The IDH-Tau-EGFR triad defines diffuse glioma pathology by controlling mesenchymal differentiation and neo-vascular fitness

Running title: Glioma genotype dictates the vascular phenotype

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SUMMARY

Classification of gliomas as wild-type or mutant *IDH1/2* tumors has profound clinical implications. However, how these two groups of gliomas progress, in a microenvironment-dependent manner, is still a pending question. Here we describe that the expression of Tau is epigenetically induced by mutant IDH1/2, whereas is almost absent from tumors with *EGFR/PTEN* mutations. Moreover, *Tau (MAPT)* expression is inversely correlated with overall survival in *EGFR*-amplified gliomas. Using orthotopic *EGFR*-related models, we have observed that Tau overexpression or microtubule stabilizers impair the mesenchymal transformation of glioma cells, with profound changes in tumor vasculature and a significant decrease in tumor burden. However, epithelial-to-mesenchymal transformed *EGFR*-mutant cells, acting as pericytes, induce neo-vasculogenesis and favor aggressive glioma growth, a process that is no longer sensitive to Tau. Altogether our data indicate that the genomic background controls glioma aggressiveness by modifying the vascular microenvironment.

KEYWORDS

Glioma; Tau (MAPT); microtubule stabilizers; IDH1/2 mutations; EGFR; TAZ, NF-kB, epithelial-to-mesenchymal-transition (EMT); glioma-derived-pericytes (GDPs), Blood-Brain-Barrier (BBB), tumor-microenvironment (TME).

INTRODUCTION

Diffuse gliomas are classified and graded according to histological criteria. They include low and intermediate-grade gliomas (herein called Lower-Grade Gliomas, LGG), which comprise World Health Organization (WHO) grades 2 and 3, and the highly aggressive glioblastomas (GB) (WHO grade 4) (Louis et al., 2007; Brat et al., 2015). Unfortunately, GB is the most common tumor pathology of the central nervous system, with 5-year survival rates of 5%. Patients with GB have a median survival of 16.6 months, which has been attributed in part to limitations in the treatments related to the tumor localization and the lack of knowledge about the physiology of this neoplasia. Moreover, the presence of cancer stem cells (CSCs) within aggressive gliomas has also been linked to the elevated resistance to therapy of these tumors that relapse in almost 100% of the cases (Bao et al., 2006; Schonberg et al., 2014; Liau et al., 2017).

In recent years, efforts have been made in order to characterize the genetic modifications associated with gliomas, some of them have been incorporated into a new classification of the WHO for this type of tumors (Louis et al., 2016). In this sense, mutations in the *IDH1/2* (*Isocitrate dehydrogenase 1/2*) genes have a deep implication in the final phenotype of glioma as tumors with point mutations in any of these proteins have a more favorable prognosis (Yan et al., 2009; Balss et al., 2008). IDH1/2 mutated proteins induce the accumulation of the oncometabolite, 2-D-hydroxyglutarate (2-HG), which interfere with iron-dependent hydroxylases, including the TET family of 5'-methylcytosine hydroxylases. The TET enzymes catalyze the removal of DNA methylation, so its inhibition by mutant IDH1/2 increases DNA methylation and generates a CpG island methylator phenotype (G-CIMP), which is associated with a general suppression of gene expression (Turcan et al., 2012; Figueroa et al., 2010). Moreover, 2-HG inhibits histone demethylases, which further contributes to this phenotype (Lu et al., 2012).

GBs are characterized by their abundant and aberrant vasculature. In fact, florid vascular proliferation is a pathological hallmark of glioma progression. Studies in mouse glioma models and human samples suggest that in early steps of gliomagenesis, tumor cells depend on vascular co-option. Later on, there is a vascular regression that results in tumor hypoxia, which leads to a robust angiogenic response, mediated in part by an increase in VEGF (Vascular Endothelial Growth Factor) secretion. Although this process has been extensively described, it is now clear that neovascularization in gliomas occurs also through other processes like vasculogenesis: recruitment of endothelial precursors from the blood, and vascular mimicry: formation of functional vessel-like networks by tumor cells that transdifferentiate into endothelial cells and mural cells (also called pericytes) (Hardee and Zagzag, 2012). In any case, the outcome of the neoplastic

angiogenic process in gliomas is an abnormal vascular network, with dilated and tortuous vessels. This, together with defects in pericyte recruitment and coverage, contributes to the formation of an abnormal blood-brain-barrier (BBB), which is also a hallmark of aggressive gliomas (Park et al., 2016; Batchelor et al., 2014).

Several cytoskeletal proteins have shown a close relationship with tumor progression. Tau protein, encoded by the gene *MAPT* (microtubule-associated protein tau), is well-known for its relevance in Alzheimer´s disease (AD). In healthy conditions, it is crucial for the regulation of the dynamic instability of microtubules during neural development and for the synaptic transmission (Avila et al., 2012; Wang and Mandelkow, 2016). Tau binds to the same domains of other microtubule-stabilizing agents like taxanes (placlitaxel) (Kar et al., 2003). For that reason, the expression of Tau has been associated with the chemosensitivity of breast cancer cells to this type of compounds (Bonneau et al., 2015). However, Tau and taxanes also modulate protein and organelle trafficking (Sonee et al., 1998; Barreda and Avila, 2011; Li et al., 2012), which could be relevant for cancer cells. Interestingly, a possible co-morbidity of dementias and GB has been suggested (Lehrer, 2010), which has been associated with common mitochondrial modifications (Sanchez-Valle et al., 2017). Considering all these data, and the fact that *Tau (MAPT)* is also expressed in other cell types such as oligodendrocytes, astrocytes or even glioma cells (Lopes et al., 1992; LoPresti et al., 1995; Miyazono et al., 1993; Zaman et al., 2019), we sought to explore the possible role of this protein in the pathology of gliomas.

RESULTS

High levels of *Tau* (*MAPT*) correlate with better clinical prognosis in LGG and GB.

We performed a Western Blot (WB) analysis in a panel of GB and we found that there are indeed high levels of Tau in several samples (Figure 1A). In fact, the amount of Tau in the immunoreactive gliomas is comparable to that observed in healthy tissue (Normal Tissue, NT) (Figure 1A). Immunohistochemical (IHC) staining of glioma samples confirmed that Tau protein is clearly expressed in the cytoplasm of tumor cells, with a different pattern to that observed in the NT (Figure 1B). Moreover, the quantification of the staining revealed that Tau was enriched in LGG compared to GB (Figure 1C). Using the TCGA-LGG+GB cohort, which includes transcriptome data for 664 patients, we performed an in silico analysis and we found that higher expression of the *Tau (MAPT)* gene is associated with an increased overall survival of glioma patients (Figure 1D). Similar results were obtained from patient samples within the REMBRANDT-LGG+GB cohort (329 patients) (Figure 1E) as well as in other four glioma cohorts (Suppl. Figure S1A-D). Furthermore, Tau expression is retained in xenografts derived from primary

tumors (Figure 1F). In agreement with the in silico data, those GB cells expressing lower levels of Tau are much more aggressive than the higher-expressing Tau lines when they are injected in the brain of immunodeficient mice (Figure 1G). To search for a relationship between *Tau (MAPT)* and the development of this disease, we evaluated the expression of this gene in a full set of diffuse glioma tumors (TCGA-GBM+LGG cohort), divided according to the conventional WHO histopathological classification. We observed that as the levels of *Tau* decrease, tumor aggressiveness increases (Figure 1H) (Suppl. Figure S1E, F). We did not find differences between grade 2 and 3 oligodendrogliomas (Figure 1H), although the prognostic value of the WHO grading within these tumors is less clear (Olar et al., 2015). Furthermore, we carried out a comparative study of patients depending on their clinical evolution and we found a marked decrease in the expression of *Tau* in the relapsing tumors (Figure 1I). These data suggest that Tau downregulation must be important for the reappearance of the tumor after surgical resection, which is a critical step in the mortality related to this pathology.

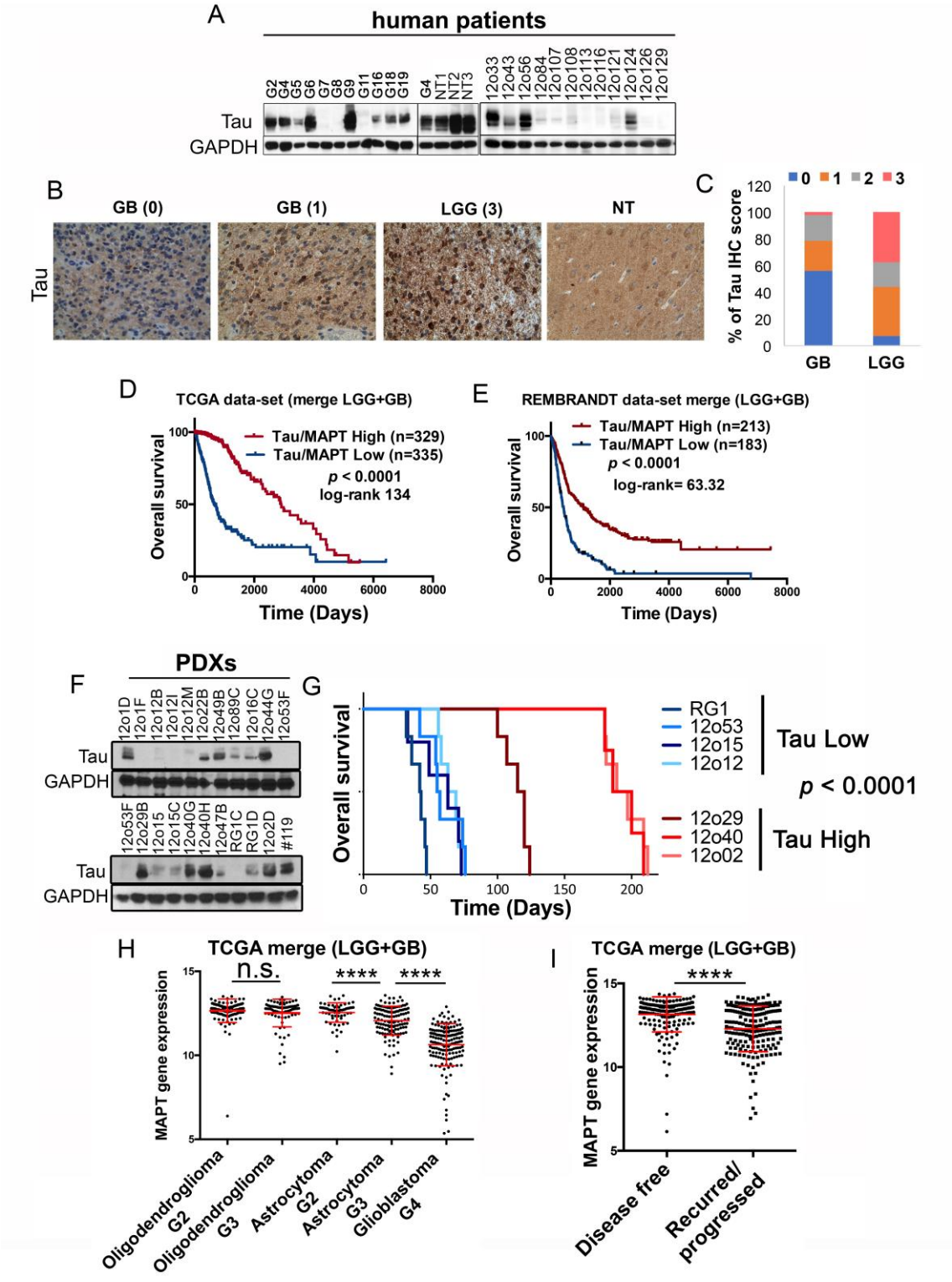


Figure 1. Tau is expressed in gliomas and it is associated with the clinical evolution of the disease. (A) WB analysis of Tau expression in tumor tissue extracts from patients diagnosed with GB. Normal brain tissue (NT) was used for expression control and GAPDH staining as a loading control. (B) Representative pictures of the IHC Tau staining of several gliomas and normal brain tissue (NT). The Tau IHC score is represented between brackets. (C) Percentage of tumors with

