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Active WNT vampirization by glioblastoma network leads to brain tumor growth and neurodegeneration

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Running title: Glioma-induced neurodegeneration

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Summary

Glioblastoma (GB) is the most lethal brain tumor due to its high proliferation, aggressiveness, infiltration capacity and resilience to current treatments. Activation of the Wingless-related-integration-site (WNT) pathway is associated with a bad prognosis. Using *Drosophila* and primary xenograft models of human GB, we describe a mechanism that leads to the activation of WNT signaling [Wingless (Wg) in *Drosophila*] in tumor cells. GB cells display a network of tumor microtubes (TMs) which enwraps neurons, accumulates Wg receptor Frizzled1 (Fz1), and, thereby, actively depletes Wg from the neurons. Consequently, GB cells proliferate due to β-catenin activation, and neurons degenerate due to Wg signaling extinction. This novel view explains both neuron-dependent tumor progression, and also the neural decay associated with GB.

Keywords

Neuron, Glia, Cancer, Synapse-loss, wingless, Frizzled, beta-catenin, Glioblastoma, Cytoneme, Wg-depletion.

Introduction

The evolution of glioblastoma (referred here as GB) is accompanied by broad neurological dysfunctions, including neurocognitive disturbances, that compromise quality of life during the short life span of patients, one year usually [1]. These tumors are often resistant to standard treatments which include resection, radiotherapy and chemotherapy with temozolomide [2]. Numerous studies are focused on new molecular targets to treat GBs [3-6] ; however, none of them has proven effective yet, which is in stark contrast to the considerable progress made in other tumor types. It is therefore necessary to explore new biological concepts that can lead to additional therapeutic strategies against GBs.

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The WNT canonical pathway is activated upon the ligand “Wingless-related integration site” (WNT) binding to specific receptors (LRPs and FZD) in the plasma membrane. As a consequence, the destruction complex (APC and Axin) is inactivated and β-catenin (armadillo in *Drosophila*) is released. Further, β-catenin translocates into the cell nucleus where it promotes the expression of target genes (*i.e. Cyclin D1* and *Myc*) [7-8]. The WNT pathway is conserved through metazoans and it plays a central role in brain development [9], adult neuronal physiology [10] and synaptogenesis [11]. Perturbations in WNT signaling are associated with neural deficits, Alzheimer´s disease and brain cancer, most notably GB [12]. WNT and FZD signaling can be deregulated in glioblastoma [13-14] (reviewed in [15]). In particular, one of the hallmarks of bad prognosis is the accumulation of ß-catenin in tumoral cells [16-17], indicating an activation of WNT/FZD pathway [18].

GB cells extend ultra-long membrane protrusions that interconnect tumor cells [19]. These tumor microtubes (TMs) are associated with the worst prognosis in molecular subtypes of human gliomas. TMs contribute to invasion and proliferation, resulting in effective brain colonization by GB cells. Moreover, TMs constitute a multicellular network that connects GB cells over long distances, a feature that likely provides resistance against radiotherapy, chemotherapy and surgery [19-20]. Considering the many cytological similarities of TMs and tunneling nanotubes (TNTs) [21], it seems that TMs in aggressive gliomas are the in vivo correlate of TNTs described in cell culture. In addition, TMs seem akin to a basic mechanism of cell-cell connection and molecular communication called "cytonemes" in *Drosophila* [22]. *Growth Associated Protein-43* (*GAP43*) is essential for the development of TMs and, thus, the tumor cell network which is associated with GB progression [19]. However, many aspects of this paradigmatic finding in glioma biology are still unexplored, including its impact on neighboring neurons.

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Here, we report that *Drosophila* glial cells develop a TM network upon oncogenic transformation, akin to what is known from refined mouse glioma models, and from patients [19]. These TMs share characteristics in common with *Drosophila* epithelial cytonemes [22-23] which are also dependent on *Gap43* expression. TMs relocate Frizzled (Fz1) receptor in the glia-neuron interphase, depleting Wingless (Wg) from neighboring neurons. As a consequence, the number of glioma cells increases, while neuronal synapse number decreases and neurodegeneration ensues. The concept of a Wg/Fz1 signaling equilibrium between glioma cells and neurons is relevant because it redefines GB as a neurodegenerative disease, and because it reveals a potential novel strategy against GB.

Results

Drosophila glioma network

To study the mechanisms of communication among malignant glial cells and neighboring neurons, we used a previously described *Drosophila* GB model [24], which consists in the co-overexpression of constitutively active forms of EGFR (dEGFRλ) and the PI3K catalytic subunit p110α (PI3K92E) driven by the glial specific *repo-Gal4.* This combination stimulates malignant transformation of post-embryonic larval glia, leading to lethal glial neoplasia [24-25] which is measured by the increase of glial cell number (Repo positive) compared with a control brain (Figure 1A-C). Based on this *Drosophila* GB model we have evaluated the impact of glial tumor cell proliferation on neighboring neurons.

To visualize the glial network, a myristoylated form of red fluorescent protein (expressed via *UAS-myrRFP*), which accumulates in the plasma membrane, was expressed in glial cells. RFP signal in control brains shows glial soma and the network

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formed in WT larval brains (Figure 1D). Relative to the control brain, the glioma brain shows a significant enlargement in glial membrane projections (Figure 1E).

To define the nature of these projections, we expressed a *UAS-Ihog-RFP* construct in glial cells. Ihog (*interference hedgehog*) is a type 1 membrane protein shown to mediate the response to the active Hedgehog (Hh) protein signal [26], which accumulates in the epithelial cell filopodia, termed cytonemes [27]. This red fluorescent tagged form of Ihog-RFP in epithelial cells labels cellular processes (filopodia) in the basal region of the wing imaginal discs [27-28]. The expression of *UAS-Ihog-RFP* under the control of *repo-Gal4* allows the visualization of cytonemes in glial cells (Figure 1F) as part of a wild type glial interconnecting network. The accumulation of Ihog in the cellular projections of transformed glial cells allowed labeling the tumor microtube-like processes of these cells. Since the term tumor microtube (TM) is the now established terminology to describe thin membrane protrusions from malignant glioma cells in human and murine tumors, we decided to also use this term from here on [19]. In glioma, TMs are observed across the brain (Figure 1G) suggesting a larger volume of TMs. Quantifications (Figure 1H) including the ratio of glioma cells number vs the volume of glioma TMs network show that TMs growth is higher in glioma cells and therefore that the network expands more than the proportional glial number in GB.

A detailed analysis of this glioma network revealed that glial TMs wrap clusters of neurons in individual GB “nests” (Figure 1J compared with I, Figure S1 A-B´´ and see video S1 and S2) comparable to previously described perineuronal nests in patients [29]. To further identify the identity of the TMs, we took advantage of the LifeAct-GFP reporter as a validated marker of human TMs [19] (Figure S1C). Additionally we characterized the TMs with previously described markers for cytonemes in *Drosophila* [28] and orthologues markers of human TMs [19,28] (Figure S1D-G). Moreover, we performed functional experiments where the TMs are defective after the downregulation of *neuroglian* (*nrg-RNAi*) as it has been previously described in

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epithelial cytonemes [30] (Figure S1 H-I). This characteristic morphology suggests that glioma cells build an organized TMs network around the neurons which segregates neuronal populations.

Next we sought to clarify whether similar molecular machineries are involved in human and *Drosophila* TMs. GAP43 is necessary for TMs formation and function, and drives microtube-dependent tumor cell invasion, proliferation, interconnection, and radioresistance [19]. We have reproduced this specific and unique characteristic of human GB in the *Drosophila* model. To determine whether the *Drosophila* glial network is susceptible to *GAP43* depletion, as it has been described in human tumor cells [19], we knocked down igloo-the *Drosophila Gap43* homologous gene [31], in glioma cells. Confocal images of larval brains show that the glioma network does not develop upon *Gap43* silencing and, as a consequence, glial TMs do not enwrap neurons showing a phenotype similar to the control (Figure 1K and video S3).

In addition, we obtained scan electron microscopy (SEM) images to visualize glia morphology (Figure 2A-G). High magnification SEM images show an enlargement of glial cells surface in glioma samples (Figure 2D). Higher magnifications show perineuronal nests of TMs which surround neurons (Figure 2E-F). Longitudinal sections of glial cells show the intercalation of glioma TMs in neuronal tissue (G). Moreover, 3D reconstructions of Control and glioma brains with the glial network marked with ihog-RFP and LifeAct-GFP showed similar phenotypes (Figure 2H-I and Videos S4-S5).

To exclude the possibility that suppression was due to a titration of GAL4 activity caused by introducing an additional UAS-transgene, we also tested whether co-expression of *UAS-lacZ* or *UAS-yellow-RNAi* had any rescuing ability in glioma brains. The observed phenotypes such as the number of glial cells and the GB nests were unchanged in the presence of an additional UAS-transgene (Figure S2A-B). This

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observation indicates that in *Drosophila* glioma Gap43 reproduces the function previously described in human samples.

The genetic disruption of *Gap43* prevents the tumoral network of TMs and halts the overgrowth of glioma cell membranes. Moreover, the direct consequence on flies developing a glioma is larval/pupal lethality. Upon *Gap43* knockdown, however, the glioma-induced lethality is prevented allowing the emergence of adults (Figure 1L). Interestingly, *Gap43* knockdown in wild type glial cells neither affects the normal development of neurons and glia, nor their viability (Figure S2C-G). Taking all data together, transformed glial cells take advantage of the Gap43-dependent tumoral network to proliferate and enwrap neurons and, as a consequence, cause death. Thus, the dependency of TMs on GAP43 is conserved between flies and gliomas originated from human tumor cells.

Wingless signaling in glioma

GB is characterized by the deregulation of many key signaling pathways involving growth, proliferation, survival, and apoptosis, such as p53, pRB, EGFR, PI3K, STAT3 or WNT [32-33]. WNT signaling has long been suggested as a hallmark in gliomagenesis associated with the proliferation of stem-like cells in human GBs [34]. WNT signaling promotes tumoral cell proliferation and dissemination as well as resistance to chemo and radiotherapy (reviewed in [15,35]). To assess the prevalence of WNT genes/pathway deregulation in GB, we searched for mutations related to WNT pathway in the Collection of Somatic Mutations in Cancer (COSMIC). We analyzed data from 922 samples of grade IV astrocytomas (GB) searching for gain of expression or duplications. First, we analyzed *Wnt* family genes, which encode the ligands of the WNT pathway. In particular, we analyzed WNT6 that is related to the self-renewal ability of GB cells [36], WNT3A and WNT1 overexpression that is detected in glial stem cells [37] and WNT5A and WNT2 that were shown to induce migration of GB cells [38-39].

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Our analysis of the COSMIC database revealed that WNT1, WNT3A, WNT6 or WNT5A genes do not appear mutated in GB samples and only 6 cases showed mutations in WNT2 (0,6%) (Figure S3A). WNT signaling is a hallmark of GBs, but as no evident changes in WNT expression were found, we searched for mutations in FZD genes, that encode the receptors of WNT. In particular, we analyzed FZD2 and FZD9 that are linked to self-renewal ability in GB [36,39-40] and FZD4, a positive WNT regulator, which is a causative effector for invasive phenotypes of GB cells [41]. We did not find any case of a GB patient with a gain of expression in FZD2 or FZD4, and barely 4 cases (0,4%) showed a mutation for FZD9. The total number of mutations related with WNT or FZD genes accounts for 5% of the total GB samples analyzed (Supp. Table 1). In addition, we analyzed data from GB in The Cancer Genome Atlas (TGAC) for WNT ligands, FZD receptors and transcriptional targets of WNT pathway databases Figure S3C-D through the Xena Functional Genomics Explorer (https://xenabrowser.net/). We analyzed expression levels in primary GB tissue and non tumoral tissue, transcriptional targets for WNT pathway (Figure S3B) are upregulated in GB samples. However, WNT ligands from the canonical WNT pathway are not upregulated (Figure S3C) and among FZD receptors, only FZD7 shows significant changes in GB tissue (Figure S3D). Taking all these data together, in spite of WNT pathway activation in GB tissue, there is no correlation between overexpression of WNT ligands and GB development.

Nevertheless, previous reports indicate that WNT targets, such as FoxM1 which promotes nuclear localization of β-catenin, or β-catenin itself are related with Glial Stem Cells (GSC) maintenance and tumorigenesis. Indeed, they are standard biomarkers for GB bad prognosis [16,42-45]. β-catenin activation (translocation to the nuclei) is a downstream event of WNT pathway. It has been identified in 19% of surgical samples from adult GB patients and in 30% of surgical samples from pediatric patients. Moreover, WNT inhibition leads to suppression of tumor growth, proliferation in cultures, and a modest induction of cell death [34].

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Given the discrepancy between the presence of WNT pathway markers and the lack of mutations in GB samples, we decided to study *Wingless* (*wg*) expression, the fly homologue to human WNT, in our glioma model. The results showed that Wg protein is homogeneously distributed in control larval brains with a slight increase in glial membranes (Figure 3A-A´´, C) but glioma brains showed accumulation of Wg in tumoral cells (Figure 3B-B´´, C), in line with human GBs [38,46]. To determine whether Wg could be signaling to the glial cells, we assessed the presence of Frizzled (Fz) receptors in glial membranes. Monoclonal antibodies were used to visualize the Fz1 and Fz2 receptors in control samples, Fz1 receptors are localized homogeneously across the brain and partially accumulated in glial membranes (Fz1, Figure 3D-3D´´ and Fz2, showing the expected pattern in the wing disc [47] Figure S4A and localized homogeneously in the brain S4B-B´´´). And glioma samples where Fz1 is highly accumulated in glial membranes (Figure 3E-E´´, H). No changes for Fz2 were detected in glioma brains (Figure S4C-C´´´). In addition, a detailed analysis of glioma brains revealed that Fz1 protein specifically accumulated in TMs ($iHog^{+}$) (Figure 3E''). Furthermore, we used a Fz1-GFP protein reporter to determine the localization of Fz1 protein in more detail (supp. Figure S4D-D´´). The results indicate that Fz1 is preferentially accumulated in glial cells located at the border of the tumor, in direct contact with neighboring neurons. These data suggest that the glioma TMs network contributes to Wg/Fz1 signaling.

To evaluate the contribution of Fz1 to the progression of the glioma, we knocked down the Fz1 receptor (using *UAS-Fz1-RNAi*) in transformed glial cells. First we validated *UAS-Fz1-RNAi* tool in epithelial tissue from larvae wing imaginal discs. Even though Fz1 expression in wing imaginal disc is discrete, upon *UAS-Fz1-RNAi* expression in the posterior compartment (marked with GFP), there is a reduction in Fz1 protein signal compared with the anterior compartment from the same tissue (Figure S5A). Brains showed a significant reduction of Fz1 protein expression (Figure 3F, H). However, no

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change was observed in glioma TMs network development (Figure 3F'). Furthermore, we inhibited the development of the glioma network by expressing *UAS-Gap43-RNAi* and stained the brains for Fz1. Under these conditions the network does not expand, Fz1 does not accumulate in TMs and it shows a homogeneous distribution similar to control brains (Figure 3G-G´, H). These results indicate that Fz1 accumulation in TMs is a consequence of the glioma network but the glioma network formation does not require Fz1 accumulation in the TMs.

Glial Fz1 interacts with neuronal Wg

The abnormal distribution of Wg and Fz1 in glioma brains could be due to either an increase in gene expression or to redistribution of the protein. First, to determine *wg* and *Fz1* transcription levels in glioma, we performed quantitative PCR experiments from brain RNA extracts. The results showed that *wg* and *Fz1* transcription levels are comparable in control and gliomas (supp. Figure S6A). To consider the mRNA translation or protein stability and degradation, we measured total Fz1 and Fz2 protein levels in Western blot experiments. Control and glioma brain protein extracts were blotted and incubated with anti-Fz1 or Fz2 antibodies; Tubulin was used as a loading control (Supp. Figure S6B). Quantification of the membranes showed no significant changes for Fz1 protein levels in glioma (Supp. Figure S6B). Moreover, we performed *in situ* hybridization experiments to detect Wg and Fz1 mRNA expression in control and glioma brains. The results are consistent with the qPCRs results (Figure S6A) showing no differences for *wg* or *Fz1* transcription between controls and gliomas (Figure S6C).

The data indicate that in spite of a higher signal for Wg and Fz1 proteins in glioma cells by immunofluorescence, there are no changes in gene expression. We hypothesized that Fz1 is transported and accumulated along glioma TMs, which contact neighboring neurons and receive Wg from them.

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According to this hypothesis, the glial membranes would be in close proximity to neuronal membranes to allow Fz1-Wg interaction. To assess this, we performed GRASP experiments [48]. This technique determines intimate physical interaction among glia and neurons, in the range of 20-40nm (synaptic distance) which is compatible with protein-protein interaction [48]. Each split fragment of the green fluorescent protein (GFP) was expressed in neurons (*elav-lexA*) or glial (*repo-Gal4*) cells, respectively (see Material and Methods). Only upon intimate contact between the two split fragments GFP fluorescence is reconstituted. Control samples (Figure 4A-C´´) showed a discrete signal corresponding to the physiological interaction between glia and neurons, nevertheless, upon glioma induction a massive GFP signal from GRASP reporter was detected (Figure 4D-F´´). This result indicates that, in a glioma condition, there is a significant increase of glia-neuron membrane interaction.

We had observed specific signal localization for Wg and Fz1 in glioma membranes (myr-RFP/ihog-RFP) (Figure 3B-B´´, E-E´´). However, to determine if Fz1 (receptor) and Wg (ligand) co-localized in glioma brains we conducted co-staining with Wg and Fz1 antibodies. The confocal images showed Fz1 and Wg accumulation in glioma TMs compared to controls (ihog-RFP marked) (Figure 4G-G´´-H-H´´). Moreover, a detailed inspection of the images revealed that Wg protein accumulated at the border between neuron and glioma cells (Figure 4H-H´´), which is consistent with our hypothesis of glial cells receiving Wg from neighboring neurons. To confirm the physical interaction of Fz1 and Wg proteins we performed proximity ligation assays (PLA) (see Materials and Methods) [49]. This quantitative method reports the interactions between two proteins with a resolution of 40nm [50]. Control brains showed a discrete number of puncta showing the physiological interactions (Figure 4I-I´´, K). However, glioma brains showed a fivefold increase in the number of puncta (Figure 4-J´´, K) indicating a significant increase in Fz1-Wg number of interactions. These results confirm that glioma cells accumulate Fz1 receptor in TMs, and then this specific receptor interacts

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with Wg. But Wg is not upregulated in the glioma brain so we hypothesize that Wg comes from neighboring neuronal membranes and it accumulates in glioma cell membranes.

