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Modelling Glioblastoma angiogenesis in *Drosophila* larvae

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ABSTRACT

Glioblastoma multiforme (GBM) is the most common malignant type of cancer of the Central Nervous System (CNS) and is characterized as highly angiogenic. Poor prognosis combined with short lifespan after diagnosis (~18 months) and resistance to the most invasive treatments highlight the need to understand the disease pathology at the molecular level. Angiogenesis plays an important role on tumor cell survival and malignancy, but the precise mechanisms that control the interactions between glial cells and blood vessels remain unknown. *Drosophila melanogaster* has emerged as an excellent model for GBM studies because glia-specific overexpression of EGFR/Ras and PI3K-Akt, the major pathways upregulated in human GBMs, causes low- and high-grade gliomas. Our goal is to use *Drosophila* larvae to understand the mechanisms governing GBM tracheogenesis, the functional and molecular equivalent of angiogenesis.

In this study we used immunofluorescence and confocal microscopy to characterize glia-tracheae interactions in established *Drosophila* GBM models. Specifically, we used the Gal4 – UAS system to overexpress the Ras^{V12} oncogene, the constitutively active catalytic subunit of PI3K, $Dp110^{CAAX}$, alone or in combination, in glial cells of *Drosophila* larvae. We found that the mitotic index in $Dp110^{CAAX}$ overexpressing brains was moderately increased, whereas brains overexpressing Ras^{V12} alone or in combination with $Dp110^{CAAX}$ exhibited strikingly increased mitosis compared to control brains. The levels of brain mitosis correlated with increased central brain size. In addition, brains overexpressing Ras^{V12} alone had significantly increased number of apoptotic cells compared to controls, $Dp110^{CAAX}$, and $Dp110^{CAAX}$; Ras^{V12} brains. To understand glia-trachea interactions, we needed to develop a model to visualize the trachea in GBM-bearing larvae. Since we were using the Gal4 – UAS system to overexpress the tumor-causing genes, we needed a different method to highlight the brain tracheal cells. We tested available trachea-specific QF-QUAS and LexA-LexAop drivers, but they did not allow brain trachea visualization. Therefore, we reverted to using the QF -QUAS system to overexpress Ras^{V12} in glial cells and the Gal4-UAS system to visualize the trachea, a combination that proved successful. The mitotic activity of this model agreed with our results from the brains overexpressing oncogenes using the Gal4-UAS system. Finally, our 3D imaging suggests increased tracheogenesis in brains overexpressing Ras^{V12}.

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COMPOSITION OF THE EXAMINATION COMMITTEE

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1. INTRODUCTION

1.1 Glioblastoma multiforme

Glioblastoma multiforme (GBM) is the most common primary type of cancer found in the Central Nervous System (CNS). It displays resistance to common treatments such as chemotherapy and radiotherapy due to the formation of tumor microtubes (Furnari et al. 2007, Read 2011, Portela-Esteban, Casas-Tintó 2021). Glial tumors are subdivided into two categories, the low-grade gliomas, also called gliomas, and the high-grade gliomas or glioblastomas. High-grade gliomas are highly heterogeneous at the molecular, genetic and cytopathological levels and thus they are termed 'multiform' (Conte et al. 2021, Portela-Esteban, Casas-Tintó 2021, Furnari et al. 2007) .

Despite GBM heterogeneity, some common mutations are found among patients, including those in components of the Epidermal Growth Factor Receptor (EGFR) and the Phosphatidyl-inositol-3 kinase (PI3K) pathways (Read 2011, Portela-Esteban, Casas-Tintó 2021). In *Drosophila,* similar to humans, mutations of the EGFR and PI3K pathway alone are usually not sufficient to cause GBM, but rather low-grade glioma (Figure 1). Nevertheless, it was previously suggested in *Drosophila* that Ras overexpression exhibits a stronger phenotype, resembling high-grade glioblastoma (Read et al. 2009). GBM arises as a result of multiple mutations that lead to synchronous activation of multiple pathways. The EGFR pathway is essential for the normal differentiation, proliferation, and migration of the Neural stem cells (NSCs) and the precursor cells of glial cells. Mutations that cause amplification of the EGFR lead to overactivation of downstream pathway components including Ras, that induce proliferation and migration (Read 2011).

The PI3K pathway controls mitotic activity, growth and migration of a variety of neuronal cells physiologically (Read 2011). The most common mutation affecting the PI3K pathway arise in the catalytic subunit of PI3K, p110α leading to the activation of the receptor and the downstream kinase Akt (Read 2011). PTEN antagonises PI3K resulting in controlled activation of the PI3K pathway. Approximately 75% of GBM cases bear loss of function PTEN mutations, that induce the pathway (Read 2011).

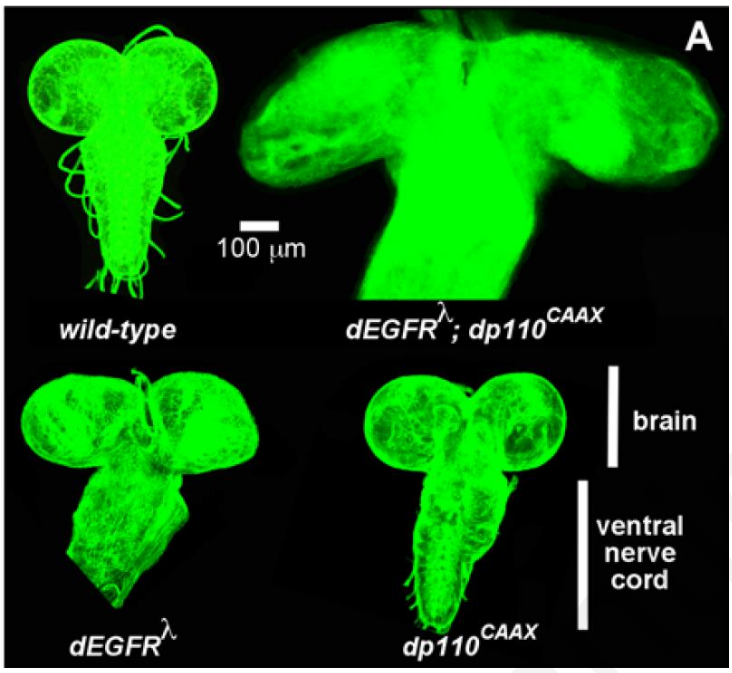

Figure 1: Low- and high-grade gliomas in 3rd instar larva Drosophila Brain in comparison with control brain (Read et al. 2009)

Other Receptor Tyrosine Kinases (RTKs) have been found overexpressed in GBMs, such as the PDGFR-VEGFR (PVR), which is the *Drosophila* homolog of the human Platelet Derived Growth Factor Receptor -Vascular Endothelial Growth Factor Receptor (PDGFR/VEGFR), the Breathless (Btl) homolog of the Fibroblast Growth Factor Receptor (FGFR), and the Insulin Receptor (InR). PVR overexpression has been shown to increase the migrating ability of glial cells, whereas InR and/or Btl overexpression was associated only with increased proliferation (Witte et al. 2009).

Apoptosis or programmed cell death is an essential homeostatic mechanism that contributes to the control of tissue growth and elimination of damaged cells (Ribeiro et al. 2004). Inhibition of apoptosis is a hallmark of cancer cells, but that's not the case for GBMs, where cancer cells undergo spontaneous apoptosis. The apoptotic rate of high-grade gliomas is higher than the low-grade gliomas (Eisele, Weller 2013, Kuriyama et al. 2002). According to the World Health Organization (WHO) higher levels of pro-apoptotic agents were found in patients with GBMs and higher levels of anti-apoptotic agents were found in patients with low-grade gliomas (Eisele, Weller 2013). Some studies tried to correlate apoptotic index/proliferation ratio to patient's

survival and use it as a tool for survival prediction. Unfortunately, the results were controversial since in some cases the higher apoptotic index occurred in patients with the shortest survival and the lower apoptotic index occurred in patients with the longest survival (Kuriyama et al. 2002, Ribeiro et al. 2004). The exact mechanism leading to spontaneous apoptosis of GBM cells and reasons for this unexpected event are still under investigation (Eisele, Weller 2013, Ribeiro et al. 2004, Kuriyama et al. 2002).

1.2 Angiogenesis and GBMs

Angiogenesis is the formation of new blood vessels from preexisting ones, and aberrant angiogenesis is considered a hallmark of cancer (Ahir et al. 2020). During development, angiogenesis is an essential process that supplies the developing tissue with oxygen and nutrients. This process is regulated by the balance between pro-angiogenic and anti-angiogenic stimuli including transcription factors and growth factors (Ahir et al. 2020, Tamamouna, Pitsouli 2018).

GBMs are characterized as highly angiogenic tumors. Increased GBM vascularization is considered as one of the main factors that contribute to poor patient prognosis (Ahir et al. 2020). Hypoxia is the condition where a part of tissue or several cells have insufficient oxygen supply. As the tumor grows, and the number of cancer cells increases, the preexisting vessels are unable to supply all the cells with oxygen leading to the formation of a hypoxic tumor core (Das, Marsden 2013, Pecorino 2012). Any cell that is located approximately 100μm from the nearest blood vessel can become hypoxic, as oxygen can be diffused within the range of 100-200μm (Pecorino 2012, Tamamouna, Pitsouli 2018).

