}$ is the number of injected cells and $\tau$ is the passage duration. Since the composition of the tumour quickly acquires the stationary composition given by Eqs. (23a,b,c) during the passage, the total number of S and P cells upon harvesting is given by $(\phi^{S} + \phi^{P})n_{i-1}^{\text{inj}}\gamma(\tau_{i-1})$. Therefore, to inject an average of $n_{i}^{\text{inj}}$ cells at the beginning of passage $i$, the probability $p_{i}^{\text{inj}}$ must be chosen as

$$
p_{i}^{\text{inj}} = \frac{n_{i}^{\text{inj}}}{(\phi^{S} + \phi^{P})n_{i-1}^{\text{inj}}\gamma(\tau_{i-1})}.
$$

Then, the system again evolves according to Eq. (20) till $t_{i+1}^{\text{harv}}$ and the same procedure is repeated for the next passage.

### 5.4 Example

Fig. S1 and Fig. 2d in the main text show numerical examples of the simulation. The upper panel displays different trajectories of barcode frequencies across three passages. Because of stochastic cell fate decisions, clones stochastically grow or shrink during a passage. Therefore, individual trajectories may emerge above and drop below a detection threshold (shaded area in Fig. S1) several times over the course of time (see yellow trajectory in Fig. S1 for an example). While the majority of clones are lost, a few clones grow very large by chance, acquiring several hundreds of cells. After each passage, all barcode frequencies abruptly drop due to harvesting and reinjection of a small sample of the tumour ($\sim 5\%$). From many realizations of the system, statistical properties of the clones such as barcode frequency distributions and correlations can be obtained: the lower panel of Fig. S1 shows, e.g., the average barcode frequency. Note that the average barcode frequency is strongly affected by the majority of clones becoming extinct very quickly while only a few
Figure S1  Numerical examples of barcode frequency trajectories across three serial passages on a logarithmic scale. The shaded area indicates an example detection threshold. The yellow curve shows a clone that emerges above and drops below the detection threshold several times. The lower plot shows the average over all trajectories. Parameters are given in Table S3.

clones become large. Fig. 2e in the main text shows the first incomplete moment of the barcode frequency distribution, revealing a negative binomial distribution over many decades as discussed in Section 2. We now use these simulations to systematically compare experimental data with our theory.

6 Comparison of theory and experiments

We now compare our theory with experiments. First, we discuss biologically sensible parameter ranges for our model. We then compare barcode frequency distributions and number of barcodes that survive the serial transplantations with experiments, highlighting that many qualitative key features of our theory are actually independent of the specific choice of parameters.
6.1 Parameter estimates

Can all experiments be characterized by the same set of parameters? Experimental data show a considerable degree of variation in the growth of different xenografts: for instance, referring to Table S4, the tertiary xenograft of the transplantation series labelled $1;2;1$ grows by 42-fold over a duration of 55 days, while the tertiary xenograft of $1;2;3$ grows by only 26-fold over the longer duration of 78 days, with both xenografts having been derived from the same secondary xenograft $1;2$. Whether or not there are potential sources for these variations among replicate experiments, this example already indicates that it is not possible to characterize all experiments with a single set of parameters. Rather, it suggests a corresponding degree of variation for the proliferation and differentiation rates of stem-like cells and progenitors as well as the apoptosis rate of the differentiating progeny. Here we aim at constraining plausible parameter ranges using experimental data.

An estimate for the apoptosis rate $\Gamma'$ of the differentiating progeny can be inferred from the steady-state composition of the tumour: we used Ki67 staining of xenograft samples to determine the relative amount of proliferating cells as 50% on average, see Extended Data Fig. 3d. Based on this estimate we fixed the relative amount of progenitor cells among the progenitor population, $\psi = n^P/(n^D + n^P)$, as $\psi \approx 0.5$. Using Eqs. (23) and (24), the apoptosis rate $\Gamma'$ can be expressed in terms of $\psi$ and the other parameters as

$$\Gamma' = \frac{\lambda}{2} \left( \frac{1}{\psi^{-1} - 1} - \epsilon \omega \right).$$

