Oncogenic addiction of glioma cells to the regulation of vesicular trafficking by Kish/TMEM167A

Oncogenic addiction of glioma cells to 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 towards recycling in glioma cells. *kish/TMEM167A* downregulation in gliomas restored endosomal system to a physiological state and altered lysosomal function, fueling EGFR towards degradation by the proteasome. These endosomal effects mirrored the endo/lysosomal response of glioma cells to BrefeldinA (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 a novel oncogene addiction for gliomas, which depends on modifications of the vesicle transport system reliant on *kish/TMEM167A*. Non-canonical genes in GB could be a key for future therapeutic strategies targeting EGFR-dependent gliomas.

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 p110a catalytic subunit of PI3K (Furnari et al., 2007; Gray et al., 2001). 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). Similar results are described in *Drosophila* models, where a combination of *EGFR* and *PI3K* mutations 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, 2014) (Zahonero & Sanchez-Gomez, 2014). Moreover, it has been shown that NHE9 (a Na^+/H^+ exchanger) limits luminal acidification in glial cells to circumvent EGFR turnover, and thus prolongs downstream signaling pathways that drive glioma growth and migration (Kondapalli et al., 2015). Therefore, modulators of RTK vesicular transport are promising targets in these tumors. In fact, therapeutic strategies to induce non-apoptotic cell death in glioma cells are based on endosomal “mis-trafficking” (Pasupuleti, Grodzki, & Gorin, 2015). However, inhibitors of the endo/lysosomal system are still pretty far from preclinical development as they target too common cellular mechanisms with potentially high toxicity at the organism levels (Stasyk & Huber, 2016).

In this work, we have taken advantage of the *Drosophila* glioma model, which allows us to look for novel oncogene addiction mechanisms of tumor cells, as we can simultaneously test the effect of target genes in glioma cells and normal glia. On these premises, we have identified kish/TMEM167A, a protein associated with vesicle transport and secretion (Gershlick, Schindler, Chen, & Bonifacino, 2016; Wendler et al.,

2010), which is necessary for glioma growth in human cells and *Drosophila* models, but not for glial cells during fly development. Mechanistically, *TMEM167A/kish* downregulation alters the endo-lysosomal system, resulting in a change of EGFR localization and the induction of proteosomal EGFR degradation, which prevents tumoral growth. Moreover, *TMEM167A/kish* downregulation mirrors part of the effects of BrefeldinA (BFA), a lactone antiviral that alters the morphology and function of the Golgi apparatus and endosomal compartments in different cell types (Lippincott-Schwartz et al., 1991). We propose vesicle transport as a key property of tumoral cell biology and a target for EGFR-associated GB; this novel strategy takes advantage of a general feature of GB cells, therefore, it could be relevant for a plethora of de novo and recurrent GBs.

Results

kish or *gryzun knockdown prevent glioma progression*

We used the *Drosophila* glioma model to search for genes implicated in vesicle transport necessary for tumor progression. Glioma induction results in 100% lethality thus, we stablished survival as the read-out to perform the screening. 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) (Marta, se puede poner la figura al revés, primero kish y luego gryzun?).

To validate our results we marked glial cells with GFP. The number of glial/glioma cells (GFP+) was quantified in adult control brains, gliomas and gliomas expressing *kish or gryzun RNAi*. The results showed an increase of glial cell number in glioma brains when compared to control brains and a prevention of glioma cell number increase upon *kish* or g*ryzun* knockdown (Figure 1B-F) (solo se muestra kish, cambiad la figura o el texto). Additionally, we observed a significant increase in total GFP fluorescence in animals developing a glioma, which upon *kish* or g*ryzun* silencing was comparable to control levels in glioma brains (Supporting Information Figure S1).

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 100% of the individuals reached adulthood (Figure1A). Altogether, these data suggest that *kish* or g*ryzun* downregulation are harmless for normal glia development but they can inhibit glioma growth.

kish/TMEM167A, but not gryzun/TRAPCC11, interference inhibits human glioma growth

The human orthologue of *kish* is *TMEM167A* and its 5-exon gene is located in chromosome five. It codifies for a small trans-membrane protein (72 amino acids) with unknown function. The human orthologue 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 towards TMEM167A or TRAPCC11 or a control shRNA (all Doxycycline-inducible), in the well-known U87 glioma cell line. These cells were grown in the absence of serum as floating neurospheres. In this defined media, cells re-express stem cell markers, display high tumorogenic potential and respond to glioma molecules, like EGFR (Pozo et al., 2013). U87 infected cells were injected into the flank of immunodeficient mice and once tumors were visible, RNA interference was induced and tumor size was measured twice a week. The results show that shTRAPCC11 RNAi silenced the expression of the target gene, but did not reduce significantly the growth of the tumors (Supporting Information Figure S2). However, there was a strong reduction in xenograft growth upon *shTMEM167A* knockdown (Figure 2A-D). Final tumor size was significantly smaller after *shTMEM167A* induction (Figure 2C) and, xenograft growth inhibition correlated well with the level of reduction in *TMEM167A* expression (Figure 2D). Based on these results, we continued our studies on the function of kish/TMEM167A on glioma progression.

