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Investigating the Role of Neural Stem Cells in Aggressive Gliomas

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Abstract

In the United States alone, there are more than 1.8 million people diagnosed with cancer every year. This number increases exponentially as the scope is expanded to look at the number of people affected worldwide (National Cancer Institute, 2020). Given that a large number of genetic mutations have been identified, and there is a wide variety of cancers and cancer promoting networks. The current treatments have been extensively researched and explored, but there is ultimately no cure for this aggressive and unrelenting disease. One extremely invasive type of cancer is Glioblastoma Multiforme (GBM), which is a specific type of brain cancer. The exact growth patterns of these tumors are unknown, but it is known that GBM is formed from excess glial stem cells, which are produced by neuroblasts (neural stem cells). One understudied area is if glioma tumors arise from neuroblasts already present in the brain, or in response to tumor promoting signals new neuroblasts are created to induce and promote GBM tumor metastasis. These aggressive tumors grow rapidly and aggressively, which makes their origins and pathways of growth extremely difficult to locate and track. *Drosophila melanogaster*, or the common fruit fly, is the model organism for this study. The power of *Drosophila* lies in the multiple genetic tools available for experimental design, and the conservation of genes and cell-biological processes between flies and humans, which means that findings from *Drosophila* studies can be easily verified in mammalian models and human patients. We have developed a GBM model in flies using the GAL4-UAS system, where two genotypically different flies will be crossed to induce these tumors in developing *Drosophila* larval brains. This study will explore the origins of GBM tumors and the nature of cell-biological and growth promoting pathways that promote uncontrolled growth of glial cells and neuroblasts within the brain.

Acknowledgements

First, I would like to especially thank my mentor and advisor Dr. Madhuri Kango-Singh for her guidance and instruction throughout the years in helping this project to transform and grow. I would also like to thank Arushi Rai, Karishma Gangwani and Rohith BN for their assistance in the lab with procedural measures, such as dissections, staining and imaging with the confocal microscopes. Additionally, thank you to Dr. Amit Singh lab for their help throughout this process in supplying materials and other assistance. I would like to thank everyone who was a part of the DSF and ISE CoRPs Programs for allowing me to further my research project during summer sessions at UD. I would also like to thank all of my family and friends who have been so supportive and encouraging throughout this process. Lastly, thank you to the University of Dayton Honors Program for providing me with the opportunity to compose and write an Honors Thesis during my undergraduate schooling.

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Introduction

GBM in patients: the mysteries surrounding origin of glia in the GBM tumors

Many patients suffering from GBM struggle to understand the entirety of this diagnosis as there is a great deal of mystery surrounding this cancer. Median survival rates of GBM patients range from 8%-12%, which demonstrates an extremely poor prognosis and a greater fear within patients who receive GBM as a diagnosis (Ghosh, 2017). GBM is a dangerous illness, which has no cure as the disease grows very rapidly and can be hard to track. This is especially true in brain cancer. Figure 1 shows a simplified view of the difference between normal cells and cancer cells. Besides the rapid cell division cycles that result in formation of a mass, loss of cell shape and polarity contribute to changes that facilitate metastasis that results in the spread of cancer. Tumor recurrence is a peculiar feature of GBM, where post-surgery and chemoradiation therapy the tumor recur at the sight of the original tumor. These recurrent GBM tumors are therapy resistant with poor prognosis.

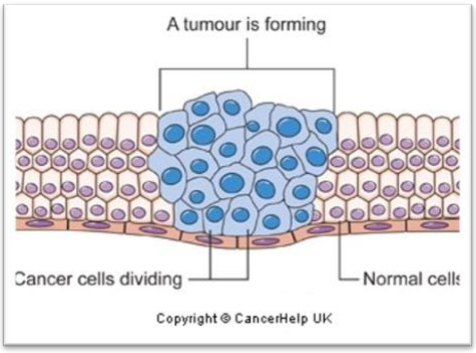

Figure 1: Simple explanation of tumor-forming cells versus normal cells in cancerous tumors. This image shows the differences between growth patterns in cancerous cells that lead to tumor formation versus normal cells.
Photo credit: [13]

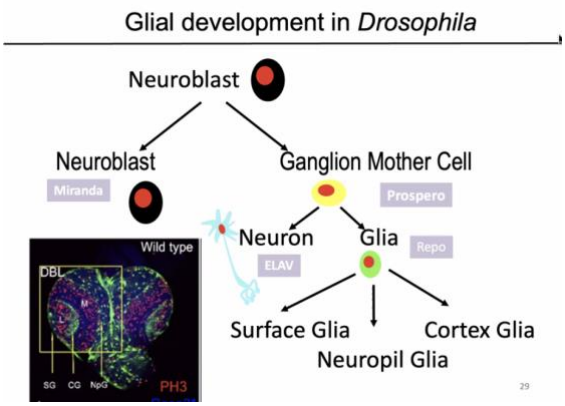

Figure 2. The division from neuroblast to glial cell. This image shows the process of a neuroblast dividing into an identical neuroblast and ganglion mother cell. This will then divide into 2 neurons, 2 glia or 1 neuron and 1 glia.
Photo credit: [Dr. I. Waghmare]

In the context of GBM, the normal development of glial cells has helped understand the processes that may contribute to the growth of the tumor. The loss of cell division control results in the rapid expansion of cell types like glia and neurons. Normally, in the *Drosophila* CNS, the process of cell division begins with a neuroblast (Figure 2), which divides to generate (replicate) another neuroblast as a copy of itself and also produces a differentiated cell called the ganglion mother cell (GMC). The GMC then divides to produce either two neurons, two glial cells, or one neuron and one glial cell (Freeman et al., 2006; Homem at al., 2012; Homem et al., 2015). When mutations occur within gliogenesis, problems arise and result in an excess amount of glial cells, ultimately forming a cancerous tumor (Read et al., 2011).

