Supporting Information

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SI Materials and Methods

Transgenic Animals, Procedures, and Genotyping. All mouse procedures were performed in accordance with Tufts University's recommendations for the care and use of animals and were maintained and handled under protocols approved by the Institutional Animal Care and Use Committee. The strategy for the construction of the EGFR-based genetically engineered mouse strains is depicted in Fig. S1 and detailed as follows: wild-type and vIII isoforms of human EGFR cDNAs were inserted in the CAGGS-Col1α1 vector plasmid (a kind gift from M. Werning and R. Jaenisch, Whitehead Institute, Cambridge, MA). After DNA sequencing for integrity, plasmid DNAs were coelectroporated along with pCAGGS-Flpe plasmid into C2 ES cells (a gift from M. Werning and R. Jaenisch). Clonal selection was achieved using hygromycin, and individual clones were screened by Southern blot hybridization (1) with probes described elsewhere (2) (Fig. S1*A*). ES clones with properly knocked-in EGFR transgenes in the Col1α1 locus (Fig. S1*B*) were used to produce chimeric mice, which were then mated to produce founder animals. Germline-transmitted $EGFR^{WT}$ and $EGFR^{vIII}$ founder males were mated to InkΔ2/3 (3) and conditional PTEN knockout strains (4). Note that homozygous null mice for the Ink4a/Arf locus develop lymphomas and subcutis sarcomas at a median age of 30 weeks and have never been reported to develop glioma in their lifespan (3); nonrecombined $PTEN^{2lox}$ mice are normal and viable; and CNS-specific deletion of PTEN alone is inconsequential (ref. 5 and data not shown). In addition, targeting into the 3′ region of Col1α1 gene does not produce an observable phenotype, as we have knocked in several other minigenes to that locus without phenotypic consequences of integration (data not shown). The combinations of strain indicated in the text were produced by crossbreeding. Compound EGFR transgenic mice that were not exposed to Cre recombinase did not exhibit phenotypic features that are consistent with spontaneous tumor formation (data not shown). The EGFR transgenic strains were genotyped using the following primer set: Col frt A1 (5′GCA CAG CAT TGC GGA CAT GC3′), Col frt B (5′CCC TCC ATG TGT GAC CAA GG3′), and Col frt C (5′GCA GAA GCG CGG CCG TCT GG3′) for the collagen 1α1 locus genotype. The EGFR alleles were occasionally genotyped using Flp1N 4230–4250 (5′CCC CCT GAA CCT GAA ACA TAA3′) along with hEGFR-390rev (5′ATG GGC AGC TCC TTC AGT CCG3′) or hEGFR-1110rev (5′TAA ATG CCA CCG GCA GGA TG3′) for the WT or vIII allele, respectively. The PCR cycling parameters for the genotypes are 94° 5 min, 35 cycles at 94° for 30 sec, 55° for 30 sec, and 72° for 30 sec followed by a 10-min extension at 72°. Genotyping protocols for InkΔ2/3 knockout animals and conditional PTEN knockout strains were carried out as described elsewhere (3, 4).

Nomenclature of Strains. Throughout the manuscript, homozygous mice with 2 copies of EGFR wild-type knockin alleles are indicated as $EGFR^{WT/WT}$ and hemizygous with one copy as $EGFR^{WT/+}$. Animals with one or both copies of the knockin allele of $EGFR^{vIII}$ are indicated as $EGFR^{vIII/+}$ and $EGFR^{vIII/vIII}$. Mice with one copy each of EGFR WT and vIII knockin alleles are indicated as $EGFR^{WT/vIII}$.

Stereotactic Injections. Adult animals (3 months of age and older) of the indicated genotype were anesthetized with an IP injection of ketamine/xylazine (ketamine 100−125 mg/kg, xylazine 10–12.5 mg/kg). The animals were mounted in a Stoelting stereotaxic frame (Harvard Apparatus Inc.) with nonpuncturing ear bars. The incision site was shaved and sterilized with betadine surgical scrub, and a single incision was made from the anterior pole of the skull to the posterior ridge. A 1-mm burr hole was drilled at the stereotactically defined location of the striatum (2.1 mm rostral to the bregma, 1.5 mm lateral to the midline, and at 2 mm depth to the pia surface) and either a 1-μl Hamilton syringe or a pulled glass pipette mounted onto a Nanoject II injector (Drummond Scientific Company) was used to inject the adeno-CMV Cre virus (Gene Transfer Vector Core, University of Iowa, Iowa City, IA) at a rate of 0.1 μl/min. Following retraction of the syringe or pipette, the burr hole was filled with sterile bone wax, the skin drawn up and sutured, and the animal placed in a cage with a padded bottom atop a surgical heat pad until ambulatory.

Primary Cultures. Primary cultures of tumors or newborn mice astrocytes were established as follows: tumors or P0 neonate cortices were excised and minced in 0.25% trypsin (wt/vol) 1 mM EDTA and allowed to disaggregate for 15 min at 37 °C. The resulting cell suspension was then strained through a 70-μm cell strainer (Falcon). The single suspension of cells was washed in PBS twice and plated on 0.2% gelatin-coated tissue culture plates. Cells were fed every 24 hr with fresh media that consisted of DMEM supplemented with 10% heat-inactivated FBS and antibiotics. The primary cultures of astrocytes were routinely stained for markers of astrocytic lineages by immunofluorescence (data not shown).