To determine glioma proliferation, we analyzed the number of mitosis in the dissected U87-glioma cells. The quantification of these results show a significant reduction in BrdU incorporation, which suggests a decrease in proliferation after *TMEM167A* downregulation (Supporting Information Figure S3A). This correlates with the observed decrease in BrdU incorporation when U87 infected cells were incubated with Dox *in vitro* (Supporting Information Figure S3B).

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. Tumor growth was monitored by the expression of the RFP reporter in an IVIS equipment (Figure 2E). 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 2F).

Kish/TMEM167A is upregulated in human GB

To determine the contribution of *TMEM167A* to GB, we analyzed the data from the The Cancer Genome Atlas database (TCGA). Our *in silico* analysis showed that *TMEM167A* gene is highly expressed in several tumors of the nervous system, including gliomas (Supporting Information Figure S4A). 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 GB patients (Figure 2G). 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 gliomas, showed an association with ER functions, protein transport and extracellular matrix (Supporting Information Figure 4B). Altogether, these results suggest that TMEM167A has a pro-oncogenic function in GB that that could affect vesicular trafficking.

kish modulates vesicular trafficking in gliomas

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). The images

were quantified 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 to normal brains), with no significant changes in the other endosomal compartments (Figure 3I-L, M-P).

EGFR signaling is affected by kish RNAi

Interference of *kish* expression rescued glioma cells proliferation and lethality when EGFR and PI3K 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 EGFR activity. Figure 4A shows that Ras-induced gliomas are lethal during development, similar to EGFR-induced gliomas. However, *kish* depletion in Ras-induced gliomas could not rescue this lethality (Figure 4A) (se podría hablar de gryzum en el texto, pero queda raro, yo lo quitaría de la figura para no liar). These results suggest that *Kish* is required for EGFR protein biology in glioma but is no longer necessary when downstream components of this pathway are activated.

EGFR localization in glioma depends on kish

As we mentioned above, EGFR signaling and recycling depend on the endo-lysosomal system. In order to determine if *kish* could be altering the localization of the receptor, we used specific antibodies to visualize the total amount of EGFR protein in *Drosophila* brains. As expected, glioma brains have a significant higher amount of EGFR (measured as positive puncta). However, glioma samples expressing *kish* RNAi showed a reduction

of total EGFR similar to control levels (Figure 4B). Additionally, we determined *EGFR* mRNA levels by quantitative RT-PCR and we observed no significant differences between glioma and glioma expressing *kish* RNAi (Supporting Information Figure 5A), indicating that *kish* interference does not reduce UAS-*EGFR* transcription.

Next, we decided to study the relevance of kish in EGFR vesicular trafficking. We quantified the co-localization of EGFR protein with each of the endosomal or lysosomal markers. The results indicate that glioma cells accumulate EGFR protein in early endosomes, where EGFR is active. In addition, there is an increase of EGFR protein in the recycling endosomes (Figure 4C-D; Supporting Information Figure 5B-M) (quitar el asterisco de la gráfica 4C, ya está puesto en la 4B). Both results together are compatible with an increase in EGFR signaling in fly glioma cells, as it has been described in mouse models and human tumors. On the contrary, *kish* knockdown delocalized the preferential endosomal position of EGFR and increased its localization in lysosomes (Figure 4C-D; Supporting Information Figure 5B-M), suggesting a perturbation of the endo-lysosomal system and EGFR trafficking. At this point, we were wondering if *kish* knockdown was altering the endo-lysosomal system to promote the degradation of EGFR 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 +) compared to controls (Figure 4E, F, J). Upon *kish* knockdown in normal and glioma brains, lysotracker-positive lysosomes were significantly reduced (Figure 4H-J), suggesting that *kish* is necessary for the acidification of the lysosomes.

These results suggest that glioma cells displace endosomal EGFR trafficking towards an accumulation in early endosomes and recycling endosomes, favoring EGFR signaling. Besides, *kish/TMEN167A* silencing reduces EGFR accumulation in early/late and recycling endosomes, and it fuels receptor localization to non-degradative lysosomes.

Kish knockdown stimulates EGFR degradation in the proteasome

The data indicate that glioma samples expressing *kish-RNAi* have a reduction of total EGFR. These cells accumulate EGFR in the lysosomes but these are not degradative so we can still detect it. We wondered where were the rest of the EGFR 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 (Ellgaard, Molinari, & Helenius, 1999; Schroder & Kaufman, 2005; Shen, Zhang, & Kaufman, 2004). At this point, we hypothesized that EGFR could be targeted to degradation in the proteasome due to *kish* knockdown in glioma brains.

