A restricted cell population propagates glioblastoma growth after chemotherapy

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Glioblastoma multiforme is the most common primary malignant brain tumour, with a median survival of about one year. This poor prognosis is due to therapeutic resistance and tumour recurrence after surgical removal. Precisely how recurrence occurs is unknown. Using a genetically engineered mouse model of glioma, here we identify a subset of endogenous tumour cells that are the source of new tumour cells after the drug temozolomide (TMZ) is administered transiently to arrest tumour growth. A nestin-ATK-IRES-GFP (Nes-ATK-GFP) transgene that labels quiescent subventricular zone adult neural stem cells also labels a subset of endogenous glioma tumour cells. On arrest of tumour cell proliferation with TMZ, pulse-chase experiments demonstrate a tumour re-growth cell hierarchy originating with the Nes-ATK-GFP transgene subpopulation. Ablation of the GFP+ cells with chronic ganciclovir administration significantly arrested tumour growth, and combined TMZ and ganciclovir treatment impeded tumour development. Thus, a relatively quiescent subset of endogenous glioma cells, with properties similar to those proposed for cancer stem cells, is responsible for sustaining long-term tumour growth through the production of transient populations of highly proliferative cells.

We have extensively studied a series of mouse strains harbouring conditional alleles of the tumour suppressors Nf1, p53 (also known as Trp53) and Pten, which spontaneously develop malignant gliomas with 100% penetrance, and have identified the source cells of these tumours as deriving from the subventricular zone (SVZ). Thus, we proposed that adult neural stem cells (NSCs) are the likely source of these tumours. We wished to determine whether a Nes-ATK-GFP transgene, originally devised to mark adult NSCs, would also mark endogenous glioma cells. Elements of the nestin gene can drive transgene expression specifically in adult NSCs (Fig. 1a). The transgene also harbours a cassette containing a modified version of the herpes simplex virus (HSV) thymidine kinase (ATK), allowing for temporally regulated ablation of dividing neural progenitors by systemic ganciclovir (GCV) administration, and an IRES-GFP cassette to mark Nes-ATK-expressing cells in the absence of GCV. The Nes-ATK-GFP transgenic mice showed the expected expression in both glial fibrillary acidic protein (GFAP)-positive adult NSCs and early doublecortin (DCX)-positive neural progenitor cells (NPCs) in the major adult NSC niche: the SVZ of the lateral ventricle (Fig. 1b, c). To validate that the GCV-activated Nes-ATK-GFP transgene could effectively eliminate endogenous neural stem/progenitor cells, 1-month-old transgenic mice were treated with GCV for 2 weeks. In control mice, the rostral migratory stream (RMS), formed by NPCs migrating from the SVZ to the olfactory bulb, is visualized by Nissl staining. In contrast, the RMS was severely diminished in Nes-ATK-GFP mice after GCV treatment (Fig. 1b, bottom). Consistent with this observation, DCX immunostaining indicated the absence of NPCs in the SVZ of GCV-treated Nes-ATK-GFP animals (Fig. 1c, bottom). Because, in the presence of GCV, HSV TK targets only proliferating cells, the GFAP+ NSCs that remained quiescent were unaffected, although they also express the transgene, as indicated by GFP (Fig. 1c, bottom). Thus, our Nes-ATK-GFP transgene is specifically expressed in SVZ quiescent NSCs, demonstrating that many GFP+ cells more distal in the RMS. White arrowheads in bottom panel (Nes-ATK; GCV) indicate DCX+ GFP- cells in the SVZ stem cell niche. White arrowheads in middle panel (Nes-ATK; PBS) indicate DCX+ GFP- cells more distal in the RMS. White arrowheads in bottom panel (Nes-ATK; GCV) indicate GFP+/GFAP- but DCX- quiescent NSCs.