different Tau IHC score in lower and high-grade gliomas (LGG n=22 and GB n=55). (D and E) Kaplan-Meier overall survival curves of patients from the TCGA (LGG + GB) (n=664) and the Rembrandt (LGG + GB) (n=396) cohorts. Patients in each cohort were stratified into 2 groups based on high and low *Tau (MAPT)* expression values. (F) WB analysis of Tau expression in tumor tissue extracts from subcutaneous patient-derived xenografts (PDX). (G) Kaplan-Meier overall survival curves of mice that were orthotopically injected with different primary GB cell lines. (H) Analysis of *Tau (MAPT)* mRNA expression by RNAseq in gliomas (TCGA cohort, n=692) grouped according to the WHO classification (histological type and grade). (I) Analysis of *Tau (MAPT)* mRNA expression by RNAseq in glioma (n=386) grouped according to the clinical evolution of the tumors. ****, P ≤0.0001.

***Tau (MAPT)* expression is associated with *IDH1/2* mutations in diffuse gliomas.**

IDH1/2 mutations are one of the most frequent mutations found in gliomas, mainly in LGG and GB tumors of the proneural (PN) subtype. By contrast, gene modifications in *PTEN* and *EGFR* are normally found in high-grade gliomas (Suppl. Figure S2A). Our in silico analysis showed that *IDH1/2* mutations define a distinct subset of GB with a better outcome (Figure 2A). Conversely, the absence of *IDH1/2* mutations in LGG marks a distinct *IDH*-wild-type subgroup characterized by poorer prognosis (Figure 2A), which is in agreement with the published data (Verhaak et al., 2010; Eckel-Passow et al., 2015). Considering the *Tau* expression data (Figure 1), we decided to study if there was a correlation between the expression of this gene and the distribution of the most common somatic non-silent mutations in gliomas. We found that *IDH1* and *ATRX* mutations, which normally occur in the same tumors, accumulate in tumors with high *Tau* expression, whereas *EGFR* and *PTEN* mutations are frequent in tumors with low *Tau* expression (Fig. 2B). The correlation between high *Tau* levels and *IDH* mutations was validated using a Volcano Plot analysis (Figure 2C). We also observed an increased expression of *Tau* in the G-CIMP GB subtype (Suppl. Figure S2B), as well as a strong inverse correlation between the expression of wild-type *IDH1* and *Tau* in the TCGA cohort (Suppl. Figure S2C). Moreover, when we quantified the amount of Tau IHC staining in wild-type and mutant *IDH* gliomas we found a clear enrichment of high and medium stained sections in the second group (Figure 2D). All these data suggest that *Tau* transcription is epigenetically regulated in *IDH* mutant gliomas. In order to test this hypothesis, we overexpressed wild-type IDH1 or the most common mutated form of IDH1 (IDH1 R132H) in a primary GB cell line. As expected, the expression of mutated IDH1 generated tumors that grew significantly slower than the wild-type IDH1-expressing ones (Figure 2E). Interestingly, when we dissected out the intracranial xenografts we observed a clear increase in Tau protein levels in the mutant tumors (Figure 2F). To strengthen

these data we used a recently published glioma model, in which the IDH1 mutation is expressed in an *ATRX* mutant background (Koschmann et al., 2016) (Núñez et al., 2019) and we found that it also slowed down tumor growth (Figure 2G). Moreover, we observed that Tau was clearly induced in the presence of IDH1 R132H (Figure 2H).

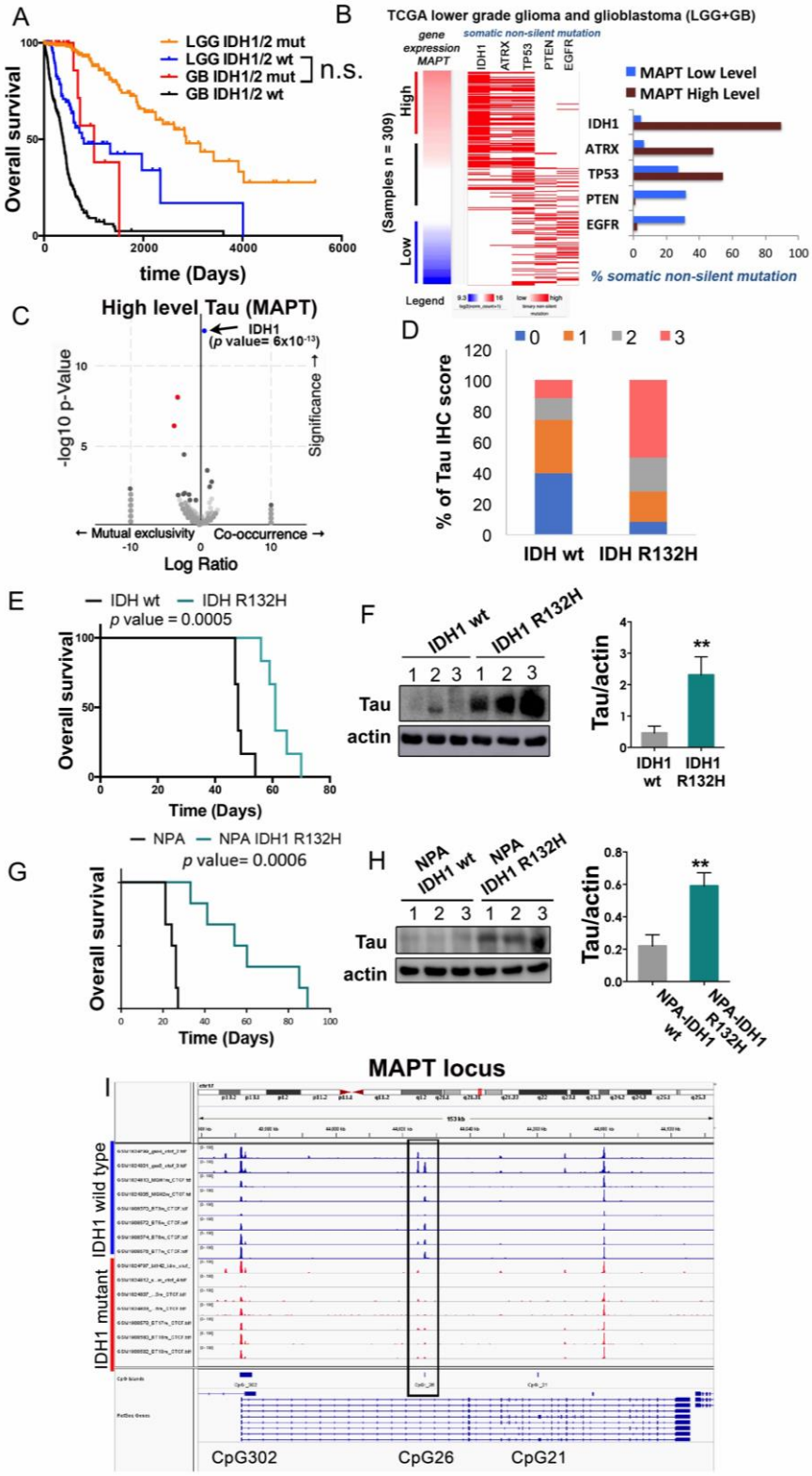


Figure 2. *Tau* expression is regulated by IDH1/2 mutations. (A) Kaplan-Meier overall survival curves of LGG (n=451) and GB (n=299) patients from the TCGA cohort, stratified based on the status of *IDH1/2*. (B) Analysis of non-silent somatic mutations in genes commonly modified in diffuse glioma grouped based on high or low expression of *Tau (MAPT)*. (C) Volcano plots showing mutated genes with differential distribution in glioma comparing tumors with high and low level of *Tau*. The arrow points to *IDH1* mutations. (D) Quantification of the IHC analysis of Tau expression in wt (n= 35) and mutant (n=36) *IDH1* gliomas. (E) Kaplan-Meier overall survival curves of mice that were orthotopically injected with RG1 cells overexpressing either IDH1wt or IDH1 R132H. (F) WB analysis of Tau expression in intracranial tumors from E. Quantification is shown on the right. Actin levels were used for normalization. (G) Kaplan-Meier overall survival curves of mice that were orthotopically injected with NPA or NPA-IDH1 R32H cells (H) WB analysis of Tau expression in intracranial tumors from G. Quantification is shown on the right. Actin levels were used for normalization. (I) CTCF-binding profiles for the *Tau (MAPT*) locus in IDH-mutant and IDH wild-type tumors, normalized by average signal. **, P ≤0.01.

IDH mutations promote *Tau* expression by an epigenetic mechanism.

