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A Kinome-Wide RNAi Screen in *Drosophila* Glia Reveals That the RIO Kinases Mediate Cell Proliferation and Survival through TORC2-Akt Signaling in Glioblastoma

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Abstract

Glioblastoma, the most common primary malignant brain tumor, is incurable with current therapies. Genetic and molecular analyses demonstrate that glioblastomas frequently display mutations that activate receptor tyrosine kinase (RTK) and Pi-3 kinase (PI3K) signaling pathways. In *Drosophila melanogaster*, activation of RTK and PI3K pathways in glial progenitor cells creates malignant neoplastic glial tumors that display many features of human glioblastoma. In both human and *Drosophila*, activation of the RTK and PI3K pathways stimulates Akt signaling along with other as-yet-unknown changes that drive oncogenesis. We used this *Drosophila* glioblastoma model to perform a kinome-wide genetic screen for new genes required for RTK- and PI3K-dependent neoplastic transformation. Human orthologs of novel kinases uncovered by these screens were functionally assessed in mammalian glioblastoma models and human tumors. Our results revealed that the atypical kinases RIOK1 and RIOK2 are overexpressed in glioblastoma cells in an Akt-dependent manner. Moreover, we found that overexpressed RIOK2 formed a complex with RIOK1, mTor, and mTor-complex-2 components, and that overexpressed RIOK2 upregulated Akt signaling and promoted tumorigenesis in murine astrocytes. Conversely, reduced expression of RIOK1 or RIOK2 disrupted Akt signaling and caused cell cycle exit, apoptosis, and chemosensitivity in glioblastoma cells by inducing p53 activity through the RpL11-dependent ribosomal stress checkpoint. These results imply that, in glioblastoma cells, constitutive Akt signaling drives RIO kinase overexpression, which creates a feedforward loop that promotes and maintains oncogenic Akt activity through stimulation of mTor signaling. Further study of the RIO kinases as well as other kinases identified in our *Drosophila* screen may reveal new insights into defects underlying glioblastoma and related cancers and may reveal new therapeutic opportunities for these cancers.

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Introduction

Glioblastoma (GBM), the most common primary malignant brain tumor, infiltrates the brain, grows rapidly, and is refractory to current therapies. Signature genetic lesions in GBM include amplification, mutation, and/or overexpression of receptor tyrosine kinases (RTKs), such as EGFR and PDGFRα, as well as activating mutations in components of the PI-3 kinase (PI3K) pathway (reviewed in [1]). More than 40% of GBMs show EGFR gene amplification, and these amplification events are often accompanied by mutations in EGFR [1]. The most prevalent mutant form of EGFR is ΔEGFR (EGFRvIII, de2-7EGFR, EGFR*), an intragenic truncation mutant that displays constitutive kinase activity [2]. ΔEGFR and other constitutively active mutant forms of EGFR found in GBMs potently drive tumor cell survival, migration, and proliferation [2,3]. The most frequent mutation in the PI3K pathway in GBM is loss of the PTEN lipid phosphatase, which results in unopposed signaling through PI3K and robust stimulation of Akt, especially in the context of EGFR activation [1]. In mouse models, co-activation of these pathways in glia, glial progenitor cells, and/or neuro-glial stem cells induces GBM [4,5,6,7]. However, the full range of signaling events acting downstream of or in combination with EGFR and PI3K to drive oncogenesis remain to be determined. While several normal effectors of RTK and PI3K signaling, such as Ras, Akt, and mTor, are used by EGFR and PI3K in GBM and are required for gliomagenesis [1], constitutive activation of RTK and PI3K pathways may evoke changes distinct from those induced by

Author Summary

Glioblastomas, the most common primary brain tumor, harbor mutations in receptor tyrosine kinases (RTKs), such as EGFR, and components of the Pi-3 kinase (PI3K) signaling pathway. However, the genes that act downstream of RTK and PI3K signaling to drive glioblastoma remain unclear. To investigate the genetic and molecular basis of this disease, we created a glioblastoma model in the fruit fly *Drosophila melanogaster*. To identify new genes involved in glioblastoma development, we performed a screen for the genes required for tumor cell proliferation using our *Drosophila* glioblastoma model and then functionally assessed the activity of human versions of novel genes identified in this screen. Our results revealed that the RIO kinases become overexpressed in human glioblastomas but not in normal human glial or neuronal cells. We found that overexpression of the RIO kinases promotes and maintains signals that drive tumor cell proliferation and survival in RTK- and PI3K-dependent human glioblastoma, and reduction of RIO kinase expression decreased proliferation and prompted cell death and chemosensitivity in glioblastoma cells. Therefore, disruption of the RIO kinases may provide new therapeutic opportunities to target glioblastoma and other RTK- or PI3K-dependent cancers.

normal developmental signaling. Notably, treatments with pharmacologic inhibitors of EGFR or mTor are cytostatic at best in a subset of patients, indicating that other, unidentified factors or compensatory signals affect the survival and growth of tumor cells [8].

To uncover new factors required for EGFR- and PI3K-mediated gliomagenesis, we developed a GBM model in *Drosophila melanogaster* [9]. *Drosophila* offers several advantages for modeling cancers like GBM. Flies have orthologs for 75% of human disease genes [10], including nearly all known gliomagenic genes; signaling pathways are highly conserved; versatile genetic tools are available for cell-type specific gene manipulation [11,12]; and *Drosophila* neural cell types are homologous to their mammalian counterparts [13,14]. While a *Drosophila* model cannot address all aspects of human GBM, our model recapitulates important pathologic features. Specifically, constitutive activation of EGFR-Ras and PI3K signaling in *Drosophila* glial progenitor cells gives rise to proliferative, invasive neoplastic glia that create transplantable malignant tumors [9]. These tumors are induced through activation of a synergistic genetic network composed of downstream pathways commonly mutated and/or activated in human GBMs, such as Akt and mTor signaling [9]. However, activating these known downstream pathways alone or in combination is not sufficient to induce glial neoplasia in *Drosophila*, indicating that additional, as yet unidentified, genetic pathways are involved in transformation. Thus, we undertook genetic screens using our *Drosophila* GBM model to discover new genes underlying EGFR and PI3K mediated neoplastic transformation, and tested whether human orthologs of the genes identified in *Drosophila* represent new human genes involved in GBM.

Our analyses in both *Drosophila* and human systems uncovered that the RIOK1 and RIOK2 kinases drive the survival and proliferation of GBM cells. RIOK1 and RIOK2 are members of the RIO (right open reading frame) family of atypical protein kinases, named for yeast (*S. cerevisiae*) Rio1p and Rio2p, respectively [15]. The RIOK1 and RIOK2 proteins are highly conserved, and are present in all phylogenetic kingdoms, from yeast to mammals among eukaryotes. Kinases in this family are characterized by the presence of the RIO kinase domain, a kinase fold structurally homologous to eukaryotic serine-threonine protein kinase domains, but that lacks classic activation and substrate binding loops (reviewed in [15]). While these kinases undergo autophosphorylation and phosphorylate nonspecific substrates *in vitro*, the actual *in vivo* substrates of RIO kinases are unknown [15]. In both yeast and human cells, RIOK1 and RIOK2, which are not functionally redundant, are required for processing of the 18S rRNA and cytoplasmic maturation of the 40S ribosomal subunit, although neither kinase is an integral component of the ribosome [16,17,18,19]. Recent studies demonstrate that, in yeast, Rio2p also transiently associates with immature ribosomes to block translation initiation, although how RIOK2 is regulated in this context is unclear [20]. To date, several studies have provided suggestive evidence that the RIO kinases could be involved in RTK and PI3K signaling: RIOK2 becomes rapidly phosphorylated in response to EGFR stimulation; Rio2p binds to Tor2p, an ortholog of the mTor kinase, and RIOK1 is required for the proliferation and survival of Ras-dependent cancer cells [21,22,23]. However, to date, no specific function has been ascribed to RIOK1 or RIOK2 in the context of EGFR or PI3K signaling.