Figure 1 | Characterization of the Nes-ATK-GFP transgene. a, Diagram of the Nes-ATK-GFP transgene. b, c, GCV administration ablates NSCs in wild-type mice. b, Representative Nissl staining of the SVZ region in wild-type mice treated with GCV (WT; GCV), Nes-ATK transgenic mice treated with PBS (Nes-ATK; PBS) and Nes-ATK transgenic mice treated with GCV (Nes-ATK; GCV); black arrows indicate the stem cell RMS, which is greatly reduced in Nes-ATK mice treated with GCV. c, GFP (transgene), GFAP (quiescent NSCs) and DCX (committed neural progenitors) immunostaining of the SVZ stem cell niche. White arrowheads in middle panel (Nes-ATK; PBS) indicate DCX+ GFP- cells more distal in the RMS. White arrowheads in bottom panel (Nes-ATK; GCV) indicate GFP+/GFAP- but DCX- quiescent NSCs. d, Representative GFP immunostaining in sections from two untreated gliomas of Mut7;Nes-ATK mice. From tumour to tumour, varying numbers of GFP+ cells were observed. TOPO-3 stains nuclei. e, Representative GFP and Ki67 co-immunostaining in two untreated gliomas of Mut7;Nes-ATK mice. White arrowheads highlight GFP+ but Ki67- cells, demonstrating that many transgene GFP+ cells are quiescent. f, Percentage of Ki67+ cells in GFP+, GFP-, and overall tumour population in gliomas from untreated Mut7;Nes-ATK mice. g, Kaplan–Meier survival curve of untreated Mut7 and Mut7;Nes-ATK animals. No difference in per cent survival was observed. h, Representative haematoxylin and eosin staining of Mut7 and Mut7;Nes-ATK brains without treatment. Infiltrative malignant gliomas are present in the cortex (red arrowhead) of both genotypes. ***P < 0.001.
and proximal progenitor cells, and chronic GCV administration effectively blocks neurogenesis by ablating quiescent cells as they enter the cell cycle.

We bred the Nes-ATK-GFP transgene into hGFAP-Cre;Nes;Pten1/2;P531/2 mice (Mut7) glioma-prone mice. Mut7 mice develop malignant glioma, with full penetrance, by somatic deletion of three of the most frequently mutated tumour suppressors in glioblastoma multiforme: p53, NF1 and Pten. All Mut7;Nes-ATK-GFP mice also developed gliomas and we observed only a subset of tumour cells expressing GFP (Nes-ATK-GFP positive; Fig. 1d, e). These cells also co-expressed the neural stem cell marker Sox2 (not shown). We next examined tumour cell proliferation and found that Mut7;Nes-ATK-GFP tumours exhibited a significant proportion of Ki67− cells are relatively quiescent in comparison to a highly proliferative Ki67+ cells, and conversely, the subset of GFP− tumour cells rarely co-stained for Ki67 (Fig. 1e, f). These data indicate that most transgene GFP+ tumour cells are relatively quiescent in comparison to a highly proliferative (Ki67+) subpopulation reminiscent of the SVZ, where GFP+ stem cells are quiescent compared to GFP− progenitors (Fig. 1c). Furthermore, as in wild-type mice, in which the Nes-ATK-GFP transgene does not affect SVZ neurogenesis in the absence of GCV administration (Fig. 1b, c), introduction of the transgene into the Mut7 genetic background did not affect tumour development or enhance survival (Fig. 1g). Like Mut7 mice, Mut7;Nes-ATK-GFP mice developed malignant glioma with 100% penetrance and with similar kinetics (Fig. 1g, h).