To check this hypothesis, we block 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 EGFR degradation, which is reverted upon proteasomal blockage (Figure 5A-D, G). Additionally, we blocked the proteasome genetically through the expression of a dominant negative form of VCP (*UAS-VCP*^{QQ}) (Rumpf, Lee, Jan, & Jan, 2011) and we obtained similar results. Again, EGFR degradation stimulated by *kish* knockdown was reverted upon proteasomal blockage by VCP^{QQ} (Figure 5A-B, E-G). These data confirm that most of the reduction of EGFR after *kish* downregulation is mediated by the proteasome.

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 addiction mechanism that could be therapeutically exploited. To further 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 S6). We performed a biased drug screening by feeding the drugs or the vehicle (DMSO) during the whole development to control flies and flies with glioma.

The results showed that 20% of glioma flies reached adulthood after BFA treatment as compared to 0% survival in the corresponding vehicle control, whereas the rest of the compounds did not have a protective effect (Supporting Information Figure S6). 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 control brain (Figure 6A-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 endo-lysosomal compartment (Lippincott-Schwartz et al., 1991) (Supporting Information Figure S6). To determine the cellular effect of BFA on glioma cells, we performed an analysis of the vesicle trafficking system in glioma brains after DMSO or BFA feeding. 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) (Fig 6D). In addition, BFA provokes a reduction of recycling endosomes (Rab11) and a significant increase of lysosomes (dArl8) as compared to glioma cells exposed to DMSO. These changes are very similar to the ones observed after kish downregulation (Figure 3).

To analyze BFA effect in human gliomas we injected U87 cells in immunodeficient mice and, once tumors became visible, we performed daily injections of this compound. Figure 6E shows that there was a strong reduction in glioma growth in the presence of BFA. Interestingly, both BFA and shTMEM167A induce a significant decrease in the amount of EGFR-positive U87 cells (Figure 6F-G), similar to what happens in flies after kish downregulation (Figure 4B). (falta cambiar el orden e incluir la citometría en células U87). 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 S7A-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.

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 that ends up being partially degraded by the proteasome.

~~A recent work has demonstrated a key function for TSSC1 (tumor suppressing sub chromosomal transferable fragment candidate gene 1) in the endosomal trafficking (Blomen et al., 2015). TSSC1 interacts with *TMEM167A* and several other golgi/vesicular proteins and it has been involved in the retrograde transport at the trans-Golgi network (TGN) and in the recycling to the plasma membrane (Gershlick et al., 2016).~~ (esto no sé si ponerlo, creo que lía más) 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 mal-functioning 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.

~~Surface levels of EGFR are controlled by the rates of EGFR endocytosis, recycling, and lysosomal degradation. Typically, prolonged exposure to EGF results in degradation of EGFR, which serves to prevent excessive stimulation of the cell with mitogens. Nonetheless, degraded EGFR is replaced by newly synthesized protein from the ER to maintain homeostasis. Biosynthetic EGFR trafficking was presumed to be a constitutive and unregulated process. However, recent findings suggest regulatory mechanisms that integrate EGFR degradation and transport, thus maintaining EGFR levels at the plasma membrane (Scharaw et al., 2016). This process is mediated by members of the inner coat protein complex II (COPII) in response to EGF stimulation (Scharaw et al., 2016). We already know that there is a significant correlation of messenger level of the receptor and *TMEM167A* in glioma xenografts, hence, it would be reasonable to explore if ligand stimulation of EGFR induces *TMEM167A* expression.~~ Creo que ahora no viene mucho a cuento.

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. On the other hand, monensin (which blocks receptor recycling) or Phenothiazine (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, Bonifacino, & 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). ~~In fact, BFA is a well-known inhibitor of the transport of molecules out of the ER/Golgi, commonly used as an inhibitor of secretion and exosome formation (Oh-Hashi, Tanaka,~~

~~Koga, Hirata, & Kiuchi, 2012; Tseng et al., 2013).~~ (he quitado lo de la secrección de la figura y de la discusión, para simplificar). 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 could mimic this vesicular effect of BFA, being toxic for glioma cells but not for normal glial cells. This confirms 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). In addition, the non-oncogenic addiction could include other receptors such as PDGFRA, another key pathogenic factor gliomas, as its stability and signaling depend on the vesicular trafficking regulation (Chen et al., 2014). Besides, MET receptor mutants require endocytic trafficking to generate oncogenic signaling (Joffre et al., 2011). 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, consequently to understand the oncogenic function of this protein. What we know from the fly models is that *kish* is expendable when downstream targets of the receptors (Ras) are active. Regardless, the novel approach presented here takes 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.

Material and methods

Fly stocks

Flies were raised in standard fly food at 25ºC. A list of the fly stocks used in this study is detailed in the supplementary Materials and methods.

Viability assays

Flies were crossed and progeny was raised at 25ºC under standard conditions. The number of adult flies emerged from the pupae were 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.

Patients and tumor samples

TCGA GB patients data-set (n = 588) from The Cancer Genome Atlas (TCGA) were downloaded from the cBioPortal (http://www.cbioportal.org/) and UCSC Xena browser (https://xenabrowser.net).

