



Published in final edited form as:

*Cancer Res.* 2011 December 1; 71(23): 7198–7206. doi:10.1158/0008-5472.CAN-11-1514.

## Chronic Activation of Wild Type Epidermal Growth Factor Receptor and Loss of Cdkn2a Cause Mouse Glioblastoma Formation

Jaime Acquaviva<sup>1,§</sup>, Hyun Jung Jun<sup>1,§</sup>, Julie Lessard<sup>1</sup>, Rolando Ruiz<sup>3</sup>, Haihao Zhu<sup>1</sup>, Melissa Donovan<sup>3</sup>, Steve Woolfenden<sup>1</sup>, Abraham Boskovitz<sup>2</sup>, Ami Raval<sup>2</sup>, Roderick T. Bronson<sup>4</sup>, Rolf Pfannl<sup>2</sup>, Charles A. Whittaker<sup>6</sup>, David E. Housman<sup>5,6</sup>, and Alain Charest<sup>1,2,3,5,\*</sup>

<sup>1</sup>Molecular Oncology Research Institute, Tufts Medical Center, Boston, MA 02111, USA

<sup>2</sup>Department of Neurosurgery, Tufts University School of Medicine, Boston, MA 02111, USA

<sup>3</sup>Genetics Program, Tufts University School of Medicine, Boston, MA 02111, USA

<sup>4</sup>Department of Pathology, Harvard Medical School, Boston, MA 02115, USA

<sup>5</sup>Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

<sup>6</sup>David H. Koch Institute for Integrative Cancer Research at MIT, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

### Abstract

Glioblastoma multiforme (GBM) is characterized by overexpression of EGFR and loss of the tumor suppressors Ink4a/Arf. Efforts at modeling GBM using wild-type EGFR in mice have proven unsuccessful. Here, we present a unique mouse model of wild-type EGFR-driven gliomagenesis. We used a combination of somatic conditional overexpression and ligand-mediated chronic activation of EGFR in cooperation with Ink4a/Arf loss in the CNS of adult mice to generate tumors with the histopathological and molecular characteristics of human GBMs. Sustained, ligand-mediated activation of EGFR was necessary for gliomagenesis, functionally substantiating the clinical observation that EGFR-positive GBMs from patients express EGFR ligands. To gain a better understanding of the clinically disappointing EGFR targeted therapies for GBM, we investigated the molecular responses to EGFR tyrosine kinase inhibitor (TKI) treatment in this model. Gefitinib treatment of primary GBM cells resulted in a robust apoptotic response, partially conveyed by MAPK signaling attenuation and accompanied by BIM<sub>EL</sub> expression. In human GBMs, loss-of-function mutations in the tumor suppressor PTEN are a common occurrence. Elimination of PTEN expression in GBM cells post-tumor formation did not confer resistance to TKI treatment, demonstrating that PTEN status in our model is not predictive. Together, these findings offer important mechanistic insights into the genetic determinants of EGFR gliomagenesis and sensitivity to TKIs and provide a robust discovery platform to better understand the molecular events that are associated with predictive markers of TKI therapy.

\*Correspondence: Alain Charest, Tufts Medical Center, MORI, 800 Washington St, box 5609, Boston, MA, 02111. [alain.charest@tufts.edu](mailto:alain.charest@tufts.edu), 617-636-8876 (phone), 617-636-5277 (Fax).

<sup>§</sup>These authors contributed equally to this work.

No potential conflicts of interest were disclosed.

## Introduction

GBMs are classified on the basis of histopathological features, clinical presentation and molecular characteristics (reviewed in (1, 2)). The hallmark features of GBM are uncontrolled cellular proliferation, extensively diffuse infiltration, and a propensity for hypoxia and necrosis that engenders robust angiogenesis and a perennial resistance to therapeutic intervention. The epidermal growth factor receptor (EGFR) plays a crucial role in GBM pathogenesis (3). The importance of this pathway is highlighted by the fact that wild type EGFR (EGFR<sup>WT</sup>) and its ligands are over expressed and activated in >65% of GBM tumors (4–6). While initiation of this tumor subtype requires the overexpression of EGFR<sup>WT</sup> along with the concomitant loss of the Cdkn2a (p16INK4A/p19ARF) tumor suppressor locus (7), the role of EGFR signaling in tumor maintenance and sensitivity to TKIs is less well studied, especially in animal models. Most of our knowledge of EGFR signaling is based on *in vitro* studies of acute, ligand-mediated activation of the receptor within minutes. This paradigm differs from clinical observations, as EGFR is thought to be chronically active in GBM due to autocrine/paracrine expression of ligands including EGF, TGF $\alpha$  and HB-EGF (8–11). As such, studies based on clinically relevant mechanisms of response to EGFR inhibition remain largely unexplored, especially in an *in vivo* model system.

The oncogene addiction hypothesis stipulates that a cancer cell is physiologically dependent on the continued activity of an oncogene for maintenance of the malignant phenotype (12). Although the mechanistic details of oncogene addiction are likely to be cancer specific, it appears that oncogenic kinases transduce excessive survival signals through pathways that are controlled by canonical growth and survival pathways (e.g. Akt and MAPK). Treatment of oncogenically-addicted cancer cells with kinase-inhibitory drugs suppresses these signals, resulting in an oncogenic shock characterized by the cessation of proliferation through a variety of mechanisms including growth arrest, differentiation, senescence and apoptosis (reviewed in (13)). *In vitro*-based studies demonstrate that inhibition of receptor tyrosine kinases in GBM can lead to the rapid onset of apoptosis, suggesting that GBMs also experience this addiction to oncogenic stimuli (14) although the mechanistic details of oncogene addiction in GBMs remain ill defined.

The use of EGFR TKIs for GBM treatment has proven surprisingly ineffective, resulting in gains of a few months of progression-free survival, with no significant gain in overall survival (reviewed in (15)). Retrospective studies demonstrated that patients who responded to TKI therapy had tumors harboring EGFR<sup>VIII</sup> mutant receptors and an intact PTEN gene whereas the non-responding patients had PTEN-null tumors (16, 17). This finding led to the suggestion that loss of PTEN uncouples PI3K-Akt signaling from the control of EGFR activity. By freeing tumors from their addiction to oncogenic EGFR, PTEN deletion may provide a means of resistance to TKI therapy. However, these observations were not sustained in follow up phase I/II trial studies (18, 19) demonstrating a vast complexity in the molecular mechanisms of EGFR TKI therapy response.

Overcoming resistance to targeted therapeutics in patients will require an in depth understanding of the molecular mechanisms of tumor cell resistance. Accurate and realistic model systems can serve as a surrogate paradigm to predict clinical testing, representing a rapid, inexpensive and powerful approach to this problem. However, there are currently no mouse models of malignant glioma that use wild-type EGFR as an oncogenic driver of tumorigenesis, making such studies impossible (20). Here we describe and validate a novel genetically engineered mouse (GEM) model of EGFR<sup>WT</sup>-driven GBM. We established that a strict spatiotemporal expression of EGFR<sup>WT</sup> and chronic autocrine stimulation with a ligand, combined with the loss of clinically relevant tumor suppressor genes efficiently

induces gliomagenesis. Using this novel mouse model, we reveal that these GBM tumor cells are oncogenically addicted to EGFR. Treatment with an EGFR TKI results in a rapid BIM<sub>EL</sub>-mediated apoptotic response. We further demonstrate that loss of PTEN post-tumor formation does not uncouple PI3K-Akt survival signaling from EGFR control and does not induce TKI resistance. These findings are consistent with the clinical observation that PTEN status is not a predictor of EGFR TKI sensitivity.

## Materials and Methods

### EGFR conditional mice and procedures

Procedures were performed in accordance with Tufts University's recommendations for care and use of animals and were maintained and handled under protocols approved by IACUC. Conditional expression of EGFR<sup>WT</sup> was achieved as previously described (21). Viral vector construction, production, and stereotactic injections are described in Supplementary Materials & Methods section.