Wg/Fz1 pathway targets are active in glioma and inactive in neurons

Wg targets are indicators of Wg/Fz1 activity in the recipient cell. Armadillo/βCatenin is a cytoplasmic protein which, upon activation of Wg pathway, translocates into the nucleus and activates transcription of target genes [12,51]. To determine if Fz1 is signaling in gliomas as a consequence of Wg-Fz1 interaction, we stained control and glioma brains for Armadillo (Arm) with a specific antibody that detects preferentially its cytoplasmic inactive form (Cyt-Arm) [52]. Cyt-Arm was homogeneously distributed along the brain in control samples (Figure 5A, D). However, glioma brains showed accumulation of Cyt-Arm in the cytoplasm of the neurons and a reduction in glioma cells (Figure 5B, D). This result suggests that, in glioma brains, Wg/Fz1 pathway is inactive in neurons and active in glioma cells. More importantly, this data shows that the network expansion and the accumulation of Fz1 in the TM projections have an effect on neighboring neurons. Further, we dismantled the glioma network expressing *UAS-Gap43-RNAi* or down[53]regulated Fz1 (*UAS-Fz1-RNAi*) and stained for Cyt-Arm. Under this condition, Cyt-Arm was homogeneously distributed along the brain similar to the control (Figure 5C, D and S7A) demonstrating that the network is required to promote Wg signaling in the transformed glia.

To further confirm that Wg pathway is active in the glial transformed cells and silenced in neurons, we used several additional Wg pathway reporters, namely *arm-GFP, nkd-lacZ, tsh-lacZ, fz4-GFP* and *dally-LacZ* [54-55]. The results showed that all these reporters were active in glioma cells and inactive in neurons compared to control brains (Figure 5E-H´´ and Figure S7B-G´´) which confirm that the Wg/Fz1 pathway is inactive in neurons and active in glioma cells.

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Since we observed specific signal localization and accumulation of Wg and Fz1 in glioma membranes and consequently the activation of the pathway in glioma cells, we wondered if these results were conserved in human glioma cells. Therefore, we used a primary patient-derived glioblastoma culture xenograft model using S24 cells kept under stem-like conditions (see Materials and Methods), which reproduce previously described Scherer modes, perivascular migration and spread [29,56]. GFP-marked S24 cells were injected in the brain of NMRI nude mice, and brains were removed and analyzed 90 days later (as previously described in [19]). To validate our data, we performed a series of more diffuse tumor parts (history grade II-like tumor periphery where normal brain has just been colonized) and denser tumor parts (grade III-IV like, from central areas) GB images from S24 xenografts brain sections.

We stained the samples for β-catenin and WNT1 proteins in S24 xenograft brain sections and compared them to control samples (Figure 5-I-N and Figure S8G-H). We observed a significant increase of both proteins in GB cells, in line with previous observations. These data indicate that WNT1 is deposited in GB cells and activates WNT pathway; consequently β-Catenin is upregulated to mediate GB cells malignancy [16]. Moreover, images of the WNT1 and βCatenin staining of grade II and III GB images from S24 xenografts brain sections and quantification of the pixel intensity indicates that the accumulation of WNT1 and activation of βCatenin correlates with the growth of the tumor (Figure S8A-F).

To determine Fz1's contribution to the proliferation of *Drosophila* gliomas we quantified the number of transformed glial cells upon *Fz1* downregulation. A specific Repo antibody was used to mark glial cell nuclei in the brains. The data show a significant increase of glial cell number in glioma brains, which is prevented by *Fz1* downregulation or TM network dismantlement (Figure 6A-E). In addition, we studied the adult survival by quantifying the number of flies with glioma and/or *Fz1-RNAi* expression. First, Fz1 downregulation in normal glial cells allows 80% adult survival.

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337 On the other hand, the glioma caused 100% lethality in larval or pupal stages. By
338 contrast, glioma lethality was reverted upon *Fz1* downregulation in repo cells, and 70%
339 of the animals reached adulthood (Figure 6F). In conclusion, Fz1 is not necessary for
340 the glioma network overgrowth but Wg/Fz1 signaling is necessary for the increase in
341 the number of tumoral cells and the associated lethality.

342

343 **Wg/Fz1 pathway disruption in adult brains**

344 To discard that these results are restricted to a developmental process, we performed
345 similar experiments in adult brains. We included a tub>Gal80^{TS} system to repress Gal4
346 activation during all developmental stages (see Materials and Methods), glioma was
347 induced 4 days after adult eclosion when adult brain is fully differentiated. Analysis of
348 adult flies survival indicate that animals with glioma start dying at day 9 after the glioma
349 induction until the 14th day, significantly earlier that control flies. (Figure S9A). 7 days
350 after glioma induction we stained for Arm, Fz1 and Wg control and glioma adult brain
351 samples and quantified (n>15) the average pixel intensity ratio (Glial cells membranes
352 RFP+ vs neuronal tissue (Figure S9B-H). The results reproduce the findings from larval
353 brains: Fz1 and Wg proteins are accumulated in glioma tissue and Cyt-Arm signal
354 shows an increase of wg-pathway activity in glioma cells. Therefore, there are no
355 differences between larval and adult brains regarding Wg-pathway in glioma cells and
356 neurons. Moreover, we analyzed *Drosophila* adult negative geotaxis behavior (climbing
357 assay), as an indication for possible motor defects associated with neurodegeneration.
358 The results showed symptoms of neurodegeneration in glioma flies compared to
359 controls (Video S6).

360 **Gliomas cause Neurodegeneration**

361 Previous results suggest that a neurodegeneration process is taking place in glioma
362 brains. To determine whether the glioma is causing neurodegeneration we quantified

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the number of active zones (synapses) in the neuromuscular junction (NMJ). This well-established system has been used for decades to study neurodegeneration in *Drosophila* [57-60], motor neuron soma is located in the central nervous system, but synapse counting can be accurately done in synaptic buttons located in adult or larva muscular wall. Adult NMJs Quantification of confocal images stained with anti-bruchpilot (Nc82) revealed a significant reduction in the number of synapses in glioma adult (Figure S9I) or larval NMJs compared to controls (Figure 7A-B, F) and therefore, a neurodegenerative process, which is prevented by Fz1 downregulation or TM network dismantlement (Figure 7C-D, F).

Wg expression in glioma cells is dispensable for tumor progression

Fz1 receptor is accumulated in glioma TMs and contribute to Wg depletion and pathway equilibrium disruption. To determine whether the source of Wg is neuronal or glial, we silenced *wg* expression in neurons exposed to glioma, or in glioma cells. First we validated *UAS-wg-RNAi* tool in epithelial tissue, wing imaginal discs. Activation of *UAS-wg-RNAi* in the posterior compartment (marked with GFP) cause a reduction of specific monoclonal anti wg (Figure S5B). Pan-neuronal *wg* silencing (*elav>wg RNAi*) is lethal in line with our hypothesis regarding the requirement of Wg in neuronal biology. Besides, *wg* knockdown (*wg-RNAi*) in glioma cells (*Repo>PI3K; EGFR; wg-RNAi)* does not prevent glioma cell number increase (Figure 7G-I, K) nor glioma TMs volume expansion (Figure 7G-I, L), these results suggest that *wg* expression in glioma cells is not relevant for glioma progression.

Moreover, to stress on the contribution of Fz1 receptor as mediator for Wg depletion, we generated glioma cells and silenced *Fz1* expression, in addition we expressed a constitutively active form of *armadillo* (*UAS-armS10*) [61] to activate Wg pathway downstream Wg-Fz1 in these gliomas (Figure7E-F and J-L). We counted the number

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of synapses and observed that it is comparable to glioma + *Fz1RNAi* (Figure 7A-F). This result suggests that the reduction in the number of synapses is specifically mediated by Fz1 accumulation. To confirm that the Fz1 depletion and Arm signaling produces a glioma like condition we counted the number of glial cells and network volume (Figure 7G-L). We observed that in this case the number of glioma cells increased and expanded TMs (Figure 7J-L) similar to a glioma (Figure 7JH).

Wg/Fz1 pathway disruption causes neurodegeneration

Neuronal development and physiology are dependent on Wg/Fz1 signaling and disruptions in this signaling pathway lead to synapse loss, an early symptom of neurodegeneration (reviewed in [62-65]. To determine if an imbalance in Wg distribution caused by glioma cells can affect the neighboring neurons, we aimed to determine the contribution of Fz1/Wg pathway to neuronal physiology. To inhibit Wg/Fz1 pathway signaling we expressed *UAS-fz1-RNAi* or *UAS-wg-RNAi* in motor neurons under the control of a *D42-Gal4* driver [66] and quantified the number of active zones (synapses) in the neuromuscular junction (NMJ). Quantification of confocal images stained with anti-bruchpilot (Nc82) revealed a significant reduction in the number of synapses and therefore, a neurodegenerative process (Figure 8A-D). These data suggest that Wg/Fz1 signaling pathway activity in neurons is necessary in for synaptogenesis.

So far, we have demonstrated that glioma cells cause a disruption of Wg/Fz1 signaling in neurons (Figure 5) and our data suggest that this is dependent on Fz1 accumulation in glioma cells (Figure 3 and 4H). Next, we decided to restore this signaling equilibrium by overexpressing Fz1 receptor in neurons surrounded by glioma cells. To avoid crossed expression we generated Fz1 transgenic flies based on the LexA-LexAop expression system [67] , which is independent of the Gal4-UAS system used to generate the glioma. We validated this newly generated tool in *Drosophila* brains. *LexAop-Fz1* was activated in neurons under the control of *elav-LexA* and monoclonal anti-Fz1

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showed higher signal in neurons and functional activity revealed by anti-Arm staining (Figure S5C-D). Oversized glioma brains showed the expected glioma network compartmentalizing neurons in the brain (Figure 8E-F´´). However, Fz1 overexpression in neurons restored homogeneous Fz1 protein distribution in the brain (Figure 8G, H), rescued brain size (Figure 8G-G´´) and neuron distribution and morphology (Figure 8H´-H´´). In addition, Fz1 equilibrium restoration partially rescued lethality and most animals reached adulthood. To verify Fz1 activation of the pathway we stained for Wg and Cyt-Arm (Figure S10). As previously shown, glioma brains showed a heterogeneous distribution for Wg protein (Figure S10A-A´´) and, as a consequence, an imbalance in pathway activation reported by Arm accumulation (Figure S10 C-C´´). As expected, neuronal Fz1 overexpression in glioma brains restored Wg distribution and Arm signal to a control situation (Figure S10B-B´´, D-D´´)

To further determine the effect of Wg/Fz1 signal restoration in neurons, we quantified the number synapses in their NMJs. Neuronal morphology is disrupted by glioma (Figure 8I, J) and restored by Fz1 overexpression in neurons neighboring glioma cells (Figure 8J, K). Moreover, synapse number reduction upon glioma induction is restored by Fz1 overexpression in neurons (Figure 8L). All these results indicate that the Wg/Fz1 pathway disruption caused by glioma is responsible of the synapse loss. Restoration of the signaling equilibrium between glia and neurons prevents synapse loss and therefore, neurodegeneration.

Glioma depletes Wg from neuronal membranes

The actual mechanisms of Wingless delivery have been under debate. This protein was initially described as a diffusible secreted protein [68]. Recent studies have proved that Wg secretion is not necessary for *Drosophila* development [69]. A membrane-tethered version of Wg protein [70] (Wg^{NRT}) can substitute the endogenous gene, mimic Wg normal functions and produce viable organisms [69]. We took advantage of this tool

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441 to determine the cellular mechanisms mediating glioma Wg retrieval from neurons. We
442 created a genetic combination to substitute one copy of endogenous Wg with one copy
443 of *wg*^{NRT} exclusively in neurons (by using the LexAop system). In addition, upon LexA
444 system activation, neurons are marked with membranous GFP (CD8-GFP) while the
445 rest of the cells are wild type. Moreover, this Wg^{NRT} is tagged with an HA which allows
446 to discriminate from the endogenous Wg protein. We induced a glioma and marked the
447 glial cells in red (ihog-RFP) (see materials and methods and Figure 9G). First, we
448 studied normal control brains that express Wg^{NRT} exclusively in neurons in the absence
449 of glioma. We stained with anti-HA and observed a positive signal both in neurons and
450 glial cells but not in the control without the elav-LexA driver, indicating that the HA
451 signal is not a false positive (Figure 9A-B, H). Finally, we performed the experiment in
452 the glioma model to examine the interaction of glioma cells with Wg^{NRT} expressing
453 neurons (Figure 9E-F-I) and stained with anti-HA. The images show a homogeneous
454 signal for HA throughout the brain in glia and in neurons (See figure H-I magnifications
455 for more detail), suggesting that glioma cells can deplete this non-
456 secretable/membrane tethered version of Wg from neurons. We have quantified the
457 number of HA^{+} puncta in glia and neuron from control and glioma samples. The results
458 suggest that glial cells sequester Wg^{NRT} from neuronal membranes at comparable
459 rates under both physiological and glioma conditions (Figure 9J). Noticeably, however,
460 there was a detectable reduction in the number of glioma cells, similar to previous
461 results when Wg/Fz1 equilibrium in glial vs neurons was restored (Figure 8C, E and
462 Figure 8G-H´´). Since Wg^{NRT} is anchored to neuronal membranes, it would be expected
463 to reduce the total Wg signaling in glioma cells thereby decreasing cell
464 proliferation/survival, thus resulting in a rather normal sized brain (Figure 9A, C, E).
465 Moreover, heterozygous *wg*^{NRT}*/wg* prevented glioma network progression (Figure
466 9D´vs F´). In conclusion, glioma cells produce a network TMs that reach neighboring
467 neurons, increasing intimate membrane contact that facilitates neuronal-Wg
468 sequestering mediated by the Fz1 receptor in glioma TMs. Since Wg/Fz1 signaling in

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glioma mediates cell number and tumor progression, targeting this cellular interaction may be a new candidate for future therapies.

Discussion

In addition to cell autonomous features of tumor cells, including founder mutations, recent evidences indicate that microenvironment signals contribute to glioma progression. Neuroligin-3 (NLGN3) is a synaptic protein cleaved and secreted after neuronal activity which promotes PI3K-mTOR signaling stimulating glioma growth. Thus, NLGN3 mediates an autocrine/paracrine loop in glioma cells which perpetuates tumoral features [71] (reviewed in [72]). Also, neural precursor cells (NPC) from the subventricular zone (SVZ) produce chemoattractants (SPARC/SPARCL1, HSP90B and pleiotrophin) which facilitate glioma invasion of the SVZ through Rho/ROCK signaling [73].

We showed recently that TM network formation determines GB tumor malignancy, confers radiotherapy resistance and influences patient's prognosis [19]. TMs stability in GB is sensitive to *Gap43* expression in tumoral cells [19]. Also, *Tweety homologue-1* (*TTHY1*) expression in GB cells, mediated by NLGN3 regulates TM formation [74].

This study shows that TMs intercalate among neurons and enwrap them in perineuronal nests [29] structures establishing an intimate link glioma-neuron. Then GB cells make direct contact via TMs and deprive neurons of WNT.

Expression data from human cancer databases indicate that glioma cells do not upregulate *Wnt* expression, neither upregulate its receptors. Instead, the results in the fly model show that glioma cells relocate Fz1 receptor in the TMs allowing to vampirize Wg from neurons. Consistent with these data, in the patient-derived GB xenograft model, where WNT1 is deposited in GB cells and the WNT pathway is activated, β-

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494 catenin is upregulated. The available data suggest that GB TMs grow towards the
495 source of Wg. However, as TMs expand upon Fz1/Wg signaling, the question
496 regarding the exact order of events remains open. Do TMs require some initial stimuli
497 from the source of Wg to grow? Alternatively, do TMs initiate growth triggered by glial
498 internal signals and directed through a gradient of neuron-secreted attractants?
499
500 Concerning the mechanism of Wg vampirization, we have expressed a non secretable,
501 HA- tagged version of membrane- tethered Wg^{NRT} in neurons. In that experiment Wg is
502 detected within glial cells demonstrating that GB cells can take Wg directly from the
503 neuronal membrane. However, further studies are still required to determine the
504 precise mechanism of neuronal Wg depletion by GB cells TMs.
505
506 It is widely observed that brain tumors and related ailments can cause cognitive decline
507 and neuronal dysfunction (reviewed in [75]). High-grade glioma patients continue to
508 display cognitive deficits after surgery, radiotherapy or chemotherapy [76-78]. The most
509 common deficits concern memory, executive functions and general attention beyond
510 the effects of age, education and gender [79]. Nevertheless, the molecules mediating
511 neuronal degeneration need to be determined.
512
513 Synapse loss is an early step in neurodegeneration [80-82] which is consistent with the
514 cognitive defects observed in GB patients. Nonetheless, cognitive defects can be
515 observed also in patients with excess of synapses as in the case of fragile X syndrome
516 [83-84]. GB cells can stimulate aberrant synapses associated with seizures [85] which are
517 compatible with cognitive dysfunctions. Neuronal death is a later event in
518 neurodegenerative processes such as Alzheimers´s disease [86-88]. In GB patients,
519 neuronal death is under debate and this issue should be addressed with further data.
520 There is preclinical work [88-89] supporting Glioma-induced neuronal death due to
521 glutamate cytotoxicity, in addition clinical studies from [90] support neuronal death in GB

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patients. However, it is certainly very difficult to draw clear conclusions from clinical samples or clinical courses, considering that therapy, antiepileptics and the pure space occupation plus the edema contribute to the neuronal dysfunction, degeneration and cell death.

In particular, neuronal cell loss is typically found at and around glioblastomas, and neurocognitive disturbances are a frequent finding in glioma patients. Although evidences from our experience and from neuropathology expertise, this is an open debate which requires further attention.

The data also reveal that reestablishing Wg signaling equilibrium by Fz1 overexpression in neurons, not only restores synapse number but also blocks GB progression. Functional disruption of the equilibrium between GB glia and neurons is described here for the first time. Possibly, this mechanism could be valid for other molecules related to tumor progression such as Notch, Hedgehog or TGF. Moreover, cytoneme-like structures play a role in development and other cell types [91-93]. Hence, we propose that cytoneme-like structures in physiological conditions and TMs in pathological GB conditions could redistribute limited amounts of signaling molecules among competing cell types, therefore long range redistribution of signaling molecules could be a general mechanism to compete for different resources.

This study integrates for the first time the oncogenic nature of glioma with the neuronal degeneration caused by Wg depletion. This innovative concept of glioma-induced neurodegeneration opens the possibility of combined treatments to fight GB progression and associated neurodegeneration at the same time. Our data demonstrate that making the neurons more competitive for secretable factors such as Wg already has an impact in GB tumor growth, although it remains to be demonstrated what type of drugs can carry out these actions.

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The rapid transformation of GB cells and the heterogeneity of mutations in these tumors are a handicap for genetic therapies and monoclonal therapies. In our view, cellular features such as the network shared by GB cells emerge as an alternative to tackle tumor progression. Among the possible new strategies, TMs dynamic and cellular transport of receptors to the TMs could be a target to prevent GB proliferation and neurodegeneration. Gap43 has emerged as a functional component of GB network formation. Recent studies indicate that other proteins such as Flotilin [28,94], participate in cytoneme dynamics. The discovery of molecules regulating TM/cytoneme biology arises as potential targets for cancer treatment.

