DROSOPHILA GLIOBLASTOMA MODEL TO STUDY SIGNALING PATHWAYS

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DROSOPHILA GLIOBLASTOMA MODEL TO STUDY SIGNALING PATHWAYS

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ABSTRACT

DROSOPHILA GLIOBLASTOMA MODEL TO STUDY SIGNALING PATHWAYS

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Objective: Glioblastoma (GBM) is a highly aggressive and malignant brain tumor that has limited treatment options and has an extremely poor prognosis (Waghmare et al. 2014). The amplification of Epidermal Growth Factor Receptor-VIII (EGFR-VIII) and activation of the Phosphatidyl Inositol 3-Kinase (PI3K) pathway are common genetic alterations observed in GBM patients (An et al. 2018). Our objective is to model GBM in *Drosophila melanogaster* and study the signaling pathways that promote GBM growth and inhibit cell death. Specifically, we aim to investigate the roles of MAPK, Hippo, and WNT signaling pathways in regulating GBM growth and Cactus expression, which regulates the JNK pathway.

Methods: Our project involves genetic crosses that produce larvae with GBM, followed by brain dissections and immunohistochemistry to study changes in signaling pathways that promote GBM growth. Specifically, we are studying the early time points to understand the roles of signaling pathways like MAPK, Hippo, and WNT in promoting GBM growth and/or inhibiting cell death. By comparing our GBM models to experimental controls, we aim to generate initial data for designing further genetic experiments to identify specific signaling interactions that affect cell death and proliferation. We will use two lines, (1) y w UAS PI3K92E; +; Repo-Gal4

UAS GFP/TM3B,Sb, and (2) y w; UAS EGFRλtop/TM6C, to generate glioma in *Drosophila*, and investigate whether the Hippo pathway regulates Cactus, which also regulates the JNK pathway.

Significance: The proposed research has significant implications for understanding the

molecular mechanisms underlying GBM growth and identifying key molecules and pathways that drive this deadly disease. Using *Drosophila* as a model system allows for efficient genetic manipulation and provides a cost-effective way to study complex biological processes. Additionally, the results of this study will contribute to our understanding of GBM.

Dedicated to my Family

ACKNOWLEDGMENTS

I extend my deepest gratitude to my mentor, Dr. Kango-Singh, for her unwavering support and guidance in shaping this thesis. I am thankful to Dr. Tom Williams and Dr. Amit Singh for their valuable insights as committee members. Special appreciation goes to the dedicated undergraduate and graduate students who contributed to this research. Their collaborative efforts were crucial to the project's success. I am also grateful for the support of my family and friends. This work wouldn't have been possible without the financial assistance from the University of Dayton. Thank you all for being an integral part of my academic journey.

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LIST OF ABBREVIATIONS AND NOTATIONS

Akt	Protein kinase B
EGFR	Epidermal growth factor receptor
GBM	Glioblastoma multiforme
MAPK	Mitogen-activated protein kinase
mTOR	Mechanistic target of rapamycin kinase
P53	Tumor protein p53
PI3K	Phosphatidylinositol 3-kinase
Pten	Phosphatase and tensin homolog

CHAPTER 1

INTRODUCTION AND PROBLEM STATEMENT

Introduction

Glioblastoma multiforme (GBM) presents a formidable challenge within the realm of oncology, characterized by its aggressive nature and limited therapeutic options (Sahoo et al., 2024). As per the data from the American Society of Neurological Surgeons, GBM is the most common type of CNS cancer that accounts for 47% of all cases (https://www.aans.org/). GBM has an incidence rate of 3.2 per 100,000 population, and survival is poor. For example, in the first year after diagnosis about 40% patients survive however, two years after diagnosis the survival reduces to 17%. In general, GBM occurs more frequently in males than in females, and media age of occurrence is 64years (Tamimi et al., 2017).

The symptoms of GBM may include severe headache, vomiting, blurry vision, seizures (new onset), inability to think or learn, speech difficulty and changes in mood and personality. Imaging techniques like Computed Tomography (CT) or Magnetic Resonance Imaging (MRI) are used to detect the location of the tumor and also to guide the surgeon for tumor removal and/or biopsies. Current standard of care for patients follows the Stupp protocol, where patients with GBM undergo surgery followed by radiation and chemotherapy with Temozolomide, followed by several rounds of adjuvant therapy(Stupp et al., 2005). However, these therapies often extend patient survival by a very short time.

Genetic and genomic alterations found in GBM patients:

Analyses of GBM tumors has led to the identification of alterations in a variety of genetic pathways that are involved in DNA damage repair, apoptosis, cell migration, angiogenesis and the cell cycle. Most tumors show mutations due to Loss of Heterozygosity (LOH), amplification of genes, deletions or point mutations. Activating mutations in PI3K pathway (constitutive activation of PI3K, or AKT or loss of function of pathway inhibitors like PTEN) and MAPK pathway (e.g., gene amplification of the EGFR-VIII) or dominant negative mutations in P53 are frequently associated with GBM (see, Table 1)(Haque et al., 2011).

Table 1 Gene alterations in GBM

Gene alterations	Frequency of mutations (%)
LOH 10q	50-70
EGFR amplification	40-60
P16Ink4a deletion	30
TP53 mutation	50-60
PTEN mutation	60
MDM2 polymorphism	40-60
MGMT Hypermethylation	50-60
IDH1 mutation	40-50
CDK4 amplification	20-30
PDGFRA amplification	20-30

Primary *de novo* GBM accounts for more than 80% of GBM, occurs in older patients (mean age = 64 years), and typically shows epidermal growth factor receptor (EGFR) over expression, PTN (MMC I) mutation, CDKN2A (p16) deletion, and less frequently MDM2 amplification(Ohgaki et al., 2004; Stupp et al., 2005).

Secondary GBM develops from lower grade astrocytoma (or oligodendrogliomas), occurs in younger patients (mean age = 45 years), and often contains TP53 mutations as the earliest detectable alteration. Mutations in isocitrate dehydrogenase-1 (IDH1) and IDH2 are present in 70–80% of low-grade glioma and secondary GBM, and in only 5–10% of primary GBM ((Appin et al., 2013; Hartmann et al., 2010; Yan et al., 2009).

Other studies with transcriptomics (bulk RNA expression profiling or scRNA seq) combined with FISH to perform spatial profiling severe tumor heterogeneity (Shireman et al., 2023). These studies also revealed the many complex cell types present in the GBM, its microenvironment as in the normal brain and contributed to the tumor promoting inter cellular interactions. These studies have provided insights about the regional transcriptional programs of GBM, mapped the microenvironment with respect gene expression and cellular states/plasticity in GBM (Shireman et al., 2023)

However, a key challenge in GBM is tumor recurrence, which is thought to cause more aggressive tumor growth that is refractory/ non-responsive to treatment strategies (Birzu et al., 2020). Thus, better understanding of the basic cancer biology will help improve our understanding of the changes that cause GBM, and lead to identification of possible targets for treatment are needed.

Signaling pathways linked to GBM

Molecular analysis of GBM from patient biopsies and model system research showed that multiple signaling pathways are altered or upregulated in GBM. Specifically, Wnt, Transforming growth factor -beta (TGFb), VEGF, Epidermal Growth Factor Receptor (EGFR), cyclin-dependent kinase 2A (CDKN2A), nuclear factor-κB (NF-κB), phosphatidylinositol-3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) are implicated in initiation, progression or aggressive behavior of GBM (Khabibov et al., 2022). Interestingly, several of these pathways are involved in development are coopted by cancer cells to modify their behaviors.

Central to the understanding of GBM pathogenesis are the intricate signaling pathways, among which the PI3K and EGFR pathways stand out prominently (Khabibov et al., 2022). The PI3K pathway, through its involvement in regulating cellular processes such as survival, proliferation, and metabolism, plays a crucial role in GBM progression. Similarly, the EGFR pathway, known for its role in promoting cell growth and differentiation, is frequently dysregulated in GBM, contributing to tumor proliferation and resistance to apoptosis. Despite significant strides in elucidating the individual roles of these pathways, the complexity of GBM pathobiology demands a deeper comprehension of their interactions with other molecular cascades.

