**Oncogenic dependence of glioma cells on kish/TMEM167A regulation of vesicular trafficking**

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**Abstract**
Genetic lesions in glioblastoma (GB) include constitutive activation of PI3K and EGFR pathways to drive cellular proliferation and tumor malignancy. An RNAi genetic screen, performed in Drosophila melanogaster to discover new modulators of GB development, identified a member of the secretory pathway: kish/TMEM167A. Downregulation of kish/TMEM167A impaired fly and human glioma formation and growth, with no effect on normal glia. Glioma cells increased the number of recycling endosomes, and reduced the number of lysosomes. In addition, EGFR vesicular localization was primed toward recycling in glioma cells. kish/TMEM167A downregulation in gliomas restored endosomal system to a physiological state and altered lysosomal function, fueling EGFR toward degradation by the proteasome. These endosomal effects mirrored the endo/lysosomal response of glioma cells to Brefeldin A (BFA), but not the Golgi disruption and the ER collapse, which are associated with the undesirable toxicity of BFA in other cancers. Our results suggest that glioma growth depends on modifications of the vesicle transport system, reliant on kish/TMEM167A. Noncanonical genes in GB could be a key for future therapeutic strategies targeting EGFR-dependent gliomas.

**Keywords**
EGFR, glia, glioma, kish/TMEM167A, lysosomes, proteasome, vesicular trafficking

**1 INTRODUCTION**

Glioblastoma (GB) is the most common and aggressive cancer of the central nervous system, affecting 3/100,000 people per year (Thakkar et al., 2014). GB has a glial origin characterized by its rapid cell proliferation and its great infiltration capacity. GB evolution correlates with neurological dysfunction and results in the death of the patients in an average period of 14.6 months. The in-depth study of this type of brain cancer is especially relevant as it is resistant to current treatments including surgery, radiotherapy, and chemotherapy (Aldape, Zadeh, Mansouri, Reifenberger, & von Deimling, 2015).

The most frequent genetic lesions in human GB include mutations and/or amplification of the epidermal growth factor receptor (EGFR) gene, present in almost 50% of GB. However, the strategies targeting the tyrosine-kinase activity of this receptor have not resulted in clinical improvements. This might be due to kinase-independent functions of the receptor, to the existence of other alterations in escaping clones, or to the acquisition of secondary mutations in the pathway (Zahonero & Sanchez-Gomez, 2014). In fact, overexpression of other receptor tyrosine kinases (RTKs), or inhibition of neurofibromatosis 1 (NF1) function are also very common in gliomas. They all drive the chronic stimulation of Ras signaling to drive cellular proliferation and migration (Furnari et al., 2007; Gray, Lewis, Maher, & Ally, 2001). Other frequent genetic lesions include the loss of phosphatase and tensin homolog (PTEN), which antagonizes the phosphatidylinositol-3 kinase (PI3K) signaling pathway, and activating mutations in PI3KCA, which encodes the p110α catalytic subunit (Furnari et al., 2007; Gray et al., 2001; von Deimling, Louis, & Wiestler, 1995). Gliomas often show constitutively active AKT, a major PI3K effector. However, EGFR-Ras or PI3K mutations alone are not sufficient to transform glial...
cells, rather multiple mutations that co-activate EGFR-Ras and PI3K/AKT pathways are required to induce a glioma in mouse models (Holland et al., 2000; Read, Cavenee, Furnari, & Thomas, 2009). In Drosophila models, a combination of constitutively active mutant forms of dEGFR (dEGFR\textsuperscript{C}) and dPI3K (Dp110\textsuperscript{CAAX}) effectively causes a glioma-like condition that shows features of human tumors, including glial expansion and invasion (Kegelman et al., 2014; Read, 2011; Read et al., 2009). Moreover, this model has proved to be valuable in finding new kinase activities relevant to the glioma progression (Read et al., 2013).

Vesicle transport plays a central role in cell biology as it provides membranous platforms to assemble specific signaling complexes and to terminate signal transduction (Stasyk & Huber, 2016). In GB, for example, members of the small GTPases involved in cytoskeletal dynamics and vesicle trafficking, are overexpressed and promote tumor progression (Kim et al., 2014; Wang & Jiang, 2013). Several growth factor receptors and adhesion molecules are clients of the vesicular transport, both for proper membrane localization and for degradation in the lysosomes (Mattissek & Teis, 2014). In fact, defective endocytic downregulation of EGFR has been associated with cancer, in particular with gliomas (Jones & Rappoport, 2016). Mechanistically, TMEM167A/kish during fly development. During this genetic combination to the adulthood, we used the thermosensitive repression system Gal80\textsuperscript{T5}. Individuals maintained at 17 °C did not activate the expression of the UAS constructs, after switching the flies to 29 °C, the protein Gal80\textsuperscript{5} is degraded and the expression system UAS/Gal4 activated. Moreover, we used a UAS-GFP\textsuperscript{NLS} reporter to monitor repo cells.

A list of the fly genotypes is detailed in the Supporting Information "Materials and Methods".