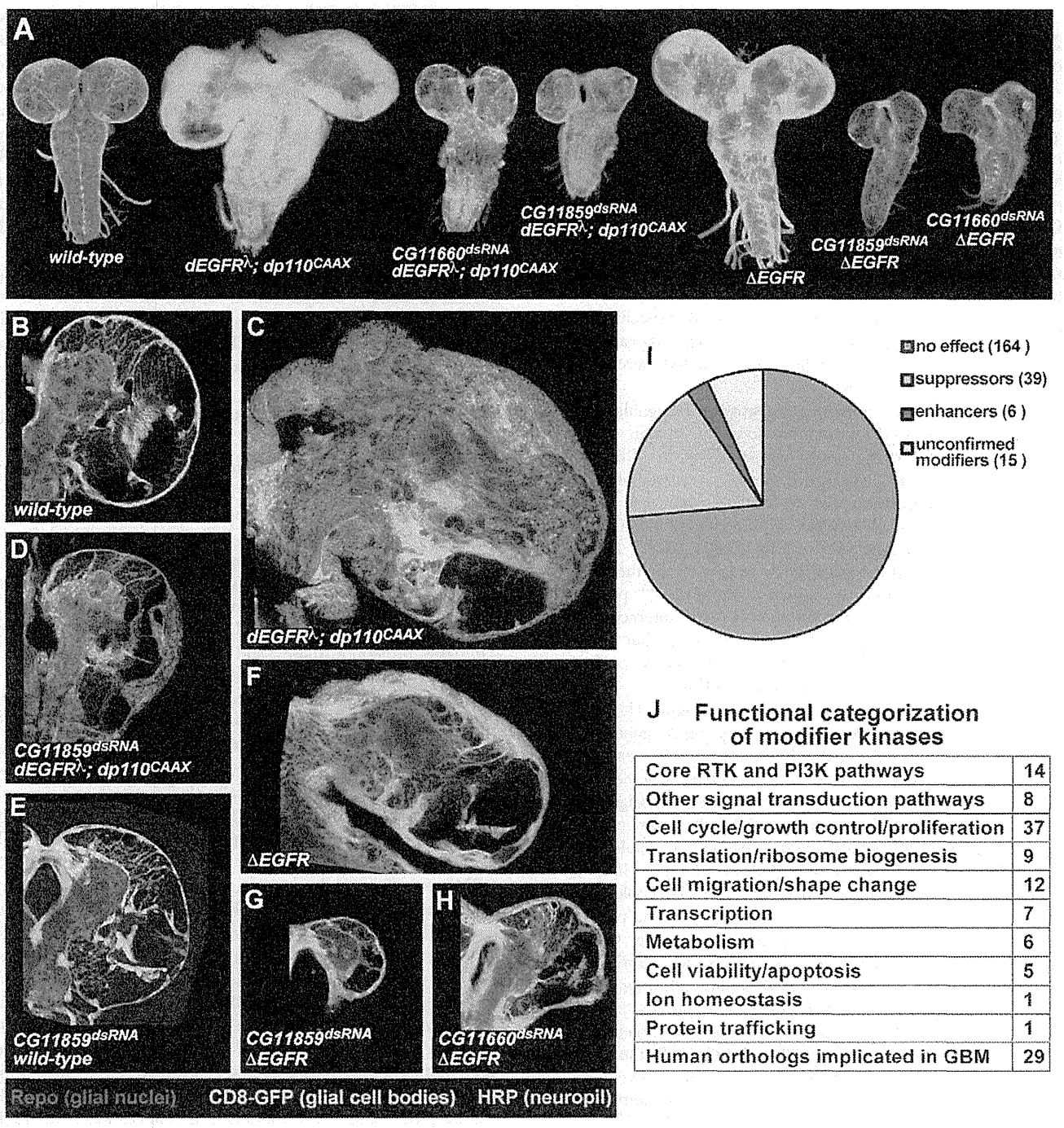
In this manuscript we demonstrate that RIOK1 and RIOK2 become overexpressed in GBM tumor cells relative to normal brain cells; that RIOK1 and RIOK2 overexpression occurs in response to constitutive Akt signaling; that RIOK2 forms a complex with RIOK1, mTor, and other signaling components to drive activation of Akt signaling and tumorigenesis; and that, in GBM cells, RIOK1 or RIOK2 loss causes a reduction in Akt signaling and provokes p53-dependent apoptosis, cell cycle exit, and chemosensitivity through the RpL11-dependent ribosomal stress checkpoint. Our data demonstrate that the RIO kinases play a key role in Akt-mediated transformation of GBM cells.

Results

A kinome-wide screen for modifiers of glial neoplasia

To discover new genes involved in glial pathogenesis, we performed a genetic screen using our *Drosophila* GBM model. Co-overexpression of constitutively active forms of *Drosophila* EGFR (dEGFRλ) and the PI3K catalytic subunit p110α ($dp110^{CAAX}$) stimulates malignant transformation of post-embryonic larval glia, inducing lethal glial neoplasia (Figure 1A–1C) (characterized in detail in [9]). Using this larval *Drosophila* GBM model, we performed an RNAi-based modifier screen for genes that suppress (inhibit) or enhance (worsen) neoplastic phenotypes caused by constitutive EGFR and PI3K signaling. In this scheme, which is an enhancer-suppressor screen, modifier kinases that block/inhibit fly glial neoplasia when their expression is reduced are referred to as 'suppressors,' and modifier kinases that exacerbate neoplasia when their expression is reduced are referred to as 'enhancers.' This is in keeping with standard *Drosophila* nomenclature, in which genes are classified by their loss-of-function phenotypes. As a side-note, in this context, the term suppressor does not refer to mammalian tumor suppressors.

To enrich for new pathway components, we screened nearly all of the kinases encoded in the *Drosophila* genome (Table S1). Our choice to screen kinases was based on four considerations: (1) kinases regulate a broad array of biological and cellular processes, including those underlying oncogenesis; (2) kinases are highly conserved between *Drosophila* and humans such that every *Drosophila* kinase has a clear human ortholog; (3) as a group, they are well characterized, facilitating functional analysis; and (4) drug discovery efforts are focused on development of specific kinase inhibitors.



J Functional categorization of modifier kinases

Core RTK and PI3K pathways	14
Other signal transduction pathways	8
Cell cycle/growth control/proliferation	37
Translation/ribosome biogenesis	9
Cell migration/shape change	12
Transcription	7
Metabolism	6
Cell viability/apoptosis	5
Ion homeostasis	1
Protein trafficking	1
Human orthologs implicated in GBM	29