Immunoblots. Western blots were performed as follows: cell lysates were prepared using radioimmunoprecipitation (RIPA) buffer supplemented with 5 mM Na_3VO_4 (freshly made) and Complete™ protease inhibitor mixture (Roche). Forty micrograms of total cell lysates were separated by SDS-PAGE and electrotransfered to polyvinylidene fluoride (PVDF) membrane (Immobilon P; Millipore). Blots were blocked in Tris-buffered saline 0.1% (vol/vol) Tween-20 (TBS-T), 1% (wt/v) BSA, and 5% (wt/v) nonfat dry milk (Bio-Rad) for 1 hr on a shaker. Primary antibodies were added to blocking solution at 1:1,000 dilution and incubated overnight at 4 °C on a shaker. Blots were washed several times with TBS-T BSA, and secondary antibodies were added at 1:10,000 dilution into TBS-T BSA and incubated for 1 hr at room temperature on a shaker. After several washed, enhanced chemiluminescence (ECL) reactions were performed as described by the manufacturer (Western Lightning Kit; Perkin-Elmer). The following primary antibodies used in these studies were obtained from Cell Signaling Technology: *p*-Stat3 (Tyr-705; 9145S), Stat3 (9132S), *p*-S6 kinase Thr-421/Ser-424 (9204S), *p*-c-Kit (Tyr-719; 3391S), *p*-p38 MAPK (Thr-180/Tyr-182; 9211S), *p*-AKT (Thr-308; 9275S), *p*-AKT (Ser-473) (9271S), AKT (9272S), *p*-S6 ribosomal protein (Ser-235/236; 2211S), *p*-GSK-3β (Ser-9; 9336S), *p*-EGFR (Tyr-845; 2231S), *p*-EGFR (Tyr-1068; 2234S), *p*-EGFR (Tyr-1173; 4407S), *p*-EGFR (Tyr-1148; 4404S), *p*-EGFR (Tyr-1045; 2237S), *p*-EGFR (Tyr-992; 2235S), EGFR (2232S), MEK1/2 (9122S), *p*-MEK1/2 (Ser-217/221; 9121S), *p*-p42/44 MAPK (Erk1/2; Thr-202/Tyr-204; 9101S), *p*-PDK1 (Ser-241; 3061S), PDK1 (3062S), S6 ribosomal protein (2217S), antiphosphotyrosine 4G10 (Millipore, 05–1050), anti-α-tubulin (DM1A; T9026-Sigma), and antidynamin (6C9; Sigma).

Histology and Immunohistochemistry. Deeply anesthetized animals were transcardially perfused with cold PBS. Brains were excised, rinsed in PBS, and serial coronal sections cut using a brain mold. Half of the sections were used to isolate primary cultures of tumor cells as described previously, and the other half were postfixed in 4% paraformaldehyde overnight. Formalin-fixed tissues were embedded in paraffin, sectioned at 5–10 μM, and stained with H&E (Sigma) for histopathological analysis. For immunohistochemistry (IHC), cut sections were deparaffinized and rehydrated through xylenes and graded alcohol series and rinsed for 5 min under tap water. Antigen target retrieval solution (Dako, S1699) was used to unmask the antigen (microwaved for 10 min at low power then cooled down for 30 min) followed by 3 washes with PBS for 5 min each. Quenching of endogenous peroxidase activity was performed by incubating the sections for 30 min in 0.3% H_2O_2 in methanol followed by PBS washes. Slides were preincubated in blocking solution [5% (vol/vol) goat serum (Sigma) in PBS 0.3% (vol/vol) Triton-X100] for 1 hr at room temperature, followed by mouse-on-mouse blocking reagent (Vector Labs, Inc.; MKB-2213) incubation for 1 hr. Primary antibody for the EGFR (mouse mAb anti-EGFR, 31G7, 1:100, Zymed, Lab, Inc.) was incubated for 24 hr. Secondary antibodies used were biotinylated anti-rabbit or anti-mouse (Vector Labs, Inc.; 1:500) for IHC. All antibodies were diluted in blocking solution. All immunobinding of primary antibodies was detected by biotin-conjugated secondary antibodies and Vectastain ABC kit (Vector Labs, Inc.) using DAB (Vector Labs, Inc.) as a substrate for peroxidase and counterstained with hematoxylin. The following primary antibodies were used: anti-GFAP (Dako), anti-S100 (Dako), anti-EGFR (31G7; Zymed), and anti-NeuN (MAB377; Chemicon).

Magnetic Resonance Imaging. MR imaging was performed on a 4.7 T Bruker Pharmascan MRI scanner. T2-weighted images (TR = 3,500 ms, TE = 75 ms, 12 signals acquired, acquisition time 11 min and 12 sec, matrix size 256 × 256, field of view 2.5 × 2.5 cm, slice thickness 1.0 mm, 16 sections acquired) as well as pre- and postcontrast T1-weighted images (TR = 800, TE = 13; 4 signals acquired, acquisition time of 6 min 57 sec, matrix size 256 × 192, field of view 2.5 × 2.5 cm, slice thickness 1.0 mm, and 18 sections were acquired) were obtained after the i.v. administration of 0.3 mmol/kg of DTPA-Gd (Magnevist).

1. Ausubel F, *et al.* (2001) in *Current Protocols in Molecular Biology*, ed Ausubel F, *et al.* (Wiley, New York).
2. Beard C, *et al.* (2006) Efficient method to generate single-copy transgenic mice by site-specific integration in embryonic stem cells. *Genesis* 44:23–28.
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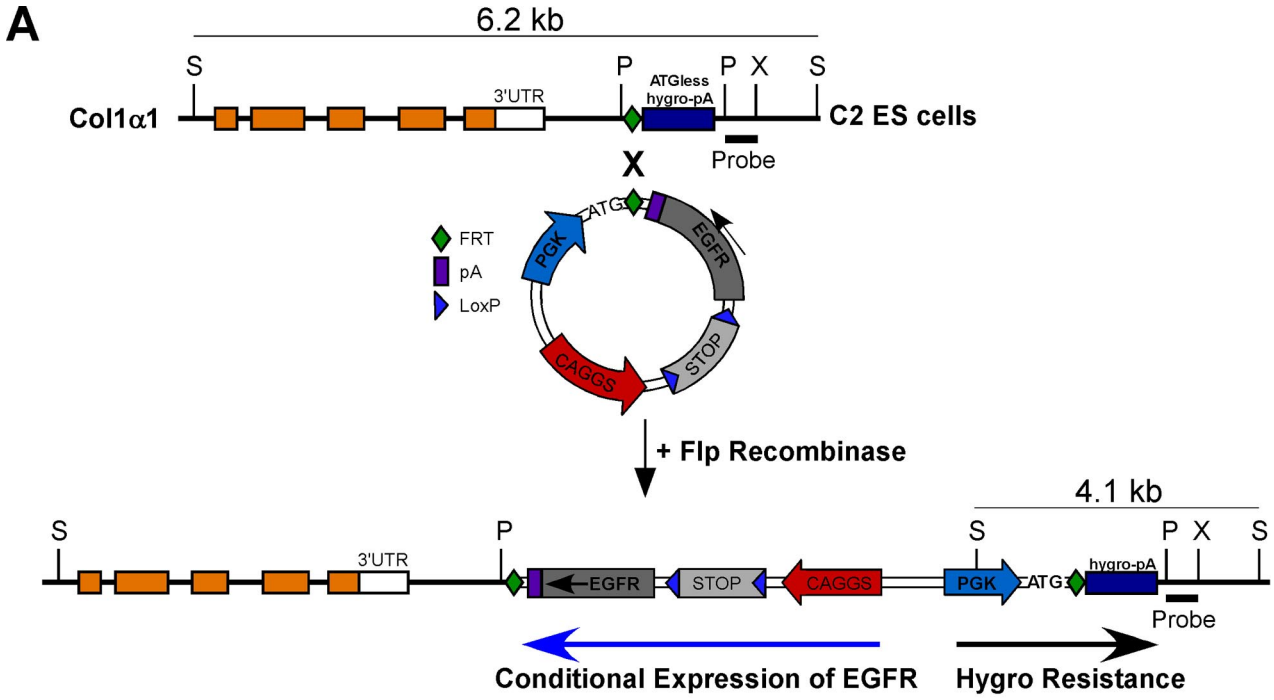
6. Sarkaria JN, *et al.* (2006) Use of an orthotopic xenograft model for assessing the effect of epidermal growth factor receptor amplification on glioblastoma radiation response. *Clin* Cancer Res 12:2264–2271.