***Drosophila* models of GBM**

Drosophila melanogaster, or the common fruit fly, is used as the model organism for this study. This organism has a short reproduction time, distinct stages in its life cycle, is capable of producing a plethora of offspring and is relatively inexpensive. Most importantly, the pathways in *Drosophila melanogaster* model many of the same pathways seen in humans. It has been revealed that 75% of the known human disease genes have a recognizable match in the genome of fruit flies, consolidating its legitimacy as a model organism for medical research (Jennings, 2011). This is especially important to

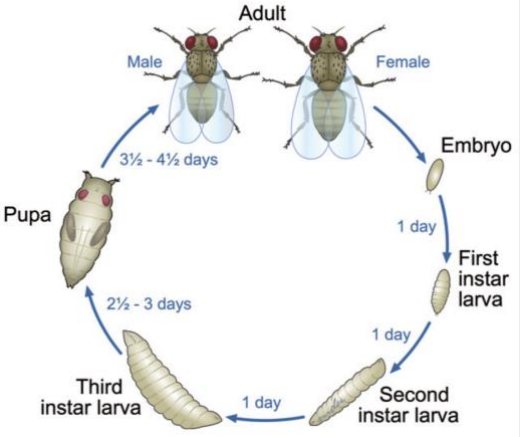

Figure 3: Life cycle of *Drosophila melanogaster* fruit fly. This image shows the 5 growth stages. This growth begins with embryo and ends with the pupal stage. The stage denoted by "Third instar larvae" will be the majority of this study.
Photo credit: [22]

note as we look at the similarities relative to the production of neural stem cells (NSCs) and formation of neuroblasts (Egger, 2008). In order to see these pathways in the fruit fly model, our study mainly focuses on the third instar larvae. It is during this stage that large invasive CNS tumors are formed. This stage, along with the entirety of the life cycle of these flies is provided in Figure 3.

Advantages of *Drosophila* models: genetics, tools and techniques for manipulating gene expression, introduce the gal4 UAS system

In order to produce the Glioblastoma Multiforme (GBM) tumors in the *Drosophila melanogaster* model, the UAS-GAL4 pathway is used. This pathway allows for the gene of interest to be misexpressed, or in this case overexpressed, through the binding of GAL4 to UAS (Busson, 2007). This process is shown in Figure 4 as the GAL4 driver line is expressed in the male flies, while the UAS responder line will contain the gene of interest, which will be represented by the female (Busson, 2007). In this study, two flies are crossed, one from the GAL4 driver line and the other from the UAS-target gene line in order to produce progeny that express the gene of interest under the control of the GAL4 driver. This allows tissue- and cell-specific expression of genes of interest. In the current study, we crossed *UAS-Pten*^{RNAi} and *UASRas*^{V12} females (referred to as *Pten*^{RNAi}, *Ras*^{V12} throughout this text) to the *repoGAL4* males. In the F1 progeny from this cross, we will find larvae where both the GAL4 and UAS are expressed together, and

a glioma will form in the CNS (brain) of these larvae. We will select these larvae for further studies described in the sections below.

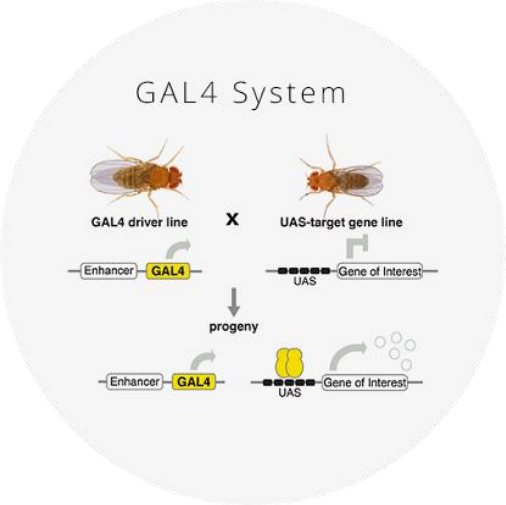

Figure 4: Explanation of the workings of GAL4-UAS system. A GAL4 enhancer is crossed with a UAS-target gene of interest in order to express the gene of interest within the progeny.
Photo credit: [6]

Drosophila GBM model for loss of Pten and Ras activation

Pten is a tumor suppressor gene, while Ras is an oncogene that is implicated in several types of cancer. Both these genes are conserved in flies and are required for the regulation of pathways controlling protein synthesis (Figure 5), cell cycle entry and cell cycle progression (Figure 5). These processes are central to normal development and are misregulated in cancer (Read et al., 2011).