Author contributions

Conceptualization, S.C.T. M.P. and FW; Methodology, S.C.T., VR, M.P., M.L.P., E.S. and N.F.; Investigation, S.C.T., FW, VR, M.P. M.L.P, E.S and N.F.; Writing – Original Draft, M.P, FW and SCT.; Writing – Review & Editing, S.C.T. and M.P.; Funding Acquisition, S.C.T and FW.; Supervision, S.C.T.

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Experimental Procedures

Fly stocks

Flies were raised in standard fly food at 25ºC.

Fly stocks from the Bloomington stock Centre: *UAS-GFP^{nls}* (BL4776), *UAS-lacZ* (BL8529), *UAS-myr-RFP* (BL7119), *UAS-Gap43-RNAi* (BL29598), *arm-GFP* (BL8555), *nkd04869a-lacZ* (BL25111), *D42-Gal4* (BL8816), *GFP-fz1-GFP* (BL59780), *repo-Gal4* (BL7415), *puc-lacZ, UAS-CD8-GFP* (BL32186), *tub-gal80^{ts}* (BL7019), *elav-lexA (BL52676), lexAop-CD8-GFP* (BL32205), *lexAop-flp* (BL-55819), *UAS-armS10 (BL4782), sqh-GFP (BL57145), UAS-CD4-spGFP1-10, lexAop-CD4-spGFP11* (obtained from BL58755), *en-Gal4, UAS-GFP (from BL25752)*. Fly stocks from the Vienna *Drosophila* Resource Centre: *UAS-fz1-RNAi* (v105493), *fz4-GFP* (v318152), *UAS-mmp1-RNAi* (v101505), UAS-*wg-RNAi* (v104579), UAS-*yellow-RNAi* (v106068), *UAS-nrg-RNAi* (v107991). *GFP-sls (MLC, ZCL2144 from http://flytrap.med.yale.edu), UAS-dEGFR$^{\lambda}$, UAS-PI3K92E* (dp110^{CAAX}) (A gift from R. Read), *UAS-ihog-RFP* (a gift from I. Guerrero), *tsh-lacZ* and *dally-lacZ* (gifts from M. Milan), *lexAop*-fz1 (generated in this study), *FRT Wg FRT NRT–Wg-HA, pax –Cherry (a gift from A. Baena-López) and puc-lacZ* (a gift from E. Martín-Blanco), *lifeact-GFP* (a gift from I. Guerrero), for electron microscopy studies, we used the *UAS-HRP:CD2* as reporter [95], *UAS-GPI-YFP* [96], *UAS-GMA-GFP* [97].

Drosophila glioblastoma model

The most frequent genetic lesions in human gliomas include mutation or amplification of the Epidermal Growth Factor Receptor (EGFR) gene. Glioma-associated EGFR mutant forms show constitutive kinase activity that chronically stimulates Ras signaling to drive cell proliferation and migration [98-99]. Other common genetic lesions include loss of the lipid phosphatase PTEN, which antagonizes the phosphatidylinositol-3 kinase (PI3K) signaling pathway, and mutations that activate PI3KCA, which encodes the p110a catalytic subunit of PI3K [98-99]. Gliomas often show constitutively active Akt, a

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611 major PI3K effector. However, EGFR-Ras or PI3K mutations alone are not sufficient to
612 transform glial cells. Instead, multiple mutations that coactivate EGFR-Ras and
613 PI3K/Akt pathways are required to induce a glioma [29]. In Drosophila, a combination of
614 EGFR and PI3K mutations effectively causes a glioma-like condition that shows
615 features of human gliomas including glia expansion, brain invasion, neuron
616 dysfunction, synapse loss and neurodegeneration [24,100-101]. Moreover, this model has
617 proved to be useful in finding new kinase activities relevant to glioma progression.[25] To
618 generate a glioma in *Drosophila* melanogaster adult flies, the Gal4/UAS system [102] was
619 used as described above (*repo*-Gal4>UAS-*EGFRλ*,UAS-*dp110*. To restrict the
620 expression of this genetic combination to the adulthood, we used the thermo sensitive
621 repression system Gal80^{TS}. Individuals maintained at 17ºC did not activate the
622 expression of the UAS constructs, but when flies were switched to 29ºC, the protein
623 Gal80^{TS} changed conformation and was not longer able to bind to Gal4 to prevent its
624 interaction with UAS sequences, and the expression system was activated.

625 **Generation of Transgenic flies**

626 *LexAop-Frizzled1* construct was generated by RECOMBINA S.L. Fz1 (*CG17697*) CDS
627 was synthesized by overlapping g-block assembly. The complete 17665bp fragment
628 was amplified using the high fidelity Phusion taq polymerase (Thermo fisher Scientific)
629 and *Eco.Friz.Fw* and *Xba.Friz.Rv* primers. PCR amplicon was cloned in pJET entry
630 vector (Thermo Fisher Scientific), then Frizzled fragment was released with *EcoRI*/*XbaI*
631 restriction enzymes and sub-cloned into destination *pLOTattB* plasmid.

632 *Eco.Fz1.Fw* 5´-GAATTGGGAATTCATGTGGCGTCAAATCCTG-3´

633 *Xba.Fz1.Rv* 5´-TCTAGACTAGACGTACGCCTGCGCCC-3´

634 Transgenic flies were injected and *Frizzled1* fragment was inserted in the chromosome
635 2L by the *Drosophila* microinjection service (CBMSO-CSIC) using the following stock:

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636 *y[1] M{vas-int.Dm}ZH-2A w[*]; M{3xP3-RFP.attP'}ZH-22A* (BL24481). Transgenic flies
637 were selected individually by eye color (w+) and balanced with *CyO*.
638
639 **Antibodies for Immunofluorescence**
640 Third-instar larval brains, were dissected in phosphate-buffered saline (PBS), fixed in
641 4% formaldehyde for 30min, washed in PBS + 0.1 or 0.3% Triton X-100 (PBT), and
642 blocked in PBT + 5% BSA.
643 Antibodies used were: mouse anti-Wg (DSHB 1:50), mouse anti-Repo (DSHB 1:50),
644 mouse anti-Fz1 (DSHB 1:50), mouse anti-Fz2 (DSHB 1:50), Rabbit anti-Fz1 [103], 1:300),
645 mouse anti-Arm (DSHB 1:50), mouse anti-β-galactosidase (Sigma, 1:500), rabbit anti-
646 GFP (Invitrogen A11122, 1:500), mouse anti-GFP (Invitrogen A11120, 1:500), mouse
647 anti-Nc82 (DSHB 1:20), Rabbit anti-Hrp (Jackson Immunoresearch 111-035-144,
648 1:400) , mouse anti-HA (12CA5 Roche 11583816001 1:100), rat anti-HA (Roche
649 11867423001, 1:200).
650 Secondary antibodies: anti-mouse Alexa 488, 647, anti-rabbit Alexa 488, 647
651 (Thermofisher, 1:500). DNA was stained with 2-(4-amidinophenyl)-1H-indole-6-
652 carboxamidine (DAPI, 1µM).
653
654 **Cell culture, fixation and histology of S24 Xenograft model**
655 The S24 cell line was derived as a primary glioblastoma culture (Lemke et al., 2012;
656 Osswald et al., 2015). For the S24 glioma model, 50.000 S24:GFP cells (stably
657 transduced by lentivirus) were injected into the cortex in 8-10 week old male NMRI
658 nude mice (Charles River, Sulzfeld, Germany, n=2). Cells were cultivated under serum-
659 free conditions in DMEM-F12 as sphere cultures (Thermo Fisher Scientific Inc.,
660 Waltham, MA, USA) supplemented with 2% B-27 (Thermo Fisher Scientific Inc.,
661 Waltham, MA, USA), 5 µg/ml human insulin (Sigma-Aldrich Corporation, St. Louis, MO,
662 USA), 12.8 ng/ml heparin (Sigma-Aldrich), 0.4 ng/ml EGF (R&D Systems Inc.,

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Minneapolis, MN, USA) and 0.4 ng/ml FGF (Thermo Fisher Scientific Inc., Waltham, MA, USA). Animals were sacrificed 90 days after glioma cell injection with age-matched wild-type NMRI nude mice (n=2) which were used as control.

Brains were fixed with transcardial perfusion with 40 ml PBS and 40 ml 4 % PFA. The brain was removed and postfixed in 4 % PFA for 4 hrs at room temperature. Afterwards the brains were stored in PBS at 4 °C in the dark.

For histology, S24:GFP tumor-bearing brains were coronally cut on a vibratome (Sigmann Elektronik, Hüffenhardt, Germany) into 100 µm sections. The sections were permeabilized with 1 % TX100 for 3 hrs and counterstained with primary antibodies against beta-catenin (abcam, ab32572) and WNT1 (abcam, ab15251) for 3 hrs in 0.2% TX100 and 5 % FCS. Sections were washed three times with 0.2% TX100 and 5 % FCS and counterstained with secondary antibodies couples to Alexa-647 and Alexa-546 (Invitrogen) as well as DAPI for 3 hrs. The sections were washed three times in 1 × PBS, pH=7.4, and mounted on coverslips using self-made moviol. Images were acquired on a confocal laser-scanning microscope (Leica SP8, Leica, Germany) using a × 63 immersion oil objective (numeric aperture=1.4). z-Stacks were acquired with a pixel size of 141 nm and 300-nm z-steps.

All animal experiments were approved by the regional animal welfare committee (permit number: G132/16 Regierungspräsidium Karlsruhe).

Western blots

For western blots, we used NuPAGE Bis-Tris Gels 4–12% (Invitrogen), and the following primary antibodies: mouse anti-Fz1 (DSHB 1:500), mouse anti-Fz2 (DSHB 1:500) and mouse anti-tubulin (1:10,000 Sigma), we use Tubulin as a loading control instead of actin because the tumor microtubes are Actin positive and tubulin negative as previously described [19]. There were 3 biological replicates and Relative Fz1

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688 Average pixel intensity was measured using measurement tool from Image Studio Lite
689 Ver 5.2 and normalized against Tubulin.
690
691 **Proximity ligation assay**
692 DUO92101 Duolink® In Situ Red Starter Kit Mouse/Rabbit with DUO92013 Duolink In
693 Situ Detection Reagents FarRed (Sigma).
694 The interaction between Wg and Fz1 in Drosophila larval brains was detected *in situ*
695 accordingly to the instructions of the manufacturer. Briefly, primary antibody incubation
696 against Wg (mouse anti-Wg (DSHB 1:50) and Fz1 (Rabbit anti-Fz1 [103], 1:300)) were
697 applied using the same conditions as immunocytochemistry staining. Duolink
698 secondary antibodies against the primary antibodies were then added. These
699 secondary antibodies were provided as conjugates to oligonucleotides that were able
700 to form a closed circle via base pairing and ligation using Duolink ligation solution when
701 the antibodies were in close proximity [49] at a distance estimated to be <40 nm. The
702 detection of the signals was conducted by rolling circle amplification using DNA
703 polymerase incorporating fluorescently labeled nucleotides into the amplification
704 products. The resulting positive signals were visualized as bright fluorescent dots, with
705 each dot representing one interaction event. As negative control one of the primary
706 antibodies was not added therefore, no positive signals were obtained from that assay).
707 The tissues were visualized using a confocal microscope system (LEICA TCS SP5).
708
709 **In situ hybridizations**
710 Protocol was performed according to [104]. Imaginal discs and brains were dissected and
711 fixed in 4% formaldehyde for 20 min at room temperature, washed in PBS-0.1% Tween
712 (PBT) and re-fixed for 20 min at room temperature with 4% formaldehyde and 0.1%
713 Tween. After three washes in PBT, discs were stored at −20°C in hybridization solution
714 (HS; 50% formamide, 5× SSC, 100 µg/ml salmon sperm DNA, 50 µg/ml heparin and
715 0.1% Tween). Disc were pre-hybridized for 2 h at 55°C in HS and hybridized with

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digoxigenin-labelled RNA probes at 55°C. The probes were previously denaturalized at 80°C for 10 min. After hybridization, discs were washed in HS and PBT, and incubated for 2 h at room temperature in a 1:4000 dilution of anti-DIG antibody (Roche). After incubation, the discs were washed in PBT and the detection of probes was carried out using NBT and BCIP solution (Roche). The discs were mounted in 70% glycerol. Images were acquired with a Leica DM750 microscope and Leica MC170HD camera and LASv4.8 software. The probes were generated from the cDNAs RE026007 (*wg)* and LD32066 *(fz1)* from the Expression Sequence Tags (EST) collection of the Berkeley Drosophila Genome Project.

TEM

Transmission electron microscopy (TEM) was performed in CNS of 3rd instar larvae with horseradish peroxidase (HRP) genetically driven to glial cells. Brains were fixed in 4% formaldehyde in PBS for 30 min at room temperature, and washed in PBS, followed by an amplification of HRP signal using the ABC kit (Vector Laboratories) at room temperature. After developing with DAB brains were washed with PBS and fixed with 2% glutaraldehyde, 4% formaldehyde in PBS for 2h at room temperature. After washing in phosphate buffer the samples were postfixed with OsO4 1% in 0.1 M phosphate buffer, 1% K3[Fe(CN)6] 1h at 4ºC. After washing in dH2O, Brains were incubated with tannic acid in PBS for 1min at room temperature then washed in PBS for 5min and dH2O 2x5min. Then the samples were stained with 2% uranyl acetate in H2O for 1h at room temperature in darkness followed by 3 washes in H2O2d. . Brains dehydrated in ethanol series (30%, 50%, 70%, 95%, 3x100% 10 min each at 4ºC). Infiltration: samples were incubated in EtOH : propylene's OXID (1:1;V.V) for 5 min, propylene's OXID 2x10min, propylene's OXID:Epon (1:1) for 45 min, Epon 100% in agitation for 1 h and Epon 100% in agitation overnight. Then change to Epon 100% for 2-3 h. Finally encapsulate the samples in BEEM capsules and polymerize 48h at 60ºC[105].

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744

745 **Imaging**

746 Fluorescent labeled samples were mounted in Vectashield mounting media with DAPI
747 (Vector Laboratories) and analyzed by Confocal microscopy (LEICA TCS SP5/SP8).
748 Images were processed using Leica LAS AF Lite and Fiji (Image J 1.50e). Images were
749 assembled using Adobe Photoshop CS5.1.

750

751 **Quantifications and Statistical Analysis**

752 Relative Wg, Fz1, Arm, WNT1 and βCatenin staining within brains was determined
753 from images taken at the same confocal settings. Average pixel intensity was
754 measured using measurement log tool from Fiji 1.51g and Adobe Photoshop CS5.1.
755 Average pixel intensity was measured in the glial tissue and in the adjacent neuronal
756 tissue (N=~10 for each sample) and expressed as a ratio. Total average pixel intensity
757 of WNT1 and βCatenin staining within mice brains was measured in the glioma (N=6)
758 and control samples (N=6), to quantify this, single sections were taken from similar z-
759 positions in both control and glioma samples. Glial network volume was quantified
760 using Imaris surface tool (Imaris 6.3.1 software). The number of Proximity ligation
761 assay puncta, HA^+ puncta, $Repo^+$ cells and the number of synaptic active sites was
762 quantified by using the Imaris 6.3.1 software.
763 The Western Blot bands were quantified by using the Image Studio Lite 5.2 software.
764 Data was analyzed and plotted using GraphPad Prism v7.0.0. A D'Agostino & Pearson
765 normality test was performed and the data found to have a normal distribution were
766 analyzed by a two-tailed t-test with Welch-correction. In the case of multiple
767 comparisons a One-way ANOVA with bonferroni post test was used. The data that did
768 not pass the normality test were submitted to a two-tailed Mann-Whitney U-test or in
769 the case of multiple comparisons a Kruskal-Wallis test with Dunns post test. Error bars
770 represent Standard Error of the Mean, significance was ***p≤0.0001, ** p≤0.001 *
771 p≤0.01, ns=non-significant.

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772

773

774 **Viability assays**

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

779 **Survival assay**

780 Males *Tub-Gal80; Repo-Gal4* were crossed with males bearing a control construct
781 (*UAS–LacZ*) or glioma (*UAS–PI3K^{dp110}; UAS-EGFR$^{\lambda}$)* and raised at 17°C. Progeny
782 bearing a glioma (experimental) or LacZ (control) chromosomes were put at 29ºC and
783 viability was calculated as the percentage of surviving flies with respect to the starting
784 number of flies as follows: viability = observed (nº of flies)/starting nº of flies × 100. Six
785 independent vials for glioma (*n*= 6) and control (*n*= 6) were analyzed, with each vial
786 with 10 flies.

787

788 **qRT-PCRs**

789 Total RNA was isolated from larvae brains (Trizol, Invitrogen) and cDNAs were
790 synthesized with M-MLV RT (Invitrogen). The following specific probes from applied
791 Biosystems were used: Wingless Dm01814379_m1 and Frizzled1 Dm01793718_g1,
792 RpL32 Dm02151827_g1 was used as housekeeping.

793 qRT-PCR was performed using Taqman Gene Expression (Applied Biosystems) using
794 a 7500 Real Time PCR System (Applied Biosystems) with cycling conditions of 95°C
795 for 10 min and 40 cycles of 95°C for 15 s and 55°C for 1 min. Each experimental point
796 was performed with samples from two independent crosses and three replicates per
797 experimental point, and differences were assessed with a 2-tailed Student *t* test.

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798 Results were normalized using the housekeeping RpL32 and the ΔΔ cycle threshold
799 method and are expressed as the relative change (-fold) of the stimulated group over
800 the control group, which was used as a calibrator. qRT-PCR results were analyzed with
801 7500 v2.0.6 software (Applied Biosystems).

802

803 **Figure Legends**

804 **Figure 1: Co-activation of EGFR-Ras and PI3K in *Drosophila* glia causes an**
805 **expansion of the glial network that is susceptible to Gap43 depletion.**

806 Brains from 3rd instar larvae. Glia are labeled with GFP (green) driven by *repo-Gal4*.
807 Each brain is composed of 2 symmetrical hemispheres. (A-C) In *repo>dEGFR$^{\lambda}$;*
808 *dp110^{CAAX}* (*glioma*) larvae (B), both brain hemispheres and the VNC are enlarged and
809 elongated and the number of glial cells is increased relative to *wt* control (A). The
810 quantification of the number of glial cells is shown in (C). Arrows indicate glial nuclei.

811 (D–E) Optical sections of larval brain to visualize the glial network, glial cell bodies and
812 membranes are labeled in red (myrRFP). (D) RFP signal in control brains shows glial
813 somas and the network in *wt* brain. (E) The glioma brain shows a dramatic increase in
814 the membrane projections and in the size of the network. Nuclei are marked with DAPI
815 (blue). Scale bars sizes are indicated in this and all figures.