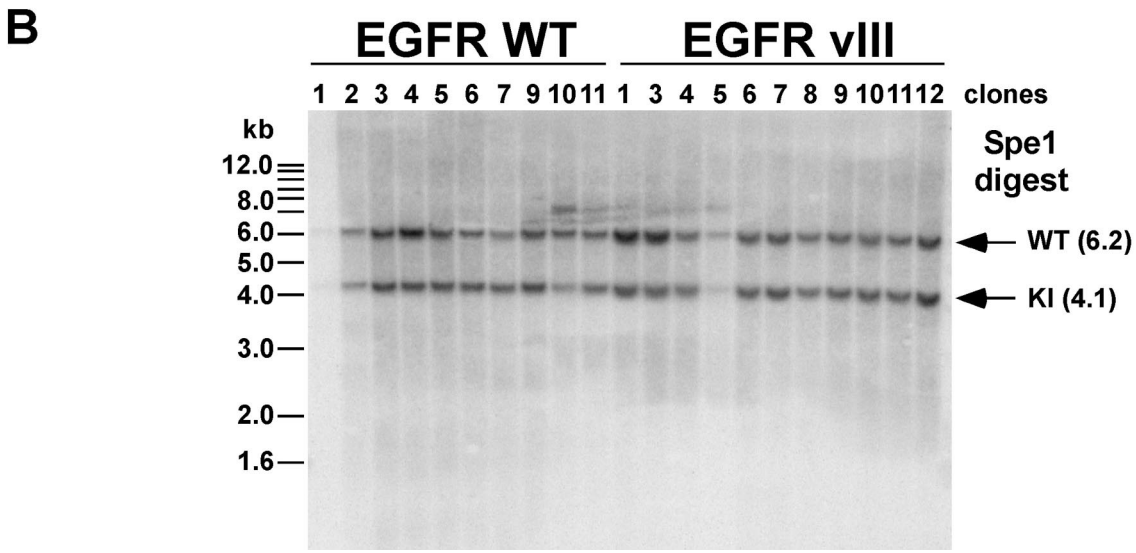


Fig. S1. Collagen 1α1 locus Flp-in system. (*A*) Schematic representation of the modified Col1α1 locus in C2 ES cells. In these cells, a promoter and ATG-less hygromycin resistance cassette has been inserted in the 3′ region of the collagen 1α1 gene. Transient transfection of a targeting plasmid with a Flpe recombinase plasmid in C2 ES cells result in the restoration of a functional hygromycin resistance cassette and the introduction of a CAGGS-loxSTOPlox-EGFR segment. Exons are shown as orange boxes and 3′ UTR as an open box. P, PstI; S, SpeI; X, XhoI. Note that the figure is not drawn to scale. (*B*) Results of Southern analysis of hygromycin-resistant flp-in clones for EGFR WT and EGFRvIII alleles using a 3′ internal probe (indicated).