Inhibitors of PI3K EGFR were not efficacious in inhibiting GBM, nor are several tyrosine kinase inhibitors (Khabibov et al., 2022, Brar et al., 2022). Thus, new targets need to be identified downstream of the PI3K EGFR pathways that

specifically drive GBM growth. The crosstalk between the PI3K and EGFR pathways, as well as their interplay with other signaling networks such as the MAPK and mTOR pathways, remains a focal point of investigation. Understanding these interactions is essential not only for unraveling the underlying mechanisms driving GBM progression but also for devising more effective therapeutic strategies. Moreover, the emergence of therapy resistance poses a significant obstacle in GBM treatment, underscoring the urgent need for innovative approaches.

Model systems in GBM research

The use of genetic models in glioma research offers a powerful tool for dissecting the genetic basis of tumor initiation, progression, and therapy resistance. By introducing specific genetic alterations that mimic those found in human gliomas, researchers can recapitulate key aspects of the disease in experimental settings. These models enable the study of gene-gene interactions, as well as the identification of genetic modifiers that influence tumor behavior. Furthermore, genetic models provide a platform for testing targeted therapies and investigating mechanisms of drug response and resistance, ultimately facilitating the development of more effective treatment strategies for glioma patients.

Whole animal models represent an invaluable resource for studying glioma biology in the context of a living organism. By implanting glioma cells or tumor fragments (xenografts) into the brains of animals such as mice or rats, researchers can assess tumor growth, invasion, and response to therapy in a physiologically

relevant environment(Tentler et al., 2012). These models allow for the evaluation of complex interactions between tumor cells and the surrounding microenvironment, including immune cells, blood vessels, and stromal elements. Additionally, whole animal models enable longitudinal studies over time, providing insights into the dynamic nature of glioma progression and therapeutic responses that cannot be captured in cell culture or isolated tissue models.

Given the complexity of GBM, and the urgent need to explore the molecular networks that drive GBM growth, several preclinical *in-vitro* and *in-vivo* model systems have been developed (Paolillo et al., 2021, Rybin et al., 2021, Gómez-Oliva et al., 2021). In addition to the conventional xenograft based assays, dissociation of tumor cells to was refined to develop 3-D neurospheres/organoid models alongside genetic models like mouse models of GBM (Miyai et al., 2017), other animal models were developed in dog, zebrafish and fruitflies (Schuhmacher and Squatrito, 2017). In our lab we use the fruit fly (*Drosophila melanogaster*) to study the role of signaling pathways in glioma growth.

Model systems and Drug/inhibitor screens

High-throughput drug or inhibitor screens represent a powerful approach for identifying novel compounds with therapeutic potential against glioma (Bialkowska and Yang, 2012). By screening large libraries of small molecules or biologics in glioma cell lines or animal models, researchers can identify compounds that selectively target key pathways involved in tumor growth and survival. These screens may uncover novel drug candidates, repurpose existing drugs for glioma treatment, or reveal synergistic drug combinations that enhance therapeutic

efficacy (Munnik et al., 2022). Moreover, drug screens can provide valuable insights into the molecular mechanisms underlying glioma biology and therapy resistance, guiding the development of more rational and personalized treatment strategies for patients with this devastating disease (Munnik et al., 2022).

Drosophila as a preclinical cancer model

With its vast repository of genetic tools *Drosophila* is a popular model system for generating cancer models. For example, cancers affecting the blood cells, muscle, germ cells, intestinal system and the brain are modeled in flies (Waghmare et al., 2014, Snigdha et al., 2019, Mirzoyan et al., 2019).

Drosophila as a preclinical glioma model

Utilizing *Drosophila melanogaster* as a preclinical model for studying glioma provides researchers with a unique platform to investigate the intricate mechanisms underlying this complex disease (Read et al., 2009, Teresa Witte et al., 2009). *Drosophila*'s genetic tractability allows for precise manipulation and analysis of specific genes and pathways involved in glioma progression (Snigdha et al., 2019, Waghmare et al., 2014). Additionally, its relatively short generation time enables rapid experimentation and screening of potential therapeutic targets (Munnik et al., 2022). By leveraging the evolutionary conservation of key signaling pathways, such as PI3K, EGFR, MAPK, and mTOR, researchers can gain valuable insights into the fundamental processes driving glioma development and identify novel strategies for intervention.

Drosophila models allow the study of early changes in a genetically tractable system.

The use of genetically tractable systems, such as *Drosophila melanogaster*, allows for the study of early changes in glioma development and progression that are otherwise challenging to investigate in mammalian models or human patients. By manipulating specific genes or pathways implicated in gliomagenesis, we can induce tumor formation and track the subsequent molecular and cellular changes that occur during early stages of tumorigenesis. These studies provide insights into the initial events that drive glioma initiation, as well as the molecular mechanisms underlying tumor growth, invasion, and metastasis. Furthermore, genetically tractable systems offer a platform for testing experimental interventions aimed at intercepting or reversing early-stage glioma progression, with the potential to inform the development of preventive or therapeutic strategies for individuals at high risk of developing this deadly disease. Thus, by harnessing the power of *Drosophila* genetics, specific molecular pathways implicated in GBM can be manipulated, facilitating the dissection of complex signaling networks and the identification of key regulators.

The proposed research aims to leverage the *Drosophila* model to delve deeper into the molecular mechanisms driving GBM progression and therapy resistance. Specifically, we seek to delineate the intricate signaling networks involving PI3K, EGFR, and other pathways in GBM pathogenesis. Through genetic interventions, we aim to elucidate how these pathways interact to promote tumor growth and evade therapeutic interventions. Furthermore, by identifying novel

molecular targets and testing potential therapeutic agents in the *Drosophila* model, we aim to accelerate the translation of basic research findings into clinically relevant strategies for potential GBM treatment.

This proposed endeavor builds upon the groundwork laid by prior researchers who have harnessed *Drosophila* as a model system to explore diverse biological processes, including cancer. By scrutinizing the signaling pathways fostering GBM growth and impeding cell death, particularly the roles of MAPK, Hippo, and WNT pathways, the study aims to enrich the current state of knowledge(Cheng et al., 2016; Minata et al., 2019). Focusing on early time points, the project endeavors to unravel the intricate signaling interplays unfolding during GBM initiation and progression.

The significance of this proposed research is underscored by its potential to advance our understanding of the biological changes that promote GBM. Through the identification of specific signaling pathways and interactions propelling tumor growth while stifling cell death, the study could unveil fresh targets that can be tested for therapeutic value. Furthermore, in the future, the utilization of the *Drosophila* model system may offer a cost-effective and efficient means of screening potential drug candidates for GBM treatment.

In the following sections, I briefly summarize the signaling pathways under study for my research.

(A) MAPK pathway:

The Mitogen-Activated Protein Kinase (MAPK) pathway represents one of the most intricate and versatile signaling cascades in cellular biology, orchestrating a myriad of essential processes fundamental to cell behavior, tissue development, and organismal homeostasis (Braicu et al., 2019). Within the intricate landscape of *Drosophila melanogaster*, this pathway intricately weaves through a series of molecular events, comprising a cascade of protein kinases including Raf, MEK (MAPK/ERK kinase), and ERK (extracellular signal-regulated kinase) (Shilo, 2014). The activation of the MAPK pathway is a meticulously choreographed response triggered by a plethora of extracellular signals, ranging from growth factors to environmental stresses. This activation cascade initiates with Raf phosphorylating and activating MEK, which subsequently phosphorylates and activates ERK. Once activated, ERK translocates into the nucleus where it exerts its regulatory influence on a plethora of transcription factors and other targets, thus dictating the expression of genes pivotal for cell proliferation, differentiation, and survival (Braicu et al., 2019). The MAPK pathway, being conserved across species, is indispensable for a myriad of biological phenomena ranging from embryonic development to immune responses and has been intricately linked to the pathogenesis of numerous diseases, including cancer.