816 (F-K) Glia are labeled with *UAS-ihog-RFP* to visualize active cytoneme/TM structures
817 in glial cells as part of an interconnecting network. (F-G) In control brains the active
818 glial cytonemes are shown by *repo>ihog-RFP* in red. In glioma brains, the TMs grow
819 and expand across the brain, quantification of the network volume and the network/glial
820 cell ratio is shown in H. (I-K) Higher magnifications of control brains (I) showing the glial
821 cytonemes (red) compared with the glioma brains where the TMs overgrow and
822 enwrap neuronal clusters (J). Upon Gap43 downregulation the glial network does not

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overgrow or enwrap neuronal clusters (K) and shows a pattern and size similar to the control. Nuclei are marked with DAPI. Arrows indicate glial cytonemes/TMs. (L) A viability assay shows that the lethality induced by the glioma is fully rescued upon knockdown of *Gap43*. Error bars show S.D. *** P<0.0001. Scale bar size are indicated in this and all figures.

Figure 2: *Drosophila* Tumor Microtubes

Transmission electron microscopy (TEM) images of a 3rd instar larval brains expressing HRP in the glial cells. (A-B) HRP deposits label cell membranes, thus identifying glial cells. Coloured images from control brains showing glial cells identified by HRP staining (magenta) and HRP-negative neurons (cyan). (C) Schematic diagram, a glioma cell labeled with HRP (magenta) showing that glioma cells produce a network of TMs that grow to surround neighboring neurons (cyan). (D-G) Several magnifications of Glioma brains showing TMs that grow and enwrap neighboring neurons (cyan). Detail of several layers of a glioma membrane enwrapping a neuron (F) and a longitudinal section of a TM (G), arrows indicate glial membranes. (H-I) Control and glioma brains from 3rd instar larvae. Glia is labeled with *UAS-Ihog-RFP* driven by *repo-Gal4* to visualize TMs in glial cells as part of an interconnecting network (red). Glial network is marked with lifeActin-GFP reporter (green) and nuclei are marked with DAPI (blue). Imaris 3D reconstructions are shown in H´´´-I´´´).

Figure 3: Wingless/Fz1 accumulate in glioma cells

Larval brain sections with glial cell bodies and membranes labeled in red (myrRFP) and stained with Wg antibody show homogeneous expression in the control brains (A) in green. In the glioma brains Wg accumulates in the glial transformed cells (B), quantified in (C). Arrows indicate Wg staining in glial membranes. (D-F) Glial cells are labeled with *UAS-Ihog-RFP* to visualize the glial network, and stained with Fz1 (green).

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(D) Fz1 is homogeneously distributed in control brains, with a slight accumulation in the Ihog+ structures. (E) Fz1 accumulates in the TMs and specifically in the projections that are in contact with the neuronal clusters. (F) Upon knockdown of *fz1* in glioma brains, the tumoral glial network is still formed but Fz1 is not detectable. (G) Knockdown of *Gap43* in glioma brains restores a normal glial network and Fz1 shows a homogeneous distribution along the brain section. Arrows indicate Fz1 staining in glial membranes. (H) Quantification of Fz1 staining ratio between $iHog^{+}$ and $iHog^{-}$ domains. Nuclei are marked with DAPI. Error bars show S.D. * P<0.01 *** P<0.0001 or ns for non-significant.

Figure 4: Fz1 in glia interacts with neuronal Wg

GRASP technique was used and both halves of green fluorescent protein tagged with a CD4 signal to direct it to the membranes (CD4-spGFP) were expressed in neurons (*elav-lexA*) and glial (*repo-Gal4*) cells respectively. Only upon intimate contact GFP protein is reconstituted and green fluorescent signal is visible. (A-C) Control brains showed a discrete signal corresponding to the physiological interaction between glia and neurons. (D-F) In glioma brains a massive GFP signal from GRASP reporter is detected. Arrows indicate GRASP reconstitution GFP signal. (G-H) Control and Glioma brains stained with Wg (red) and Fz1 (green). Gliomas show Fz1 and Wg accumulation in glioma TMs (ihog-RFP in blue), specifically Wg protein accumulates at the border between neuron and glioma cells. Arrows indicate Wg-Fz1 co-localization at the Glia-neuron interphase. (I-K) Proximity ligation assays (PLA) were performed in control and glioma brains to quantify the interactions between Wg and Fz1. (I) Control brains showed a discrete number of puncta (green) showing the physiological interactions. (J) Glioma brains showed a five-fold increase in the number of puncta, quantified in (K). Nuclei are marked with DAPI (blue). Arrows indicate PLA^{+} puncta. Error bars show S.D. *** P<0.0001.

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Figure 5: Wg signaling pathway is active in glioma cells, and the glioma inactivates it in neuronal clusters in glioma brains.

Larval brain sections with glial cytonemes labeled in red and stained with Arm (green). (A) Cytoplasmic-Armadillo (Cyt-Arm) is homogeneously distributed in control sections. (B) In glioma brains Cyt-Arm accumulates in the neurons cytoplasm where it is inactive. (C) Knockdown of *Gap43* in glioma brains restores a normal glial network and Arm does not accumulate showing a homogeneous distribution similar to the control. Arrows indicate Cyt-Arm staining at the Glia-neuron interphase. (D) Quantification of Cyt-Arm staining ratio between Ihog+ and Ihog$^-$ domains. Glial cell bodies and membranes are labeled with myrRFP (red). (E-H) Wg signaling pathway reporters *arm-GFP* (E-F) and *nkd-lacZ* (stained with anti-bGal (G-H) in control and glioma brains show activation of the pathway in glioma cells compared with the reporter activation mostly in neurons in the control brains. Arrows indicate cells with reporter activation. (I-N) Confocal immunofluorescence single plane images of S24 GBSC NMRI nude mice brains (glioma) and NMRI nude mice (control) brains stained with human anti-βCatenin (I-J) and WNT1 (L-M) both show in grey (red in the merged image) an increase in the glioma samples. The corresponding quantification of the pixel intensity is shown in K and N. Green signal from tumor cell GFP expression allows specific detection of S24 GBSC related structures in the mouse brain (I´, L´). Arrows indicate glioma or control cells. Nuclei are marked with DAPI. Error bars show S.D. *** P<0.0001 and ns for non-significant.

Figure 6: Glioma network is responsible for the increase in the number of glial cells.

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Larval brain sections with glial cell nuclei stained with Repo (green). The number of glial cells is quantified in the following genotypes: (A) Control, (B) Glioma showing an increase in Repo+ cells. (C) Upon knockdown of *Fz1* in glioma brains, the number of glial cells is partially restored (D) knockdown of *Gap43* in glioma brains restores the number of glial cells similar to the control. (E) Quantification of the number of Repo+ cells. Nuclei are marked by DAPI (blue). (F) Viability assay showing the lethality induced by the glioma that is partially rescued upon knockdown of *fz1*. Error bars show S.D. *** P<0.0001, and ns for non-significant.

Figure 7: Gliomas cause Neurodegeneration and Wg expression in glioma cells is dispensable for tumor progression

Neurons from the larval neuromuscular junction are stained with Nc82 showing the synaptic active Zones. (A-F) Upon glioma induction (B) the number of synapses (grey) is reduced when compared with the control (A). The number of synapses is restored upon knockdown of *Fz1* (C), *Gap43* (D) or *armS10; Fz1-RNAi* (E).The quantification of synapse number in all genotypes is shown in (F). (G-L) *wg* knockdown (I) in glioma cells (*wg-RNAi*) or *armS10; Fz1-RNAi* (J) does not prevent glioma cell numbers increase nor glioma TMs volume expansion quantified in (K-L). Error bars show S.D. *** P<0.0001, ** P<0.001 and ns for non-significant.

Figure 8: Knockdown of the Wg signaling pathway results in neurodegeneration and restoration of the glia-neuron Wg/Fz1 signaling equilibrium inhibits glioma progression.

Neurons from the larval neuromuscular junction are stained with Nc82 showing the synaptic active Zones. (A-D) Upon knockdown of *wg* (C) or *Fz1* (B) the number of synapses (grey) is reduced when compared with the control (A). Arrows indicate synapses. The quantification of synapse number in all genotypes is shown in (D).

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926 (E-H) Larval brain sections with glial network labeled with *UAS-Ihog-RFP* in red and
927 stained with Fz1 (grey or blue in the merge). Neurons are labeled with *lexAop-CD8-*
928 *GFP* driven by *elav-lexA*. Fz1 overexpression in neurons (green) restore homogeneous
929 Fz1 protein distribution (blue) in the brain, rescue brain size and neuron distribution (G-
930 H) compared to (E-F) where the *elav-lexA* is not present in the glioma brains, Nuclei
931 are marked with DAPI in (E-F) (green). Arrows indicate Fz1 staining in the glial
932 membranes at the Glia-neuron interphase of glioma brains and its restored localization
933 in G-H.

934 (I-L) Neurons from the larval neuromuscular junction are stained with Nc82 showing the
935 active Zones. Upon glioma induction the number of synapses (grey) is reduced (J)
936 when compared with the control (I). The number of synapses is restored upon
937 overexpression of Fz1 specifically in the neurons (K). Arrows indicate synapses. The
938 quantification of synapse number is shown in (L). Error bars show S.D. *** P<0.0001, *
939 P<0.01 or ns for non-significant.

940

941 **Figure 9: Glioma depletes Wg from neuronal membrane**

942

943 Larval brain sections with glial network labeled with *UAS-Ihog-RFP* in red and stained
944 with HA (blue). (A-B, H) Control brains express Wg^{NRT} and the anti-HA (grey or blue in
945 the merge) staining shows a positive signal in both neurons (green) and glial cells
946 (red). (C-D) Glioma samples that do not express Wg^{NRT} in neurons do not show HA
947 signal. (E-F, I) Glioma brains with membrane anchored Wg (Wg^{NRT} grey or blue in the
948 merge) in neurons (green), show a homogeneous signal for HA (grey or blue in the
949 merge) in both glioma cells and neurons, quantified in (J), and the glial network size is
950 restored in these animals (K). Arrows indicate HA^{+} staining in glial or neuronal
951 membranes. Error bars show S.D. ns for non-significant. (G) Schematic diagram of this

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952 experiment: a neuron labeled with GFP (green) and a glioma cell labeled with ihog-
953 RFP (red) showing that glioma cells produce a network of TMs that grow to reach
954 neighboring neurons. Intimate membrane contact facilitates neuronal-Wg (blue)
955 sequestering mediated by Fz1 receptor (black) from glioma. In this experiment neurons
956 express a membrane anchored version of Wg (Wg^{NRT} represented as Wg in blue with a
957 purple anchor) which is more difficult for the glioma to retrieve from the neuron.

958

959 **Supplementary Figures and VideosFigure S1 (Related to Figure 1): TMs enwrap**
960 **neurons in GB and cytoneme markers co-localize with glioma network**

961 Brains from 3rd instar larvae. Glia is labeled with *UAS-Ihog-RFP* or *UAS-myr-RFP*
962 driven by *repo-Gal4* to visualize TMs in glial cells as part of an interconnecting network,
963 nuclei are marked with DAPI (blue). (A-B) Neurons are stained in green (Hrp) and
964 enwrapped by glial TMs in glioma brains (see magnification (B) yellow arrowheads).
965 (C-G) Glial network is marked with several cytoneme markers: (C) lifeact-GFP reporter
966 (green and glial nuclei are marked with Repo, magenta), (D) GMA-GFP (green), (E)
967 GPI-YFP (green), (F) GFP-MLC (green), (G) sqh-GFP (green) in a glioma brain. (H-I)
968 Downregulation of neuroglian (*nrg-RNAi*) in glioma brains results in defective TMs.
969 Nuclei are marked with DAPI. Scale bar size are indicated in this and all figures.

970

971 **Figure S2 (Related to Figure 1): *Gap43* Knockdown does not show effects in the**
972 **number of synapses in the NMJ, in the glial network or in the viability of the flies**

973 (A-B) Glial cells are stained with Repo (green) and the number of glial cells are
974 quantified in the following genotypes: *Control*, *Glioma* showing an increase in Repo+
975 cells, *Glioma;lacZ* and *Glioma;yellow-RNAi* showing a similar number of Repo+ cells to
976 Glioma alone. (C-D) Upon *Gap43* knockdown by *RNAi* in normal brains, the glial

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977 network is similar to the control. Glial cells are marked by Repo in green. Nuclei are
978 marked by DAPI. (E-F) Neurons from the larval neuromuscular junction are stained
979 with Nc82 showing the synaptic active sites. Upon knockdown of *Gap43* the number of
980 synapses marked by Nc82 (green) is similar to the control. (F) Graph showing the
981 quantification of the synapse number. (G) A viability assay shows that the knockdown
982 of *Gap43* does not alter the viability of male and female flies. Error bars show S.D. ***
983 P<0.0001 or ns for non-significant.

984 **Figure S3 (Related to Figure 3): Cases of human GB patients with mutations in**
985 **WNT or FZD.**

986 Complete analysis of mutations in human GB samples from COSMIC database
987 http://cancer.sanger.ac.uk/cosmic (A) and Cancer Genome Atlas (TGAC) for
988 transcriptional targets of WNT pathway (B), WNT ligands (C) and FZD receptors (D),
989 data are represented in percentage out of 902 or 922 samples. The total number of
990 cases with mutations in any WNT or FZD gene is shown in red. Genes from WNT and
991 FZD family without mutations in GBs is shown in the bottom.

992

993 **Figure S4 (Related to Figure 3): Fz2 remains normal in glioma brains.**

994 (A) Wing imaginal control disc stained with Fz2 (green) showing the expected
995 endogenous localization pattern. Brains from 3rd instar larvae displayed at the same
996 scale. Glia is labeled with *UAS-Ihog-RFP* driven by *repo-Gal4* to visualize active
997 filopodia/TMs in glial cells, and stained with Fz2 (green). (B) Fz2 is homogeneously
998 distributed in control sections, (C) Fz2 is homogeneously distributed in glioma brain
999 sections, similar to the control. Nuclei are marked by DAPI (blue). Scale bar size is
1000 indicated in the figure. Glial cell bodies and membranes are labeled with *UAS-myrRFP*
1001 (red) driven by *repo-Gal4* and stained with GFP antibody to visualize a tagged form of

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endogenous Fz1 protein. (D) Fz1-GFP accumulates specifically in glial transformed cells that are in contact with neuronal clusters. Nuclei are marked by DAPI (blue).

Figure S5 (Related to Figure 3 and 8): Validation of tools, RNAis and antibodies

(A) *UAS-Fz1-RNAi* tool was validated in epithelial tissue, wing imaginal discs. Upon *UAS-Fz1-RNAi* expression in the posterior compartment (marked with GFP), there is a reduction in Fz1 protein signal compared with the anterior compartment from the same tissue. (B) *UAS-Wg-RNAi* tool was validated in epithelial tissue, wing imaginal discs. Upon *UAS-Wg-RNAi* expression in the posterior compartment (marked with GFP), there is a reduction in Wg protein signal compared with the anterior compartment from the same tissue. (C) *lexAop-Fz1* tool was validated in brain tissue. Upon ectopic Fz1 expression in the neurons (driven by *ELAV-LexA* marked with GFP), there is an increase in Fz1 protein signal compared with the rest of the brain tissue. (D) Upon ectopic Fz1 expression in the neurons (driven by *ELAV-LexA>lexAop-Fz1* marked with GFP), there is an increase in active Arm protein signal compared with the rest of the brain tissue.

Figure S6 (Related to Figure 4): Wg and Fz1 transcription levels are similar between controls and gliomas

(A) qPCRs with *RNA* extracted from control and glioma larvae showing no change in the transcription (*mRNA* levels)of *wg* or *fz1*. (B) Western blot of samples extracted from control and glioma larvae showing no change in the amount of Fz1 or Fz2 protein. Error bars show S.D. ns for non-significant. (C) In situ hybridization experiments for Wg and Fz1 in controls and gliomas showing no change in the transcription (*mRNA* levels) of *wg* or *fz1.*

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Figure S7 (Related to Figure 5): Wg signaling pathway is active in glioma cells

Larval brain sections with glial network labeled in red and stained with Cyt-Arm (green). (A) Knockdown of *Fz1* in glioma brains showing a homogeneous Cyt-Arm distribution similar to the control. Quantification of Cyt-Arm staining ratio between Ihog+ and Ihog$^-$ domains is shown in principal Figure 5D. (B-G) Glial cell bodies and membranes are labeled with myrRFP or ihog-RFP (red) driven by *repo-Gal4*. Wg signaling pathway reporters *tsh-lacZ* stained with anti-bGal (B-C), *fz4-GFP* (D-E) and *dally-lacZ* stained with anti-bGal (F-G) show activation of the pathway in glial transformed cells.

Figure S8 (Related to Figure 5): Wg signaling pathway is active in human glioma cells

(A-D) A series of grade II and III GB images from S24 xenografts brain sections stained with WNT1 and ß-Catenin show an increase of these signals in grade III when compared with grade II brain sections, indicating that the accumulation of WNT1 and ß-Catenin correlates with the progression of the GB, quantified in (E-F) (G-H) Technical immunohistofluorescence negative control in NMRI nude control mice brains stained only with the corresponding secondary antibodies showing the background unspecific signal. Nuclei are marked by DAPI (blue).

Figure S9 (Related to Figure 3, 5 and 7): Adult *Drosophila* gliomas

(A) Survival curve of adult control or glioma flies after a number of days of glioma induction and progression. (B-H) Adult brain sections 7 days after glioma induction with Glial cells are labeled with *UAS-myr-RFP* to visualize the glial network and stained with Cyt-Arm, Fz1 and Wg antibodies. (B-C, D) Cyt-Arm staining specifically marks the mushroom bodies and it is homogeneously distributed in the rest of the brain tissue in control sections and accumulates in the neurons cytoplasm where it is inactive in

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glioma brains. Quantification of Cyt-Arm staining ratio between RFP+ and RFP$^-$ domains (D). (B´-C´, E) Fz1 staining show homogeneous localization in the control brains (B´) in blue. In the glioma brains Fz1 accumulates in the glial transformed cells (C´), quantified in (E). (F-H) Wg is homogeneously distributed in control brains, with a slight accumulation in the RFP+ structures. Wg accumulates in the glioma network similar to the larval brains quantified in (H). (I) Graph showing synapse number quantification of adult NMJs from control flies and glioma-bearing flies.

Figure S10 (Related to Figure 8): Restoration of the glia-neuron Wg/Fz1 signaling equilibrium inhibits glioma progression

(A-D) Brains from 3rd instar larvae displayed at the same scale. Glia is labeled with *UAS-Ihog-RFP* driven by *repo-Gal4* to visualize active filopodia in glial cells, and stained with Wg or Arm (green). Neurons are labeled with *lexAop-CD8-GFP* driven by *elav-lexA* (blue). Fz1 overexpression in neurons (blue) restore homogeneous Wg (grey or green in the merge) (B) and Cyt-Arm (D) protein distribution (green) in the brain, compared to (A, C) where the *elav-lexA* is not present in the glioma brains, Nuclei are marked by DAPI (blue) in (A, C).