### Histology and immunodetection

Brains were either used to isolate primary cultures or processed for histology (Supplementary Materials and Methods). Immunodetection of cytologic markers by IHC and proteins were performed using antibodies and standard protocols (see Supplementary Materials and Methods).

### Survival assays and inhibitor treatments

Cell viability was measured by trypan blue exclusion and XTT assays. Cells were treated with gefitinib (LC Labs) or PD325901 (LC Labs) for 16–24hr and the total number of cells was reported.

### Statistical analysis

Statistical analyses were performed using the two-tailed, unpaired Student's t test in Prism 5.0 (GraphPad Software).

## Results

### Ligand-mediated activation of wild-type EGFR in the context of tumor suppressor loss in mice induces tumors with histopathological characteristics of human GBM

Many studies have reported the presence of autocrine and/or paracrine expression of EGFR and its ligands in GBM tumors (8–11). We validated these observations using The Cancer Genome Atlas (TCGA) public database by performing a gene set enrichment analysis (GSEA) in GBMs with an amplified EGFR gene locus that overexpress wild-type and point-mutant EGFR versus non EGFR-expressing tumors to determine whether EGFR ligands are indeed preferentially expressed in EGFR positive tumors (Supplementary Fig. 1A–C). Our analysis reveals that human tumors that overexpress EGFR preferentially have a relatively high expression of EGF ligand when compared to GBM tumors with low EGFR expression levels ( $p=0.000076$ ). These results, combined with previously reported evidence of ligand-receptor co-expression in GBM, demonstrate that physiologically relevant overexpression of EGFR is associated with ligand expression. This strong correlation between ligand and receptor overexpression suggests that EGFR<sup>WT</sup> signaling can be chronically active in GBMs.

We recently supported these observations experimentally *in vivo* by demonstrating that overexpression of EGFR<sup>WT</sup> alone is insufficient to promote gliomagenesis (21). In order to

model EGFR<sup>WT</sup>-driven GBM in mice, we developed a strategy to co-express transforming growth factor alpha (TGF $\alpha$ ), an EGFR ligand expressed in human GBMs (8, 11, 22–26), and EGFR<sup>WT</sup> in the adult mouse brain. We used a Cre/Lox conditional EGFR<sup>WT</sup> transgenic strain in which overexpression of human EGFR<sup>WT</sup> is Cre-dependent (21). Robust EGFR<sup>WT</sup> expression is triggered by the removal of a floxed translational and transcriptional stop cassette (LSL), which attenuates the activity of an artificial ubiquitous promoter (CAG). To simultaneously express Cre recombinase and TGF $\alpha$ , we created a bicistronic lentiviral vector that expresses TGF $\alpha$  and Cre (TGF $\alpha$ -IRES-iCre). A construct expressing eGFP in lieu of TGF $\alpha$  serves as a control vector (eGFP-IRES-iCre) (Fig. 1A). We induced the co-expression of TGF $\alpha$  (or eGFP) and EGFR<sup>WT</sup> by performing stereotactic intracranial injections of matched titers (Supplementary Fig. 2A,B) of TGF $\alpha$ -IRES-iCre and eGFP-IRES-iCre viruses in cohorts of conditional CAG-LSL-EGFR<sup>WT</sup>;Ink $\Delta$ 2/3<sup>-/-</sup> compound mice and monitored tumor formation and survival over time. Mice co-expressing EGFR<sup>WT</sup> and TGF $\alpha$  in a p16<sup>Ink4a</sup>/p19<sup>Arf</sup> null background developed brain tumors with a median survival of 10 weeks post-injection (Fig. 1B). Neither expression of EGFR<sup>WT</sup> in the absence of ligand, nor expression of TGF $\alpha$  in the absence of transgenic EGFR<sup>WT</sup> resulted in tumor formation in p16<sup>Ink4a</sup>/p19<sup>Arf</sup> null mice, supporting the hypothesis that receptor and ligand co-expression are required for EGFR<sup>WT</sup>-driven gliomagenesis in mice.

TGF $\alpha$ -EGFR<sup>WT</sup>;Ink $\Delta$ 2/3<sup>-/-</sup> tumors share many histopathological features with human GBMs (Fig. 1C). They are highly cellular, very proliferative (numerous mitoses) and are composed of cells displaying pleomorphic nuclei present on a fibrillary background (Fig. 2A). In addition, the tumors include giant multinucleated cells and areas of pseudopallisading necrosis, both prominent features of human GBM (Fig. 2B–C). Moreover, these tumors are highly infiltrative with leptomeningial spread (Fig. 2D) and diffuse infiltration into normal parenchyma (Fig. 2E). Tumor cells are also found in the perivascular space and can be observed at significant distance from the bulk mass (Fig. 2F). Immunohistochemical staining of TGF $\alpha$ -EGFR<sup>WT</sup>;Ink $\Delta$ 2/3<sup>-/-</sup> tumors for EGFR revealed robust membrane expression, while staining for markers associated with astrocytic (GFAP and S100) and neuronal (NeuN) differentiation revealed that the neoplastic cells express markers of astrocytic lineage (Fig. 2G). In addition, the levels of EGFR expression seen in these tumors is similar to those observed in human GBMs with an amplified EGFR locus (Supplementary Fig. 3) and these cells recapitulate growth and histopathological features of the original tumors when orthotopically allografted in mice (Supplementary Fig. 4A,B).

### Signaling through constitutively activated EGFR<sup>WT</sup> in GBM cells

Signaling events resulting from a chronic activation of EGFR<sup>WT</sup> in GBM have yet to be studied in detail. To understand EGFR<sup>WT</sup> signaling events in this context, we established a series of primary cultures from TGF $\alpha$ -EGFR<sup>WT</sup>;Ink $\Delta$ 2/3<sup>-/-</sup> tumors. These primary cultures are stable, demonstrate unrestricted growth in low serum conditions (Supplementary Fig. 5) and produce and secrete TGF $\alpha$  (Supplementary Fig. 6A). This TGF $\alpha$ -driven growth is markedly reduced when the cultures are incubated with an anti-TGF $\alpha$  antibody (Supplementary Fig. 6B) thus indicating that TGF $\alpha$ -EGFR<sup>WT</sup>-driven signaling is sufficient to support tumor cell growth and maintenance. We next used these primary GBM cells to ascertain the effects of EGFR inhibition on cell growth and signaling.

To decipher EGFR signaling in our GBM tumor cultures, we surveyed EGFR's phosphotyrosine levels by immunoblot analysis. The phosphorylation levels of the canonical tyrosine residues 845, 1045, 1068, 1148 and 1173 decreased dramatically upon EGFR kinase inhibition (Fig. 3A). In contrast, the levels of pTyr<sup>992</sup> increased upon gefitinib treatment. Next, we determined the levels of activation and the effect of EGFR inhibition on the canonical EGFR signaling pathways driven by MAPK and PI3K-Akt. We found that the MAPK pathway (Mek1/2-Erk1/2) is highly active in TGF $\alpha$ -EGFR<sup>WT</sup>;Ink $\Delta$ 2/3<sup>-/-</sup> tumor

cells and inhibition of EGFR with gefitinib dramatically reduces Mek1/2-Erk1/2 signaling (Fig. 3B). Surprisingly, the PI3K-Akt pathway is not activated in TGF $\alpha$ -EGFR<sup>WT</sup>;Ink $\Delta$ 2/3<sup>-/-</sup> tumor cells (Supplementary Fig. 7).

We then ascertained the effect of EGFR kinase inhibition on cell growth. We first calculated the IC<sub>50</sub> values for gefitinib using an *in vitro* cell growth assay (Supplementary Fig. 8) and determined that treatment with 10  $\mu$ M of gefitinib for 24 hr results in maximal growth inhibition. Gefitinib treatment of TGF $\alpha$ -EGFR<sup>WT</sup>;Ink $\Delta$ 2/3<sup>-/-</sup> tumor cultures (T1-3) resulted in a 50–80% reduction in viability (Fig. 4A). We surmised that the decrease in viability might be due to an increased rate of apoptosis. Using flow cytometry, the levels of apoptosis in TGF $\alpha$ -EGFR<sup>WT</sup>;Ink $\Delta$ 2/3<sup>-/-</sup> tumor cultures, as measured by the percentage of cells expressing cleaved caspase-3, increased dramatically after 24 hours of gefitinib treatment (Fig. 4B,C). This increase in apoptosis is confirmed by detecting the presence of cleaved-PARP (Fig. 4D) and can be observed as early as 4 hours after gefitinib treatment (Supplementary Fig. 9). A similar apoptotic response was brought about by identical concentrations of erlotinib (data not shown).