IDH1/2 mutant proteins generate a G-CIMP phenotype, which is supposed to block the transcription of many genes but to activate certain others, especially those involved in the tumorigenesis of LGG, like the *PDGFRA* oncogene, through the disruption of the binding site of the CTCF insulator protein (Flavahan et al., 2016). Interestingly, we found a significant correlation between *Tau (MAPT)* and *PDGFRA* expression in the TCGA samples (Suppl. Figure S2D). This discovery led us to hypothesize that a similar mechanism might be operating for the induction of both genes. To test this hypothesis, we first analyzed the presence of CpG islands in the *Tau (MAPT*) promoter region by using the xena genome browser. We identified three of them in the 5´region of transcription initiation site (Suppl. Figure S2E), which correlates with the sites previously described (Caillet-Boudin et al., 2015). Using epigenetic data from the TCGA (LGG cohort) we compared the methylation of the whole promoter region. We observed that the presence of *IDH* mutations is associated with increased methylation in two of the CpG islets of the *Tau (MAPT)* gene (Suppl. Figure S2F, G). However, when we analyzed the ChIP-seq data of the CTCF binding to the different clusters, we clearly observed that binding to the CpG:26 site was lost in the *IDHmut* tumors (Figure 2I). In order to confirm the epigenetic regulation of Tau expression by IDH mutant function, we treated primary GB cells with 2-HG and we observed a dose-dependent accumulation of *Tau* mRNA (Suppl. Figure S2I), an effect that was reversed in the presence of azacytidine (Suppl. Figure S2J). Altogether, these results strongly suggest that the methylation of the CpG:26 region in response to IDH mutations disrupts the chromosomal insulator topology and the binding of CTCF to the *Tau* promoter, inducing its transcription.

TAU opposes EGFR signaling in GB.

The data presented so far suggest that Tau, which is induced in response to IDH mutations, could be controlling glioma aggressiveness. In order to understand this mechanism, we performed a DAVID gene analysis of the pathways co-upregulated with *Tau* in gliomas. As expected, we found an association with microtubule and neurogenic-related processes (including axonal and dendrite control and synapse regulation), suggesting that the role of Tau in tumor cells must be related to its main function in neurons (Suppl. Figure S3A) (Avila et al., 2012; Wang and Mandelkow, 2016). However, the other Tau-associated processes were somehow linked to receptor tyrosine kinases (RTK) signaling: vesicle-transport, MAPK/GTPase regulation and Pleckstrin related processes (Suppl. Figure S3A). Moreover, our in silico analysis indicated that *IDH* mutations (Figure 2B, Suppl. Figure S2A), as well as higher levels of Tau (Figure 3A-C) are mutually exclusive with *EGFR* and *PTEN* mutations. These data suggest that Tau could be modulating this pathway in gliomas. In order to test this hypothesis, we first separated TCGA-glioma pools in three groups: tumors with *EGFR* mutations (*EGFR*mut), tumors without genetic alteration in EGFR (*EGFR*wt), and tumors without mutations but with gene amplification (*EGFR*amp). We then estimated the prognostic value of *Tau (MAPT)* expression in these three groups and we found that it had no clinical relevance in *EGFRwt* gliomas (Figure 3D), which include *IDH*wt and *IDH*mut tumors, or in the presence of mutations in *EGFR* (Figure 3E). However, our data revealed that the expression of *Tau* has a clear implication in the survival of *EGFR*amp gliomas (Figure 3F). Interestingly, similar results were obtained when we consider the genetic status of *PTEN* and *EGFR* simultaneously (Suppl. Figure S3B-D). These results suggest that in the group of *EGFR*amp gliomas (and/or in tumors with the loss of one *PTEN* copy), there is a synthetic lethal interaction between IDH/Tau function and the EGFR/PI3K pathway. It is important to emphasize that *IDH* mutations are rarely found in *EGFR* amplified gliomas, although they are present in a percentage of gliomas with *EGFR* gains (Figure 3C). Therefore, we hypothesized that Tau might inhibit EGFR signaling, and that this could be particularly relevant in the presence of certain level of activation of this signaling pathway. However, in the presence of mutations in the receptor or if there is a constitutive activation of *EGFR* signaling, Tau function might be no longer relevant.

To test this hypothesis, we overexpressed Tau in two mouse glioma models: SVZ-EGFRwt and SVZ-EGFRvIII and we performed intracranial allografts. These two models were generated by transforming subventricular zone (SVZ) progenitors from p16/p19 ko mice with lentivirus expressing either the wild-type EGFR or the variant III (vIII) isoform or the receptor. The vIII isoform harbors a deletion of the exons 2 to 7 of *EGFR*, which leads to the expression of a truncated version of the receptor, lacking part

of the extracellular domain, responsible for ligand binding. The vIII mutation is present in half of the *EGFRamp* gliomas and has a strong tumorigenic potential (Zahonero and Sanchez-Gomez, 2014; Furnari et al., 2007; Liu et al., 2015). Overexpression of Tau clearly inhibited the growth of SVZ-EGFRwt mouse gliomas (Figure 3G) but had no effect on SVZ-EGFRvIII tumors (Figure 3H). To obtain an independent confirmation of these data, we overexpressed Tau in two human primary cell lines, the first one, RG1, harbors an *EGFR* amplification and the second one, 12O150, harbors the amplification and a point mutation of this gene (Table1, STAR methods). We observed that Tau expression impaired the growth of the *EGFR*amp cell line (Figure 3I) but had no effect on the growth of the *EGFR*mut cells (Figure 3J). When we analyzed the dissected tumors, we observed that in the presence of Tau overexpression, there was a reduction of phospho-EGFR levels in SVZ-EGFRwt tumors, as well as in *EGFR*amp xenografts, whereas there was no change in SVZ-EGFRvIII gliomas or in *EGFR*mut xenografts (Figure 3K, L). In agreement with this, the in silico analysis showed that higher levels of phosphorylated EGFR are observed in tumors with lower *Tau* expression (Suppl. Figure S3E). Interestingly, Tau overexpression did not inhibit the growth or the self-renewal capacity of RG1 (*EGFR*amp) (Suppl. Figure S3F) or SVZ-EGFRwt (Suppl. Figure S3G) cells grown *in vitro*. Altogether, these data suggest that Tau might control tumor aggressiveness by opposing wild-type EGFR signaling, an event that must be especially relevant in the context of the tumor microenvironment (TME).

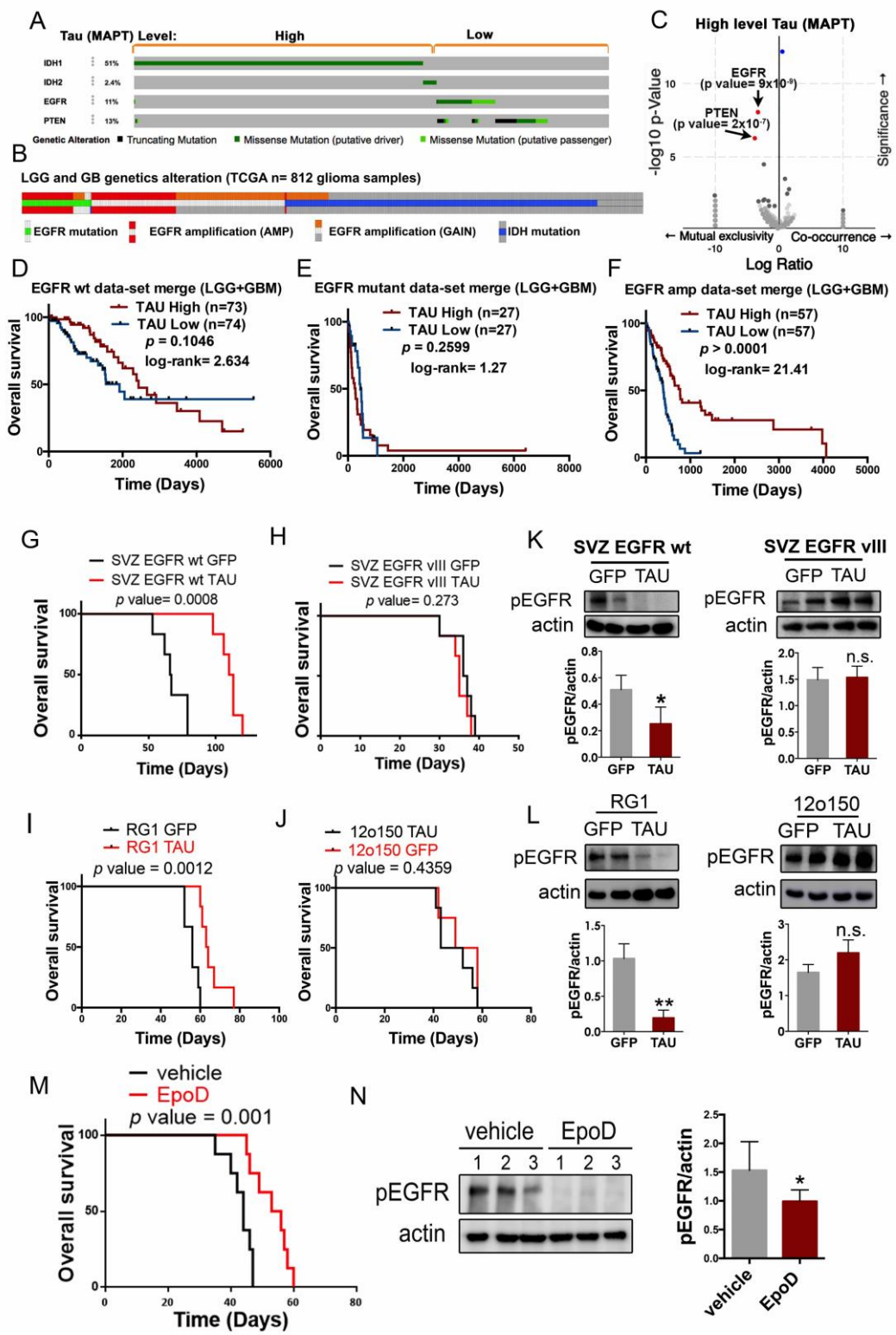


Figure 3. Tau opposes EGFR signaling in gliomas. (A) Distribution of somatic non-silent mutations in *IDH1/2*, *EGFR* and *PTEN* in a glioma cohort (TCGA, n=812). (B) Distribution of somatic non-silent mutations in *IDH1/* and *EGFR*, together with *EGFR* amplifications and gains in a glioma cohort (TCGA, n=811). (C) Volcano plots showing mutated genes with differential distribution in gliomas, comparing tumors with high and low level of *Tau*. The arrows point to *PTEN* and *EGFR* mutations. (D-F) Kaplan-Meier overall survival curves of patients from the

GB+LGG TCGA cohort. Patients were separated based on the *EGFR* status: tumors without alterations (D), tumors with point mutations (E) and tumors with copy number alterations (F). Then, they were stratified into 2 groups based on *Tau (MAPT)* expression values. (G-J) Kaplan-Meier overall survival curves of mice that were orthotopically injected with SVZ-EGFRwt (G), SVZ-EGFRvIII (H), RG1 (*EGFR*amp) (I) or 12o150 (*EGFR*mut) (J) cells overexpressing either GFP or Tau (n=6). (K-L) WB analysis of phosphorylated EGFR (pEGFR) in orthotopic allografts (K) or xenografts (L) expressing GFP or Tau. Actin levels were used for normalization. Quantification is shown on the bottom. (M) Kaplan-Meier overall survival curves of mice that were orthotopically injected with RG1 cells and subsequently treated with intraperitoneal injections (2/week) of EpoD (1μg/Kg) (n=8). (N) WB analysis of pEGFR in orthotopic allografts from animals treated with control or EpoD. Actin levels were used for normalization. Quantification is shown on the right. **, P ≤0.01; *, P ≤0.05. n.s., non-significant.