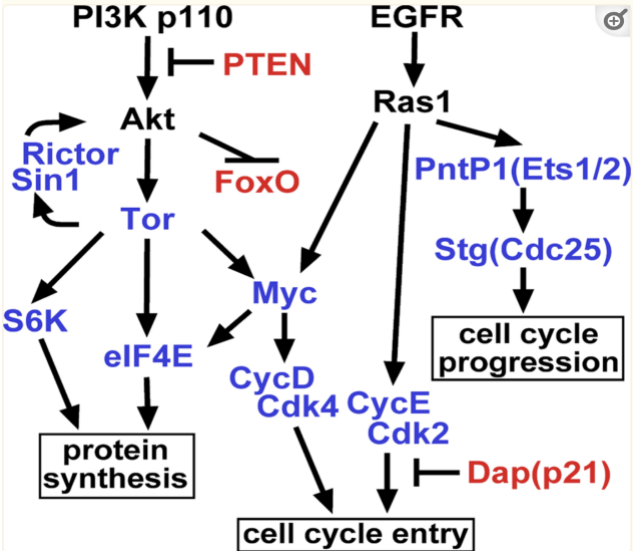

Figure 5: The EGFR-PI3K pathway is demonstrates here. The key components involved in glial neoplasia are initiated by EGFR and PI3K in *Drosophila*. Positive regulators are in blue and negative regulators are in red. Arrows indicate pathway connections, although these connections are not necessarily direct.
Photo credit: [23]

In *Drosophila melanogaster*, the glial-specific co-activation of EGFR-Ras and PI3K pathways result in the stimulation of glial cells and the formation of glial tumors (Read et al., 2011). Mutations in these genes result in the loss of tumor suppressor functions, which result in tumor-like growths in the fly brain (Read et al., 2011). These

disruptions result in abnormal cell division as the central nervous system is experiencing loss-of-function mechanisms. The EGFR-PI3K pathway, which is shown in Figure 5, is complex and consists of several genes that ultimately regulate cell cycle processes like cell cycle entry and progression. When disrupted, it results in continued cell division cycles and the consequences are detrimental. As shown, the EGFR pathway largely relies on the functioning of the Ras gene, while the PI3K pathway relies on the Pten gene function. Ultimately, a misregulation of either or both can result in the formation of harmful GBM tumors in the *Drosophila* fruit fly model.

CNS development in flies: Cues from normal development for studying glioma growth

The central nervous system (CNS) of the *Drosophila melanogaster* fly consists of an extremely complex network of neural circuits. The CNS begins to develop in the embryo of the fly, which then continues throughout the larval stage and finally ends with the formation of the CNS in the adult fly brain. The CNS develops from stem cell-like precursor cells, which are called neuroblasts (Goodman and Doe, 1993). This allows for the control of neurogenesis and regulation of cell division, which are very important processes in the differentiation of neuroblasts. Temporal transcription factors (TTFs) are found in NBs of the CNS and are crucial neural progenitor cells in cell division (Sato, 2022). The expression of TTFs connect to the formation of tumors in glioma models and they contribute to upregulation, downregulation, gain of function, and loss of function. Each of these changes can lead to hindrance of normal brain development and result in expression seen in glioma models.

Neurogenesis: Type I and Type II neuroblasts in fly CNS- how glia form from stem cell divisions in the neuroblasts or neural stem cells

GBM is an extremely aggressive type of brain tumor that yields a high mortality rate and poor prognosis. In *Drosophila melanogaster*, the conservation of genes of the Epidermal Growth Factor Receptor (EFGR) and PI3K pathways yield the GBM tumor model utilized in other studies (Gangwani et al., 2020; Read et al., 2011). This study will look into the GBM tumor produced through a loss of Pten and Ras activation. This model is studied in the larval central nervous system (CNS), which consists of two brain lobes and a ventral nerve cord, which is shown in Figure 6. The NSCs to be studied are located in the optic lobe (OL) neuroepithelium, the central brain (CB) neuroblasts, and the ventral nerve cord (VNC) neuroblasts (Gangwani et al., 2020). The CB and VNC contain type I neuroblasts as the majority, which asymmetrically divide to self-renew and generate a GMC which differentiate into either neurons or glial cells as shown in Figure 6 (Nguyen et al., 2022). The CB also contains type II neuroblasts, which asymmetrically divide to self-renew and to generate an intermediate neural progenitor (INP), which divides to eventually produce GMCs as shown in Figure 7 (Nguyen et al., 2022). The two types of neuroblasts differ in their expression, but the varying locations of these NSCs determine when they are produced in the larval life cycle. The neuroblasts in the CB and VNC are produced during embryogenesis, while the neuroblasts in the OL arise during mid-larval development (Nguyen et al., 2022). Work from our lab showed that in the fly glioma, several additional neuroblasts are present in the glioma. The purpose of this research is to explore if these additional neuroblasts represent stem cells that are already present in the brain or represent new stem cells formed by *de-novo* processes. To answer

these questions, we used immunohistochemical approaches in *Drosophila melanogaster* CNS.

What is the role of Notch signaling in glial cell differentiation?

Notch is a cell-surface receptor which transduces short-range signals with neighboring cells by interacting with a ligand to influence cell division, fate, and death (Kopan, 2012). Once binding occurs between the receptor and ligand, then cleavage and release of the Notch intracellular domain (N^{ICD}) will occur and N^{ICD} travels to the nucleus to regulate transcriptional complexes (Kopan, 2012). This is a key player in cell differentiation as Notch is essentially a gene in the process of neurogenesis (formation of neurons) in early development. The expression of Notch and its target genes helps separate different cell types in the central and peripheral nervous system. Notch signal transduction ultimately leads to differentiation of progenitor cells into different cell lineages and yields the promotion and inhibition of different pathways (Urbanek, 2017). Gain and loss of