Figure 1. A kinome-wide screen for modifiers of EGFR- and PI3K-dependent glial neoplasia. (A) Optical projections of whole brain-nerve cord complexes from late 3rd instar larvae approximately 130 hrs old, displayed at the same scale. Dorsal view; anterior up. GFP labels glia (green). Each brain is composed of 2 hemispheres and a nerve cord. Knockdown of strong suppressor loci decreased brain size, even relative to wild-type controls, as in *CG11859^{dsRNA};dEGFRλ;dp110^{CAAX}* and *CG11660^{dsRNA};dEGFRλ;dp110^{CAAX}* animals. Glial-specific overexpression of ΔEGFR drives increased glial cell numbers, brain enlargement, and lethality, and knockdown of strong suppressor loci grossly decreased brain size relative to controls, as in *CG11859^{dsRNA};ΔEGFR* and *CG11660^{dsRNA};ΔEGFR* animals. (B–H) 3 μm optical projections of brain hemispheres from late 3rd instar larvae approximately 130 hrs old, displayed at the same scale. Frontal sections, midway through brains. Anterior up; midline to left. Glial cell nuclei labeled with Repo (red); glial cell bodies labeled with GFP (green). Brains counter-stained with anti-HRP (blue), which reveals neuropil at high intensity and neuronal cell bodies at low intensity. Dark areas contain unstained neuronal precursor cells. *dEGFRλ;dp110^{CAAX}* (C) and *ΔEGFR* (F) brains showed a dramatic increase in glial cell number (red nuclei, green) relative to wild-type (B). Upon suppression, as in *CG11859^{dsRNA};dEGFRλ;dp110^{CAAX}* (D), *CG11859^{dsRNA};ΔEGFR* (G), and *CG11660^{dsRNA};ΔEGFR* (H), there are few excess glia (red nuclei), and remaining glial cells show abnormal development (green). Reduction in both glial (green) and neuronal cell types (low intensity blue) account for reduced brain size upon CG11660 and CG11859 knockdown in the context of *dEGFRλ;dp110^{CAAX}* or *ΔEGFR*, which suggests that remaining abnormal glia do not properly support neuronal cell survival. Modifier constructs were also tested for effects in wild-type glia, as in *CG11859^{dsRNA}* animals (E). (I) Breakdown of screen results by kinases tested. Unconfirmed modifiers are defined by only one RNAi construct each. (J) Functional classifications of confirmed modifiers. Individual kinases noted in Table S6.
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We tested 553 conditional RNAi constructs targeting 223 of the 243 kinases in the fly genome (Tables S1, S2) [24,25]. RNAi constructs were expressed specifically within the glial lineage and were tested for their phenotypic effects on proliferation, migration, morphology, and/or viability of dEGFRλ;dp110^{CAAX} neoplastic glia. The specificity of modifier loci was confirmed by testing multiple RNAi constructs, dominant negative constructs, and/or available mutant alleles to determine if they produced analogous phenotypes in the *dEGFRλ;dp110^{CAAX}* model (Tables S2, S3). Control assays were performed for the effects of modifier RNAi constructs when expressed specifically in other cell types, including normal glia, neuroblasts (*Drosophila* neural stem cells), and neurons in order to distinguish those RNAi constructs that caused non-specific toxicity from RNAi constructs that caused specific changes in neoplastic glia (Table S4, Text S1). Constructs that caused early organismal lethality in all cellular contexts tested were excluded from analysis (Tables S2, S4).

To test whether modifiers act in oncogenic signaling downstream of specific EGFR mutations found in human GBM, we created flies that overexpress human ΔEGFR. Glial-specific expression of ΔEGFR caused lethal glial neoplasia phenotypes, alone and in combination with dp110^{CAAX}, that were similar to dEGFRλ, and these phenotypes required EGFR kinase activity and core PI3K effectors, such as *dAkt* (Figure 1A–1F, Figure S1, Table S5). We tested modifier RNAi constructs for the ability to alter glial-specific ΔEGFR and *ΔEGFR;dp110^{CAAX}* phenotypes (Table S5); the results mirrored their genetic interactions with *dEGFRλ;dp110^{CAAX}* (Table S2, S5), indicating that modifiers identified in the screen are common to neoplastic phenotypes conferred by both *Drosophila* and human EGFR.

We identified a total of 45 modifier genes (Figure 1I, Tables S2 and S3). Suppressor RNAi constructs targeting 39 genes reduced neoplasia and induced smaller brain size and lower glial cell numbers relative to *dEGFRλ;dp110^{CAAX}* controls, whereas enhancer RNAi constructs targeting 6 genes worsened tumorigenesis and neoplasia and induced increased glial cell numbers, and/or aberrant glial morphologies (Figure 1A–1D, 1I, Tables S2 and S3). Modifiers that suppressed glial neoplasia include genes identified in previous studies, including *dAkt* [9] (Tables S2, S5). A small subset of suppressor constructs caused strong phenotypes in the context of constitutive EGFR-PI3K signaling. Constructs targeting three modifier loci, *Raf*, *Src42A*, and *Taf1*, rescued *dEGFRλ;dp110^{CAAX}* animals to adult viability, allowing neoplastic glia to differentiate and function normally despite the presence of dEGFRλ and dp110^{CAAX} (Table S2). Constructs targeting two of the strongest modifiers, *CG11660* and *CG11859* (the *Drosophila* orthologs of the RIOK1 and RIOK2 kinases, respectively), caused severe reduction in brain size and glial cell number when combined with *dEGFRλ;dp110^{CAAX}*, *ΔEGFR;dp110^{CAAX}*, and/or *ΔEGFR*, as compared to wild-type control animals (Figure 1A–1H, Figure S1). dRIOK2 knockdown gave a stronger effect (Figure 1D, 1G). In contrast, RNAi constructs targeting dRIOK1 and dRIOK2 did not produce a dramatic growth reduction when targeted to normal glia (Figure 1E, Table S4), indicating that dRIOK1 or dRIOK2 knockdown does not simply cause nonspecific cellular toxicity.

Modifier kinases were classified by bioinformatic annotations using Flybase, Gene Ontology, KEGG pathways, the STRING database [26], and comparisons with other *Drosophila* RNAi screens. These classifications (Figure 1J, Figure S2, Table S6) show that kinases with core functions in the RTK and PI3K pathways were highly represented, validating our model and screening methodology [27,28,29]. Notably, very few of our modifiers have emerged from *Drosophila* RNAi screens for cell viability (Table S7) [30], indicating that most of the modifiers are not generically required for cell survival. The largest group of modifiers have roles in cell proliferation (Figure 1J, Table S6), and many of these yielded reduced cell lineages upon knockdown in neuroblasts (Table S4) [31], consistent with known requirements for RTK and PI3K signaling in neural progenitor cells [9,32]. Several of the modifiers involved in cell proliferation, such as *warts* (Table S2), are also components of the hippo pathway, a pathway with a documented role in glial cell proliferation [33]. Broad comparisons with orthologs from species such as yeast (*S. cerevisiae*), revealed modifiers kinases implicated in protein translation, such as dRIOK1 and dRIOK2 [16,20], or in cell shape change and migration. Finally, comparison to human kinases shows that the majority of our modifier kinases have orthologs previously implicated in GBM (Figure 1J, Figure S2, Table S7), leaving a set of 16 novel modifiers, including dRIOK1 and dRIOK2.

Overexpression of RIOK kinases in human GBM correlates with Akt activity

Novel modifier kinases identified in our *Drosophila* screens may represent human kinases directly involved in GBM pathogenesis. Kinases that block fly glial neoplasia when their expression is reduced are of interest because their human orthologs may be promising new targets for therapeutic inhibition. There are 27 human orthologs for the 16 novel *Drosophila* modifier kinases (Table S8). To determine if any of these human kinases are expressed or mutated in GBM, we analyzed tumor genomic databases, proteomic atlases, and GBM cell lines, which provided suggestive evidence that 12 modifier orthologs are subject to genetic alteration and/or elevated gene or protein expression in GBMs (see Text S1, Tables S9 and S10, Figures S3 and S4). Among these, RIOK1 and RIOK2 showed increased protein expression consistent with involvement in GBM. Given that loss of the dRIOKs strongly and specifically blocks growth and survival of EGFR and PI3K mutant glia, and that recent publications suggest that the RIO kinases may contribute to EGFR and/or mTor signaling [21,22], the functional roles of RIOK1 and RIOK2 in GBM were of particular interest.