Tau is a well-known microtubule binding protein. This interaction is responsible for its main physiological and pathological functions (Avila et al., 2012; Wang and Mandelkow, 2016) and can be reproduced by taxanes and other microtubule-stabilizing agents. Interestingly, Tau and taxanes have been linked to EGFR signaling inhibition in different cancers (Hu et al., 2015; Wu et al., 2013). In order to determine if the microtubule-stabilizing activity of Tau could be responsible for the effect observed in gliomas, we treated RG1(*EGFR*amp)-injected mice with a Taxol derivative, Epothilone D (EpoD), which has been proved to reach the brain (Zhang et al., 2012). Systemic treatment with EpoD significantly diminished tumor burden (Figure 3M) and reduced the amount of phospho-EGFR in the tumors (Figure 3N), supporting the notion that the microtubule-related function of Tau is responsible for its effect on EGFR signaling and tumor growth in gliomas.

Vascular and proliferative differences between SVZ-EGFRwt and SVZ-EGFRvIII tumors.

The results presented so far suggest that IDH mutated proteins, by modulating *Tau* expression, are controlling glioma aggressiveness, at least in part through the inhibition of EGFR signaling. However, in the presence of EGFR mutations, the IDHmut/Tau axis has no longer an effect. Therefore, we reasoned that by characterizing the differences between wild-type and mutant *EGFR* gliomas we could have a better idea of the function of Tau in these two types of tumors.

We had previously observed a clear difference in tumor burden depending on the expression of either wt or vIII receptor in the tumor cells (Figure 3G, H and Figure 4A), which correlates with the worse prognosis *EGFR*mut gliomas, in comparison with tumors

harboring the wild-type (amplified or not) receptor (Figure 4B). In order to decipher how the *EGFR* genotype affects the final outcome of the tumors, we performed a thorough analysis of the mouse SVZ gliomas. We decided to analyze these models because they have no masking effect of concomitant mutations in other genes, which are present in the different primary GB cell lines (Table1, STAR methods). The IHC and immunofluorescent (IF) analysis of the tumors revealed a highly compact and proliferative growth after EGFRvIII expression, whereas cells in SVA-EGFRwt tumors appeared detached from each other and they were less proliferative (Figure 4C). Interestingly, we observed a higher density of enlarged-dilated tumor vessels in the mutant tissues (Figure 4D, E), which indicates that there are differences in the vascular phenotype induced by the two different tumor cells. In agreement with this, the in silico analysis revealed that several angiogenesis-related signatures were significantly induced in *EGFR*mut vs *EGFR*amp human gliomas (Suppl. Figure S4A-D), whereas other signatures, for example inflammation, were similar in both type of tumors (Suppl. Figure S4E-F). We then narrowed down the set of differentially expressed genes (including several secreted factors) and we confirmed by qRT-PCR analysis that they were significantly induced in SVZ-EGFRvIII compared to SVZ-EGFRwt tumors (Figure 4F). These data suggest that *EGFR* mutant gliomas have a stronger angiogenic capacity, which could be related to their higher tumoral capacity.

The leaky BBB of aggressive gliomas facilitates the diagnosis of GB by magnetic resonance imaging (MRI) of gadolinium (Gd) extravasation. However, to our surprise, the MRI analysis revealed a much higher contrast enhancement in SVZ-EGFRwt compared to SVZ-EGFRvIII-injected brains (Figure 4G), especially at later times after the Gd injection (Figure 4H). This observation clearly indicates that the tumor vessels are much leakier in the presence of EGFR wild-type cells. In agreement with this, we observed a stronger extravasation of Evans Blue (Supp. Figure S4G) and IgG (Supp. Figure S4H) in SVZ-EGFRwt tumors. Altogether, these data suggest that the presence of the EGFR mutation in the tumor cells is associated with a complex angiogenic response that give rise to highly vascularized tumors. By contrast, the angiogenic signals induced by overexpression of EGFRwt result in a less dense vasculature network, with a severely compromised BBB.

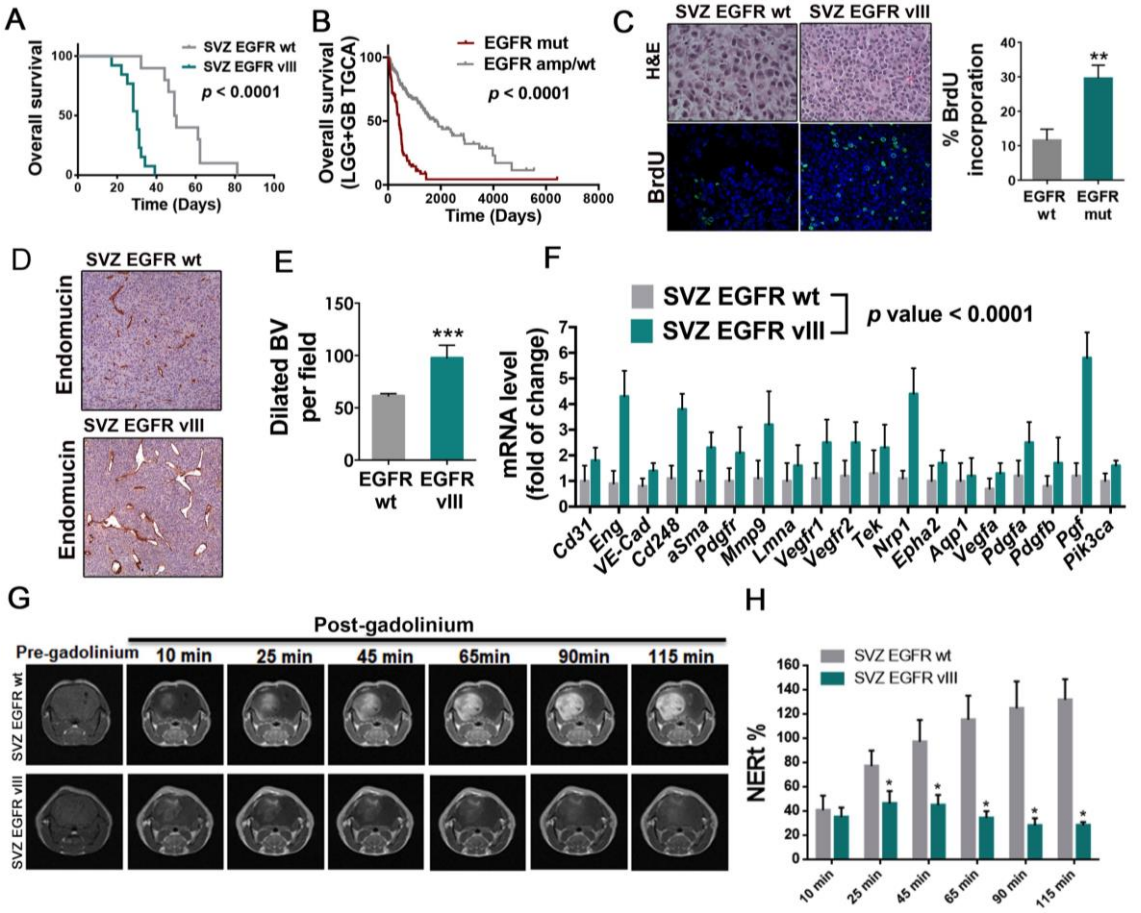


Figure 4. SVZ-EGFRvIII gliomas show increased angiogenesis but decreased vascular permeability, in comparison with SVZ-EGFRwt tumors. (A) Kaplan-Meier overall survival curves of mice that were orthotopically injected with SVZ-EGFRwt or SVZ-EGFRvIII cells (n=10/13). (B) Kaplan-Meier overall survival curves of patients from the GBM+LGG TCGA cohort, separated based on the *EGFR* status (n=272). (C) Representative images of hematoxylin and eosin (H&E) (Top) and BrdU IF (Bottom) staining of sections from SVZ gliomas. Quantification of the percentage of BrdU+ cells is shown on the right. (D) Representative images of the IHC staining of SVZ glioma sections using an Endomucin antibody. (E) Quantification of the number of dilated vessels in D. (F) qRT-PCR analysis of angiogenesis-related genes in SVZ-EGFRwt and SVZ-EGFRvIII tumors. The level of *Actin* was used for normalization. (G) Representative contrast-enhanced MRI images of SVZ-EGFRwt and SVZ-EGFRvIII-containing brains at different time points after Gd injection. H) Quantification of the Gd extravasation in G. ***, P ≤0.001; **, P ≤0.01; *, P ≤0.05.

Glioma-derived-pericytes from EGFRmut tumors are able to attach to the endothelium, stabilizing the vasculature.

The expression of the GFP reporter by the SVZ tumor cells led us to observe that the SVZ-EGFRvIII cells were located in close contact with the endothelium, whereas most SVZ-EGFRwt cells appeared detached from the vessels (Supp. Figure S5A). We

reasoned that this different behavior could explain the changes in BBB permeability observed between wt and vIII-expressing gliomas (Figure 4).

BBB stability in gliomas depends on two main cellular components: endothelial cells and perivascular cells (pericytes), which envelop the surface of the vascular tube and stabilizes the blood flow (Bergers and Song, review Neuro Oncol 2005). It has been shown that pericytic functions are being performed in a great percentage by CSCs in gliomas, which undergo a transdifferentiation process, acquiring mesenchymal and mural cell features (Scully et al., 2012; Cheng et al., 2013; Zhou et al., 2017). In agreement with this notion, the IF staining of SVZ tumors revealed that some GFP+ cells also express αSMA, both in SVZ-EGFRwt and SVZ-EGFRvIII gliomas (Supp. Figure S5B). This observation suggests that EGFR-expressing CSCs are able to transdifferentiate into mesenchymal/pericytic cells (herein called Glioma-Derived-Pericytes, GDPs). Furthermore, we observed that there was a specific expression of pericyte-related markers (*Pdgfrb, αSma, Ng2, Cd248*) in GFP+ cells, sorted out after SVZ tumor dissociation, both in SVZ-EGFRwt (Figure 5A) and SVZ-EGFRvIII tumors (Figure 5B). Moreover, by using human-specific primers, we observed the expression of these markers in *EGFR*amp and *EGFR*mut tumor cells from xenografts (Figure 5C, D). Therefore, we hypothesized that GDPs are present in all the EGFR-related tumors, regardless of the expression of the wild-type or the mutant isoform of the receptor.