To validate these results, we treated animals with actively growing orthotopically allografted TGF $\alpha$ -EGFR<sup>WT</sup>;Ink $\Delta$ 2/3<sup>-/-</sup> GBM cells with erlotinib and assessed tumor response (Fig. 5A–C). Within 48 hours of treatment, the levels of phospho-EGFR are no longer detectable, the levels of BrdU incorporation in GBM cells are drastically decreased and there is a marked increase in the number of apoptotic cells as measured by TUNEL assay (Fig. 5A–C).

### Elevation of BIM<sub>EL</sub> expression upon attenuation of EGFR signaling

Acute inhibition of constitutively activated EGFR using TKIs has been shown to result in an apoptotic response that is mediated by an increase in the expression of the pro-apoptotic protein BIM (27) as a result of the attenuation of MAPK signaling (28, 29). To gain insight into the mechanisms responsible for the EGFR TKI-mediated apoptosis observed in our EGFR<sup>WT</sup>-driven tumor cell cultures, we measured the expression of BIM in cells treated with gefitinib or the Mek1/2 inhibitor PD325901 and performed cell growth assays. Fig. 6A,B demonstrates that inhibition of EGFR causes an increase in the expression of the long form of BIM (BIM<sub>EL</sub>) and that BIM<sub>EL</sub> expression is partly mediated by the Mek1/2-Erk1/2 signaling axis. Inhibition of Mek1/2 led to a more modest apoptotic response as measured both molecularly and physiologically.

### Modulation of PTEN expression in established tumor cultures has no therapeutic consequence

Loss of function of the tumor suppressor gene PTEN is a common occurrence in GBM tumors. Originally, studies in patients indicated that GBM tumors with an intact PTEN were more sensitive to EGFR kinase inhibitors than those with PTEN deficiencies (16). However, these observations were not sustained in follow up studies suggesting that PTEN status has little predictive value for EGFR TKI treatment response. To determine whether PTEN contributes to the sensitivity of our GBM tumor cell cultures to EGFR kinase inhibition, we eliminated PTEN expression in our TGF $\alpha$ -EGFR<sup>WT</sup>;Ink $\Delta$ 2/3<sup>-/-</sup> tumor cells using a potent shRNA (Supplementary Fig. 10) and analyzed these cultures for their sensitivity to gefitinib treatment.

Elimination of PTEN expression in these cells resulted in the activation of the PI3K-Akt signaling axis as measured by the appearance of activated Akt (Fig. 7A). However, this newly acquired PI3K-Akt signaling remains dependent on the activity of EGFR, since gefitinib treatment completely eliminated Akt phosphorylation. PTEN knockdown in TGF $\alpha$ -



EGFR<sup>WT</sup>;InkΔ2/3<sup>-/-</sup> tumor cells did not result in an increased resistance to gefitinib treatment, as measured by similar levels of apoptosis in control and PTEN knockdown cells when ascertained by immunoblot (Fig. 7A) or cell viability assay (Fig. 7B).

## Discussion

Numerous *in vitro* studies demonstrated that overexpression of wild-type EGFR leads to cellular transformation only in the presence of ligands (30–36). This requirement for co-expression of receptor and ligand for oncogenic transformation is also exemplified in animal models where simple overexpression of non-mutant EGFR in different tissues, including glioma, results in hyperplasia rather than tumor formation (37–39). We have recently demonstrated that somatic overexpression of EGFR<sup>WT</sup> in the CNS of mice is incapable of forming glioma (21). Moreover, autocrine/paracrine co-expression of EGFR and its ligands EGF, TGF $\alpha$  or HB-EGF has been shown in various human tumors, including gliomas (8–10, 22–26, 40), an observation that we validated using the TCGA public database. Together, these studies propose the notion that physiologically relevant overexpression of EGFR<sup>WT</sup> is not an oncogenic event in and of itself and that co-expression of a ligand may be required to initiate tumorigenesis. In this report, we present experimental data that support this hypothesis by demonstrating for the first time the need for an EGFR ligand (TGF $\alpha$ ) to initiate GBM tumor formation with EGFR<sup>WT</sup> in the context of p16Ink4a/p19ARF nullizygoty in the mouse.

Expression of TGF $\alpha$  in gliomas is well established (8, 11, 22–26, 32, 33) and it has been demonstrated that there are no differences between EGF- or TGF $\alpha$ -stimulated EGFR signaling events (41) thus offering a compelling rationale for its use in our studies. Moreover, soluble EGFR ligands are produced as membrane-bound pro-peptides that are proteolytically cleaved to release an active ligand from their membrane tethers. We found that mature, active TGF $\alpha$  but not EGF (data not shown) can be expressed from an artificial cDNA corresponding to the post-proteolytic product.

Our GEM model offers a unique system to study the potential effects that TGF $\alpha$  may exert on the parenchyma. It is conceivable that the expression of exogenous TGF $\alpha$  influences the tumor microenvironment in a way that would promote GBM cell growth. Given the emergence of data demonstrating the importance of the microenvironment on tumor behavior (42), it is likely that TGF $\alpha$  impacts tumor growth beyond its autocrine role and our model represents a relevant stage to research this phenomenon.

GBM tumors are now categorized into four subgroups termed Proneural, Neural, Classical and Mesenchymal, based on well-defined molecular characteristics (recently reviewed in (1)). The combination of EGFR overexpression and Cdkn2a loss is found in nearly 65% of all GBMs and is a key molecular component that defines the classical GBM subgroup (6). Our knowledge of EGFR signaling is mostly derived from *in vitro* studies of acute, short-term stimulation of the receptor with exogenous ligands. Although informative in many respects, including the establishment of EGFR signaling networks (3), this paradigm falls short in clinical relevancy in that it does not address signaling events that emanate from chronically activated receptors. More importantly, these short-term *in vitro* studies are inadequate to determine the cellular effects of inhibition of a chronically active receptor. Our model, which is based on relevant genetic aberrations, recapitulates hallmark histopathological features of GBMs including uncontrolled cellular growth, massive invasion and infiltration of tumor cells in surrounding normal parenchyma and pseudopallisading necrosis. Moreover, our model establishes a clinically relevant baseline upon which studies of oncogenic EGFR<sup>WT</sup> signaling can be performed.

Activation of EGFR leads to the creation of phosphotyrosine (pTyr) residues on the receptor itself and on substrate proteins. These pTyr sites are beacons for a host of SH2 and PTB domain-containing signaling proteins capable of phosphotyrosine-dependent sequence-specific recognition and binding, resulting in the transmission of highly precise signals (reviewed in (43)). Knowledge of these sites is an invaluable tool in determining the signaling events that emanate from a receptor. In our studies, the inhibition of EGFR kinase activity with gefitinib resulted in a drastic decrease in the levels of the canonical pTyr residues we surveyed, with the exception of pTyr<sup>992</sup>, which was increased. Decreases in levels of phosphorylation at tyrosine residues 1068, 1148, and 1173 are expected to result in an attenuation of MAPK signaling (44). Tyrosine 845 is a target of Src family kinases (44) and a reduction in the levels of phosphorylation at tyrosine residue 845 indicate a reduction in Src activity. Phosphorylation on tyrosine 1045 creates a binding site for the ubiquitin ligase c-Cbl (44). A decrease in the levels of phosphorylation on tyrosine 1045 would possibly lead to a lower rate of receptor degradation.