Glioma model

U87 cells from the ATCC were grown (*in vitro* and *in vivo*) as previously described (Pozo et al., 2013). 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 doxycycline (Dox) (Sigma-Aldrich) in vitro or by adding 2 mg/ml Dox in the drinking water in vivo.

Imaging

Fluorescent labelled 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 analysed by using Imaris 6.3.1 Bitplane scientific Solutions software. Images were assembled using Adobe Photoshop CS5.1.

qRT-PCRs

RNA (from flies, mouse tumor tissue, human samples and cultured cells) was converted to cDNA and subjected to qPCR using SYBR Green for detection. Gene expression data were analyzed by the ΔΔCt method.

Statistics

Data was analysed and plotted using Microsoft Excel 2013 and GraphPad Prism 6, using two-tailed Student's t test or Anova 1 way statistical analysis. The data in the graphs are presented as the means ± SEM.

The survival of nude mice was analyzed by the Kaplan-Meier method and evaluated woth a 2-sided log-rank test. * represents *P value* ≤ 0.05; ** *P value* ≤ 0.01; *** *P value* ≤ 0.001. Statistical values of *P value* >0.05 were not considered significant, (n.s.).

Detailed experimental procedures and data analysis are in the Supplemental Material.

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Figure legends

Figure 1. *Kish or gryzum* knockdown prevent EGFR-dependent glioma progression.

(A) The viability assay shows that the lethality induced by the glioma is partially rescued upon inhibition of *kish* or *gryzun* expression. (A minimum of 50 animals were quantified for each genotype). (B-D) Confocal images from *Drosophila* adult brains displayed at the same scale. Glia are labeled with nuclear GFP (green) driven by *repo-Gal4*. Each brain is composed of 2 symmetrical optic lobes attached to the central brain. The quantification of the number of glial cells is shown in (F) N=9 for each genotype. Scale bar size=50µm (falta la barra de escala en la figura).

Figure 2. *TMEM167A* downregulation impairs human glioma growth.

(A) Representative images of heterotopic tumors, visualized with IVIS ILUMINA through RFP expression in tumor cells. (B) Control and shTMEM167Aa-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) Quantification of tumor volume at the final endpoint. (D) *TMEM167A* levels determined by qRT-PCR in tumor tissue. *HPRT* expression was used for normalization. (E) Representative image of intracranial U87 tumor growth, visualized with IVIS ILUMINA through RFP expression in tumor cells. (F) The Kaplan-Meier survival curve indicates a significant reduction in tumor burden after *TMEM167A* downregulation (N=6 for each). (G) *TMEM167A* expression in GB samples, compared to normal brain tissue (TCGA dataset GB).

Figure 3. *Kish* affects vesicular trafficking in gliomas

(A-L) Confocal images of larval brain sections from 3rd 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) Quantification of the total number of vesicles in all genotypes. An average of N=5 brains were analyzed for each genotype in each condition. Scale bar size=5μm.

Figure 4. EGFR stability and distribution in glioma depends on kish.

(A) The viability assay shows that the lethality induced by the glioma is partially rescued upon inhibition of *kish* or *gryzun. UAS-dp110^{CAAX}* and *UAS-Ras^{V12}* expressing glioma is also lethal but the *kish* or *gryzun* inhibition is not efficient to rescue viability (A minimum of 50 animals were quantified for each genotype). (B-D) Control, glioma and glioma *kish-RNAi* brains were stained with anti-EGFR (green) and vesicle markers (red) for Early endosomes (Rab5), Late endosomes (Rab7), Recycling endosomes (Rab11) and Lysosomes (dArl8). (B) Quantification of the total number of EGFR puncta. (C) The co-localization of EGFR with vesicle markers was assessed in control, glioma and glioma *kish-RNAi* brains. The statistical significance of EGFR colocalization in endosomal vesicles is shown in (D). (E-I) Confocal images of larval brain sections from 3rd instar larvae. Control, glioma, *kish-RNAi* and glioma;*kish-RNAi* brains were stained with Lysotracker to assess vesicle acidification. (J) Quantification of the total number of Lysotracker+ puncta in all genotypes. N=9 for each genotype. Scale bar size=10μm.