Video S1: Control Network

3D video reconstruction of control brains with glia labeled with *ihog-RFP* (*repo>ihog-RFP*) in red (grey in the 3D reconstruction) to visualize cytoneme structures in glial cells as part of an interconnecting network.

Video S2: Glioma TMs

3D video reconstruction of Glioma brains with glia labeled with *ihog-RFP* (*repo>ihog-RFP*) in red (grey in the 3D reconstruction) to visualize TMs structures in glial cells as

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1077 part of an interconnecting network. In glioma brains, the TMs expand across the brain
1078 and form perineuronal nests.

1079

1080 **Video S3: Glioma Gap43-RNAi Network**

1081 3D video reconstruction of Glioma; Gap43-RNAi brains with glia labeled with *ihog-RFP*
1082 (*repo>ihog-RFP*) in red (grey in the 3D reconstruction) to visualize TM structures in
1083 glial cells as part of an interconnecting network. Upon Gap43 downregulation the glial
1084 network does not overgrow or enwrap neuronal clusters and shows a pattern and size
1085 similar to the control.

1086

1087 **Video S4: Control LifeActin**

1088 3D video reconstruction of control brains from 3rd instar larvae. Glia is labeled with
1089 *UAS-Ihog-RFP* driven by *repo-Gal4* to visualize cytonemes in glial cells as part of an
1090 interconnecting network (red). Glial network is marked with lifeActin-GFP reporter
1091 (green) and nuclei are marked with DAPI (blue).

1092

1093 **Video S5: Glioma LifeActin**

1094 3D video reconstruction of gliomal brains from 3rd instar larvae. Glia is labeled with
1095 *UAS-Ihog-RFP* driven by *repo-Gal4* to visualize TMs in glial cells as part of an
1096 interconnecting network (red). Glial network is marked with lifeActin-GFP reporter
1097 (green) and nuclei are marked with DAPI (blue). Glial TMs enwrap clusters of neurons
1098 in individual GB perineuronal nests.

1099

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Video S6: Climbing assay

Video of *Drosophila* adult negative geotaxis behavior analysis (climbing assay), as an indication for possible motor defects associated with neurodegeneration. The results showed symptoms of neurodegeneration in glioma flies (right tube) compared to controls (left tube).

