

## Modeling Glioma Growth and Invasion in *Drosophila melanogaster*<sup>1</sup>

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### Abstract

Glioblastoma is the most common and most malignant intrinsic human brain tumor, characterized by extensive invasion and proliferation of glial (astrocytic) tumor cells, frequent activation of tyrosine kinase receptor signaling pathways, relative resistance to chemotherapy and radiotherapy, and poor prognosis. Using the Gal4-UAS system, we have produced glioma models in *Drosophila* by overexpressing homologs of human tyrosine kinase receptors under control of the glia-specific promoter *reversed polarity* (*repo*). Glial overexpression of activated epidermal growth factor receptor (EGFR) resulted in enhanced proliferation and migration of larval glial cells with increased numbers in the eye imaginal disc, diffuse tumor-like enlargement of the optic stalk, and marked ectopic invasion of glial cells along the optic nerve. Glial overexpression of the downstream kinase *PI3K* showed similar pathology. Overexpression of activated *pvr* (platelet-derived growth factor receptor/vascular endothelial growth factor receptor homolog) led to migration of glial cells along the optic nerve, whereas expression of activated *htl* (fibroblast growth factor receptor 1 homolog) and *INR* (insulin receptor) showed markedly elevated numbers of glial cells in the optic stalk. The EGFR/phosphatidylinositol 3-phosphate kinase (PI3K) phenotype was partly reverted by the administration of the EGFR tyrosine kinase inhibitor gefitinib and completely rescued by the PI3K inhibitor wortmannin and the Akt inhibitor triciribine. We suggest that *Drosophila* models will be useful for deciphering signaling cascades underlying abnormal behavior of glioma cells for genetic screens to reveal interacting genes involved in gliomagenesis and for experimental therapy approaches.

*Neoplasia* (2009) 11, 882–888

### Introduction

Glioblastomas represent the most common and most malignant intrinsic human brain tumors. These astrocytic neoplasms are characterized by a high proliferative activity, diffuse invasion of brain tissue, and relative resistance to conventional cytotoxic chemotherapy and radiotherapy, leading to poor prognosis with median survival of 12 to 18 months [1]. New types of “personalized” therapies targeting the underlying molecular alterations of individual glioblastomas have increasingly been considered and used in ongoing clinical studies. Promising molecular targets for therapeutic intervention have included the tyrosine kinase receptors epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), and vascular endothelial growth factor receptor (VEGFR) and their downstream signaling cascades, the phosphatidylinositol 3-phosphate kinase (PI3K)/AKT and Ras/mitogen-activated protein kinase pathways [2,3].

Experimental *in vivo* models of glioblastoma are required for evaluating new types of experimental therapies as well as for better understand-

ing the biology of these fatal neoplasms. Most models are based on transplantation of glioma cell lines into mouse or rat brains or on the use of transgenic mice developing gliomas [4–6]. Advantages of rodent models include their wide distribution among institutions with accumulated experience for decades, the existence of a blood-brain barrier, and the possibilities of applying therapeutic agents and studying the interaction of tumor and brain tissue. However, there are potential problems of rodent models, such as restrictions of host genotype (only syngenic or

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<sup>1</sup>This research was funded by Deutsche Forschungsgemeinschaft (grant Pa 328/7) and Innovative Medizinische Forschung, Medical Faculty of Münster (grant JE 210704).

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Received 6 April 2009; Revised 3 June 2009; Accepted 4 June 2009

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DOI 10.1593/neo.09576

immunodeficient animals being susceptible to tumor growth), the artificial nature of tumorigenesis after transplantation, a high number of genetic changes in tumor cells, the long duration of experiments, high cost, and ethical considerations.

*Drosophila melanogaster*, as a model organism, offers several advantages, including easy handling, rapid generation time, low cost, and a wide armamentarium of genetic techniques [7]. Many molecular pathways are conserved between invertebrates and humans, such as tyrosine kinase receptor signaling cascades. Furthermore, *Drosophila* can be used in neuropharmacological experiments because this organism is amenable to external/food application, inhalation, or injection of substances in a large number of wild type or mutant animals [8–12]. Fly models of the hereditary tumor syndrome tuberous sclerosis as well as neurofibromatosis types 1 and 2 have been established [13–16]. Furthermore, several mutants interfering with asymmetric cell division of neuroblasts exhibit neuronal/neuroblastic tumors that are referred to as “hyperplastic” in case of preserved architecture such as *malignant brain tumor (l(3)mbt)* [17] or “neoplastic” with loss of architecture and invasion such as *brain tumor (brat)*, *raps*, *numb*, *pros*, and *mira* [18].