The analysis of the GFP/αSMA double IF staining (Supp. Figure S5B) suggests that in SVZ-EGFRvIII tumors, GDPs are always localized around the vessel lumen, whereas in SVZ-EGFRwt tumors, many GDPs do not surround any vessel. To further corroborate this observation, we double-stained the tumor sections with endomucin and αSMA antibodies. We confirmed that GDPs appeared in close contact with endothelial cells in SVZ-EGFRvIII tumors (Figure 5E), whereas in the SVZ-EGFRwt tumors, some αSMA+ cells were in contact with the vessels (acting as mural cells), whereas most of them appeared delocalized (arrows in Figure 5E), with no clear contact with the endothelium. This observation was not specific to the mouse glioma model, as many αSMA+ also appeared delocalized in *EGFR*amp xenografts, whereas in *EGFR*mut xenografts, they were always in close contact with endothelial cells (Figure 5F). These data suggest that glioma cells overexpressing EGFR, either the wt or the mutant isoform, are able to undergo the transdifferentiation process, acquiring the expression of pericytic-related markers. However, only in the presence of EGFR mutations, these pericytes are effectively recruited to the endothelial cells. Moreover, glioma cells expressing mutant EGFR seem to have a stronger capacity for GDP formation, as there is an increased GDP to endothelium ratio in *EGFR*mut xenografts compared to *EGFR*wt tumors (Suppl. Figure S5C). Altogether, our results suggest that GDPs in *EGFR*mut gliomas participate

in the stability of the tumor vasculature, preventing excessive BBB leakiness and favoring the function of the vessels. These phenomena might explain why the cells are more aggressive and give rise to more compact and proliferative tumors (Figure 4).

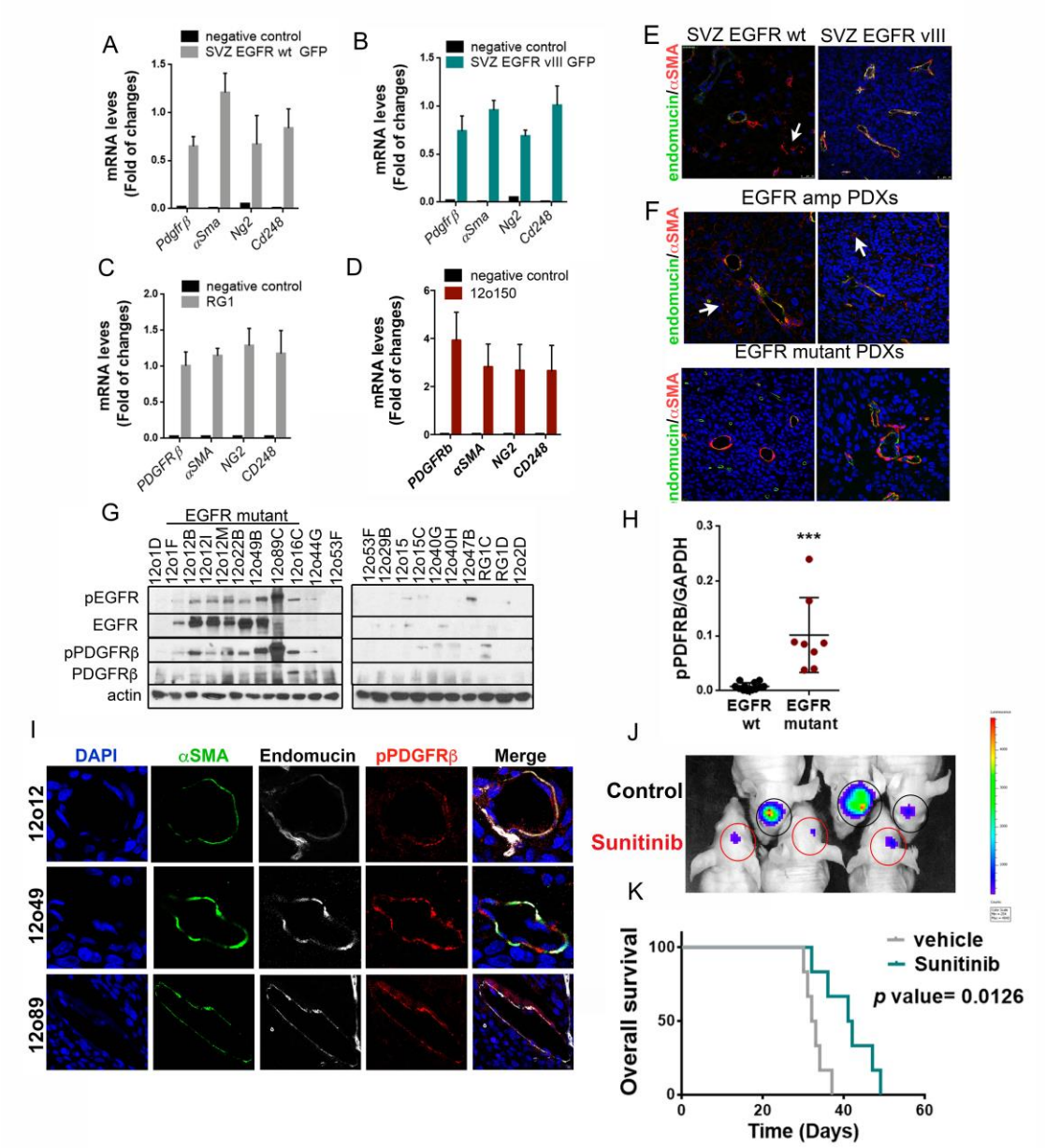


Figure 5. *EGFR*amp and *EGFR*mut glioma cells transdifferentiate into pericytes but their location depends on the genetic status of the receptor. (A-B) qRT-PCR analysis of pericytic–related genes in GFP-sorted cells from SVZ-EGFRwt and SVZ-EGFRvIII tumors. The expression of *Actin* was used for normalization. (C-D) qRT-PCR analysis of pericyte–related genes (using human-specific primers) in *EGFR*amp and *EGFR*mut xenografts. The expression of *HPRT* was used for normalization. Human (A-B) or mouse (C-D) cDNA was used as a negative control. (E-F) Representative images of endomucin and αSMA IF staining of sections from SVZ gliomas and orthotopic xenografts. Arrows point towards examples a αSMA-positive cells that do not localize close to endomucyin-positive cells. (G) WB analysis of EGFR, phospho-EGFR, PDGFRβ and

phospho-PDGFRβ in tumor tissue extracts from a panel of subcutaneous GB xenografts. The expression of GADPH was used for normalization. (H) Quantification of the relative amount of phospho-PDGFRβ in G. (I) Representative images of αSMA, endomucin and pPDGFRβ IF staining of sections from orthotopic *EGFR*mut xenografts. (J) Representative pseudo-color images of Fluc bioluminescence of intracranial SVZ-EGFRvIII tumors treated with Sunitinib or vehicle (control). (K) Kaplan-Meier overall survival curves of mice that were orthotopically injected with SVZ-EGFRvIII and treated with Sunitinib or vehicle (n=6). ***, P ≤0.001.

In order to characterize the mechanisms behind the distinct behavior of *EGFR*amp and *EGFR*mut GDPs in the tumors, we performed a molecular analysis, testing several endothelial and pericytic markers *in vitro* and *in vivo*. We detected a clear upregulation of phospho-EGFR in the SVZ-EGFRvIII tumors, suggesting a stronger activation of the pathway. This was paralleled by a strong increase in the levels of phospho-PDGFRβ (platelet derived growth factor receptor-β), with no differences in the amount of total PDGFRβ, phospho-AKT or βcatenin (Suppl. Figure S5D, E). This increase was detected as well in several *EGFR*mut xenografts, but not in tumors with wild-type *EGFR* (with or without amplification) (Figure 5G, H). Interestingly, PDGFRβ activation was not observed in GL261 tumors (Suppl. Figure S5D, E), a commonly used mouse glioma model that does not carry any alteration of the *EGFR* gene, even though it shows many glioma-related vascular abnormalities (Park et al., 2016). Activation of PDGFRβ is crucial for proper integration of the mural cells in the vessel wall (Abramsson et al., 2003; Chatterjee and Naik, 2012; Franco et al., 2011; Franco et al., 2011). In agreement with this, we observed phospho-PDGFRβ staining in pericytes of different *EGFR*mut PDX (Figure 5I). Moreover, systemic treatment of SVZ-EGFRvIII injected mice with sunitinib, a PDGFRβ inhibitor (Navis et al., 2011), reduced tumor growth and increased overall survival (Figure 5J, K). Thus, the data presented here suggest that mutant EGFR signaling favors the recruitment of pericytes to the glioma vessels through the activation of PDGFRβ, supporting the maturation and function of the vasculature and favoring tumor growth.

Tau overexpression inhibits tumor-to-pericyte trans-differentiation, favoring vascular normalization of *EGFR*amp gliomas.

Considering how the status of *EGFR* determines the vascular phenotype of gliomas (Figures 4 and 5) and the fact that Tau overexpression inhibits the growth of the wild-type, but not the mutant, EGFR-related gliomas (Figure 3), we decided to study the possible effect of Tau overexpression in tumor vessels. For that we analyzed the tumors formed by GFP-RG1 (*EGFR*amp) and Tau-RG1 cells and we found a significant reduction in the number of dilated blood vessels (Figure 6A-B), and a decrease in

proliferation (Figure 6C-D) in the second ones. Moreover, we observed a strong decrease in the amount of cells labelled with αSMA or CD248 antibodies after Tau overexpression, especially those that appear delocalized (Figure 6E). This was accompanied by a significant inhibition of the expression of several human pericytic markers (Figure 6F) that did not occur after Tau overexpression in *EGFR*mut (12O150) xenografts (Suppl. Figure S6A). Interestingly, Tau overexpression did not affect the expression of mouse pericityc genes (Figure 6G), suggesting that it inhibits the glioma-to-pericyte trans-differentiation but it does not influence the amount, or the differentiation capacity, of host pericytes. In fact, the images in Figure 6E suggest that the remaining pericytes in Tau expressing tumors are located next to endothelial cells, functioning as mural cells. In parallel to these results, we observed the lack of hemorrhages in *EGFR*amp tumors overexpressing Tau, an event that was frequent in control tumors (Figure 6H). This was accompanied by a reduction in the extravasation of IgG after Tau overexpression (Figure 6I), which suggest that there is a stabilization of the tumor vasculature, accompanied by the recovery of the BBB integrity. In agreement with this hypothesis, tumors overexpressing Tau had a clear reduction in the levels of Ang2 (Angiopoietin 2) (Figure 6J). Ang2 is a key regulator of angiogenesis that binds to the endothelial-specific receptor tyrosine kinase 2 (TIE2) and acts as a negative regulator of ANG-1/TIE2 signaling (Thomas and Augustin, 2009; Huang et al., 2010). In GB, Ang2 inhibition has been associated with normalization of tumor vessels (Park et al., 2016; Peterson et al., 2016) and the Ang2/Ang1 ratio has a negative prognostic value (Sie et al., 2009). In agreement with this, we observed a significant decrease in Ang2/Ang1 ratio (measured by qRT-PCR using mouse primers) after Tau overexpression in the *EGFR*amp model (Figure 6K), but not in the *EGFR*mut xenografts (Suppl. Figure S6B).