Oxygen levels must be maintained within normal levels (normoxia) for the tissue to function properly. A key molecule that controls oxygen homeostasis is the Hypoxia Inducible Factor-1α (HIF-1α). HIF-1 is a heterodimer transcription factor comprised by the HIF-1α and HIF-1β subunits and its activity depends on the formation of the heterodimer in hypoxic environments. During normoxia, HIF-1α is hydroxylated by prolyl-4-hydroxylase (PHD) (Figure 2) with the addition of -OH to specific proline residues (Tamamouna, Pitsouli 2018). The hydroxylated HIF-1α is recognized by the von-Hippel-Lindau (VHL) ubiquitin ligase, which binds to HIF-1α leading it to proteasomal degradation (Figure 2). During hypoxia, PHD is unable to hydroxylate HIF-1α.

The non-hydroxylated form of HIF-1α is not recognized by VHL and therefore HIF-1α is not degraded (Figure 2). HIF-1α is free to bind to HIF-1β forming the HIF-1 heterodimer. The heterodimer is able to enter the nucleus and together with the co-activator p300 can recognize and bind to Hypoxia Response Elements (HREs) to activate expression of pro-angiogenic genes including VEGF, and FGF (Figure 2) (Tamamouna, Pitsouli 2018). VEGF and FGF are the most common upregulated pro-angiogenic growth factors among GBM patients (Ahir et al. 2020).

In *Drosophila*, oxygen is transported by tracheae, which are functionally homologous to mammalian blood vessels. In accordance, we refer to the formation of new tracheae from pre-existing ones as tracheogenesis. Trachea and blood vessel formation are controlled by evolutionarily conserved signalling pathways. The *Drosophila* homolog for HIF-1α is encoded by *similar* (*sima*), for HIF-1β by *tango* (*tgo*), for PHD by *fatiga* (*Fga*), and for VHL by *dVHL* (Tamamouna, Pitsouli 2018). In the *Drosophila* developing brain the main growth factor that controls trachea development is Branchless (Bnl), the FGF homolog, that binds to its receptor Breathless (Btl), the homolog of FGFR (Pereanu et al. 2007).

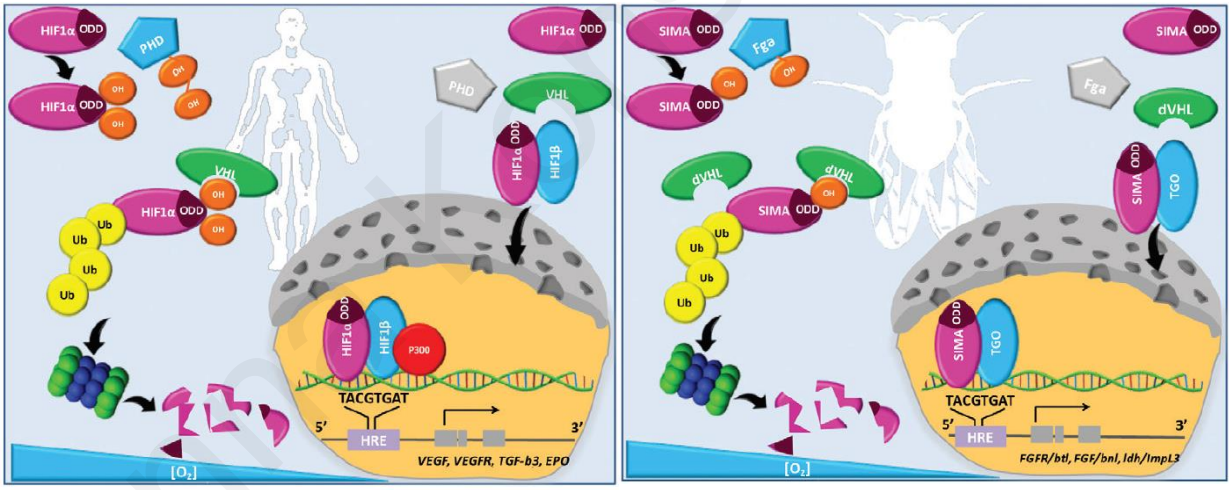

Figure 2: The HIF-1α/ sima pathway. The light blue gradient at the bottomo of each image represents the levels of oxygen (Tamamouna, Pitsouli 2018)

1.3 *Drosophila melanogaster* as a model organism for the study of Glioblastomas

Drosophila melanogaster is one of the easiest model organisms to cultivate and the cost for its maintenance is low. The short life cycle (~10 days in 25°C) (Figure 3) in combination with the

high number of offspring in each generation allows the performance of more experiments in a shorter amount of time (Hales et al. 2015). In addition, *Drosophila* offers a variety of genetic tools, such as transgenic flies, ectopic gene expression, tissue specific overexpression, genetic mosaics, gene silencing using interference RNA (RNAi) etc (Wolpert et al. 2015). Finally, many signaling pathways like EGFR/RTK-Ras, PI3K, Notch, Wnt, Jak-STAT, Hedgehog and TGF-β are conserved between humans and flies (Read 2011). Specifically, approximately 75% of the human disease related genes have a functional homolog in the fruit fly (Pandey, Nichols 2011).

Drosophila has proven to be an excellent model organism to study GBM, because many neuronal and glial subtypes of the mammalian brain have homolog subtypes in the fly (Read 2011). For example, the *Drosophila* neuropile glia is homologous to the astrocyte glia of the mammalian brain which has been found to be the primary type of glia involved in the mammalian GBM (Read 2011).

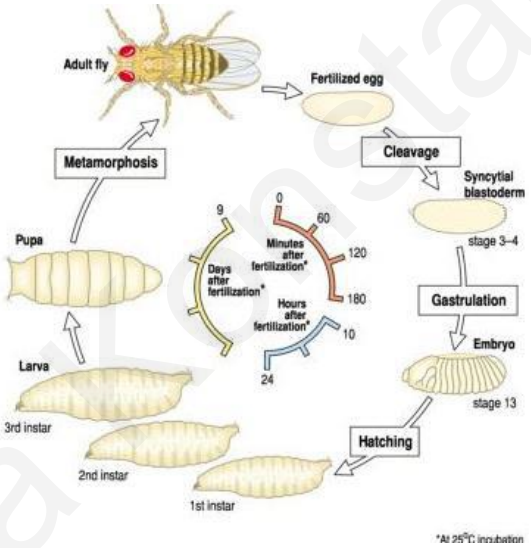

Figure 3: Life cycle of *Drosophila,* from the embryo to the adult at 25°C (Wolpert et al. 2015)

1.4 *Drosophila* brain anatomy and types of glial cells

The CNS of the *Drosophila* larval brain is composed of two spherical and symmetrical hemispheres and the Ventral Nerve Cord (VNC), on which the hemispheres are connected (Figure 1) (Read 2011, Witte et al. 2009). The neurons of the Peripheral Nervous System (PNS) will emerge from the neurons of the VNC (Witte et al. 2009). The hemispheres are also called brain

lobes since on their surface lies the center of the brain that is necessary for the processing of visual information (Apitz, Salecker 2014, Li et al. 2014). This center is the optic lobe and is located at the surface of the brain lobes in a horse-shoe shape (Figure 1) (Apitz, Salecker 2014).

Glial cells can be defined as the non-neuronal cells of the CNS and are derived from the precursors of the neuronal cells (Yildirim et al. 2019). Glial cells may not have the role of neuronal cells, but it has been shown that their role in the development and homeostasis of the CNS is essential (Yildirim et al. 2019, Kremer et al. 2017). In the developing brain, glial cells control the NSC proliferation rate to maintain the cell populations of the CNS (Kremer et al. 2017). In addition, glial cells are responsible for the removal of dying neurons, ionic homeostasis through neurotransmitter regulation, metabolic homeostasis of the neurons etc (Kremer et al. 2017, Yildirim et al. 2019). Finally, they act as the primary immune cell population of the CNS (Kremer et al. 2017).

The *Drosophila* CNS has 3 types of glial cells, the surface glia, the cortex glia and the neuropile glia (Figure 4) (Hartenstein 2011). Surface glia cells are divided into two subtypes, the perineurial glia, which constitute the outer layer of glial cells and the subperineurial glia that are located beneath the perineurial layer. These two subtypes of glial cells form the blood brain barrier (Figure 4) (Hartenstein 2011, Yildirim et al. 2019). The perineurial glia are small cells with many projections that allow them to form the outer layer of the blood brain barrier and do not directly contact the neurons. The subperineurial glia are flat, large cells, highly polyploid due to endoreplication that form septate junctions providing the inner part of the blood brain barrier (Yildirim et al. 2019).

Cortex glia are located just beneath the subperineurial glia and are in contact with neurons (Figure 4) (Yildirim et al. 2019). They are small round cells that build a network surrounding NSCs and neurons that are connected via septate and adherent junctions (Hartenstein 2011, Yildirim et al. 2019). Cortex glia have a fundamental role for the development of neurons in the optic lobe and in the activation of neuroblast stem cells (Yildirim et al. 2019). In the developing brain, cortex glia cells are responsible for neuronal homeostasis of essential metabolic molecules (Yildirim et al. 2019, Hartenstein 2011).