The larval brain is composed of two hemispheres and the ventral ganglion where peripheral nerves originate (Figure 1A). In the *Drosophila* central nervous system, approximately 10% of cells are of glial nature, which are classified as either midline glia or lateral glia, the latter being positive for the glial marker *reversed polarity (repo)* [19]. During larval development, photoreceptor neurons are specified within the eye imaginal disc and project their axons along the basal surface of the disc into the optic stalk, bridging the eye imaginal disc with the optic lobes. *Repo*-positive glial cells that originate from the optic stalk populate the eye disc in a stereotyped manner, so that the number of glial cells is predictable at certain time points of development [20], and changes of proliferation or migration are easily detectable. The larval visual system is located adjacent to the eye imaginal disc and consists of a pair of 12 cells, the Bolwig organs. These send axonal projections (Bolwig nerves, BNs) that enter the brain through the optic stalks and are physiologically not accompanied by glial cells [21,22], enabling convenient detection of glial overmigration along these axonal projections.

Only very recently, we and others have started to explore the usefulness of *Drosophila* in modeling gliomas by inducing EGFR/PI3K signaling in larval glia [23,24]. We here report that not only *Drosophila* lines overexpressing EGFR and/or PI3K but also lines overexpressing other tyrosine kinase receptors, including PDGFR/VEGFR, fibroblast growth factor receptor (FGFR), and insulin receptor homologs, show increased proliferation and/or overmigration of glial cells in larval eye structures, recapitulating histologic key features of human gliomas. Moreover, we demonstrate that these experimental gliomas can be reverted by drugs targeting the EGFR signaling pathway.

## Materials and Methods

### Fly strains and genetics

All crosses were performed on standard food at room temperature unless indicated otherwise. We used fly strains *repo Gal4* (III), generated by a random Gal4 P-element insertion into the *repo* locus [25], *UAS  $\lambda$ top*, the activated form of *torpedo/Egfr* [26,27], *UAS  $\lambda$ pvr*, the constitutively active form of *pvr* [28], as well as *UAS  $\lambda$ htl* [29]. *UAS PI3K<sup>92E</sup>*, generated by P-element insertion, *UAS INR*, and *UAS CD8GFP*, which targets green fluorescent protein to the plasma membrane, were obtained from Bloomington Stock Center (Bloomington, IN).

### Crossings

Flies homozygous for *UAS  $\lambda$ top*, *UAS PI3K<sup>92E</sup>*, *UAS CD8GFP*, *UAS  $\lambda$ pvr*, *UAS  $\lambda$ htl*, *UAS INR* and flies harboring *UAS  $\lambda$ top* as well as *UAS PI3K<sup>92E</sup>* were crossed against a stock carrying a *repo Gal4* transgene.

### Immunohistochemistry

Fluorescent immunostaining was performed on third instar larva brains and eye imaginal discs. Specimens were mounted in Vectashield (Vector Laboratories, Burlingame, CA). Anti-*repo* antibodies were obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). Rabbit and mouse anti-GFP (both 1:500; Invitrogen, Eugene, OR) and goat anti-HRP Cy5 (1:200; Dianova, Hamburg, Germany) antisera were used according to the manufacturer's instructions. Fixation and treatment of tissues for immunohistochemistry were performed according to standard procedures.

Cy2, Cy3, or Cy5 (1:200; Dianova GmbH) and Alexa 488, 568, or 647 (1:500; Molecular Probes, Carlsbad, CA) were used as secondary antibodies.

### Pharmacological Inhibition of Ectopically Expressed Genes

Substances used were gefitinib (Biomol, Hamburg, Germany), wortmannin (Biaffin, Kassel, Germany), and triciribine (Biaffin). One gram of *Drosophila* medium (Carolina Biological Supply, Burlington, NC) was mixed with 5 ml of H<sub>2</sub>O. The inhibitors were made soluble in 100 mM DMSO. Flies harboring double insertions *UAS PI3K<sup>92E</sup>* and *UAS  $\lambda$ top* were set for 24 hours on normal food (at 25°C) and then on prepared food for 24 hours (at 25°C). Effects of inhibition were analyzed by immunohistochemistry.