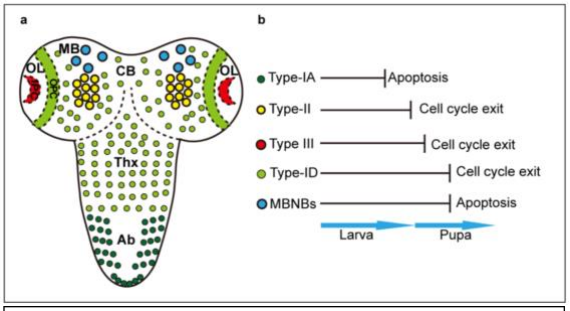

Figure 6: Structure of larval *Drosophila* brain.
The central brain is denoted by CB and the optic lobes are each denoted by OL.
Photo credit: [19]

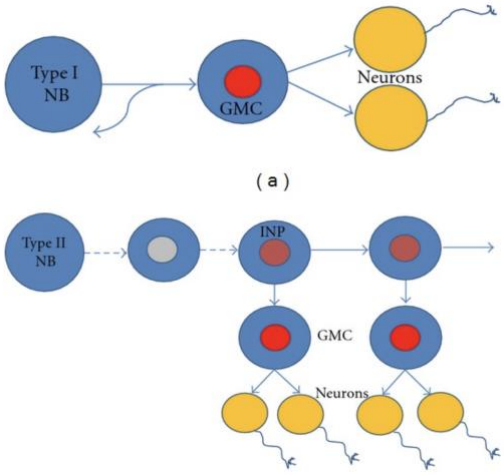

Figure 7: Differentiation between the division of Type I NB and Type II NB. The larger lineage of Type II NBs is shown through the visual presence of the intermediate neural progenitor (INP).
Photo credit: [24]

functions among cells is ultimately resulting from the activity and signal transduction of the Notch gene. The Notch signaling pathway is necessary for developmental processes in a number of organ systems, including hematopoiesis, somitogenesis, vasculogenesis, and neurogenesis. These processes involve maintenance of stem cell self-renewal, proliferation, specification of cell fate or differentiation, and apoptosis. Recent studies have led to the recognition of the role of the Notch pathway in early neurodevelopment, learning, memory, and late-life neurodegeneration. The role of the Notch pathway is important in early development, but also later neuroglial development (Lütolf et al., 2002; Nye et al., 1994; Tanigaki et al., 2001). Recent findings have revealed a more complex role for Notch and gliogenesis. Notch has demonstrated a potential role in neural progenitor maintenance in glial specific cell types due to the cell's potential to act as neural stem cells (Lasky et al., 2005). We plan to use the Notch cell signaling gene as an important marker for NBs to explore whether NSCs arise from preexisting neuroblasts or from new neuroblasts produced during gliomagenesis. The expression of Notch in the glioma models in the third larval instar (which is well past the developmental stage where larval neuroblasts are born), but not the control models, will demonstrate that new NBs are being made. If Notch is not expressed in the glioma model or the control model, then this will indicate NBs are produced from preexisting cells and the induction of glioma does not induce new NB formation.

Prospero, Miranda, Deadpan and N^{ICD}: Biomarkers used in this study and their roles in normal development

Prospero, Miranda, Deadpan, and N^{ICD} are antibodies used in the immunohistochemistry protocol for our experiments. Each of these antibodies is paired

with ELAV (which marks the neurons in the CNS) to highlight different stages of cell differentiation throughout brain development. ELAV is a marker for neurons and is common to all of the immunohistochemical studies. Each of these genes has a different purpose and role in neural differentiation. The gene Prospero promotes the expression of neural differentiation genes and represses NSC identity genes and cell cycle proliferation genes. Asymmetric cell division results in the separation into GMC daughter cells, which then allows Prospero to enter the nucleus of GMCs. The expression of Prospero is maintained in the glial cells but not in neurons. Miranda has been identified as a protein that interacts with Prospero (Shen et al., 1997). Miranda encodes a protein that can bind to Prospero products and also results in division into GMC daughter cells. In the larval central nervous system, Deadpan expression results in an increase in the size of the brain lobes in comparison to wild-type brains, while also playing an important role in NB self-renewal and differentiation. The loss of Deadpan leads to the early loss of NBs and shortened NB lineages, which is believed to be mediated by Prospero. Over-expression of Deadpan promotes ectopic self-renewing divisions and maintains NB self-renewal. Lastly, Notch functions as a receptor for membrane-bound ligands Delta and Serrate to regulate cell-fate determination (Acar et al., 2008; Brückner et al., 2000; Okajima et al., 2003; Wilkin et al., 2004). Notch also regulates NB self-renewal, identity and proliferation.

In summary, Prospero is a marker for Ganglion mother cells in the neurogenic lineage. Miranda is a marker of stem cells used to assess their number and placement. Deadpan is a Notch target required for the maintenance of neuroblasts. N^{ICD} will mark activated Notch in the initiation of neural differentiation. In terms of the stains, Prospero

and ELAV will be compared to the second stain with Miranda and ELAV in order to show the presence of mature stem cells. Deadpan and ELAV will be used in order to show whether or not normal epithelial cells are becoming NSCs. Lastly, N^{ICD} and ELAV will be used to show the formation of new stem cells in the brain.

Outstanding question or gap in information to be addressed

The question being addressed through this research is where exactly do NSCs come from, especially in relation to their contributions in the formation of harmful glioma tumors. More specifically, it is addressed and asked whether these NSCs arise from preexisting neuroblasts or from new neuroblast production from neural development. Given our current understanding of this important process, the goal of this research is to uncover the answer through the use of glioma models. This information will contribute to further questions being asked and lend information towards understanding the origin of such daunting GBM tumors.