Hence, given numerical values for the other parameters $\omega$, $\epsilon$, and $\lambda$, this fixes the value of $\Gamma'$. For the loss-and-replacement rate $\lambda$ of the progenitors, we choose an upper bound of $\lambda = 1.5$/day, motivated by the fact that in mammalian cells, the typical S phase duration is already 5 to 6 hours which constrains the cell cycle speed. In Section 3, we have seen that progenitors have to divide much faster than stem-like cells ($\lambda \gg \omega$) in order to generate the characteristic negative binomial form of the barcode frequency distribution. Therefore, we restrict the stem cell division rate $\omega$ to values of at least an order of magnitude less, $\omega \lesssim 0.3$/day. In our model, overall growth of the tumour crucially depends on the rate $\epsilon \omega$ of symmetric stem cell divisions (see Sections ?? and 5.3). Considering fast death of the differentiating progeny ($\Gamma' \gg \epsilon \omega$) and a small ratio of symmetric divisions ($\epsilon \ll 1$), Eq. (27) enables us to estimate the symmetric division rate of the stem cells as $\epsilon \omega \approx \tau^{-1} \ln \gamma \psi$, where $\tau$ is the passage duration, $\gamma$ is the fold-change in cell number from injection to harvesting and $\psi$ is the amount of proliferating cells introduced above. Given the range of values for $\gamma$ and $\tau$ shown in Table S4, we obtain an estimate for the range of $\epsilon \omega$ of 0.02...0.06/day. Since $\epsilon < 1$, this automatically yields a lower bound of $\omega \gtrsim 0.02$/day for the stem cell division rate. In our model, the ratio $\epsilon$ of symmetric
stem cell divisions sets the relative size of the stem cell pool and the progenitor pool, see Eq. (23). Assuming that the stem-like cells form a minority population, we here restrict $\varepsilon \lesssim 20\%$.

A summary of the thus inferred parameter ranges is given in Table S2. To show that these estimates for the parameter ranges are consistent with the clonal behaviour observed in experiments, we now compare numerical solutions of the model with experimental data.

### 6.2 Barcode frequency distributions

A direct quantitative comparison of barcode frequency distributions is currently not possible because of limitations in experimentally determining absolute barcode frequencies. However, the characteristic functional shape of the barcode frequency distributions is independent of absolute barcode frequencies and can be compared with experiments. To assess the barcode frequency distributions generated by our model, we obtain their first incomplete moment $\mu$ as defined in Eq. (8) from Eq. (21). Fig. 2e in the main text shows examples for $\mu$ for each passage, obtained from a numerical simulation of $2 \times 10^6$ realizations of the system. The linear behaviour over many decades of barcode frequencies indicates a negative binomial size distribution as discussed in Section 2. In fact, we find these negative binomial distributions within a large range of parameters. This linear behaviour is preceded by a short non-linear transient behaviour for very small barcode frequencies that are likely below the experimental detection threshold.

### 6.3 Barcode survival

The survival of barcodes is reflected by the number of detected barcodes across passages. In experiments, the number of detected barcodes depends on the detection threshold and the fraction of sequenced cells. To obtain a measure for clone survival that is independent of these experimental constraints, we make use of the fact that barcode frequency distributions have the negative binomial form Eq. (7), which entails a characteristic barcode frequency $n_0$. This enables us to define the number
of clones that exceed a specified fraction \( \theta \) of the characteristic barcode frequency \( n_0 \) as \( \sum_{n>\theta n_0} h(n) \), where \( h(n) \) is the number of clones with size \( n \). The ratio of clones derived from initially injected barcoded cells that exceed the size \( \theta n_0 \) at a given passage therefore serves as a measure for barcode survival,

\[
\beta_\theta = \frac{1}{N_B} \sum_{n>\theta n_0} h(n) ,
\]

(30)

where \( N_B \) is the number of uniquely barcoded cells injected before the first passage, given by \( N_B = \eta n_{1 \text{ inj}} \) with \( \eta \) being the labelling efficiency and \( n_{1 \text{ inj}} \) being the number of cells injected.

Fig. S2 shows the results from simulations\(^4\) covering the parameter ranges indicated in Table S2, along with the corresponding experimental data\(^5\). Density bars show the distribution of values for \( \beta_{1/2} \), dots show experimental data points\(^6\). Clearly, most of the values obtained in the biologically plausible parameter range also capture the experimentally obtained values. Moreover, simulations show a systematic decline of the barcode survival probability with increasing passage number.