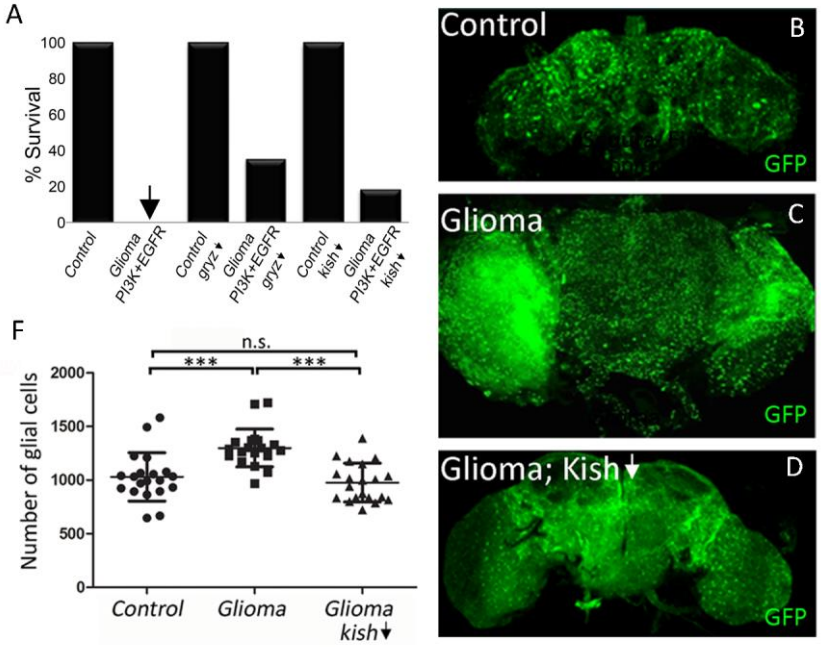
Figure 5. *Kish* knockdown stimulates EGFR proteasomal degradation

(A-F) Confocal images of larval brain sections from 3rd instar larvae. Glioma and glioma *kish-RNAi* brains were stained with EGFR (grey). (A-B) brain samples exposed to the vehicle DMSO. (C-D) brain samples exposed to the proteasomal inhibitor (MG132 at a concentration of 50 μM). (E-F) brain samples with expression of the *VCP^{QQ}* transgene, which results in proteasomal inhibition. (G) Quantification of the total number of EGFR + puncta in all genotypes. N=12 for each genotype. Scale bar size=10 μm.

Figure 6. BFA impairs glioma growth and modulates vesicle transport in glioma cells.

Adult flies brain displayed at the same scale. Glia are labeled with GFP (green) driven by *repo-Gal4*. (A-C). In *repo>dEGFR$^{\lambda}$; dp110^{CAAX}* (glioma) brains exposed to BFA (B), the number of glial cells is similar to *wt* control (A). The quantification of the number of glial cells is shown in (C) N=6 for each genotype. Scale bar size=50 μm. (D) Control and BFA-treated glioma brains were stained with vesicle markers for Early endosomes (Rab5), Late endosomes (Rab7), Recycling endosomes (Rab11) and Lysosomes (dArl8). Quantification is shown in the graph. An average of N=5 brains were analyzed for each genotype in each condition. (E) U87 cells were implanted into the flank of nude mice. One group of mice was treated with BFA every day during seven days (0.240mg/day). Tumor size was measured once every 2 days and represented as fold change. (N=6 for each). (F-G) Quantification of EGFR levels in the membrane (meassure by flow citometry) in control U87 cells and cells treated with BFA (1μg/μl) (N=3) (F) and shControl and shTMEM167A cells in the presence of Dox (N=3) (G).