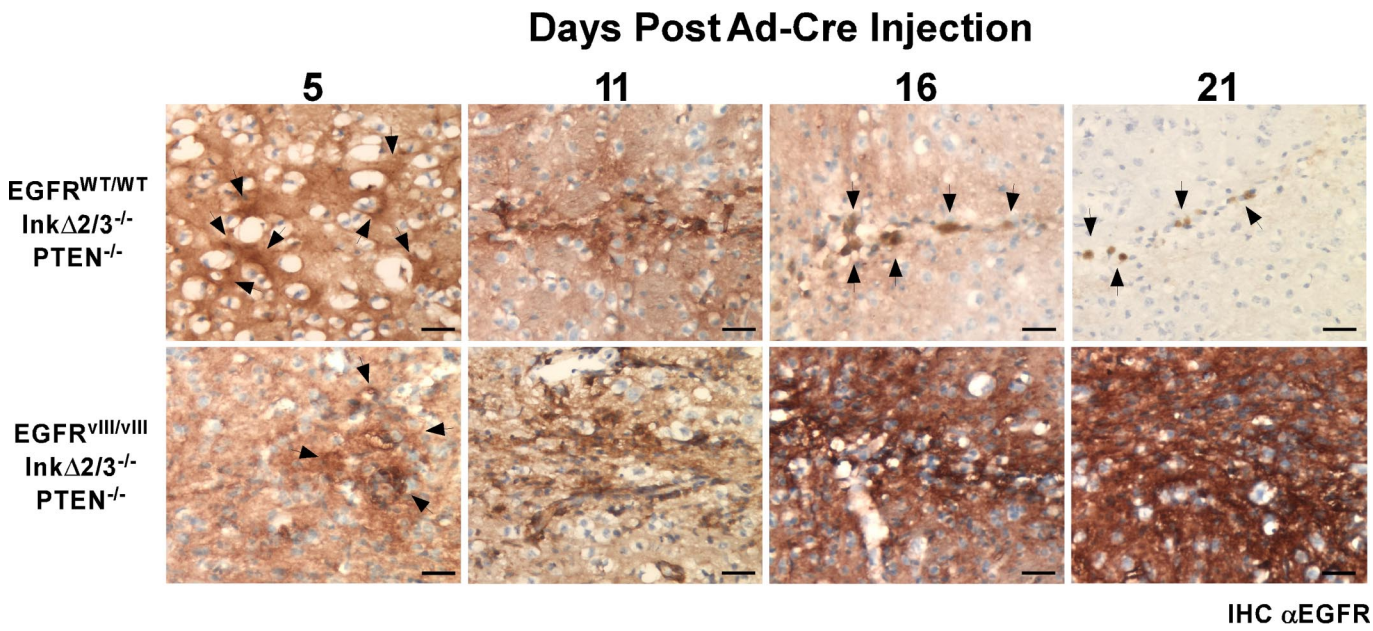


Fig. S2. Expression of EGF receptors in vivo post-Ad-Cre injection. Col1α1-EGFR$^{WT/WT}$ and Col1α1-EGFR$^{vIII/vIII}$ mice on InkΔ2/3 and PTEN null backgrounds were injected with Ad-Cre virus and EGFR expression detected by immunohistochemistry (DAB stain, brown) 5, 11, 16, and 21 days after injections. Sections were counterstained with hematoxylin. Cells expressing EGFR are identified by arrows. EGFR expression is seen along the needle track. By day 21 post-Ad-Cre administration, EGFR$^{WT/WT}$-expressing cells (arrows) are virtually inexistent, whereas EGFR$^{vIII/vIII}$ cells are flourishing. (Scale bars: 30 μM.)

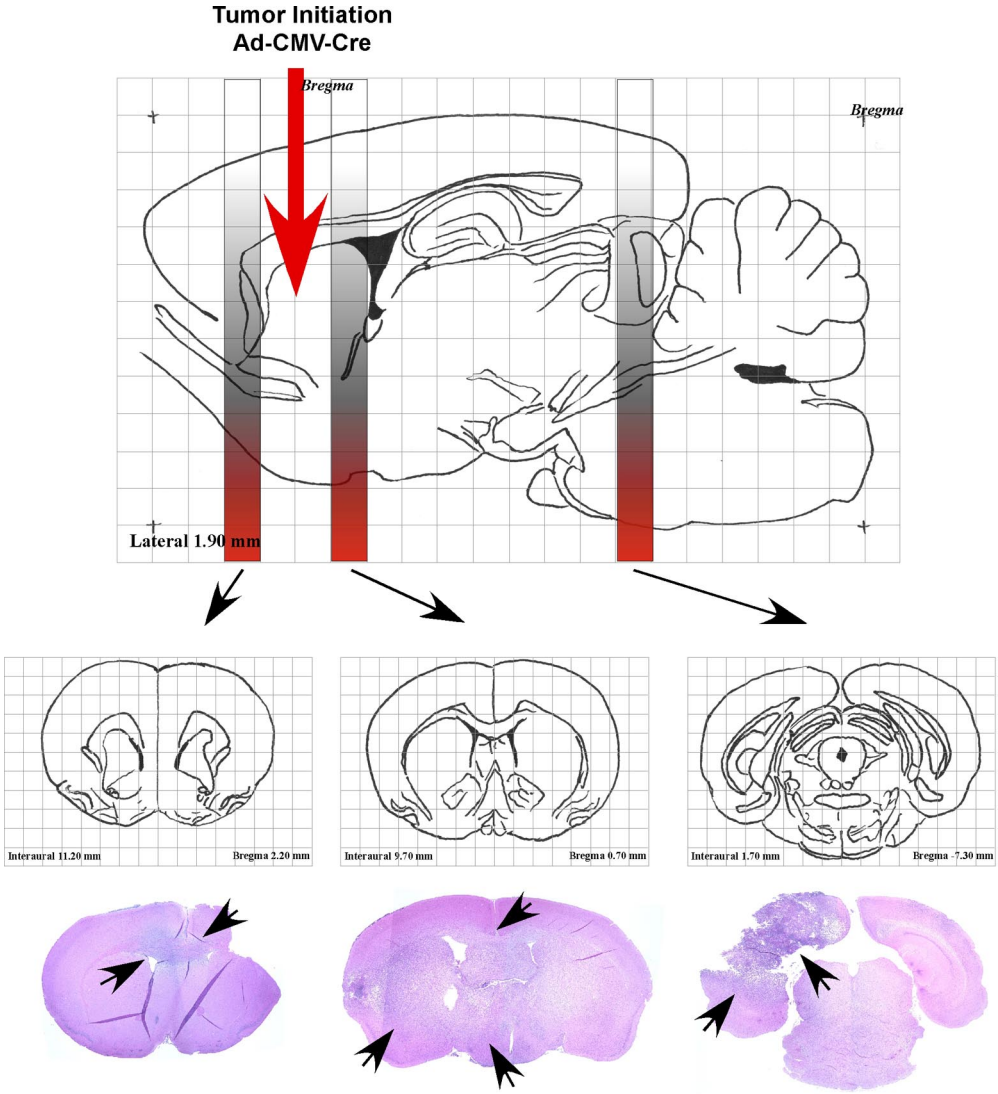


Fig. S3. EGFR tumors are highly infiltrative. Sagital schematic representation of a mouse brain indicating the location of the viral injection and showing the 3 different planes of sections analyzed. Below are photomicrographs of H&E strained sections corresponding to the planes, illustrating the extent of tumor cell migration and infiltration. The tumor is indicated with black arrows.