Hypothesis

Glial cells are formed from neuroblasts in the brain and are post-mitotic cells, meaning once they are formed, they are not capable of dividing any further. Thus, gliomas form from the presence of excess glial cells in the brain, but these cells are not actively dividing. In normal cells, neural stem cells (NCSs) divide to produce a neuroblast and ganglion mother cell, which will divide again to produce either a neuron or glial cell (Gangwani et al., 2020). It is unsure whether NCSs are produced from neuroblasts that are preexisting in the brain or if new neuroblasts are generated to induce the formation of brain tumors, such as those of GBM. **My hypothesis** is that these excess glial cells are being formed from new neuroblasts that are generated in aggressive glioma

tumors. Through dissections of the glioma larval brain and performing various antibody staining and imaging, we will be able to determine whether (a) these tumor-inducing neuroblasts already exist within the brain producing normal glial cells or (b) they are newly generated to create and continue the growth of GBM tumors. The concentration, production and migration of these NSCs will be visualized through immunohistochemistry protocol involving the entirety of the third instar larval brain.

After the desired cross has been amplified and there are enough larvae, antibody staining is performed utilizing immunohistochemistry protocol. This immunofluorescent antibody staining allows for fine neuronal processes to be better visualized, which is essential in exploring the growth patterns of NSCs and neuroblasts (Manning et al., 2016). In all experiments, glial cells are tagged with GFP (green), and neurons are marked by the expression of ELAV antibody (blue). In this study, there are four proposed rounds of staining listed below:

(i) Prospero and ELAV, which will then be compared to the second stain with

(ii) Miranda and ELAV in order to show the presence of mature stem cells.

(iii) N^{ICD} and ELAV will be used to show the formation of new stem cells in the brain.

(iv) Deadpan and ELAV in order to show whether or not normal epithelial cells are becoming NSCs.

Materials and Methods

The first step was to utilize the GAL4-UAS system extensively in order to produce a genetic cross that will induce the GBM tumors in the third instar stage in *Drosophila melanogaster*. The virgin females were taken from the *UASPten*^{RNAi},

UASRas^{V12} stock and mated with the *RepoGal4 UASGFP* males. All fly lines and experiments were maintained on standard fly medium (Cornmeal, Agar, Molasses, Yeast, etc.) at room temperature or at 25°C. The flies were curated following standard protocols, and appropriate males and virgin females will be collected for experimental crosses. Third instar larvae were dissected (n=15 to 20) from the cross and control samples for immunohistochemistry following previously established protocol (Kango-Singh et al., 2002). All samples (experimental and control) were processed under identical conditions, and the dissected larval brains mounted in Vectashield (Vector Labs, USA).

The established immunohistochemistry protocol involves two rounds of staining, one with a primary antibody and the other with a secondary antibody. After performing the initial dissection, a 1.5ml microfuge tube is used to fix samples for 20 minutes in 150µL PBS and 50µL PFA 16%. Then, 1000µL PBST is added to the solution in the microfuge tube and placed on a nutator for 15 minutes. The solution is carefully removed from the microfuge tube and the process of adding 1000µL PBST and suctioning it out is repeated. Next, the primary antibody is added, and tissues are incubated in the fridge (at 4ºC) for anywhere from 12 to 24 hours. If less than 10 brain samples are present, then 20µL of primary antibody is used. If there are 10 or more, 25µL is used. The primary antibodies used were mouse anti-Prospero (1:100), rat anti-Miranda (1:250), rat anti-Deadpan (1:100), and mouse anti-N^{ICD} (1:100). After incubation for 12 to 24 hours, the primary antibody is removed and stored in a new tube. Then, 1000µL PBST is added to the solution in the microfuge tube and placed on a nutator for 15 minutes. This process occurs twice. Then, the secondary antibody is added to the tube, which is then wrapped in foil wrap and placed onto the nutator for 2 hours. If there are 15 brains or less, then 60µL

of secondary antibody is used. If 15 or more brains are present in the solution, then 80μL of secondary antibody is used. The secondary antibodies used were anti-mouse IgG conjugated to Cy3, anti-rat IgG conjugated to Cy3, anti-mouse IgG conjugated to Cy5, and anti-rat IgG conjugated to Cy5. Following incubation with the secondary antibody, the samples were washed twice for 15 min with 1000μL PBST. Lastly, mounting, cleaning, and fixation of the brain samples on the microscope slides occurs so the slides can be viewed, and images are taken.

We assessed the samples for staining quality on the Olympus BX51 fluorescence microscope, and imaged good slides using an Olympus Fluoview 3000 laser scanning confocal microscope. The data was collected at 20X and 40X magnification, and images processed in Adobe Photoshop CS for further analyses. Good slides are determined to be those that have at least 8 brains on a slide with 6 of them being intact brains with the potential to provide high resolution data. This high-resolution data was taken only from samples that met criteria to be imaged and provided images that could provide the potential to see pattern and migration of NSCs.

Lastly, analysis was completed to determine if the protein expression was affected. If protein expression was affected, it was determined whether the protein levels went up or down. Quantification of these changes was accomplished by measuring signal intensity for both control and experimental samples.

Results

Results were obtained using a stepwise approach, which first began with researching the purpose of different antibodies to understand what was being shown with each. This allowed for the determination of how many stem cells were present in control

wild type brains versus experimental brains with tumors. After exploring the immunohistochemistry stains, other important cell signaling markers were explored.