A range of GBM cells and cell lines were examined to determine how RIOK1 and RIOK2 expression correlated with tumor cell genotype and phenotype. Our analyses showed that RIOK1 and RIOK2 were expressed in PTEN-null U87MG GBM cells and were upregulated in U87MG cells engineered to express ΔEGFR at levels detected in tumors [34] (Figure 2A). RIOK1 and RIOK2 were also upregulated in GBM tumors with EGFR overexpression/mutation as well as activated Akt (Figure 2B), although these correlations were not clear in all specimens. Primary neurosphere cultures, which are composed of neural stem cell-like human GBM cells propagated in EGF-supplemented media [35,36,37], can maintain mutations/gene expression found in their parent tumors [37]. Neurosphere cultures showed strong RIOK1 and RIOK2 expression (Figure 2C); these included neurosphere lines with ΔEGFR, as well as neurosphere lines displaying PDGFRα overexpression, PTEN loss, and/or other mutant forms of EGFR (Figure 2C). In a panel of standard GBM cell lines, RIOK1 and RIOK2 showed strong expression in cell lines known to harbor PTEN and/or EGFR mutations, and RIOK1 and RIOK2 expression was comparatively lower in a GBM cell line with intact PTEN (Figure S5) [38,39]. In contrast, RIOK1 and RIOK2 were nearly undetectable in mixed glial cultures freshly derived from adult human cortex (Figure 2D). Thus, RIOK1 and RIOK2 overexpression appeared to be correlated with RTK mutation/overexpression and/or PTEN loss in GBM tumor cells.

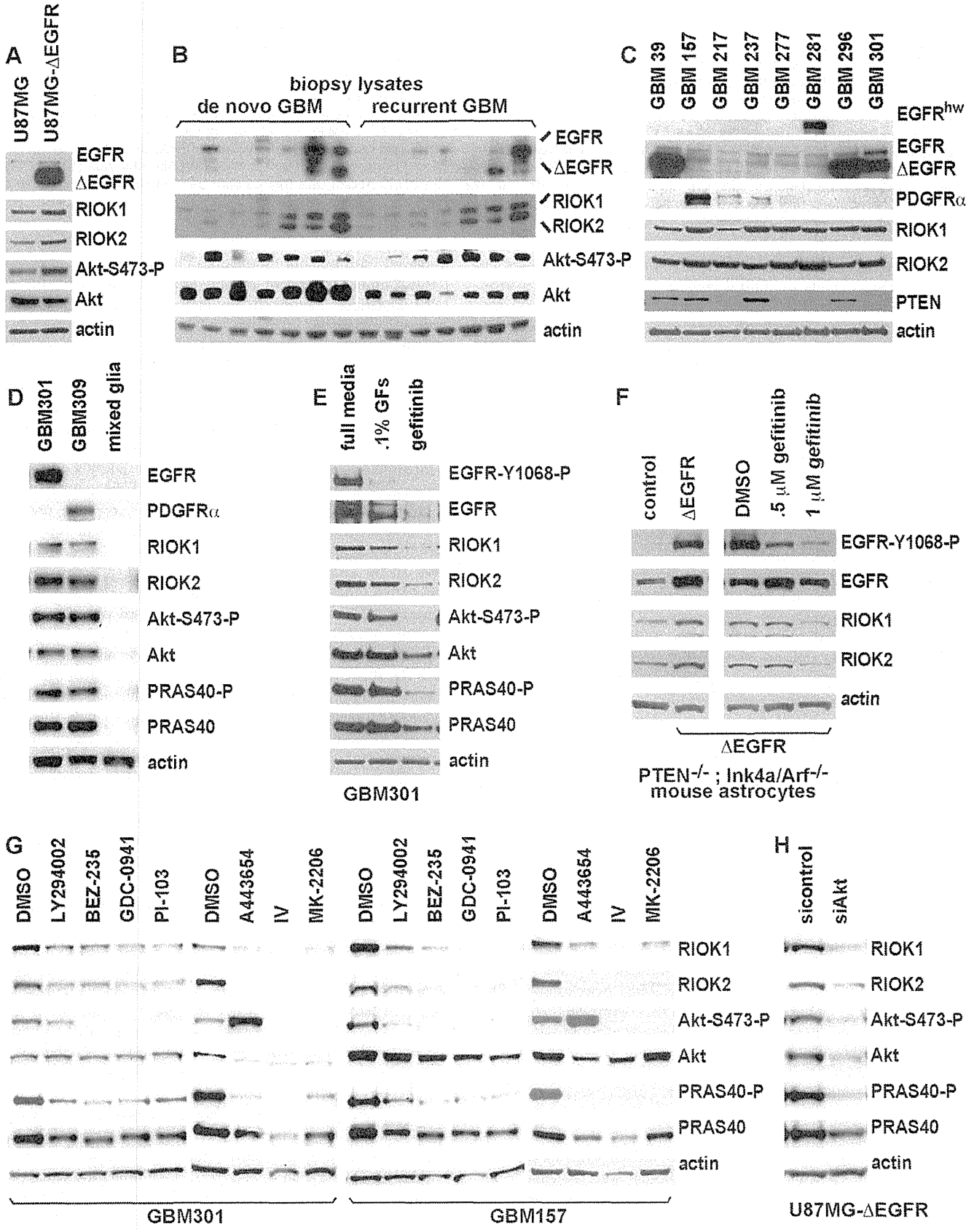

Figure 2. Expression of RIO kinases is associated with EGFR and Akt activity in GBM cells. (A) U87MG and U87MG-ΔEGFR cells cultured with .1% serum for 36 hrs to enrich ΔEGFR signaling. ΔEGFR runs below full-length EGFR. (B) Biopsies of new (de novo) and recurrent GBMs. RIOK1 (~75 kDa) and RIOK2 (~63 kDa) antibodies used serially on the same blot. Serine-473 phosphorylation is a proxy for Akt activation. (C) Primary

neurosphere GBM cultures. GBM39 is isolated from a ΔEGFR-positive serial xenograft [41]. Others are low-passage cultures established from fresh tumors; expression of ΔEGFR, EGFR, or PDFGRα derives from parent tumors [35]. $EGFR^{hw}$ is a high molecular weight (>200 kDa) mutant version detected in GBM 281. (D) RIOK expression and Akt signaling in neurospheres compared with a fresh culture of mixed human glia and astrocytes (established from normal adult cortex) grown under the same conditions. (E) GBM301 treated for 24 hrs with growth factor withdrawl (.1% GFs, .1% of the normal growth factor dosage) or 5 μM gefitinib. GBM301 cells are ΔEGFR-positive, EGFR-amplified, and PTEN-negative (see C). (F) Extracts from *PTEN*$^{-/-}$; *Ink4a/arf*$^{-/-}$ astrocytes transduced with empty vector (left) or ΔEGFR (right), or grown with .5% serum and treated with gefitinib for 24 hrs (far right). EGFR inhibition evidenced by reduced Tyrosine-1068 phosphorylation. (G) Neurosphere cultures treated for 24 hours with DMSO or indicated inhibitors. P110 inhibitors: 50 μM LY294002, 1 μM BEZ-235, 2 μM GDC-0941 [71], 2 μM PI-103 [71]. Akt inhibitors: 1 μM A443654, 10 μM Akt inhibitor IV [40], and 8 μM MK-2206 [72]. Inhibition of PI3K-Akt signaling evidenced by reduced phosphorylation of PRAS40, a direct Akt substrate, and reduced Akt and/or PRAS40 protein levels. With A443654, increased Akt-Ser473 phosphorylation occurs despite Akt inhibition [40]; decreased Akt-Ser473 phosphorylation occurs with Akt inhibitor IV and MK-2206 [72]. (H) U87MG-ΔEGFR cells treated with pan-Akt siRNAs compared to cells treated with nontargeting control siRNAs, harvested 72 hours post-transfection.
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To determine if elevated RIOK1 and/or RIOK2 expression in GBM cells depends on EGFR and/or PI3K signaling, neurosphere lines and U87MG-ΔEGFR cells were treated with relevant inhibitors (Figure 2E and 2G, Figure S6). RIOK1 and RIOK2 levels decreased upon either growth factor withdrawal or gefitinib treatment of primary neurosphere cultures (Figure 2E), indicating that their up-regulation can be EGFR-dependent. Consistent with this, *Pten*$^{-/-}$; *Ink4a/arf*$^{-/-}$ mouse astrocytes transformed by ΔEGFR showed increased RIOK1 and RIOK2 levels, which were reduced by gefitinib treatment (Figure 2F). RIOK1 and RIOK2 protein levels also decreased in neurosphere cells and U87MG-ΔEGFR cells treated with inhibitors of the p110 PI3K catalytic subunit, such as BEZ-235, and inhibitors of Akt, such as A443654 (Figure 2G) [40]. siRNA-mediated Akt knockdown or restoration of PTEN function in U87MG-ΔEGFR cells also reduced RIOK protein levels (Figure 2H, Figure S7). Treatments with p110 and Akt inhibitors also demonstrated that p110 and Akt signaling is required for RIOK1 and RIOK2 expression in PDGFRα-overexpressing neurospheres (Figure 2G). Taken together, these data indicate that RIOK1 and RIOK2 overexpression in GBM cells is driven by Akt activity downstream of RTK mutation/overexpression and/or PTEN loss.