The deepest layer of a *Drosophila* larval brain is called neuropile due to the existence of the neuropile compartments that are hosting dendrites, axon branches and synapses. Neuropile is constructed by neuropile glia cells that form the septa, dividing the neuropile into compartments (Figure 4) (Technau 2008). During development neuropile glia and cortex glia restricts the growth of one another establishing the cortex-neuropile boundary (Coutinho-Budd et al. 2017). Neuropile glia are subdivided into two categories, the ensheathing glia and the astrocyte-like glia. Ensheathing glia are flat cells that cannot penetrate into the neuropile compartments (Figure 4) (Hartenstein 2011). Ensheathing glia act as phagocytes in the brain, therefore they are responsible for clearance of the apoptotic neurons, a process essential especially during metamorphosis (Hartenstein 2011, Doherty et al. 2009). Astrocyte-like glia are polarized cells that form brunches that penetrate into the neuropile compartments building a reticulum of glial cells allowing them to come in direct contact with terminal axons, dendrites and synapses (Figure 4) (Awasaki et al. 2008). Astrocyte-like glia provide guidance cues to axons and sustain normal neuronal function as they remove the transmitter from the synaptic cleft (Spindler et al. 2009, Jackson, Haydon 2008, Yildirim et al. 2019). The morphological, structural, and molecular features of these cells are conserved between mammals and *Drosophila* (Yildirim et al. 2019).

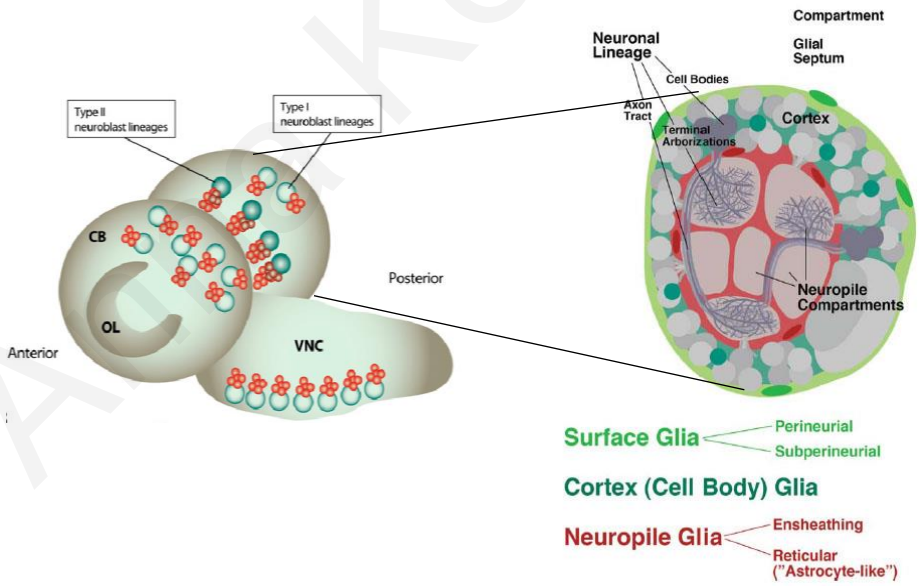

Figure 4: Image of a whole brain on the left, (Neumuller, Knoblich 2009) and a cross section of a brain lobe on the right (Hartenstein 2011)

1.5 Thesis Aims

GBM is the most common malignant type of cancer of the CNS and is characterized as highly angiogenic. Increased GBM vascularization is considered a key factor contributing to poor patient prognosis and resistance to treatments. Angiogenesis plays an important role in tumor cell survival and malignancy, but the precise mechanisms that control the interactions between the glial cells and the blood vessels remain elusive (Ahir et al. 2020). *Drosophila* GBM models have been established since 2009 (Read et al. 2009) and they offered great insights in various mechanisms underlining the disease. Our long-term goal is to use *Drosophila* larvae to understand the mechanisms governing tracheogenesis, the functional and molecular equivalent of angiogenesis, in GBM.

To assess brain tracheogenesis and its potential remodeling during tumorigenesis, we need to generate tumors and simultaneously label the cerebral trachea. Therefore, in the first part of my thesis, I took advantage of previously described mutations leading to gliomas and I examined brain growth, mitosis and apoptosis upon glial-specific overexpression of (a) the oncogenic form of Ras, Ras^{V12}, (b) the active catalytic subunit of PI3K, p110α, and (c) their combination in third instar larvae. To visualize the tracheae in these Gal4-UAS-dependent gliomas, I tried fluorescent labelling using the alternate binary systems LexA-LexAop and QF-QUAS. Because these approaches proved unsuccessful, in the second part of my thesis, I switched to the QF-QUAS system to overexpress Ras^{V12} for glioma generation in combination with the Gal4-UAS for trachea visualization. This approach enabled effective glioma generation and simultaneous trachea visualization. Using this model, I observed increased tracheogenesis in Ras^{V12} brain tumors. Further work using this model aims to genetically manipulate tracheogenesis in order to assess tumor growth and aggressiveness.

2. MATERIALS AND METHODS

2.1 Binary expression systems for tissue-specific overexpression (the GAL4 – UAS, QF– QUAS and LexA-LexAop systems)

The GAL4-UAS system is a binary system borrowed from *Saccharomyces cerevisiae* and it is used as the main tool for tissue specific expression of genes in *Drosophila melanogaster* (Brand, Perrimon 1993). The transcriptional activator GAL4 recognizes and binds to the Upstream Activating Sequence (UAS) allowing the expression of the downstream gene (Brand, Perrimon 1993). Tissue-specific promoters are fused upstream of the transcriptional activator GAL4 and the UAS is cloned upstream of potential genes of interest (genes to be overexpressed). Such constructs are introduced in flies using transgenesis and can be combined to allow tissue-specific overexpression (Brand, Perrimon 1993). Specifically, when two such strains are crossed, the GAL4 that is only expressed in a specific tissue will recognize the UAS activating the expression of the gene of interest only in this specific tissue (Nicholson et al. 2008). Using this binary system, we can ectopically overexpress any gene of interest in any specific tissue (Brand, Perrimon 1993). The QF-QUAS system is a binary system borrowed from *Neurospora crassa* and its function is comparable with the GAL4-UAS system since QF is a transcriptional activator that recognizes QUAS and activates the expression of the downstream gene (Potter et al. 2010). The third binary system used in *Drosophila* is the LexA-LexAop from bacteria like *Escherichia coli*. The transcriptional activator LexA recognizes and activates the LexA operator (LexAop) leading to the expression of the gene downstream of the operator. Each one of these three binary systems functions individually from the others (del Valle Rodríguez et al. 2012).

2.2 *Drosophila* strains

Strain ID	Genotype	Source
6326	w^{1118}	BDSC
-	*w; UAS-srcGFP/CyO; repo-GAL4/TM6C*	MSc Eirini Charalambous
7415	*w; repo-Gal4/TM3*	BDSC
5788	*w; UAS-Ras*^{V12}*(II)*	BDSC
25908	*ywUAS-Dp110*^{CAAX}	BDSC
-	*ywUAS-Dp110*^{CAAX}*; UAS-Ras*^{V12}	This study
-	*yw; btlGAL4UAS-srcGFP/CyO; QF2-repo/TM6C*	This study
66477	*yw;Pin/CyO;repo-QF2/TM6B*	BDSC
64775	*yw; wg*$^{Sp-1}$*/CyO,y*$^{+}$*; QUAS-Ras85D*^{V12}	BDSC
-	*w;btlGal4UAS-srcGFP/CyO*	(Pitsouli, Perrimon 2010)
32207	*w;I(2)****/CyO;LexAbtl*	BDSC
66620	*w;26XLexAop2-mcD8GFP(III)*	BDSC
-	*w; QF6QUAS-mtd-Tomato/TM6C*	(Tamamouna et al. 2021)

Table 1: All the genotypes used for this study and the information of the source of them

2.3 Brain dissections and Immunofluorescence

Dissections of late 3rd instar larval brains (~120 hours after fertilization) (Figure 3) are performed in 9-well plates in 1X PBS (Table 2). Brain dissection (within 25 minutes) is followed by fixation for 30 minutes at room temperature (RT) with 4% Formaldehyde (FA) (Table 2). Fixative is removed by 3 rinses with 1X PBS. The fixed samples are incubated with PBT (Table 2) for 2-3hrs at RT or overnight (12-16hrs) at 4^{o}C to ensure blocking of non-specific binding and to increase the penetration of the antibody in the deepest layers of the tissue. Then, the primary antibodies (Table 3) are added and incubated overnight at 4^{o}C. The samples are washed 3 times for at least 15 minutes each at RT with PT (Table 2). Samples are then incubated with the appropriate secondary

antibodies (Table 3) at RT in the dark for 1,5-2hrs followed by 3 washes for at least 15 minutes each at RT in the dark with PT. Finally, the samples are mounted on glass slides with Vectashield (Table 2) to prevent photobleaching, then covered with a coverslip and shielded with nail polish.

Reagent	Components/Source
10X PBS	1.3M NaCl, 0.07M Na_2HPO_4, 0.03M NaH_2PO_4
16%FA	16% Methanol-free EM-grade Formaldehyde (FA, Polysciences, Inc)
PBT	1x PBS, 0.3% Triton X-100, 0.5% BSA
PT	1x PBS, 0.2% Triton X-100
Vectashield	Vector Laboratories, Inc.