EGFR pTyr<sup>992</sup> is a substrate for the tyrosine phosphatase SHP-2 (45). The observed increase of pTyr<sup>992</sup> levels upon gefitinib treatment may result from a shift in the balance between the activities of EGFR and SHP-2. On the other hand, binding of a high affinity SH2 or PTB domain-containing protein to pTyr<sup>992</sup> may be increased upon gefitinib treatment, which would then result in protection of this residue from the activity of phosphatases. Regardless of the mechanism involved, phosphorylation on Tyr<sup>992</sup> creates a binding site for the SH2 domains of phospholipase C- $\gamma$ , RAS-GAP and Vav2 (45–47). Our results suggest that a sustained increase in signaling from these effector proteins in our cells may result from gefitinib treatment. Alternatively, other as of yet unidentified signaling molecules may be recruited and activated by this increase in pTyr<sup>992</sup>. We demonstrate that inhibition of a chronically activated receptor has different consequences than that of an acutely stimulated receptor. Under these clinically relevant parameters, there is a renewed interest in studying downstream signaling upon inhibition of EGFR kinase activity.

The canonical Mek1/2-Erk1/2 and PI3K-Akt signaling axes are well-described effector pathways for EGFR. We demonstrate that in our tumor cells, MAPK signaling is utilized by EGFR (Fig. 3). However, to our surprise, we did not detect PI3K-Akt activation (as measured by the levels of phospho-Akt). This result is surprising given the longstanding notion that EGFR strongly signals through PI3K. Perhaps in the chronic setting of our *in vivo* GBM model, tumor cells select for non-PI3K-dependent pro-survival signals. The cells from this tumor model are addicted to EGFR activity for maintenance, as inhibition of EGFR with gefitinib results in a rapid (4–8 hrs) induction of apoptosis, which is associated with the appearance of BIM<sub>EL</sub> expression. We further demonstrate that the increased BIM<sub>EL</sub> expression is partly mediated by MAPK activity since inhibition of Mek1/2 leads to a partial apoptotic response and attenuated BIM<sub>EL</sub> expression as compared to gefitinib treatment. These results indicate that EGFR signals through additional, as of yet unidentified pathways that when inhibited, feed into the mechanism of BIM<sub>EL</sub> expression. BIM is a pro-apoptotic protein known to interact with and inhibit the anti-apoptotic activity of Bcl-2, Bcl-X(L) and Mcl-1 (reviewed in (29)). Our observations are reminiscent of examples in non-small cell lung cancers (NSCLC) that are addicted to oncogenic EGFR, where TKI treatment results in apoptosis (reviewed in (48)). The mechanistic details connecting loss of EGFR kinase activity and initiation of apoptosis still remain unclear but reported data in NSCLC suggest that the apoptosis is mediated by a Mcl-1/Bim axis (49).

Loss of PTEN is commonly associated with GBMs. Molecularly, loss of PTEN is thought to uncouple PI3K activity from the control of EGFR, thus rendering tumor cells insensitive to EGFR TKI therapy. However, this simplistic molecular view of PTEN function does not harmonize with clinical data and reveals the complexities associated with PTEN-modulated

signaling events. Here we demonstrate that eliminating PTEN post-tumor formation does not uncouple PI3K from EGFR and does not confer resistance to EGFR TKI treatment. Our results are in line with the clinical observations that PTEN status does not predict response to EGFR TKI treatment.

The results presented here demonstrate that chronic activation of wild-type EGFR is necessary for gliomagenesis and that the resulting tumors are addicted to EGFR activity. Our model is the first wild-type EGFR glioma model, which provides a paradigm for studies of signaling events in the clinically relevant context of human GBMs with amplification and overexpression of wild-type, non mutated EGFR. Loss of PTEN post-tumor formation does not confer resistance to TKI therapy, reaffirming that patient selection for EGFR TKI therapy may not be based on PTEN status alone.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

Grant Support. This research was supported (in part) by NIH grant NCI U01 CA141556 (A.C.), by American Cancer Society Research Scholar Award 117409 (A.C.), by NIH grant U54 CA119349 (D.H. and A.C.) and by T32 training grant HD049341 (M.D.) and NIH R25GM066567 (R.R.).

The authors thank Drs. Bill Chiu and Ken Hung for their helpful comments and Dr. Robert R. Langley (Department of Cancer Biology, The University of Texas M.D. Anderson Cancer Center) for the generous gift of the human TGF $\alpha$  cDNA, Dr. Jason Coleman (Picower Institute, MIT) for the pTyf vector backbone plasmid and iCre cDNA, and Dr. John Alberta (Dana Farber Cancer Institute) for the Olig2 antibody.

## References

1. Brennan C. Genomic profiles of glioma. *Curr Neurol Neurosci Rep.* 2011; 11:291–7. [PubMed: 21465149]
2. Furnari FB, Fenton T, Bachoo RM, Mukasa A, Stommel JM, Stegh A, et al. Malignant astrocytic glioma: genetics, biology, and paths to treatment. *Genes Dev.* 2007; 21:2683–710. [PubMed: 17974913]
3. Huang PH, Xu AM, White FM. Oncogenic EGFR signaling networks in glioma. *Sci Signal.* 2009; 2:re6. [PubMed: 19738203]
4. <http://cancergenome.nih.gov>.
5. McLendon R, Friedman A, Bigner D, Van Meir EG, Brat DJ, Mastrogianakis M, et al. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature.* 2008; 455:1061–8. [PubMed: 18772890]
6. Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell.* 2010; 17:98–110. [PubMed: 20129251]
7. Attolini CS, Cheng YK, Beroukheim R, Getz G, Abdel-Wahab O, Levine RL, et al. A mathematical framework to determine the temporal sequence of somatic genetic events in cancer. *Proc Natl Acad Sci U S A.* 2010; 107:17604–9. [PubMed: 20864632]
8. Tang P, Steck PA, Yung WK. The autocrine loop of TGF- $\alpha$ /EGFR and brain tumors. *J Neurooncol.* 1997; 35:303–14. [PubMed: 9440027]
9. Ekstrand AJ, James CD, Cavenee WK, Seliger B, Pettersson RF, Collins VP. Genes for epidermal growth factor receptor, transforming growth factor alpha, and epidermal growth factor and their expression in human gliomas in vivo. *Cancer Res.* 1991; 51:2164–72. [PubMed: 2009534]
10. Mishima K, Higashiyama S, Asai A, Yamaoka K, Nagashima Y, Taniguchi N, et al. Heparin-binding epidermal growth factor-like growth factor stimulates mitogenic signaling and is highly expressed in human malignant gliomas. *Acta Neuropathol.* 1998; 96:322–8. [PubMed: 9796995]