### Confocal Laser Scanning Microscopy and Cell Counting

Images were taken with a confocal laser scanning microscope (Axiovert 200M or Axio Imager Z.1 with LSM 510 META scanning module; Zeiss, Oberkochen, Germany). Numbers of *repo*-expressing glial cells were scored in different developmental stages. Cell counts were performed on confocal image stacks with sections taken at 0.6- $\mu$ m distance (resolution, 512  $\times$  512 pixels; pinhole, 100 mm; scan speed, 8). Glial nuclei were counted using a three-dimensional image processing program (Volocity 4.0; Improvision, Waltham, MA). For each age group, 15 imaginal discs were investigated. Volocity quantitation was set with an intensity range of 60 to 255 and a minimal volume of 25  $\mu$ m<sup>3</sup>. Only complete confocal stacks were counted. White/black balance was performed using Photoshop 7.0 (Adobe, Dublin, Ireland).

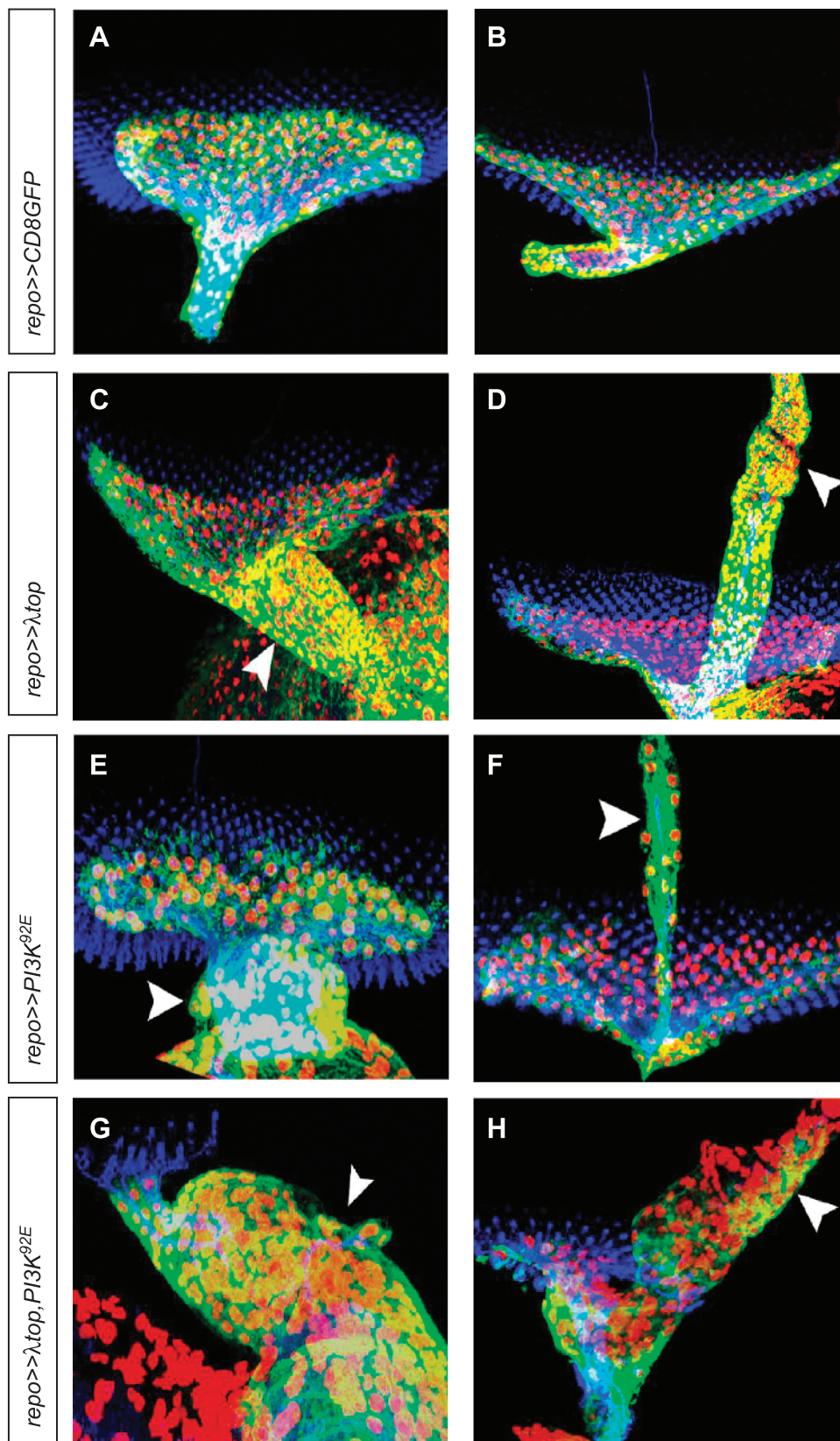
### Statistical Analysis

Quantitative data were expressed as arithmetic means  $\pm$  SEM. One-way analysis of variance was performed followed by Scheffé procedure;  $P < .05$  was considered significant. Statistical analysis was performed using SPSS version 15.0 (SPSS, Inc, Chicago, IL).

## Results

### Ectopic Expression of Activated Egfr and PI3K<sup>92E</sup> in Eye Imaginal Discs of Third Instar Larva

Activated *Egfr* as well as activated *Egfr* in combination with wild-type *PI3K* was expressed in *Drosophila* glial cells using the *Gal4/UAS* system and the *repo* promoter, directing expression to lateral glial cells. Expression of  *$\lambda$ top*, the activated form of *torpedo/Egfr* (*top/Egfr*) led to increased numbers of glial cells in the optic stalk (Figure 1C). Overmigration of glial cells along BNs of the third instar larva was also observed (Figure 1D). Similarly, expression of *PI3K<sup>92E</sup>* resulted in