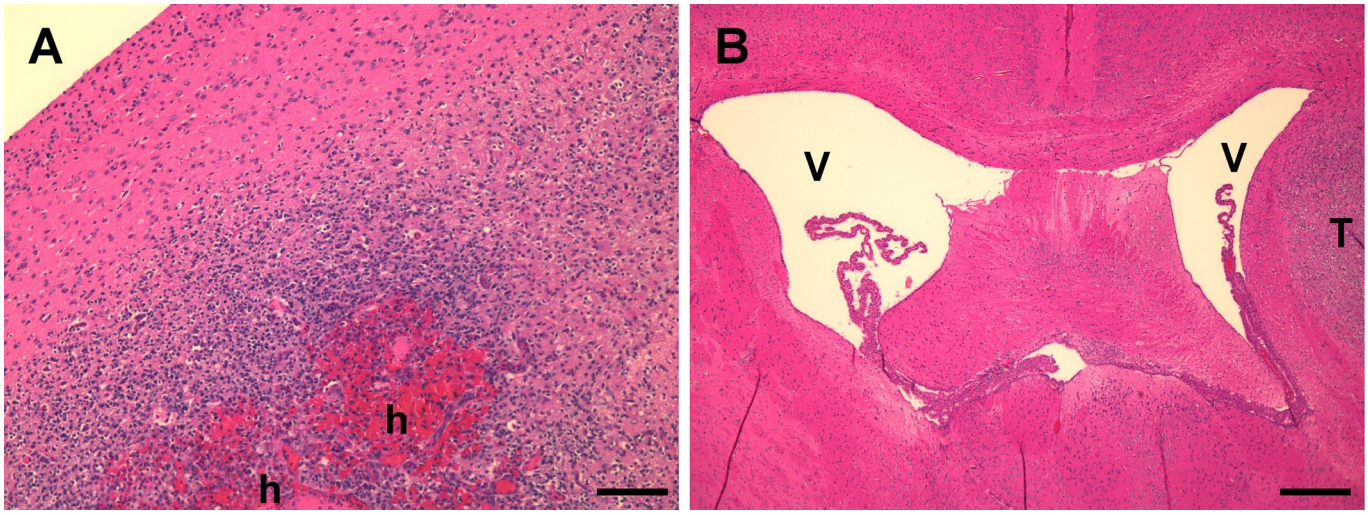


Fig. S4. Histopathology of EGFR GBM tumors. Photomicrographs of H&E-stained sections of representative $EGFR^{vIII/+}$; $Ink\Delta2/3^{-/-}$; $PTEN^{-/-}$ GBM tumors. (*A*) Tumors contain area of varying degree of hemorrhaging (h). (*B*) A GBM tumor (T) exerts pressure on the lateral ventricle (V). (Scale bar: 125 μM.)

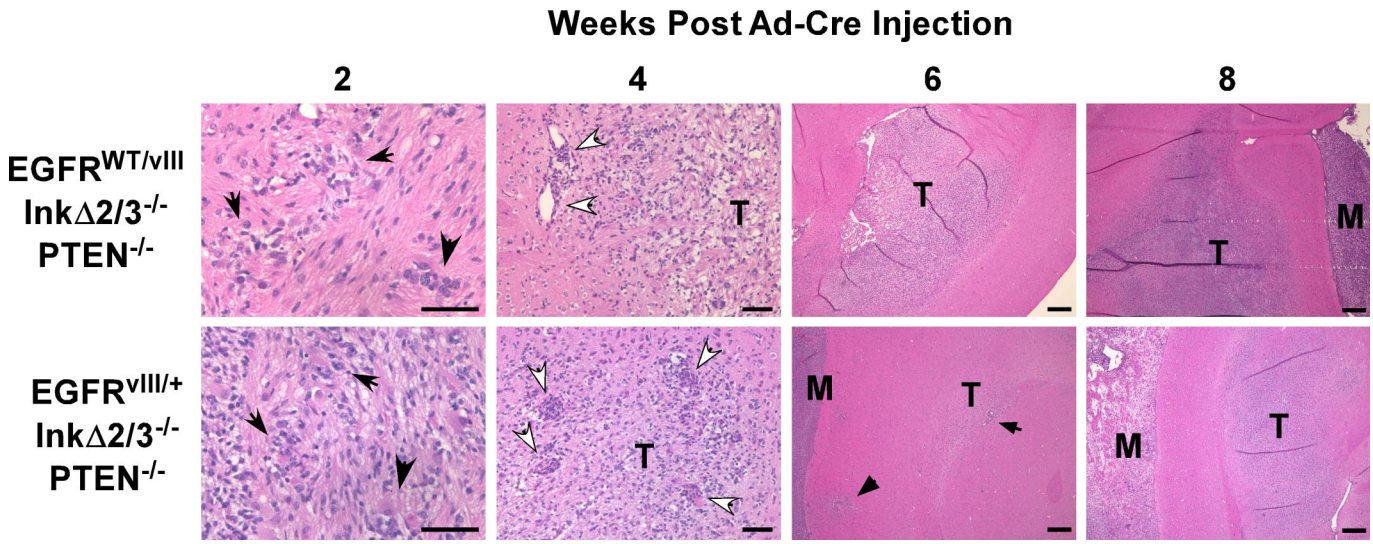


Fig. S5. Characteristics of mutant EGFR GBM tumor growth over time. Photomicrographs of H&E-stained GBM tumor sections at 2, 4, 6, and 8 weeks postinjection of Ad-Cre into the CNS of $EGFR^{WT/vIII}$; $Ink\Delta2/3^{-/-}$; $PTEN^{-/-}$ and $EGFR^{vIII/+}$; $Ink\Delta2/3^{-/-}$; $PTEN^{-/-}$ mice. At 2 weeks, small clusters of neoplastic cells are seen (black arrows); at 4 weeks, GBM tumors (T) are larger, and tumor cells have infiltrated the resident vasculature (white arrowheads). (Scale bar: 62.5 μM.) At 6 and 8 weeks, tumors grow in size and show meningeal infiltration (M). (Scale bar: 250 μM.)