### 6.4 Correlations of barcode frequencies across passages

We now make use of the fact that unique barcoding enables us to identify clones throughout different passages and replicate experiments. A characteristic feature of the clonal dynamics that includes this longitudinal data is the correlation of the size of a uniquely labelled clone across passages, see Extended Data Fig. 4f and Fig. 2h in the main text. To quantify these correlations, we define the normalized

\( h(x) \) with \( x \) being the relative barcode frequency are generated by binning the experimentally obtained barcode frequencies with a bin size of \( (x_{\text{max}} - x_{\text{min}})/100 \) where \( x_{\text{max}} \) and \( x_{\text{min}} \) are the largest and smallest relative barcode frequencies, respectively. We then fitted the resulting barcode frequency distributions using the negative binomial form \( p(x) = N_0^{-1} e^{-x/x_0} / x \) with \( n_0 \) and the normalisation constant \( N_0 \) as fit parameters. Since the detection threshold from sequencing may distort the distributions for small barcode frequencies, we truncate the barcode frequency distributions from below (within the first 20 data points) such that the coefficient of determination \( R^2 \) of the fit is maximized. This yields the characteristic barcode frequency \( x_0 \) and \( \beta_\theta \) is readily obtained as \( \beta_\theta = N_B^{-1} \sum_{x>\theta x_0} h(x) \). The standard error \( \sigma_{x_0} \) on \( x_0 \) obtained from the fit is used to calculate positive and negative errors for \( \beta_\theta \) as \( \sigma_{\beta_\theta} = N_B^{-1} \sum_{x>\theta x_0} (x_0 \pm \sigma_{x_0}) h(x) \).

The value \( \theta = 1/2 \) was chosen because the corresponding threshold \( n_0/2 \) lies well above the detection threshold from sequencing and at the same time takes into account most of the acquired data.

---

\(^4\)A total of 108 parameter sets equally distributed in the parameter ranges for \( \omega, e, \) and \( \lambda \) indicated in Table S2 have been used to sample the parameter space. The parameter \( \Gamma \) was fixed according to Eq. (29). Each simulation consists of 100,000 realizations of clones using the passage times and number of injected cells reported in Table S4.

\(^5\)We obtain \( \beta_\theta \) from experimental data as follows. Barcode frequency distributions \( h(x) \) with \( x \) being the relative barcode frequency are generated by binning the experimentally obtained barcode frequencies with a bin size of \((x_{\text{max}} - x_{\text{min}})/100 \) where \( x_{\text{max}} \) and \( x_{\text{min}} \) are the largest and smallest relative barcode frequencies, respectively. We then fitted the resulting barcode frequency distributions using the negative binomial form \( p(x) = N_0^{-1} e^{-x/x_0} / x \) with \( n_0 \) and the normalisation constant \( N_0 \) as fit parameters. Since the detection threshold from sequencing may distort the distributions for small barcode frequencies, we truncate the barcode frequency distributions from below (within the first 20 data points) such that the coefficient of determination \( R^2 \) of the fit is maximized. This yields the characteristic barcode frequency \( x_0 \) and \( \beta_\theta \) is readily obtained as \( \beta_\theta = N_B^{-1} \sum_{x>\theta x_0} h(x) \). The standard error \( \sigma_{x_0} \) on \( x_0 \) obtained from the fit is used to calculate positive and negative errors for \( \beta_\theta \) as \( \sigma_{\beta_\theta} = N_B^{-1} \sum_{x>\theta x_0} (x_0 \pm \sigma_{x_0}) h(x) \).

The value \( \theta = 1/2 \) was chosen because the corresponding threshold \( n_0/2 \) lies well above the detection threshold from sequencing and at the same time takes into account most of the acquired data.
Figure S2  Fraction $\beta_{1/2}$ of initially injected barcodes growing above half of the characteristic barcode frequency $n_0/2$ as defined in Eq. (30) for all experimental trajectories given in Table S4. Density bars show the distribution of simulation results pooled over the parameter ranges indicated in Table S2. Dots show experimental data. The plot titles indicate the experimental trajectory as given in the first column of Table S4.

cross correlation of the barcode frequency for passages $i$ and $j$ as

$$
C_{ij} = \frac{\langle n_i n_j \rangle - \langle n_i \rangle \langle n_j \rangle}{\sqrt{\langle n_i^2 \rangle - \langle n_i \rangle^2} \sqrt{\langle n_j^2 \rangle - \langle n_j \rangle^2}},
$$

(31)
Table S3  Parameter values used for the numerical examples in Figs. S1 and S4 and Figs. 2 and 3 in the main text. The parameters $\omega$, $\epsilon$, $\lambda$, and $\Gamma$ are introduced in Sec. 4.1; the parameter $\varphi$ is introduced in Sec. 6.5. These parameter sets are used to illustrate the model behaviour and have therefore been chosen to be located in the center of the biologically plausible parameter ranges indicated in Table S2.