**Figure 1.** Eye imaginal discs of third instar larvae with overexpression of activated Egr and/or wild-type PI3K in glial cells. Immunofluorescence staining of (A–H) eye imaginal discs of third instar larvae. Nuclei of glial cells are red ( $\alpha$ -Repo), glial cytoplasm is green ( $\alpha$ -GFP), and photoreceptor neurons are blue ( $\alpha$ -HRP). Images are projections of confocal image stacks. (A) Wild-type eye imaginal disc with optic stalk. (C, E, G) Ectopic gene expression resulted in a thickened optic stalk due to increased numbers of glial cells (arrowheads). (B) Wild-type eye imaginal disc with BN. (D, F, H) Ectopic gene expression resulted in overmigration of glial cells along BN (arrowheads).

enlargement of the optic stalk and overmigration of glial cells along BN (Figure 1, *E* and *F*). Ectopic coexpression of *λtop* and *PI3K<sup>92E</sup>* resulted in thickening of the optic stalk (Figure 1*G*) and in massive ectopic migration along BN (Figure 1*H*), being more pronounced than in the single transgenic lines. Quantitative analysis revealed that glial overexpression of *λtop* significantly increased glial cell number in the eye imaginal disc in all stages in comparison to wild type (Figure 2). However, overexpression of *PI3K<sup>92E</sup>* resulted in reduced glial cell numbers in later stages of development (Figure 2), presumably because most glial cells were misplaced in optic stalk and BN. Consequently, no differences with the wild type were established after activation of both *λtop* and *PI3K<sup>92E</sup>* with respect to the number of glial cells in the eye disc.

### Pharmacologically Induced Inhibition of Ectopically Activated Genes

To determine whether the effects of transgene overexpression can be rescued by pharmacological inhibitors, we used flies simultaneously overexpressing *UAS λtop* and *UAS PI3K<sup>92E</sup>*. First, the effect of gefitinib (an EGFR-specific tyrosine kinase inhibitor) on the larval eye imaginal disc was investigated. After applying the lowest concentration (0.5 μM) of gefitinib in *Drosophila* food, the optic stalk was still thickened, and large numbers of glial cells were migrating along BN. Using the highest concentration of gefitinib (250 μM), the number of glial cells in the optic stalk remained high, whereas the number