However, the role of factors that act downstream of Akt was less clear (mTor inhibitors did not always reduce RIOK levels, see Figure S6), suggesting that RIOK1 or RIOK2 levels may be directly regulated by Akt. Given that mRNA expression levels of RIOK1 and RIOK2 did not show significant upregulation in tumor samples with PTEN and/or EGFR alterations (Table S9), and given that RIOK1 and/or RIOK2 levels decline after short-term treatments with Akt inhibitors (Figure S7), we hypothesized that Akt signaling may regulate RIOK2 and/or RIOK1 levels by modulating protein stability post-translationally. Consistent with this, addition of a proteosome inhibitor, MG132, prevented the reduction in RIOK1 and/or RIOK2 protein levels observed upon A443654 treatment or PTEN add-back (Figure S7). RIOK2 has several mapped serine phosphorylation sites (www.phosphosite.org), including putative Akt target sites (Figure S8). However, mutation of this single site did not abolish detection of RIOK2 by a phospho-Akt-substrate antibody (Figure S9). Thus, Akt-mediated regulation of RIO kinase levels does not hinge on phosphorylation at a single residue in RIOK2, and likely involves a more complex mechanism that requires more investigation.

To confirm that the RIOKs are expressed in GBM, we performed immunohistochemistry (IHC) for RIOK2 on a group of typed tumor specimens (RIOK1 antibodies were unsuitable for IHC). Xenograft specimens of GBM39, which is ΔEGFR-positive [41], showed RIOK2 expression in tumor cells, with cells displaying diffuse cytoplasmic and sub-surface RIOK2 localization (Figure 3A). In contrast, murine stromal cells had little or no RIOK2 immunoreactivity, although the antibody can detect mouse RIOK2. ΔEGFR-positive and EGFR-overexpressing specimens displayed strong, but sometimes heterogeneous, cytoplasmic RIOK2 immunoreactivity, ranging from the giant cell to the small cell populations (Figure 3B–3E, Figures S10 and S11), with the strongest expression in mitotic cells and densely cellular pseudopallisades (Figure 3C and 3D, Figure S10). Heterogeneity in RIOK2 expression may possibly reflect heterogeneity of RTK expression in tumors [42,43], or may reflect upregulation of RIOK2 in actively cycling cells given the increased immunoreactivity observed in mitotic cells. Cytoplasmic localization of RIOK2 in GBM is consistent with observations of RIO kinase localization in yeast and human cells [16,17,18]. In contrast, RIOK2 did not show appreciable immunoreactivity in neural cells in matched normal control brain (n = 14) (Figure 3F, 3H), and did not show immunoreactivity in tumor stroma (Figure 3B), demonstrating that RIOK2 upregulation is tumor-specific. Akt signaling in tumors was assessed by staining for Akt phosphorylated at Serine-473 (Akt-S473-P, example shown in Figure 3I), and EGFR status of tumors was primarily assessed with staining for EGFR phosphorylated on Tyrosine-1068 (EGFR-Y1068-P, example shown in Figure 3J), which indicates EGFR activation. Statistical analysis demonstrated that RIOK2 expression was significantly correlated with EGFR status in tumor specimens (Figure 3L), although some EGFR-negative tumors also showed RIOK2 immunoreactivity (Figure 3G, 3L), while some EGFR-negative tumors did not (Figure 3K). The correlation of RIOK2 expression with EGFR activity is likely secondary to Akt-mediated regulation of RIOK2: RIOK2-expressing specimens positive EGFR-Y1068-P always showed staining for Akt-S473-P (n = 20). Indeed, all specimens that showed RIOK2 immunoreactivity, whether EGFR-positive or EGFR-negative, showed staining for Akt-S473-P (Figure 3L).

RIOK2 overexpression in astrocytes induces invasive glial tumors and TORC2-Akt activation

To determine if elevated levels of RIOK2 drives oncogenic changes in mammalian cells, we tested the effects of RIOK2 overexpression in astrocytes. *Pten*$^{-/-}$; *Ink4a/arf*$^{-/-}$ murine astrocytes, which are immortalized by tumor suppressor mutations common in GBM, express little endogenous RIOK2 and are not gliomagenic in intracranial grafts assays [44]. In two experiments, mice intracranially grafted with *Pten*$^{-/-}$; *Ink4a/arf*$^{-/-}$ astrocytes overexpressing RIOK2 showed symptoms of hydrocephaly and neurological deficits 3 weeks following implantation, unlike mice grafted with *Pten*$^{-/-}$; *Ink4a/arf*$^{-/-}$ control astrocytes. Histological analysis showed that control *Pten*$^{-/-}$; *Ink4a/arf*$^{-/-}$ astrocytes yielded no intracranial tumors in 12 total animals tested, whereas *RIOK2*^{overexpression}; *Pten*$^{-/-}$; *Ink4a/arf*$^{-/-}$ astrocytes formed invasive high-grade glial tumors composed of invasive spindle-shaped cells in 7 out of 10 total animals tested ($p<.001$ by chi-squared test) (Figure 4A, 4B).

In GBM, ΔEGFR drives strong Akt activation in the context of PTEN loss, and can drive gliomagenic transformation of *Pten*$^{-/-}$;