TMZ is a DNA alkylating agent that is currently the primary chemotherapy administered to glioblastoma multiforme patients as it has transient tumour growth-arrest properties. We found that TMZ eradicates proliferative cells in the endogenous murine gliomas. TMZ was administered over several days to tumour-bearing mice followed by BrdU injection 2 hours after final treatment, and the mice were killed 2 hours thereafter (Fig. 2a). The TMZ-treated mice showed a marked reduction of BrdU incorporation in both tumours and NSC niches (Fig. 2b, c). Similar to treated glialoma patients after TMZ treatment, the murine tumours reinitiated cell division and growth. Thus, TMZ targets proliferating cells but tumour recurrence is inevitable. To examine the details of tumour recurrence, we traced the first wave of tumour cell proliferation after completion of the drug regimen in tumour-bearing mice by pulse chase using the BrdU analogues CldU and IdU. CldU and IdU were injected 1 day and 3 days, respectively, after the final TMZ injection (Fig. 2d). Given the variable but relatively low proportion of GFP+ cells in all tumours (Fig. 1d, e), we reasoned that if cell proliferation reinitiated randomly in tumour cells after TMZ treatment, then the number of GFP+ cells that would incorporate CldU and/or IdU should be low to insignificant. However, if renewed tumour cell proliferation was hierarchical and derived from the GFP+ cells, a biased incorporation of nucleotide analogues into the relatively small GFP+ cohort of tumour cells would result. Examination of tumour sections with immunohistochemistry revealed that the large majority of cells incorporating CldU and IdU after relatively short times also contained GFP expression (Fig. 2e; GFP percentage in CldU+ cells in all tumours (Fig. 1d, e), we reasoned that if cell proliferation reinitiated randomly in tumour cells after TMZ treatment, then the number of GFP+ cells that would incorporate CldU and/or IdU should be low to insignificant. However, if renewed tumour cell proliferation was hierarchical and derived from the GFP+ cells, a biased incorporation of nucleotide analogues into the relatively small GFP+ cohort of tumour cells would result. Examination of tumour sections with immunohistochemistry revealed that the large majority of cells incorporating CldU and IdU after relatively short times also contained GFP expression (Fig. 2e; GFP percentage in CldU+ population = 77 ± 14; GFP percentage in IdU+ population = 83 ± 10). Moreover, IdU+ cells retained both GFP and CldU, indicating that after TMZ eradication of a majority of pre-existing proliferating tumour cells, the re-emergent population of proliferating cells derived from Nes-ATK-GFP-expressing cells and not from random tumour or other specific subsets of cells (Fig. 2e; CldU percentage in IdU+ population = 86 ± 9). When the second, IdU, pulse was prolonged to a 7-day chase after the CldU pulse, most IdU cells double-labelled for CldU (85 ± 7%) but lost GFP expression (Supplementary Fig. 1a–c). Thus, as in the normal SVZ stem cell niche, over time the GFP-expressing glioma cell population gives rise to cells that progressively lose stem cell properties (that is, nestin expression and relative quiescence) and concomitantly shut down the Nes-ATK-GFP transgene (Supplementary Fig. 1c, d). Using endogenous lineage tracing of renewed cell division within tumours, we have identified the relatively quiescent Nes-ATK-GFP-expressing tumour cells as the primary source of proliferating and eventually non-dividing tumour cell derivatives (Supplementary Fig. 1c, d). In addition, the data show that in our endogenous glioma models, TMZ targets the proliferating derivatives but not the GFP+ quiescent cells.

One prediction would be that eradication of the GFP+ cells in the endogenous tumours should considerably decrease the emergence of new dividing tumour cells. We tested this prediction at two stages: early endogenous tumour development and advanced tumour development. First, Mut7;Nes-ATK-GFP mice were treated with GCV beginning at 8 weeks of age—the earliest time of detectable pre-tumorigenic anomalies. To capture most quiescent GFP+ cells in a dividing state, GCV treatment lasted for 10 weeks, requiring three osmotic mini-pump placement surgeries that unfortunately were accompanied by complications, resulting in considerable variance of drug delivery. Drug-delivery efficacy was qualitatively measured by examination of the normal stem cell niche response to drug (Fig. 1b, c) as manifested by residual RMS (Supplementary Fig. 2a). Accordingly, tumour incidence varied from mouse to mouse, with some perishing...
early with large tumours. However, as a group, the GCV-treated cohort showed a clear survival advantage (Fig. 3a). After 10 weeks of treatment, the only surviving mice were among those treated with GCV (Supplementary Fig. 2b), and analysis of their brains revealed only low-grade lesions (Supplementary Fig. 2c). When GCV was effectively delivered, survival was substantially prolonged and tumour progression was severely impaired. Thus, elimination of Nes-ATK-GFP cells at early/pre-tumorigenic stages prevents development of high-grade gliomas.

A second GCV regimen was started in 10-week-old tumour-bearing mice. Most 10-week-old Mut7 mice do not show neurological symptoms, yet histological examination of large cohorts showed that all mice harbour astrocytomas of differing grades (Supplementary Fig. 3). We subjected 10-week-old Mut7:Nes-ATK-GFP or Mut7 mice to GCV or saline for 2 months. GCV treatment of Mut7:Nes-ATK-GFP mice improved survival by approximately 30 days compared to saline-treated tumours (bottom) showing the marked decrease in Ki67+ cells in tumour regions with highest number of Ki67+ cells. Representative Ki67 staining of control and GCV-treated tumours. The percentage is significantly decreased in GCV-treated mice; a clear survival advantage (Fig. 3a). After 10 weeks of treatment but had no such effect on the Mut7 mice. n = 5–7 for the GCV-treated mice; n = 4–7 for the saline-treated mice; P values determined using log-rank test). βMed sur., change in median survival.