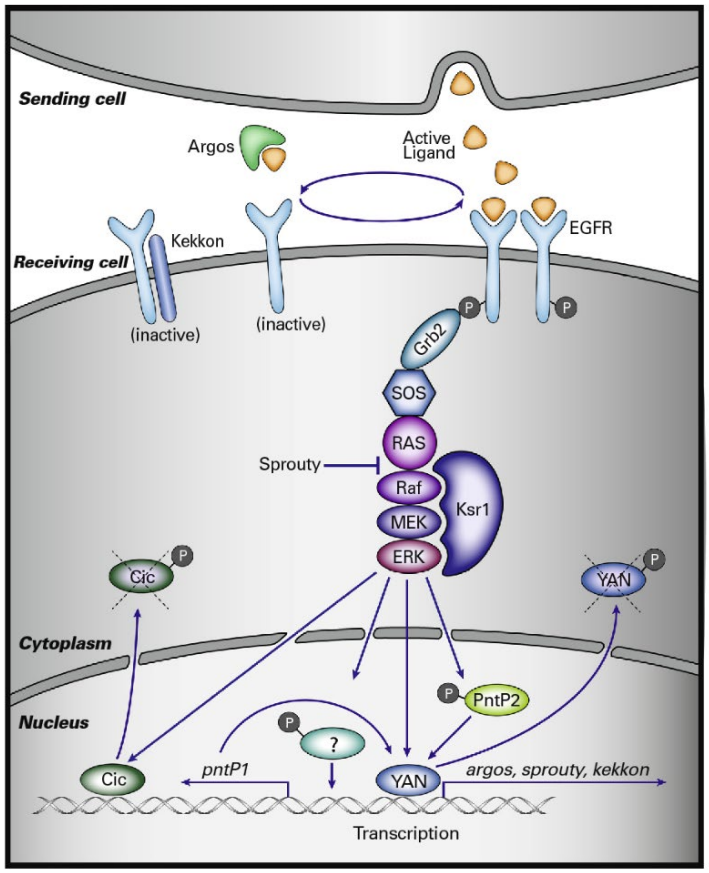

Figure 1 The *Drosophila* MAPK pathway

The MAPK pathway in *Drosophila*, composed of Raf, MEK, and ERK, responds to various extracellular signals, directing crucial cellular processes like proliferation and differentiation. Activation involves sequential phosphorylation, culminating in ERK's nuclear translocation to regulate gene expression. Its conservation across species underscores its significance in development and disease, particularly cancer.

(B) Hippo Growth Regulatory Pathway:

The Hippo Pathway emerges as a pivotal signaling cascade governing cell growth, proliferation, and apoptosis (Kango-Singh and Singh, 2009)(Meng et al., 2016). Within this project, the Hippo pathway assumes particular significance owing to its implication in the regulation of Cactus, which, in turn, modulates the JNK pathway (Snigdha et al., 2021). The JNK pathway, a stress-responsive signaling cascade, also influences cell death and proliferation (La Marca and Richardson, 2020). By probing the Hippo pathway within the context of GBM in *Drosophila*, the study endeavors to deepen our understanding of the molecular mechanisms underpinning the onset and progression of this disease. Specifically, the investigation aims to elucidate the Hippo pathway's role in fostering GBM growth and hindering cell death, alongside exploring its potential interactions with other signaling pathways, such as MAPK and WNT.

The Hippo growth regulatory pathway serves as a fundamental mechanism governing tissue growth and organ size in multicellular organisms, including *Drosophila melanogaster* (Kango-Singh and Singh, 2009)(Meng et al., 2016). This signaling cascade orchestrates intricate cellular processes such as proliferation, apoptosis, and differentiation, thereby maintaining tissue homeostasis. Understanding the Hippo pathway is paramount in the context of investigating Glioblastoma (GBM) using *Drosophila* models, as its dysregulation has been implicated in various cancers, including GBM (Bhat et al., 2011).

At its core, the Hippo pathway comprises a series of kinases, scaffold proteins, and transcriptional coactivators that function in a tightly regulated manner

(Irvine and Harvey, 2015). In *Drosophila*, the key components include the protein kinases Hippo (Hpo) and Warts (Wts), along with the scaffold proteins Salvador (Sav) and Mob-as-tumor-suppressor (Mats) (Justice et al., 1995; Kango-Singh et al., 2002; Lai et al., 2005; Pantalacci et al., 2003; Tapon et al., 2002; Udan et al., 2003; Xu et al., 1995). Activation of the Hippo pathway typically occurs in response to upstream signals, such as cell-cell contact, mechanical cues, or tissue damage. Upon pathway activation, the kinase Hpo phosphorylates and activates Wts, which subsequently phosphorylates the transcriptional coactivator Yorkie (Yki). Phosphorylated Yki is sequestered in the cytoplasm, preventing its translocation into the nucleus and subsequent activation of target genes involved in cell proliferation and survival. Thus, the Hippo pathway acts as a potent suppressor of cell proliferation by inhibiting the transcriptional activity of Yki.

In the context of GBM research using *Drosophila* models, the Hippo pathway assumes particular significance due to its potential role in regulating glioma growth and progression. Previous studies have suggested that the Hippo pathway may interact with other signaling pathways such as the MAPK and JNK pathways (Doggett et al., 2011, La Marca and Richardson, 2020). Specifically, the Hippo pathway may regulate the expression of the *Drosophila* IkBa ortholog, Cactus, which in turn modulates the JNK pathway (Liu et al., 2016, Snigdha et al., 2021). Studying the Hippo pathway in the context of GBM may provide insights into tumor promoting mechanisms and possible therapeutic targets, thereby offering new avenues for the treatment of this devastating disease.

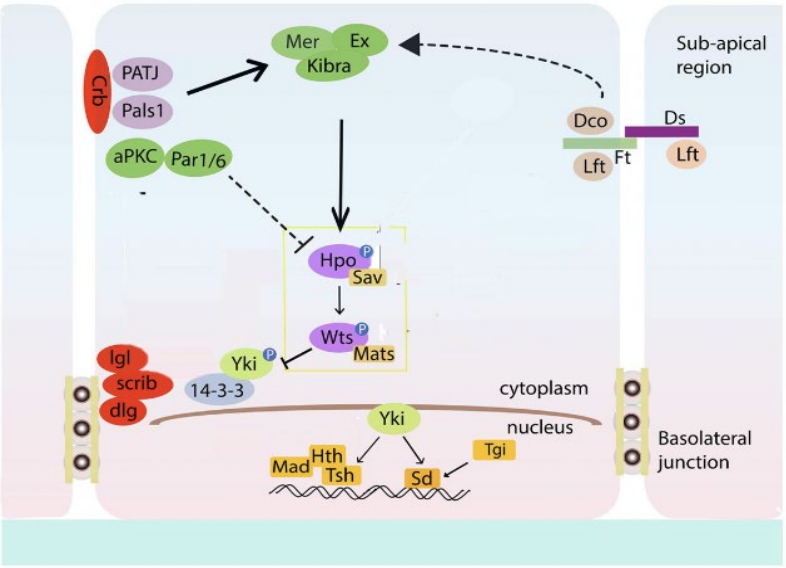

Figure 2 **The Hippo pathway in** ***Drosophila***

The upstream components (Ex, Mer. Kibra) and receptor-ligands (Ft-Ds) transmit a signal to the kinase cascade (Hippo and Warts) which restrict the transcriptional coactivator Yki by phosphorylation. P-Yki is degraded via the proteasomal system. However, loss of Hippo signaling relieves Yki of Wts mediated inhibition allowing it to enter the nucleus and associate with transcription factors like Scalloped (Sd) or Mad, Hth, or Tsh. Once Yki is bound to cognate TFs, it initiates transcription of pathway specific genes.

(C) JNK pathway: The c-Jun N-terminal Kinase (JNK) pathway, akin to a vigilant sentinel, stands as a sentinel against an onslaught of stressors, meticulously choreographing cellular responses to an array of environmental insults ranging from oxidative stress to ultraviolet radiation and inflammatory cytokines. In the intricate tapestry of *Drosophila* biology, the JNK pathway unfurls through a cascade of protein kinases including JNKKK (JNK kinase kinase), JNKK (JNK kinase), and JNK itself. Activation of this pathway embarks on a phosphorylation cascade initiated by

JNKKK, followed by phosphorylation and activation of JNKK, ultimately culminating in the activation of JNK. Activated JNK binds to the AP-1 family transcription factors (Jra and Fos in *Drosophila*), to induce expression of target genes involved in autoregulation of the JNK pathway (the dual-specificity phosphatase Puckered) or invasion (MMP1), and other stress response genes.