Table 2: Reagents used for dissections and Immunofluorescence

Primary Antibodies	Recognizing cells	Secondary Antibody
Rabbit-anti-phospho histone H3 (pH3 1:4000)	Mitotic cells	Donkey- anti-rabbit Alexa 555 (1:1000)
Mouse – anti- Repo (8D12 1:20)	Glial cells	Donkey- anti- mouse Alexa 555/647 (1:1000)
Rat - anti- Elav (7E8A10 1:100)	Neuronal cells	Donkey- anti- rat Alexa 555/647 (1:1000)
Rabbit-anti-*Drosophila* caspase protein 1 (Dcp-1 1:100) (Cleaved)	Apoptotic cells	Donkey- anti- rabbit Alexa 555 (1:1000)

Table 3: Primary and Secondary antibodies used in this study

2.4 Image and data analysis

Confocal images were acquired using a Leica TCS SP2 AOBS Laser Scanning Confocal Microscope with the following settings: XYZ acquisitions, 20X oil objective, Average 4 and 1024x1024 px Format, which equals 750x750 μm. Images were processed and analysed using ImageJ (NIH)(Schneider et al. 2012). The scale of the images was set by Analyze → Set scale → Distance in pixels: 1024, Known distance: 750, Pixel aspect ratio: 1, Unit of length: μm. The surface of each central brain was measured automatically by selecting each central brain with Polygon selection → Analyze → Measure. Nuclei were measured using the automated counting of cells (Single colour images). For this feature the image must be in grey scale (8-bit or 16-bit) then for the following steps were used Image → Adjust → Threshold in order to mark the nuclei → Apply. This will provide a binary version of the image. In many cases the nuclei of different cells are merged, to avoid counting them as one before counting Process → Binary → Watershed can separate the nuclei by adding one pixel between them. After the image is processed and in binary form central brains were selected using Polygon selection → Analyze → Measure Particles → Size (μm^2) 0.6-Infinity, Circularity 0.0-1.0, Show: Outlines.

2.4.1 Statistical analysis

The statistical analysis of the data was performed in Microsoft Excel by calculating the mean and the standard deviation from the mean. The statistical significance of the results was calculated via the Student's t-test, every p-value bellow 0.05 was considered significant. The box plots were generated in Microsoft Excel using all the values collected by the image analysis.

3. RESULTS

Drosophila GBM models have been developed and characterized since 2009. The authors used the Gal4-UAS system, and specifically, the Reverse polarity (Repo)-Gal4 driver to overexpress the most frequently upregulated genes in GBM patients in a tissue-specific manner (Read et al. 2009). They showed that glial-specific overexpression of either activated *EGFR* or *Dp110α* in glial cells causes low-grade gliomas in 3rd instar larvae, but the simultaneous overexpression of both causes high-grade gliomas, reminiscent of human GBM (Figure 1). Additionally, they suggested that overexpression of *Ras* alone, which is a downstream component of the EGFR pathway, can cause a more severe phenotype, since it can activate the PI3K pathway endogenously (Read et al. 2009).

Given the above, we started our research using the known *repo-Gal4*-UAS model to overexpress *Dp110a, Ras^{V12}*, and their combination in glial cells to confirm previous observations and further study the effect of Ras^{V12} overexpression. We focused our study on the central brain and not the whole brain, because the optic lobes are mostly formed by optic sensory neurons. In addition, the astrocyte-like glia, which are considered key in GBM (Maher et al. 2001), are located in the central brain of the *Drosophila* larva.

3.1Overexpression of Ras^{V12} alone in glial cells is sufficient to cause neoplasia

To analyze the growth of gliomas in *Drosophila* 3rd instar larval brains, we tested mitotic activity in control brains and brains overexpressing Ras^{V12}, *Dp110α* alone and in combination using the pH3 antibody (Figure 5). As expected, we observed that in all genotypes the highest mitotic activity was observed in the optic lobe, because mostly differentiated neurons and glial cells populate the central brain (Figure 5 A-D'). Quantification of $pH3^+$ cells of the central brain, where astrocyte-like glia resides, showed that brains overexpressing Ras^{V12} alone or in combination with *Dp110α* exhibited significantly higher mitotic activity compared with wild type brains. Interestingly, we did not observe any significant differences between these two genotypes. The brains overexpressing the active catalytic subunit of PI3K exhibited increased number of $pH3^+$ cells compared with the wild type brains, but significantly lower when compared with the brains overexpressing Ras^{V12} (Figure 5E). The size of the brains overexpressing Ras^{V12} alone and in combination have no significant differences when compared to each over but in both genotypes

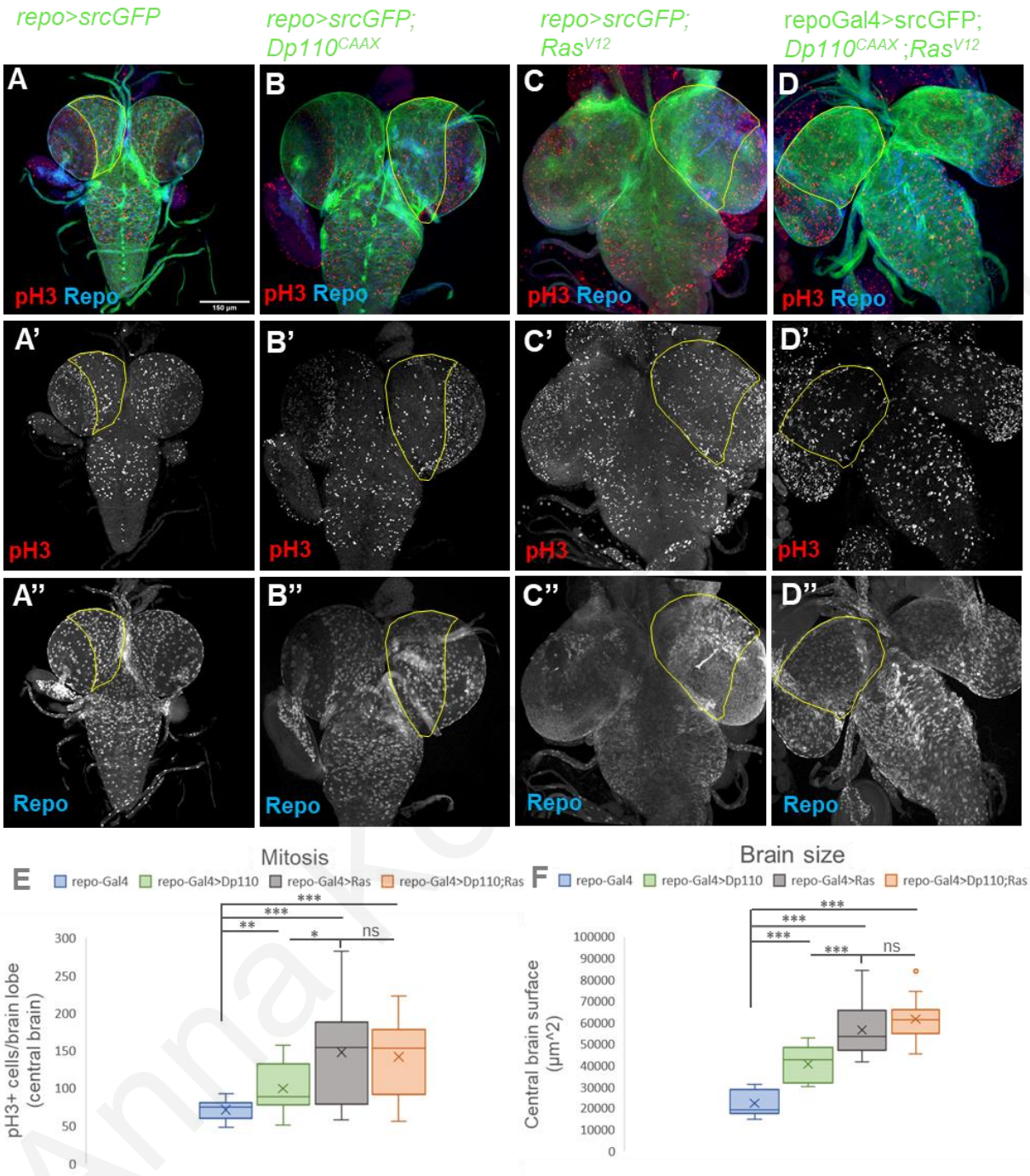

Figure 5: Overexpression of *Ras^{V12}* alone in glial cells is sufficient to cause neoplasia. (A-D) Maximum projections of whole brains stained with anti-pH3 and anti-Repo antibodies. Wild type brain (A) and brains overexpressing *Dp110α* (B), *Ras^{V12}* (C), and *Dp110α; Ras^{V12}* (D). (A'-D') Grey scale of pH3^{+} cells that represent A-D respectively. (A''-D'') Grey scale glial nuclei that represent A-D respectively. (E) Quantification of pH3^{+} cells in the central brain. (F) Central brain surface measurements.

$p<0.05$ = *, $0.05< p \leq 0.01$ = **, $0.01< p \leq 0.001$ = ***

the brains were significantly enlarged compared with the wild type brains (Figure 5F). Brains expressing *Dp110α* were enlarged compared with the wild type brains but significantly smaller than the brains overexpressing Ras^{V12} alone (Figure 5F).