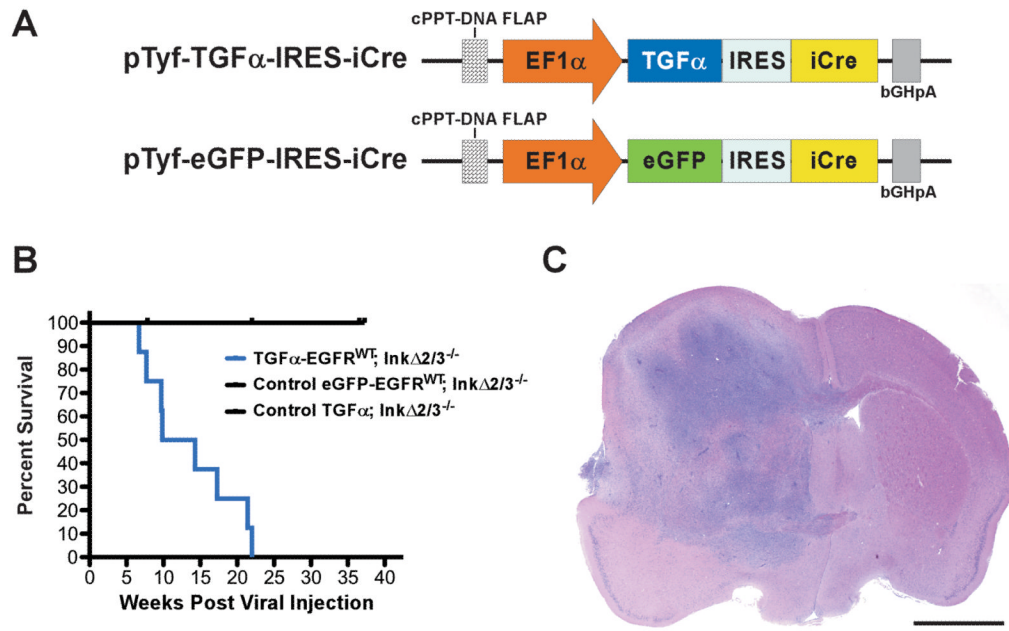


11. Schlegel U, Moots PL, Rosenblum MK, Thaler HT, Furneaux HM. Expression of transforming growth factor alpha in human gliomas. *Oncogene*. 1990; 5:1839–42. [PubMed: 2284103]
12. Weinstein IB. Cancer. Addiction to oncogenes--the Achilles heal of cancer. *Science*. 2002; 297:63–4. [PubMed: 12098689]
13. Sharma SV, Settleman J. Exploiting the balance between life and death: targeted cancer therapy and “oncogenic shock”. *Biochem Pharmacol*. 2010; 80:666–73. [PubMed: 20211150]
14. Pillay V, Allaf L, Wilding AL, Donoghue JF, Court NW, Greenall SA, et al. The plasticity of oncogene addiction: implications for targeted therapies directed to receptor tyrosine kinases. *Neoplasia*. 2009; 11:448–58. 2 p following 58. [PubMed: 19412429]
15. Huang TT, Sarkaria SM, Cloughesy TF, Mischel PS. Targeted therapy for malignant glioma patients: lessons learned and the road ahead. *Neurotherapeutics*. 2009; 6:500–12. [PubMed: 19560740]
16. Mellingerhoff IK, Cloughesy TF, Mischel PS. PTEN-mediated resistance to epidermal growth factor receptor kinase inhibitors. *Clin Cancer Res*. 2007; 13:378–81. [PubMed: 17255257]
17. Mellingerhoff IK, Wang MY, Vivanco I, Haas-Kogan DA, Zhu S, Dia EQ, et al. Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors. *N Engl J Med*. 2005; 353:2012–24. [PubMed: 16282176]
18. Brown PD, Krishnan S, Sarkaria JN, Wu W, Jaeckle KA, Uhm JH, et al. Phase I/II trial of erlotinib and temozolomide with radiation therapy in the treatment of newly diagnosed glioblastoma multiforme: North Central Cancer Treatment Group Study N0177. *J Clin Oncol*. 2008; 26:5603–9. [PubMed: 18955445]
19. van den Bent MJ, Brandes AA, Rampling R, Kouwenhoven MC, Kros JM, Carpentier AF, et al. Randomized phase II trial of erlotinib versus temozolomide or carmustine in recurrent glioblastoma: EORTC brain tumor group study 26034. *J Clin Oncol*. 2009; 27:1268–74. [PubMed: 19204207]
20. Hambardzumyan D, Parada LF, Holland EC, Charest A. Genetic modeling of gliomas in mice: New tools to tackle old problems. *Glia*. 2011
21. Zhu H, Acquaviva J, Ramachandran P, Boskovitz A, Woolfenden S, Pfannl R, et al. Oncogenic EGFR signaling cooperates with loss of tumor suppressor gene functions in gliomagenesis. *Proc Natl Acad Sci U S A*. 2009; 106:2712–6. [PubMed: 19196966]
22. Maruno M, Kovach JS, Kelly PJ, Yanagihara T. Transforming growth factor-alpha, epidermal growth factor receptor, and proliferating potential in benign and malignant gliomas. *J Neurosurg*. 1991; 75:97–102. [PubMed: 2045927]
23. Ramnarain DB, Park S, Lee DY, Hatanpaa KJ, Scoggin SO, Otu H, et al. Differential gene expression analysis reveals generation of an autocrine loop by a mutant epidermal growth factor receptor in glioma cells. *Cancer Res*. 2006; 66:867–74. [PubMed: 16424019]
24. Samuels V, Barrett JM, Bockman S, Pantazis CG, Allen MB Jr. Immunocytochemical study of transforming growth factor expression in benign and malignant gliomas. *Am J Pathol*. 1989; 134:894–902. [PubMed: 2705509]
25. van der Valk P, Lindeman J, Kamphorst W. Growth factor profiles of human gliomas. Do non-tumour cells contribute to tumour growth in glioma? *Ann Oncol*. 1997; 8:1023–9. [PubMed: 9402177]
26. Yung WK, Zhang X, Steck PA, Hung MC. Differential amplification of the TGF-alpha gene in human gliomas. *Cancer Commun*. 1990; 2:201–5. [PubMed: 2165797]
27. Costa DB, Halmos B, Kumar A, Schumer ST, Huberman MS, Boggon TJ, et al. BIM mediates EGFR tyrosine kinase inhibitor-induced apoptosis in lung cancers with oncogenic EGFR mutations. *PLoS Med*. 2007; 4:1669–79. discussion 80. [PubMed: 17973572]
28. Ewings KE, Hadfield-Moorhouse K, Wiggins CM, Wickenden JA, Balmanno K, Gilley R, et al. ERK1/2-dependent phosphorylation of BimEL promotes its rapid dissociation from Mcl-1 and Bcl-xL. *EMBO J*. 2007; 26:2856–67. [PubMed: 17525735]
29. Ewings KE, Wiggins CM, Cook SJ. Bim and the pro-survival Bcl-2 proteins: opposites attract, ERK repels. *Cell Cycle*. 2007; 6:2236–40. [PubMed: 17881896]
30. Ciardiello F, McGeady ML, Kim N, Basolo F, Hynes N, Langton BC, et al. Transforming growth factor-alpha expression is enhanced in human mammary epithelial cells transformed by an

activated c-Ha-ras protooncogene but not by the c-neu protooncogene, and overexpression of the transforming growth factor-alpha complementary DNA leads to transformation. *Cell Growth Differ.* 1990; 1:407–20. [PubMed: 1981145]