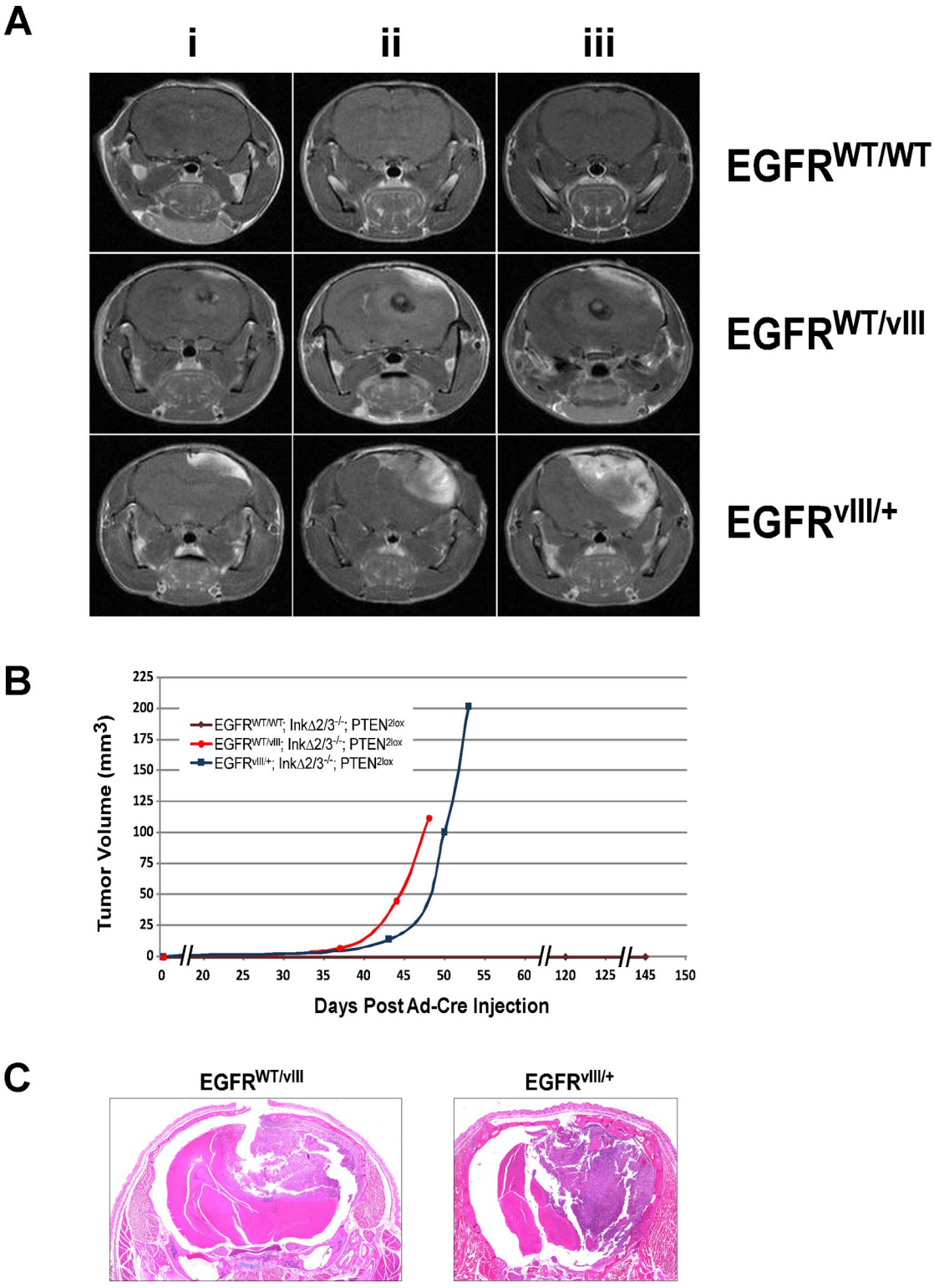


Fig. S6. Growth rate of GBM tumors by longitudinal serial MR imaging. (*A*) Mice of the indicated genotypes on an InkΔ2/3$^{-/-}$; PTEN$^{-/-}$ background were serially imaged over time by MRI. Gad-enhanced T1-weighted images of EGFR$^{WT/WT}$; i = 37, ii = 120, and iii = 145 days post-Ad-Cre injection. EGFR$^{WT/vIII}$; i = 37, ii = 44, and iii = 48 days post-Ad-Cre injection, and EGFR$^{vIII/+}$; i = 43, ii = 50, and iii = 53 days post-Ad-Cre injection. (*B*) Quantitative measurement of tumor volumes from MRI imaging over time. For each mouse, tumor area from serial 1-mm sections were multiplied by the slice thickness and then added to produce a final volume (*C*) Photomicrographs of H&E-stained coronal sections of EGFR$^{WT/vIII}$; InkΔ2/3$^{-/-}$; PTEN$^{-/-}$ and EGFR$^{vIII/+}$; InkΔ2/3$^{-/-}$; PTEN$^{-/-}$ tumors shown in (*A*).