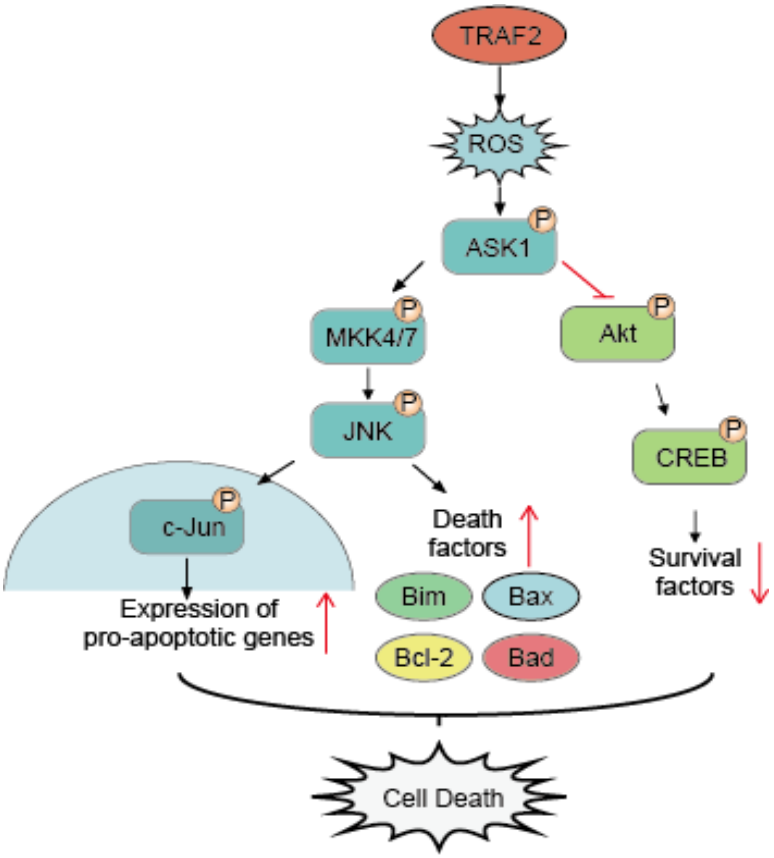

Figure 3 The JNK pathway

JNK pathway is a stress response pathway with both pro-survival and pro-apoptosis outputs.

(D) Wingless pathway: The Wingless (Wg) pathway, akin to a master orchestrator, conducts a symphony of cellular responses crucial for tissue morphogenesis, cell fate determination, and tissue polarity during development (Swarup and Verheyen, 2012). In the intricate milieu of *Drosophila* developmental biology, the Wg pathway unfolds through a series of intricate molecular events initiated by the binding of the Wg ligand to its receptor complex, consisting of Frizzled and the Arrow co-receptor. This binding event triggers a cascade of intracellular signaling events culminating in the disassociation of the destruction complex, comprising Axin, APC (Adenomatous Polyposis Coli), and GSK3β (Glycogen Synthase Kinase 3β). Consequently, β-catenin (also known as Armadillo in *Drosophila*) is released from this complex, accumulates in the cytoplasm and translocate into the nucleus, where it collaborates with TCF/LEF (T-cell factor/lymphoid enhancer factor) transcription factors to modulate the expression of genes critical for various cellular processes. The Wg pathway, a pivotal player in the developmental repertoire, is indispensable for embryonic development, tissue regeneration, and adult tissue homeostasis.

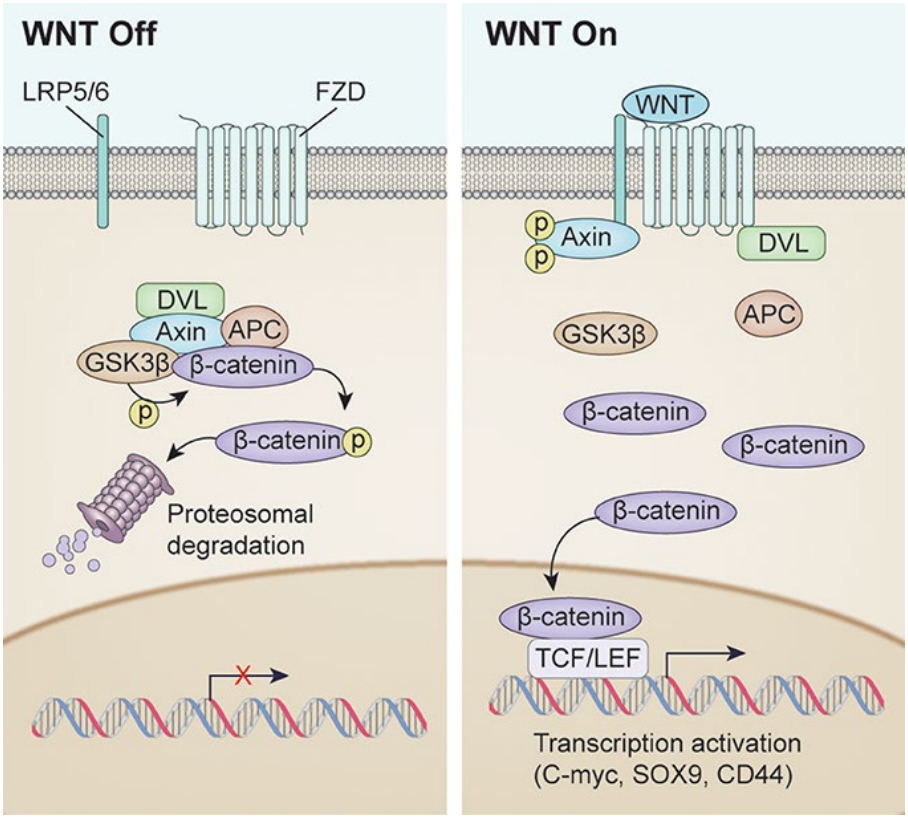

Figure 4 The Wnt/Wingless pathway in flies and mammals

In the intricate milieu of *Drosophila* developmental biology, the Wg pathway unfolds through a series of intricate molecular events initiated by the binding of the Wg ligand to its receptor complex, consisting of Frizzled and the Arrow co-receptor. This binding event triggers a cascade of intracellular signaling events culminating in the disassociation of the destruction complex, comprising Axin, APC (Adenomatous Polyposis Coli), and GSK3β (Glycogen Synthase Kinase 3β).

Hypothesis and objectives (goals) of this research:

The hypothesis of this study is that the Hippo and JNK pathways play critical roles in regulating the growth and survival of glioma cells in *Drosophila melanogaster*. Specifically, we hypothesize that modulation signaling levels of these pathways will impact the proliferation and survival of glioma cells, ultimately influencing tumor growth. Through genetic manipulation and antibody staining techniques, we aim to investigate the crosstalk between the Hippo and JNK pathways in glioma progression and determine how their interaction influences tumor behavior. Additionally, we seek to identify specific molecular targets within these pathways that could serve as potential targets for GBM treatment.

The objectives of this study are twofold: First, we aim to elucidate the molecular mechanisms underlying glioma growth by investigating the interplay between the Hippo and JNK pathways in *Drosophila* models of GBM. By comparing two genetically distinct glioma models and assessing changes in gene expression and pathway activity, we aim to identify key regulators of tumor growth and survival. Second, we aim to evaluate the therapeutic potential of targeting the Hippo and JNK pathways in glioma cells. Through genetic manipulation of pathway activity and assessment of cell proliferation and survival, we aim to determine the impact of pathway modulation on tumor behavior. Overall, our study seeks to advance our understanding of GBM pathogenesis.

CHAPTER 2

MATERIALS AND METHODS

Experimental Approach:

The experimental approach encompasses the generation of *Drosophila* models replicating glioblastoma (GBM) through genetic manipulation, followed by extensive immunohistochemical analysis to probe changes in signaling pathways associated with GBM growth and cell death inhibition.

Generation of *Drosophila* Models:

Initially, specific fly mutants and transgenic lines listed in FlyBase will be meticulously chosen to establish desired genotypes. This involves strategic genetic crosses combining mutant or transgenic lines carrying relevant genetic alterations, such as the *UAS-PI3K92E*^{CA} and *UAS-EGFR*$^{\lambda Top}$ mutations, with driver lines like *RepoGAL4* that specifically drive gene expression in the glial cells in the *Drosophila* CNS. *UASGFP* is coexpressed to help visualize the amount of glia in the brain.

To generate larvae bearing glioma, flies of the appropriate genotypes were crossed, and the cross was maintained in a fly incubator at 25ºC. These crosses will yield progeny (F_1) harboring the desired genotypes necessary for modeling GBM in *Drosophila*.