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Portela_Fig8

Active Zones Nc82

Control

A

10 µm

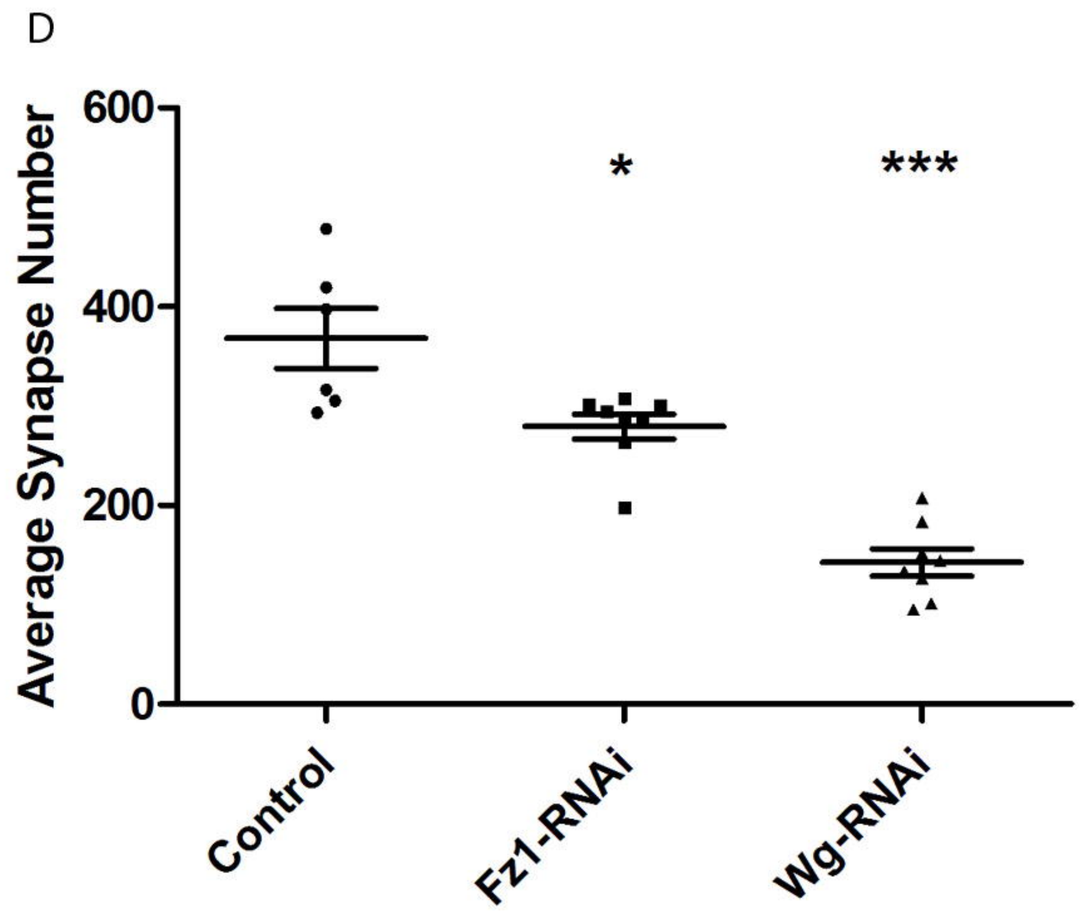
$Fz1^{RNAi}$

B

Wg^{RNAi}

C

D



Fz1 | Repo-ihog-RFP | Fz1 ihog-RFP DAPI

Glioma

E

20 µm

E´

E´´

F

10 µm

F´

F´´

Glioma; Neuron>Fz1

Active Zones Nc82

Control

I

10 µm

Glioma

J

K

Fz1 | Repo-ihog-RFP | Fz1ihog-RFP ELAV-GFP

G

20 µm

G´

G´´

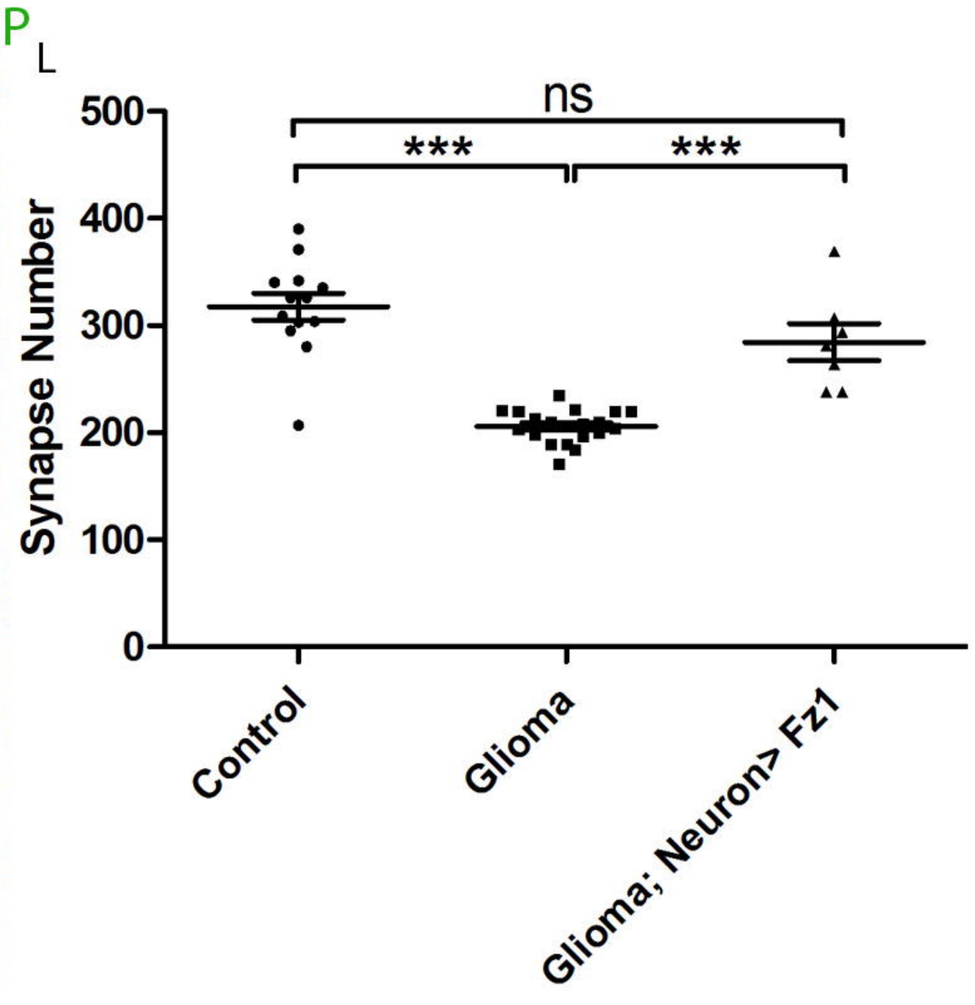
H

10 µm

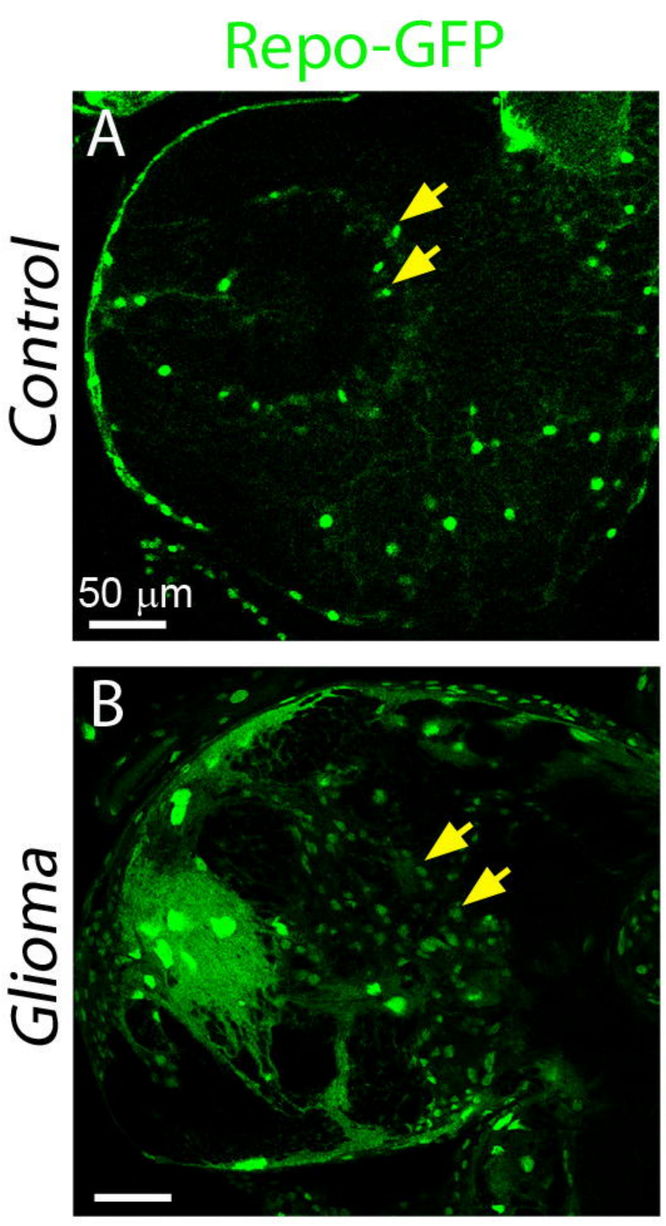
H´

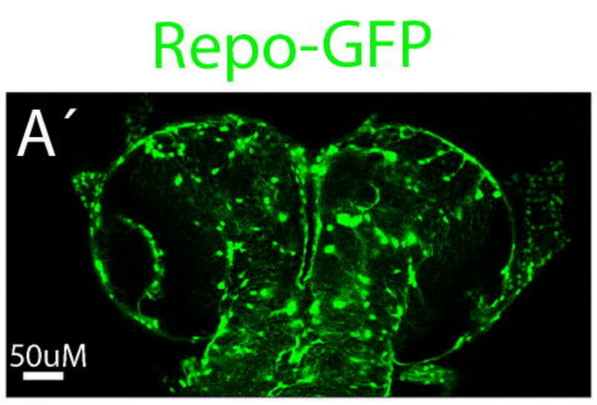
H´´

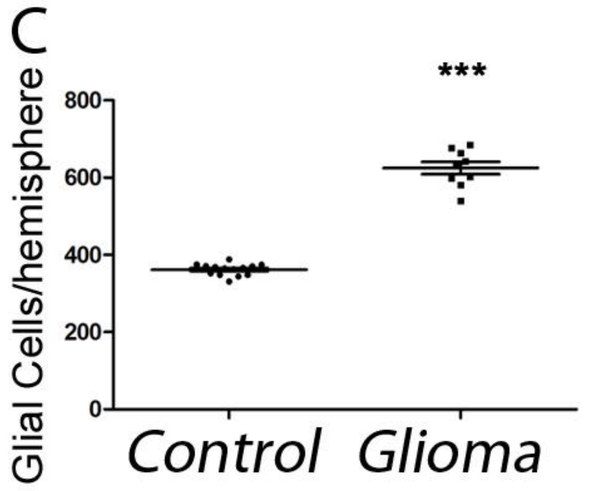
L

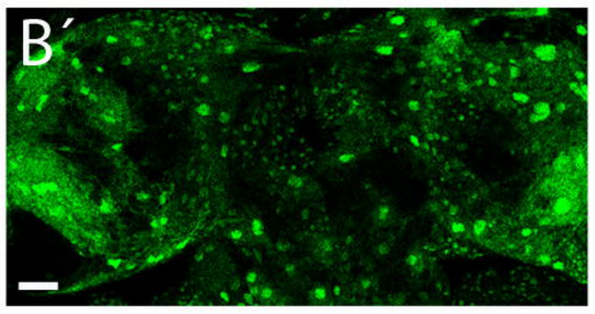


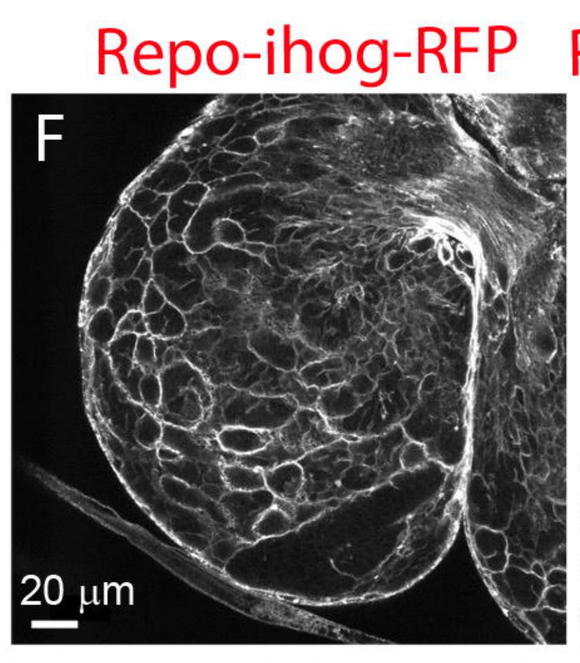
Portela_Fig1

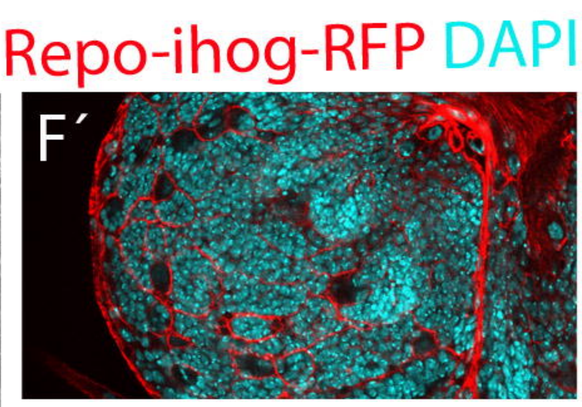


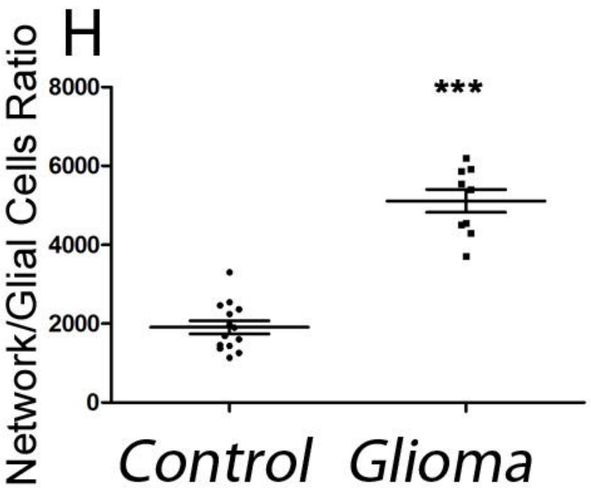


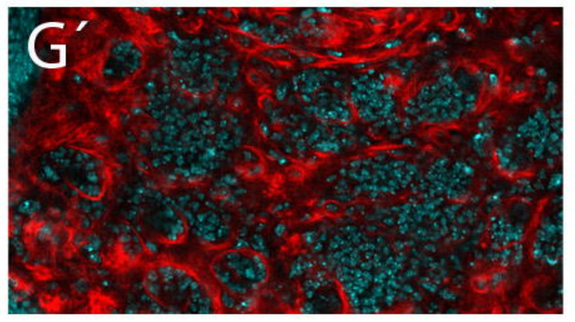


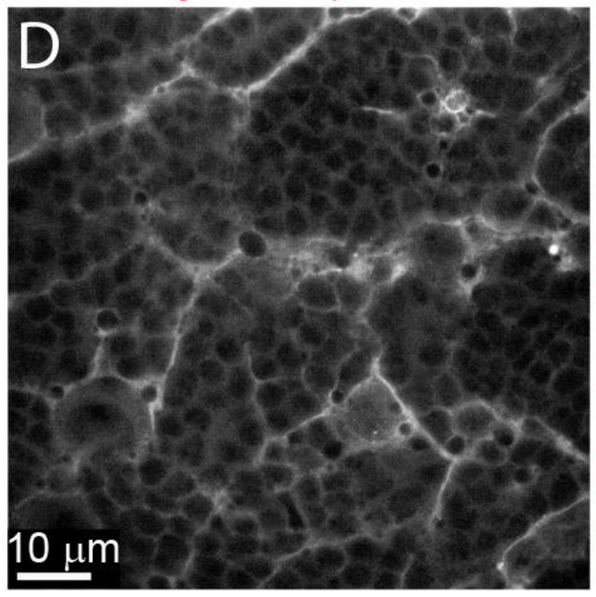


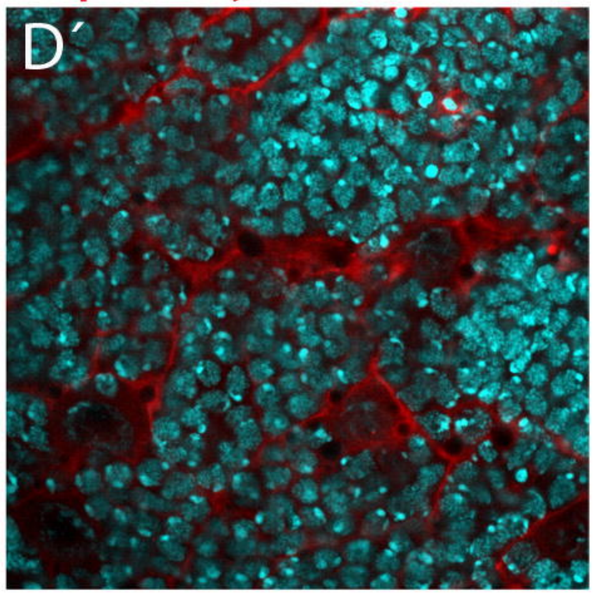


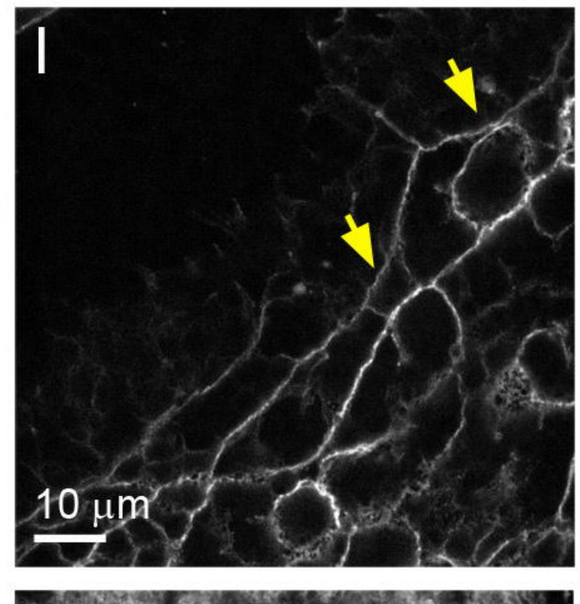


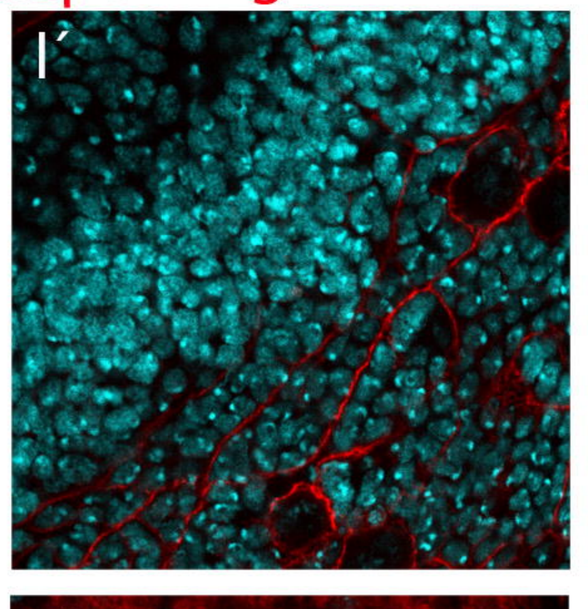


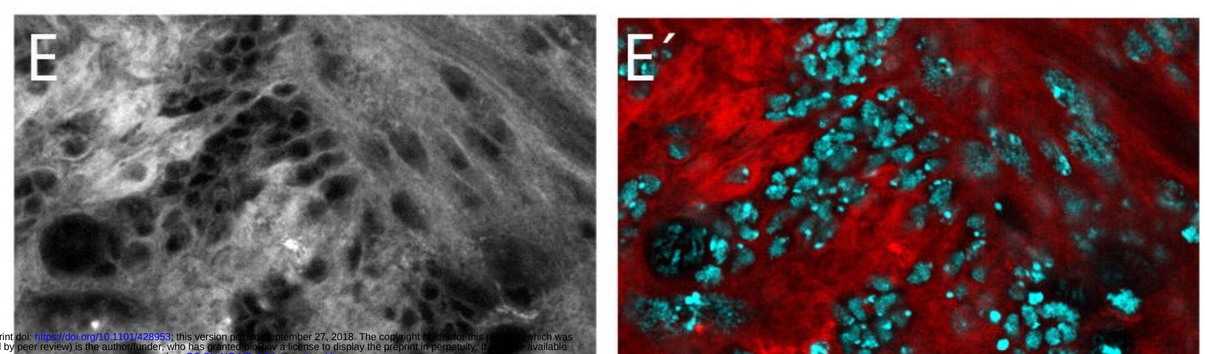












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Portela_Fig 2

Control

Glioma

A

6µm

B

2µm

Neuron

Glia

C

D

6µm

E

2µm

F

500µm

G

500µm

LifeActin-GFP

Repo-ihog-RFP

DAPI

IMARIS 3D

H

H´

H´´

H´´´

50 µm

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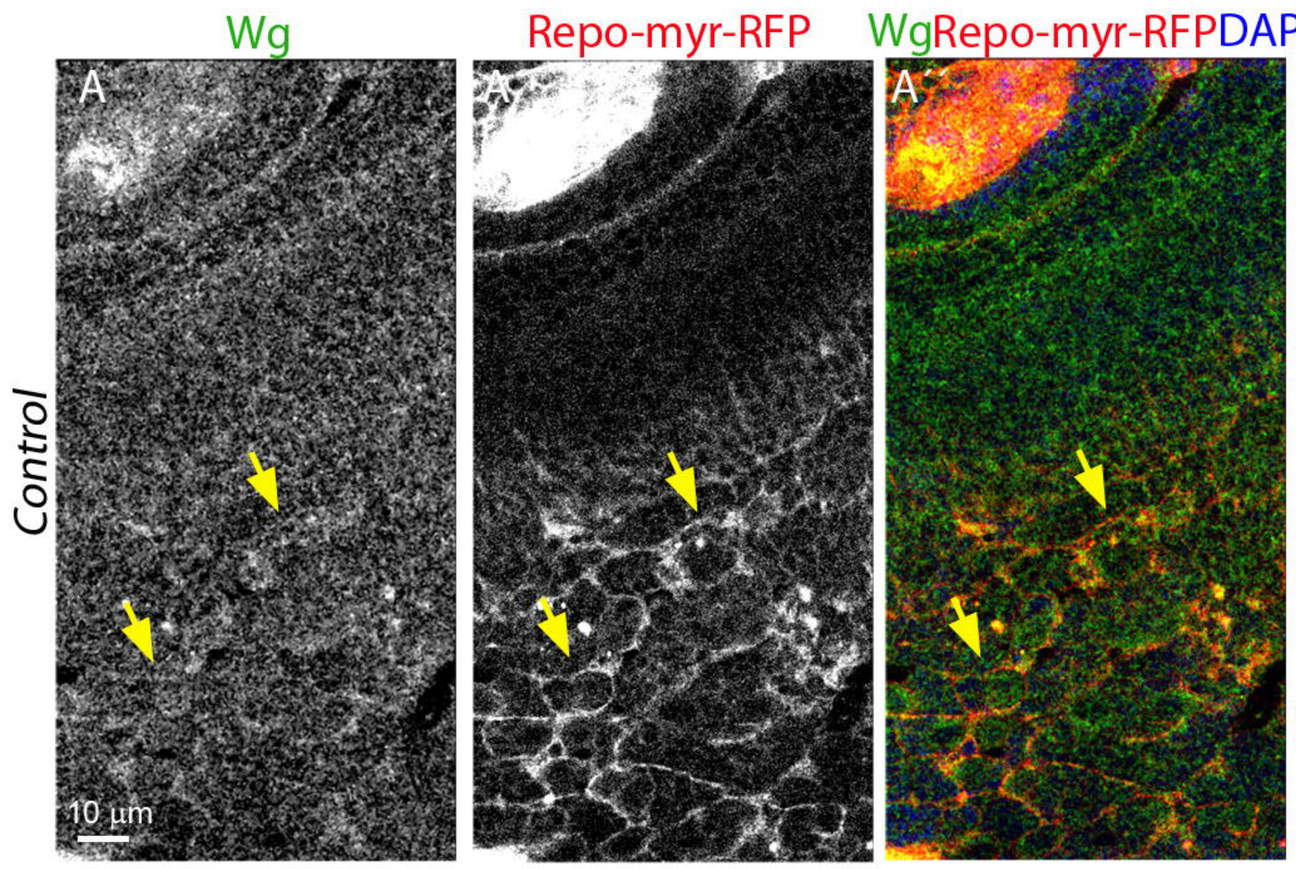
I