The residual tumours in these tumour-bearing Mut7:Nes-ATK mice treated with GCV beginning at 10 weeks of age did not have the classic glioma feature of invasiveness but instead were circumscribed with well-defined boundaries (Fig. 3d, e and Supplementary Fig. 2d, e). The residual tumours in these tumour-bearing mice treated with GCV or saline for 2 months. GCV treatment of Mut7:Nes-ATK-GFP mice improved survival by approximately 30 days compared to saline-treated tumours. Despite effective depletion of GFP-expressing tumour-propagating cells with GCV treatment, a considerable residual tumour mass remained (Fig. 3d). We therefore attempted a therapeutic strategy to eliminate both the rapidly proliferating tumour cells and the quiescent proliferating tumours. The percentage is significantly decreased in GCV-treated Mut7:Nes-ATK mice (n = 3) versus control (n = 4). Data are mean ± s.e.m.; **P < 0.01, Student’s t-test.

The HSV TK system has been widely used as a method to induce endogenous cell suicide. A potentially confounding phenomenon is the ‘bystander effect’, whereby HSV-TK-expressing cells not only commit suicide in the presence of GCV, but can also induce the death of neighbouring non-TK-expressing cells. However, several NSC-specific HSV-TK-expressing transgenic mouse models have been reported and no bystander effect has been described. To examine whether tumour development was appreciably impaired by the bystander effect, we turned to transplantation assays.

First, we determined whether GCV treatment would also blunt Mut7:Nes-ATK-GFP tumour growth in a transplantation assay. Primary gliomas from Mut7:Nes-ATK-GFP and Mut7 mice were dissociated and directly injected subcutaneously into nude mice in the presence of continuous GCV or saline treatment (Supplementary Fig. 4a). Neither GCV nor saline affected the tumour growth of Mut7-derived cells (Supplementary Fig. 4b). In contrast, similar to a previous report, mice transplanted with Mut7:Nes-ATK tumour cells and then treated with GCV developed significantly smaller tumours that appeared to be poorly vascularized compared to saline-treated controls (Supplementary Fig. 4c–f). Thus, the Mut7 tumour cells transplant efficiently in immunocompromised mice and, as in the endogenous setting (Fig. 3), GCV treatment severely impairs tumour development after transplantation. To test for the bystander effect, we transduced primary Mut7 cells with a lentivirus harbouring either a control RFP cassette or an HSV TK cassette (Supplementary Fig. 5a). Mixed ratios of the two cell populations were injected subcutaneously into nude mice and allowed to seed tumours for 4 weeks, after which GCV was administered for 2 weeks (Supplementary Fig. 5b). The data indicate that the presence of a 10%, 20% or 50% initial ratio of TK-expressing tumour cells did not impair tumour development of the non-TK-expressing cells in the presence of GCV (Supplementary Fig. 5c, d). In the extreme case when equal numbers of TK+ (105) and TK− (105) cells were injected compared to 105 TK− cells alone, tumour development was appreciably impaired in both cohorts, demonstrating that GCV toxicity to 50% of the tumour cells did not extend into the TK− tumour cell population (Supplementary Fig. 5c, d). In our endogenous tumours, such a bystander effect would require the relatively rare GFP+ tumour-propagating cells to be widely toxic in order to have a considerable paracrine effect on tumour properties. Instead, these studies indicate that GCV administration does not have an appreciable effect on cells outside those expressing the TK gene, consistent with other recently reported studies. We conclude that eradication of the Mut7:Nes-ATK-GFP endogenous tumour cells through GCV treatment effectively disrupts the continued production of tumour cells, as predicted from the preceding data.

Despite effective depletion of GFP-expressing tumour-propagating cells with GCV treatment, a considerable residual tumour mass remained (Fig. 3d). The residual tumours in these tumour-bearing mice treated with GCV beginning at 10 weeks of age did not have the classic glioma feature of invasiveness but instead were circumscribed with well-defined boundaries (Fig. 3d, e and Supplementary Fig. 2d, e). Although the Mut7:Nes-ATK-GFP transgene is only expressed in a subset of cells in the untreated tumour (Fig. 1e), tumours in GCV-treated Mut7:Nes-ATK mice showed a marked reduction of Ki67+ cells and stem cell markers (Fig. 3e–g). These data further support the hypothesis that chronic GCV administration progressively ablates the relatively quiescent GFP+ tumour cell population and the remaining GFP− tumour cells eventually exhaust their proliferative and infiltrative potential.

