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Riley Gulbronson Date

Exploring Innate Immunity Pathways in the Sex-Based Differences of Glioblastoma using *Drosophila melanogaster* Models

By

Riley Gulbronson

Master of Science

Graduate Division of Biological and Biomedical Sciences

Cancer Biology and Translational Oncology

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By

Riley Gulbronson

B.S. Emory University, 2019

Advisor:

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An abstract of a thesis submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Master of Science in the Graduate Division of Biological and Biomedical Science, Cancer Biology and Translational Oncology, 2020

Abstract

Exploring Innate Immunity Pathways in the Sex-Based Differences of Glioblastoma using *Drosophila melanogaster* Models

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Glioblastoma multiforme is the most common, primary malignant brain tumor in adults, accounting for 15% of all central nervous system cancer diagnoses. Previous studies have characterized a sex-based difference in human glioblastoma patients, in which biological males have a 60% higher chance of developing glioblastoma than do biological females. This sex-dependent disparity carries over into survival rates as well, where, regardless of age, race, or geographical location, biological males tend to have a worse prognosis. Even so, the median survival rate after a standard treatment of surgical resection, radiotherapy, and adjuvant chemotherapy with temozolomide, remains dismal at 15 months. A novel focus for developing glioblastoma treatment is the controlled manipulation of glial-based innate immune responses. Endogenous innate immunity is also stratified based on sex, with biological females having a more robust response to infection or injury than biological males. Considering these parallel sex-based differences, we aim to further investigate the impact of innate immune response pathways on gliomagenesis.

Using a *Drosophila* model of glioblastoma, we conducted morphological screens of genes within the Toll signaling pathway, known to play a major role in *Drosophila* innate immune responses. Glial-specific RNAi-gene knockdown constructs and gene overexpression constructs identify *Toll-9* and *spatzle*, which encode a Toll-pathway receptor and ligand, as candidate genes for further investigation for sex-based differences in neoplastic glial growth in our *Drosophila* glioblastoma model. These results provided us with a framework for future investigations into innate immunity genes that enhance or suppress the glioblastoma phenotype.

The data collected during this exploratory investigation provide preliminary support for the interaction between innate immunity signaling pathways and sex-based differences in glioblastoma. Further characterization of the sex-based phenotypic influences of downstream signaling effectors of the Toll signaling pathway may prove useful for identifying new therapeutic targets for glioblastoma treatments.

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INTRODUCTION AND BACKGROUND

Clinical Characteristics of Glioblastoma Multiforme

The disease burden of Glioblastoma multiforme (GBM) is great, as it is the most common primary brain tumor in adults [5]. The highly aggressive, malignant, and invasive nature of these undifferentiated and intractable tumors leads the World Health Organization (WHO) to classify them as grade IV gliomas [6]. Usually originating in the cerebral hemispheres, GBM, as well as its treatment, has been known to impair neurological and cognitive function in patients [7].

The American Cancer Society estimates that 23,890 new central nervous system (CNS) tumor diagnoses will be made in 2020 [8], with brain tumors accounting for 85% of these and GBM, specifically, accounting for nearly 15% and representing the highest number of malignant diagnoses [9]. The median age of diagnosis is 65 with incidence peaking at around 85 [9, 10], and the median survival after diagnosis is 15 *[5, 11]*. Unfortunately, there has been little improvement upon this poor prognosis over the last 15 years [12].

The current standard of care for GBM patients includes surgical resection of the tumor mass, radiotherapy, and adjuvant chemotherapy with temozolomide, a DNA damaging agent that interferes with replication [5]. Even with this aggressive treatment, recurrence is almost inevitable with over 90% of patients relapsing after just 6 months [13-15].

Unfortunately, both primary and recurrent GBM tumors continue to remain resistant to current treatments, making it imperative to develop new, effective therapeutic strategies for GBM and related cancers. To do this, we need to determine how aspects of tumor cell biology and tissue pathology can be manipulated to actively stimulate the death or elimination of tumor cells.

Glioblastoma Biology

GBM occurs sporadically and there are few well-established risk factors associated with diagnosis [16], making it imperative to understand the underlying genomics and biology of GBM. The Cancer Genome Atlas (TCGA) and other large-scale genomic sequencing projects identified common genetic mutations found in GBM tumor samples that are thought to be essential for tumorigenesis; these include genomic amplification, activating mutations, and overexpression of receptor tyrosine kinases (RTKs) and their signaling pathway components [17, 18]. One of the most frequent is the mutation of epidermal growth factor receptor (EGFR) into its constitutively active variant III ($EGFR^{vIII}$). The constitutive activation of EGFR and other RTKs furthers GBM tumorigenesis by promoting proliferation, migration, tumor cell survival, which are among the hallmarks of cancer [19-24].

Sex-Based Differences in Glioblastoma

In nearly all cancers that affect both males and females, there exists an incidence disparity based on sex; males are 1.5 to 3 times more likely to develop cancer in any organ or tissue, and often have poorer responses to treatment and overall survival [8, 25]. Accounting for age, race, geographic location, and tumor histology, sex-based differences in gliomagenesis and associated fatality can be seen throughout the world as well [8, 25, 26]. With little exception, we see increased initiation rates and poorer prognosis in males with high grade gliomas (though lower grade gliomas exhibit little difference based on sex) [25]. The male to female incidence rate for GBM, specifically, is 1.6:1 [9, 26]

While many investigations of sex-based differences in disease consider sex hormone concentration variability [25, 27], sex differences in GBM are observed in all age groups and cannot be the consequence of sex hormone activation alone [26, 28, 29]. An investigation into the role of EGFR pathway components in sex-based differences of GBM identified p53, RB, and EGF itself as promoters of transformational disparities [29]. As previous attempts to target EGFR in GBM treatment have overall proven unsuccessful [30], the possibility of utilizing these sexually dimorphic downstream components of the signaling pathway could be crucial in addressing gliomagenesis and GBM proliferation.

Other possible mechanisms by which sex could affect cancer rates and outcome include differing metabolism and growth rates, developmental variability, genetic and epigenetic processes, and differences in immunity response [25, 31]. Identifying a molecular basis for the sex-based difference in GBM incidence and prognosis may reveal fundamental, sex-specific components of disease risk which may ideally lead to sex-based therapeutics and individualized cancer treatments.

Innate Immunity in Cancer Treatment

Innate immunity, is critical in protecting against nonspecific, invading cells and pathogens [32, 33]. This is in contrast with adaptive or acquired immunity, which builds up over time and exposure to provide increased protection against subsequent reinfection. These systems work together to provide comprehensive protection, with the innate immune response taking immediate effect in response to a biological threat before being recruited by the specialized, adaptive immune response to provide

subsequent direction and remove targeted pathogens. In humans, this recruitment usually requires 4 to 7 days, depending on the pathogen [33].

Many current therapeutic approaches to treat cancer and related diseases are predominantly focused on early stimulation of adaptive immunity, with the goal of creating an anti-tumor immune response [34, 35]. As the field of active cancer immunotherapy and the focus on cancer and innate immunity interactions are still relatively new and developing, the molecular mechanisms of how the innate immune responses endogenously recognizes tumorigenesis are poorly understood [35]. While cancer treatments involving T-cell responses and immune checkpoints and signaling pathways have led to remarkable success in some cancer patients, the majority do not benefit from these treatments, emphasizing the need to identify additional molecular pathways that could be exploited [36, 37].

Innate Immunity and Toll-Like Receptors in Glioblastoma

The focus of GBM treatment has recently shifted toward modulation of the microglial immune response, though the unique tumor microenvironment (TME) made this a challenge. Innate immunity is considered the primary cellular defense mechanism that responds to microenvironment variation. During neural inflammation, degeneration, and injury, innate immunity signaling pathways are expressed even in resident, non-immune cells like glia, though in most cases, the signaling response differs slightly from that of that canonical immune cells [38]. Toll-like receptors (TLRs) are major contributors to these innate immune responses. Because of this, they serve as promising potential targets for therapeutic treatment of GBM [39, 40].

The role of TLRs in non-immune cells of homeostatic brain tissue is comprehensively characterized, but their effects on an altered tumorigenic microenvironment are not as well understood. Some TLRs can have pro- or anti-inflammatory roles in tumorigenesis [40]. In tissue-based non-immune cells, the fate of TLR activity depends on interactions with other signaling partners [38]. Known interactions exist between TLRs and the EGFR signaling pathway [41, 42], which has an established role in GBM initiation and progression, and also exhibits a sex-based difference.

Sex-Based Differences in Glial-Based Innate Immunity

Innate immunity is also stratified based on sex, as explained by the well-established observation that females have a more robust immune response than males [43]. This advantage is demonstrated, not only in GBM and other cancers [25], but in bacterial, fungal, and parasitic resistance as well [44]. Within the CNS, immune responses that result in the release of proinflammatory cytokines are increased in both female humans and rat models, as compared to males [45].

Multiple studies have observed a sex-based difference in microglia expression and function and have characterized the role this difference plays in many neurological and neurodegenerative diseases [43, 45]; however, studies have not adequately addressed the same in astrocytes and other non-immune glial cells. As the role of microglia is encompassed in the multifunctional glial cells of *Drosophila melanogaster*, for which there is no evidence of microglia in the CNS or elsewhere [46], future investigations using *Drosophila* models may aid in further characterizing these sex-based differences.

Drosophila melanogaster as a Model for Glial Neoplasia

Drosophila models are an extremely effective tool when it comes to understanding the mechanisms of cancer and tumorigenesis [47]. The short lifespan, large number of progeny, and fully sequenced genome of *Drosophila* provide myriad advantages over traditional animal models. Speaking to their use as a cancer model, *Drosophila* are known to develop neoplastic tumors that share key features of vertebrate neoplasms and demonstrate the hallmarks of cancer: evasion of growth suppressors and reproductive immortality resulting in rapid, autonomous growth, invasion into adjacent tissue and metastasis into distant tissue, sustained proliferative signaling and lack of contact inhibition, and potential lethality to the host [47]. *Drosophila* models also exhibit unlimited growth after transplantation, a crucial characteristic of malignant tumors [48].

When comparing *Drosophila* genes to those associated with human disease, 30% are similar enough that the associated proteins have identical functions [49]. This extensive homology allows *Drosophila* to act as a model for neurological diseases as well. The *Drosophila* CNS, like that of humans, consists of a central nerve cord and two bilaterally symmetrical brain hemispheres, all composed of glial cells and neurons [50]. Several known RTK gliomagenesis pathways are remarkably conserved between *Drosophila* and vertebrates. Many mammalian RTKs have *Drosophila* orthologs with not only sequence homology (70%), but functional homology as well. Single functional orthologs such as EGFR (*dEGFR*), PIK3CA (*dp110*), PTEN (*dPTEN*), RAS (*dRas*), RAF (*dRaf*), and AKT (*dAkt*), allow for complex genetic analysis to be simplified [51]. In many cases, genes

essential for human gliomagenesis were first discovered in the simplified *Drosophila* genome.

There are also many existing tools for targeted mutagenesis or RNA interference (RNAi) available for almost all *Drosophila* genes. Models such as the binary UAS-GAL4 system allow for cell-type specific tissue manipulation, including that of the CNS, as well as complex genetic manipulation through the expression of multiple transgenes within a particular cell type [52-56].

In order to investigate signaling cooperation during glial neoplasia and tumorigenesis, Read *et al.* developed a *Drosophila* GBM model. As seen in published mouse models, co-overexpression of epidermal growth factor receptor (EGFR) and phosphoinositide 3-kinase (PI3K) signaling in glial cells results in glial neoplasia in *Drosophila*. By expressing the constitutively active forms of *Drosophila* EGFR (dEGFR) and dp110, the catalytic subunit of PI3K ($dp110^{CAAX}$), it is possible to induce lethal, malignant, glial neoplasia in larval *Drosophila* brains [2] (Figure 2).