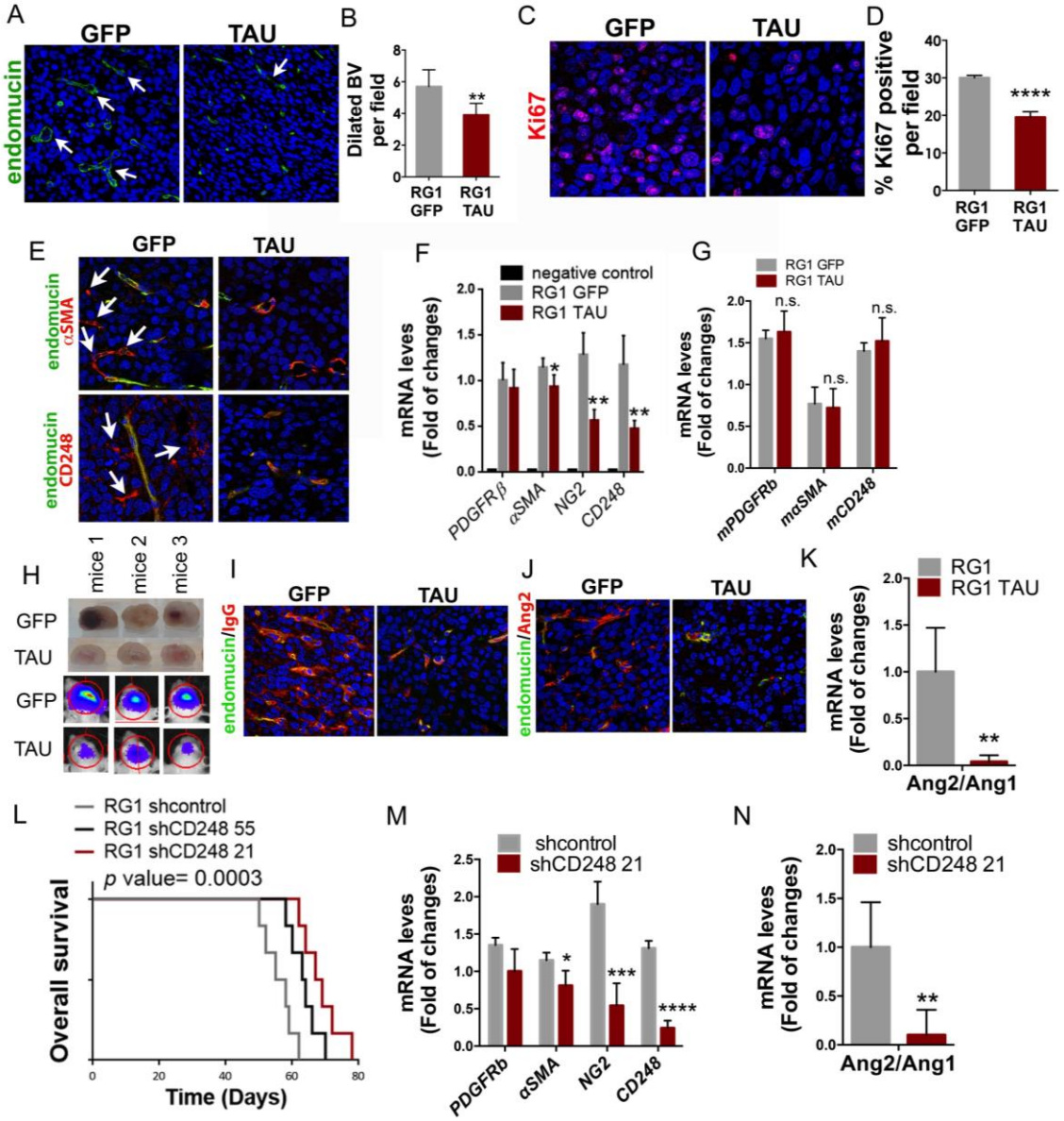


Figure 6. Tau overexpression blocks the appearance of GDPs in *EGFR*amp gliomas and normalizes the tumor vasculature. (A) Representative images of endomucin IF staining of sections from RG1 xenografts expressing GFP or Tau. (B) Quantification of dilated blood vessels in A (indicated by arrows). (C-D) Representative images of Ki67 IF staining of sections from RG1 xenografts expressing GFP or Tau (C) and quantification in (D). (E) Representative images of endomucin and αSMA (top) or endomucin and CD248 (bottom) IF co-staining of sections from RG1 xenografts expressing GFP or Tau. (F) qRT-PCR analysis of pericytic–related genes (using human-specific primers) in RG1 GFP and Tau xenografts (mouse cDNA was used as a negative control). The expression of *HPRT* was used for normalization. (G) qRT-PCR analysis of pericytic–related genes (using mouse-specific primers) in RG1 GFP and Tau xenografts. The expression of *actin* was used for normalization. (H) Representative images of sectioned mouse brains (top) and pseudo-color images of Fluc bioluminescence (bottom), after the injection of GFP or Tau-expressing RG1 cells. (I) Representative images of endomucin and IgG IF co-staining of RG1 tumor sections. (J) Representative images of endomucin and Ang2 IF co-staining of sections from

RG1 xenografts expressing GFP or Tau. (K) Ratio of Ang2/Ang1 expression measured by qRT-PCR in RG1 gliomas. The expression of *HPRT* was used for normalization. (L) Kaplan-Meier overall survival curves of mice that were orthotopically injected with RG1 cells expressing shcontrol, shCD248 55 or shCD248 21 (n=6). (M) qRT-PCR analysis of pericytic-related genes in RG1 tumors expressing shControl or shCD248 21. (N) Ratio of Ang2/Ang1 expression measured by qRT-PCR in RG1 gliomas after *CD248* downregulation. The expression of *HPRT* was used for normalization. *, P ≤0.05; **, P ≤0.01. n.s., non-significant.

The data presented here suggest that Tau inhibits the appearance of GDPs in *EGFR*amp gliomas, which normally appear delocalized in the tumors. Moreover, Tau induces a vascular normalization and a reduction in the tumor growth. Therefore, we hypothesized that the delocalized GDPs could be responsible for the formation of abnormal and leaky tumor vessels in *EGFR*amp gliomas. In order to confirm this theory, we depleted GDPs by an alternative mechanism. For that we downregulated *CD248* expression in RG1 cells with different shRNA sequences (Suppl. Figure 6C). Then, we selected the most effective ones and we injected the interfered cells into the brains of immunodeficient mice, which contain normal host pericytes. We observed a significant delay in tumor growth after *CD248* downregulation (Figure 6L). CD248, also called endosialin, aids in supporting tumor microvasculature and it is expressed in pericytes, but only within malignant solid tumors, including high-grade gliomas (Cheng et al., 2013; Simonavicius et al., 2008). *CD248* downregulation in the tumor cells reduced the expression of other pericytic markers *in vitro* (Suppl. Figure S6D) and *in vivo* (Figure 6M). Moreover, tumors formed after *CD248* downregulation showed a reduced Ang2/Ang1 ratio (Figure 6N), suggesting that the depletion of GDPs normalizes the tumor vessels. These results confirm that the aggressive behavior of *EGFR*amp gliomas depends, at least in part, on the presence of the GDPs in the tumors and the subsequent vascular abnormalities induced by them. By repressing the trans-differentiation capacity of glioma cells, Tau overexpression (or *CD248* inhibition) blocks the disruption of the BBB, normalizes the vasculature and reduces tumor burden.

Tau blocks the mesenchymalization of *EGFR*amp, but not *EGFR*mut, glioma cells.

In order to understand how Tau could be affecting the process of glioma-to-pericyte trans-differentiation, we performed a bioinformatic analysis, which showed that high levels of *Tau (MAPT)* expression correlated positively with the PN signature, whereas it exhibited a strong inverse correlation with the mesenchymal (MES) subtype (Figure 7A, Suppl. Figure S7A-B). We also analyzed the established signatures for different cellular processes and we observed that *Tau* expression inversely correlates

with the NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells) and the inflammatory pathways, but not with others, like the hypoxic or the PDGFRα signatures (Figure 7A-B). These in silico observations were confirmed by the analysis of the *EGFR*amp (RG1) xenografts overexpressing Tau, which showed a clear inhibition of the NF-κB subunit phosphorylation (Figure 7C-D). The activation of this pathway has been associated with inflammation, although it can also respond to other stimuli, like growth factors (including EGF), and has an important role in different cancers (Mantovani, 2010). In fact, aberrant constitutive activation of NF-κB has been observed in GB (Nogueira et al., 2011; Bredel et al., 2011), where it induces MES differentiation and radiation resistance (Bhat et al., 2013; Kim et al., 2016). This is especially important as pericytes have a mesenchymal profile. In fact, they have been considered as the perivascular mesenchymal stem cells (Bergers and Song, 2005; Appaix et al., 2014) and a major source of epithelial mesenchymal transition (EMT) factors in gliomas (Mader et al., 2018). In agreement with this, we observed that, parallel to the NF-kB pathway inhibition in the tumors, Tau overexpression induced a strong downregulation of TAZ in *EGFR*amp xenografts (Figure 7C-D). However, Tau did not affect the expression of other mesenchymal genes (Figure 7E) and it had no effect on TAZ expression or NF-kB activation in *EGFR*mut tumors (Suppl. Figure S7C-D). TAZ is a transcriptional regulator and a master regulator of the MES phenotype (Bhat et al., 2011; Carro et al., 2010; Guichet et al., 2018). However, the bioinformatics analysis revealed that most of the pathways upregulated with TAZ in gliomas are associated with angiogenesis and tumor vasculature (Suppl. Figure S7E). Interestingly, the mesenchymal impairment observed in Tau expressing tumors was accompanied by a strong induction of the expression of OLIG2 (Figure 7F), a transcription factor that is frequently used as a *bona fide* PN marker (Lu et al., 2016). Moreover, the IF analysis of RG1 gliomas confirmed that Tau overexpression represses the nuclear expression of TAZ, whereas it induces the nuclear expression of OLIG2 in the tumor cells and not in the surrounding stroma (Figure 7G). These data were also confined in silico, where Tau correlated inversely with TAZ (Suppl. Figure S7F) and positively with Olig2 (Suppl. Figure S7G) expression. Altogether, our results indicate that Tau overexpression induces a change in the glioma phenotype, repressing MES regulators and inducing PN promoters in the tumor cells through the regulation of the EGFR/TAZ/NF-kB pathway. It is important to remark that this regulation only happens in the context of EGFR amplification and in the absence of receptor mutations. Moreover, this effect is not due to changes in the TME, but rather the direct consequence of the inhibition of the EMT in the glioma cells, which enables them to acquire pericyte markers and properties.