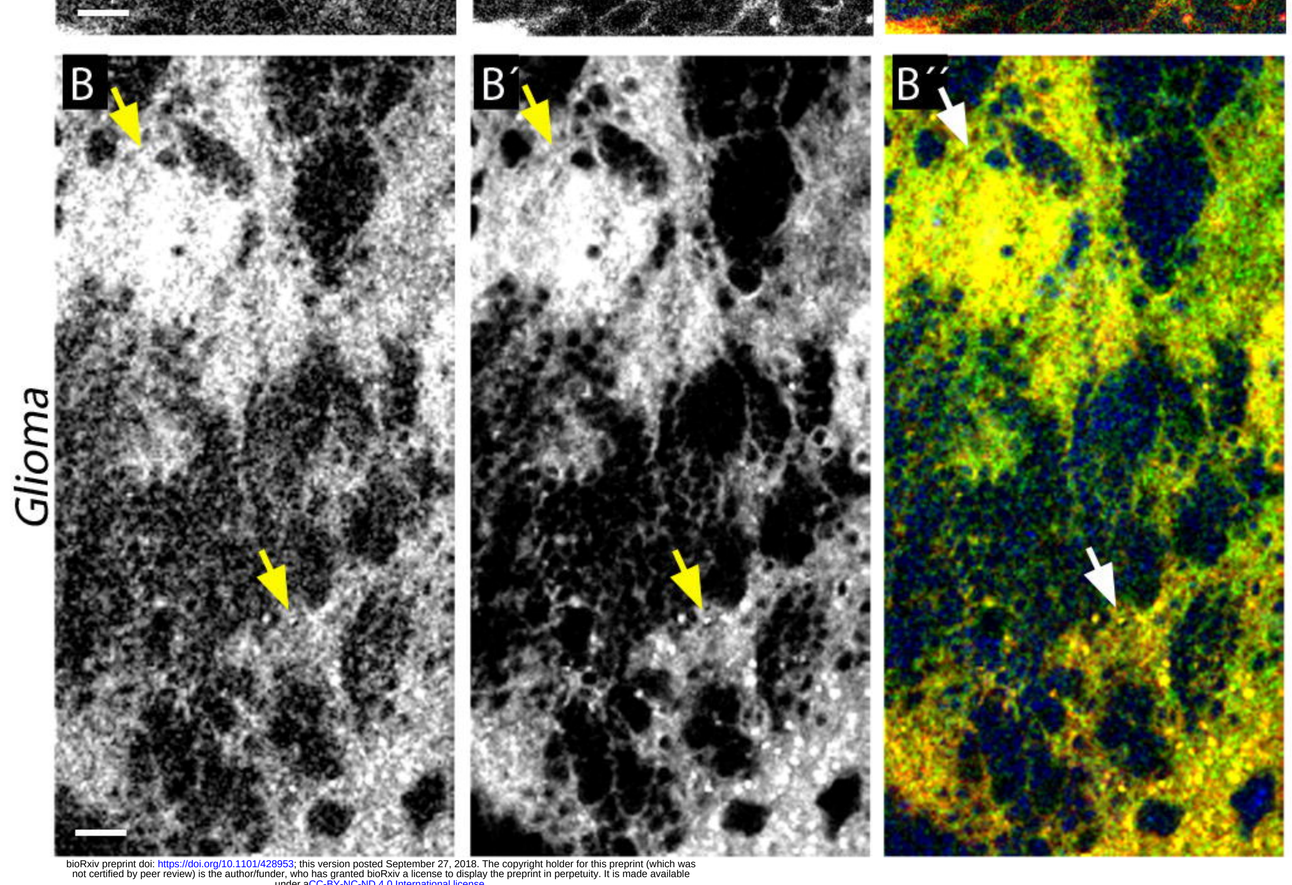
I´

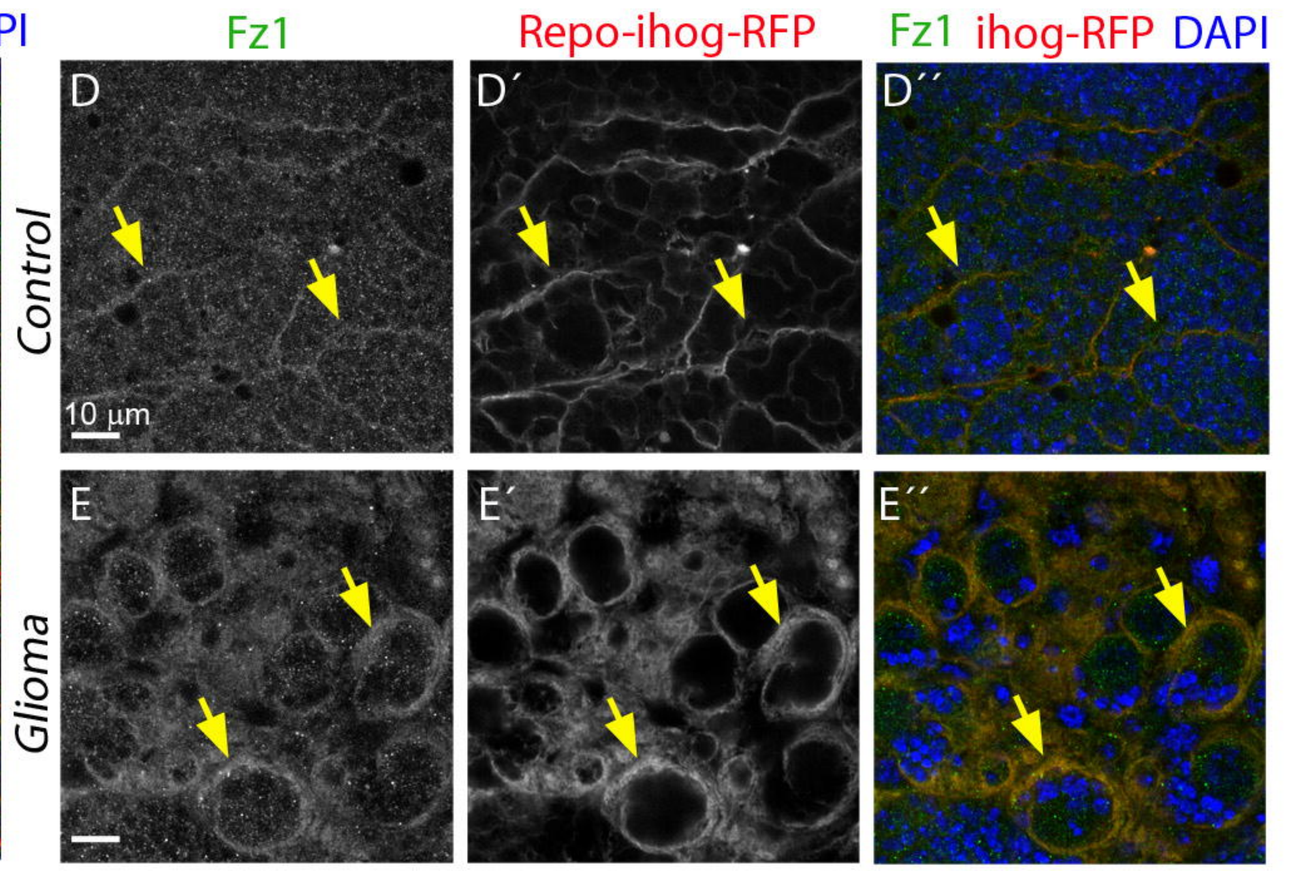
I´´

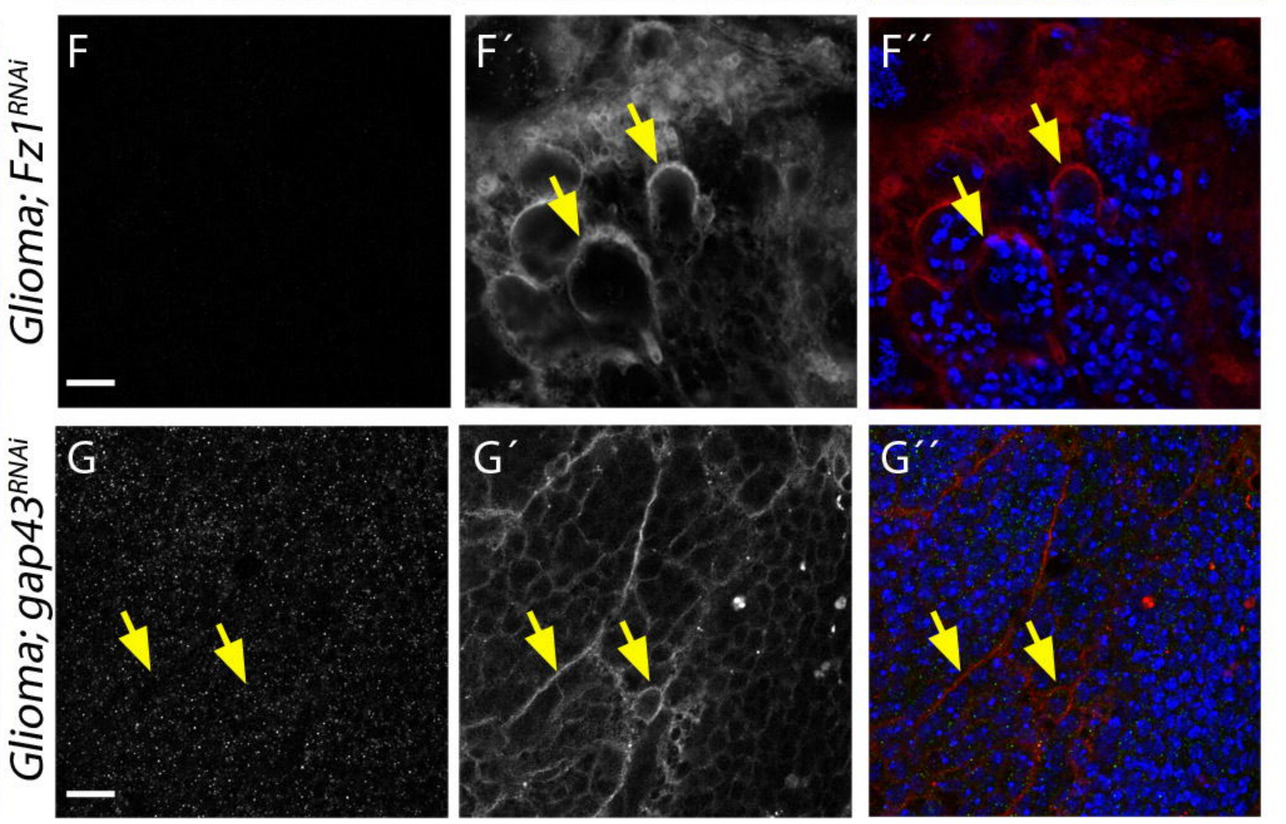
I´´´

Portela_Fig3









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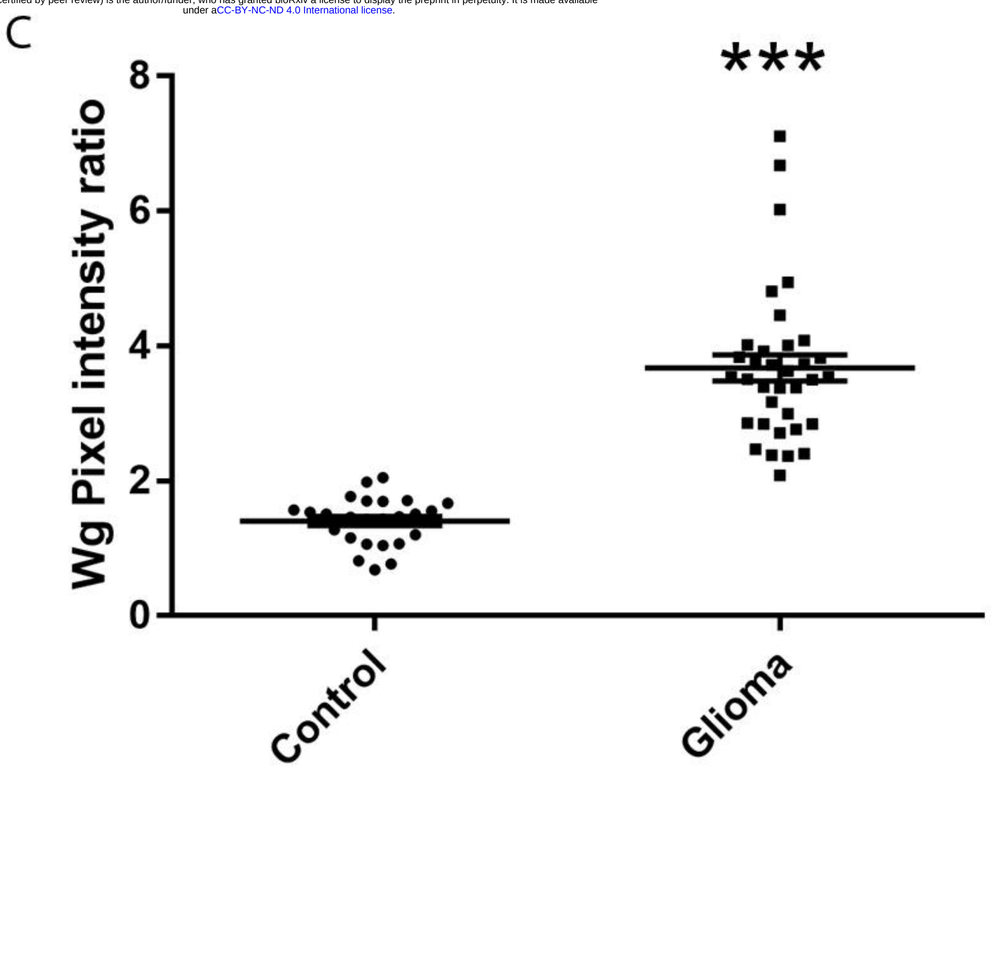
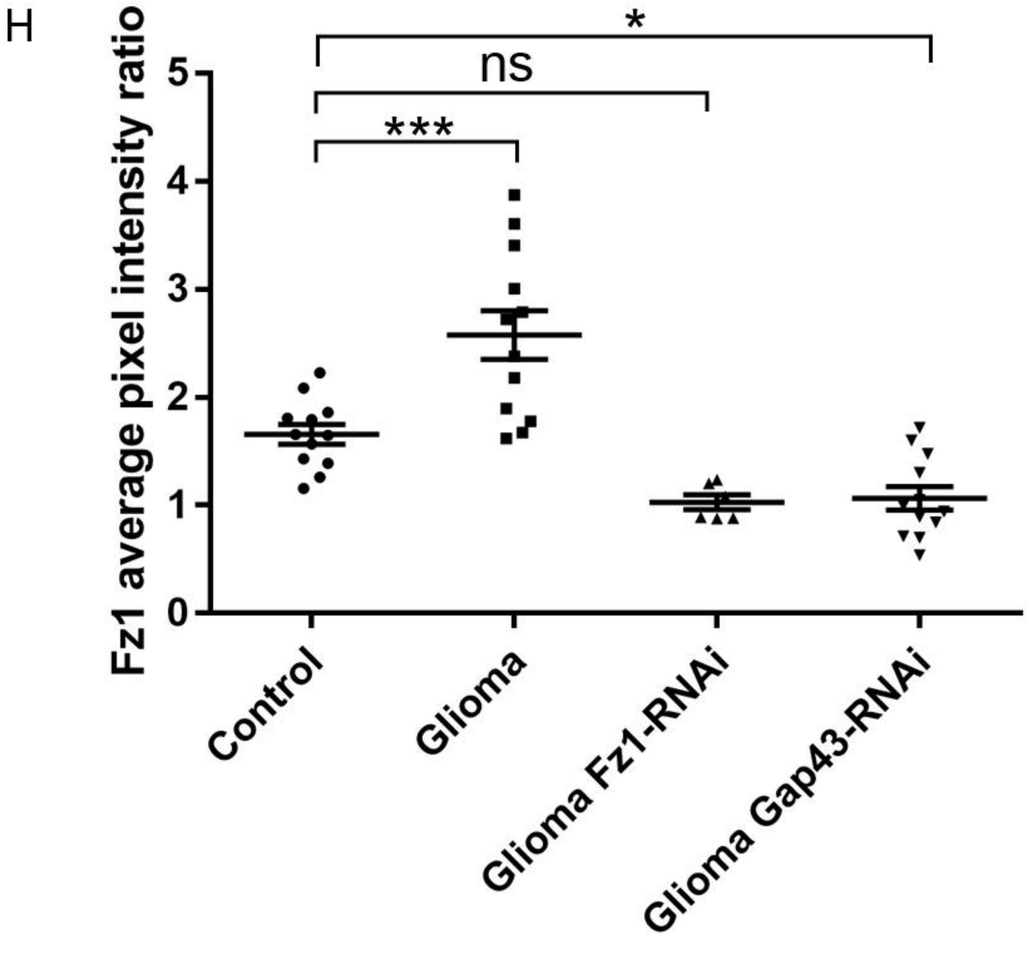




Portela_Fig4

ELAV-GRASP | Repo-ihog-RFP | DAPI

Control

A A′ A″

100 µm

B B′ B″

10 µm

C C′ C″

10 µm

Glioma

D D′ D″

E E′ E″

F F′ F″

Fz1 | Wg | Repo-ihog-RFP

Control

G G′ G″

10uM

Glioma

H H′ H″

PLA | Repo-ihog-RFP | DAPI

Control

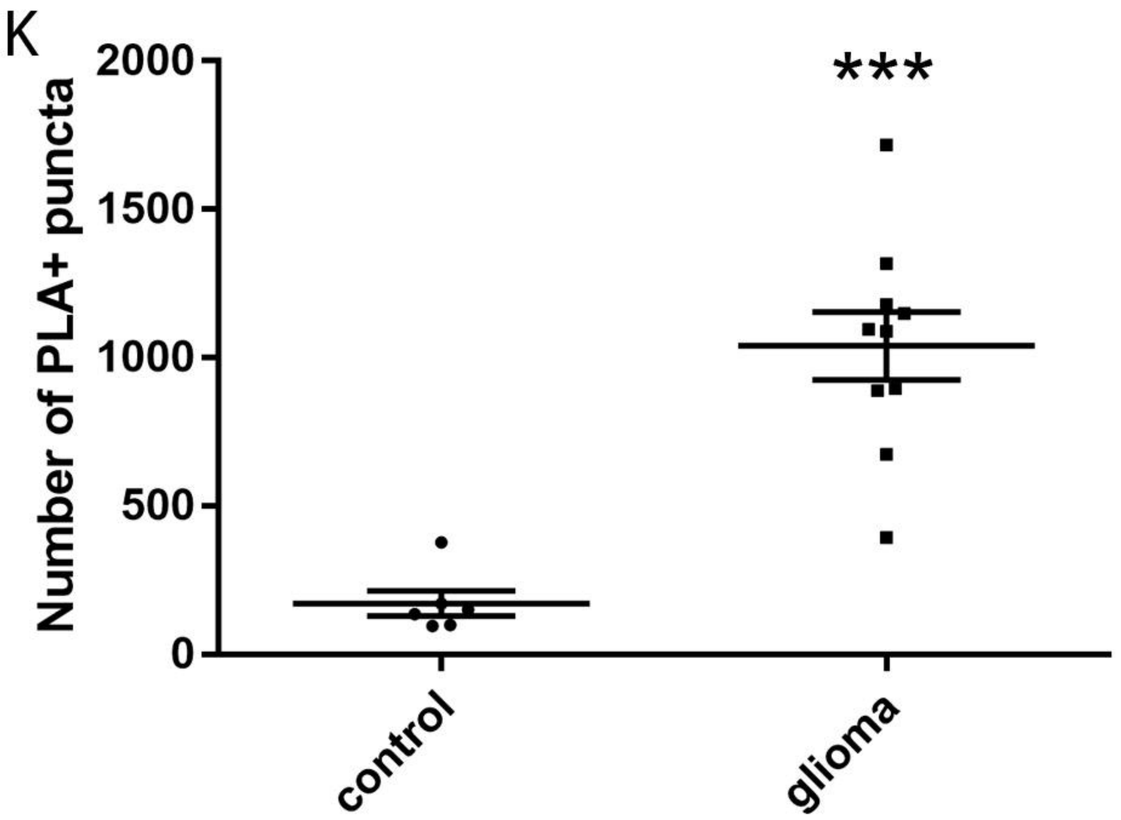
I I′ I″

1.0 µm

Glioma

J J′ J″

K



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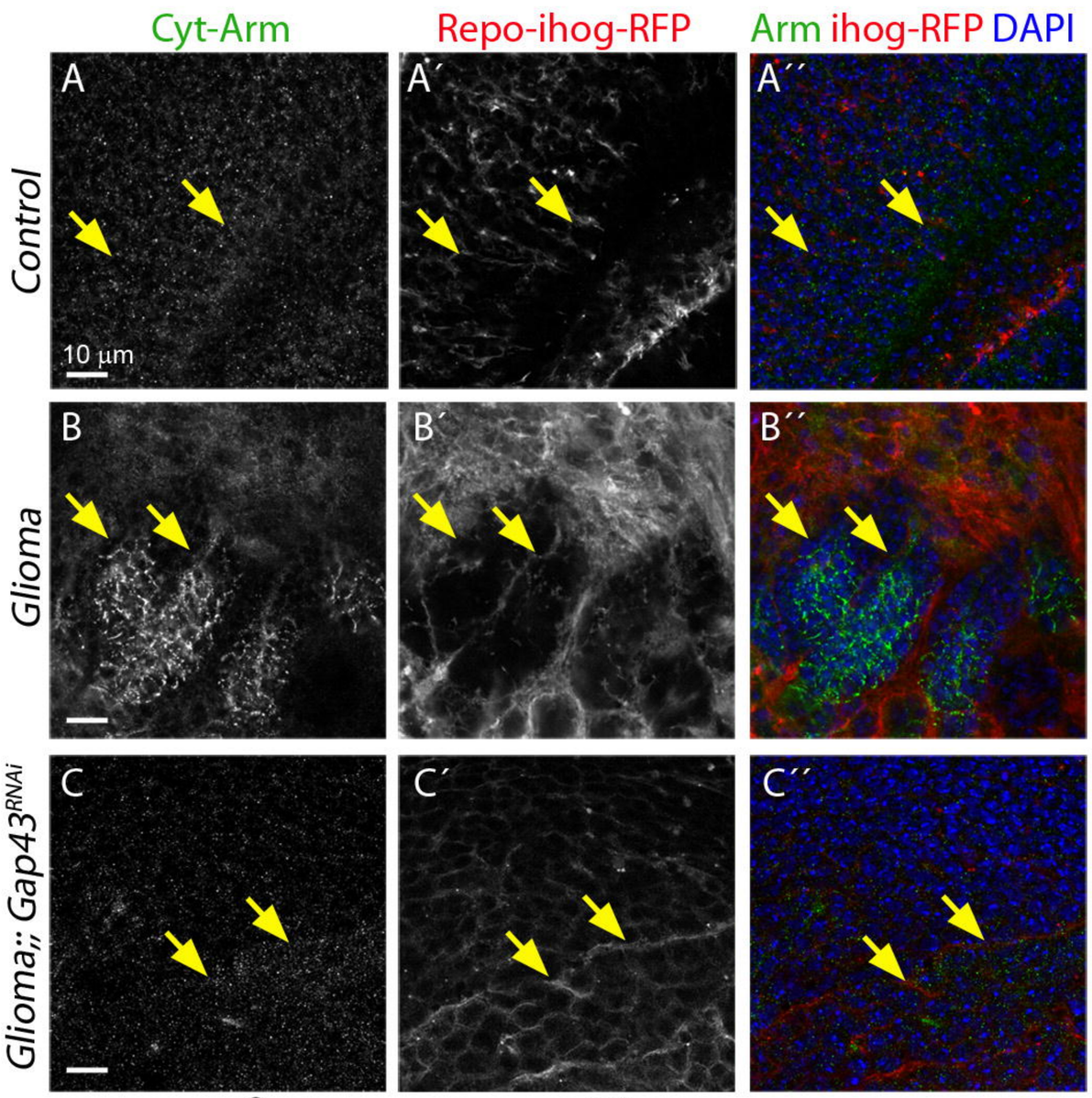
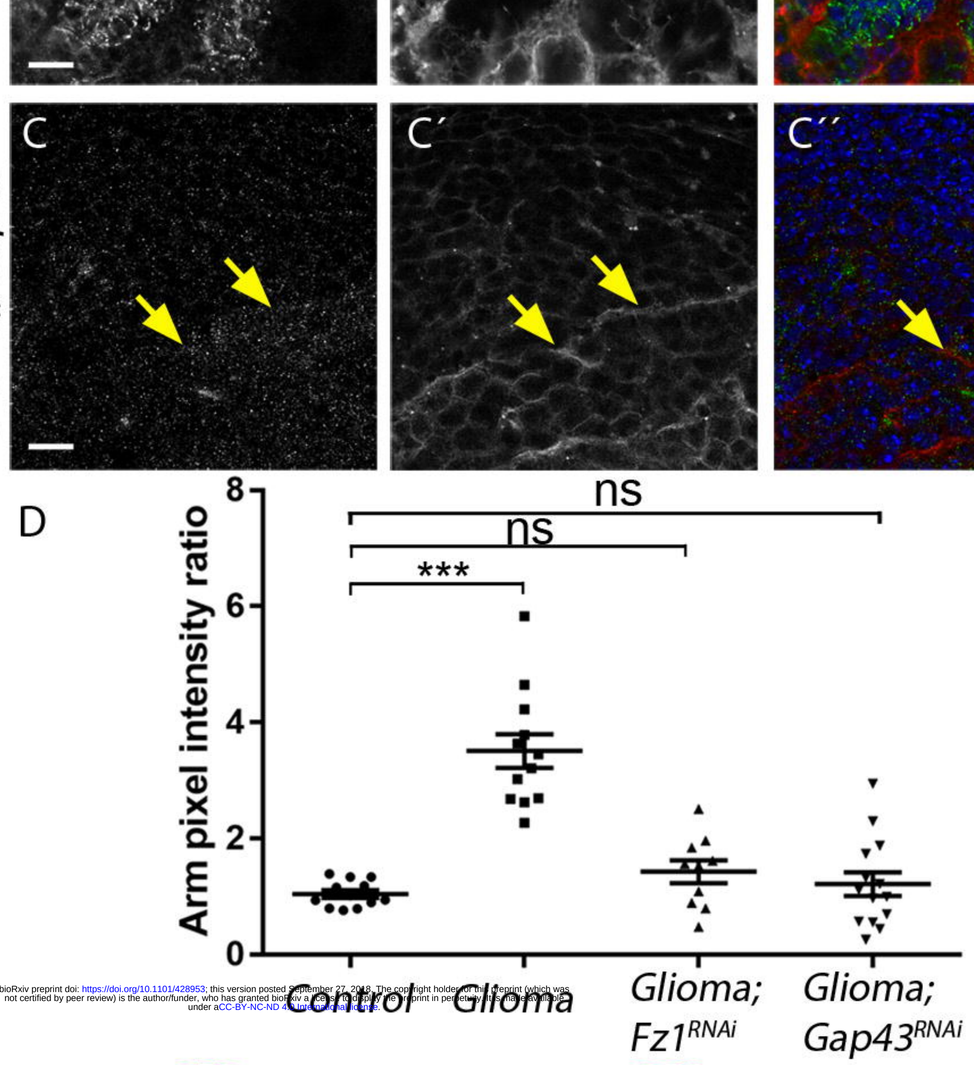
Portela_Fig5