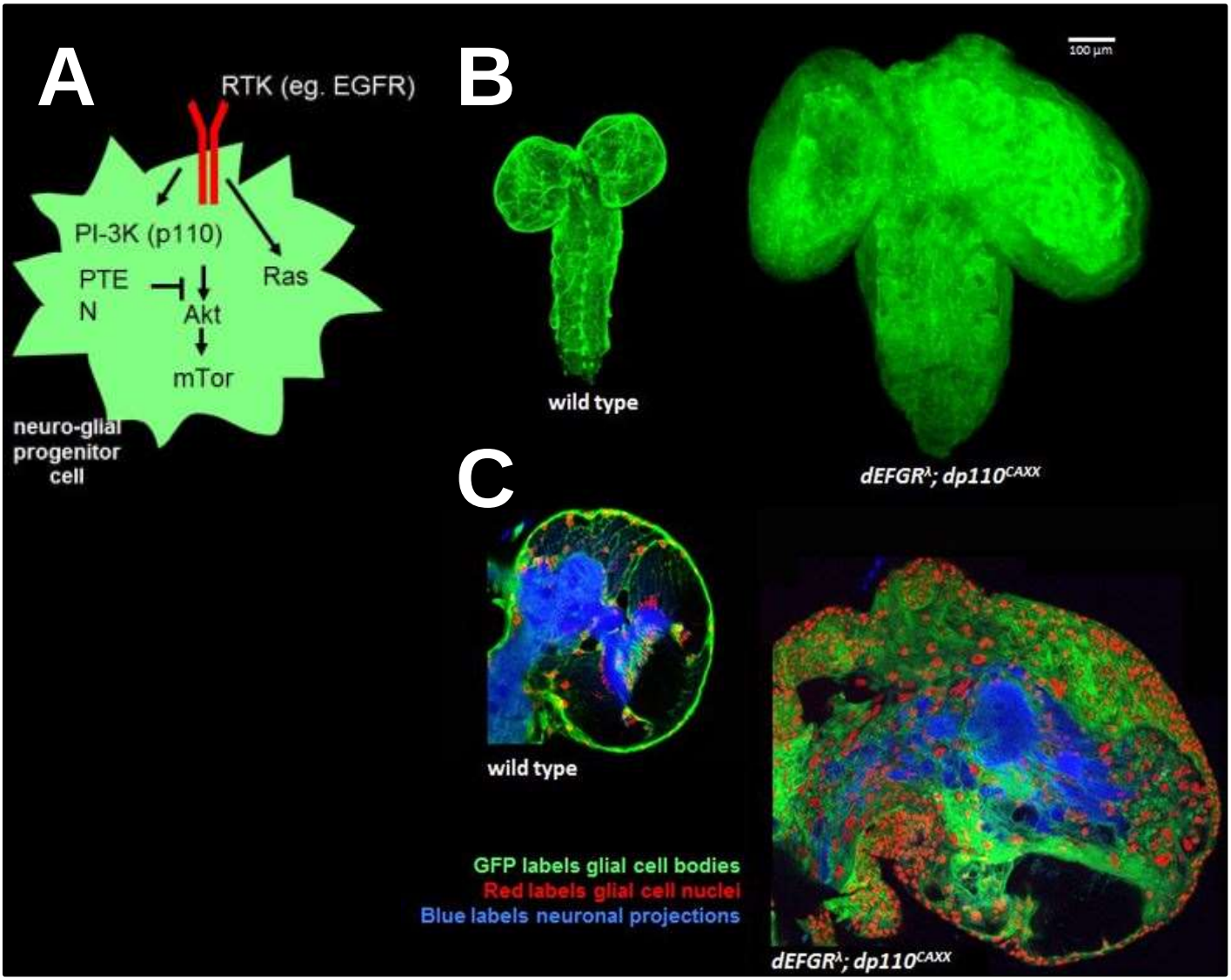

Figure 1: A *Drosophila* model of glioblastoma. (A) Activation of EGFR and PI3K signaling in glial progenitor cells drives glial neoplasia and creates invasive, malignant tumors. (B) Glial-specific *repo-Gal4* is used to co-overexpress constitutively active versions of dEGFR and dp110. This co-activation of EGFR and PI3K signaling in a glial specific manner results in glial neoplasia in *Drosophila* larval brains. Representative 10x images of age matched *Drosophila* larval brains and of 40x brain hemispheres (C) are presented. Repo (red) labels glial cell nuclei; CD8-GFP (green) labels glial cell bodies; anti-horseradish peroxidase (blue) counterstains for neurons and neuropil.

Innate Immunity in *Drosophila melanogaster* and the Toll Signaling Pathway

Drosophila melanogaster is also a powerful model when studying the role of immunity in disease development. Though they lack an adaptive immune system [57] the defensive, innate immunity responses of flies are highly conserved at the molecular level in humans and other vertebrates [32]. The absence of adaptive immune responses also allows for the study of innate immunity aspects that might otherwise be unavailable [57]. Because of this, many of the known innate immunity effectors in humans were originally found in *Drosophila* models; particularly those cell intrinsic pathways of the central nervous system.

This homology between *Drosophila* and humans allows for the exciting possibility to increase understanding of innate immunity in diseased organisms [32]. For instance, in humans, Toll-like receptors (TLRs) that usually participate in the detection of viral or bacterial infections have also been identified as aiding in the detection of tumor cells and consequential immune response [36]. TLRs that are expressed in the CNS in neurons, glia, and macrophage-like microglia, are strongly implicated in cell death, injury, inflammation, and neurodegeneration [58, 59]. TLRs and their associated pathways were originally discovered in *Drosophila* as part of the homologous Toll signaling pathway (Figure 2). First identified as embryonic developmental players, the Toll pathway was subsequently classified as an essential component in innate immunity responses [60, 61].

Our results show that activation of the Toll pathway has a strong and unexpected anti-tumor effect in *Drosophila* GBM models. This interaction is intriguing, as the ancient system of humoral and cellular responses that defend tissues against infection

and injury is conserved in humans [62-64]. Investigation of the Toll signaling pathway in the context of *Drosophila* GBM could illuminate pro-apoptotic TLR signaling as an attractive therapeutic target for human disease. The interaction may also help us understand the mechanisms underlying the sex-based differences in both innate immunity and GBM initiation and progression [65].

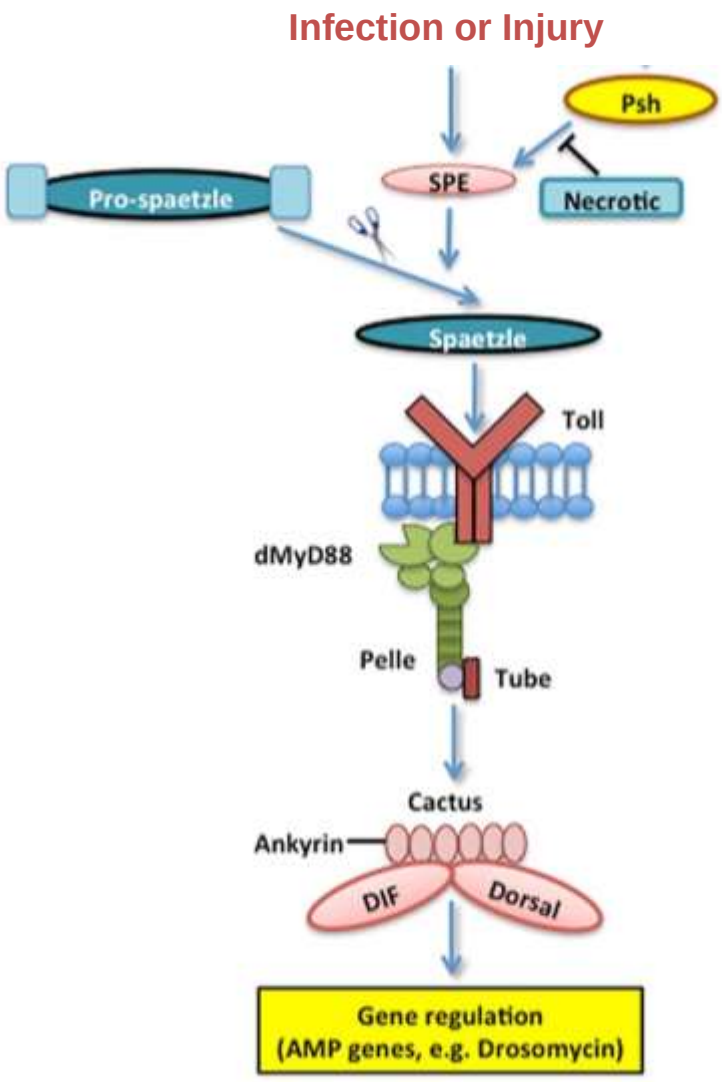

Figure 2: The Toll Signaling Pathway. The Toll signaling pathway in *Drosophila melanogaster* acts in both an antifungal and an antibacterial manner. The Spatzle-Processing Enzyme (SPE) cleaves the Spatzle cytokine to trigger the Toll signaling response. Activation results in the recruitment of myeloid differentiation primary response 88 (dMyD88), Pelle, and Tube, adapter proteins in the cytoplasm, to promote signaling to Cactus. Inactively, Cactus is bound to the Nuclear Factor kappa B (NF-κB) transcription factors Dorsal-related Immunity Factor (DIF) and Dorsal. Upon phosphorylation, Cactus degrades and releases DIF and Dorsal, which transcribe for the upregulation of antimicrobial peptide (AMP) genes, such as Drosomycin. Figure adapted from [3]

Scope of the Thesis

The following project uses *Drosophila melanogaster* models to explore the role of cell intrinsic, innate immunity players in the observed sex-based difference in glioblastoma growth. Given the parallels between sexually differentiated innate immune responses and of glioblastoma incidence and prognosis, we hypothesize that there are common signaling components at play. Using *Drosophila melanogaster* as a model system, I aim to identify GBM-interacting innate immunity genes of interest and further explore their role within a *Drosophila* GBM model.

A morphological screen of RNAi-targeted genes provides me with the basis for further investigation. Subsequent experimentation with a selection of these identified candidate genes will provide further clarification on the interaction between GBM, innate immunity, and sex. Using dsRNA overexpression constructs, RNAi-targeted knockdown constructs, and other existing tools for *Drosophila* genetic manipulation, I will come to make preliminary conclusions as to if and how each gene interacts with our *dEGFR$^{\lambda}$; dp110^{CAAX}* model.

MATERIALS AND METHODS

Drosophila melanogaster Stock Maintenance

Drosophila melanogaster stocks were maintained at room temperature and without controlled humidity. True breeding stocks were transferred (without anesthetization) to new food vials approximately every 30 days to prevent contamination and maintain viability.

Virgin, female *Drosophila* were collected at least once every day to ensure enough individuals were available for successful crosses. Virginity is essential, as *Drosophila* have the ability to store sperm from previous mating to be used to fertilize future progeny [66]. Male *Drosophila* homologous for the genotype of interest are also collected in preparation for a cross (Figure 3.A).

All *Drosophila* stocks came from Bloomingdale Stock Center and Vienna Drosophila Resource Center unless otherwise noted. UAS-spz^{Act} stocks were provided by the Laura A. Johnston Lab in the Columbia University Department of Genetics and Development.