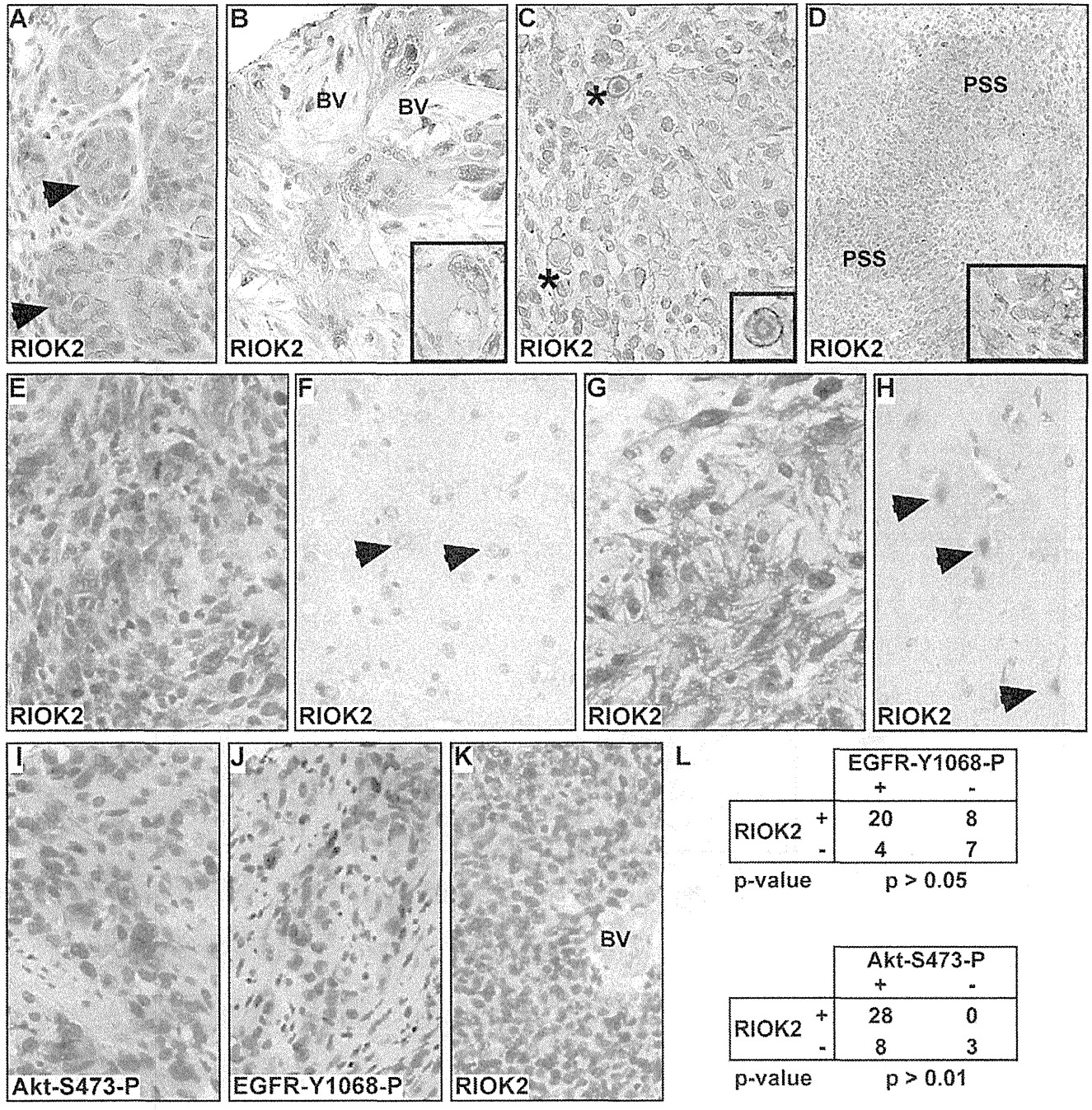

Figure 3. RIOK2 overexpression in GBM tumors is associated with Akt signaling. (A–E) Immunohistochemistry for RIOK2 (reddish brown) showing cytoplasmic and submembraneous enrichment for RIOK2 in tumor cells. Hematoxilin counterstain, (A) GBM39 tissue, from a subcutanteous xenograft, showing RIOK2 staining in tumor cells (arrows), which formed lobules delineated by RIOK2-negative host stromal cells. (B) ΔEGFR-positive human GBM with RIOK2-positive giant cell component (inset shows a conspicuous giant cell), and RIOK2-negative tumor stroma composed of abnormal blood vessels ("BV"). (C) ΔEGFR-positive human GBM, abnormal mitotic cells with high RIOK2 staining denoted with asterisks and shown in inset close-up. (D) ΔEGFR-positive human GBM, lower magnification to highlight enriched RIOK2 in pseudopallisades ("PSS"), inset shows enriched RIOK2 staining present in dense cellular regions of pseudopallisades. (E) RIOK2 expression in an EGFR-overexpressing human GBM with (F) matched normal control tissue from the same surgical specimen, arrows denote normal astrocytes (recognized by their open nuclei). (G) RIOK2 expression in an EGFR-negative/Akt-S473-P-positive GBM shown alongside (H) another example of normal control brain tissue. Arrows denote normal neuronal cells (recognized by their basophilic cell bodies) with low/undetectable RIOK2 expression. (I) and (J) examples of Akt-S473-P and EGFR-Y1068-P immunoreactivity in RIOK2-positive GBM tumor specimens. (K) a RIOK2- negative GBM with a negative abnormal blood vessel ("BV"). (L) Statistical analysis of RIOK2-positive and negative tumor specimens showing a significant correlation between RIOK2 expression and phosphorylation of EGFR at Tyrosine-1068 and phosphorylation of Akt at Serine-473. More stains from tumors shown in Figures S10 and S11.
doi:10.1371/journal.pgen.1003253.g003