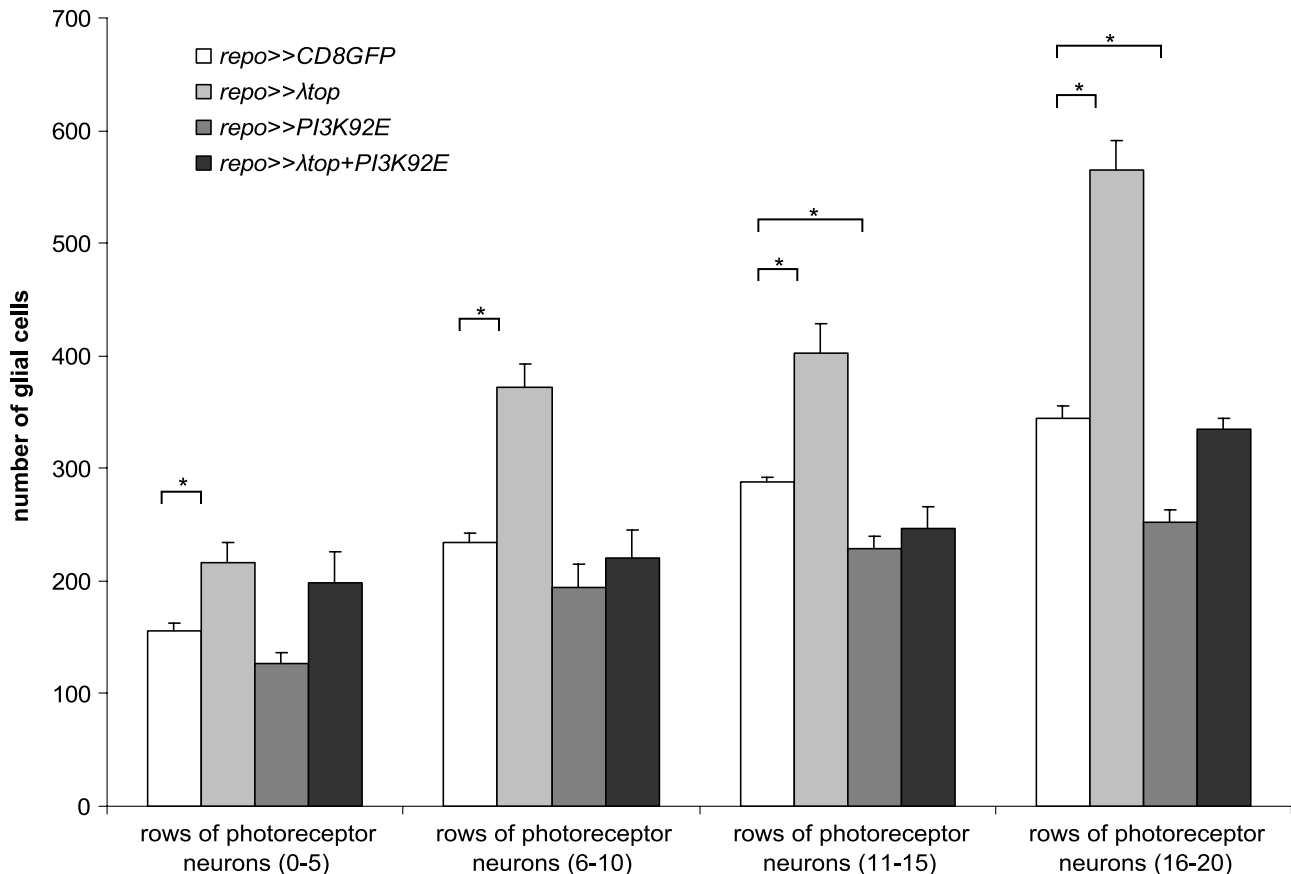
of glial cells abnormally migrating along BN was considerably reduced (Figure 3*D*).

Even at low concentrations (0.5 μM), the Akt inhibitor triciribine markedly decreased glial overmigration along BN (Figure 3*E*), whereas higher concentrations showed complete rescue (Figure 3, *F–H*).

Low concentrations of 0.5 μM wortmannin (a specific PI3K inhibitor) were not sufficient to reduce optic stalk mass (Figure 3*I*), whereas higher concentrations resulted in wild-type morphology optic stalks (Figure 3, *K* and *L*). In contrast, BN (Figure 3, *I–L*) showed wild-type configuration with all applied concentrations, indicating that even the lowest concentration of wortmannin was sufficient to rescue abnormal migration along BN.