UAS-GAL4 Genetic System for *Drosophila* Crosses

The binary UAS-GAL4 genetic model system was utilized to allow for cell-type specific gene manipulation in Drosophila. (Figure 3.B) The GAL4 transcriptional activator was expressed under the control of a glial cell specific promoter, which allowed for transcriptional activation of candidate UAS-controlled, RNAi, transgenes. UAS-GFP was also incorporated in order to visualize glial cells using fluorescence and confocal microscopy.

Crosses were carried out between approximately 20 to 30 virgin females and 6 to 10 males and maintained at 25°C for a 72-hour preincubation period, allowing for mating to occur. Crosses were then transferred to newly prepared cross vials every 24

hours and returned to the incubation environment; this ensured that all larvae of a particular were no more than 24 hours apart in age.

Drosophila Larval Brain Dissection, Fixation, and Preservation

Drosophila crosses were maintained at 25°C for 120 hours and 144 hours for *repo>CD8-GFP* and *repo>dEGFR$^{\lambda}$; dp110^{CAAX}* cross progeny, respectfully, until third instar larval progeny were present. All third instar larvae of the same genotype suitable for dissection were removed from cross vials and separated by sex (Figure 3.A, C-D). Larval brains were dissected under a Zeiss LSM 700 stereomicroscope and collected in phosphate-buffer saline (PBS).

Once enough larval brains were collected for immunohistochemical (IHC) staining, samples of the same genotype were separated by sex and placed in wells with 4% paraformaldehyde. Dissected larval brain samples fixated for approximately 60 minutes and washed 3 times with 1x phosphate-buffer saline + 0.3% Triton (PBST). After the final wash, PBST with .03% sodium azide was added for preservation and stored in the refrigerator. This process was repeated daily for both sexes of all genotypes and all samples were age matched.

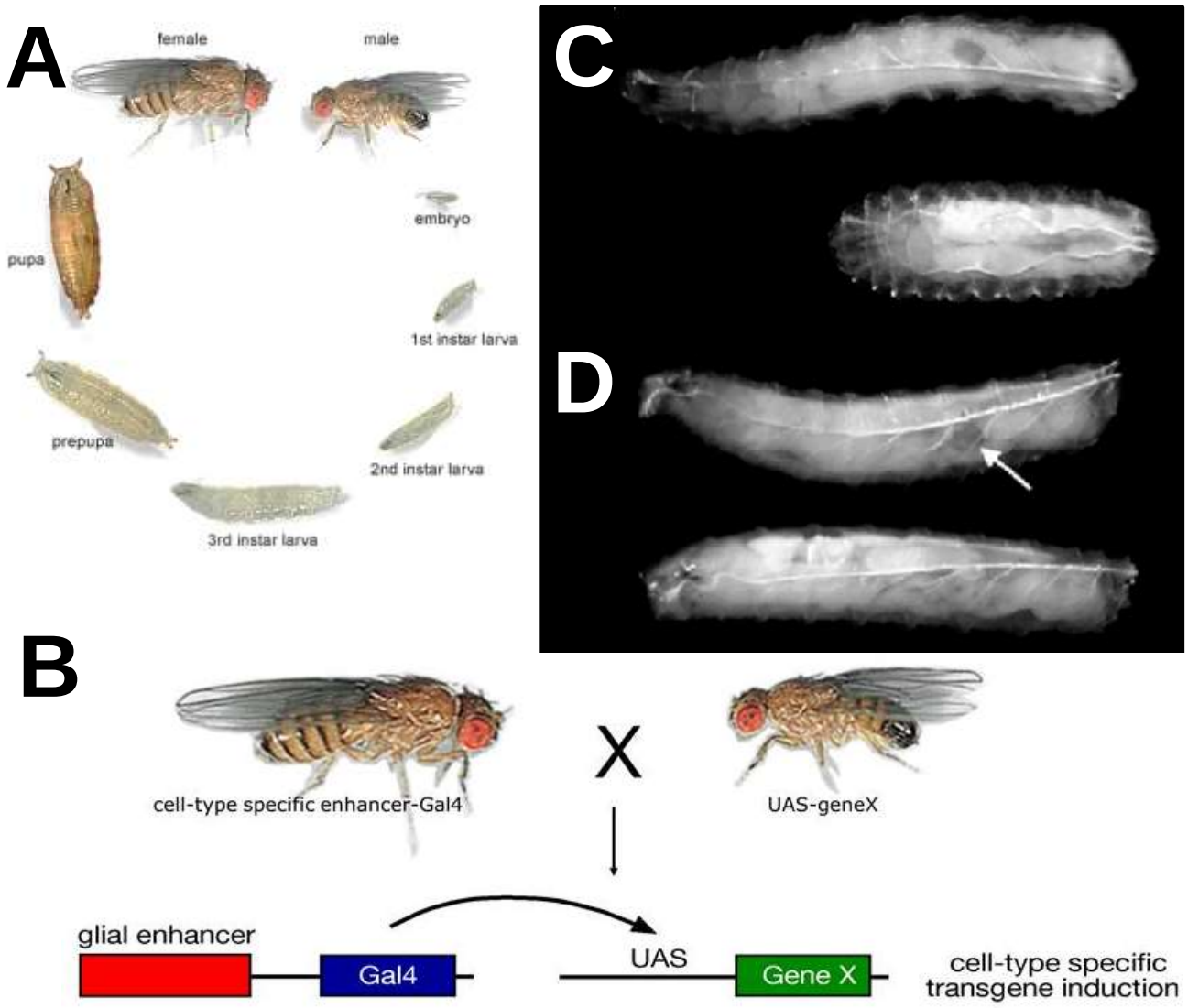

Figure 3: *Drosophila* identification and UAS-Gal4 genetic crossing system. (A) Morphological differentiation between male and female adult *Drosophila* is necessary for virgin female collection and cross preparation. Identification of third instar larvae within the *Drosophila* lifecycle is necessary during dissection. Figure adapted from [1]. (B) The binary UAS-Gal4 genetic crossing system allows for cell-type specific gene manipulation in *Drosophila*. Under a glial-specific promoter, *repo*, the transcription factor, GAL4, binds to its target sequence, UAS. This allows for glial-specific translation of a gene of interest in the F1 progeny. (C) Our *Drosophila* model was maintained over a Tm6BTb balancer chromosome with tub-Gal80. Because of this, crosses resulted in two classes of F1 progeny: one with the desired genotype (upper), the other with the Tm6BTb balancer (lower). Only larvae with the desired genotype were collected for dissection. Figure adapted from [4]. (D) Differentiation of male (upper) and female (lower) third instar larvae is based on visibility of male gonads (arrow). Figure adapted from [4]. [License Number: 4811081426013]

Immunohistochemical Staining of *Drosophila* Larval Brains

Dissected larval brains were stained to allow for visualization of glial cell bodies, glial cell nuclei, and neuronal cell bodies. The PBST and sodium azide in which dissected larva brains were stored was aspirated and the samples were washed with PBST 3 times to remove any remaining azide. After the final wash, samples were incubated with primary antibody stain anti-Repo (1:10, 9E10, Developmental Studies Hybridoma Bank (DSHB)) for up to 48 hours, followed by incubation with secondary antibody stain (anti-mouse-Cy3; 1:200) for 24 hours. Anti-HRP-Cy5/Alexa-647 (1:50) was used to visualize neuropil. Samples were refrigerated during storage.

Confocal Microscopy and Image Analysis

Whole *Drosophila* larval brains were mounting using bridge slides after IHC preparation. Larval brains were imaged using a Zeiss LSM 700 confocal microscope and the ZEN 2012 software from Zeiss.

Three-dimensional image analysis was performed using volume rendering algorithms from the IMARIS 9.2 software and with assistance from the Emory University Integrated Cellular Imaging Core.

Drosophila Larval Brain RNA Extraction and Preparation

Age matched *Drosophila* larval brains of each genotype and sex were dissected and placed in TRIzol RNA purification medium (Thermo Fisher, 15596026). Samples were stored at -80°C. To isolate RNA, chloroform was added to create layers of biomaterial, including RNA, DNA, proteins, and lipids. The RNA containing aqueous

phase was removed and purified through alcohol-based precipitation, resulting in an RNA pellet that was resuspended in nuclease free water.

RNA samples were analyzed for purity by Bioanalyzer (EIGC) and samples with RIN scores of 7 or greater were selected for RNA sequencing.

RNA Sequencing

Drosophila RNA samples were submitted to Admera, Inc. for library generation and RNAsequencing. Due to circumstances beyond our control, data collection and analysis could not be completed before the presentation of this thesis.

RESULTS

A Morphological Screen of Innate Immunity Genes that Potentially Modify Glial Neoplasia in *Drosophila*

To determine whether known innate immunity genes play a role in GBM initiation and proliferation, we performed a morphological screen using our *Drosophila* GBM model [2]. We used this model, in conjunction with RNAi-based modifiers, to identify known innate immunity genes that suppress or enhance neoplastic glial phenotypes.

"Enhancer-suppressor" modifier screens are widely used within *Drosophila* research to identify novel genes and characterize known components of signaling pathways. As screening takes place within the developing tissue of a live organism, this approach is *in vivo* and more closely resembles physiologically relevant conditions, making it advantageous over *in vitro* studies.

Diagrammed in Figure4 is the design for our morphological screen. We crossed *repo>dEGFR$^{\lambda}$; dp110^{CAAX}* flies with flies homozygous for UAS-RNAi constructs. The *repo>dEGFR$^{\lambda}$; dp110^{CAAX}* line was maintained over a Tm6BTb balancer chromosome with tub-Gal80, which suppressed *repo-GAL4* driven overexpression of *dEGFR$^{\lambda}$; dp110^{CAAX}* transgenes in the parent females. This cross resulted in two classes of F1 progeny: one with *repo>dEGFR$^{\lambda}$; dp110^{CAAX}* and expressing green fluorescent protein (UAS-CD8GFP), the other with the Tm6BTb balancer expressing no GFP. We observed and scored the brains of live *Drosophila* larvae under a Zeiss V16 fluorescent microscope without dissection. To control for the effects of UAS-containing transgenes in the *repo>dEGFR$^{\lambda}$; dp110^{CAAX}* model, all experimental cross progeny were compared to control cross progeny that contained UAS-lacZ.

If the *repo>dEGFR$^{\lambda}$; dp110^{CAAX}*; *RNAi* larvae showed a less severe phenotype than the *repo>dEGFR$^{\lambda}$; dp110^{CAAX}*; *lacZ* controls, we classified the RNAi-targeted gene as a "suppressor." If the *repo>dEGFR$^{\lambda}$; dp110^{CAAX}*; *RNAi* larvae showed a more severe phenotype than the *repo>dEGFR$^{\lambda}$; dp110^{CAAX}*; *lacZ* controls, we classified the RNAi-targeted gene as an "enhancer." An important note: in this context, the classification of "suppressor" is not synonymous with "mammalian tumor suppressor. In keeping with standard *Drosophila* nomenclature, genes are classified by their loss-of-function phenotypes [67]. We tested 26 RNAi constructs that were expressed specifically within the glia of the *repo>dEGFR$^{\lambda}$; dp110^{CAAX}* glioma model. Of these, 13 constructs and 8 associated genes were found to be phenotypic enhancers, while 7 constructs and 1 associated gene were found to be phenotypic suppressors. 5 constructs and 4 associated genes yielded inconclusive results (Table 1).