2.2 Viability assays

Flies were crossed and progeny was raised at 25 °C under standard conditions. The number of adult flies emerged from the pupae was counted for each genotype. The number of control flies was considered 100% viability and all genotypes are represented relative to controls. Experiments were performed in triplicates.

2.3 Immunofluorescent staining

Third-instar larval or adult brains, were dissected in phosphate-buffered saline (PBS) (Sigma), fixed in 4% formaldehyde for 30 min, washed in PBS + 0.3% Triton X-100 (PBT), and blocked in PBT + 5% BSA (Sigma). Antibodies used were as follows: Mouse Repo (DSHB 1:50), mouse dEGFR (1:100 AE4 DSHB), rabbit dArl8 (1:100 DSHB), rabbit Rab5, Rab7 and Rab11 (1:50 a gift from Marcos González-Gaitan), rabbit GFP (Invitrogen A11122, 1:500), mouse GFP (Invitrogen A11120, 1:500). Secondary antibodies were as follows: Anti-mouse Alexa 488, 647, anti-rabbit Alexa 488, 647. DNA was stained with 2-(4-aminophenyl)-1H-indole-6-carboxamidine (DAPI, 1 µM).

U87 Cells were grown with doxycycline (Dox) in serum over coverslips pretreated with Matigel (BD Biosciences, Franklin Lakes, NJ) and then fixed in 4% paraformaldehyde for 10 min. Cells were blocked for 1 hr in 2% BSA and 1% Triton X-100 in PBS and incubated o/n with rabbit anti-Manosidase II (1:100) (Merck-Millipore, Burlington, MA) and rabbit anti-Calnexin (1:200) (ThermoFisher, Waltham, MA). Secondary antibody used was rabbit-Cy5 (1:100) (Jackson ImmunoResearch, West Grove, PA) and DNA was stained with DAPI.

Vibratome sections from xenografted brains were blocked for 1 hr in 2% (w/v) BSA (Sigma) and 0.2% Triton X-100 in PBS and then stained with primary antibodies overnight.
incubated o/n with rabbit anti-EGFR (1:100) antibody (Cell Signaling) in PBS-BT. Secondary antibody used was rabbit Cy5 (1:100) (Jackson ImmunoResearch) and DNA was stained with DAPI.

2.4 | Patients and tumor samples

Glioma (N = 694) and normal brain (N = 1,136) patient's data from The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) data set were downloaded from the cbioPortal (http://www.cbioportal.org/) and UCSC Xena browser (https://xenabrowser.net).

2.5 | Glioma model

We grew U87 cells from the ATCC (in vitro and in vivo) as previously described (Pozo et al., 2013). We obtained GB1 cells by dissociation of human GB surgical specimens from patients treated at Hospital Ramón y Cajal (Madrid, Spain). The lentiviral vectors pTRIPZ, pTRIPZ-shTMEM167A (a and b) and pTRIPZ-shTRAPPC11(c) (Open-Biosystems) were used to produce conditionally interfered cells. shRNA expression was induced by 1 μg/ml of Dox (Sigma-Aldrich, St. Louis, MO) in vitro or by adding 2 mg/ml Dox in the drinking water in vivo.

2.6 | Glioma cell culture

U-87 MG cells were obtained from the ATCC. GBM1 cells were obtained by dissociation of a human GBM surgical specimen from Hospital 12 de Octubre (Madrid, Spain), after patient's written consent and with the approval of the ethic committee. We digested fresh tissue samples enzymatically using Accumax (Millipore), and then we purified isolated cells by a Ficoll gradient (GE Healthcare, Chicago, IL). U87 MG and GBM1 cells were grown as previously described (Pozo et al., 2013) in complete medium: Neurobasal (Fisher) supplemented with B27 (1:50) (Fisher); Glutamax (1:100) (Fisher); Penicillin-streptomycin (1:100) (Lonza, Basel, Switzerland); 0.4% heparin (Sigma-Aldrich); 40 ng/ml EGF and 20 ng/ml bFGF2 (Peprotech, Rocky Hill, NJ). We passed cells after enzymatic disaggregation using Accumax (Millipore). The lentiviral vectors pTRIPZ, pTRIPZ-shTMEM167A (a and b), and pTRIPZ-shTRAPPC11 (Open-Biosystems) were used to produce conditionally interfered cells. shRNA expression was induced by 1 μg/ml of Dox (Sigma-Aldrich) in vitro or by adding 2 mg/ml Dox in the drinking water of the mice.

2.7 | Mouse Xenografts

All the protocols with animals were reviewed and approved by the Research Ethics and Animal Welfare Committee at our institution (Instituto de Salud Carlos III, Madrid), in agreement with the European Union and national directives.