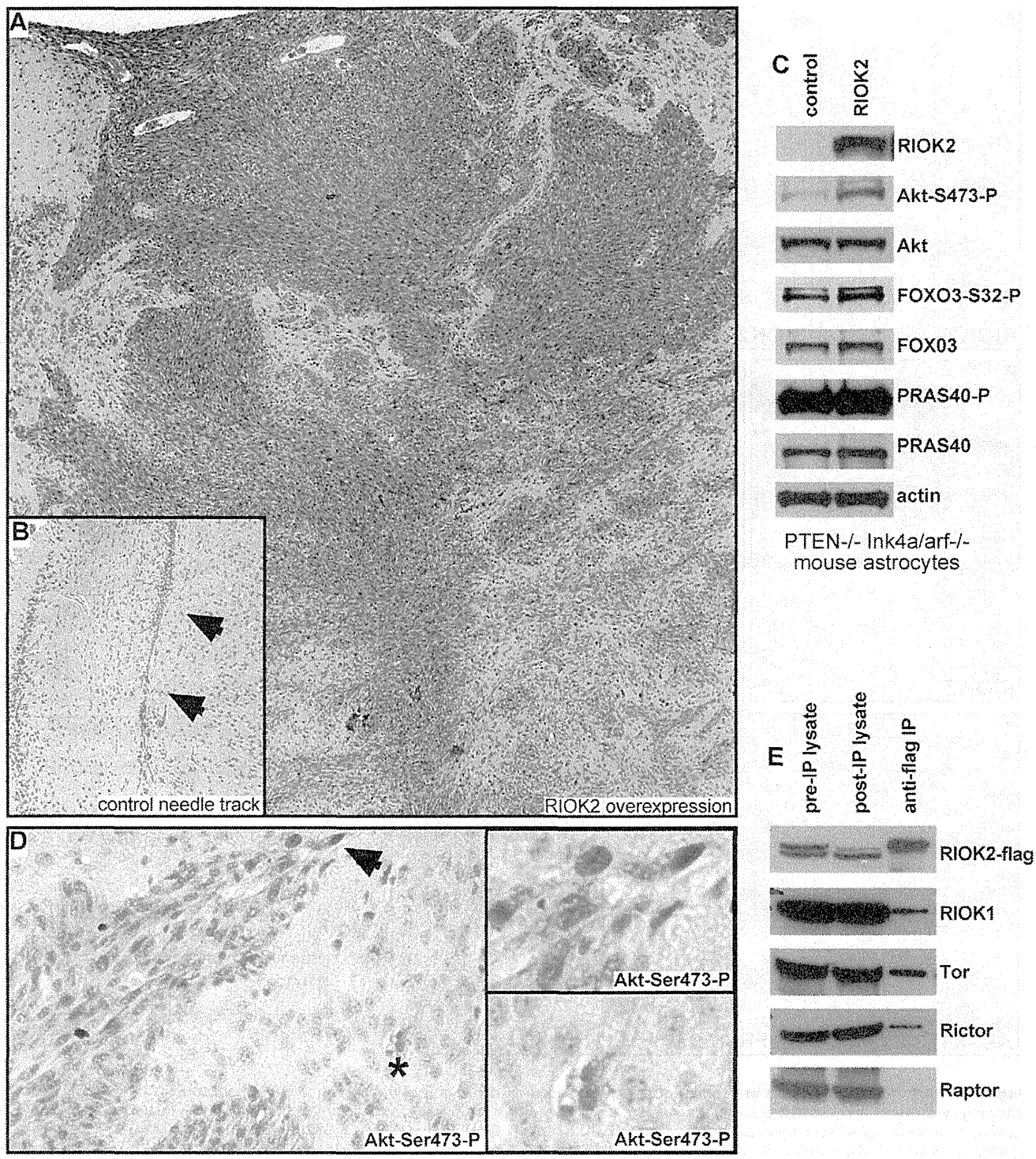

Figure 4. Overexpression of RIOK2 in murine astrocytes promotes tumorigenesis and TORC2-Akt signaling. (A) H&E stain showing high-grade glioma derived from *RIOK2*^{overexpression}; *Pten*$^{-/-}$; *Ink4a/arf*$^{-/-}$ astrocytes grafted into the mouse brain. Tumor cells (purple) generate masses composed of spindle-shaped cells as well as infiltrative neoplastic cells that show invasion into the parenchyma and along blood vessels, animals sacrificed ~19 days following injection. (B) representative needle tract (arrows) in a mouse brain grafted with control *Pten*$^{-/-}$; *Ink4a/arf*$^{-/-}$ astrocytes engineered with empty vector; note the slight concentration of astrocytic cells along the needle tract but no tumor mass or infiltrates. (C) Western blots of *RIOK2*^{overexpression}; *Pten*$^{-/-}$; *Ink4a/arf*$^{-/-}$ astrocytes compared to *Pten*$^{-/-}$; *Ink4a/arf*$^{-/-}$ astrocytes with empty vector, grown *in vitro*. (D) Immunoreactivity for Akt phosphorylated at Serine-473 (reddish brown) in a tumor derived from *RIOK2*^{overexpression}; *Pten*$^{-/-}$; *Ink4a/arf*$^{-/-}$ astrocytes, tumor margin shown, with many surrounding normal cells (purple nuclei, faint staining). Arrow indicates strong staining in invasive cells at tumor margin, asterisk indicates more distant individual invasive cells; both shown in close-up (right). (E) Epitope tagged RIOK2 (RIOK2-flag, runs slightly larger than endogenous untagged RIOK2) was overexpressed in 293T cells and immunoprecipitated along with associated proteins. Blots were probed for indicated proteins, whole lysates from both before (pre-IP) and after (post-IP) are included as a control for protein expression.
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Ink4a/arf$^{-/-}$ astrocytes [6,45]. Thus, we wondered whether RIOK2 overexpression also drives astrocyte transformation by activating Akt. Consistent with this, *RIOK2*^{overexpression}; *Pten*$^{-/-}$; *Ink4a/arf*$^{-/-}$ astrocytes displayed increased phosphorylation of Akt at Serine-473 (Figure 4C), and tumor tissue from *RIOK2*^{overexpression}; *Pten*$^{-/-}$; *Ink4a/arf*$^{-/-}$ cells showed specific staining for Akt-Ser473-P (Figure 4D). Phosphorylation of Serine-473 is required for Akt activity towards select substrates such as FOXO3 [46], a direct Akt substrate that governs GBM cell tumorigenicity [47]. FOXO3 also displayed increased phosphorylation *RIOK2*^{overexpression}; *Pten*$^{-/-}$; *Ink4a/arf*$^{-/-}$ astrocytes (Figure 4C). However, we did not observe increased phosphorylation of all Akt targets, including PRAS40, with RIOK2 overexpression, suggesting that the effect of RIOK2 on phosphorylation of Akt targets was selective to Serine-473 dependent substrates [46].

Akt is phosphorylated on Serine-473 by mTor-complex-2 (TORC2), a multi-protein complex composed of the mTor kinase and several other signaling components, including Rictor [46], a protein that becomes elevated in glioblastomas that also drives gliomagenesis when overexpressed in astrocytic cells [48,49]. In yeast proteomic analyses, Rio2p has been shown to bind to Rio1p and Tor2, the yeast mTor ortholog that forms the equivalent of TORC2 [22]. From human cells overexpressing RIOK2, RIOK2 co-immunoprecipitated with RIOK1, mTor, and Rictor, a protein which is definitive of the TORC2 complex (Figure 4E) [46]. mTor also associates with another complex, mTor-complex-1 (TORC1), which phosphorylates other mTor substrates, such as EIF-4E, and is composed of signaling components including the Raptor protein [46]. Raptor did not co-immunoprecipitate in the RIOK2-RIOK1-mTor-Rictor complex (Figure 4E), suggesting that RIOK2 specifically associates with TORC2. Taken together, these data suggest that RIOK2 directly binds to TORC2 to stimulate phosphorylation of Akt at Serine-473 and activation of Akt towards select substrates, such as FOXO3, and that this process may directly involve RIOK1 recruitment.

Requirement for RIOK kinases for proliferation and survival in GBM cells

Given that knockdown of their *Drosophila* cognates yields growth reduction of neoplastic glia, we tested RNAi constructs targeting human orthologs of novel suppressor kinases for their requirement in GBM cell survival and proliferation (Text S1, Figure S12). Among these, RIOK1 or RIOK2 knockdown yielded strong effects, inhibiting U87MG-ΔEGFR and U87MG proliferation (Figure 5A, 5B). ΔEGFR-positive neurosphere cultures, such as GBM301 and GBM39, also showed a pronounced apoptotic response to RIOK2 or RIOK1 knockdown, with RIOK2 loss yielding stronger effects (Figure 5C, 5D). In U87MG cells, which are dependent on Akt signaling for growth [50], RIOK1 or RIOK2 RNAi provoked G2 cell cycle arrest and reduced proliferation (Figure 5A–5B). The phenotypes caused by RIOK1 and RIOK2 knockdown were observed in other GBM cell lines that are PTEN and/or EGFR mutant, such as A172 (Figure 6, Figure S14, data not shown). Of note, RIOK2 knockdown typically triggered a reduction in RIOK1 expression, regardless of the RIOK2 RNAi constructs used, suggesting that RIOK2 regulates RIOK1 protein levels (Figure 5D, Figure S13, see also Figure 6).

Given that the RIO kinases have been found to stimulate ribosome maturation, we initially suspected that functional reduction of RIOK1 or RIOK2 may cause generic cellular toxicity. However, in testing multiple GBM cell lines that showed strong RIOK expression, we found that a subset of GBM cells were far less affected by knockdown of RIOK1 and RIOK2. GBM6, a ΔEGFR-positive neurosphere line, did not undergo apoptosis upon RIOK1 or RIOK2 knockdown (Figure 5D). Moreover, LNZ308 cells, which are PTEN mutant [38], did not show cell cycle defects or strong reduction of RIOK1 expression with RIOK2 knockdown (Figure 5B). Both GBM6 and LNZ308 are also mutant or null for p53, whereas GBM cells that show cell cycle defects and apoptosis upon RIOK loss, such as U87MG, are wild-type for p53 [38,41]. We observed a similar lack of apoptosis upon RIOK1/RIOK2 loss in other p53 mutant/null GBM cells, such as U373 (data not shown). This implies that the survival and proliferation defects induced by RIOK1 and RIOK2 loss rely on p53. Consistent with this, concomitant knockdown of p53 with RIOK1 or RIOK2 in U87MG cells blocks the apoptosis observed upon RIOK2 or RIOK1 knockdown alone (Figure 5E).