Overall, we compared two glioma models:

Model 1 in which we downregulated Pten and activated the Ras oncogene in the glial cells to coactivate the MAPK and PI3K pathways. The genotype of this model is:

ywhsFLP/UAS Pten^{RNAi}; Sp/ UAS Ras^{V12}; repoGAL4 UAS GFP/+

Model 2 in which we co-expressed constitutely active PI3K and EGFR in the glial cells to coactivate the PI3K and PI3K MAPK pathways. The genotype of this model is:

ywhsFLP/UAS PI3K92E^{CA}; Sp/ +; repoGAL4 UAS GFP/UAS EGFR^{lTop}

Table 2 List fly lines used in this study:

Genotype	MKS lab stock number	Comments
yw hsFLP; Sp/CyO; repo GAL4 UASGFP/TM6B	REPO	Serves as control
UASPI3K92E^{CA}; Sp/CyO; RepoGAL4 UAS GFP/TM6B	MKS 1772 MKS 1772 x MKS 1633	(a) Serves as a control for PI3K alone (b) Used to generate glioma by crossing to *w; +; UAS EGFR$^{\lambda Top}$*
w; +; UAS EGFR$^{\lambda Top}$	MKS 1633	Used *for EGFR$^{\lambda Top}$* controls*:* Crossed to *yw hsFLP; Sp/CyO; repo GAL4 UASGFP/TM6B*

Dissection and Immunohistochemistry:

Following the establishment of desired genotypes, larvae expressing the GFP marker will be carefully selected for subsequent experiments. Larval central nervous systems (CNS), particularly brain tissues, will be meticulously dissected from GFP-positive larvae to ensure the isolation of targeted tissues. These dissected tissues will then be fixed in 4% Paraformaldehyde to maintain their structural integrity and preserve molecular components. Following fixation, the tissues will be washed in 1XPBST (PBS containing 2% Triton X-100), and blocked using 1% normal goat serum.

Immunohistochemical Staining:

Immunohistochemistry will be employed to scrutinize changes in signaling pathways associated with GBM growth and cell death inhibition within the dissected tissues. Primary antibodies targeting specific signaling molecules or pathway markers will be added to the tissue sample following blocking and the sample will be incubated overnight at 4°C. These primary antibodies will include: mouse anti-DIAP1(from DSHB, dilution1:250), mouse anti-MMP1 (from DSHB, dilution 1:200), mouse anti-pJNK (from Cell Signaling, dilution 1:250) and mouse anti-Wg (from DSHB, dilution 1:200) , representing key components of the Hippo, JNK and Wg pathways, respectively.

Following primary antibody incubation, the tissues will undergo a series of washes with 1XPBST to remove excess antibody solution. Subsequently, the tissues will be incubated for 2h with secondary antibodies (from Jackson

Immunoresearch Inc.) conjugated with fluorescent dyes such as Cy-3 or Cy-5. These secondary antibodies will bind to the primary antibodies, enabling the visualization of targeted molecules or pathway markers. Following this step, the samples will be washed in PBST to remove unbound and excess secondary antibody and mounted in Vectashield (from Vector Labs).

Confocal Imaging and Analysis:

Confocal imaging will be performed on immunohistochemically stained tissue samples using the Olympus Fluoview 3000 Laser Scanning Confocal Microscope with high-resolution capabilities. This imaging technique allows for three-dimensional visualization of fluorescently labeled structures within the tissue samples, capturing detailed spatial distribution and intensity of fluorescence corresponding to the targeted signaling molecules or pathway markers. The acquired confocal images will undergo comprehensive analysis to quantify fluorescence levels corresponding to targeted signaling molecules or pathway markers. Z-projections of the optical sections will be used to generate images for X-Y and other planes. We will then extract relevant quantitative data, including signal intensity and distribution patterns. Statistical analyses will then be applied to compare fluorescence levels between experimental and control groups, facilitating the identification of significant changes associated with GBM growth and cell death inhibition. Overall, the comprehensive immunohistochemical analysis coupled with confocal imaging and thorough data analysis will provide

valuable insights into the roles of key signaling pathways, including Hippo, JNK, MAPK, and Wnt, in glioma growth and survival in *Drosophila* models of GBM.

CHAPTER 3

RESULTS

Fly glioma models (Model 1 and Model 2) form lethal invasive neoplasms

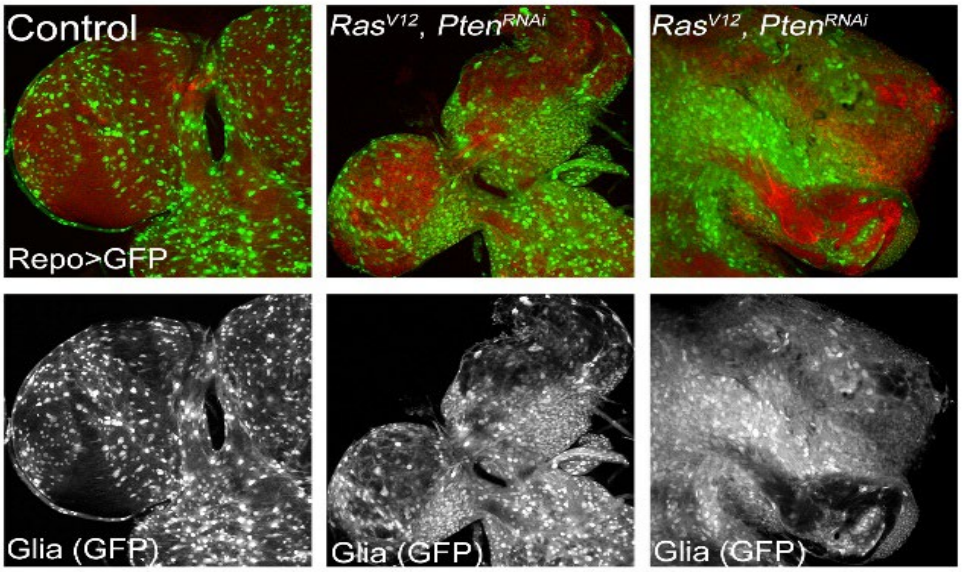

Figure 5 Glioma model show Aggressive growth.

Panels show comparison of (a) wild-type control brain (*repoGAL4>UAS GFP*) with (b) glioma brain (*repoGAL4> UAS GFP, UASPten^{RNAi}, UASRas^{V12}*). Note the size of the brain lobes, and the number of glial cells (GFP, green).

Panels show comparison of (a) wild-type control brain (*repoGAL4>UAS GFP*) with (b) glioma brain (*repoGAL4> UAS GFP, UASPten^{RNAi}, UASRas^{V12}*). Note the size of the brain lobes, and the number of glial cells (GFP, green).

The mature third instar larval brain shows several characteristics, for example, two well-developed dorsal lobes and a long ventral nerve cord. The dorsal lobes are the region of sensory input (as the optic nerve and several olfactory and other inputs) are integrated in these structures (Hartenstein et al., 2008). The outer

regions of the dorsal lobes form the optic center of the brain, and the remaining region form the central brain region. The VNC, on the other hand, is the center from which motor signals are disseminated.

Repo-GAL4 is expressed in the glia all through CNS development, and in the mature third instar stage the glia are distributed in characteristic pattern in the central brain, the VNC and the outer proliferation centers of the optic lobes (Fig. 4A, green). We first tested the effects of coactivation of the PI3K and MAPK pathways in the larval CNS and observed that the two glioma models resulted in the formation of large, lethal, and invasive neoplasms (Fig. 4, 5). The activation of these pathways is sufficient to recapitulate several characteristics of glioma. The *repoGAL4>UASGFP*, *UASPten^{RNAi}, UASRas^{V12}* glioma (model 1) phenotypes usually showed a dramatic increase in the number and distribution of glia in the central brain and the ventral nerve cord (VNC) of the larva (Fig. 4B). A higher magnification image of the dorsal lobe shows that the glial neoplasms form a growth front near the optic lobes causing these structures to often look elongated and distorted compared to the wild-type brain (Fig. 4C).

We then compared the *repoGAL4>UASGFP*, *UASPten^{RNAi}, UASRas^{V12}* glioma (model 1) with the *repoGAL4>UASGFP, UASPI3K^{92E}, UASEGFR$^{\lambda Top}$* (Model 2) (Fig. 5). In the wild type, the *repo>GFP* expresses GFP in all the glial cells in the larval brain. These serve as control for normal size, glial cell numbers and overall development of the brain (Fig, 5 A).