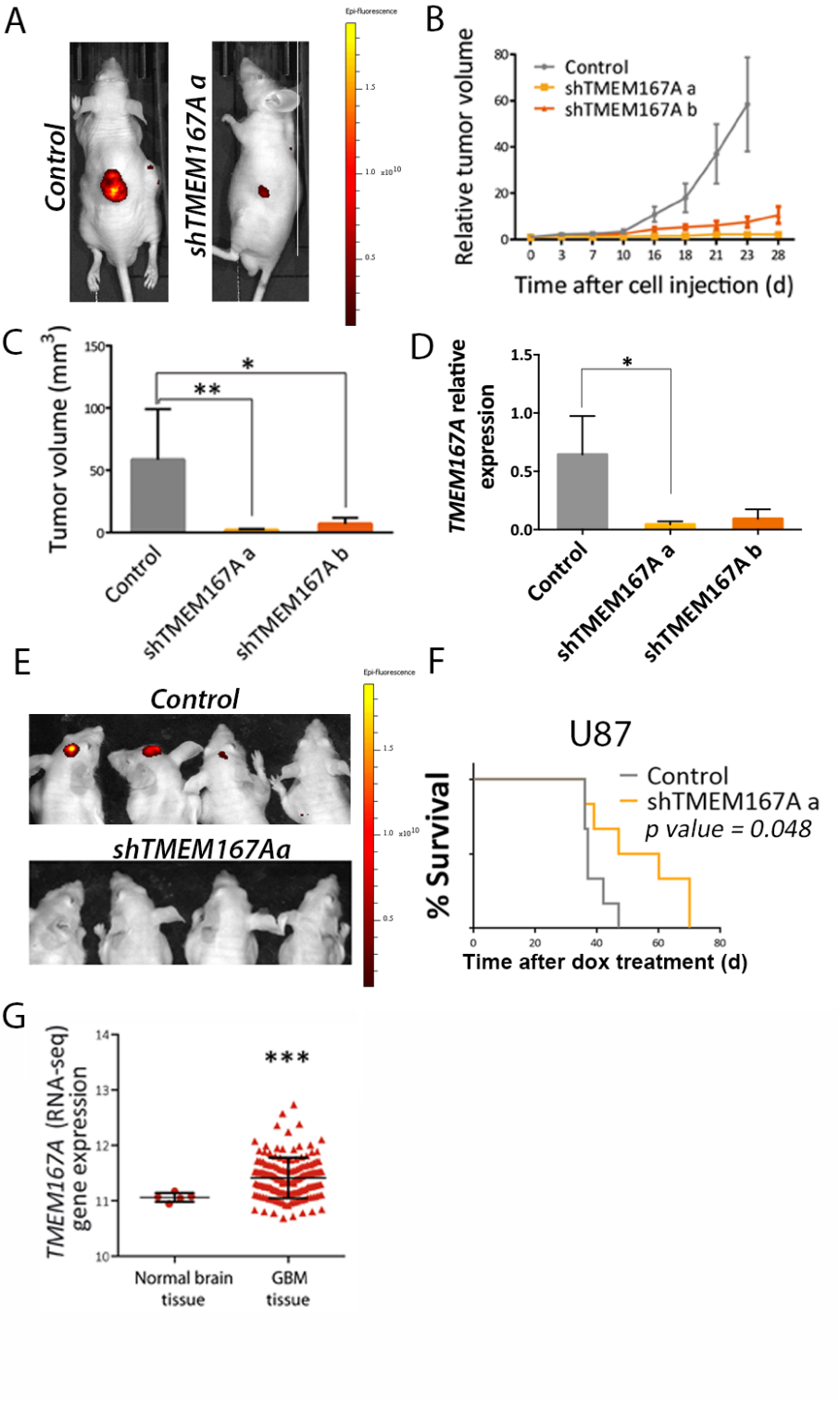
Portela_Fig1



E

Glioma Gryzun RNAi

Portela_Fig2



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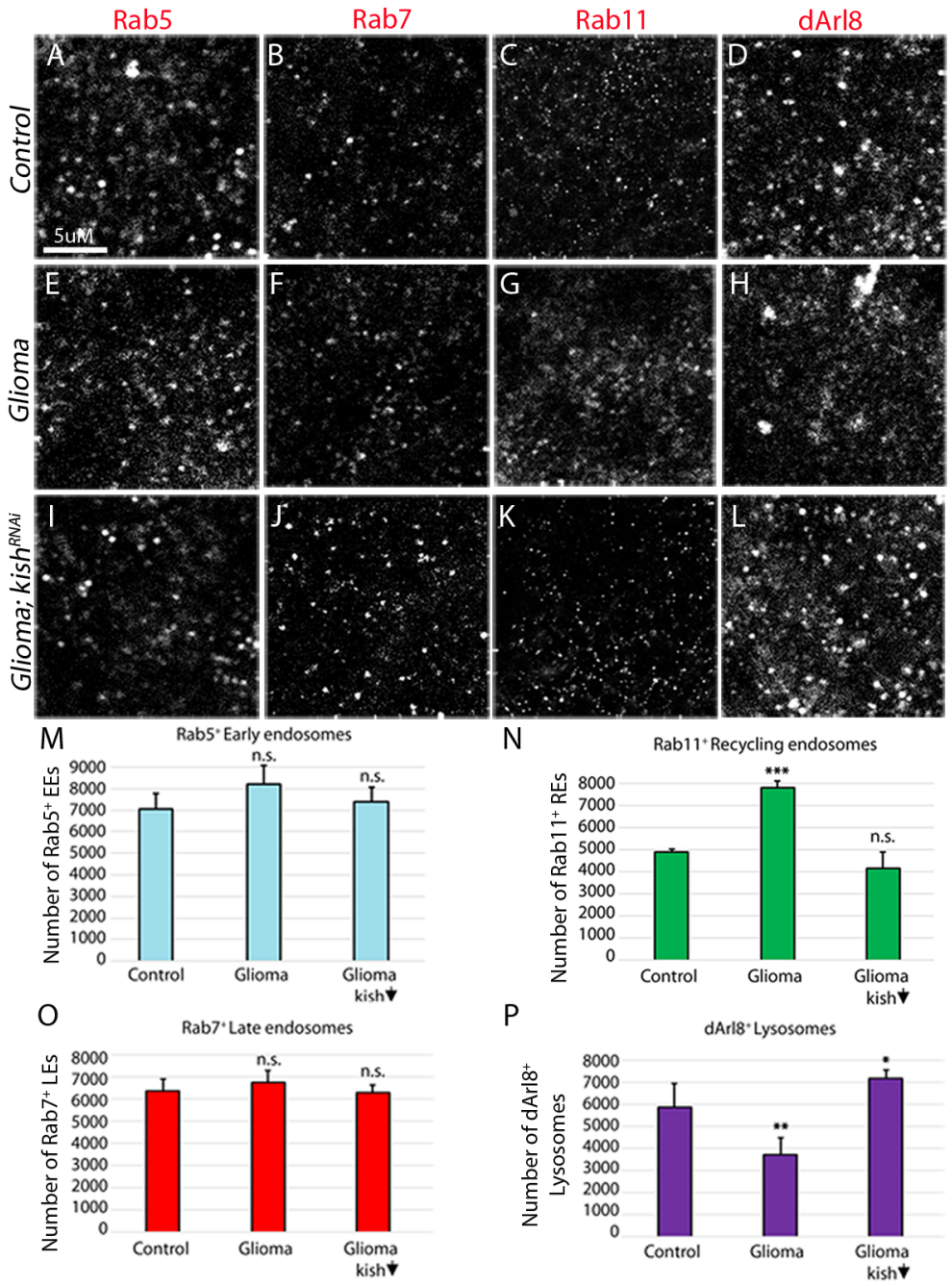
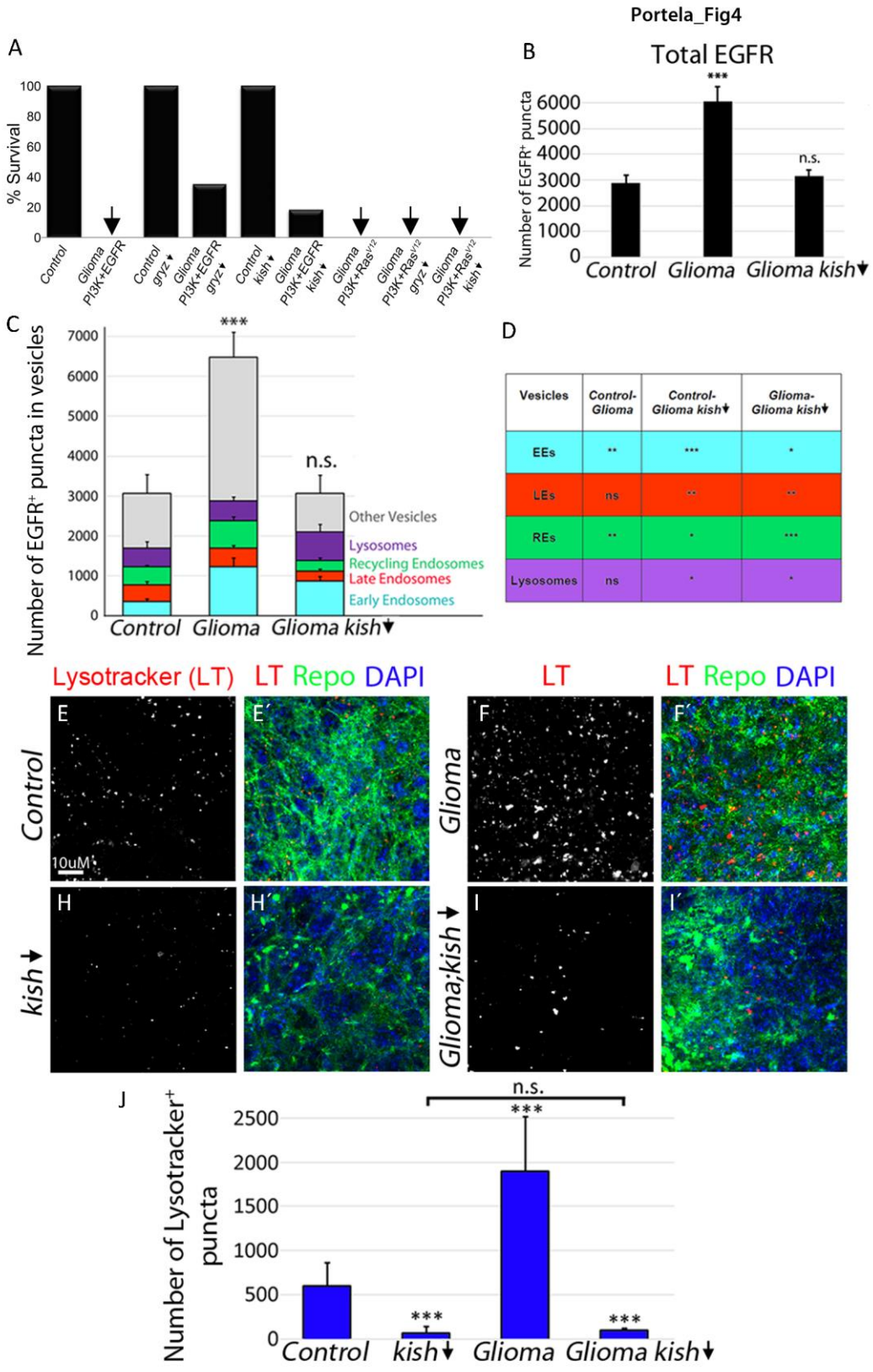
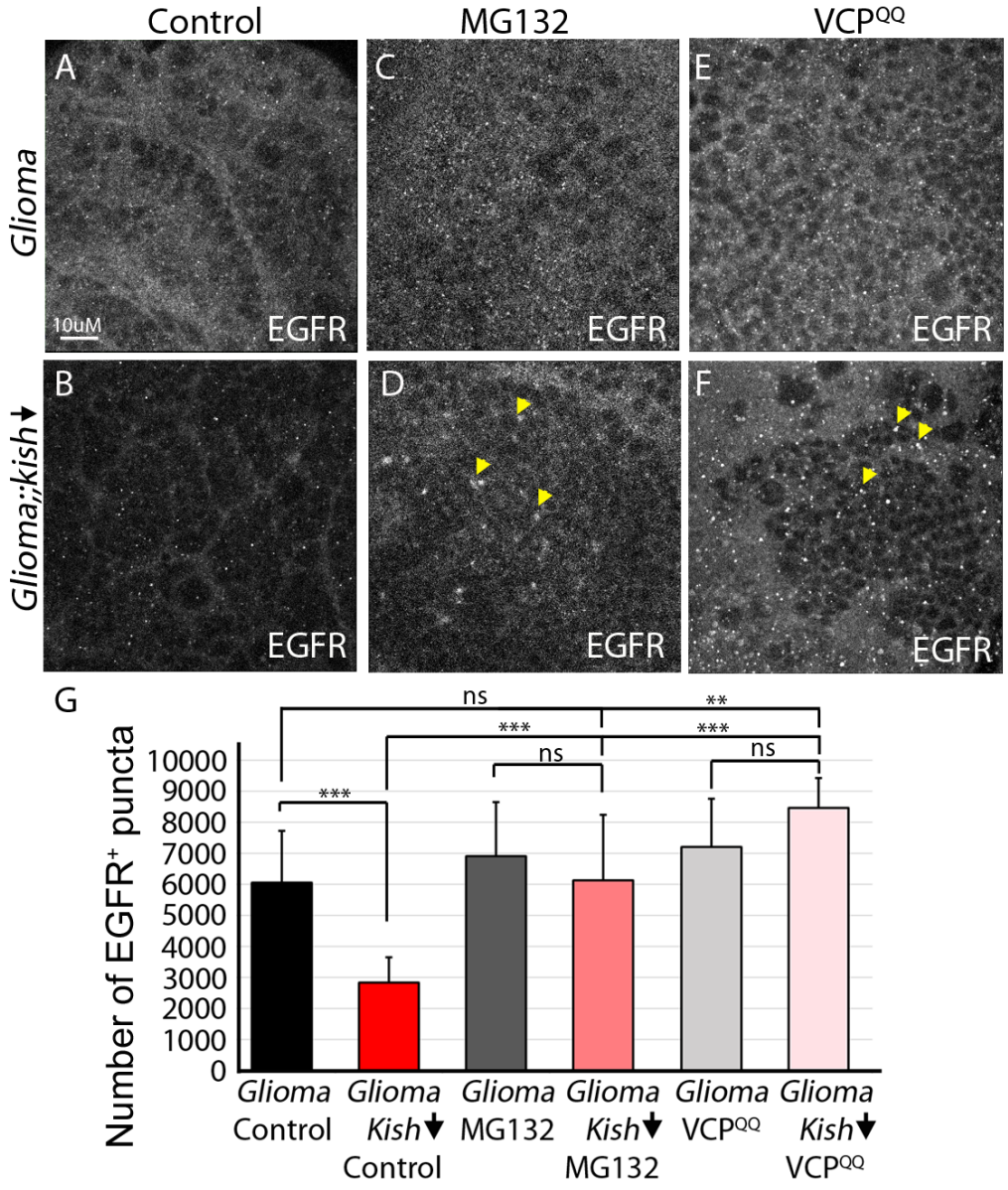



Portela_Fig4
A
% Survival
Control
Glioma PI3K+EGFR
Control gryz▼
Glioma PI3K+EGFR gryz▼
Control kish▼
Glioma PI3K+EGFR kish▼
Glioma PI3K+Ras^{V12}
Glioma PI3K+Ras^{V12} gryz▼
Glioma PI3K+Ras^{V12} kish▼
B
Total EGFR
Number of EGFR+ puncta
***
n.s.
Control
Glioma
Glioma kish▼
C
Number of EGFR+ puncta in vesicles
***
n.s.
Other Vesicles
Lysosomes
Recycling Endosomes
Late Endosomes
Early Endosomes
Control
Glioma
Glioma kish▼
D
Vesicles
Control-Glioma
Control-Glioma kish▼
Glioma-Glioma kish▼
EEs
LEs
REs
Lysosomes
ns
Lysotracker (LT)
LT Repo DAPI
LT
LT Repo DAPI
Control
Glioma
kish▼
Glioma;kish▼
10uM
E
E´
F
F´
H
H´
I
I´
J
Number of Lysotracker+ puncta
n.s.
***
Control
kish▼
Glioma
Glioma kish▼

Portela_Fig5



Portela_Fig6