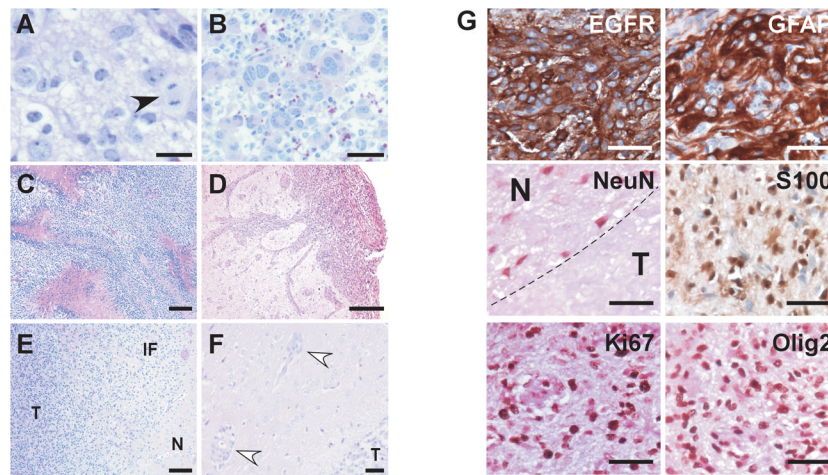
31. Di Fiore PP, Pierce JH, Fleming TP, Hazan R, Ullrich A, King CR, et al. Overexpression of the human EGF receptor confers an EGF-dependent transformed phenotype to NIH 3T3 cells. *Cell.* 1987; 51:1063–70. [PubMed: 3500791]
32. Di Marco E, Pierce JH, Aaronson SA, Di Fiore PP. Mechanisms by which EGF receptor and TGF alpha contribute to malignant transformation. *Nat Immun Cell Growth Regul.* 1990; 9:209–21. [PubMed: 2196461]
33. Di Marco E, Pierce JH, Fleming TP, Kraus MH, Molloy CJ, Aaronson SA, et al. Autocrine interaction between TGF alpha and the EGF-receptor: quantitative requirements for induction of the malignant phenotype. *Oncogene.* 1989; 4:831–8. [PubMed: 2755700]
34. Rosenthal A, Lindquist PB, Bringman TS, Goeddel DV, Derynck R. Expression in rat fibroblasts of a human transforming growth factor-alpha cDNA results in transformation. *Cell.* 1986; 46:301–9. [PubMed: 3459590]
35. Shankar V, Ciardiello F, Kim N, Derynck R, Liscia DS, Merlo G, et al. Transformation of an established mouse mammary epithelial cell line following transfection with a human transforming growth factor alpha cDNA. *Mol Carcinog.* 1989; 2:1–11. [PubMed: 2786419]
36. Watanabe S, Lazar E, Sporn MB. Transformation of normal rat kidney (NRK) cells by an infectious retrovirus carrying a synthetic rat type alpha transforming growth factor gene. *Proc Natl Acad Sci U S A.* 1987; 84:1258–62. [PubMed: 3469667]
37. Andl CD, Mizushima T, Nakagawa H, Oyama K, Harada H, Chruma K, et al. Epidermal growth factor receptor mediates increased cell proliferation, migration, and aggregation in esophageal keratinocytes in vitro and in vivo. *J Biol Chem.* 2003; 278:1824–30. [PubMed: 12435727]
38. Cheng J, Huang H, Zhang ZT, Shapiro E, Pellicer A, Sun TT, et al. Overexpression of epidermal growth factor receptor in urothelium elicits urothelial hyperplasia and promotes bladder tumor growth. *Cancer Res.* 2002; 62:4157–63. [PubMed: 12124355]
39. Ding H, Shannon P, Lau N, Wu X, Roncari L, Baldwin RL, et al. Oligodendrogliomas result from the expression of an activated mutant epidermal growth factor receptor in a RAS transgenic mouse astrocytoma model. *Cancer Res.* 2003; 63:1106–13. [PubMed: 12615729]
40. Iyer AK, Tran KT, Griffith L, Wells A. Cell surface restriction of EGFR by a tenascin cytotactin-encoded EGF-like repeat is preferential for motility-related signaling. *J Cell Physiol.* 2008; 214:504–12. [PubMed: 17708541]
41. Lipeski LE, Boylan JM, Gruppuso PA. A comparison of epidermal growth factor receptor-mediated mitogenic signaling in response to transforming growth factor alpha and epidermal growth factor in cultured fetal rat hepatocytes. *Biochem Mol Biol Int.* 1996; 39:975–83. [PubMed: 8866014]
42. Inda MM, Bonavia R, Mukasa A, Narita Y, Sah DW, Vandenberg S, et al. Tumor heterogeneity is an active process maintained by a mutant EGFR-induced cytokine circuit in glioblastoma. *Genes Dev.* 2010; 24:1731–45. [PubMed: 20713517]
43. Scott JD, Pawson T. Cell signaling in space and time: where proteins come together and when they're apart. *Science.* 2009; 326:1220–4. [PubMed: 19965465]
44. Oda K, Matsuoka Y, Funahashi A, Kitano H. A comprehensive pathway map of epidermal growth factor receptor signaling. *Mol Syst Biol.* 2005; 1:2005 0010. [PubMed: 16729045]
45. Agazie YM, Hayman MJ. Molecular mechanism for a role of SHP2 in epidermal growth factor receptor signaling. *Mol Cell Biol.* 2003; 23:7875–86. [PubMed: 14560030]
46. Rotin D, Margolis B, Mohammadi M, Daly RJ, Daum G, Li N, et al. SH2 domains prevent tyrosine dephosphorylation of the EGF receptor: identification of Tyr992 as the high-affinity binding site for SH2 domains of phospholipase C gamma. *EMBO J.* 1992; 11:559–67. [PubMed: 1537335]
47. Tamas P, Solti Z, Bauer P, Illes A, Sipkei S, Bauer A, et al. Mechanism of epidermal growth factor regulation of Vav2, a guanine nucleotide exchange factor for Rac. *J Biol Chem.* 2003; 278:5163–71. [PubMed: 12454019]
48. Engelman JA, Settleman J. Acquired resistance to tyrosine kinase inhibitors during cancer therapy. *Curr Opin Genet Dev.* 2008; 18:73–9. [PubMed: 18325754]

49. Faber AC, Wong KK, Engelman JA. Differences underlying EGFR and HER2 oncogene addiction. *Cell Cycle*. 2010; 9:851–2. [PubMed: 20160489]
50. Shimshek DR, Kim J, Hubner MR, Spergel DJ, Buchholz F, Casanova E, et al. Codon-improved Cre recombinase (iCre) expression in the mouse. *Genesis*. 2002; 32:19–26. [PubMed: 11835670]



**Figure 1.**

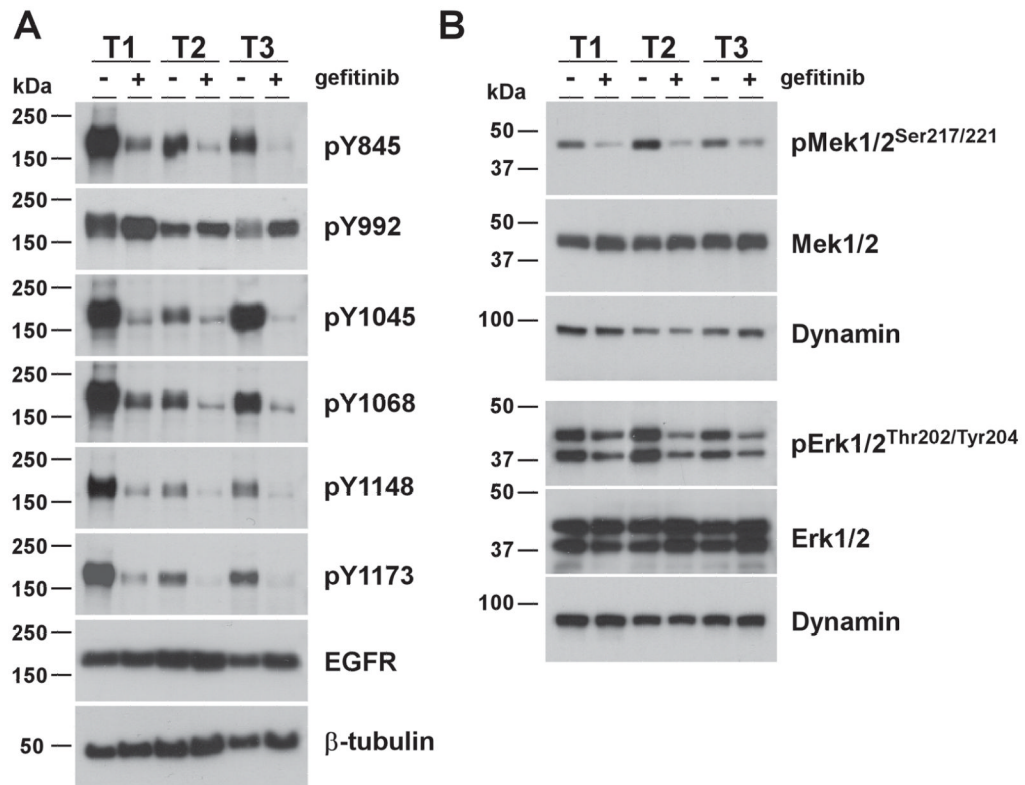
Activated EGFR<sup>WT</sup> cooperates with loss of tumor suppressor genes to form brain tumors that have characteristics of GBM. **A**, Schematic representation of the pTyf lentiviral transducing vectors. This bicistronic vector is derived from a previously described self-inactivating virus (46) modified to contain the human TGF $\alpha$  cDNA followed by a poliovirus1 IRES and improved Cre (iCre) cDNA (50) driven by the human elongation factor-1 $\alpha$  (EF1 $\alpha$ ) promoter. For control experiments, TGF $\alpha$  is replaced with the eGFP gene. The presence of a central polypurine tract (cPPT)-DNA FLAP element upstream of the multiple cloning site significantly improves the transduction efficiency in CNS tissues (48, 49). **B**, Survival (Kaplan-Meier) analysis of conditional EGFR mice. Cohorts of mice of the indicated genotypes were stereotactically injected in the striatum with titer-matched pTyf-TGF $\alpha$ -IRES-iCre or pTyf-eGFP-IRES-iCre and monitored for survival over time. **C**, Photomicrograph of an H&E-stained coronal section of a TGF $\alpha$ -EGFR<sup>WT</sup>; Ink $\Delta$ 2/3<sup>-/-</sup> brain tumor. Scale bar; 2.0 mm.