In human cells, RIOK1 and RIOK2 transiently associate with immature cytoplasmic 40S ribosomal subunits to promote their maturation and stimulate rRNA processing, like their yeast counterparts [18,19,22,51]. Defects in ribosome biogenesis and rRNA processing can activate a p53-dependent ribosomal-stress checkpoint to suppress growth and induce cell cycle arrest and apoptosis, a process that relies on p53 upregulation and transcriptional activation mediated by release of the RpL11 ribosomal protein (reviewed in [52,53]). In U87MG cells and other GBM cell lines, RIOK1 or RIOK2 knockdown induced up-regulation of p53 and the p21 cdk inhibitor, a p53 transcriptional target (Figure 5F, Figure S13, S14), and coincident knockdown of RpL11 and RIOK1 or RIOK2 blocked induction of p53 and p21 (Figure 5F and Figure S14). Therefore, RIOK1 or RIOK2 loss leads to p53 activation, which requires the p53-RpL11-dependent ribosomal stress checkpoint.

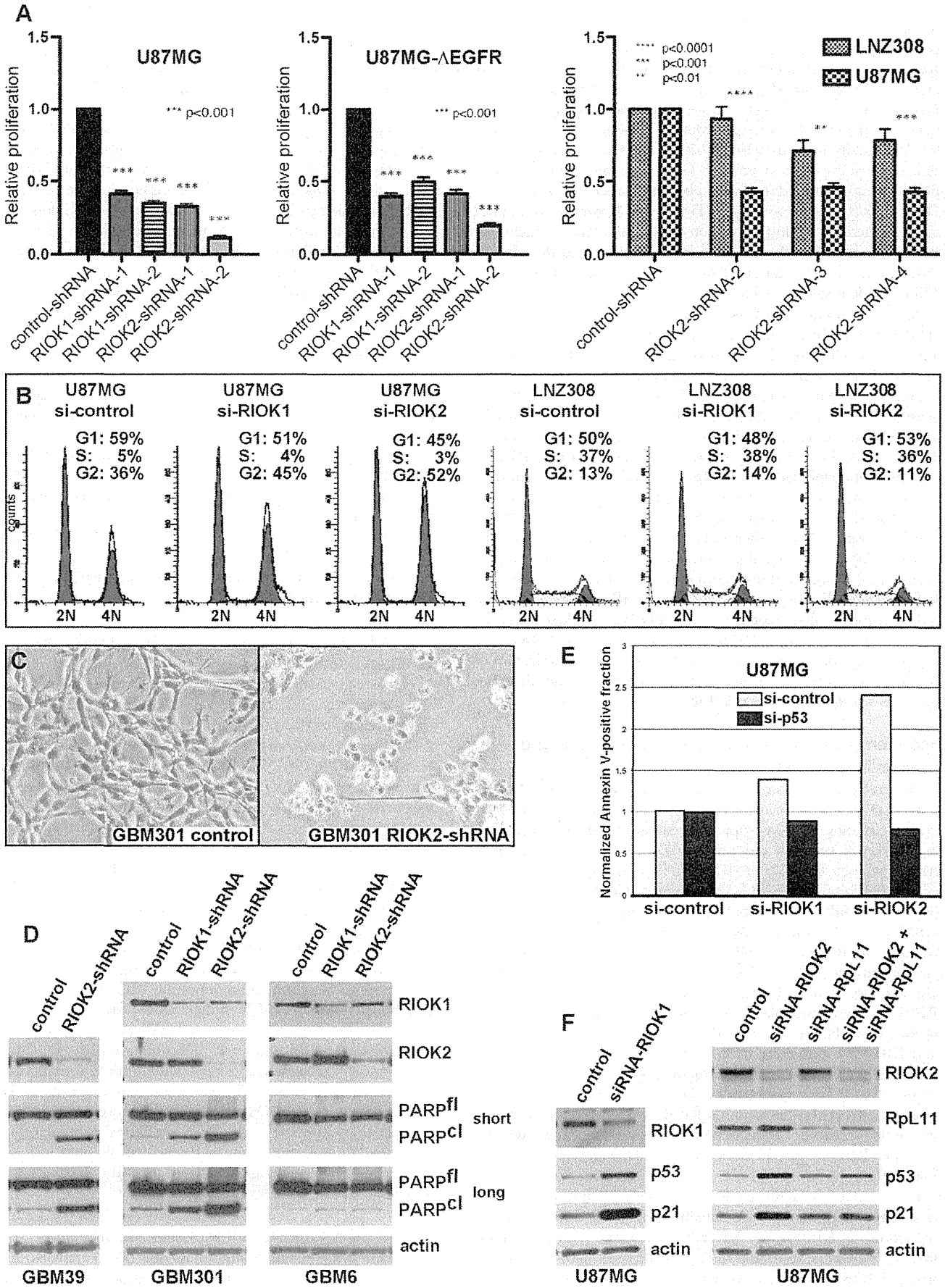
Loss of RIOK function chemosensitizes GBM cells in a p53-dependent manner

p53 is often downregulated in GBM tumors and tumor cells with activated Akt, PTEN loss, and/or EGFR mutation/overexpression [54]. Yet, the majority of tumors with PTEN loss or EGFR mutation/amplification have intact p53 loci (EGFR: 85–92%, PTEN: 92–95%) [55,56]. Given that RIOK loss upregulates p53 levels, we tested whether knockdown of RIO kinases could potentiate the response of GBM cells to treatments with DNA-damaging agents such as doxorubicin, which cooperates with p53 to provoke apoptosis, [57,58] and temozolomide, which is a DNA alkylator used to treat GBM. In GBM cells wild-type for p53, such as GBM301 and U87MG, knockdown of RIOK1 or RIOK2 potentiated apoptotic responses to doxorubicin and/or temozolomide (Figure 6A–6B, Figure S15). In contrast, cells mutant for p53, such as LNZ308 cells, did not show apoptosis upon RIOK1 or RIOK2 knockdown and doxorubicin-temozolomide treatments (Figure 6A). Therefore, inhibition of the RIO kinases chemosensitizes EGFR- and/or PTEN mutant GBM cells.

Our results suggest that elevated p53 activity can potentiate elimination of EGFR and/or PTEN mutant GBM cells. One way to increase p53 levels and activity is with nutlin-3, a small molecule which is know to cause cell cycle arrest and sensitivity to DNA-damaging agents in U87MG cells [59]. However, nutlin-3 did not provoke the same cell cycle defects observed with RIO kinase knockdown, despite inducing high levels of p53 and p21 (Figure S16). Thus, other changes induced by RIO kinase loss must contribute to cell cycle arrest and apoptosis.

Loss of RIOK function antagonizes Akt signaling

We tested for signaling alterations that occur upon RIOK1 or RIOK2 knockdown that would explain reduced proliferation and survival of GBM cells. The caspase inhibitor ZVAD was used to


A
U87MG
U87MG-ΔEGFR
LNZ308
U87MG
Relative proliferation
*** p<0.001
**** p<0.0001
** p<0.01
control-shRNA
RIOK1-shRNA-1
RIOK1-shRNA-2
RIOK2-shRNA-1
RIOK2-shRNA-2
RIOK2-shRNA-3
RIOK2-shRNA-4
B
U87MG si-control
U87MG si-RIOK1
U87MG si-RIOK2
LNZ308 si-control
LNZ308 si-RIOK1
LNZ308 si-RIOK2
G1: 59% S: 5% G2: 36%
G1: 51% S: 4% G2: 45%
G1: 45% S: 3% G2: 52%
G1: 50% S: 37% G2: 13%
G1: 48% S: 38% G2: 14%
G1: 53% S: 36% G2: 11%
counts
2N 4N
C
GBM301 control
GBM301 RIOK2-shRNA
E
U87MG
si-control
si-p53
Normalized Annexin V-positive fraction
si-control
si-RIOK1
si-RIOK2
D
control
RIOK2-shRNA
RIOK1-shRNA
RIOK1
RIOK2
PARPfl
PARPcl
short
long
actin
GBM39
GBM301
GBM6
F
siRNA-RIOK1
siRNA-RIOK2
siRNA-RpL11
siRNA-RIOK2 + siRNA-RpL11
RpL11
p53
p21
U87MG