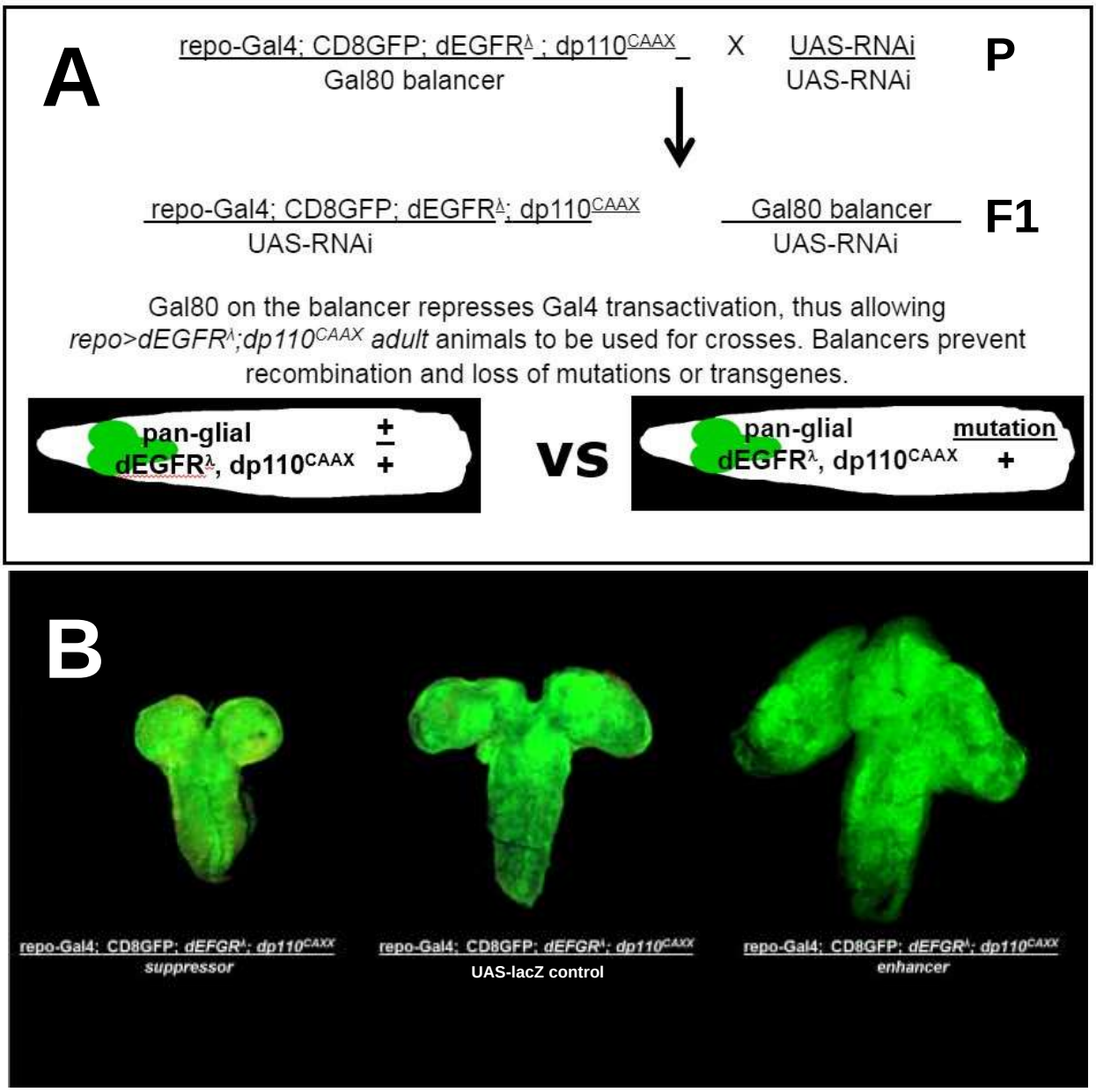

Figure 4: A morphological enhancer-suppressor screen for modifiers of the *dEGFR$^{\lambda}$; dp110^{CAAX}* phenotype. (A) Diagram of parental (P) genetic crosses and F1 progeny genotypes. The phenotypes of the progeny were compared to determine if *Drosophila* with RNAi-targeted genes showed enhancement or suppression, relative to those with the UAS-lacZ control. (B) Examples of *dEGFR$^{\lambda}$; dp110^{CAAX}* modifier phenotypes, which were scored independently by two investigators under a Zeiss V16 fluorescent microscope. "Enhanced" flies had a worsened phenotype and "suppressed" flies had a milder phenotype.

Drosophila Gene Name	Description	*Drosophila* Stock Numbers	Huamn Orthologs	Observed Interaction
pelle	encodes forf a tyrosine kinase that functions in the Toll pathway; plays key roles in developmental processes and innate, antifungal immune responses	VDCR 103774	RAK4	phenotypic enhancement
SARM	encodes for intracellular signaling molecule required for activation of pro-degenerative pathways; involved in regulating neuronal death after injury; predicted to have adaptive signaling activity	VDCR 22620, 22622, 39803, 102044, 104812, 105369, 105521	SARM1	phenotypic suppression
Sos	involved in neuron differntiation; playys key roles in transmembrane RTK signaling pathways	BSC 34833	SOS1	inconclusive
spatzle	encodes for a protien that causes the dimerization of the product of *Tl*, and the subsequent activation of the Toll pathway, which plays a key role in various developmental and immune processes	BSC 28538, 34699, 66924; VDCR 105107		phenotypic enhancement
spatzle 4	exhibits growth factor activity; predicted to have Toll-binding activity and to be involved in Toll pathway regulation; predicted to be involved in CNS formation; predicted to play a role in innate immune responses	VDCR 7679		inconclusive
spatzle 5	exhibits growth factor activity; involved in Toll signaling pathway; involved in CNS development and regulation of neuronal synaptic plasticity in response to neurotrophin	VDCR 102389		phenotypic enhancement
Tehao	encides for a homolog of the Toll receptor; involved in Toll signaling pathway and innate immunity responses	BSC 25933	TLR4	phenotypic enhancement
Toll	encodes for a transmembrance receptor that activates Toll intracellular signaling upon binding with *spz* ligand; involved in nervous system development and immunity responses	BSC 31044; VDRC 100078	TLRs (1,2,3,4,6,7,8,10)	inconclusive
Toll-3	not known to have a canonical role in the Toll signaling pwathway or in antimicrobial immuity response	VDCR 108034	TLRs (1,2,3,4,6,10)	phenotypic enhancement
Toll-4	encodes for a transmembrane protein related to Toll; predicted to be involved in innate immunity response	BSC 28543	TLRs (1,2,10)	phenotypic enhancement
Toll-7	encodes for a transmembrane Toll receptor that binds to viral glycoproteins and activates antiviral defenses	BSC 30488	CD180	phenotypic enhancement
Toll-9	encodes for a transmembrane Toll receptor; predicted to be involved in inflammatory response	BSC 30535, 34853	TLRs (1,2,10)	phenotypic enhancement
Tollo	encodes for a Toll-like receptor; contribute to innate immunity responses and peripheral nervous system development	VDCR 9431, 27098, 27099	TLR3	inconclusive

Table 1: Candidate innate immunity genes used in enhancer-suppressor screen to identify modifiers of the *dEGFR$^{\lambda}$; dp110^{CAAX}* phenotype. Innate immunity genes found in *Drosophila* were targeting with RNAi constructs, resulting in glial-specific gene knockdown in F1 progeny. The genes of interest are listed, along with descriptions and known human orthologs. The observed interaction is that seen between the *dEGFR$^{\lambda}$; dp110^{CAAX}*; *RNAi* phenotype and the *dEGFR$^{\lambda}$; dp110^{CAAX}*; *lacZ* control phenotype in live, larval brains. *repo>dEGFR$^{\lambda}$; dp110^{CAAX}*; *RNAi* larvae with a less severe phenotype than the control had their associated RNAi-target genes classified "phenotypic suppressors" while *repo>dEGFR$^{\lambda}$; dp110^{CAAX}*; *RNAi* larvae with a more severe phenotype than the control had their associated RNAi-target genes classified "phenotypic enhancers." If no interaction was observed, or observation data varied between investigator scores, the RNAi-targeted gene was reported to have an inconclusive phenotypic interaction.

Innate Immunity Gene Knockdowns that Enhance Glial Neoplasia in a *Drosophila* GBM Model

We identified the following genes as being phenotypic enhancers of the GBM phenotype in our *dEGFR$^{\lambda}$; dp110^{CAAX}* model.

Pelle – Gram-positive bacterial or fungal activation of Toll recruits the protein kinase Pelle, which functions to downregulate Tube and also interacts with Pellino, Dorsal, and Cactus. This protein contains a protein-protein interaction motif known as a 'death domain,' which acts to regulate apoptosis and inflammation. Pelle is also known to act as a positive regulator of innate immunity in *Drosophila [68-72]*.

spz – Spatzle directly activates the innate immunity Toll pathway by inducing the dimerization of Toll. Spatzle is also known to play a role in inducing apoptosis during cell competition, and to act as a growth factor during development [61, 64, 73].

Tehao - Tehao is expressed throughout *Drosophila* development and is known to interact with Toll, Dorsal, and Pelle within the innate immunity signaling pathway. It also works to initiate the transcription of endogenous drosomysin and metchnikowin, antifungal and antibacterial players, respectively [74].

Toll-3 – Toll-3 is not known to play any role in activating the canonical Toll signaling pathway or antimicrobial response. One study concluded that both Toll-3 and Toll-4 are expressed exclusively in males and no longer play essential roles in development due to their rapidly evolving nature [75]. Our RNAi-targeted gene knockdown screen identified Toll-3 as playing a role in antitumor immunity, its knockdown resulted in a more severe *dEGFR$^{\lambda}$; dp110^{CAAX}*phenotype. These results will be revisited.

Toll-4 – The hypothesis that Toll-4 only exits in male *Drosophila* and plays no functional role in development [75] adds an interesting twist to our morphological screen results. The screen identified Toll-4 as playing a role in antitumor immunity, as the RNAi-targeted knockdown of Toll-4 resulted in a more severe *dEGFR$^{\lambda}$; dp110^{CAAX}*phenotype. These results will be revisited.

Toll-7 – Toll -7 is expressed throughout the CNS and ensures neuronal survival and antiviral defense. Studies also show that Toll-7 plays a role, not only in development and immunity, but also in neurotrophism, or programmed neuronal death, after injury [76, 77].

Toll-9 – As it naturally occurs, Toll-9 is a constitutively active protein that binds with both Pelle and Cactus to activate the antifungal drosomycin gene. The similarities between Toll and Toll-9 were the basis for the research that identified the complex mechanisms by which Toll and TLRs function together in innate immunity responses [60, 78, 79].

Innate Immunity Gene Knockdowns that Suppress Glial Neoplasia in a *Drosophila* GBM Model

We identified the following gene as being a phenotypic suppressor of the GBM phenotype in our *dEGFR$^{\lambda}$; dp110^{CAAX}* model.

SARM – Sterile alpha and armadillo-motif containing protein (SARM) plays an important role in axonal death and neuronal degeneration after injury. It is believed to regulate signaling downstream effectors of TLRs and mediate cell autonomous cell death in a brain region-specific manner. Our morphological screen yielded contradictory results: the RNAi-targeted knockdown of SARM resulted in a less severe *dEGFR$^{\lambda}$;*

dp110^{CAAX} phenotype, indicating increased immunity against gliomagenesis. Interestingly, on other contexts, knockdown of SARM or its homologs result in increased susceptibility to infection due to decreased production of tumor necrosis factor α (TNFα), an inflammatory cytokine known to play an important role in infection and tumor resistance [80-82]. These results, which may suggest that SARM acts in opposition to the Toll receptor signaling pathway, will be revisited.