In Figure 8, we looked at the expression of a stem cell marker called Prospero, which is expressed in neuroblasts and as the brain matures at the end of larval development, it is found in more specialized cells known as the GMC cells. We looked at the expression of Prospero in the central brain region of the wildtype brain *(RepoGAL4-UASGFP*) in Figure 8A, B, C (red, gray panels). The glial cells are shown by the expression of GFP under the glial cells specific driver *Repo>GFP*. The normal distribution of glial cells is shown in Figure 8A, B, D (green, gray panels). The number of neurons found in the wildtype brain is shown by the expression of ELAV in Figure 8A, B, E. In comparison, the expression of Prospero is altered in the glioma induced brains (*RepoGAL4-UASGFP; PTEN^{RNAi}; Ras^{V12}*). The number and distribution of the Prospero positive stem cells were evaluated in Figure 8F-J. Higher magnification images show an increased number and density of Prospero in the central brain region in Figure 8F, G, H (red, gray panels). The increased number of glial cells in Figure 8F, G, I (GFP, green, gray panels) represent the overall overgrowth of glial cells that ultimately results in lethal gliomas. Larvae bearing these gliomas enter an extended larval phase, fail to pupate, and die in the pupal stage due to excessive growth of the tumor. The number of associated neurons is tracked using ELAV in Figure 8F, G, J. For each sample, a minimum of 8 discs were imaged. In the glioma larvae, a consistent increase in Prospero positive cells was seen where overgrowth of the glioma tumor occurred.

We looked at the expression of a stem cell marker called Miranda in the wildtype (*RepoGAL4-UASGFP*) (Figure 9A). Miranda is expressed in the neural stem cells in the central brain region in Figure 9A, B, C (red, gray panels). The glial cells are shown by the expression of GFP under the glial cells specific driver *Repo>GFP*. The normal distribution of glial cells is shown in Figure 9A, B, D (green, gray panels). The number of

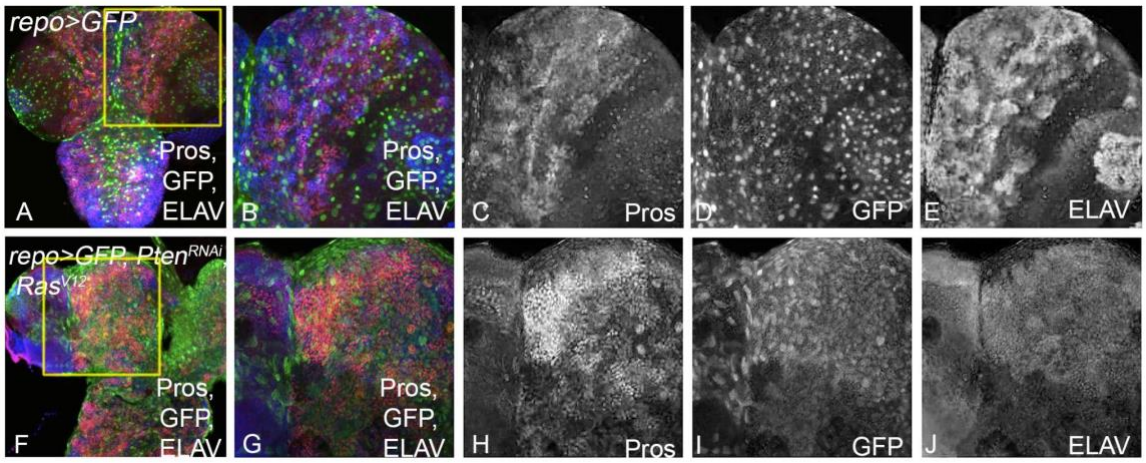

Figure 8: Expression of Prospero in Control and Experimental Glioma Larvae
Images in 20X and 40X confocal magnification showing control and glioma brains stained for glia (green), Prospero (red), and Elav (blue)

neurons found in the wildtype brain is shown by the expression of ELAV in Figure 9A, B, E. In comparison, the expression of Miranda is altered in the glioma induced brains (*RepoGAL4-UASGFP; PTEN^{RNAi}; Ras^{V12}*). The number and distribution of the Miranda positive stem cells were evaluated in Figure 9F-J. Higher magnification images show the increased number and density of Miranda in Figure 9F, G, H (red, gray panels). The increased number of glial cells in Figure 9F, G, I (GFP, green, gray panels) represent the overgrowth in the number of glial cells that ultimately results in lethal glial neoplasms. Larvae bearing these gliomas enter an extended larval phase, fail to pupate, and die in the pupal stage due to excessive growth of the tumor. The number of associated neurons is tracked using ELAV in Figure 9F, G, J. For each sample, a minimum of 8 discs were

imaged. In the glioma larvae, dependent on an increased overgrowth, a consistent increase in Miranda positive cells was noted.