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GFP$^+$ cells by sequentially administering TMZ and GCV (TMZ/GCV), respectively (Fig. 4a, b). Initially it seemed that this regimen did not prolong survival beyond that of GCV treatment alone (Fig. 4c). However, examination of the brains of the sequentially treated mice revealed only vestigial tumours in the dorsal brain that showed no transgene GFP expression, indicating effective depletion (Fig. 4d).

The cellular density of the residual TMZ/GCV-treated tumours was lower than that of tumours at the beginning of treatment (10 weeks), indicating a statistically significant reduction in tumour bulk. This contrast was markedly enhanced when treated tumours were compared to untreated control tumours (Fig. 4e), which manifested in a spectrum of survival time from 12–16 weeks. Thus, the combined treatment had dramatic inhibitory effects on dorsal tumour growth in these mice.

We were puzzled that TMZ/GCV treatment did not significantly prolong survival beyond the GCV-only treated mice, in which residual circumscribed tumour mass remained (Fig. 3d, e and Supplementary Fig. 2d, e). Analysis of the TMZ/GCV-treated brains revealed that, despite the marked inhibition of original tumour growth, these mice developed novel tumours in the ventral brain region. Six out of seven TMZ/GCV-treated mice had tumours in the brainstem region, whereas gliomas in untreated Mut7 mice and their remnants in the successfully treated mice are predominantly located in the dorsal/midbrain (Supplementary Fig. 6a). Examination of the TMZ/GCV-resistant hindbrain tumours revealed high endogenous nestin protein but no GFP expression (Supplementary Fig. 6b, c). We also searched archived material and found that a small percentage of Mut7 brains harboured both dorsal and ventral tumours that we now appreciate were independently arising and not extensions of the dorsal tumours. Appearance of ventral tumours in the presence of GCV indicates an inactive Nes-ATK-GFP transgene, and direct examination of untreated Mut7:Nes-ATK-GFP mice demonstrated that the Nes-ATK-GFP transgene was indeed silent (Supplementary Fig. 6b, e).

We next examined the expression of tumour markers in these hind-brain tumours. In contrast to typical Mut7 or Mut7:Nes-ATK-GFP gliomas, all ventral tumours in the TMZ/GCV group showed low levels of endogenous GFAP but relatively high levels of S100B (Supplementary Fig. 7a–c), including high PDGFR-α levels (Supplementary Fig. 7d). This is in contrast to the pure astrocytic tumours typically observed in this mouse model in the absence of TMZ/GCV. Thus, these data indicate that the ventral tumours that become evident after TMZ/GCV treatment are oligoastrocytic, independently arising, and distinct from the dorsal tumours. Such tumours were recently described in Mut3 mice$^{13}$, and the source was reported to be oligodendrogial progenitors. We are currently further characterizing these tumours.

Our Nes-ATK-GFP transgene labels SVZ stem cells and, fortuitously, a specific subset of glioblastoma multiforme cells that possesses many features proposed for cancer stem cells (CSCs). The CSC hypothesis holds that some tumours are composed of a hierarchical cadre of cells of which only a subset retains both self-renewal and differentiation capacity$^{14}$. In this model, only CSCs have the capacity to sustain tumour growth and are responsible for recurrence after therapy fails.

The current standard for evaluating whether solid tumours contain CSCs is an ex vivo limiting dilution tumorigenic transplantation assay into immunodeficient animals$^{14}$. However, controversy regarding the presence and frequency of solid tumour CSCs remains, probably as a reflection of the variability that accompanies such assays$^{15–18}$. Our study identifies a putative endogenous glioma stem cell located at the apex of a cellular hierarchy in tumour maintenance and recurrence after chemotherapy (Fig. 4a). Continued evaluation of these cells and their properties, including isolation and genetic lineage tracing, may yield important insights into novel therapeutic targets for this intractable disease.