An Overexpression Screen Identified Innate Immunity Pathways as Cell-Autonomous Suppressors

A previous *Drosophila* screen conducted in the lab used UAS overexpression constructs to identify secreted factors that drive glioblastoma initiation and progression. (unpublished data, N. Shah and R. Read) Using our *dEGFR*$^{\lambda}$*; dp110*^{CAAX}model and the enhancer-suppressor screen design (Figure 5.A), we that Toll-9 overexpression suppressed glial neoplasia (Figure 5.B), indicating that gain-of-function of the Toll-9 pathway may counteract glial cell transformation. These results are consistent with our RNAi-based screen wherein loss-of-function by RNAi knockdown of the Toll-9 and other Toll pathway members, such as spz, enhances glial cell transformation, particularly in males. The morphological UAS-based overexpression screen classified Toll-9 as a phenotypic suppressor, as Toll-9 overexpression resulted in a reduced brain lobe size, and thus a less severe *dEGFR*$^{\lambda}$*; dp110*^{CAAX}phenotype. In this case, the resulting brains were similar in size to wild type brains. These results suggest that Toll-9 is a key innate immunity player in the development of *Drosophila* glial neoplasia.

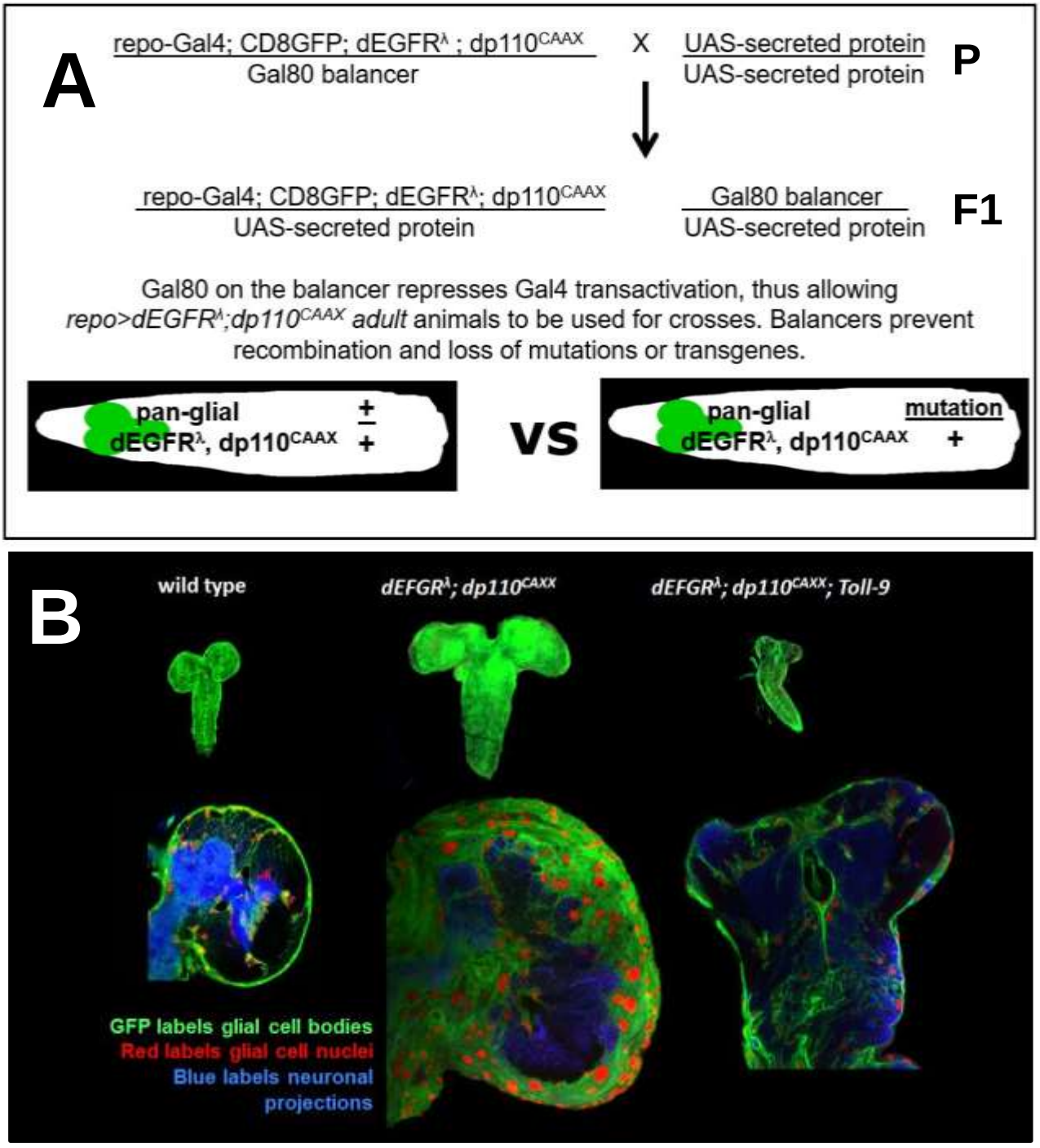

Figure 5: An overexpression screen reveals that Toll- 9 is involved in glial neoplasia. (A) Diagram of parental (P) genetic crosses and F1 progeny genotypes. The phenotypes of the progeny were compared to determine if *Drosophila* with dsRNA gene overexpression showed enhancement or suppression, relative to those with the UAS-lacZ control. (B) The results of the overexpression screen show that co-overexpression of EGFR, PI3K, and Toll-9 significantly reduces the number of neoplastic glia and suppresses the *dEGFR$^{\lambda}$; dp110^{CAAX}*phenotype, making it comparable to a wild type (repo>CD8GFP) larval brain. Age matched *Drosophila* whole larval brains (upper) and brain hemispheres (lower) are presented. Repo (red) labels glial cell nuclei; CD8-GFP (green) labels glial cell bodies; anti-horseradish peroxidase (blue) counterstains for neurons and neuropil.

Sex-Based Differences in Innate Immunity Gene Knockdowns in a *Drosophila* GBM Model

Based on the results from our morphological screens, I observed sex-based differences observed in larvae upon *spz* and *Toll-9* RNAi-targeted knockdown. To investigate these effects further, I prepared experimental *Drosophila* crosses using three RNAi constructs available in the lab for these genes and imaged the larval brains of resulting progeny to verify for sex-based differences in glial neoplasia. However, due to time constraints beyond our control, IMARIS analysis and further statistical analysis was not possible to quantify the degree to which each dsRNA construct significantly enhanced the $dEGFR^{\lambda}$; $dp110^{CAAX}$ phenotype.

Toll-9 – The morphological RNAi knockdown screen classified *Toll-9* as a phenotypic enhancer, as the absence of *Toll-9* function resulted in an increased brain lobe size, and thus a worsened $dEGFR^{\lambda}$; $dp110^{CAAX}$ phenotype, with males showing a stronger enhancement than females. Through confocal imaging of *Drosophila* larval brains, we observe a difference in larval brain lobe size based on sex when the *Toll-9* gene is knocked down via RNAi (Figure 6). This difference was observed in two separate RNAi constructs.

Spatzle – Our morphological screen of RNAi-targeted genes classified the associated gene, *spatzle*, as a $dEGFR^{\lambda}$; $dp110^{CAAX}$ phenotypic enhancer, indicating that Spatzle normally plays a role in suppressing gliomagenesis and GBM progression. Through confocal imaging of *Drosophila* larval brains, we also observe a difference in larval brain lobe size based on sex when the *spatzle* gene is knocked down via RNAi (Figure 6).

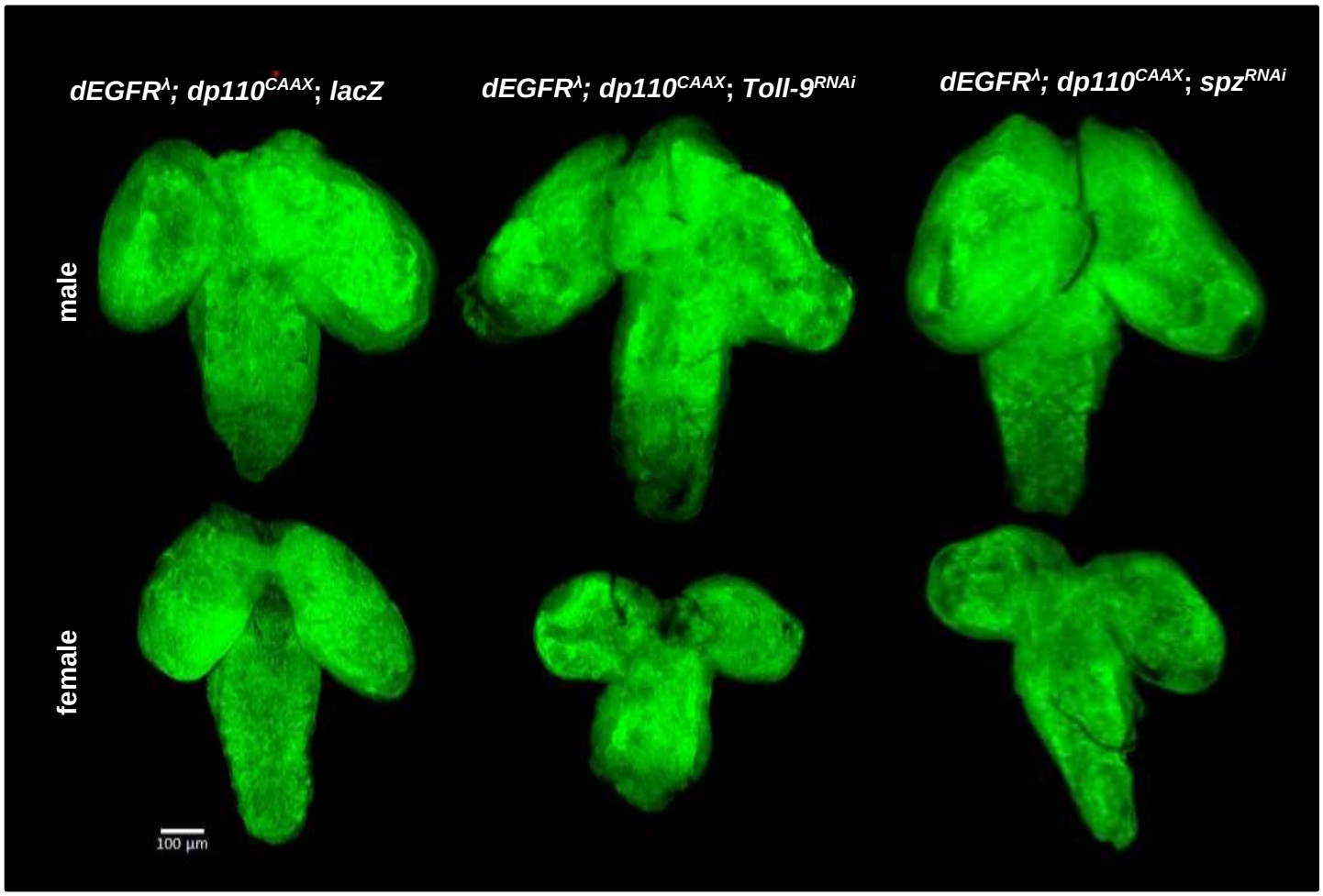

Figure 6: RNAi-targeted knockdown constructs reveal a sex-based difference in *dEGFR$^{\lambda}$; dp110^{CAAX}* phenotypic expression. Representative whole *Drosophila* larval brain images reveal the sexually differentiated effect of Toll-9 knockdown (*dEGFR$^{\lambda}$; dp110^{CAAX}; Toll-9^{dsRNA}*) and spz knockdown (*dEGFR$^{\lambda}$; dp110^{CAAX}; spz^{dsRNA}*) in our GBM model. Experimental images are compared to *dEGFR$^{\lambda}$; dp110^{CAAX}; lacZ* images to recognize the innate sex-based disparity in brain size of our model. Additional experimental replicates, image analysis, and statistics will be required before definitive conclusions can be made regarding the observed sex-based difference.