When looking at Figure 10, we noted that the Deadpan stain showed the presence of self-renewing neuroblasts in the glioma model (*RepoGAL4-UASGFP; PTEN^{RNAi}; Ras^{V12}*). The glial cells are shown by the expression of GFP under the glial cells specific

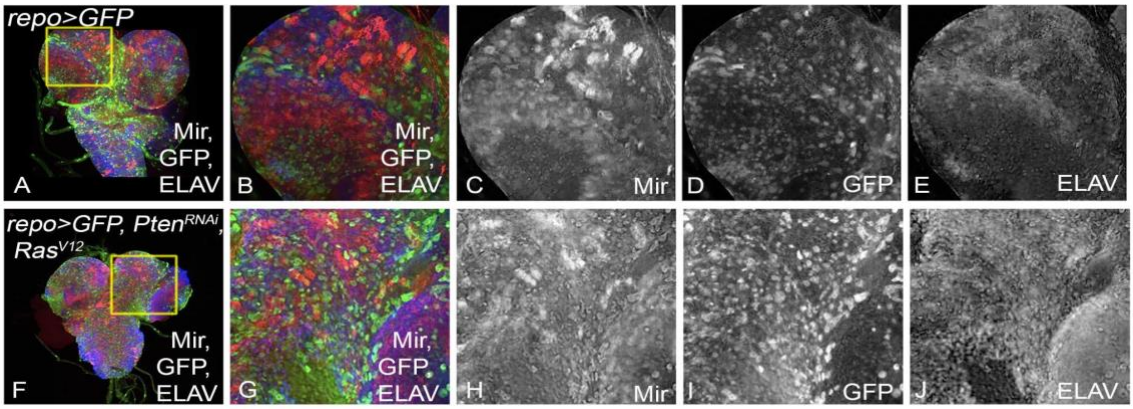

Figure 9: Expression of Miranda in Control and Experimental Glioma Larvae
Images in 20X and 40X confocal magnification showing control and glioma brains stained for glia (green), Miranda (red), and Elav (blue)

driver *Repo>GFP*. The normal distribution of glial cells is shown in Figure 10A, B, D (green, gray panels). The number of neurons found in the wildtype brain is shown by the expression of ELAV in Figure 10A, B, E. In comparison, the expression of Deadpan is slightly altered in the glioma brains (*RepoGAL4-UASGFP; PTEN^{RNAi}; Ras^{V12}*). The number and distribution of the Deadpan positive stem cells were evaluated in Figure 10F-J. Higher magnification images show the increased number of Deadpan cells in Figure 10F, G, H (red, gray panels). The increased number of glial cells in the optic lobes is especially noticeable in Figure 10F, G, I (GFP, green, gray panels). This increase represents the overgrowth of glial cells that result in lethal glial neoplasms. The number of neuroblasts is tracked using ELAV in Figure 10F, G, J. The density of ELAV-positive

cells migrates from the lower optic lobe in the wildtype brain to the upper optic lobe in the glioma induced brain. For each sample, a minimum of 8 discs were imaged. In the glioma larvae, a slight increase in Deadpan positive cells and a dramatic increase in glial cells were noted.

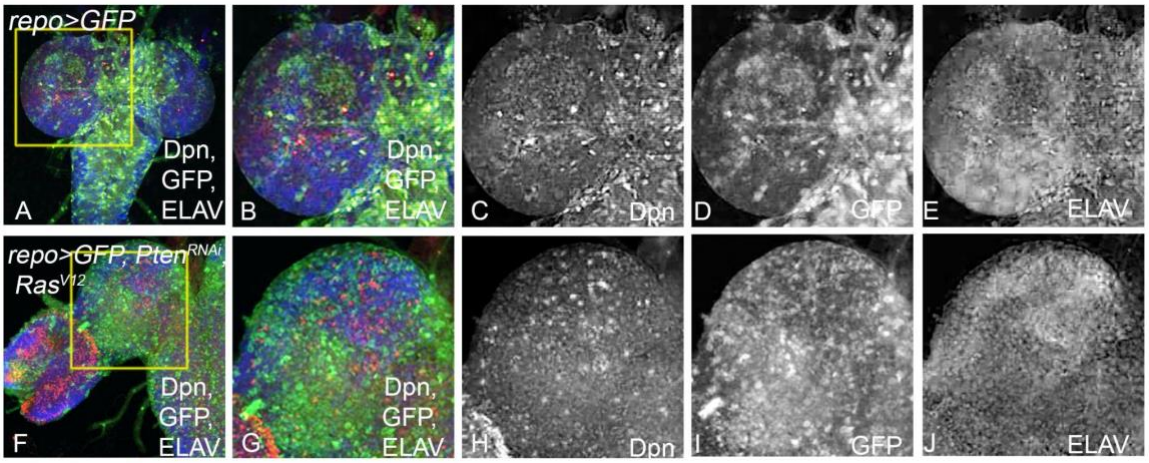

Figure 10: Expression of Deadpan in Control and Experimental Glioma Larvae
Images in 20X and 40X confocal magnification showing control and glioma brains stained for glia (green), Deadpan (red), and Elav (blue)

When looking at Figure 11 representing the N^{ICD} stains, it was noted that an increase in N^{ICD} positive cells will demonstrate the formation of new neuroblasts. The glial cells are shown by the expression of GFP under the glial cells specific driver *Repo>GFP*. The normal distribution of glial cells is shown in Figure 11A, B, D (green, gray panels). The number of neurons found in the wildtype brain is shown by the expression of ELAV in Figure 11A, B, E. In comparison, the expression of N^{ICD} is extremely increased in the glioma brains (*RepoGAL4-UASGFP; PTEN^{RNAi}; Ras^{V12}*). The number and distribution of the Deadpan positive stem cells were evaluated in Figure 11F-J. Higher magnification images show the increased number of N^{ICD} cells in Figure 11F, G, H (red, gray panels). The increased number of glial cells in the outer optic lobes is especially noticeable in Figure 11F, G, I (GFP, green, gray panels). This increase represents the overgrowth of glial cells, resulting in the formation of large glioma tumors.