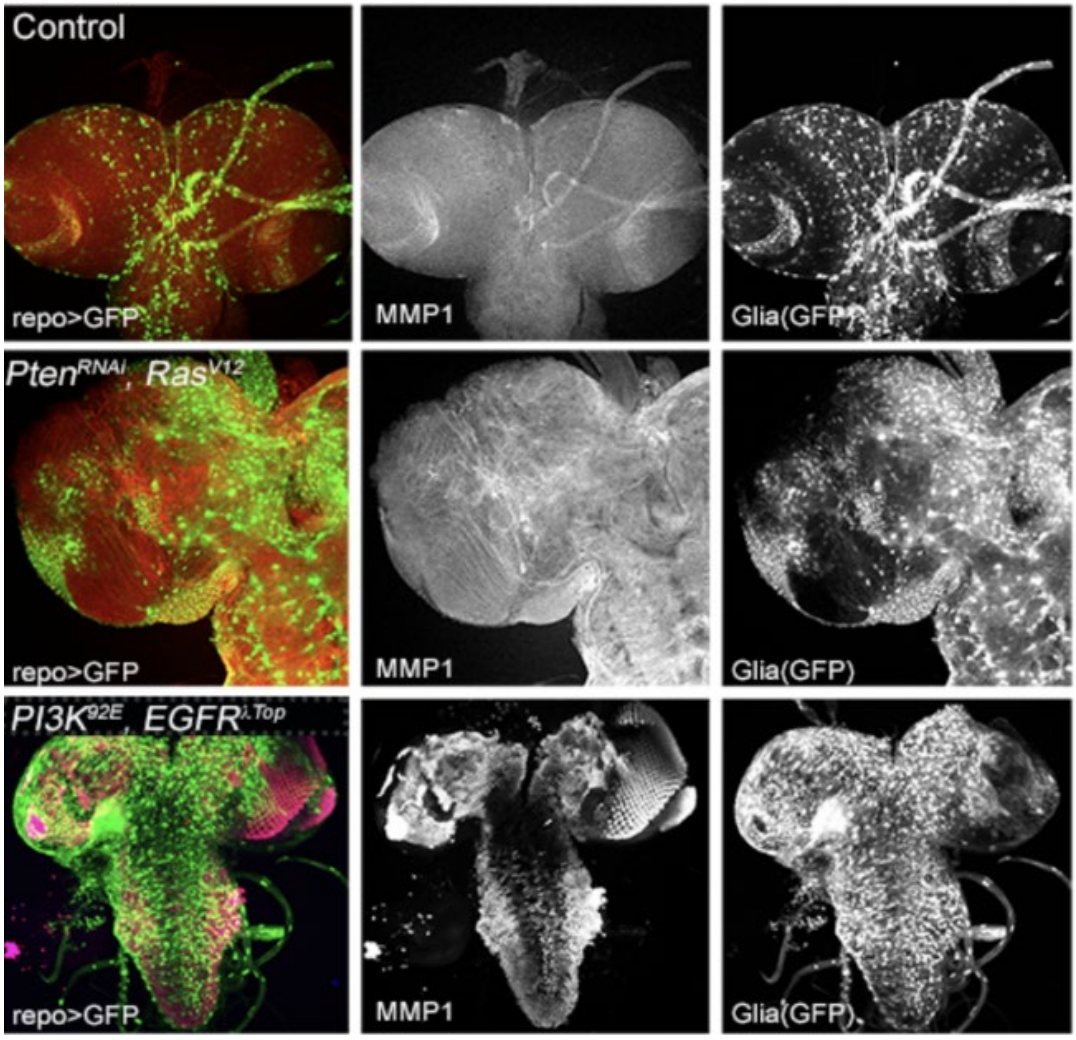

Figure 6. Comparison of Glioma Model 1 and Model 2 with normal brain

The panels show brains dissected from (a) wild-type (repo>GFP), (b) Model 1 (*Repo>GFP, Pten^{RNAi}, Ras^{V12}*), and (c) Model 2 (*Repo>GFP, PI3K^{92E}, EGFR$^{\lambda Top}$*) larvae stained for anti-MMP1 (red, and grey). The increased production of glial cells can be seen by comparing the GFP channel (green) or in right column (grey).

Repo>Pten^{RNAi}, Ras^{V12} represents Model 1 and the phenotype shows over expression of glial cells and GFP, indicating that the glioma grows to significant sizes compared to wild-type (normal) brains. The second glioma model (Model 2) is made by coexpressing PI3K^{92E}, EGFR$^{\lambda Top}$ *(Fig. 5 C).* The phenotype shows an increased number of glia and significant overgrowth *of the dorsal lobes and the ventral nerve cord* compared to normal brains. Overall, we found that both models exhibited glioma growth, but Model 2 had a slower growth rate compared to Model 1 (Fig 5). These two models will be used to study effects on *Hippo, JNK and* Wg pathway*s*. DIAP1 serves as an inhibitor of apoptosis and a target of the Hippo pathway, while MMP1 is a downstream target of the JNK pathway involved in extracellular matrix remodeling. Wg, on the other hand, is a crucial player in cell proliferation and differentiation processes.

Yorkie activity is induced during glioma growth

To study the changes in signaling pathway activity, we next tested if Yki activity is affected during glioma growth. We used the expression of the Yki-transcriptional target *Drosophila* Inhibitor of Apoptosis protein 1 (DIAP1) as a proxy for measuring Yki activity (Huang et al., 2005). DIAP1 is an important regulator of cell death (Orme and Meier, 2009) and is itself controlled by the Yki/Sd transcriptional complex that responds to Hippo pathway inputs(Huang et al., 2005). DIAP1 expression level serves as a readout of Hippo pathway activity. Increased DIAP1 expression indicates reduced Hippo pathway activity, while decreased DIAP1 expression suggests increased pathway activity. In other words, the

expression of DIAP1 depends on the activity of the transcriptional co-activator Yki. When Yki is released from Wts inhibition, it can move to the nucleus and bind TEAD class transcription factor like *Drosophila* Sd, to induce transcription of *diap1* and other target genes like *Cyclin E, Cyclin A, miRNA bantam*, and several upstream regulators of Yki like *expanded, merlin, kibra, crumbs* (Kango-Singh and Singh, 2009).

In the brain, DIAP1 is expressed in a stereotypical pattern in the outer proliferation centers of the optic lobe, and throughout the central brain and the ventral nerve cord (Fig. 7A). In control brains from *repo>GFP, EGFR$^{\lambda Top}$* or *repo>GFP, PI3K^{92E}* DIAP1 levels are moderately induced (Fig. 7). In comparison, in the Model 2 *repo>GFP, PI3K^{92E}, EGFR$^{\lambda Top}$* DIAP1 levels are the robustly induced in the central brain and in the optic lobes (Fig. 7, bottom row).

Overall these data indicate that increased Yki activity is observed in both models of glioma (Minata et al., 2019) which suggests that the Hippo pathway may be a key pathway in glioma growth