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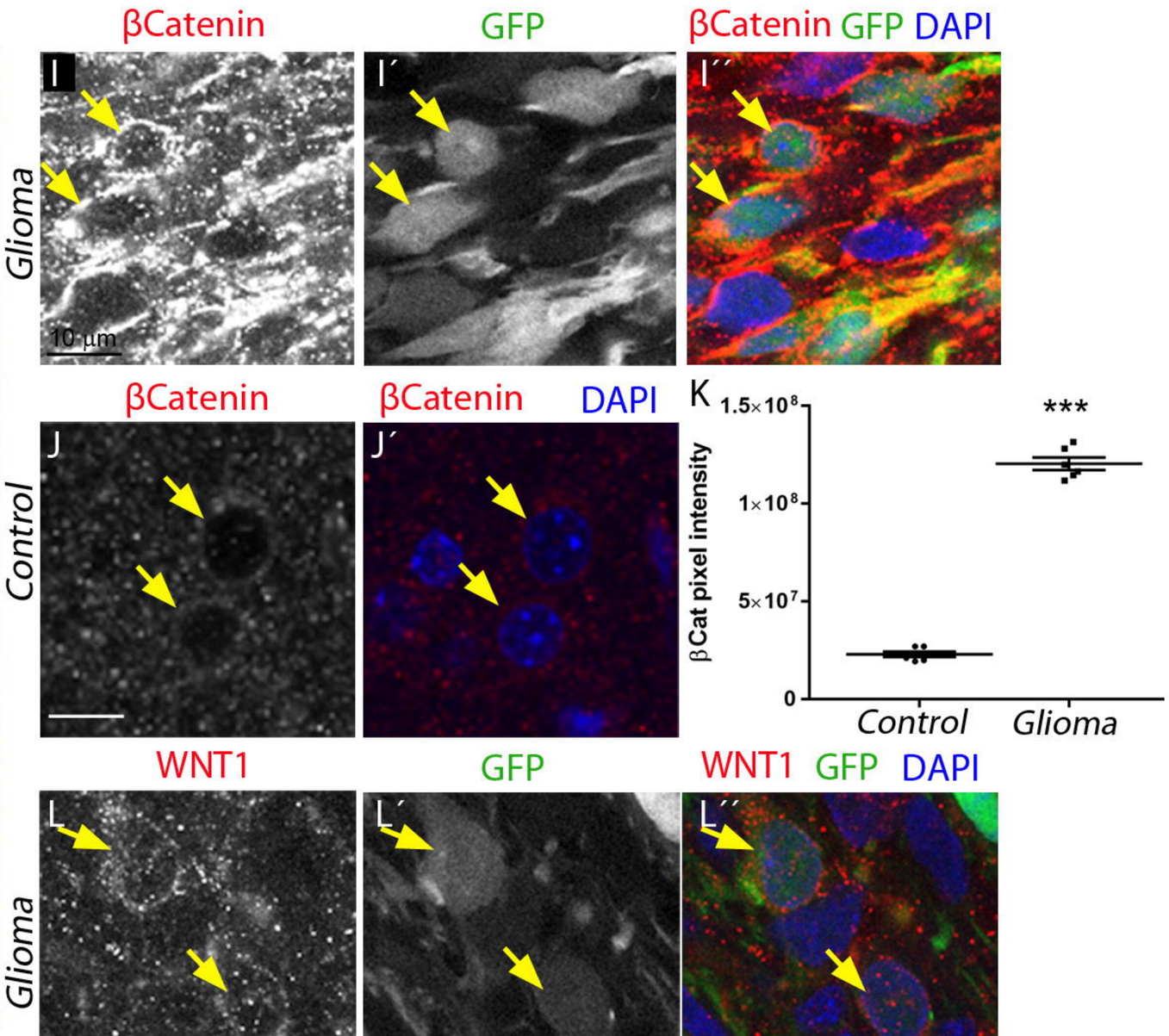
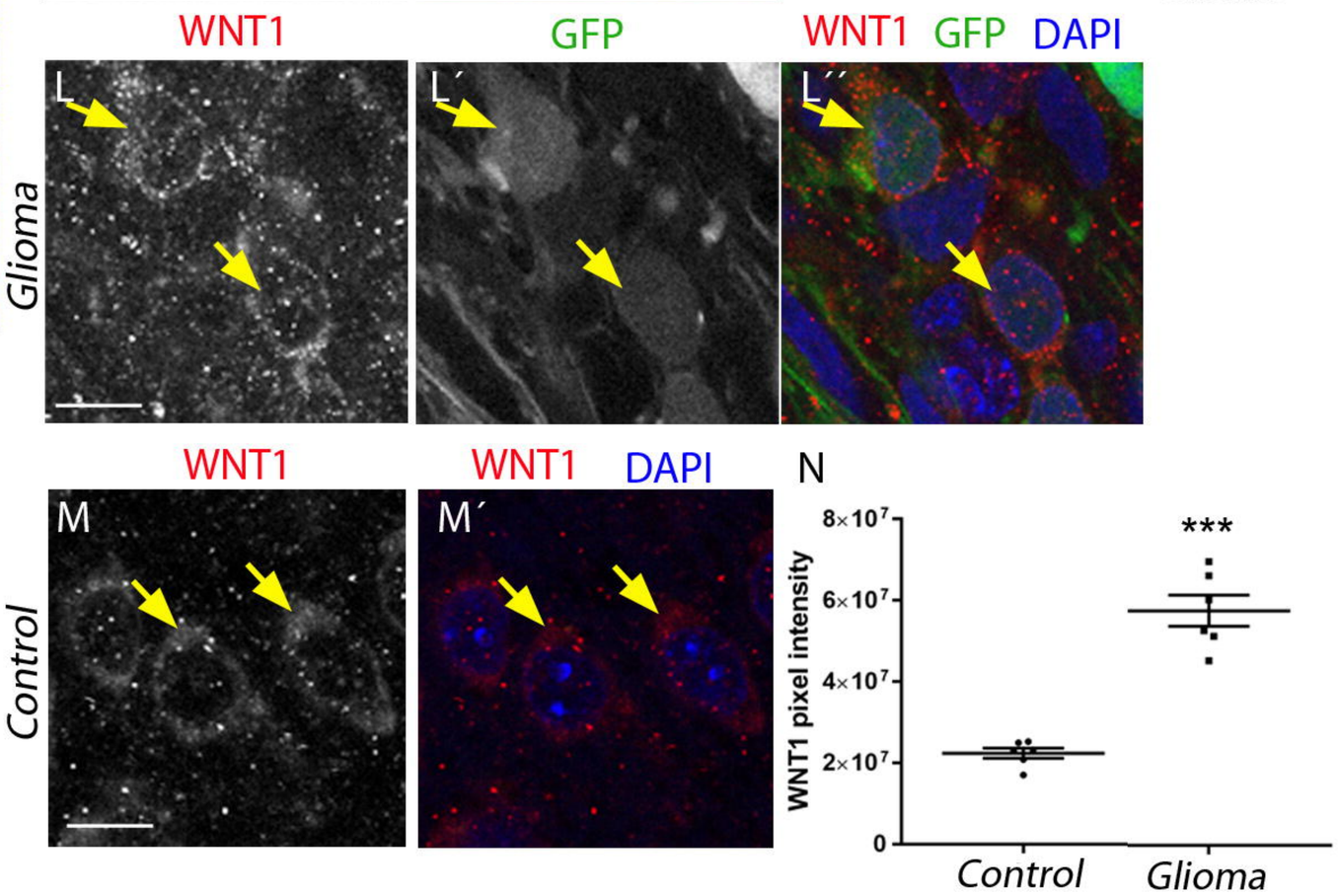
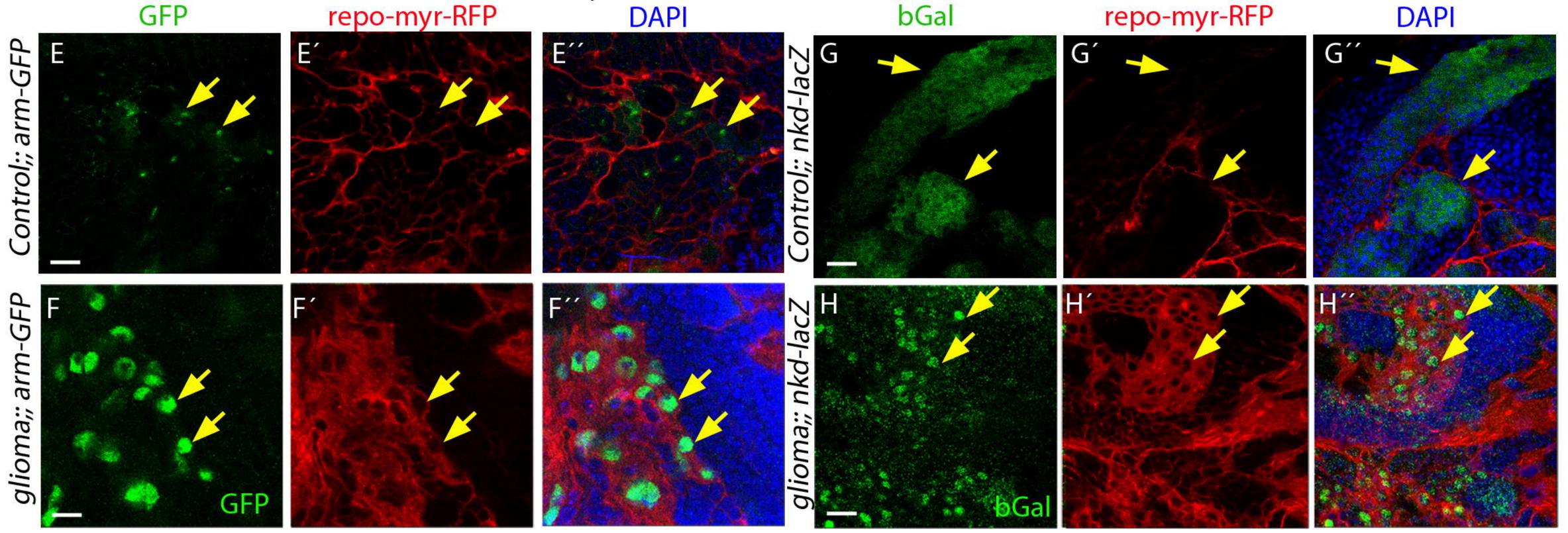
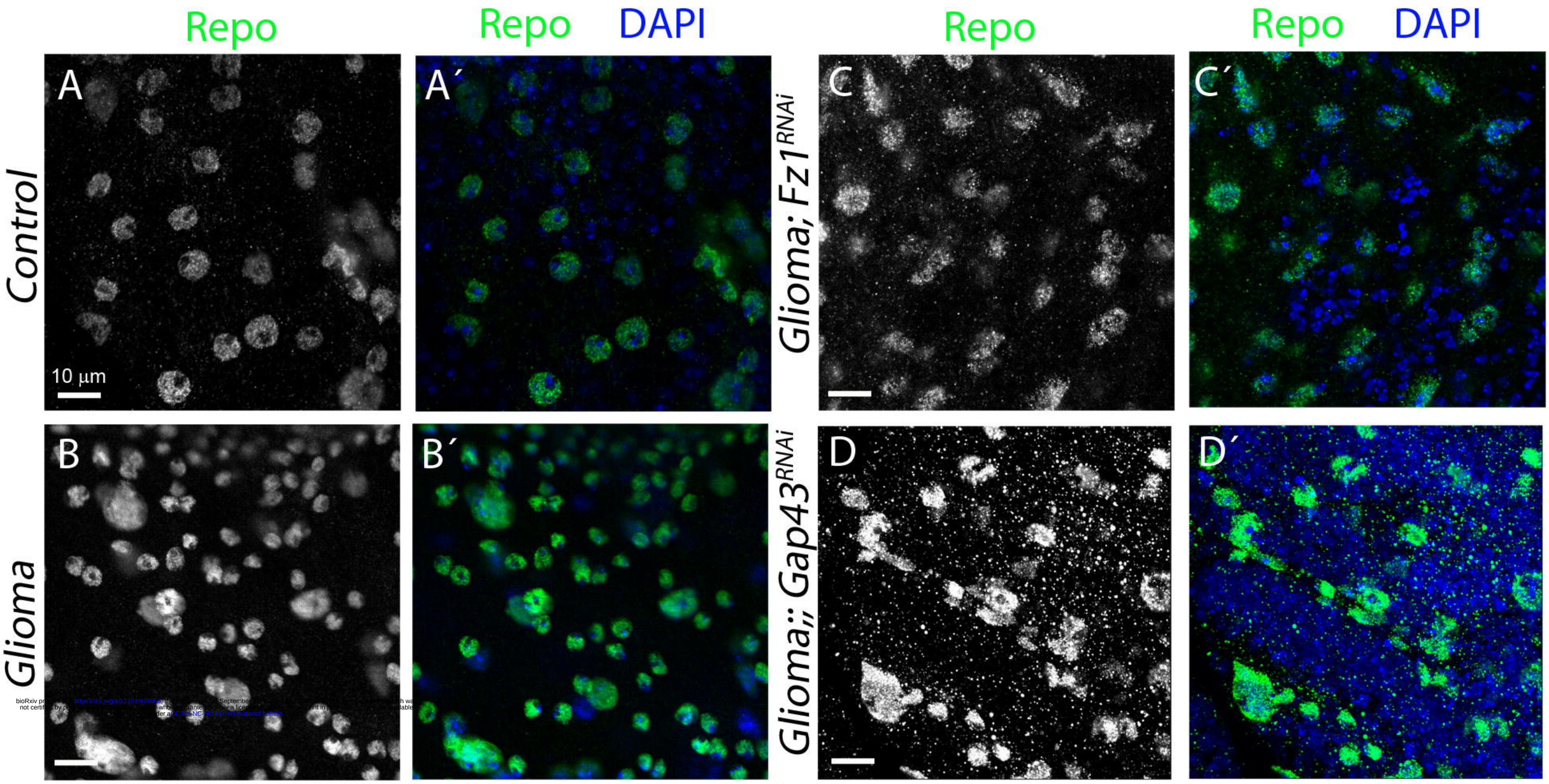






Portela_Fig6



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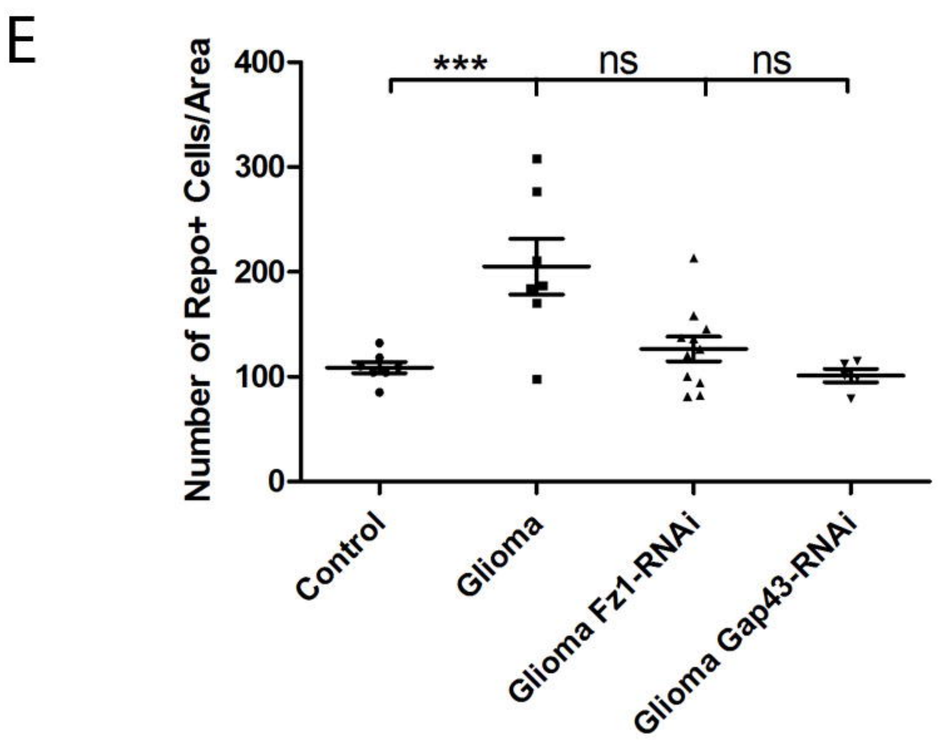

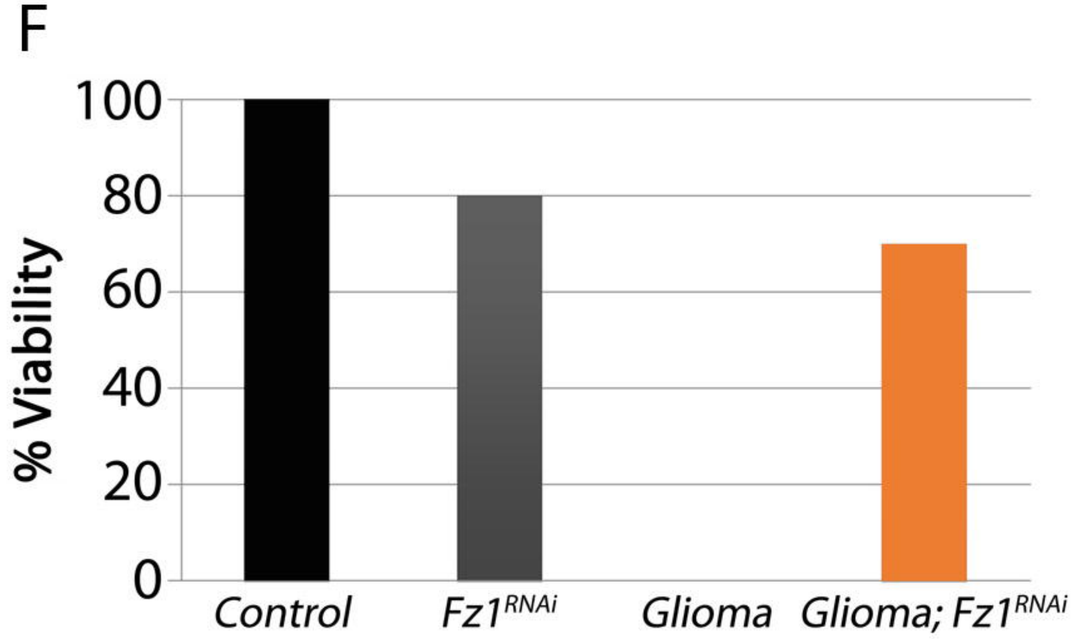

Active Zones Nc82

A
Control
10 µm
B
Glioma
C
Glioma; Fz1RNAi
D
Glioma;; Gap43RNAi
E
Glioma/armS10; Fz1RNAi

F
Average number of synapses
800
600
400
200
0
ns
**
***
**
ns
***
Control
Glioma
Glioma; Fz1RNAi
Glioma; Gap43RNAi
Glioma/armS10; Fz1RNAi

Repo
Repo-ihog-RFP
DAPI

G
G´
G´´
Control
50 µm
H
H´
H´´
Glioma
I
I´
I´´
Glioma; WgRNAi
J
J´
J´´
Glioma/armS10;Fz1RNAi

K
Average Repo+ cells
8000
6000
4000
2000
0
***
***
ns
Control
Glioma
Glioma; WgRNAi
Glioma/armS10; Fz1RNAi

L
Average volume network (uM3)
2.5×10^0 6
2.0×10^0 6
1.5×10^0 6
1000000
500000
0
**
***
**
ns
Control
Glioma
Glioma; WgRNAi
Glioma/armS10; Fz1RNAi

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HA Repo-ihog-RFP HAihog-RFP ELAV-GFP

Control; ELAV-GFP>wgNRT-HA

Glioma; ELAV-GFP>wgNRT-HA

A A´ A´´

B B´ B´´

C C´ C´´

D D´ D´´

E E´ E´´

F F´ F´´

10 µm

G

Neuron

Wg

Wg^{NRT}

Glioma Cell

Fz1

Control; ELAV-GFP>wgNRT-HA

HA Neuron HA Glia HAihog-RFP ELAV-GFP

H H´ H´´

Glioma; ELAV-GFP>wgNRT-HA

I I´ I´´

J

Number of HA+ puncta

8000
6000
4000
2000
0

ns ns

Neurons GFP Glia RFP Neurons GFP Glia RFP

Control Glioma

K

Average volume network (uM3)

2.5×10^{06}
2.0×10^{06}
1.5×10^{06}
1000000
500000
0

ns *** ns ***

Control Control WgNRT Glioma Glioma WgNRT