The number of neuroblasts is tracked using ELAV in Figure 11F, G, J. The density of

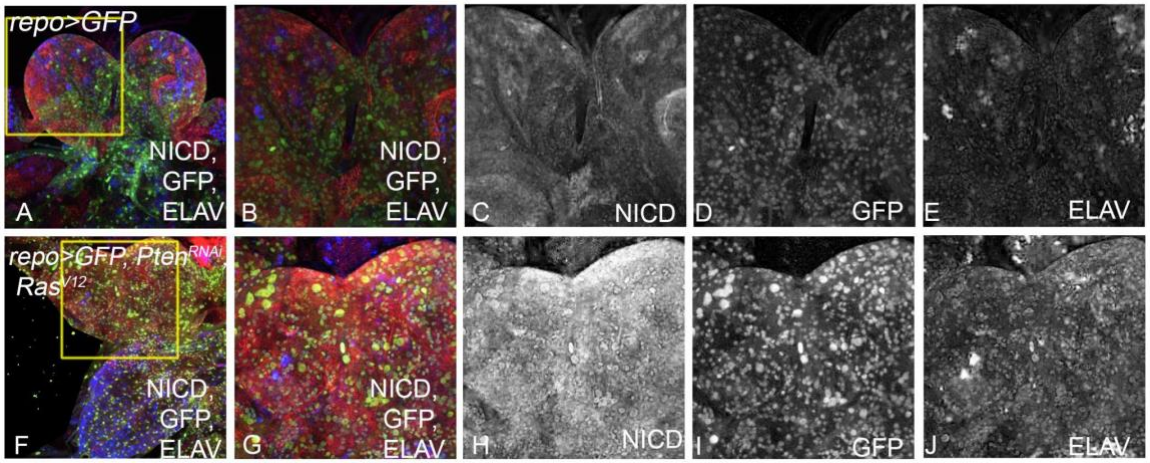

Figure 11: Expression of N^{ICD} in Control and Experimental Glioma Larvae
Images in 20X and 40X confocal magnification showing control and glioma brains stained for glia (green), N^{ICD} (red), and Elav (blue)

ELAV-positive cells in the wildtype brain is much lower than in the glioma induced brain as shown through Figure 11E and 11J. For each sample, a minimum of 8 discs were imaged. In the glioma larvae, a dramatic increase in N^{ICD} positive cells was noted.

Discussion

Overall, from these studies we observed that all markers for NB and GMCs were highly expressed and the number of neuroblasts observed in the glioma samples was higher than that in the wild-type control samples (Figures 8-11). The increase in the Prospero and Miranda positive cells indicates that during glioma growth, the stem cell precursor and the interneurons are also undergoing increased proliferation that contributes to the increased glial cell population that forms the bulk of the glioma. While increase in NBs and GMCs can be explained by the need to add new cells to the tumor mass, the increase in these precursor cells suggests that during gliomagenesis the NBs and GMCs undergo rapid and persistent rounds of cell division and spread from the central brain towards the optic regions of the dorsal lobe. Preliminary data with Deadpan

antibody shows that there are numerous Deadpan positive cells throughout the GFP positive regions (which represent the glioma tumors) suggesting that ectopic Deadpan expression is seen. This suggests that new NBs are being induced and Deadpan expression ensures their self-renewal and maintenance. We will need to evaluate this observation closely and quantify the number of Deadpan positive cells per microscopic field of view at 40X from 8 samples from the experimental group and controls to account for any variations in the expression pattern and the range of glioma phenotypes observed. This pilot data shows promising results suggesting new NB induction which will account for the large increase in both glia and neurons in the glioma samples. Similarly, preliminary analyses with our observations with N^{ICD}, showed a robust increase in the levels of N^{ICD} expression in the glioma samples. Since Notch plays a critical role in neurogenesis, we looked closely at the distribution of N^{ICD} (Figure 11). In wild-type, N^{ICD} expression is seen in the central brain regions in clusters that coincide with neuroepithelium, and no overlap is observed with the glia or neurons. However, in the glioma samples, the levels of N^{ICD} expression are high and spread all over the central brain (no longer restricted to the neuroepithelium). Further, the size of nuclei in the GFP channel which represent glia and in the ELAV channel (which mark the neurons) is different. ELAV positive nuclei are small suggesting accelerated cell division cycles before the cells can duplicate all nuclear and cytoplasmic materials. This pilot data is interesting, and these observations need to be further explored to confirm the cells in which N^{ICD} expression is induced and check the effects on both neurons and glia populations by additional experiments.

Conclusions

Taken together, our experimental evidence suggests an increased number of NBs in the glioma. The presence of these NBs suggests that new NBs may be induced from the glioma cells. Recent studies in mammalian models have shown that gliomas can undergo a switch from progenitor to stem-like cells. This is exciting as translated to the *Drosophila* model, this suggests that GMCs can switch back (and divide asymmetrically) to form NBs which could be a possible mechanism by which NBs are spread from the central brain towards the outer regions of the dorsal lobe. How cells undergo this reversal or switch remains unclear and is an area of future studies. In addition, our promising data from Deadpan and N^{ICD} experiments also shows alterations in the glioma, however, the extent to which N activation causes neurogenesis and establishes NBs remains an area of further experimentation. In the future, it will be interesting to test if the spread of the NBs in our *Drosophila* glioma model is affected by reduction in Deadpan or N^{ICD}, and if we can identify a mechanism for GMC to NB transition in the glioma cells.

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