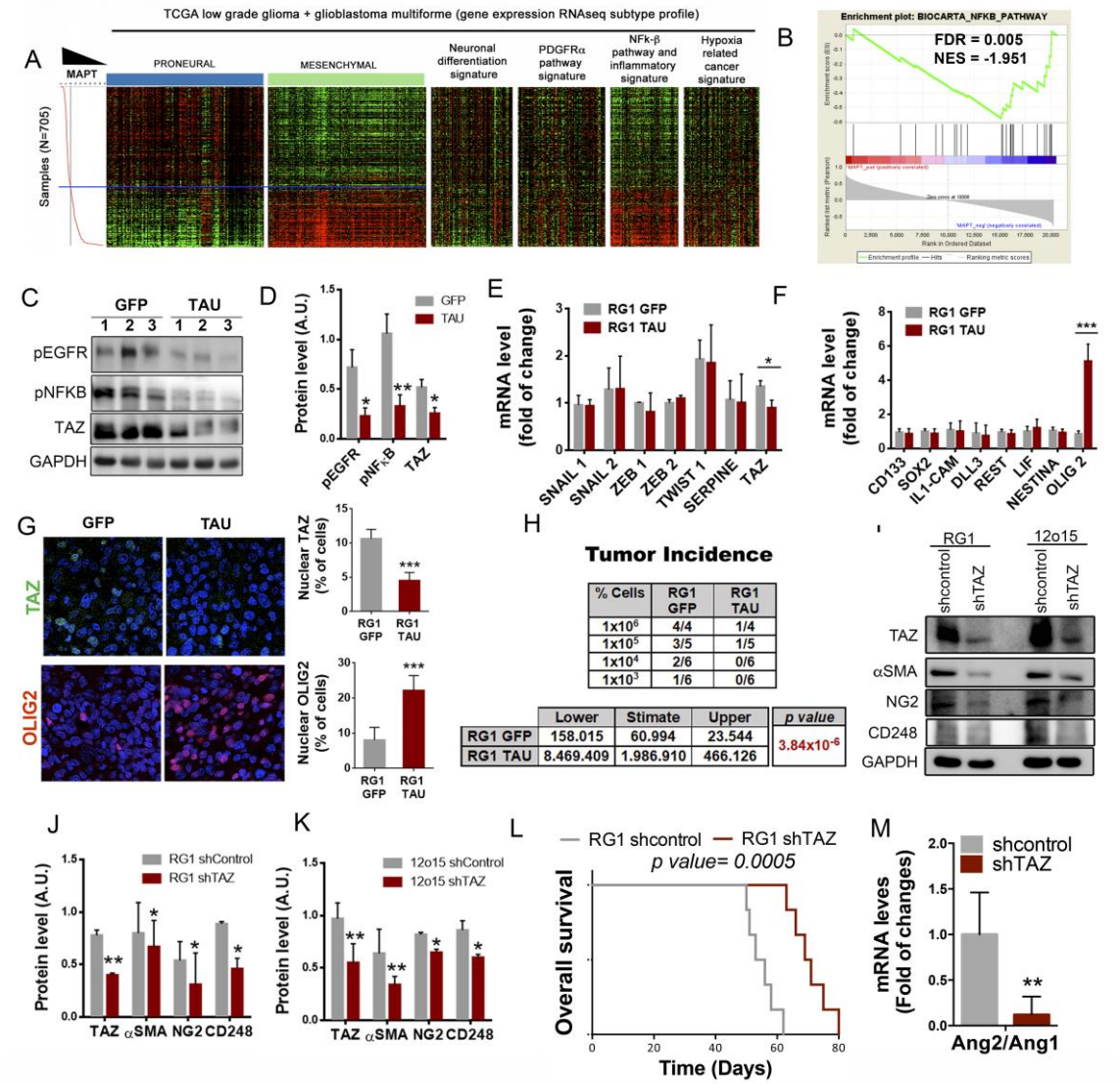
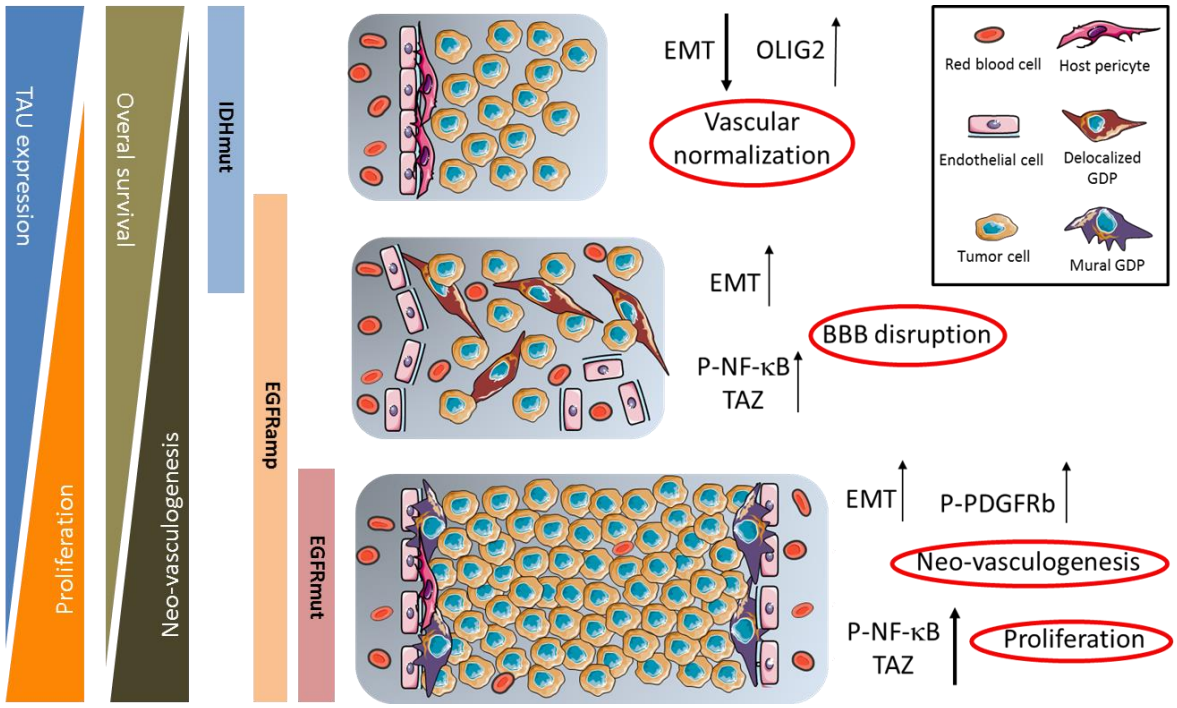


Figure 7. Tau overexpression blocks the mesenchymal features of *EGFR*-amp cells through the inhibition of NF-κB/TAZ. (A) Heatmap of PN, MES, neuronal differentiation, PDGFRα pathway, NF-κB/inflammation, and hypoxia-related cancer expression genes depending on *Tau (MAPT)* expression levels. (B) GSEA enrichment plot analysis using Tau gene expression values as template and the NF-κB pathway geneset from the Biocarta pathways database. (C-D) WB analysis of phosphorylated EGFR (pEGFR), phosphorylated pNF-κB (p65) and TAZ in RG1 xenografts expressing either GFP or Tau. GADPH levels were used for normalization. Quantification is shown in (D). (E-F) qRT-PCR analysis of EMT (E) or PN-subtype (F) related genes in RG1 xenografts expressing GFP or Tau. The expression of *HPRT* was used for normalization. (G) Representative images of TAZ (top) or OLIG2 (bottom) IF staining of sections from GFP or Tau overexpressing RG1 gliomas. Quantification is shown on the right. (H) Subcutaneous tumor growth assay (limited dilution) of RG1 cells after overexpression of GFP or Tau. The statistical analysis is shown on the bottom. (I) WB analysis of TAZ, αSMA, NG2 and CD248 in RG1 or 12O15 cells after TAZ downregulation. GADPH levels were used for normalization. Quantification is shown in (J) and (K), respectively. (L) Kaplan-Meier overall survival curves of mice that were orthotopically injected with RG1 cells expressing shcontrol or

shTAZ. (M) Ratio of Ang2/Ang1 expression measured by qRT-PCR in RG1 gliomas after *TAZ* downregulation. The expression of *HPRT* was used for normalization. *, P ≤0.05; **, P ≤0.01; ***, P ≤0.001.

EMT has been associated with the induction of stem cell properties in normal (Mani et al., Cell 2008) as well as cancer cells (Ye et al., 2015). In gliomas, MES features are linked to therapy resistance and tumor recurrence (Bhat et al., 2013; Kim et al., 2016)). Therefore, it was not surprising to observe that Tau expression severely impaired the capacity to form subcutaneous tumors of the RG1 cells in a limited dilution assay (Figure 7H, Suppl. Figure S7H). However, as we have already mentioned, no effect on self-renewal (a typical stemness feature) was observed *in vitro* after Tau overexpression (Suppl. Figure S3F-G). Therefore, EMT of glioma cells seems to be relevant for glioma initiation and growth, especially in the context of the TME. Accordingly, downregulation of TAZ by RNA interference clearly reduced the expression of pericyte markers in two *EGFR*amp cell lines (RG1 and 12o15) *in vitro* (Figure 7I-K) and slowed down RG1 tumor growth (Figure 7L). Moreover, tumors formed after TAZ downregulation showed a reduced Ang2/Ang1 ratio (Figure 7M), suggesting that there was a normalization of the tumor vessels. These results underlie the key role of the mesenchymal transformation of tumor cells in *EGFR*amp gliomas, which determines the vascular phenotype and is regulated by the amount of Tau being expressed.