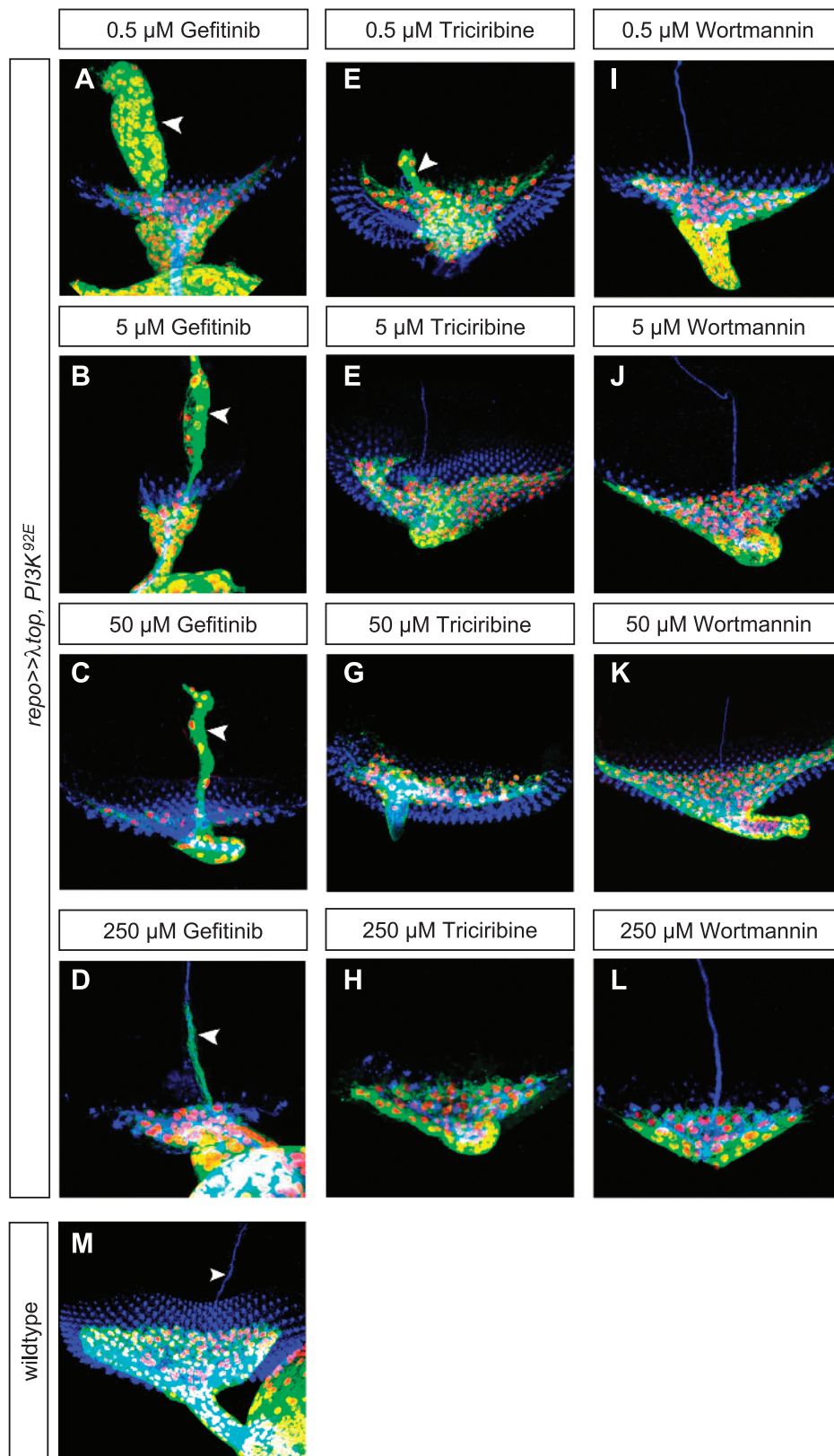
### Ectopic Expression of Other Receptor Tyrosine Kinases in Eye Imaginal Discs of Third Instar Larva

To see whether the overexpression of other receptor tyrosine kinases (RTKs) involved in gliomagenesis leads to similar results, activated *pvr*, the *Drosophila* homolog of PDGFR/VEGFR, activated *hhl*, the *Drosophila* homolog of FGFR1, as well as *INR*, the *Drosophila* insulin receptor, were expressed in *Drosophila* lateral glial cells under *repo* control. Overexpression of *λpvr* led to abnormal migration of glial cells along BN (Figure 4*A*). Expression of *λhhl* and *INR* showed extensively elevated numbers of glial cells in the optic stalk, whereas no or only little overmigration along BN was observed (Figure 4, *B* and *C*).



**Figure 2.** Number of glial cells in different genotypes in eye imaginal discs of different age. Cell numbers expressing the glial marker *repo* were morphometrically determined in different developmental stages. Age of imaginal discs was grouped according to rows of photoreceptor neurons (0-5, 6-10, 11-15, 16-20 rows). In all investigated developmental stages, overexpression of *Atop* (activated Egfr) resulted in increased glial cell numbers. In later stages, overexpression of *PI3K<sup>92E</sup>* led to a significant decrease of cell numbers (\**P* < .05).