2.7.1 | Orthotopic xenografts

Stereotactically guided intracranial injections in athymic Nude-Foxn1nu mice (Harlan Iberica) were performed injecting 100,000 U87 or GB1 cells (Control or shTMEM167A) re-suspended in 2 μl of culture medium. The injections were made into the striatum (coordinates: A-P, −0.5 mm; M-L, +2 mm; D-V, −3 mm; related to Bregma) using a Hamilton syringe and the animals were sacrificed at the onset of symptoms. Mice had 2 mg/ml Dox in their drinking water to induce shRNA expression 1 week after the injection. To visualize red fluorescent protein (RFP) reporter expression in the animals, we used an IVIS Spectrum in vivo imaging system (Perkin Elmer, Waltham, MA). We analyzed the survival of nude mice by the Kaplan-Meier method and evaluated with a two-sided log-rank test.

2.7.2 | Heterotopic xenografts

U87 (Control, shTMEM167A (a, b), and shTRAPPC11) cells (1.5 × 10⁶) were resuspended in culture media and Matrigel (BD) (1:10) and then subcutaneously injected in nude mice. When tumors reached a visible size, animals started receiving Dox in the drinking water and the tumor volume was measured with a caliper every 3 day. Tumor volume = 1/2(length × width²).

Animals were sacrificed by cervical dislocation, and the tumors induced were removed and either fixed in 4% PFA for 24 hr or immunofluorescent staining or fresh frozen for RNA extraction.

2.8 | EGFR degradation studies

Growth factor-starved U87 cells were incubated in the presence or absence of Dox (12 hr). Cycloheximide (30 μg/ml) (Sigma-Aldrich) was added to the cells and 1 hr later, EGF (100 ng/ml) was added for the times indicated. We chased the cells on ice and processed cell pellets for WB analysis as previously described (Pozo et al., 2013). Primary antibodies: rabbit anti-EGFR (1:500) (Cell Signaling), rabbit anti-phospho EGFR (1:1000) (Cell Signaling), rabbit anti-phospho-AKT (1:1000) (Cell Signaling), rabbit anti-AKT (1:1000) (Cell Signaling), mouse anti-GAPDH (1:1500) (Santa Cruz, Dallas, TX). Donkey HRP-conjugated anti-mouse, anti-rabbit, and anti-goat (Jackson ImmunoResearch) were used as secondary antibodies. We detected antibody binding with enhanced chemiluminescence with ECL (Pierce) and quantified with ImageJ-gel.

2.9 | Proteasomal inhibitor (MG132) treatment

We resuspended MG132 (Cayman chemical company 10012628, Ann Arbor, MI) in DMSO or just the vehicle DMSO (control) was added to standard cornmeal agar food to a final concentration of 50 μM. Crosses were set up in normal vials and transferred 24 hr later to MG132 or control DMSO vials. We collected, dissected, and processed third-instar larvae as described earlier.

2.10 | LysoTracker assay

We dissected third-instar larval brains in PBS, incubated with LysoTracker Red DND-99 (Invitrogen, Carlsbad, CA) for 5 min, and then fixed in 4% paraformaldehyde for 30 min, washed in PBS and mounted in Vectashield (Vector Laboratories). LysoTracker analysis after fixation of Drosophila tissues is a documented procedure (DeVorkin & Gorski, 2014a, 2014b).

2.11 | Drug studies

Flies were crossed and the progeny grew in 3 ml of standard fly food supplemented with the corresponding compounds:
Brefeldin A (TOCRIS 145E #1231 14 mg/ml) at 1:200 of, 1:500, 1:1,000, or 1:5,000.

Golgicide A (TOCRIS 195E #3584 14.2 mg/ml) at 1:200 of, 1:500, 1:1,000, or 1:5,000.

Monesin (SIGMA Aldrich M5279 34.65 mg/ml) at 1:1,000 of, 1:5,000, 1:10,000 or 1:50,000.

EXO1 (SIGMA Aldrich E8280 14 mg/ml) at 1:200 of, 1:500, 1:1,000 or 1:5,000.

Dynasore (SIGMA Aldrich D7693 3.2 g/ml) at 1:50, 1:100, 1:200 and 1:1,000.

Phenothiazine (SIGMA Aldrich P14831 10 mg/ml) at 1:200 of, 1:500, 1:1,000 or 1:5,000.

We dissolved all compounds in DMSO (14 mg/ml) and we used equivalent amount of vehicle (DMSO) for each case as control.

For studies in heterotopic tumors, we injected wild-type U87 cells and animals started receiving daily intratumoral injections of BFA (0.24 mg/day). We dissolved BFA in Tween80-H2O (TOCRIS 145E #1231). For studies in U87 cells were treated with BFA at 1 μg/μl.

2.12 | qRT-PCRs
We converted RNA (from flies, mouse tumor tissue, human samples, and cultured cells) to cDNA and subjected to qPCR using SYBR Green for detection. We analyzed gene expression data by the ΔΔCt method.

2.13 | Flow cytometry
U-87 neurospheres (Control or shTMEM167Aa) or flank tumors (Control or BFA-treated) were disaggregated into individual cells with Accumax (5 min, RT) and next they were stained with an antibody against the extracellular domain of FITC conjugated to EGFR (ICR10) (Abcam, Cambridge, UK), diluted in PBS-1% BSA (staining buffer) for 30 min on ice. Cells were washed in PBS, treated with propidium iodide (5 μg/ml, Sigma-Aldrich) and analyzed by flow cytometry (FACSalibur, Beckton Dickinson, Franklin Lakes, NJ) using the Flow-Jow software.