3.2 Overexpression of Ras^{V12} alone induces apoptosis in the central brain

We sought to examine apoptosis in these genotypes using the cleaved *Drosophila* caspase-1 (Dcp-1) antibody (Figure 6). The higher apoptotic activity in all the genotypes was observed at the optic lobes of the brains (Figure 6A-D). No significant differences were observed comparing control brains to brains overexpressing *Dp110α* alone or in combination with Ras^{V12}. On the other hand, brains overexpressing Ras^{V12} alone exhibited increased apoptosis not only when compared with the control brains but also when compared with brains overexpressing *Dp110α* alone or in combination with Ras^{V12} (Figure 6E).

3.3 Trachea of the 3rd instar larval brain can be visualized only using *btl-Gal4*

The next step to our research was to visualize the trachea of the *Drosophila* larval brain. Since we used the Gal4-UAS to overexpress the oncogenes specifically at the glial cells, we needed a second binary system to specifically overexpress a fluorescent protein in the tracheal cells of the larvae. Therefore, we tested the QF-QUAS system, as we knew from previous experiments that QF6 is a specific driver for trachea cells in the adult fly gut (Tamamouna et al, 2021). Unfortunately, QF6 was not specific to the tracheal cells of the larval brain. The QF6 driver labelled some tracheae in the VNC, but not the brain lobes, and it was also expressed in some Crustacean cardioactive peptide (CCAP) – expressing neurons (Figure 7A). After excluding QF6, we tested another binary system, the LexA-LexAop. A trachea-specific btl-LexA driver was available (Yuan et al. 2020) and thus, we tested it in the larval brains, but once again the driver was not specific for tracheal cells rather it was expressed in the Bursicon+ CCAP neurons (Figure 7B). Bursicon CCAP neurons are the CCAP neurons that express the bursicon neuropeptide and will be eliminated via programmed cell death during development (Lee et al. 2013). Since we tried those two systems and none of them was specific, we used our last option which was the Gal4-UAS system. Using

the trachea specific driver *btl-Gal*, we were able to visualize fully and with high specificity the trachea branching network of the larval brain (Figure 7C).

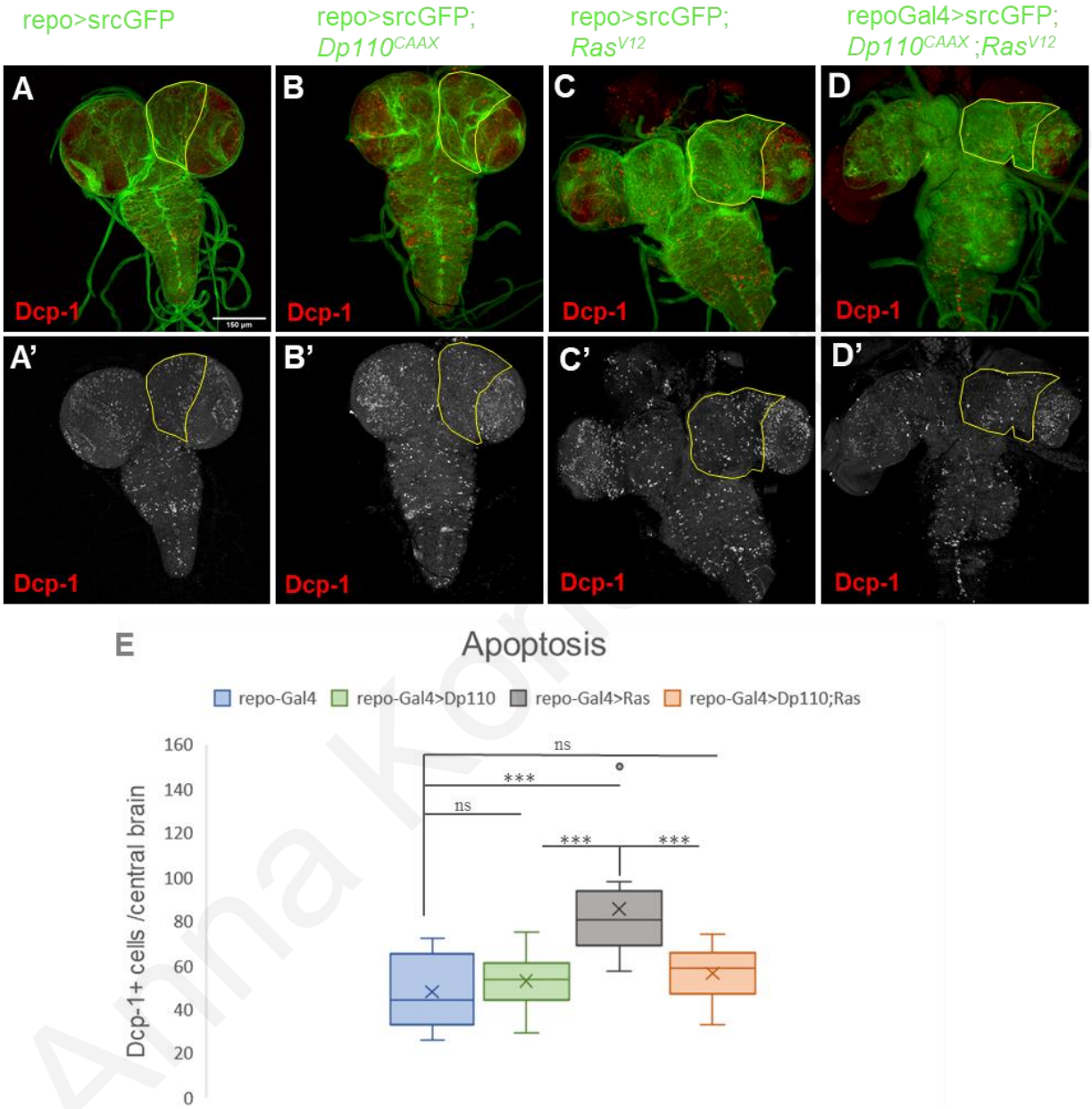

Figure 6: Overexpression of *Ras^{V12}* induces apoptosis in the central brain. (A-D') Maximum intensity projections of whole brains stained with anti-Dcp-1. Wild type brain (A) and brains overexpressing *Dp110α* (B), *Ras^{V12}* (C), and *Dp110α; Ras^{V12}* (D). (A'-D') Grey scale Dcp-1^{+} cells that represent A-D respectively. (E) Quantification of Dcp-1^{+} cells in the central brain.

p <0.05= *, 0.05< p ≤0.01 = **, 0.01< p ≤0.001 = ***

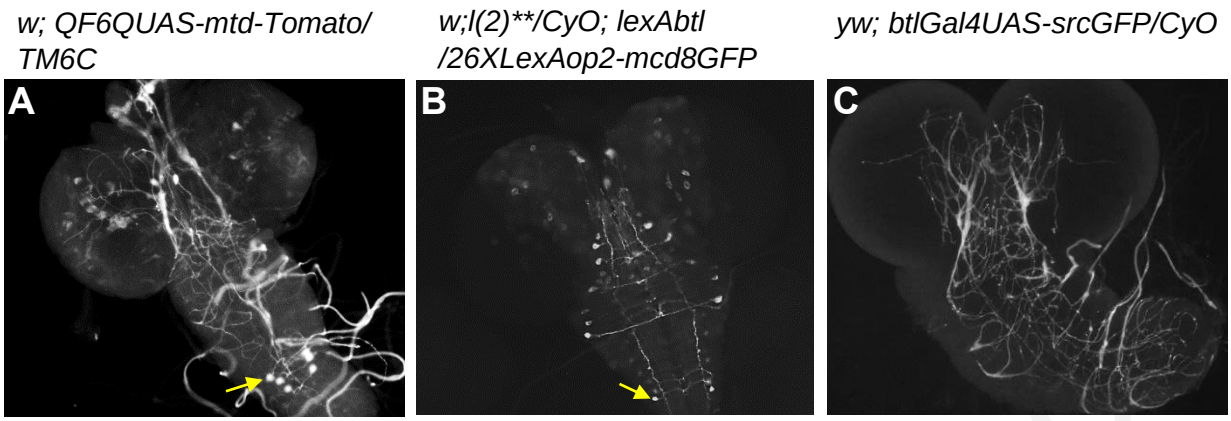

Figure 7: Tracheae of 3rd instar larvae brains can be visualized only using *btl-Gal4*. (A) Overexpression of membrane-tagged mtd-Tomato using the trachea-specific QF6 driver; the yellow arrow indicates CCAP neurons. (B) Overexpression of membrane-tagged mCD8-GFP using btl-LexA driver; the yellow arrow indicates bursicon^{+} CCAP neurons. (C) Overexpression of membrane-tagged src-GFP using the *btl-Gal4* driver. All images correspond to single channel maximum projections of the fluorescent protein label.

3.4 Overexpression of Ras^{V12} using the QF-QUAS system is sufficient to cause neoplasia accompanied by neotracheogenesis

After evaluating the three available binary systems in *Drosophila* for cell specific overexpression of genes for tracheal visualization, we concluded that only the *btl-Gal4* was specific and highlighted all the brain tracheal cells, allowing visualization of the entire tracheal network. Up to this point, for our experiments we were using *repo-Gal4* as the driver for the glial specific overexpression of genes. However, we could not use the Gal4-UAS system to create the glioma and also label the trachea. Thus, to use the btl-Gal4 for the trachea, we needed an alternative binary system for glial-specific oncogene overexpression.