Controlling for Sex-Based Differences as a Result of Other Signaling Pathways or Environmental Factors in *Drosophila*

Sex-based differences in neural development may affect signaling pathways in general. Recent National Institute of Health (NIH) guidelines have mandated that, in their design and execution, research projects should address sex-based differences that may impact any conclusions drawn from the project. In *Drosophila* specifically, we observed variability, though no statistically significant difference, in larval brain lobe size between wild type control males and females (Figure 7)

Perhaps the variation we observed is due our collection method: female *Drosophila* larvae appear to mature faster than males, and they will tend to start the wandering phase before pupal formation a few hours before male larvae do [52]. Because I collected larvae for dissection only once daily, this observed difference could have also stemmed from variability among the specific male and female animals that I dissected, some of which may have been older others (we collected eggs over a 24 hour period prior to aging the larvae to 3rd instar).

Another possibility is a sexual dimorphism within the EGFR itself. The EGFR signaling pathway is thought to play a role in the sex-based differences in glioblastoma initiation and proliferation in humans [29]. Our *Drosophila* model for GBM relies on the constitutive activation of the EGFR pathway. When observing larval brain lobe size of *dEGFR$^{\lambda}$; dp110^{CAAX} Drosophila*, we also see a difference in brain lobe size when comparing males and females (Figure 7). As our *Drosophila* GBM model was creating using FLP-FRT recombination on the x chromosome [2, 52] this difference may be a result of a sex-based sex chromosome dosage effects, though further investigation will be necessary before any conclusions are made.

To further investigate this sex-based difference, were interested in exploring the role of downstream effectors within the EFGR pathway (Figure 1.A), as there is evidence for their role in the sex-based disparities seen in humans [25, 29]. Our lab had genetic *Drosophila* constructs available to explore the PTEN lipid phosphatase and the RAS GTPase within the EGFR signaling pathway of wild type flies (Figure 8) [2]. Co-activation of a constitutively active RAS variant (Ras^{V12}) and a dsRNA-targeted knockdown of PTEN (existing on the 2nd chromosome in one construct and the 3rd chromosome in the other) had no sex-dependent effect on *Drosophila* larval brains.

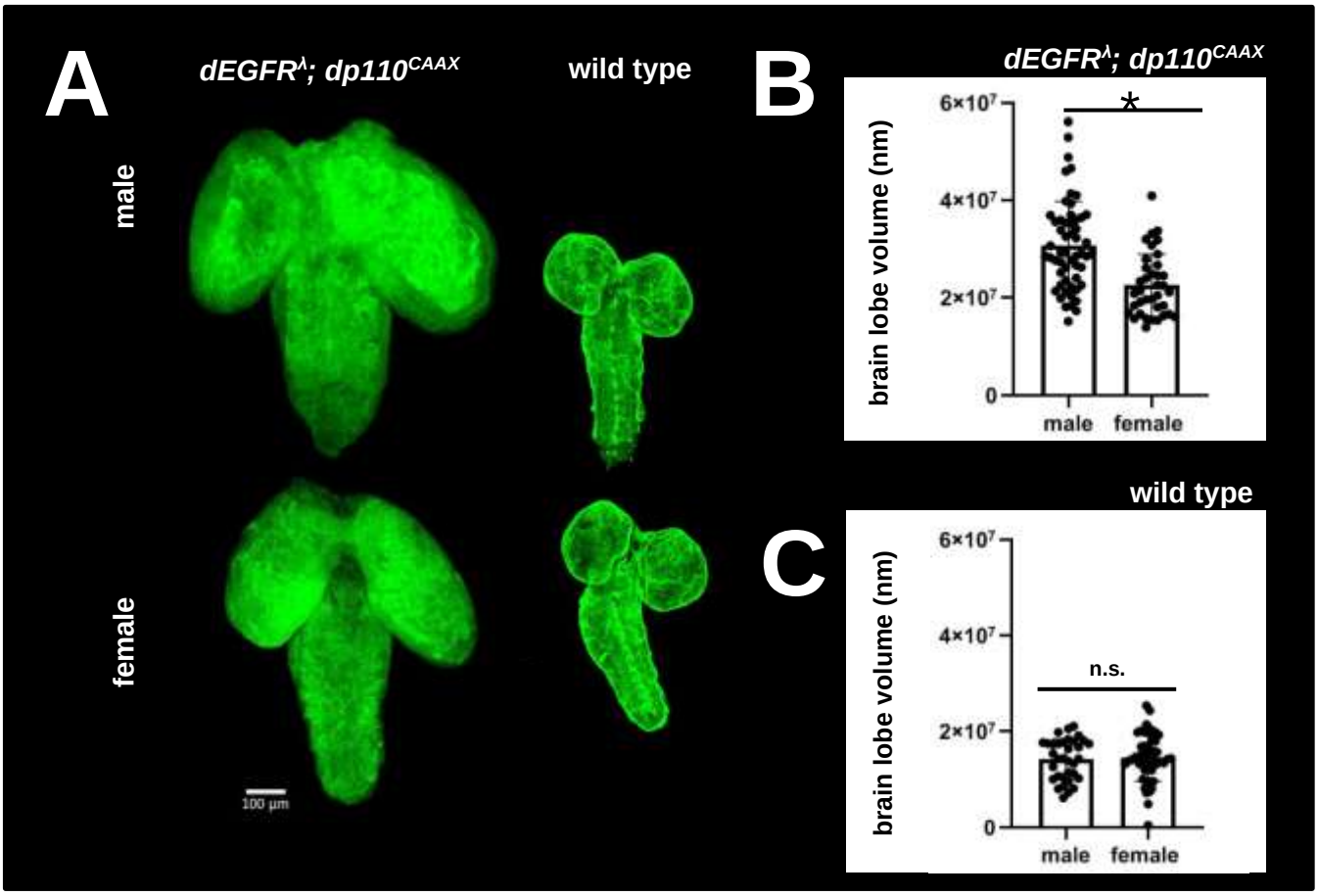

Figure 7: Analysis of larval brain images quantify an innate sex-based variability in our *Drosophila* GBM model. (A) Representative whole *Drosophila* larval brain images featuring the *dEGFR$^{\lambda}$; dp110^{CAAX}* GBM phenotype and the *dEGFR$^{\lambda}$; dp110^{CAAX}*; *lacZ* wild type phenotype. (B)IMARIS analysis of whole *dEGFR$^{\lambda}$; dp110^{CAAX} Drosophila* larval brain images reveals a statistically significant difference in brain lobe volume based on sex. Data points represent values of individual brain samples, columns represent average brain volume across samples, and error bars express the standard deviation from the mean; difference between means analyzed via independent t-test ($p = .0008$). (C) IMARIS analysis of whole wild type *Drosophila* larval brain images reveals no significant difference in brain lobe volume based on sex. Data points represent values of individual brain samples, columns represent average brain volume across samples, and error bars express the standard deviation from the mean; difference between means analyzed via independent t-test ($p = .348$)

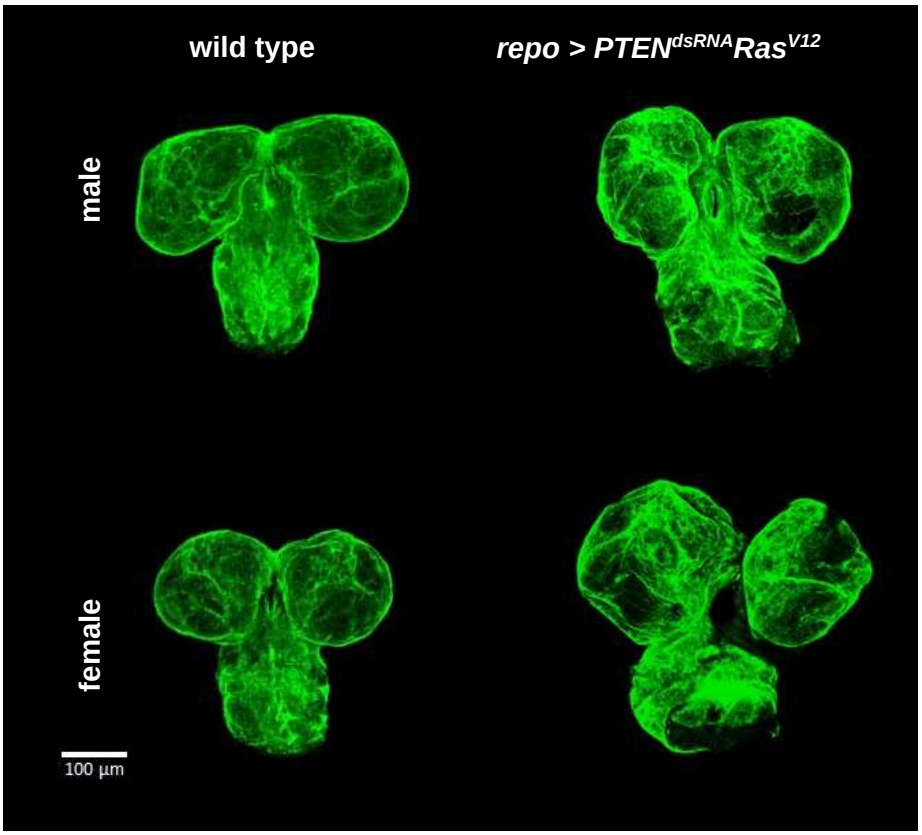

Figure 8: Co-activation of EGFR signaling components has no sex-based effect on *Drosophila* larval brains. Representative whole *Drosophila* larval brain images reveal no sex-based difference as a result of the co-expression of constitutive Ras expression (Ras^{V12}) and dsRNA-targeted overexpression of PTEN in wild type brains.

Sex-independent variability previously characterized in [2].

DISCUSSION

This project served as an exploratory introduction into the interaction between the Toll-signaling innate immunity pathway and the proliferation of glioblastoma multiforme based on biological sex in *Drosophila melanogaster* models. There is substantial evidence that a sex-based difference exists for both innate immune response and for glioblastoma incidence [25, 43]. In both cases, biological males are seen to have the lesser preferred outcome, having a less enhanced innate immunity system and a greater incidence of GBM, along with poorer prognosis [25, 43]. Given these parallels we sought to identify possible signaling pathway components that were affected in both situations.

An initial morphological RNAi-targeted screen allowed us to classify known innate immunity gene knockdown constructs as phenotypic enhancers or phenotypic suppressors of the GBM-simulating $dEGFR^{\lambda}$; $dp110^{CAAX}$phenotype. Though RNAi-targeted gene knockdown constructs were not available for every component of the Toll signaling pathway, we were able to elucidate 8 phenotypic enhancers and 1 phenotypic suppressor. As this screen looked at *Drosophila* phenotypes as a result of gene knockdown, we can assume that the opposite classifications are more descriptive of wild type gene function: those gene whose RNAi-targeted knockdown constructs enhanced the GBM phenotype are likely involved naturally in preventing gliomagenesis through innate immune responses, while those genes whose RNAi-targeted knockdown constructs suppressed the phenotype probably aid in tumor proliferation.