2.14 | Imaging
Fluorescent labeled samples (flies, tumor sections, and fixed cells) were mounted in Vectashield mounting media (Vector Laboratories) and images were acquired by confocal microscopy (LEICA TCS SP5).

Images were processed using Leica LAS AF Lite and Fiji (Image J 1.50e) and analyzed by using Imaris 6.3.1 Bitplane Scientific Solutions software. Images were assembled using Adobe Photoshop CS5.1.

2.15 | Image quantification
To quantify the number of glial cells GFP†NLS (Figures 1 and 7), the number of endosomes (Rab5†, Rab7†, Rab11†, and dArl8† puncta; Figures 3 and 5, Supporting Information Figure S4), the number of dEGFR† puncta (Figures 4, 5 and 6, Supporting Information Figure S4) and the number of Lysotracker† puncta (Figure 5), we took advantage of the "spots tool" from the Imaris 6.3.1 Bitplane scientific solutions software. We selected a minimum size and threshold for the puncta in the control samples of each experiment, then we applied these conditions to the analysis of each corresponding experimental sample. For the dEGFR-Endosomal co-localization studies (Figure 5 and Supporting Information Figure S4), we quantified the total number of an endosomal compartment (e.g., Rab† puncta) and the total number of dEGFR puncta, and then applied a co-localization filter (intensity mean of the channel of interest) using the Spots tool from the Imaris 6.3.1 Bitplane Scientific Solutions software.

2.16 | Statistics
To analyze and plot the data, we used Microsoft Excel 2013 and GraphPad Prism 6. We performed a D’Agostino & Pearson normality test and the data found to have a normal distribution were analyzed by a two-tailed t test with Welch-correction. In the case of multiple comparisons, we used a One-way ANOVA with Bonferroni post-test. The data that did not pass the normality test were subjected to a two-tailed Mann–Whitney U test or in the case of multiple comparisons a Kruskal–Wallis test with Dunns post-test. Error bars represent standard error of the mean. To analyze the survival of nude mice, we used the Kaplan–Meier method and evaluated with a two-sided log-rank test. * represents p value ≤.05; ** p value ≤ .01; *** p value ≤ .001. Statistical values of p value > .05 were not considered significant, (n.s.).

Detailed experimental procedures and data analysis are in the Supporting Information.

3 | RESULTS

3.1 | Kish or gryzun knockdown prevent glioma progression
We used the Drosophila glioma model to search for genes expressed in glial cells, implicated in vesicle transport and necessary for tumor progression. Glioma induction in flies results in 100% lethality; thus, we established survival as the read-out to perform the screening. The screening was performed silencing target genes specifically in glial cells driven by the pan-glial driver repo-Gal4 (Casas-Tinto, Arnes, & Ferrus, 2017), most of the RNAi against vesicle transport hits (including Rab family) were lethal after activation in normal glia or enhanced glioma lethality (data not shown). However, we found that the interference of two genes, kish and gryzun, rescued this lethality in 20% and 30% of the cases, respectively (Figure 1a).

To validate our results, we marked glial cells nuclei with GFPNLS. The number of glial/glioma cells (GFP+) was quantified in adult control brains when compared with control brains and a prevention of glioma RNAi. The results showed an increase of glial cell number in glioma brains when compared with control brains and a prevention of glioma cell number increase upon kish or gryzun knockdown (Figure 1b–g). In addition, we observed a significant increase in total GFP fluorescence in animals developing a glioma, which upon kish or gryzun silencing was comparable to control levels in glioma brains (Figure 1h–m).

The results show that kish and gryzun are necessary for glioma progression and that detrimental effects caused by the glioma are...
reduced upon knockdown of either of these genes in glioma cells. In addition, our results indicate that kish or gryzun RNAi expression in wild type glial cells do not induce detrimental effects on brain development as the number of glial cells is similar to control brains (Figure 1g) and 100% of the individuals reached adulthood (Figure 1a columns 3 and 5). Altogether, these data suggest that kish or gryzun downregulation are harmless for normal glia development but they can inhibit glioma growth.