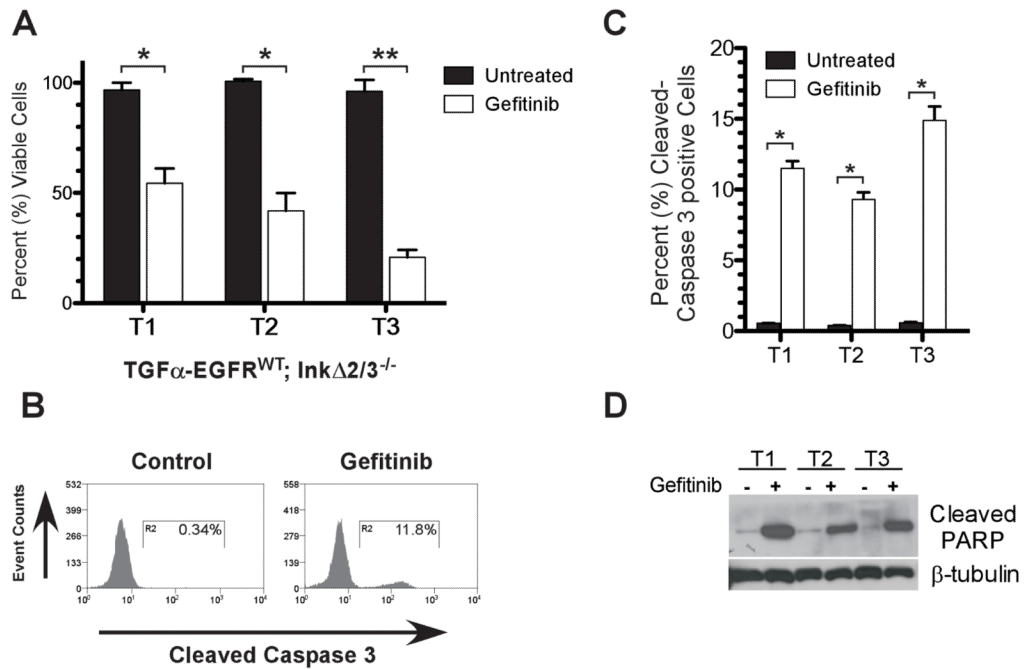
**Figure 2.**

Representative histological photomicrographs of  $TGF\alpha$ -EGFR<sup>WT</sup>;Ink $\Delta$ 2/3<sup>-/-</sup> tumors. H&E-stained paraffin-embedded tumor sections; A, Tumors are set on a fibrillary background and contain densely packed cells featuring pleomorphic nuclei with prominent nucleoli and mitoses (black arrowhead). B, Giant multinucleated cells are present within tumors. C, Tumors exhibit marked pseudopallisading necrosis. D–F, the highly infiltrative nature of  $TGF\alpha$ -EGFR<sup>WT</sup> tumor cells is depicted, (D) tumor cells migrate within meninges in the subarachnoid space and invade the Virchow-Robin space and (E) are infiltrating normal parenchyma (N) by forming a loose infiltrating front (IF) away from the bulk tumor (T) and (F) tumor cells migrate along blood vessels and invade the perivascular space (white arrow head) distant from the bulk tumor (T). G, EGFR<sup>WT</sup> GBM tumors express markers of astrocytic differentiation. Representative photomicrographs of tumors stained with cell lineage markers using IHC. Tumors stain positive for markers of astrocytic lineage (glial fibrillary acidic protein (GFAP) and S100) and negative for markers of neuronal (NeuN) lineage. GBM tumors also stain positive for human EGFR, the proliferation marker Ki67, and for Olig2. EGFR, GFAP and S100 sections were counterstained with hematoxylin and sections for the nuclear NeuN, Olig2 and Ki67 markers were counterstained with eosin. N, normal brain; T, tumor. Scale bars; 25  $\mu$ m (A), 50  $\mu$ m (B,F,G), 62.5  $\mu$ m (D), 125  $\mu$ m (C, E).