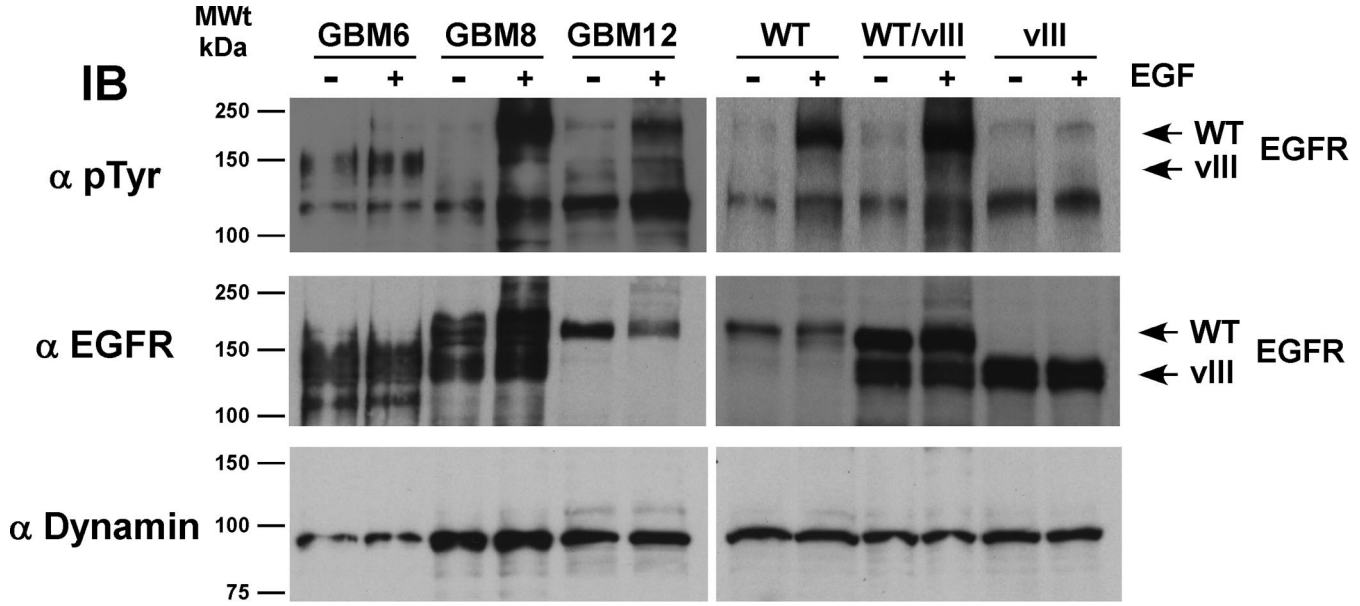


Fig. S7. EGFR expression in GBM ex vivo cultures. Immunoblot analysis of the levels of expression of the EGF receptors in cells derived from representative $EGFR^{WT/WT}$; $InkΔ2/3^{-/-}$; $PTEN^{-/-}$ and $EGFR^{WT/vIII}$; $InkΔ2/3^{-/-}$; $PTEN^{-/-}$ and $EGFR^{vIII/+}$; $InkΔ2/3^{-/-}$; $PTEN^{-/-}$ GBM tumors, and those of human GBM ex vivo cultures (6). Human GBM6 expresses EGFR vIII only, GBM8 expresses EGFR WT and EGFR vIII, and GBM12 expresses EGFR WT only. Cells were starved in 0.1% FBS media for 24 hr and treated for 5 min with 50 ng/ml of EGF. Cell lysates were immunoblotted against antiphosphotyorsine (α pTyr), membranes were stripped and reprobed for total EGFR protein (α EGFR) and dynamin as loading control.

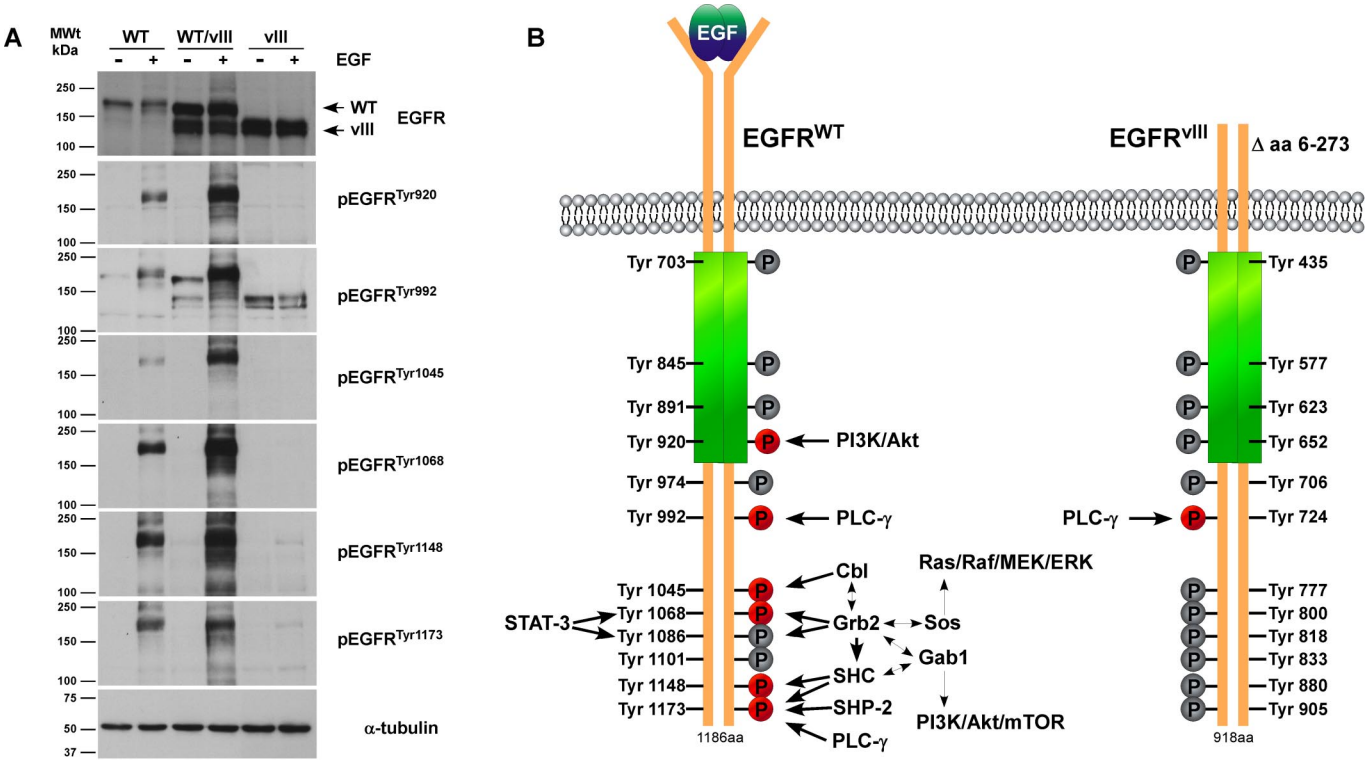


Fig. S8. EGFR autophosphorylation site preferences in GBM tumor cells. (*A*) Western blot analysis of representative ex vivo GBM tumor cells from our model expressing $EGFR^{WT}$, $EGFR^{WT/vIII}$, and $EGFR^{vIII}$ receptors all on an $Ink\Delta2/3^{-/-}$; $PTEN^{-/-}$ background. Cells were serum starved for 24 hr and stimulated with 50 ng/ml of EGF for 5 min. Immunoblots of the indicated total cell lysates were probed with phosphotyrosine residue-specific anti-EGFR antibodies. (*B*) Schematic representation of the 12 known pTyr residues on EGFR (gray circles) and their interacting signaling counterparts. Phosphorylated sites on EGFR WT and vIII in our GBM tumor cells are indicated in red. Note that the pTyr sites are the same, their numbering in EGFR vIII is different from EGFR WT due to the deletion of amino acid residues 6–273.