### METHODS SUMMARY

**Mice.** All mice were maintained on a mixed 129Sv1/C57BL/6/B6C3F1 background. Mut7 and Mut7:Nes-ATK mice were treated by crossing male hGFAP-Cre;P53$^{−/−}$ mice with female Nf1$^{−/−}$;P53$^{−/−}$;Pten$^{−/−}$;Nes-ATK mice. Genotyping for Mut7 mice was performed as reported previously$^{14}$.

**In vivo chemical administration.** Stock CldU (Sigma) and BrdU (Sigma) were dissolved in PBS at a concentration of 8.5 mg ml$^{−1}$ and 10 mg ml$^{−1}$, respectively. 11.5 mg ml$^{−1}$ IdU (MP Biomedicals) solution was made fresh each time$^{11}$. To label dividing cells, 5 ml kg$^{−1}$ stock solution was injected intraperitoneally each time according to the experimental design. GCV (Cytovene-IV, Roche Pharmaceuticals) treatment was performed as described$^{19}$. For initial characterization of Nes-ATK-GFP mice, 1-month-old Nes-ATK-GFP or control mice were administered GCV (300 mg kg$^{−1}$ d$^{−1}$) or PBS via osmotic mini-pump (Model 2002, 0.5 µl h$^{−1}$, Alzet) for 2 weeks. For treatment starting from 8 or 10 weeks, 150 mg kg$^{−1}$ d$^{−1}$ GCV or PBS was delivered through osmotic mini-pump (Model 2002, 0.25 µl h$^{−1}$, Alzet); pumps were surgically removed and replaced every 4 weeks based on the experimental requirement. TMZ (Sigma) was dissolved in DMSO and injected intraperitoneally at a dose of 82.5 mg kg$^{−1}$ d$^{−1}$ for 5 days. For the combinational therapy group, mice were first treated with TMZ for 5 days and then osmotic mini-pumps with GCV were implanted 2 days after the last TMZ injection.

**Histology and immunohistochemistry.** Mice were perfused and brains were processed as described earlier$^{9}$. Paraffin brain haematoxylin and eosin sections (5 µm) were reviewed by J.C. and D.K.B. independently. Tumour type and grades were determined by D.K.B. Fourteen-micrometre cryostat sections were used for GFP/CldU/IdU staining following reported methods$^{19}$. Primary antibodies were...
used against GFAP (DAKO, 1:2,000), Olig2 (Millipore, 1:1,000), Sox2 (Millipore, 1:5,000), nestin (BD Biosciences, 1:200), CD44 (BD Biosciences, 1:75), GFP (Rockland, 1:200, Aves Lab, 1:500), BrdU/IdU (BD Biosciences, 1:100), BrdU/IdU (AbD Serotec, 1:500), Ki67 (Novacasta, 1:1,000), PDGFR-α (Santa Cruz, 1:200). Peroxidase-peroxidase-based Vectastain ABC Kit (Vector Laboratories) or Cy2/Alexa-488, C3/Alexa555, Cy5-labelled secondary antibodies (Jackson Labs, Invitrogen) were used to visualize the primary antibody staining.

**Full Methods** and any associated references are available in the online version of the paper.

**Received 26 January 2011; accepted 7 June 2012.**

**Published online 1 August 2012.**

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METHODS

Mice. All mouse experiments were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center at Dallas. All mice were maintained on a mixed 129SvJ/C57BL/6/B6CAB background. Mut7 and Mut7;Nes-ATK mice were obtained by crossing male hGFAP-Cre;P53^6^ mice with female NF1^6^;P53^6^;Pten^6^;Nes-ATK mice. Genotyping for Mut7 mice was performed as reported previously. In vivo chemical administration. Stock CldU (Sigma) and BrdU (Sigma) were dissolved in PBS at a concentration of 8.5 mg ml⁻¹ and 10 mg ml⁻¹, respectively. 11.5 mg ml⁻¹ IdU (MP Biomedicals) solution was made fresh each time. To label dividing cells, 5 ml kg⁻¹ stock solution was injected intraperitoneally each time according to the experimental design. GCV (Cytovene-IV, Roche Pharmaceuticals) treatment was performed as described. For initial characterization of Nes-ATK-GFP mice, 1-month-old Nes-ATK-GFP or control mice were administered GCV (300 mg kg⁻¹ d⁻¹) or PBS via osmotic mini-pump (Model 2002, 0.5 μl h⁻¹, Alzet) for 2 weeks. For treatment starting from 8 or 10 weeks, 150 mg kg⁻¹ GCV or PBS was delivered through osmotic mini-pumps (Model 2004, 0.25 μl h⁻¹, Alzet); pumps were surgically removed and replaced every 4 weeks based on the experimental requirement. TMZ (Sigma) was dissolved in DMSO and injected intraperitoneally at a dose of 82.5 mg kg⁻¹ d⁻¹ for 5 days. For the combinational therapy group, mice were first treated with TMZ for 5 days and then osmotic mini-pumps with GCV were implanted 2 days after the last TMZ injection. Mice were perfused and brains were cryoprotected in 30% sucrose, embedded in OCT, and cut into 14-μm-thick frozen sections. TMZ administration and mice were perfused 2 h after BrdU injection. The brain was then paraffin-processed and cut into 5-μm-thick slices. Haematoxylin and eosin staining was performed every 70 μm to identify tumour location. Adjacent tumour sections were selected for GFAP and BrdU co-immunostaining.