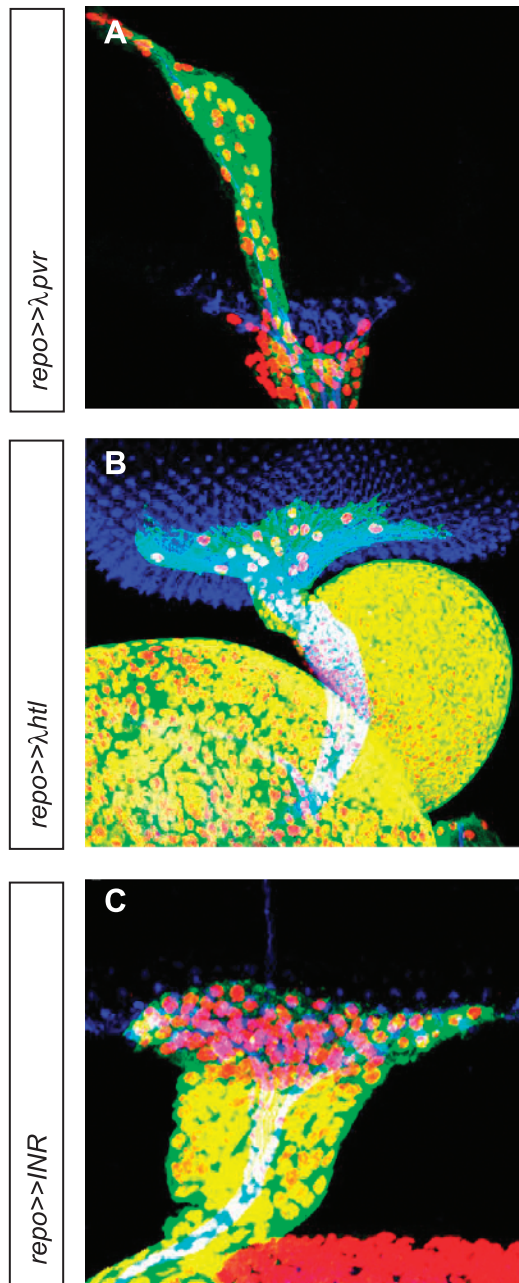




**Figure 3.** Effects of EGFR inhibitor gefitinib, Akt inhibitor triciribine, and PI3K inhibitor wortmannin on proliferation and migration induced by ectopic expression of *Atop* and *PI3K<sup>92E</sup>*. Effects of gefitinib on eye imaginal disc of larvae were tested using genotype *repo >> Atop, PI3K<sup>92E</sup>*. Increasing concentrations of gefitinib reduced ectopic migration along BN (arrowheads; B, C, D), although the wild-type phenotype was not reached. At low concentrations, the Akt inhibitor triciribine decreased glial migration along BN only partially (E, arrowhead), whereas higher concentrations resulted in complete rescue (F–H). With increasing concentrations of the EGFR inhibitor wortmannin, optic stalks showed complete rescue, corresponding to wild type. No overmigration along BN was seen even with the lowest concentration of wortmannin (I–L). M shows a wild-type eye imaginal disc.

## Discussion

We here describe invertebrate glioma models that are based on the overexpression of tyrosine kinase receptors in *Drosophila* glia. Morphologically, key features of human gliomas are recapitulated, such as diffuse enlargement of brain structures due to single cell infiltration. Invasion of brain structures along nerve tracts, which is a common feature in human gliomas [30,31], is modeled in *Drosophila* larvae



**Figure 4.** Eye imaginal discs of third instar larvae with overexpression of other tyrosine kinase receptors in glial cells. Immunofluorescence staining of (A–C) eye imaginal discs. Nuclei of glial cells are red ( $\alpha$ -Repo), glial cytoplasm is green ( $\alpha$ -GFP), and photoreceptor neurons are blue ( $\alpha$ -HRP). Images are projections of confocal image stacks. (A) Ectopic gene expression of activated *pvr*, the *Drosophila* homolog of PDGFR/VEGFR, resulted in overmigration of glial cells along BN. (B and C) Ectopic gene expression of *htl* (homolog of activated FGFR1) and *NR* (insulin receptor) resulted in a thickened optic stalk due to increased numbers of glial cells.

by migration along optic nerves (BNs), which, in the wild type, is devoid of glial cells, thereby facilitating detection of abnormal migration because all glial cells along BN are invasive. Furthermore, invasion can be easily quantitated by counting the number of glial cells in the BN and by determining their distance from the eye imaginal disc. Because of natural limitations of the species, including absence of blood vessels and an adaptive immune system, other features of malignant gliomas could not be reproduced, such as activated microglial cells and presence of immune cells, vascular proliferation, and necrotic areas, the latter being closely related to dysfunctional blood vessels in human gliomas.