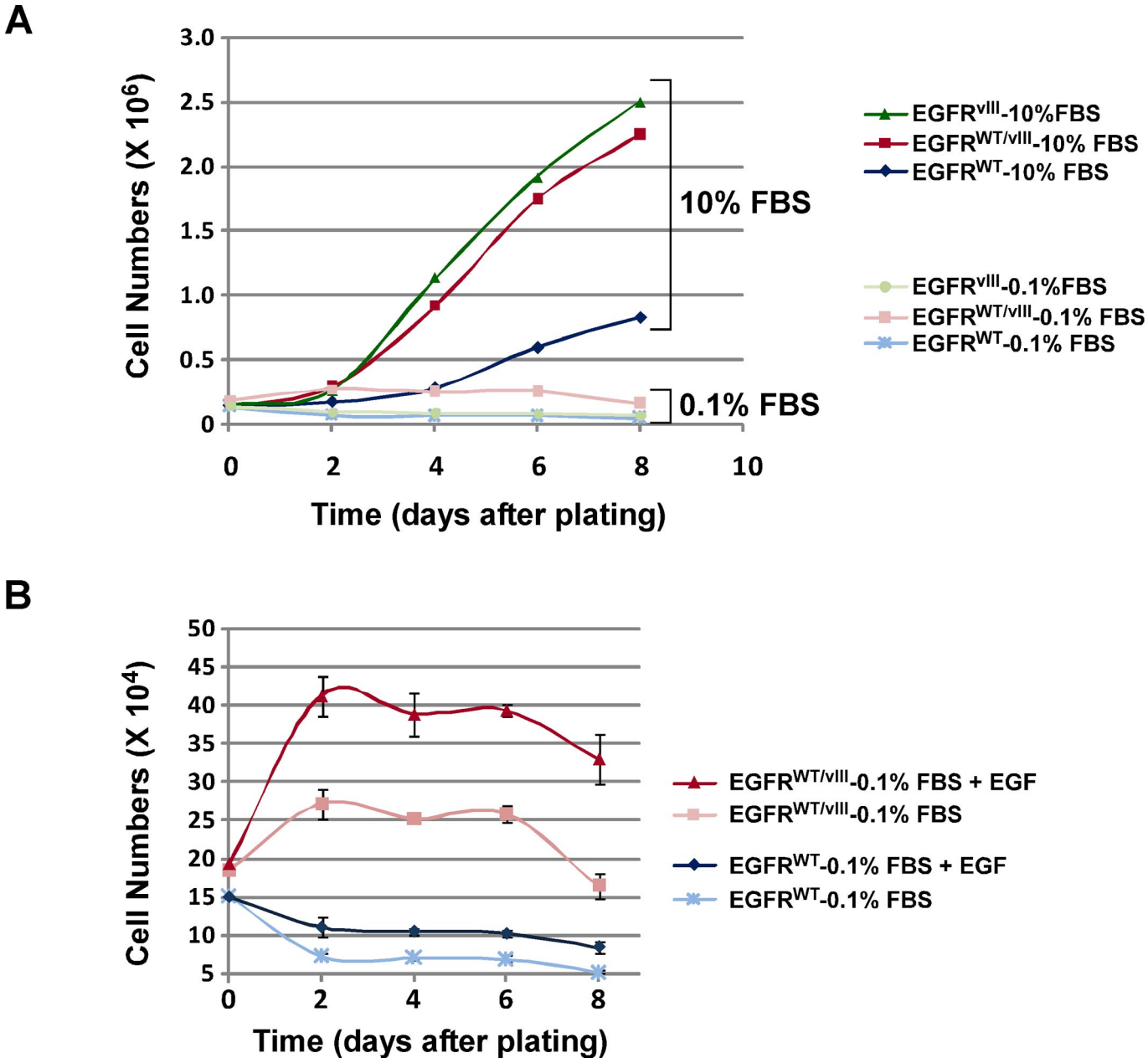


Fig. S9. Growth characteristics of GBM tumor cells ex vivo. Growth of tumor cell cultures of the indicated genotypes were assayed in vitro over an 8-day time period. (*A*) Cells were plated in triplicates in either 10% or 0.1% FBS and counted every 2 days. (*B*) Cells were plated in triplicates in 0.1% FBS or 0.1% FBS supplemented with 50 ng/ml of EGF daily and counted. Data plotted as average, error bars; SD.

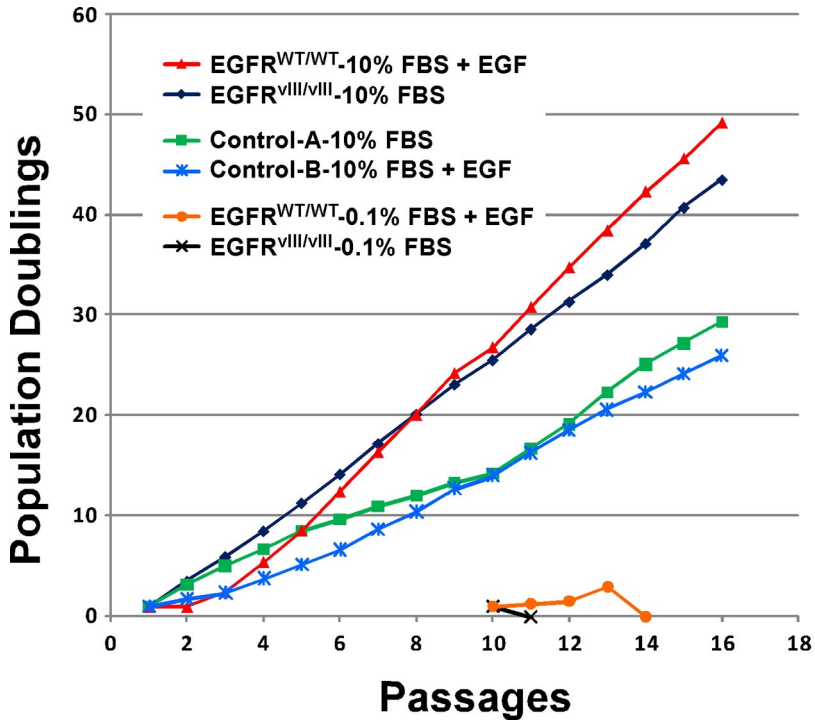


Fig. S10. Activated EGFR expressing primary astrocytes confer growth advantage in a context-dependent manner in a 3T3 growth assay. Primary astrocytes from P0 mice of the following genotypes were established: $EGFR^{WT/WT}$; $Ink\Delta2/3^{-/-}$; $PTEN^{2lox}$ and $EGFR^{vIII/vIII}$; $Ink\Delta2/3^{-/-}$; $PTEN^{2lox}$. These astrocyte cultures were infected in vitro with Ad-Cre or Ad-GFP to generate cells that express $EGFR^{WT}$ and $EGFR^{vIII}$ and controls A and B, respectively. Cell cultures were kept on a 3T3 protocol counting cells at each passage. At passage 10, $EGFR^{WT}$ and $EGFR^{vIII}$-expressing cells were plated on 0.1% FBS with the addition of 50 ng/ml of EGF daily to the former and counted at each passage.