<table>
<thead>
<tr>
<th></th>
<th>$\omega$</th>
<th>$\epsilon$</th>
<th>$\lambda$</th>
<th>$\Gamma$</th>
<th>$\varphi$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figs. 2d,e,h and S1, S4</td>
<td>0.15 d$^{-1}$</td>
<td>15%</td>
<td>1 d$^{-1}$</td>
<td>0.48 d$^{-1}$</td>
<td>0%</td>
</tr>
<tr>
<td>Figs. 3e,f</td>
<td>0.1 d$^{-1}$</td>
<td>10%</td>
<td>1.5 d$^{-1}$</td>
<td>0.74 d$^{-1}$</td>
<td>0.5%</td>
</tr>
</tbody>
</table>

where $n_i = n(t_i^{\text{harv}})$ is the barcode frequency after passage $i$. The normalized cross correlation $C_{ij}$ takes values between $-1$ and $1$, where $C_{ij} = 1$ indicates perfect correlation of barcode frequencies (i.e., small/large clones in passage $i$ correspond to small/large clones in passage $j$), $C_{ij} = 0$ indicates that barcode frequencies are completely uncorrelated, and $C_{ij} = -1$ indicates perfect anticorrelation (i.e., large clones in passage $i$ correspond to small clones in passage $j$ and vice versa).

Fig. S3 shows a comparison of the correlations for the same simulations and experimental data sets as in Fig. S2. Density bars show the distribution of values for the cross correlations $C_{ij}$, dots show experimental data points. Without a fine tuning of the parameters, the theoretically computed cross correlations not only cover the experimentally obtained values in most cases but also clearly capture the correct trend between different pairwise comparisons within a particular injection series. In the case of the GBM754 experiment, deviations from experimental results is likely due to the comparably small number of detected clones which makes the cross correlation a less reliable measure; nevertheless, that the trend of correlations is largest between the secondary and tertiary passage is correctly captured.

6.5 Effects of chemotherapy

In the main text, we observed that the clonal behaviour after treatment of xenografts with temozolomide (TMZ) can be characterized by two distinctive groups of small and large clones (termed Group A and Group B, respectively), see Fig. 3a–d. There, we hypothesized that such a behaviour is consistent with a subset of clones exhibiting a resistance to apoptosis. To assess whether our results support this scenario, we modified the simulation such that with a certain probability $\varphi$, a clone’s differentiating progeny does not die off during the second passage ($\Gamma = 0$ for the respective clones). Fig. 3e,f in the main text shows the resulting correlations of barcode frequencies for the clones resisting apoptosis (blue dots, $\Gamma = 0$) and clones following the unperturbed dynamics (green dots, $\Gamma \neq 0$) for an example simulation with parameters given in Table S3. Indeed, the resulting behaviour recapitulates the experi-
Figure S3 Barcode frequency cross correlations $C_{ij}$ as defined in Eq. (31) for all experimental trajectories given in Table S4. Density bars show the distribution of simulation results pooled over the parameter ranges indicated in Table S2. Dots show experimental data. The plot titles indicate the experimental trajectory as given in the first column of Table S4.

Experimental findings: two clusters of small and large clones, respectively, with the size of large clones being positively correlated between subsequent passages, see Figs. 3a,b in the main text. The qualitative features of these correlations robustly appear without fine-tuning and within a large range of parameters, supporting that resistance to apoptosis of a subset of clones generically leads to the observed behaviour.
7 Exome deep sequencing as a window on the mutational heterogeneity and clonal dynamics of GBM cells

To probe the mutational heterogeneity of the parent tumour and its evolution over time, we applied exome sequencing to xenografts from GBM719, focusing first on passage (p)2 and 3 of the untreated system. This analysis identified 546 mutations at p2 with variable allele frequencies (VAFs) that were above the threshold of detection, and 112 at p3. Analysis of the distribution of VAFs revealed a wide variation, with the majority clustered around the threshold value while some appeared to be clonally fixed within the population with VAFs of 0.5 or more. Note that copy number variation can amplify VAFs above the value of 0.5, the value expected for a heterozygous point mutation that has become clonally fixed across the population. Comparison of the mutational signature between p2 and p3 identified 68 mutant clones that were shared by both groups and therefore likely to be present in the parental tumour, emphasizing the mutational heterogeneity of both the parent tumour sample, and its conservation in the xenograft model.