Compared with most xenotransplantation models, our model is more similar to human glioma specimens with respect to the typical single-cell invasion pattern. Xenotransplanted glioma cell lines are often cohesive and look like metastatic sarcomas, and despite common claims, their invasion pattern is usually different from that of human gliomas. The cells in our model show diffuse invasion, and they are clearly glial so that the invasion pattern is more “human” than that of many xenotransplants. However, although adhesion molecules of glia and blood-brain barrier show considerable homologies between *Drosophila* and vertebrates, almost nothing is known about the cerebral extracellular matrix in *Drosophila*, which makes analysis of cell-matrix interactions more difficult in our model.

*EGFR* is the most frequently amplified gene in glioblastoma. It occurs in approximately 40% of primary glioblastomas and is associated with *EGFR* overexpression. Amplification of the *EGFR* gene is often associated with structural alterations, the most common being variant III (*EGFRvIII*), which is present in 20% to 50% of glioblastomas with *EGFR* amplification and constitutively activated in a ligand-independent manner, leading to cell proliferation through PI3K, RAS, and mitogen-activated protein kinase signaling pathways [1]. The PI3K signaling cascade may also be activated by *PTEN* mutations in 15% to 40% of primary glioblastomas and more infrequently by mutations of the PI3K components *PIK3CA* [32] and *PIK3RI* [33]. Because more than 80% of glioblastomas show robust Akt activation, the PI3K pathway is central in molecular pathogenesis. In fact, large-scale multidimensional genomic characterization of 91 glioblastomas revealed somatic alterations in RTK/PI3K pathways in 88% of tumors, the genes most commonly involved being *EGFR* (45%), *PTEN* (36%), and components of the PI3K complex (15%) [33]. *In vitro* evidence suggests that activation of *EGFR* inhibits apoptosis and promotes invasion of glioma cells [34,35].

Our data show that overexpression of activated *EGFR* or PI3K in *Drosophila* is sufficient to produce tumor-like overgrowth and invasion of glial cells. Furthermore, our findings demonstrate that in addition to *EGFR*/PI3K, overexpressing other RTK receptors involved in gliomagenesis leads to glioma-like lesions in *Drosophila*. Because the number of glial cells in the eye imaginal disc was increased after *EGFR* expression, but decreased after PI3K expression and unchanged after coexpressing *EGFR* and PI3K, our findings suggest that there are additional signaling pathways upstream of PI3K regulating directed migrating of glial cells. They also suggest that mutations in the same signaling cascade are not necessarily associated with identical consequences on cell biology, such as migration. It might be interesting to compare the invasion patterns of human glioblastomas with *EGFR* amplification to those with mutations of PI3K components.

New types of molecular therapies targeting *EGFR* or PI3K have recently evolved as promising experimental and clinical approaches in treating glioblastomas. Response of glioblastoma patients to the *EGFR* inhibitor erlotinib was associated with *EGFR* expression/amplification

and coexpression of EGFRvIII and PTEN in biopsy specimens [36,37]. However, only 10% to 20% of patients benefit from EGFR inhibition [38], pointing to confounding factors such as coactivation of multiple RTKs, thereby limiting the effects of therapies targeting single RTK. In fact, combined treatment with several RTK inhibitors was more successful [39]. Furthermore, glioblastomas harbor a variety of additional genetic changes that may interact with RTK targeting. *Drosophila* lines showing several coactivated RTK and inactivated tumor suppressor genes in glial cells can be produced to better model genetic changes typically encountered in glioblastomas. These invertebrate models can then be used for large-scale genetic screens in detecting interacting genes and for novel molecular-based experimental approaches.

## Acknowledgments

Statistical analysis was performed by Joachim Gerres, Institute of Medical Informatics and Biomathematics, University Hospital Münster.

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