Therefore, we developed a strain that includes *repo-QF*, for overexpressing Ras^{V12} specifically at glial cells, and *btlGal4-UASsrcGFP* for overexpressing GFP at the trachea cells. Since our results indicated that Ras^{V12} is sufficient to cause neoplasia alone, we focused our experiments on Ras^{V12}.

To validate and characterize our new glioma model, we evaluated the mitotic activity of the brains that overexpressed Ras^{V12} with the QF-QUAS system using the anti-pH3 antibody (Figure 8). We found that brains overexpressing Ras^{V12} have significantly increased number of pH3^{+} cells

compared with the wild type brains (Figure 8A-B', 8C). As expected, larvae that overexpressed *Ras*^{V12} in their glial cells have significantly enlarged brains compared with the wild type (Figure 8D). Finally, quantification of the integrated density of GFP revealed excessive neotracheogenesis in brains overexpressing *Ras*^{V12} (Figure 8E).

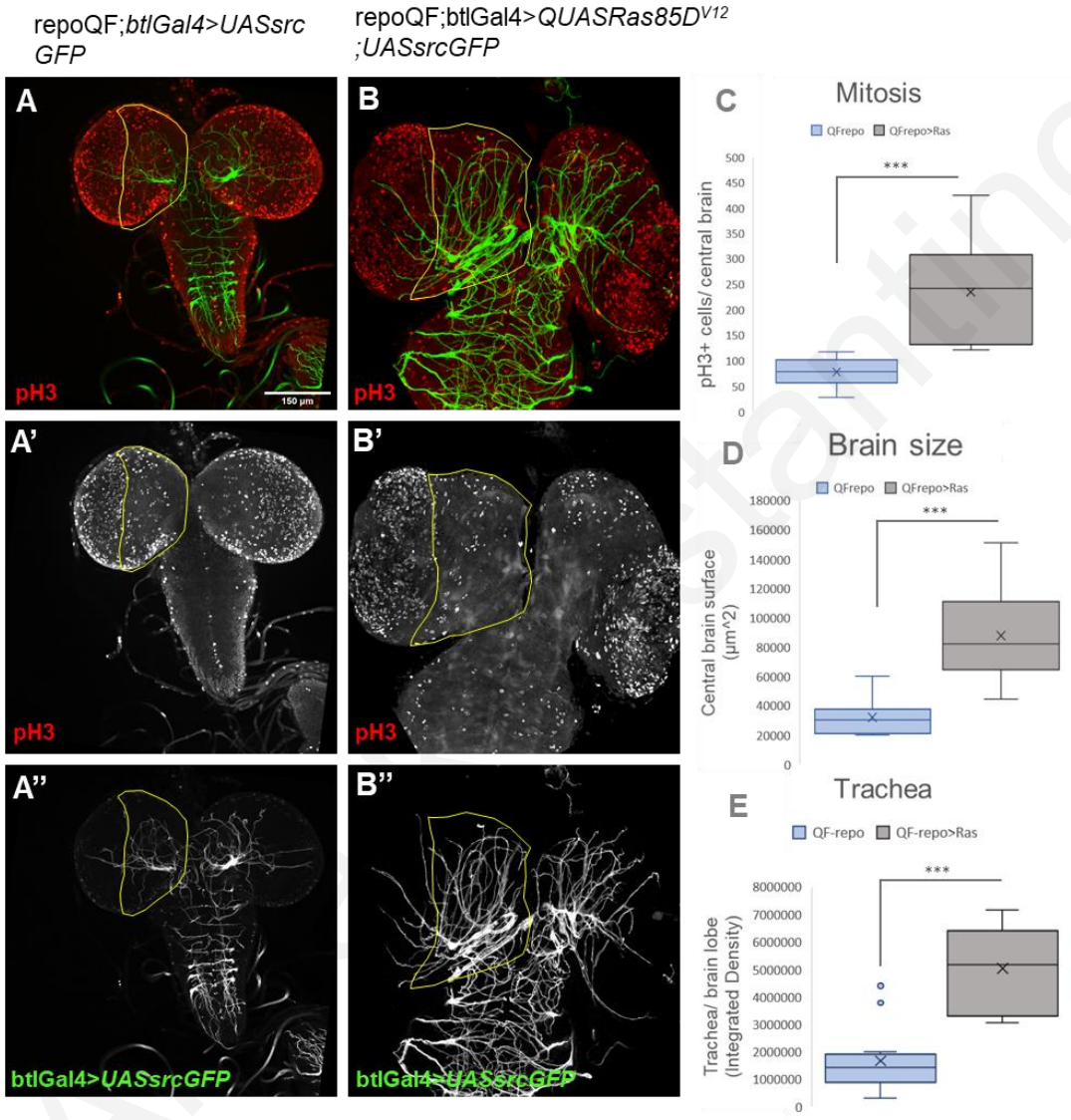

Figure 8: Overexpression of *Ras*^{V12} using the QF-QUAS system is sufficient to cause neoplasia accompanied by neotracheogenesis. (A-B) Maximum intensity projections of whole brains stained with anti-pH3. Wild type brain (A) and brain overexpressing *Ras*^{V12}(B). (A'-B') Grey scale of pH3^{+} cells that represent A-B respectively. (A''-B'') Grey scale *btl-Gal4-UASsrcGFP* showing trachea branching that represent A-B respectively. (C) Quantification of pH3^{+} cells in the central brain. (D) Measurements of the central brain surface. (E) Quantification of the cerebral trachea as Integrated Density of GFP.

$p<0.05$ = *, $0.05< p \leq 0.01$ = **, $0.01< p \leq 0.001$ = ***

3.5 Overexpression of *Ras*^{V12} using the QF-QUAS system induces apoptosis

To further characterize our new model, we tested the effect of *Ras*^{V12} overexpression in apoptosis of the central brain (Figure 9). The pattern of apoptosis agreed with our previous finding (Figure 6), as the highest apoptotic activity in all genotypes was observed in the optic lobes (Figure 9A-B'). Additionally, quantification of Dcp-1^{+} cells indicated increased apoptotic activity in brains overexpressing *Ras*^{V12} compared with wild type brains (Figure 9A'-B', C).

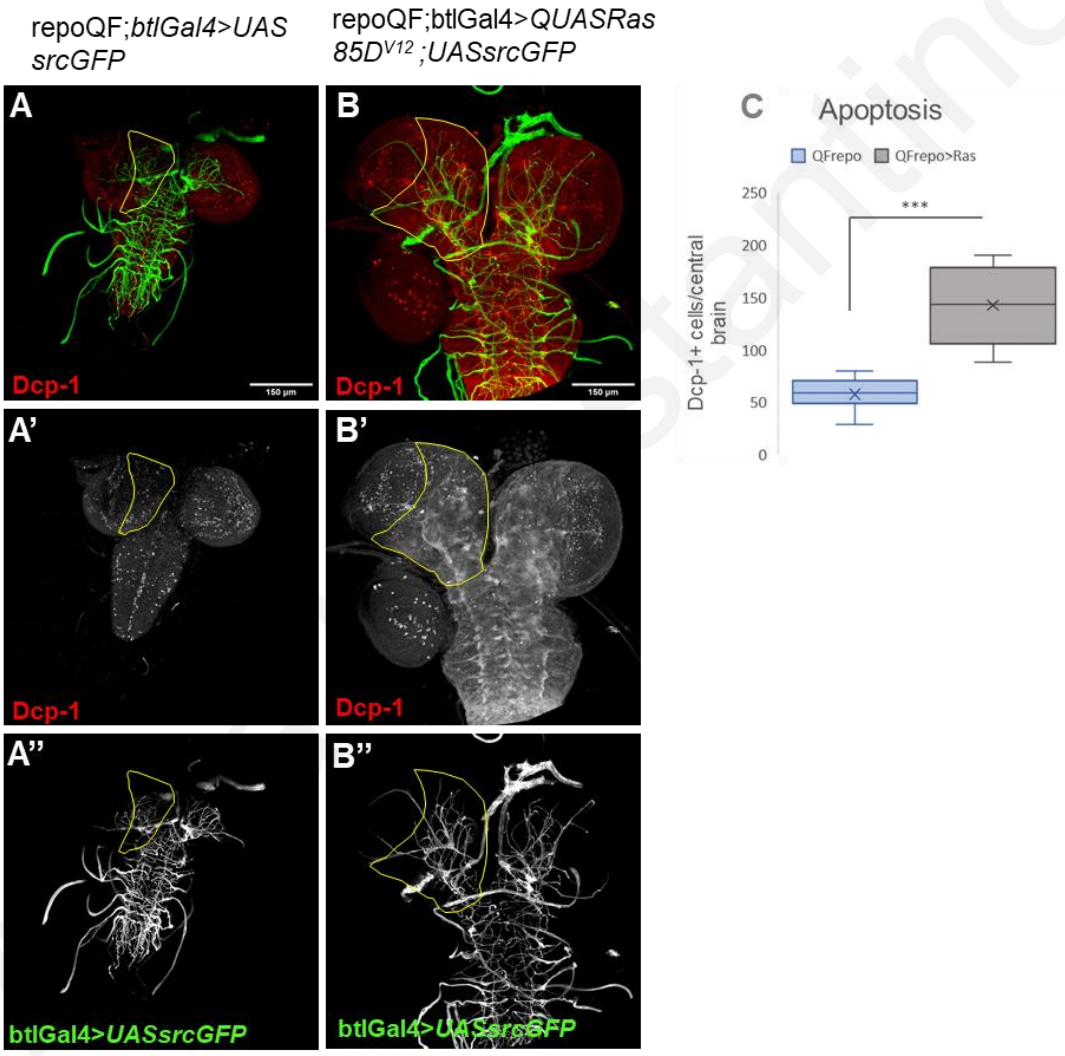

Figure 9: Overexpression of *Ras*^{V12} using the QF-QUAS system induces apoptosis in the central brain. (A-B) Maximum projection of whole brains stained with Dcp-1 antibody. Wild type brain (A) and brain overexpressing Ras^{V12} (B). (A'-B') Grey scale of anti-Dcp-1^{+} cells that represent A-B respectively. (C) Quantification of Dcp-1^{+} cells in the central brain.