As well as indicating the mutational heterogeneity of the tumour sample, the VAF also carries quantitative information on the relative abundance of point mutations within a sample and therefore carries information about the relative size of host mutant clones. Indeed, such data sets can often be used to identify cancer drivers and, in some cases, the phylogeny of mutations that drive non-neutral transformation (Williams et al., 2016; Eirew et al., 2015). However, in the present context, the current barcoding study indicates “neutral” competition between growing mutant clones suggesting that the vast majority of heterozygous point mutations, even when they occur in cancer genes, may leave the fate behaviour of tumour cells largely unperturbed. In this case, we can instead use point mutations as a surrogate clonal mark from which information on clonal dynamics of tumour cells can be inferred. However, in contrast to cellular barcoding, where the clonal mark is created at a given instant in time, mutations occur sporadically leading to modified “clonal” distributions. As a result, the VAFs obtained from exome sequencing represent a product of both the underlying fate dynamics of the mutant cells within the sample and the mutational dynamics (Simons, 2016), involving the ongoing acquisition of new point mutations and copy number variations. Nevertheless, when copy number variation is low, such approaches can be used to quantify cell fate behaviour, as exemplified by a recent study of stem cell dynamics in physiological normal human epidermis obtained from punch biopsies of eyelid epidermis (Martincorenna et al., 2015; Simons, 2016).

To develop a similar approach here, we reasoned that biopsies from primary tumours are likely to contain geographically restricted mutations (Johnson et al., 2014), further compounding the potential complexity of the VAF distribution. How-
ever, since normal cells are unlikely to survive passaging through the xenograft, we reasoned that VAFs obtained at p2 and p3 were likely to be rooted in the tumour-maintaining population. To address this data, we first considered the qualitative behaviour of the raw VAF distributions in both control (untreated) samples from p2 and p3. If, for a given locus, mutations of both alleles occur at a negligible rate, a VAF of 0.5 indicates a mutation present in the entire cell population and is therefore fixed across the population. Indeed, the VAF distributions in both samples (Extended Data Fig. 6d) exhibit an abundance of small clones as well as a smaller peak at VAFs of around 0.5, which likely corresponds to mutations that have already become fixed in the population after the respective passage.

Examining the correlations of VAFs between passages in xenografts (Extended Data Fig. 6e), we found a population of larger clones that are present after both passages, as expected for mutations that have become fixed (or almost fixed) at the end of p2. Alongside these clones, we also found both (i) clones that became extinct (or, more accurately, fell below the threshold of the deep sequencing) during repopulation and expansion in p3 as well as (ii) new clones that emerge during p3. If we assume that these new mutant clones arise from new mutations acquired during p3 (rather than from pre-existing clones that grew above the detection threshold), we can use the dynamics inferred from the barcoding to derive expected features of the VAF distribution of these newly-generated clones.

To predict the large-scale dependence of the VAF distribution, we adapted our simulation to take into account random “induction” of clones through mutations during the tertiary passage\(^7\). Model simulations suggested that the resulting VAF distribution again approximates a negative binomial form, or, equivalently, acquires an approximately exponential first incomplete moment consistent with experiment (Extended Data Fig. 6f-g, Fig. S4). Remarkably, focusing on the first incomplete moment of the 44 clones that emerge during the tertiary passage, we find that the first incomplete moment of the VAF distribution again reveals an exponential distribution (Extended Data Fig. 6g), in accordance with expectations from the barcoding study. By comparison, the TMZ-treated samples show (i) a much larger number of newly acquired mutations during p3 (Extended Data Fig. 6d) and (ii) a broad distribution