This hypothesis was supported for one associated gene, *Toll-9*, by a secondary dsRNA-targeted overexpression screen. In this case, genes whose dsRNA-targeted constructs were classified as phenotypic suppressors paralleled the probable suppression of GBM proliferation caused by naturally occurring gene expression. This

screen identified the dsRNA-targeted gene overexpression construct as a strong phenotypic suppressor. The resulting larval brain was comparable in size to that of a wild type fly, further confirming the role of the *Toll-9* as a key player in immunity against GBM.

Subsequent larval brain imaging of *dEGFR$^{\lambda}$; dp110^{CAAX}; RNAi* flies demonstrated a sex-based difference in GBM phenotype expression for both *Toll-9* knockdowns and *Spatzle* knockdowns. In all, the data collected during the course of this project provide preliminary support for the interaction between innate immunity Toll signaling pathway components and the known sex-based differences in GBM.

Additional experimentation with *Spatzle*, an associated gene found to be a phenotypic enhancer in our RNAi-targeted morphological screen, yielded contradictive results (Appendix I, Figure A1). A UAS overexpression construct for the Spatzle ligand resulted in phenotypic suppression, as compared to the *dEGFR$^{\lambda}$; dp110^{CAAX}* phenotype, though this suppression was only evident in female *Drosophila* brains. These data contradict the preliminary conclusions drawn from the original RNAi-targeted knockdown screen: If males are more sensitive to alterations in Spz-Toll signaling, as concluded previously, then we would expect to observe *dEGFR$^{\lambda}$; dp110^{CAAX}* phenotypic suppression in males when the SPZ ligand is ectopically activated. Due to circumstances outside of our control, experimental replicates and image analysis were not available to confirm the validity of these results, and further investigation will be necessary.

Other pathways that our lab has identified that contribute to neoplastic transformation of glia include the dRIOK2 kinase signaling pathway and its newly identified effector protein, Imp, which is an RNA-binding protein. Given the connection between these genes and the PI3K and Myc pathways, we investigated whether the

dRIOK2 and Imp interaction had any impact on sex-based differences in glial neoplasia in our *Drosophila* model. To address this, we used UAS-dsRNA and overexpression constructs for RIOK2 and Imp, respectively, within our Drosophila GBM model. Though we did not observe any statically significant sex-based differences in these experiments (Appendix I, Figure A2), it is possible that other RNA binding proteins could mediate sex-based differences in gliomagenesis, and could have a role in innate immune signaling pathways given their multifunctional roles in the CNS.

For the purposes of this project, *Drosophila melanogaster* served as the ideal model system. While our laboratory has the ability to bring these findings into mouse models, a fly model remains advantageous during the exploratory phase of this research. The RTK signaling pathways that play essential roles in the both neural and disease development are among the most highly conserved between *Drosophila* and humans. Flies also lack adaptive immunity, so any antitumor responses seen in our *Drosophila* GBM model will be a direct result of an innate immune response.

The continued use of fly modeling in this project will allow for a more comprehensive screen of, and investigation into the Toll signaling pathway components and their effect on gliomagenesis. It is also possible that sequencing and analysis of the submitted *Drosophila* RNA will provide further insight into the molecular players within the observed sex-based differences, in which case a fly model may result in the timely identification of therapeutic targets within this pathway, which could be further verified in corresponding mammalian model systems [83]. Until that point, the simplified, fully sequenced genome and numerous existing genetic manipulation techniques will allow for further investigation of the interaction between innate

immunity and glioblastoma and may elucidate potential conserved interactions in human disease as well.

The CNS, specifically, has a complex, functioning immune system, and GBM utilizes many strategies to evade these immune responses [37]. In order to more effectively attend to glioblastoma, and as cancer treatment, generally, moves toward individualized, therapeutic treatments, the role of tissue-based innate immune responses will become ever more relevant, as will the consideration of biological sex.

Sex is a critical factor in the incidence, prognosis, and mortality of cancer. Up until recently, females were not included as participants in medical research of any kind, including animal model studies [84]. The recent NIH guidelines mandating research projects address sex-based differences that may impact conclusions is a step in the right direction, and will likely result in new conclusions for questions that were previously presumed answered [85].

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APPENDIX I:
SUPPLEMENTARY FIGURES

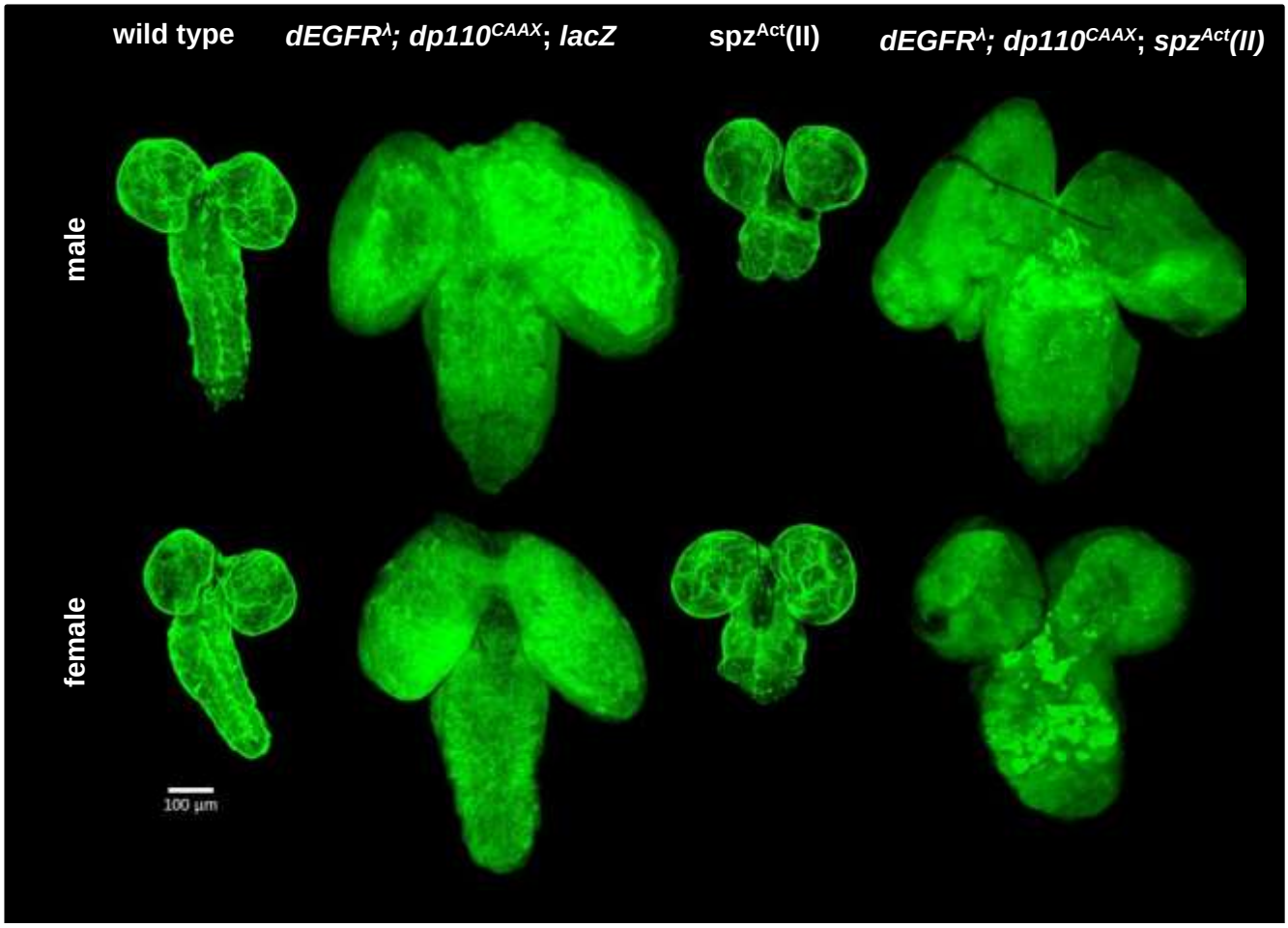

Figure A1: Overexpression of the spz^{Act}(II) *ligand in our dEGFR$^{\lambda}$; dp110^{CAAX}* model results in a confounding sex-based difference in brain lobe size. Representative whole *Drosophila* larval brain images reveal a sexually differentiated effect of SPZ ligand overexpression (*dEGFR$^{\lambda}$; dp110^{CAAX}; spz^{Act}(II)*) in our GBM model. SZP ligand overexpression in wild type *Drosophila* has no effect. Experimental images are compared to *dEGFR$^{\lambda}$; dp110^{CAAX}; lacZ* to recognize the innate sex-based disparity in brain size of our model.

Based on the images presented, we are unable to make any conclusions from this data. The sex-based difference in brain size of the *dEGFR$^{\lambda}$; dp110^{CAAX}; spz^{Act}(II)* samples is likely a result of the innate sex-based disparity of our model in conjunction with a small sample size. Due to circumstances outside of our control, we were unable to replicate this experiment.