$p < 0.05$= *, $0.05 < p \leq 0.01$ = **, $0.01 < p \leq 0.001$ = ***

4. DISCUSSION

Drosophila GBM models have been established since 2009 by R. Read. In their study they tested the overexpression of several key genes upregulated in GBM patients, including activated *EGFR*, *Dp110α*, *PTEN* and the combination of those two using the *repo-Gal4* driver. They were able to classify the tumours as low-grade and high-grade (Figure 1) according to the ability of each genotype to invade the surrounding tissue. From the single mutants they did not observe any significant invasive activity except from Ras^{V12} that had the more invasive potential resembling the invasiveness of the double mutant $EGFR\lambda;PTEN^{(-/-)}$. Therefore, they suggested that overexpression of Ras^{V12} may have a more severe phenotype due to its ability to activate PI3K endogenously. Nevertheless, they did not classify Ras^{V12} as high-grade nor as low-grade glioma due to the fact that oncogenic Ras does not suffice for the development of human high-grade gliomas, because human GBM usually requires the coactivation of multiple pathways (Read et al. 2009).

Given the above, we started our research using the repo-Gal4 driver to overexpress components of each pathway (*Dp110α*, Ras^{V12}) and the combination of them. We decided to use Ras^{V12} instead of *EGFRλ* to study its effect alone and in combination with *Dp110α*. Our results indicated that overexpression of Ras^{V12} is sufficient to cause neoplasia and we did not observe any differences between the single mutant Ras^{V12} and the double mutant $Dp110α;Ras^{V12}$ regarding their mitotic activity and their brain size (Figure 5E,F). The only difference between the double mutant and the single Ras^{V12} mutant is the number of apoptotic cells. Specifically, Ras^{V12} overexpressing brains exhibited significantly increased apoptotic activity compared to the other genotypes (control, *Dp110α*, $Dp110α;Ras^{V12}$) (Figure 6E). Even though one of the hallmarks of cancer is inhibition of apoptosis, high-grade GBM patients constitute the exception to this phenomenon, since the apoptotic activity in high-grade gliomas is higher than in low-grade gliomas (Eisele, Weller 2013). The exact mechanism underlying this phenomenon, and its contribution to the response of the patient to treatment or even the survival of the patient is still under investigation.

Our next goal was to find a way to visualize the trachea and at the same time cause neoplasia by overexpressing Ras^{V12} specifically in the glial cells of the larvae. After experimentally excluding the QF-QUAS and LexA-LexAop systems for tracheal specific overexpression we concluded that

only the Gal4-UAS system was specific enough to uncover the full cerebral trachea (Figure 7). In order to use the btl-Gal4>UAS-srcGFP to label the cerebral trachea, we needed an alternative system to generate the glia-specific tumour. Hence, we tested the QF-QUAS system for the overexpression of Ras^{V12} using the *repo-QF2* as the driver. We focused our attention on Ras^{V12} since we did not observe any differences between the overexpression of the oncogene alone and in combination with *Dp110α* regarding mitosis, but there was a robust difference between them regarding their apoptotic rate. Therefore, we used the *btl-Gal4* and the *repo-QF2* constructs to reconstruct a new *Drosophila* strain containing both (Table 1) and allowing glia-specific expression of the oncogenic Ras and GFP labelling of the trachea. Evaluation of the effect of Ras^{V12} in regard to cell proliferation and apoptosis in this new glioma model agreed with the Gal4-driven Ras model: brains overexpressing Ras^{V12} under *repo-QF2* exhibited increased mitosis accompanied with brain size increase (Figure 8C, D). In addition, brains overexpressing the oncogene had significantly higher number of apoptotic cells (Figure 9C).

Following the characterization of our model, we proceeded with a preliminary assessment of the trachea quantification using the Integrated Density of GFP as a proxy and found significantly increased trachea in the brain lobes of larvae overexpressing Ras^{V12} (Figure 8E). The 3D structure of the brains and the complexity of the cerebral tracheal network require the 3D reconstruction of the cerebral trachea to quantify accurately the branches in the X, Y and Z axes. We were able to reconstruct the 3D structure of the cerebral trachea by scanning the whole brains with confocal microscope and then using Fiji (Schindelin et al. 2012). Unfortunately, we have not yet managed to accurately quantify the cerebral tracheae due to the lack of a user-friendly tool that allows 3D measurements of branching.

In the imminent future, to further characterize our model, we will test the invasiveness of the Ras^{V12} tumours using mosaics, i.e. mosaic analysis with a repressible cell marker (MARCM), which allows the study and tracing of a number of mutant cells in an otherwise wild type brain (Germani et al. 2018). In addition, we will try to find a way to accurately quantify the cerebral trachea (i.e. IMARIS and Fiji). To fully understand the function of tracheogenesis in gliomas, we will manipulate the tracheal cells to inhibit or overactivate their branching and we will assess the tumour phenotypes. For example, we will manipulate the trachea by the overexpression and inhibition of the FGFR homologue (Breathless (Btl) in flies). The Btl pathway is essential for the

trachea growth during animal development, and it is also necessary for adult neotracheogenesis under several stress conditions including the formation of tumours in the *Drosophila* gut (Tamamouna et al. 2021).

5. ABBREVIATIONS

ABBREVIATION	MEANING
BDSC	Bloomington Drosophila Stock Center
Bnl	Branchless
BSA	Bovine Serum Albumin
Btl	Breathless
CCAP	Crustacean Cardioactive Peptide
CNS	Central Nervous System
Dcp-1	*Drosophila* caspase protein 1
EGFR	Epitermal Growth Factor Receptor
FA	Formaldehyde
fga	Fatiga
FGFR	Fibroblast Growth Factor Receptor
GBM	Glioblastoma Multiform
HIF-1	Hypoxia Inducible Factor-1
HRE	Hypoxia Response Element
InR	Insulin Receptor
NSC	Neuronal Stem Cell
PBS	Phosphate Buffered Saline
PBT	PBS BSA Triton X
PDGFR	Platelet Derived Growth Factor Receptor
pH3	phospho histon H3
PHD	Prolyl-4-hydroxylase

PI3K	Phosphatidyl-Inositol-3 Kinase
PNS	Peripheral Nervous System
PT	PBS Triton X
PVR	PDGF-VEGF Receptor
Repo	Reverse polarity
RT	Room Temperature
RTK	Receptor Tyrosine Kinase
sima	Similar
tgo	Tango
VEGFR	Vascular Endothelial Growth Factor Receptor
VHL	Von-Hippel-Lindau
VNS	Ventral Nerve Cord
MARCM	Mosaic analysis with a repressible cell marker
WHO	World Health Organization

6. BIBLIOGRAPHY

AHIR, B.K., ENGELHARD, H.H. and LAKKA, S.S., 2020. Tumor development and angiogenesis in adult brain tumor: Glioblastoma. *Molecular Neurobiology*, pp. 1-18.

APITZ, H. and SALECKER, I., 2014. A challenge of numbers and diversity: neurogenesis in the Drosophila optic lobe. *Journal of Neurogenetics*, vol. 28, no. 3-4, pp. 233-249.

AWASAKI, T., LAI, S.L., ITO, K. and LEE, T., 2008. Organization and postembryonic development of glial cells in the adult central brain of Drosophila. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, Dec 17, vol. 28, no. 51, pp. 13742-13753 ISSN 1529-2401; 0270-6474. DOI 10.1523/JNEUROSCI.4844-08.2008 [doi].

BRAND, A.H. and PERRIMON, N., 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development (Cambridge, England)*, Jun, vol. 118, no. 2, pp. 401-415 ISSN 0950-1991; 0950-1991.

CONTE, M., CASAS-TINTÒ, S. and SOLER, J., 2021. Modeling invasion patterns in the glioblastoma battlefield. *PLoS Computational Biology*, vol. 17, no. 1, pp. e1008632.

COUTINHO-BUDD, J.C., SHEEHAN, A.E. and FREEMAN, M.R., 2017. The secreted neurotrophin Spatzle 3 promotes glial morphogenesis and supports neuronal survival and function. *Genes & Development*, 20171114, Oct 15, vol. 31, no. 20, pp. 2023-2038 ISSN 1549-5477; 0890-9369. DOI 10.1101/gad.305888.117 [doi].

DAHMANN, C., 2008. *Drosophila.* Springer.

DAS, S. and MARSDEN, P.A., 2013. Angiogenesis in glioblastoma. *New England Journal of Medicine*, vol. 369, no. 16, pp. 1561-1563.