GRAPHICAL ABSTRACT



DISCUSSION

The new classification of gliomas distinguishes between *IDH1/2* wild-type and mutant gliomas, with a clear prognostic value (Yan et al., 2009). Gain-of-function IDH mutations are initiating events that induce important metabolic and epigenomic changes, which could explain the oncogenic properties of these proteins. In brain progenitor cells, for example, mutant IDH alters chromatin conformation and the transcription factor landscape, leading to a block in differentiation and tumor initiation (Bardella et al., 2016; Pirozzi et al., 2017; Lu et al., 2012). Moreover, the hypermethylation induced by IDH mutant proteins leads to the aberrant activation of some tumor promoting genes, like *PDGFRA* (Flavahan et al., 2016). In parallel, it enhances the expression of DNA damage repairing enzymes, which slows down the accumulation of other oncogenic mutations (Núñez et al., 2019). By contrast, it has been proposed that wt IDH promotes the metabolic adaptation of tumor cells to support their aggressive growth and their resistance to therapy (Bleeker et al., 2010; Calvert et al., 2017). Still, the analysis of several mouse glioma models (including the results presented here) indicates that the re-expression of the most common mutated isoform of IDH1 (R132H) in high-grade gliomas can reduce tumor burden (Figure 2A, (Calvert et al., 2017; Amankulor et al., 2017) Núñez et al., 2019), and could have an important effect on the immune infiltrate (Amankulor et al., 2017). However, the mechanism behind this tumor-suppressor function of IDH mutations has not been described yet. Here, we have identified Tau as a new positive target of IDHmut-induced DNA hypermethylation. Our results indicate that when this protein is expressed, it impairs tumor initiation, as well as tumor growth, by blocking the epithelial-to-mesenchymal transition of glioma cells and the appearance of GDPs. As a result, Tau normalizes the blood vessels, reducing BBB leakage and tumor aggressiveness. These results raise the possibility that the changes observed in the immune cells in other models could be secondary to this vascular normalization.

Although cancer and neurodegenerative diseases like AD increase exponentially with age, they have been considered classically as opposite phenomena. In fact, there have been reports of lower incidence of dementias in people who have cancer and *vice versa* (Musicco et al., 2013). However, in the case of GB, there could be a positive association between AD prevalence and tumor incidence (Lehrer, 2010). These direct and inverse co-morbidity relationships have allowed the identification of deregulated set of genes, which could link these two diseases (Sanchez-Valle et al., 2017). Here, we have explored the role of Tau, a protein that stabilizes microtubules and that is clearly implicated in several pathologies and dementias of the nervous system, in gliomas. We have found that it is overexpressed at both, messenger RNA and protein levels, in the less aggressive gliomas, where it controls the pericyte abundance, as well as vascular

normalization. Interestingly, there is an important neurovascular dysfunction at early stages of AD, associated with BBB breakdown and inflammation (Jackson et al., 2017; Kisler et al., 2017). Moreover, pericyte loss has been linked to neurodegeneration in AD (Sagare et al., 2013), so it would be interesting to determine the possible participation of Tau in those phenotypes. Another important corollary from the dual role of Tau in gliomas and AD is that some Alzheimer´s related drugs could be repurposed to treat brain tumors. In that sense, we have demonstrated that EpoD, a microtubule stabilizing agent that reduces AD pathology in mouse models (Zhang et al., 2012), slows down *in vivo* glioma growth without any apparent secondary effect. Moreover, targeting pericytes could be an interesting therapeutic approach for several neurological disorders (Cheng et al., 2018).

Our results indicate that Tau expression has an inhibitory effect on *EGFR*amp tumors, whereas it has no consequences in the presence of mutant isoforms of this receptor, whether they harbor the vIII deletion or activating point mutations. It is interesting to note that the bioinformatics analysis revealed that *IDH* mutations are mutually exclusive with *EGFR* mutations, whereas there is some overlap with *EGFR* gains. Moreover, it has been suggested that the receptor is overexpressed even in the absence of amplification (Reifenberger et al., 1996). Therefore, there is an important subgroup of gliomas where Tau expression could modulate EGFR signaling.

Regarding the tumorigenic properties of EGFR mutations in gliomas, there is plenty of literature, especially related to the commonly found vIII isoform, which apparently can maintain the signal in the absence of the ligand (Zahonero and Sanchez-Gomez, 2014; An et al., 2018) and promotes GB growth through changes in the pattern of transcription factors activated, in comparison with wild-type EGFR (Liu et al., 2015). Moreover, it has been proposed that some of these downstream targets, like NF-κB, become constitutively activated in GB after EGFRvIII expression, inducing angiogenesis through the secretion of interleukin-8 (Bonavia et al., 2012). The data presented here further supports that EGFR mutations induce a constitutive activation of NF-κB, which is no longer sensitive to microtubule modulators. By contrast, Tau overexpression or EpoD treatment clearly reduced EGFR phosphorylation and the downstream NF-kB activation in *EGFR*-amp gliomas. In this respect, drugs that interfere with microtubule function have been associated with EGFR inactivation in other cancers (Wu et al., 2013; Hu et al., 2015), as they seem to interfere with endocytic trafficking (Sonee et al., 1998; Li et al., 2012) and activate protein tyrosine phosphatases that could impair the receptor activation (Wu et al., 2013). We propose that a similar mechanism could be operating in gliomas.

In agreement with the published data, we have observed a general upregulation of several angiogeneic molecules in *EGFR*mut compared to *EGFR*amp gliomas,

although the most prominent change observed in the tumors is the activation of PDGFRβ in the pericytes located next to the vessels. This is a surprising result as it had been proposed that EGFRvIII signaling actively suppress *PDGFRβ* transcription (Akhavan et al., 2013). However, we have not seen a transcriptional difference of *PDGFRβ* between *EGFR*amp and *EGFR*mut gliomas, but rather a change in the amount of phosphorylated receptor, which only happens in the *in vivo* setting. Some of the molecules upregulated in EGFRmut tumors, like CXCR4 (Cheng et al., 2013) or Cx45 (Fang et al., 2013), could be responsible for the increased attachment of the pericytes to the endothelial cells and the subsequent activation of PDGFRβ in response to the ligand secreted by the vessels. However, we cannot discard that other molecules, differentially expressed in *EGFR*amp tumors, could contribute to the delocalized appearance of the GDPs, which do not respond to the PDGF secreted by endothelial cells. In any case, these vascular differences represent an additional explanation for the aggressiveness of *EGFR*mut gliomas and for the synergy observed between EGFR and PDGFRβ inhibitors (Akhavan et al., 2013).

Downstream EGFR/NF-kB signaling, we have found that Tau overexpression severely reduced the amount of TAZ in GB. YAP (YES-associated protein) and TAZ (also known as Transcriptional coactivator with PDZ-binding motif) are two transcriptional coactivators that play a prominent role in regulating organ size and tumorigenesis by inhibiting cell proliferation, promoting apoptosis, and regulating stem/progenitor cell expansion (Pan, 2010; Mo et al., 2014; Tang et al., 2016). Moreover, TAZ has been shown to be a key regulator of CSCs in breast cancer (Cordenonsi et al., 2011) and gliomas (Basu-Roy et al., 2015; Zhu et al., 2015; Gargini et al., 2016) and it is considered one of the master transcriptional factors inducing a GB MES signature (Bhat et al., 2011; Carro et al., 2010; Guichet et al., 2018). Moreover, TAZ is induced by NF-κB activation in gliomas and promotes radio-resistance (Bhat et al., 2013) and it has been recently proposed that its promoter is a direct target of NF-κB (Ferraiuolo et al., 2018). Therefore, Tau could be inhibiting indirectly the transcription of the *TAZ* gene. However, we cannot discard a possible regulation of TAZ protein stability downstream of EGFR/NF-κB signaling.

The results presented here reflect the relevance of the EMT process of glioma cells for tumor growth. Moreover, we can propose a key function for the mesenchymal/pericytic glioma-derived cells in the process of tumor vascularization. Our mouse and human glioma models reflect the existence of at least three types of vascular phenotypes with different levels of BBB leakage, depending on the abundance and location of the GDPs (see graphical abstract). These phenotypes would be directed by

the genetic background, in a similar way to what happens in other brain tumors (Phoenix et al., 2016). The first subtype of gliomas, with a highly normalized vasculature, would be induced by *IDH1/2* mutations. In the other extreme, the pro-angiogenic *EGFR*mut glioma cells would give rise to highly vascularized tumors with certain degree of BBB stability, due to the capacity of the GDPs to stabilize the tumor vasculature and to prevent excessive BBB leakiness, thus favoring the blood flow. That would explain why *EGFR*mut tumor cells are more aggressive and give rise to more dense and proliferative gliomas. These features are characteristic of the classical (CL) subtype of gliomas, which some authors also named as Proliferative tumors (Phillips et al., 2006). The third glioma subgroup would be induced by *EGFR* amplification or overexpression. This later group is also associated with neo-angeogenesis, although it shows a highly disrupted vasculature, which probably leads to poor oxygenation and hypoxic responses, typical of MES gliomas. We and others have previously shown that the different GB subtypes have distinct vascular phenotypes, with several angiogenic and hypoxic features being enriched in MES gliomas, whereas, in the other extreme, PN tumors are more invasive and have a better preserved BBB (Bougnaud et al., 2016; Garcia-Romero et al., 2016; Conroy et al., 2017). However, in both CL MES gliomas, tumor aggressiveness might rely on the vascular network, although for different reasons. The abundant presence of GDPs in both types of gliomas would probably make it difficult to distinguish these two subgroups based only on the expression of mesenchymal markers. However, our data suggest that there are important differences in the BBB phenotypes between the three glioma subtypes, which might translate into different MRI parameters, as it has been recently suggested (Rathore et al., 2018). This could have diagnostic as well as predictive value, as the amount of chemotherapy that can reach the tumors might depend on these vascular phenotypes (Batchelor et al., 2013; Stylianopoulos and Jain, 2013; Van et al., 2015). Moreover, the immune infiltrate and its pro- or anti-tumoral properties could also be affected by the vascular abnormalities (Fukumura et al., 2018; Missiaen et al., 2018). In that sense, future experiments are warranted in order to measure the penetrance of different anti-tumor agents and the distribution of the immune cells depending on the neo-vascular fitness.

In resume, our results place the vascular properties of gliomas at the top of the microenvironment hierarchy control. This could have a huge impact on the design of future combinatorial therapeutic approaches, which should combine agents targeting the glioma vasculature (in particular the tumor-derived pericytes) with conventional therapies, molecularly-directed drugs or immunotherapies. Moreover, these strategies should be tailor-designed for each specific glioma subtype.

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AUTHOR CONTRIBUTIONS

Conceptualization: RG, BSC, JA and PSG; Investigation: RG, BSC, EHS, ARB, BH, FJN, DGP and RGE; Formal Analysis: RG and RGE; Resources: VGE, JMS, AHL, AAS, JS and MGC; Writing-Original Draft: RG, JA and PSG; Writing-Review & Editing: VGE, AAS, JS, JMS, AHL, MGC and RGE; Funding Acquisition: RG, JA and PSG; Supervision: JA and PSG.

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

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