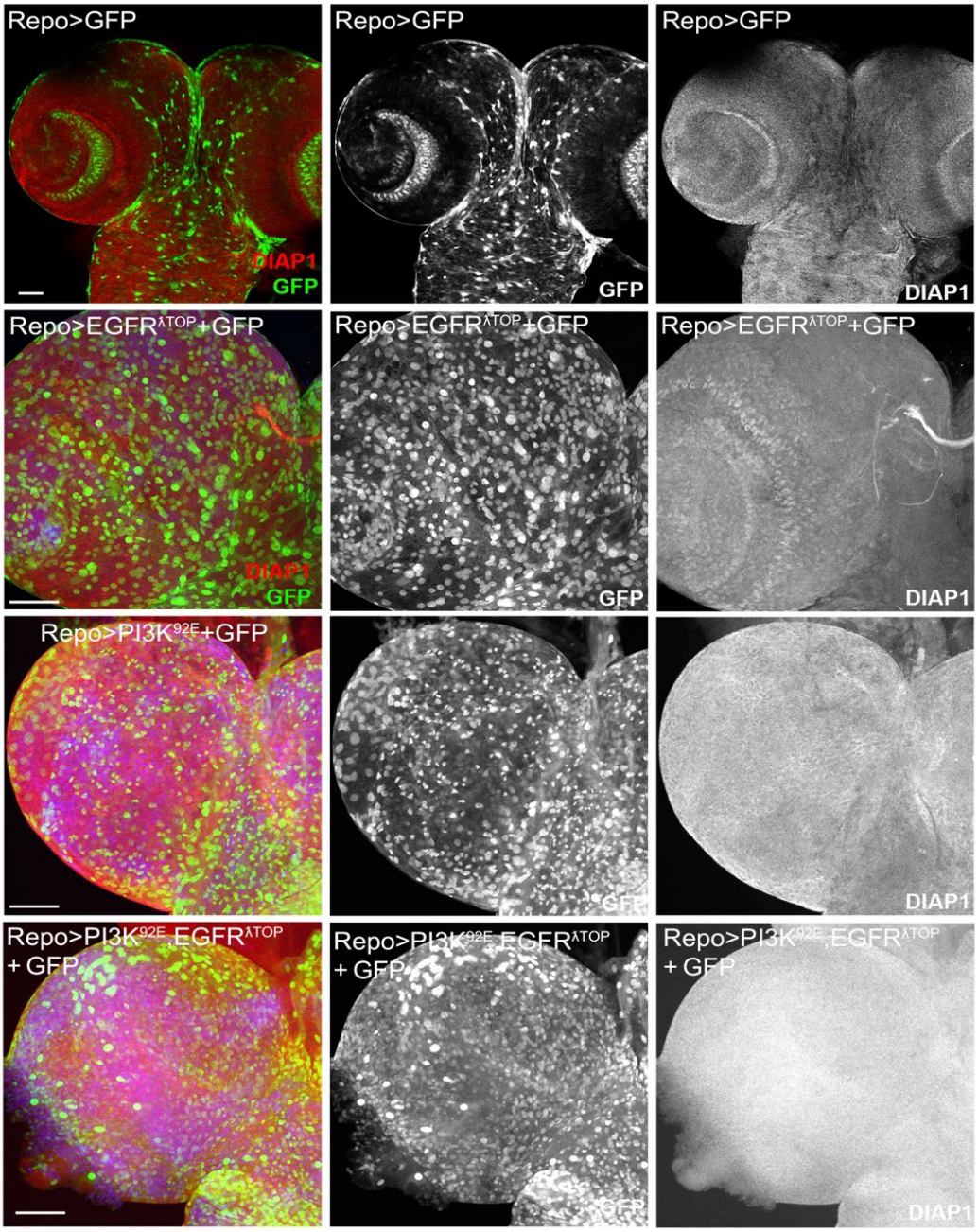

Figure 7 DIAP1 expression is induced in Glioma.

Panels show expression of DIAP1 (red, grey) in the control brain samples of the genotype *repo>GFP* (top row), *repo>GFP, EGFR$^{\lambda Top}$* (second row), *repo>GFP, PI3K^{92E}* (third row) and *repo>GFP, PI3K^{92E}, EGFR$^{\lambda Top}$* (bottom row). Note the pattern of DIAP1 expression in the optic lobe is disrupted, and robustly induced in the glioma model. The magnification and orientation of all images is identical.

JNK activity is induced in glioma models

Next, we tested the signaling activity of the JNK pathway in our glioma models (Fig. 8).

In response to stress or inflammation, the JNK pathway is activated and the *Drosophila* JNK called Basket (Bsk) is phosphorylated that causes the hetero-dimerization of *Drosophila* the AP-1 transcriptional factors *Drosophila* Jun-related antigen (Jra) and *Drosophila* Kayak (Kay) aka *Drosophila* Fos (La Marca and Richardson, 2020). Once the AP-1 complex is formed it translocate to the nucleus and activated expression of several target genes like *puckered, MMP1 or Eiger* (La Marca and Richardson, 2020). We used the anti-MMP1 antibodies to assess JNK levels. MMP1 (Matrix metalloproteinase 1) MMP1 is involved in extracellular matrix remodeling, and its expression level reflects JNK pathway activity (Külshammer et al., 2015, Uhlirova and Bohmann, 2006). Increased MMP1 expression indicates upregulated JNK pathway activity, while decreased MMP1 expression suggests decreased pathway activity.

In our glioma models, we observed increased MMP1 expression compared to control samples, indicating enhanced JNK pathway activity. This suggests that the JNK pathway may be activated in response to glioma growth. Increased JNK pathway activity may promote glioma progression by facilitating cell migration, invasion, and survival. Therefore, strategies aimed at inhibiting JNK pathway activity could potentially be explored to learn about the response of GBM to JNK inhibition.

After solidifying our pilot data, we plan to investigate the effects of these pathways on glioma stem cells. In Model 2 (Fig 5), we observed an overabundance of glial cells and an upregulation of the JNK target gene MMP1. Further studies are needed to confirm these observations and to evaluate the impact of the Hippo pathway in both models. These preliminary results suggest that our models can be useful for investigating the molecular mechanisms underlying glioma growth and identifying potential therapeutic targets.

MMP1 (Matrix metalloproteinase 1) is a downstream target of the JNK pathway, and its expression level reflects JNK pathway activity. Increased MMP1 expression indicates upregulated JNK pathway activity, while decreased MMP1 expression suggests decreased pathway activity. In our glioma models, we observed increased MMP1 expression compared to control samples, indicating enhanced JNK pathway activity. This suggests that the JNK pathway may be activated in response to glioma growth. Increased JNK pathway activity may promote glioma progression by facilitating cell migration, invasion, and survival. Therefore, strategies aimed at inhibiting JNK pathway activity could potentially reveal the signaling interactions and molecular drivers of GBM.

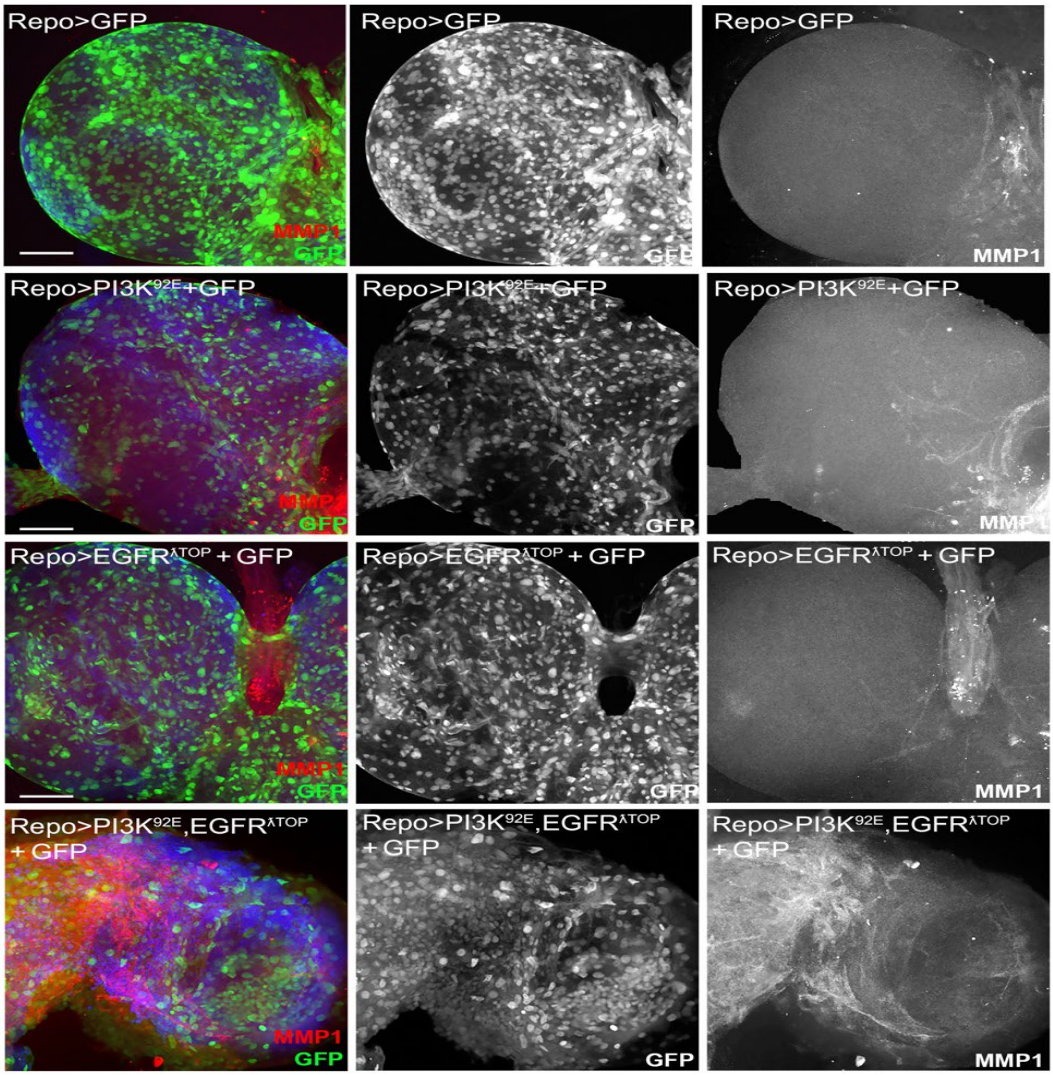

Figure 8 JNK target MMP1 is induced in the glioma.