DEL VALLE RODRÍGUEZ, A., DIDIANO, D. and DESPLAN, C., 2012. Power tools for gene expression and clonal analysis in Drosophila. *Nature Methods*, vol. 9, no. 1, pp. 47.

DOHERTY, J., LOGAN, M.A., TASDEMIR, O.E. and FREEMAN, M.R., 2009. Ensheathing glia function as phagocytes in the adult Drosophila brain. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, Apr 15, vol. 29, no. 15, pp. 4768-4781 ISSN 1529-2401; 0270-6474. DOI 10.1523/JNEUROSCI.5951-08.2009 [doi].

EISELE, G. and WELLER, M., 2013. Targeting apoptosis pathways in glioblastoma. *Cancer Letters*, vol. 332, no. 2, pp. 335-345.

FURNARI, F.B., FENTON, T., BACHOO, R.M., MUKASA, A., STOMMEL, J.M., STEGH, A., HAHN, W.C., LIGON, K.L., LOUIS, D.N., BRENNAN, C., CHIN, L., DEPINHO, R.A. and CAVENEE, W.K., 2007. Malignant astrocytic glioma: genetics, biology, and paths to treatment. *Genes & Development*, Nov 1, vol. 21, no. 21, pp. 2683-2710 ISSN 0890-9369; 0890-9369. DOI 21/21/2683 [pii].

GERMANI, F., BERGANTINOS, C. and JOHNSTON, L.A., 2018. Mosaic analysis in Drosophila. *Genetics*, vol. 208, no. 2, pp. 473-490.

GREENSPAN J., R., 2004. *Fly Pushing: The theory and practice of Drosophila genetics.* Second Edition ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York: John Inglis ISBN 0-87969-711-3.

HALES, K.G., KOREY, C.A., LARRACUENTE, A.M. and ROBERTS, D.M., 2015. Genetics on the Fly: A Primer on the Drosophila Model System. *Genetics*, Nov, vol. 201, no. 3, pp. 815-842 ISSN 1943-2631; 0016-6731. DOI 10.1534/genetics.115.183392 [doi].

HARTENSTEIN, V., 2011. Morphological diversity and development of glia in Drosophila. *Glia*, vol. 59, no. 9, pp. 1237-1252.

JACKSON, F.R. and HAYDON, P.G., 2008. Glial cell regulation of neurotransmission and behavior in Drosophila. *Neuron Glia Biology*, vol. 4, no. 1, pp. 11.

KREMER, M.C., JUNG, C., BATELLI, S., RUBIN, G.M. and GAUL, U., 2017. The glia of the adult D rosophila nervous system. *Glia*, vol. 65, no. 4, pp. 606-638.

KURIYAMA, H., LAMBORN, K.R., O'FALLON, J.R., ITURRIA, N., SEBO, T., SCHAEFER, P.L., SCHEITHAUER, B.W., BUCKNER, J.C., KURIYAMA, N. and JENKINS, R.B., 2002. Prognostic significance of an apoptotic index and apoptosis/proliferation ratio for patients with high-grade astrocytomas. *Neuro-Oncology*, vol. 4, no. 3, pp. 179-186.

LEE, G.G., KIKUNO, K., NAIR, S. and PARK, J.H., 2013. Mechanisms of postecdysis-associated programmed cell death of peptidergic neurons in Drosophila melanogaster. *Journal of Comparative Neurology*, vol. 521, no. 17, pp. 3972-3991.

LI, S., WANG, H. and GROTH, C., 2014. Drosophila neuroblasts as a new model for the study of stem cell self-renewal and tumour formation. *Bioscience Reports*, vol. 34, no. 4.

MAHER, E.A., FURNARI, F.B., BACHOO, R.M., ROWITCH, D.H., LOUIS, D.N., CAVENEE WK FAU PINHO,R.A. and DEPINHO, R.A., 2001. Malignant glioma: genetics and biology of a grave matter. *Genes & Development*, Jun 1, vol. 15, no. 11, pp. 1311-1333 ISSN 0890-9369; 0890-9369. DOI 10.1101/gad.891601 [doi].

NICHOLSON, L., SINGH, G.K., OSTERWALDER, T., ROMAN, G.W., DAVIS, R.L. and KESHISHIAN, H., 2008. Spatial and temporal control of gene expression in Drosophila using the inducible GeneSwitch GAL4 system. I. Screen for larval nervous system drivers. *Genetics*, Jan, vol. 178, no. 1, pp. 215-234 ISSN 0016-6731; 0016-6731. DOI 10.1534/genetics.107.081968 [doi].

PANDEY, U.B. and NICHOLS, C.D., 2011. Human disease models in Drosophila melanogaster and the role of the fly in therapeutic drug discovery. *Pharmacological Reviews*, 20110317, Jun, vol. 63, no. 2, pp. 411-436 ISSN 1521-0081; 0031-6997. DOI 10.1124/pr.110.003293 [doi].

PECORINO, L., 2012. *Molecular biology of cancer: mechanisms, targets, and therapeutics.* Oxford university press.

PEREANU, W., SPINDLER, S., CRUZ, L. and HARTENSTEIN, V., 2007. Tracheal development in the Drosophila brain is constrained by glial cells. *Developmental Biology*, vol. 302, no. 1, pp. 169-180.

PITSOULI, C. and PERRIMON, N., 2010. Embryonic multipotent progenitors remodel the Drosophila airways during metamorphosis. *Development (Cambridge, England)*, Nov, vol. 137, no. 21, pp. 3615-3624 ISSN 1477-9129; 0950-1991. DOI 10.1242/dev.056408 [doi].

PORTELA-ESTEBAN, M. and CASAS-TINTÓ, S., 2021. New cellular dimensions on glioblastoma progression.

POTTER, C.J., TASIC, B., RUSSLER, E.V., LIANG, L. and LUO, L., 2010. The Q system: a repressible binary system for transgene expression, lineage tracing, and mosaic analysis. *Cell*, vol. 141, no. 3, pp. 536-548.

READ, R.D., 2011. Drosophila melanogaster as a model system for human brain cancers. *Glia*, vol. 59, no. 9, pp. 1364-1376.

READ, R.D., CAVENEE, W.K., FURNARI, F.B. and THOMAS, J.B., 2009. A drosophila model for EGFR-Ras and PI3K-dependent human glioma. *PLoS Genetics*, vol. 5, no. 2, pp. e1000374.

RIBEIRO, M.d.C., COUTINHO, L.M.B. and HILBIG, A., 2004. The role of apoptosis, cell proliferation index, bcl-2, and p53 in glioblastoma prognosis. *Arquivos De Neuro-Psiquiatria*, vol. 62, no. 2A, pp. 262-270.

SCHINDELIN, J., ARGANDA-CARRERAS, I., FRISE, E., KAYNIG, V., LONGAIR, M., PIETZSCH, T., PREIBISCH, S., RUEDEN, C., SAALFELD, S. and SCHMID, B., 2012. Fiji: an open-source platform for biological-image analysis. *Nature Methods*, vol. 9, no. 7, pp. 676-682.

SCHNEIDER, C.A., RASBAND, W.S. and ELICEIRI, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, vol. 9, no. 7, pp. 671-675.

SPINDLER, S.R., ORTIZ, I., FUNG, S., TAKASHIMA, S. and HARTENSTEIN, V., 2009. Drosophila cortex and neuropile glia influence secondary axon tract growth, pathfinding, and fasciculation in the developing larval brain. *Developmental Biology*, vol. 334, no. 2, pp. 355-368.

TAMAMOUNA, V. and PITSOULI, C., 2018. The Hypoxia-Inducible Factor-1α in Angiogenesis and Cancer: Insights from the Drosophila Model. In: *Gene Expression and Regulation in Mammalian Cells-Transcription Toward the Establishment of Novel Therapeutics*IntechOpen.

TAMAMOUNA, V., RAHMAN, M.M., PETERSSON, M., CHARALAMBOUS, I., KUX, K., MAINOR, H., BOLENDER, V., ISBILIR, B., EDGAR, B.A. and PITSOULI, C., 2021. Remodeling of oxygen-transporting tracheoles drives intestinal regeneration and tumorigenesis. *bioRxiv*.

TECHNAU, M.G., 2008. *Brain Development in Drosophila melanogaster.* Gerhard M. Technau ed., New York: Springer Science&Business Media, LLC Landes Bioscience ISBN 978-0-387-78260-7.

WITTE, H.T., JEIBMANN, A., KLÄMBT, C. and PAULUS, W., 2009. Modeling glioma growth and invasion in Drosophila melanogaster. *Neoplasia*, vol. 11, no. 9, pp. 882-888.

WOLPERT, L., TICKLE, C. and ARIAS, A.M., 2015. *Principles of development.* Oxford University Press, USA.

YILDIRIM, K., PETRI, J., KOTTMEIER, R. and KLÄMBT, C., 2019. Drosophila glia: Few cell types and many conserved functions. *Glia*, vol. 67, no. 1, pp. 5-26.

YUAN, X., SIPE, C.W., SUZAWA, M., BLAND, M.L. and SIEGRIST, S.E., 2020. Dilp-2–mediated PI3-kinase activation coordinates reactivation of quiescent neuroblasts with growth of their glial stem cell niche. *PLoS Biology*, vol. 18, no. 5, pp. e3000721.