3.2 kish/TMEM167A interference inhibits human glioma growth

The human ortholog of kish is TMEM167A and its five-exon gene is located in chromosome 5. It encodes for a small trans-membrane protein (72 amino acids) with unknown function. The human ortholog of gryzun is transport protein particle complex 11 (TRAPCC11), which gene is located in chromosome 4 and has been associated with membrane trafficking and Golgi apparatus architecture (Bogershausen et al., 2013). To determine the relevance of these two genes in glioma cells, we used shRNA lentiviral plasmids directed toward TMEM167A or TRAPCC11 or a control shRNA (all Dox-inducible), in the well-known U87 glioma cell line. We grew these cells in the absence of serum as floating neurospheres. In this defined media, cells re-express stem cell markers, display high tumorogenic potential and depend on glioma oncogenic signals, like EGFR (Pozo et al., 2013). We injected U87 infected cells into the flank of immunodeficient mice and once tumors were visible, we induced RNA interference, and we measured tumor size twice a week. The results show that shTRAPCC11 RNAi silenced the expression of the target gene and reduced the growth of the tumors, although the reduction in the final tumor volume was not significant after TRAPCC11 downregulation (Supporting Information Figure S1). However, we observed a strong reduction in xenograft growth upon shTMEM167A knockdown with two different shRNA constructs (Figure 2a–d). Final tumor size was significantly smaller after shTMEM167A induction (Figure 6c) and xenograft growth inhibition correlated well with the level of reduction in TMEM167A expression (Figure 6d). Based on these results, we decided to continue our studies on the function of kish/TMEM167A on glioma progression.
FIGURE 2  TMEM167A downregulation impairs human glioma growth. (a) Representative images of heterotopic tumors, visualized with IVIS ILUMINA through RFP expression in tumor cells at day 21 after tumor injection. (b) Control and shTMEM167Aa (with two different shRNA constructs)-infected U87 cells were implanted subcutaneously in nude mice. When tumors became visible, animals were treated with Dox and tumor size was measured once every 3–4 days. The graphs are represented as fold increase in tumor volume (N = 6 for each). (c) Tumor volume quantification at the endpoint. (d) TMEM167A levels determined by qRT-PCR in tumor tissue at the final end point. We used HPRT expression for normalization. (e) Representative image of intracranial U87 tumor growth, visualized with IVIS ILUMINA through RFP expression in tumor cells at day 30 after tumor injection. We implanted control and shTMEM167Aa infected U87 (f) or GB1 (g) cells in the brains of nude mice that were treated with Dox. (f) The Kaplan–Meier survival curves indicates a significant reduction in tumor burden after TMEM167A downregulation (N = 6 for each in f; N = 5 for each in g). (h) TMEM167A expression in Glioma samples (N = 694), compared with normal brain tissue (N = 1,136) (TCGA vs. GTEx data set GB) [Color figure can be viewed at wileyonlinelibrary.com]
To determine glioma proliferation, we analyzed the number of mitosis in the dissected U87-glioma cells. The quantification of these results shows a significant reduction in BrdU incorporation, which suggests a decrease in proliferation after TMEM167A downregulation (Supporting Information Figure S2A). This correlates with the observed decrease in BrdU incorporation when U87 infected cells were incubated with Dox in vitro (Supporting Information Figure S2B).

In addition, we performed intracranial injection of U87 cells (shControl and shTMEM167Aa) in the brain of immunodeficient mice. One week after implantation, animals received Dox in the drinking water. We monitored tumor growth by the expression of the RFP reporter in an IVIS equipment (e.g., see the picture taken at Day 30 post-injection in Figure 6e). The resulting data shows that there is a reduction in tumor growth after TMEM167A downregulation, which correlates with a significant reduction in tumor burden (Figure 6f). In addition, these results were corroborated in a primary glioma cell line (GB1), the results show that the expression of shTMEM167Aa also reduced significantly the growth of the GB1 tumors in situ (Figure 6g).

**FIGURE 3** Kish affects vesicular trafficking in gliomas. (a–l) Confocal images of larval brain sections from third-instar larvae displayed at the same scale. Control, a–d; glioma, e–h; and glioma kish-RNAi i–l; brains were stained with vesicle markers for early endosomes (Rab5), late endosomes (Rab7), recycling endosomes (Rab11) and lysosomes (dArl8). (m–p) Total number of vesicles quantification in all genotypes. We analyzed an average of N = 5 brains for each genotype in each condition. Scale bar size = 5 μm [Color figure can be viewed at wileyonlinelibrary.com]
3.3 kish/TMEM167A is upregulated in human GB

To determine the contribution of TMEM167A to GB, we analyzed the data from The Cancer Genome Atlas database (TCGA). In silico analysis showed that TMEM167A gene is highly expressed in several tumors of the nervous system, including gliomas (Supporting Information Figure S3A). Although no mutations or alterations of this gene have been described in these tumors, we observed a significant upregulation of TMEM167A expression levels in tumor tissue obtained from glioma patients compared with normal brain samples (Figure 6h). Regarding its function, kish/TMEM167A is a resident protein from endoplasmic reticulum (ER) and Golgi apparatus related to secretory pathways (Wendler et al., 2010). In accordance with its vesicular trafficking role in invertebrates, the DAVID gene analysis of the pathways co-upregulated with TMEM167A in tumoral cell lines, showed an association with ER functions, protein transport and extracellular matrix (Supporting Information Figure S3B). Altogether, these results suggest that TMEM167A has a pro-oncogenic function in GB that could affect vesicular trafficking.
As kish has been associated with the control of vesicular trafficking in cell culture, we sought to determine if it could have a similar role in Drosophila glioma models. We used specific antibodies to visualize and detect the total number of early, late, recycling endosomes and lysosomes (Rab5, Rab7, Rab11 and dArl8, respectively). We quantified the images automatically (Imaris). Not surprisingly, gliomas showed a significant increase in the amount of Rab11 (recycling) endosomes and a strong reduction in dArl8 positive lysosomes in comparison with control brains. However, the number of Rab5 (early) or Rab7 (late) endosomes did not show any significant difference (Figure 3a–h, m–p). Gliomas in which kish was interfered showed a restoration of the normal number of recycling endosomes and an increase in the number of lysosomes (even compared with normal brains), with no significant changes in the other endosomal compartments (Figure 3i–l, m–p).