Panels show expression of MMP1 (red, grey) in the control brain samples of the genotype *repo>GFP* (top row), *repo>GFP, PI3K^{92E}* (second row), *repo>GFP, EGFR$^{\lambda Top}$* (third row) and glioma model *repo>GFP, PI3K^{92E}, EGFR$^{\lambda Top}$* (bottom row). Note that in control brains MMP1 expression is very low in the optic lobes and is seen in the air sacs of the brain, however, it is robustly induced in the glioma model. The magnification and orientation of all images is identical.

Assessment of Wg pathway activity in glioma models:

Wg (Wingless) is a ligand in the Wnt signaling pathway, and its expression level reflects Wg pathway activity. Increased Wg pathway activity may contribute to glioma progression by promoting cell proliferation, survival, and stemness. Therefore, strategies aimed at targeting the Wg pathway could potentially be explored to reveal the biological mechanisms of GBM growth and as therapeutic interventions for GBM.

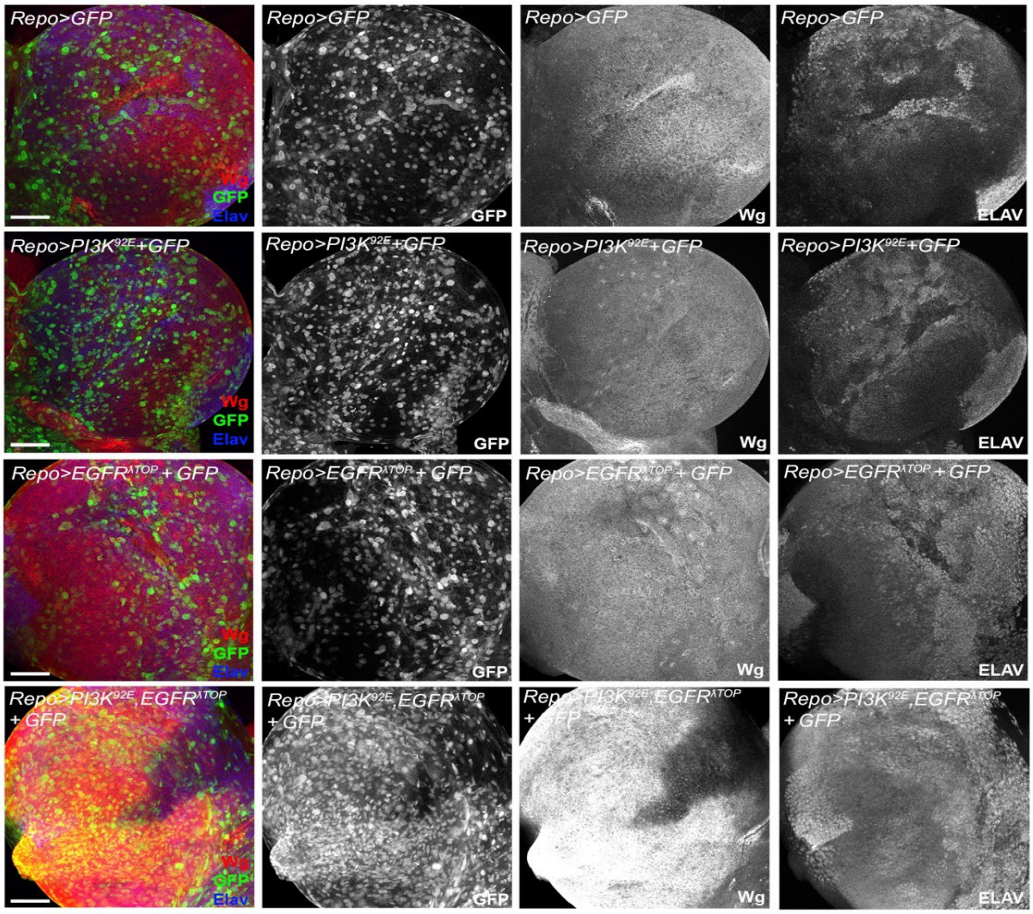

Figure 9 Wg is upregulated in Glioma.

Panels show expression of Wg (red, grey) in the control brain samples of the genotype *repo>GFP* (top row), *repo>GFP, PI3K^{92E}* (second row), *repo>GFP, EGFR$^{\lambda Top}$* (third row) and *repo>GFP, PI3K^{92E}, EGFR$^{\lambda Top}$* (bottom row). Note the pattern of Wg expression in the optic lobe is disrupted, and robustly induced in the glioma model. The magnification and orientation of all images is identical

CHAPTER 4

DISCUSSION

The investigation into glioma formation using these fly models provides valuable insights into the underlying molecular mechanisms of this deadly brain cancer. By employing *Drosophila* larvae as a model system, we have been able to replicate key aspects of glioma development observed in humans, shedding light on the complex interplay of signaling pathways involved in tumor growth and progression.

The study focuses on two distinct glioma models, referred to as Model 1 and Model 2, each generated through specific genetic manipulations targeting key signaling pathways. Model 1 involves the coactivation of the PI3K and MAPK pathways by downregulating Pten and activating the Ras oncogene in glial cells. Model 2 is created by coexpressing constitutively active PI3K and EGFR in glial cells to coactivate the PI3K and MAPK pathways.

Both models result in the formation of large and abnormal growths in the larval brain, highlighting the critical role of these pathways in glioma development (Fig. 6, 7). Interestingly, the study reveals differences in the growth of the two models, with Model 2 exhibiting a slower growth rate compared to Model 1. This suggests that the specific genetic alterations employed in each model may influence the dynamics of glioma growth, potentially reflecting varying molecular subtypes of glioblastoma multiforme (GBM) observed in humans.

Further analysis of signaling pathway activity in glioma models uncovers intriguing findings regarding the Hippo and JNK pathways (Fig. 7- 10). Enhanced activity of the Yki, indicated by increased expression of the downstream target DIAP1, suggests a potential mechanism that promotes cell proliferation in glioma progression (Fig. 7). In contrast, increased activity of the JNK pathway, evidenced by elevated expression of the downstream target MMP1, may promote glioma growth and invasion. These observations underscore the complex network of signaling pathways involved in glioma pathogenesis.

Our current data provides a framework for further investigations like investigating if Hippo, JNK and Wg pathways work independently or through a network in promoting glioma growth. Further, if these pathways form a molecular network, which pathway acts downstream and is the key driver of glioma growth. We also want to investigate if glioma growth occurs by expansion of the neuroblast stem cells (Gangwani et al., 2020) through Yki or Wg as both these genes are implicated in maintenance of stem cells. We will also test if the glioma growth is affected by genetically altering the Hippo, JNK or Wg pathways. The rich toolkit of Drosophila genetics can be used to cause additional mutations in these glioma models and study the characteristics of the resulting glioma.

The implications of these findings offer hope for the development of novel therapeutic strategies for GBM. Strategies aimed at enhancing Hippo pathway activity or inhibiting JNK pathway activity could represent promising avenues for targeted therapy, with the potential to inhibit glioma growth. Further research is

warranted to elucidate the precise mechanisms by which these signaling pathways contribute to glioma growth and progression. Additionally, validation of these findings in mammalian models and clinical samples is essential to confirm their translational relevance and potential therapeutic utility.

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