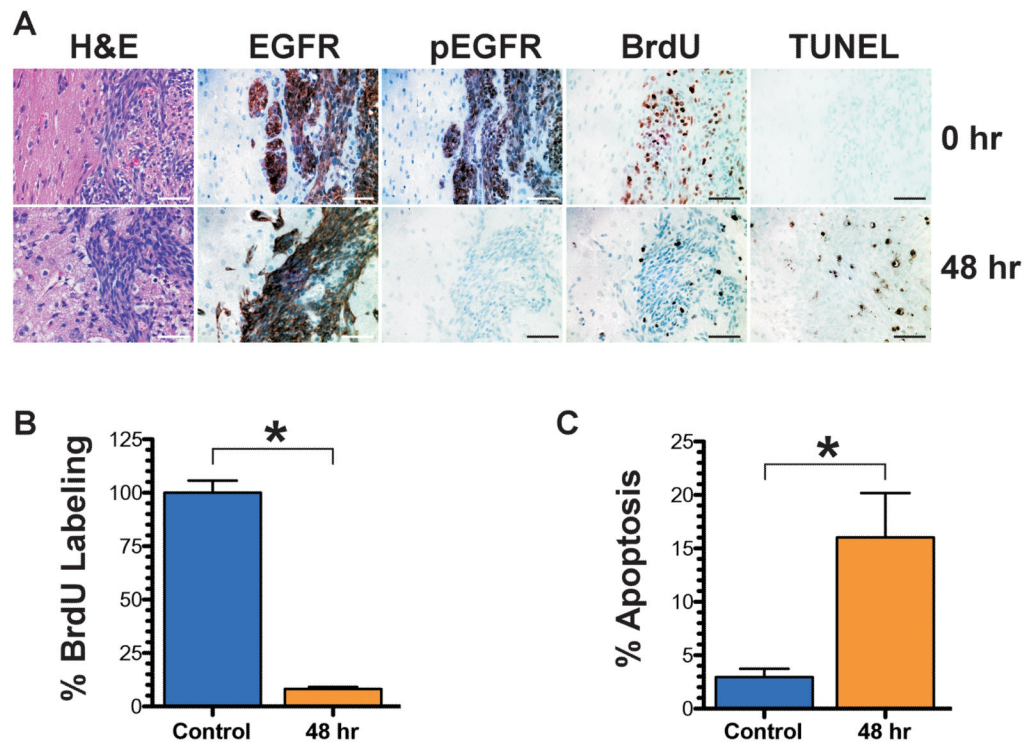




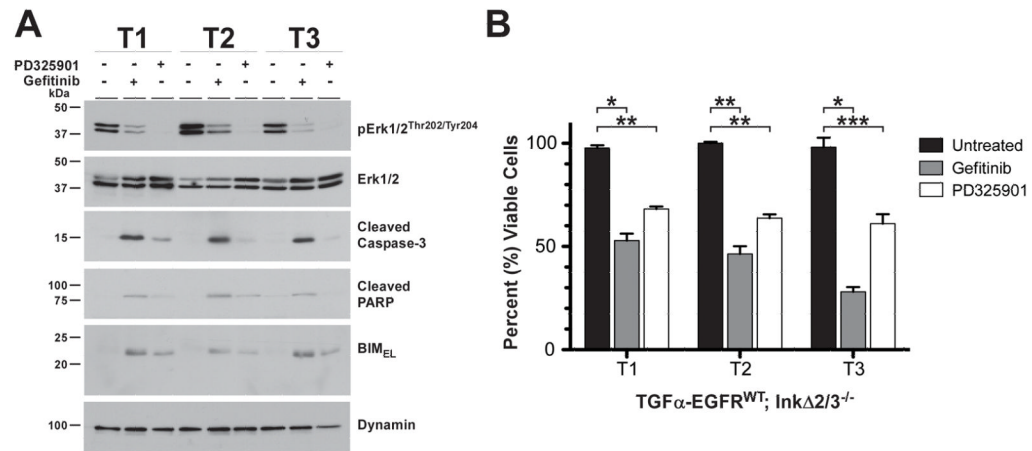
**Figure 3.** EGFR<sup>WT</sup> kinase inhibition attenuates signaling pathways. A–B, Immunoblot of total cell extracts from vehicle- and gefitinib-treated (10  $\mu$ M) TGF $\alpha$ -EGFR<sup>WT</sup>;Ink $\Delta$ 2/3<sup>-/-</sup> tumor cultures analyzed for the presence of (A) the indicated phosphotyrosine residues (B) the activation status of the canonical MAPK members Mek1/2 and Erk1/2.  $\beta$ -tubulin and dynamin are used as internal loading controls.

**Figure 4.**

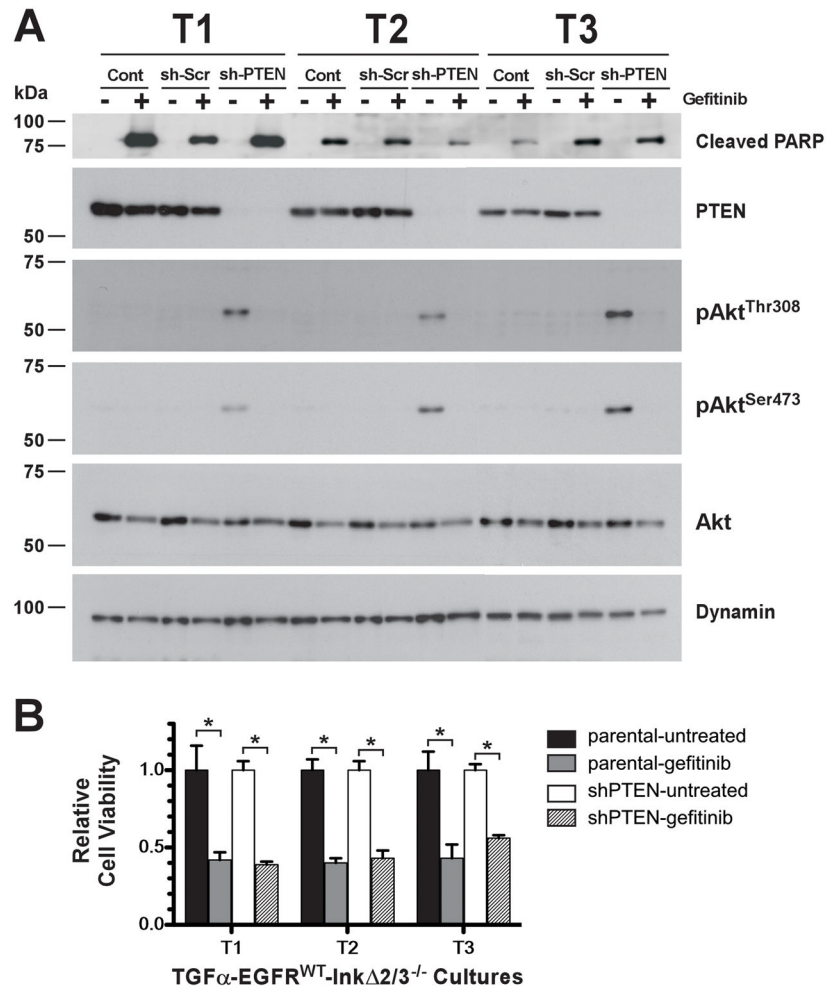
EGFR kinase inhibition in  $TGF\alpha$ -EGFR<sup>WT</sup>;Ink $\Delta$ 2/3<sup>-/-</sup> GBM tumor cells is cytotoxic. A, Tumor cells are sensitive to gefitinib treatment. Viability assay of three independent tumor cell cultures (T1-3) after vehicle or gefitinib treatment (10  $\mu$ M) for 24 hours. Data is plotted as percentage of viable cells of treated over mock treatment (mean  $\pm$  SD; n=3 in each group, \*p<0.005, \*\*p<0.0005, two-tailed t-test). B, Representative flow-cytometric analysis and C, graphical representation of  $TGF\alpha$ -EGFR<sup>WT</sup>;Ink $\Delta$ 2/3<sup>-/-</sup> GBM primary cell cultures mock- and gefitinib-treated indicating an increase in cleaved caspase-3 positive cells upon EGFR kinase inhibitor treatment (mean  $\pm$  SD; n=3 in each group, \*p<0.0001, two-tailed t-test). D, immunoblot of total cell extracts from vehicle- and gefitinib-treated (10  $\mu$ M) cultures of the  $TGF\alpha$ -EGFR<sup>WT</sup>;Ink $\Delta$ 2/3<sup>-/-</sup> GBM tumor cells analyzed for presence of the apoptotic marker cleaved PARP.  $\beta$ -tubulin is used as an internal loading control.



**Figure 5.** Orthotopic allograft tumors of  $TGF\alpha$ -EGFR<sup>WT</sup>;Ink $\Delta$ 2/3<sup>-/-</sup> GBM cells are sensitive to EGFR inhibition. A, representative photomicrographs of paraffin-embedded tumor tissue sections stained for the indicated markers from control (0 hr) and treated tumor-bearing animals 48 hr post treatment. B, graphical representation of the quantification of proliferation assayed by BrdU incorporation. The BrdU staining data is presented as the percentage of BrdU positive cells in treated tumors over control tumors. C, graphical representation of the quantification of the percentage of apoptotic cells as measured by the number of TUNEL positive cells. Quantification of apoptosis is presented as percentage of TUNEL positive cells per field of view. (mean  $\pm$  S.D., n=6 in each group \*p=0.0001, two-tailed t-test). Scale bar: 250  $\mu$ m.



**Figure 6.** EGFR<sup>WT</sup> inhibition-induced apoptosis is partly mediated by MAPK signaling attenuation. TGF $\alpha$ -EGFR<sup>WT</sup>;Ink $\Delta$ 2/3<sup>-/-</sup> GBM tumor cell cultures (T1-T3) were treated with gefitinib (10  $\mu$ M) or Mek1/2 inhibitor PD325901 (100 nM) for 24 hr and A, analyzed by immunoblot analysis for the apoptotic markers cleaved caspase-3 and cleaved PARP and for the pro-apoptotic protein BIM<sub>EL</sub> and B, analyzed for viability in a growth assay. Data is plotted as percentage of viable cells of treated over mock treatment (mean  $\pm$  SD; n=3 in each group, \*p=0.0002, \*\*p<0.0001, \*\*\*p<0.005, two-tailed t-test)..

**Figure 7.**

PTEN loss does not confer resistance to EGFR kinase inhibition. A, cells expressing a scrambled control sh-RNA (sh-Scr) or a PTEN sh-RNA (sh-PTEN) were analyzed by immunoblot for cleaved PARP, phospho Akt<sup>Thr308</sup>, Akt<sup>Ser473</sup> and PTEN expression. Total Akt and  $\beta$ -tubulin are used as an internal loading control. B, parental TGF $\alpha$ -EGFR<sup>WT</sup>;Ink $\Delta$ 2/3<sup>-/-</sup> cultured tumor cells (T1-T3) and their PTEN knockdown counterpart were treated with gefitinib (10  $\mu$ M) and assayed for cell viability. The results are presented as values relative to untreated conditions (mean  $\pm$  SD; n=3 in each group, \*p<0.005, two-tailed t-test).