3.5 | EGFR signaling require kish/TMEM167A expression

Interference of kish expression rescued glioma cells proliferation and lethality when dEGFR and dPI3K were affected in glial cells (Figure 1). To determine which mechanisms involved in glioma growth depend on kish, we induced gliomas by overexpressing a constitutively active form of Ras (downstream effector of EGFR) and PI3K. This Ras-induced glioma is independent of dEGFR activity. Figure 4a shows that Ras-induced gliomas are lethal during development, similar to dEGFR-induced gliomas. However, kish depletion in Ras-induced gliomas could
concentration of 50 μM). (e–f) Brain samples with expression of the VCPΔ transgene, which results in proteasomal blockage. (g) Total number of dEGFR+ puncta in all genotypes quantification. N = 12 for each genotype. Scale bar size = 10 μm [Color figure can be viewed at wileyonlinelibrary.com]

not rescue this lethality (Figure 4a). These results suggest that Kish is required for dEGFR protein biology in glioma but is no longer necessary when the activated components of this pathway are downstream.

To determine if the effects of kish downregulation on vesicular trafficking (Figure 3) affect to dEGFR, we quantified the total amount of the receptor (measured as dEGFR positive puncta) in glioma samples expressing kish RNAi. The results showed a reduction of total dEGFR now comparable to control levels (Figure 4b). In addition, we determined dEGFR mRNA levels by quantitative RT-PCR and we observed no significant differences between glioma and glioma expressing kish RNAi (Supporting Information Figure S4A), indicating that kish interference does not reduce UAS-dEGFR transcription but it affects total protein levels of the receptor. Human U87 tumors also show a reduction of EGFR after TMEM167A downregulation (Figure 4c). Moreover, TMEM167A downregulation in the U87 line clearly reduced the number of EGFR-positive cells (Figure 4d) and blocked signaling downstream of the receptor (pEGFR and pAKT in Figure 4e–f). Altogether, these data strongly suggest that kish/TMEM167A controls glioma growth through the regulation of EGFR stability and signaling. However, we cannot discard that in human cells, TMEM167A downregulation might be also affecting other endosomal-dependent signals.

3.6 | dEGFR localization in glioma depends on kish

To confirm the relevance of kish in dEGFR vesicular trafficking, we quantified the co-localization of dEGFR protein with each of the endosomal or lysosomal markers in Drosophila brains. The results indicate that glioma cells accumulate dEGFR protein in early endosomes, where dEGFR is active. In addition, there is an increase of dEGFR protein in the recycling endosomes (Figure Sa,b; Supporting Information Figure S4B–M). Both results together are compatible with an increase in dEGFR signaling in fly glioma cells, in line with the results described in mouse models and human tumors. On the contrary, kish knockdown delocalized the preferential endosomal position of dEGFR and increased its localization in lysosomes (Figure 5a,b; Supporting Information Figure S4B–M), suggesting a perturbation of the endolysosomal system and dEGFR trafficking. At this point, we were wondering if kish knockdown was altering the endo-lysosomal system to promote the degradation of dEGFR in the lysosome. To analyze this, it was necessary to know first if lysosomes in glioma kish knockdown brains were degradative or not. To determine the status of acidic vesicles, we evaluated internal pH with a lysotracker incorporation assay. Glioma brains showed an increase in the number of acidic vesicles, and therefore active lysosomes (lysotracker positive) compared with controls (Figure 5c, d, and g). Upon kish knockdown in normal and glioma brains, lysotracker-positive lysosomes were significantly reduced (Figure 5e–g), suggesting that kish is necessary for the acidification of the lysosomes.

These results suggest that glioma cells displace endosomal EGFR trafficking toward an accumulation in early endosomes and recycling endosomes, favoring EGFR signaling. Besides, kish/TMEM167A silencing reduces EGFR accumulation in early/late and recycling endosomes, and it fuels receptor localization to non-degradative lysosomes.

3.7 | Kish knockdown stimulates dEGFR degradation in the proteasome

The data indicate that glioma samples expressing kish-RNAi have a reduction of total dEGFR protein. These cells accumulate dEGFR in the lysosomes but these are not degradative so we can still detect it. We wondered where were the rest of the receptor from glioma; kish-RNAi brains (see Figure 4b) if they were not degraded in the lysosome. It has been previously shown that kish is required for secretion and it is localized at the ER-Golgi in Drosophila tissues (Wendler et al., 2010). Disruption of protein processing or trafficking through the ER-Golgi leads to unfolded proteins, which stimulate protein degradation via proteasome (Elgaard, Molinari, & Helenius, 1999; Schroder & Kaufman, 2005; Shen, Zhang, & Kaufman, 2004). At this point, we hypothesized that dEGFR could be targeted to degradation in the proteasome due to kish knockdown in glioma brains.