Histology and immunohistochemistry. Mice were perfused and brains were processed as described earlier. Paraffin brain haematoxylin and eosin sections (5 μm) were reviewed by J.C. and D.K.B. independently. Tumour type and grades were determined by D.K.B. Fourteen-micrometre cryostat sections were used for GFP/CldU/IdU staining following reported methods. Primary antibodies were used against GFAP (DAKO, 1:2,000), Olig2 (Millipore, 1:1,000), Sox2 (Millipore, 1:5,000), nestin (BD Biosciences, 1:200), CD44 (BD Biosciences, 1:75), GFP (Rockland, 1:200, Aves Lab, 1:500), BrdU/Iodon (BD Biosciences, 1:100), BrdU/CldU (AbD Serotec, 1:500), Ki67 (Novacastra, 1:1,000), PDGFR-α (Santa Cruz, 1:200). Horseradish-peroxidase–based Vectastain ABC Kit (Vector Laboratories) or Cy2/Alexa-488, C3/Alexa555, Cy5-labelled secondary antibodies (Jackson Labs, Invitrogen) were used to visualize the primary antibody staining. Images were taken using optical, fluorescence and confocal microscopy (Olympus and Carl Zeiss) and assembled in Adobe Illustrator (Adobe Systems Incorporated).

TMZ, BrdU analogues and pulse-chase experiments. To determine TMZ efficiency, 10- to 11-week-old Mut7 mice were first injected intraperitoneally with 82.5 mg kg⁻¹ d⁻¹ TMZ for 5 days. 50 mg kg⁻¹ BrdU was injected 2 h after the final TMZ administration and mice were perfused 2 h after BrdU injection. The brain was then paraffin-processed and cut into 5-μm-thick slices. Haematoxylin and eosin staining was performed every 70 μm to identify tumour location. Adjacent tumour sections were selected for GFAP and BrdU co-immunostaining.

For the short-term CldU chase experiments, 10- to 11-week-old Mut7;Nes-ATK mice were first treated with TMZ for 5 days. A total of three doses of CldU were injected, with 2-h intervals, the day after the final TMZ injection. A single dose of IdU was then injected 3 days after the final TMZ injection. For the long-term CldU chase experiments, 10- to 11-week-old Mut7;Nes-ATK mice were first treated with TMZ for 5 days. CldU was injected 3 times a day, with 2-h intervals, for 3 days after the final TMZ injection. A single dose of IdU was then injected 7 days after the final TMZ injection. Mice were perfused 2 h after the IdU injection and the brains cryoprotected in 30% sucrose, embedded in OCT, and cut into 14-μm-thick frozen sections. GFAP and Ki67 co-immunostaining was performed every 140 μm to locate the tumour area. Adjacent sections were selected for GFP/CldU/IdU triple immunofluorescence staining.

Quantification. Because of the heterogeneous nature of the tumours, cell density, Ki67 index and BrdU+ cell percentage were quantified using the highest staining area. Briefly, staining was checked under low magnification and the highest staining area was identified. The area was viewed at ×200 in three continuous 5-μm-thick sections and positive cells counted using the measured parameters.

For quantification of GFP/CldU/IdU triple staining, tumour areas with at least one CldU+ cell were selected, and an 8 μm Z-stack image was scanned and constructed using confocal microscopy (Olympus and Carl Zeiss). A total of ten different areas within each tumour was imaged and subjected to quantification.