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\(^7\)Considering a constant mutation rate for each locus in each cell (Simons, 2016), the probability for a mutation to occur is proportional to the instantaneous number of cells in the tumour. Therefore, knowing that in our model specified by Eq. (20), the time-dependent fold-change in cell number is given by \(γ(t) = e^{\omega t}\), see Eq. (25), we reasoned that the time-dependent probability distribution for a mutation to have occurred during the tertiary passage is given by \(p_{\text{ind}}(t) = γ(t)/\int_0^{\tau_3} γ(t) \, dt\) with \(0 ≤ t ≤ \tau_3\) where \(t = 0\) refers to the start of the passage and where \(\tau_3\) is the passage duration of the tertiary passage. Hence, for each clone, we drew a time \(t_{\text{ind}}\) from the distribution \(p_{\text{ind}}\) and simulated the respective clone for the time \(\tau_3 - t_{\text{ind}},\) i.e., the remaining time from induction during the tertiary passage to the end of the passage. We then obtained the clone size distribution and first incomplete moment from the resulting clone population.
of VAFs after p3 with a considerable subset of clones displaying VAFs larger than 0.5 (Extended Data Fig. 6d), both pointing at a treatment-induced higher genomic variability.

Although the agreement between the theoretical prediction based on the bar-coding data and experiment is encouraging, we must also exercise some caution. While correction of VAFs to account for copy number variation (CNV) is already challenging in the parent tumour, with new mutations, the challenge is even greater. When CNV occurs before the mutation, the VAF provides a faithful read-out of clone size; where it occurs afterwards, the VAF is corrupted by the amplification. The correlation between VAFs associated with shared mutations between p2 and p3 of the control xenograft suggests that CNV may be rather infrequent as compared to the clonal dynamics, consistent with the systematic behaviour of the measured clone size distribution as predicted by a conserved proliferative hierarchy. In addition, we repeated the same analysis only taking genomic regions that are predicted to be diploid within each sample based on exome sequencing. After filtering, the VAF distributions continue to conform to the negative binomial (Extended Data Fig. 6h-i). However, a more detailed quantitative analysis would require a comprehensive investigation and understanding of the interplay between tumour growth, mutational dynamics and, indeed, chemotherapy-induced mutation (Johnson et al., 2014), which are beyond the scope of the current study.
8 Remarks

Here we have introduced a theoretical model of human glioblastoma (GBM) growth based on a critical birth-death process with immigration, describing the stochastic cell fate dynamics of a proliferative hierarchy with glioblastoma stem cells (GSCs) at the apex. Our model is able to robustly capture key features of the clonal dynamics assessed experimentally: importantly, it explains the characteristic negative binomial barcode frequency distributions across all serial passages observed in experiments. Moreover, comparison of (i) the number of surviving barcodes across serial passages and (ii) correlations of barcode frequencies between serial passages show that the inferred parameter range covers the observed behaviour in the overwhelming majority of cases.

Note that the model presented here is still a minimal model in the sense that more complex alterations and refinements are conceivable. These may include a slight imbalance between loss and replacement of progenitors as well as multiple progeny compartments. Also, small amounts of cell death may occur in the stem cell and progenitor compartments. However, if the death rate was of comparable size (or larger) than the rate of symmetric proliferation, we would expect a massive loss of clones. If, on the contrary, cell death only represents a small contribution relative to the symmetric proliferation rate, it could be accounted for by an effective adjustment of the other model parameters that, e.g., determine the net tumour growth and would only be visible in subtle changes of the barcode frequency distributions that are impossible to detect in the experimentally given distributions. Importantly, these alterations do not change the basic characteristics of our model. Moreover, we have neglected the spatial aspect of tumour growth and potential ongoing driver gene mutations (Michor et al., 2006; Waclaw et al., 2015), assuming that cell division and loss-and-replacement occur at constant rates as the tumour expands within the brain. Despite its simplicity, the fact that our model is able to capture the main features of the clonal dynamics indicates a remarkably simple proliferative behaviour of human GBM despite the genomic variability of GBM cells.

References


Table S4  Experimental data sets used to compare with theory. Here, \( n_{\text{inj}} \) is the number of injected cells, \( \tau \) is the passage duration, \( s \) is the fraction of cells sequenced, \( \beta_{1/2} \) is the fraction of initially injected barcodes growing above half of the characteristic barcode frequency \( n_{0}/2 \), as defined in Eq. (30), and \( \gamma \) is the estimated fold-change in cell number between injection and harvesting, which quantifies tumour growth. In all cases, cells were harvested and injected from the ipsilateral side.