To validate this hypothesis, we blocked the proteasome with MG132, a specific, potent, reversible, and cell-permeable proteasomal inhibitor (Griciuc et al., 2010). Quantification of confocal images shows that, kish RNAi stimulates dEGFR degradation, which is reverted upon proteasomal blockade (Figure 6a–d, g). In addition, we blocked the proteasome genetically through the expression of a
dEGFR knockdown was reverted upon proteasomal block-downregulation is mediated by the proteasome. This suggests an oncogenic dependence mechanism that could be therapeutically exploited. To explore this possibility, we decided to evaluate the impact of a collection of compounds that affect exocytosis and/or endocytosis at different levels (Supporting Information Figure S5). We performed a biased drug screening by feeding the drugs or the vehicle (DMSO) during the whole development to control larvae and larvae bearing a glioma.

The results showed that 20% of glioma flies reached adulthood after BFA treatment as compared with 0% survival in the corresponding glioma larvae exposed to the vehicle control, whereas the rest of the compounds did not have a protective effect (Supporting Information Figure S5). We dissected adult brains from the survivors to determine if BFA was preventing glioma progression. Quantifications of glial cell number from confocal images showed that the gliomas grown in the presence of BFA have a number of glial cells similar to a wt control brain (Figure 7a-c), indicating that this treatment prevents glioma progression and, as a consequence, rescues viability of the animals.

The lactone antibiotic BFA reversibly blocks traffic between the Golgi and ER and within the Golgi stacks, although it also affects the endolysosomal compartment (Lippincott-Schwartz et al., 1991) (Supporting Information Figure S5). To determine the cellular effect of BFA on glioma cells, we performed an analysis of the vesicle trafficking system in glioma third-instar larval brains after DMSO or BFA feeding during the development. The results show that the BFA does not cause an effect on early endosomes (Rab5) but it does increase the number of late endosomes (Rab7) (Figure 7d). In addition, BFA provokes a reduction of recycling endosomes (Rab11) and a significant increase of lysosomes (dArl8) as compared with glioma cells exposed to DMSO. These changes are very similar to the ones observed after kish down-regulation (Figure 3).

To analyze BFA effect in human gliomas, we injected U87 cells in immunodeficient mice. When tumors became visible, we treated the mice with BFA during 7 days (0.240 mg/day) and analyzed the effect of BFA on tumor volume. The quantification of the results shows a strong reduction in glioma growth (Figure 7e). Moreover, BFA (1 μg/μl) induced a decrease in the amount of dEGFR in U87 cells (Figure 7f), similar to what happens after TMEM167A downregulation in the same cell line (Figure 4c,d) or after kish downregulation in flies (Figure 4b). However, TMEM167A downregulation did not induce any of the previously reported BFA effects on Golgi disruption or ER collapse (Lippincott-Schwartz et al., 1991) in U87 cells (Supporting Information Figure S6A-D). These results reinforce the relevance of vesicle transport regulation for GB and suggest that targeting TMEM167A or using BFA derivatives could inhibit glioma growth without undesirable toxic effects on normal cells.

3.8 | Active compounds modulate vesicle transport in glioma cells

Our results with kish/TMEM167A interference show that targeting certain components of the vesicular trafficking machinery can be detrimental for EGFR-dependent gliomas, but not for normal glia. This suggests an oncogenic dependence mechanism that could be therapeutically exploited. To explore this possibility, we decided to evaluate the impact of a collection of compounds that affect exocytosis and/or endocytosis at different levels (Supporting Information Figure S5). We performed a biased drug screening by feeding the drugs or the vehicle (DMSO) during the whole development to control larvae and larvae bearing a glioma.

The results showed that 20% of glioma flies reached adulthood after BFA treatment as compared with 0% survival in the corresponding glioma larvae exposed to the vehicle control, whereas the rest of the compounds did not have a protective effect (Supporting Information Figure S5). We dissected adult brains from the survivors to determine if BFA was preventing glioma progression. Quantifications of glial cell number from confocal images showed that the gliomas grown in the presence of BFA have a number of glial cells similar to a wt control brain (Figure 7a-c), indicating that this treatment prevents glioma progression and, as a consequence, rescues viability of the animals. The lactone antibiotic BFA reversibly blocks traffic between the Golgi and ER and within the Golgi stacks, although it also affects the endolysosomal compartment (Lippincott-Schwartz et al., 1991) (Supporting Information Figure S5). To determine the cellular effect of BFA on glioma cells, we performed an analysis of the vesicle trafficking system in glioma third-instar larval brains after DMSO or BFA feeding during the development. The results show that the BFA does not cause an effect on early endosomes (Rab5) but it does increase the number of late endosomes (Rab7) (Figure 7d). In addition, BFA provokes a reduction of recycling endosomes (Rab11) and a significant increase of lysosomes (dArl8) as compared with glioma cells exposed to DMSO. These changes are very similar to the ones observed after kish down-regulation (Figure 3).