<table>
<thead>
<tr>
<th>ID</th>
<th>Passage</th>
<th>inj. cells ( n_{\text{inj}} )</th>
<th>( \tau )</th>
<th>surv. prob. ( \beta_{1/2} )</th>
<th>growth ( \gamma )</th>
</tr>
</thead>
<tbody>
<tr>
<td>GBM719</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1)_719</td>
<td>Primary</td>
<td>( 1.25 \times 10^{5} )</td>
<td>79 d</td>
<td>( 1.44_{-0.26}^{+0.0} ) %</td>
<td>40</td>
</tr>
<tr>
<td>(1, 1)_719</td>
<td>— Secondary</td>
<td>( 3 \times 10^{5} )</td>
<td>64 d</td>
<td>( 0.32_{-0.07}^{+0.06} )%</td>
<td>7.7</td>
</tr>
<tr>
<td>(1, 2)_719</td>
<td>— Secondary</td>
<td>( 3 \times 10^{5} )</td>
<td>68 d</td>
<td>( 0.31_{-0.07}^{+0.08} )%</td>
<td>18</td>
</tr>
<tr>
<td>(1, 2, 1)_719</td>
<td>— Tertiary</td>
<td>( 3 \times 10^{5} )</td>
<td>55 d</td>
<td>( 0.13_{-0.03}^{+0.07} )%</td>
<td>42</td>
</tr>
<tr>
<td>(1, 2, 2)_719</td>
<td>— Tertiary</td>
<td>( 3 \times 10^{5} )</td>
<td>70 d</td>
<td>( 0.25_{-0.03}^{+0.04} )%</td>
<td>43.3</td>
</tr>
<tr>
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<td>— Tertiary</td>
<td>( 3 \times 10^{5} )</td>
<td>78 d</td>
<td>( 0.18_{-0.03}^{+0.04} )%</td>
<td>26</td>
</tr>
<tr>
<td>(1, 3)_719</td>
<td>— Secondary</td>
<td>( 3 \times 10^{5} )</td>
<td>89 d</td>
<td>( 0.03_{-0.01}^{+0.05} )%</td>
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<tr>
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<td>15.1</td>
</tr>
<tr>
<td>(1, 3, 2)_719</td>
<td>— Tertiary</td>
<td>( 3 \times 10^{5} )</td>
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<td>( 0.07_{-0.01}^{+0.03} )%</td>
<td>47.7</td>
</tr>
<tr>
<td>GBM754</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(1)_754</td>
<td>Primary</td>
<td>( 1.25 \times 10^{5} )</td>
<td>99 d</td>
<td>( 0.26_{-0.1}^{+0.0} )%</td>
<td>26</td>
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<td>(1, 1)_754</td>
<td>— Secondary</td>
<td>( 6 \times 10^{4} )</td>
<td>79 d</td>
<td>( 0.19_{-0.0}^{+0.0} )%</td>
<td>42.7</td>
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<tr>
<td>(1, 2)_754</td>
<td>— Secondary</td>
<td>( 6 \times 10^{4} )</td>
<td>86 d</td>
<td>( 0.1_{-0.0}^{+0.0} )%</td>
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<td>( 6 \times 10^{4} )</td>
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<td>( 0.24_{-0.01}^{+0.0} )%</td>
<td>56</td>
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<td>— Tertiary</td>
<td>( 6 \times 10^{4} )</td>
<td>73 d</td>
<td>( 0.11_{-0.0}^{+0.0} )%</td>
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<tr>
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<td></td>
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<td></td>
</tr>
<tr>
<td>(1)_742</td>
<td>Primary</td>
<td>( 2.4 \times 10^{4} )</td>
<td>78 d</td>
<td>( 0.78_{-0.0}^{+0.0} )%</td>
<td>530</td>
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<td>43 d</td>
<td>( 0.13_{-0.01}^{+0.06} )%</td>
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<tr>
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<td>47 d</td>
<td>( 0.01_{-0.01}^{+0.07} )%</td>
<td>1.8</td>
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<td>( 3 \times 10^{5} )</td>
<td>50 d</td>
<td>( 0.12_{-0.03}^{+0.22} )%</td>
<td>8.5</td>
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