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4 | DISCUSSION

The results presented here show that kish/TMEM167A, a protein previously associated with vesicle transport and secretion (Gershlick et al., 2016; Wendler et al., 2010), is necessary for glioma growth, both in human cell xenografts and Drosophila models. Mechanistically, kish/TMEM167A downregulation alters the endo-lysosomal system.
This results in a change in EGFR localization toward its degradation by the proteasome.

The quantification of the different vesicles in control and interfered cells suggest that kish/TMEM167A participates in different steps of vesicle trafficking, affecting endo-lysosomal acidification and function. Others have reported that excessive luminal alkalization by NHE9 gain-of-function circumvent EGFR turnover and prolongs downstream signaling pathways (Kondapalli et al., 2015). Moreover, the human papillomavirus type 16 E5 oncoprotein activates EGFR and PDGFR (platelet-derived growth factor receptor) with concomitant alkalization of Golgi and endosomes (Di Domenico et al., 2009). Our results suggest that TMEM167A could modulate the transport of newly synthesized proteins from the ER-Golgi to the membrane or to other organelles. Moreover, the alterations in acidic vesicles produced by TMEM167A knockdown may account for the malfunctioning of ER-Golgi trafficking. In that case, the remaining EGFR not degraded by the proteasome, would accumulate in lysosomes due to the lack of degradative capacity of these vesicles.

The screening performed in flies with exo and endocytosis regulators, shed some light into the function of kish/TMEM167A. The rescue effects of kish downregulation could only be mimicked by BFA treatment. In contrast, monensin (which blocks receptor recycling) or phe-nothiazine (which affects lysosomal function), did not inhibit glioma formation, suggesting that the main oncogenic function of kish/TMEM167A is not simply mediated by a blockade of receptor turnover or by altering lysosomal function. BFA is an inhibitor of the Arf1-guanine nucleotide exchange factor (GEF) interaction. It reversibly blocks traffic between the Golgi and ER and within the Golgi stacks, disrupting Golgi morphology (Lippincott-Schwartz, Yuan, Bonifacio, & Klausner, 1989). However, the whole endosomal compartment shows morphological changes in response to BFA treatment, with normal cycling between plasma membrane and endosomes, but with impaired traffic between endosomes and lysosomes (Lippincott-Schwartz et al., 1991). The results presented here indicate that TMEM167A downregulation does not induce Golgi disruption although it has a profound effect in the endo-lysosomal system. This suggests that kish/TMEM167A downregulation could be parallel to the vacuolar effect of BFA, without its ER/Golgi effect. Interestingly, non-tumoral cells can be made resistant to the cytotoxic effect of BFA if the Golgi appearance is preserved, even if the non-Golgi effects are still present (Yan, Colon, Beebe, & Melancon, 1994). The results presented here suggest that kish/TMEM167A downregulation mimic this vesicular effect of BFA, being toxic for glioma cells but not for normal glial cells. This is in line with previous reports indicating that EGFR ligands differentially affect endocytic receptors in neoplastic versus non-neoplastic astrocytes (Hussaini et al., 1999). Altogether, our data suggest that there is a window of opportunity for modulators of vesicular trafficking in gliomas, which would not have a deleterious effect in normal astrocytes although they would be able to inhibit tumor growth, at least for the EGFR-dependent gliomas, which account for more than 50% of them. BFA has an antitumor effect in certain cancer cell lines (Sausville et al., 1996) but it has not passed the preclinical stage of drug development due, in part, to its high toxicity. New BFA derivatives are being tested (Ohashi et al., 2012) but the results indicate that a more effective and less cytotoxic strategy would be to silence TMEM167A (Golan et al., 2015; Zimmermann et al., 2017).

There are no studies on the relevance of the biosynthetic pathway for growth factor receptors in gliomas yet, but it would be interesting to discern if TMEM167A has a general role in the membrane exposure of other receptors, or even in the secretion of relevant extracellular proteins. Future experiments will allow us to distinguish autocrine from paracrine effects of kish/TMEM167A downregulation and to reach a comprehensive understanding of the oncogenic functions of this protein. The data presented here indicate that in fly models, kish is expendable when downstream targets of the receptors (Ras) are active. However, in human glioma cells, we cannot discard that the effects of the conditional depletion of TMEM167A could depend as well on other pathways affected by changes in the endosomal system. PDGFRA, for example, is another key pathogenic receptor in gliomas whose stability and signaling depend on the vesicular trafficking regulation (Chen et al., 2014). Besides, MET receptor mutants, also present in gliomas, require endocytic trafficking to generate oncogenic signaling (Joffre et al., 2011). In any case, the novel approach presented here would take advantage of a general feature that is independent of the acquisition of secondary oncogenic mutations, and therefore potentially relevant for a plethora of de novo and recurrent GBs.

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CONFLICT OF INTEREST

Authors declare that they have no conflicts of interest.

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**SUPPORTING INFORMATION**

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