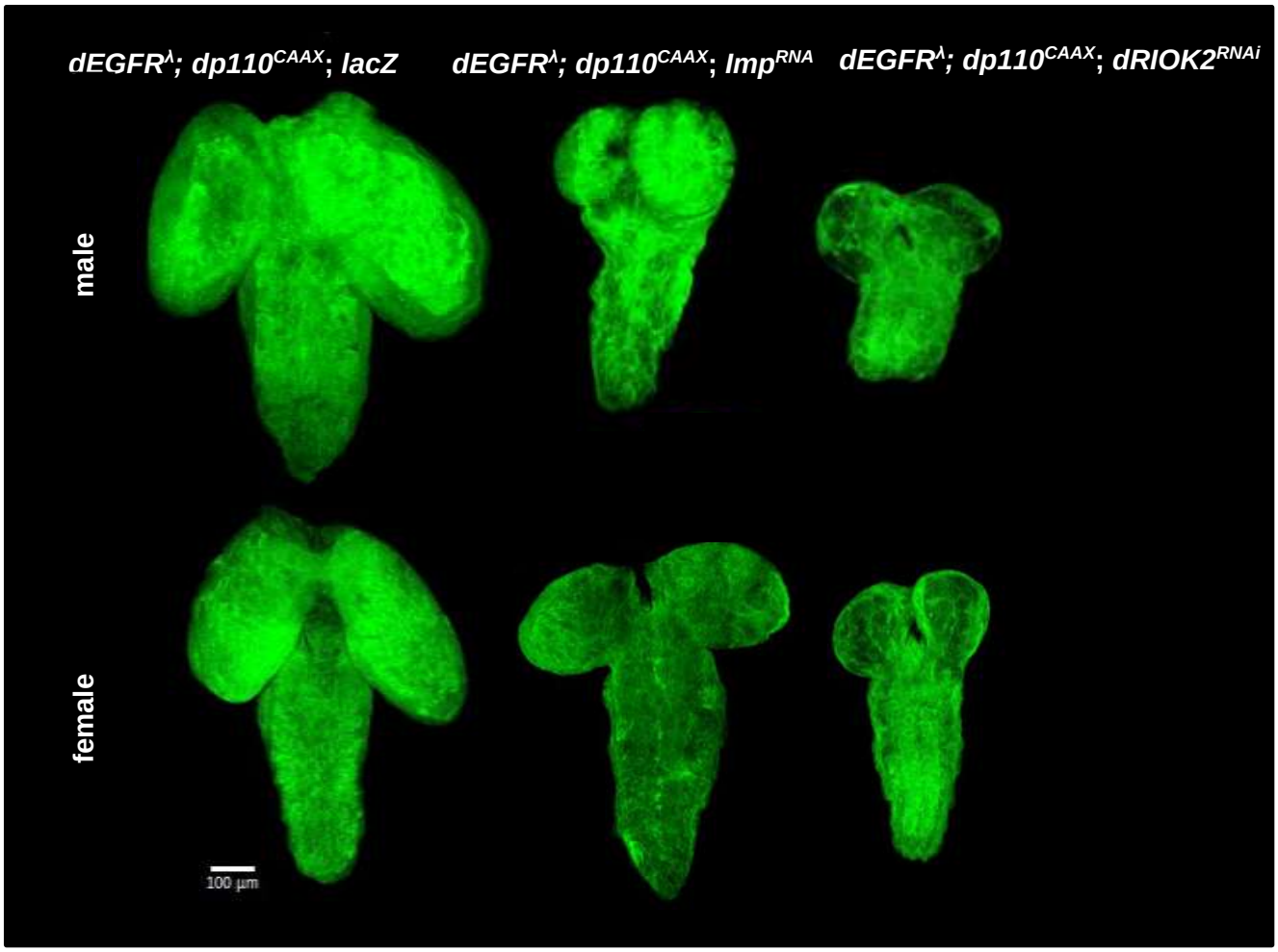

Figure A2: Expression manipulation of dRIOK2 kinase signaling pathway components does not result a sex-based difference in brain size. Representative whole *Drosophila* larval brain images reveal no sexually differentiated effect in IMP overexpression (*dEGFR$^{\lambda}$; dp110^{CAAX}; Imp^{RNA}*) or RIOK2 knockdown (*dEGFR$^{\lambda}$; dp110^{CAAX}; dRIOK2^{RNAi}*) in our GBM model. Experimental images are compared to *dEGFR$^{\lambda}$; dp110^{CAAX}; lacZ* images to recognize the innate sex-based disparity in brain size of our model.

The sex-independent effects of IMP overexpression and RIOK2 knockdown have been thoroughly characterized previously in our lab (unpublished data, A. Chen and R. Read).

APPENDIX II:
PROTOCOLS

Protocol for *Drosophila melanogaster* larval brain dissection in preparation for live tissue confocal imaging

Materials and Reagents

- 1x phosphate-buffer saline (PBS)
- 4% paraformaldehyde
- 1x phosphate-buffer saline + 0.3%Triton (PBST)
- 2% sodium azide

Equipment

- porcelain 12-well spot plate
- bamboo pick
- Zeiss V16 fluorescent microscope
- P-100, 1000 micropipettes
- Zeiss stereomicroscope
- silicone-based dissection plate
- forceps
- 96-well plate
- clear tape

Procedure

1. Pipette 750µl 1xPBS into 4 wells of a porcelain 12-well spot plate.
2. Use bamboo pick to collect *Drosophila* larvae from desired vial, placing them in one PBS-filled well of the spot plate.
    i. To ensure suitable larvae are collected, place the plate under a Zeiss V16 fluorescent microscope and ensure all larval brains express GFP.
3. Once all larvae are collected, stir larvae in PBS to remove excess food and other contaminants from animal bodies. Transfer larvae into a second PBS-filled well and stir again to clean.
4. Under a Zeiss stereomicroscope, separate larvae by sex, placing males into a third PBS-filled well and females into a fourth.
5. Transfer one well into a PBS pool on a silicone-based dissection plate.
6. Dissect larval brains by lightly grasping the larval body with one pair of forceps while another is used to grasp the animal's mouthparts; hold the larval body while quickly pulling the mouthparts away and allow CNS and surrounding tissue to spill into the PBS pool.
7. Separate and remove larval brain and enough extraneous tissue to use as a grip when transferring samples.
    i. Be sure to remove the gut and pharynx, so as to avoid possible contamination from bacteria, yeast, and food within the digestive tract.
8. Place dissected brains in a separate PBS pool on the silicone-based dissection plate.

9. Once enough larval brains are collected, transfer them to a well in a 96-well plate prepared with 100µl 4% paraformaldehyde. Allow samples to fixate for 60 minutes.
10. After fixation, aspirate the 4% paraformaldehyde using a micropipette.
    i. Be careful not to cruse the samples with the pipette tip or to aspirate them with the fixative.
11. Wash the samples by adding in 100µl PBST. Allow to sit for 10 minutes. Aspirate the PBST and immediately replace it with another 100µl.
    i. Repeat this washing step three to five times.
12. Add 2 pipette drops of 2% sodium azide for preservation.
13. Tape up well with an air-tight seal and store in 4°C refrigerator.
14. Repeat this process for the remaining larvae of the opposite sex and for all other genotypes.

Protocol for *Drosophila melanogaster* larval brain dissection in preparation for RNA extraction

Materials and Reagents

- RNAse-Zap (Thermo Fisher Scientific, AM9780)
- 1x phosphate-buffer saline (PBS)
- Trizol (Thermo Fisher, 15596026)

Equipment

- porcelain 12-well spot plate
- bamboo pick
- P-100, 1000 micropipettes
- Zeiss LSM 700 stereomicroscope
- sterile petri dish
- forceps
- sterile surgical razor blade
- 1.5 ml microcentrifuge tube

Procedure

1. Sanitize workspace and all materials and equipment with RNAse-Zap.
2. Use bamboo pick to collect *Drosophila* larvae from desired vial, placing them in one PBS-filled well of the spot plate.
    i. To ensure suitable larvae are collected, place the plate under a Zeiss V16 fluorescent microscope and ensure all larval brains express GFP.
3. Once all larvae are collected, stir larvae in PBS to remove excess food and other contaminants from animal bodies. Transfer larvae into a second PBS-filled well and stir again to clean.
4. Under a Zeiss LSM 700 stereomicroscope, separate larvae by sex, placing males into a third PBS-filled well and females into a fourth.
5. Sanitize forceps with RNAse-Zap.
6. Transfer one well into a PBS pool on a sterile petri dish.
7. Dissect larval brains by lightly grasping the larval body with one pair of forceps while another is used to grasp the animal's mouthparts; hold the larval body while quickly pulling the mouthparts away and allow CNS and surrounding tissue to spill into the PBS pool.
8. Separate and remove larval brain and enough extraneous tissue to use as a grip when transferring samples.
    i. Be sure to remove the gut and pharynx, so as to avoid possible contamination from bacteria, yeast, and food within the digestive tract.
9. Place dissected brains in a separate PBS pool on the sterile petri dish.
10. Once enough larval brains are collected, use a sterile, surgical razor blade and freshly forceps freshly sanitized with RNAse-Zap to remove all excess tissue.

11. Place brains into a final PBS pool on the sterile petri dish.
    i. When transferring brains without surrounding tissue, use forceps to collect a drop of PBS containing each brain.
12. Sanitize forceps again with RNA-Zap.
13. Transfer all brains into a 1.5 ml microcentrifuge tube prepared with 250µl of Trizol. Allow samples to dissociate.
    i. To assist with tissue dissociation, use a micropipette and a sterile tip to lyse the sample and Trizol a few times.
    ii. Samples in Trizol should be kept on ice or in -80°C freezer. DO NOT store at room temperature.
14. Repeat this process for the remaining larvae of the opposite sex and for all other genotypes.
15. Store samples in -80°C freezer.

Protocol for Immunohistochemical staining of *Drosophila melanogaster* whole larval brains

Materials and Reagents

- 1x phosphate-buffer saline + 0.3%Triton (PBST)
- Primary Antibody Stain
    - 1x phosphate-buffer saline + 0.3%Triton (PBST)
    - Natural Goat Serum (NGS) (1:10)
    - Anti-Repo (1:10)
    - 1% sodium azide (1:10)
- Secondary Antibody Stain (need recipe)
    - 1x phosphate-buffer saline + 0.3%Triton (PBST)
    - Natural Goat Serum (NGS) (1:10)
    - HRP647 (1:50)
    - anti-mouse Cy3 (1:200)
- VectaShield

Equipment

- 1.5 ml microcentrifuge tube
- P-10, 100, 1000 micropipettes

Procedure

1. Prepare primary antibody satin in 1.5 ml microcentrifuge tube. Store at 4°C until ready for use.
2. Remove dissected larval brain samples from 4°C refrigerator and aspirate PBST and 1% sodium azide.
3. Wash the samples by adding in 100µl PBST. Allow to sit for 10 minutes. Aspirate the PBST and immediately replace it with another 100µl.
    i. Repeat this washing step three to five times.
4. Replace final PBST wash with 100µl primary antibody stain.
5. Store samples in 4°C refrigerator for 48 hours.
6. Prepare secondary antibody stain in 1.5 ml microcentrifuge tube. Store at 4°C until ready for use.
7. Remove samples from 4°C refrigerator and aspirate primary antibody stain.
    i. It is possible to reuse primary antibody stain, so collect it in an additional 1.5 ml microcentrifuge tube.
8. Wash the samples by adding in 100µl PBST. Allow to sit for 10 minutes. Aspirate the PBST and immediately replace it with another 100µl.
    i. Repeat this washing step three to five times.
9. Replace final PBST wash with 100µl secondary antibody stain.
10. Store samples in 4°C refrigerator for 24 hours.
11. Remove samples from 4°C refrigerator and aspirate secondary antibody stain.
12. Suspend samples in Vectashield and store in 4°C refrigerator.

Protocol for mounting *Drosophila melanogaster* whole larval brains onto microscope slides using a bridge technique

Materials and Reagents

- VectaShield
- clear nail polish

Equipment

- Glass microscope slides
- forceps
- Zeiss stereomicroscope
- surgical razor blade
- glass cover slips

Procedure

1. Drop Vectashield on center of microscope slide and transfer dissected larval brains using forceps.
2. Under a Zeiss stereomicroscope, use forceps and surgical razor blade to remove and discard excess tissue from larval brain.
3. Align brains in a single vertical line down the center of the microscope slide. Ensure all brains are oriented in the same way.
4. Prepare 'bridge' by breaking a glass coverslip in half and placing one half on either side of the line of brains.
5. Add additional VectaShield if necessary and check to ensure correct alignment and orientation of brains. Remove any remaining air bubbles from the Vectashield using a surgical razor blade.
6. Slowly place a full cover slip on top of 'bridge.'
7. Seal edges of the cover slip and 'bridge' to ensure no VectaShield leaks. Allow nail polish to dry completely before storage.
8. Store samples at 4°C

Protocol for RNA extraction from *Drosophila melanogaster* whole larval brain live tissue

Materials and Reagents

- RNAse-Zap (Thermo Fisher Scientific, AM9780)
- *Drosophila* brain tissue samples suspended in Trizol (Thermal Fisher, 15596026)
- Chloroform
- isopropyl alcohol
- 70% ethanol
- Nuclease free water

Equipment

- centrifuge
- 1.5 ml microcentrifuge tubes
- heat block at 55°C
- P-10, 100, 1000 micropipettes

Procedure

1. Sanitize workspace and all materials and equipment with RNAse-Zap.
2. Precool centrifuge to 4°C.
3. Remove RNA samples suspended in Trizol from the -80°C freezer. Allow to thaw at 30°C for 5 minutes.
4. Add 250µl chloroform to 1.5ml microcentrifuge tube containing RNA sample and Trizol. Shake sample for 15 seconds to incorporate.
5. Spin sample (and appropriate balance) in prechilled centrifuge at 14,000 RPMs for 10 minutes.
    i. At the end of the centrifugation, observe: 1) a pink/reddish organic phase at the bottom, 2) a small white interphase with amphipathic proteins soluble in both water and lipid, and 3) and upper clear aqueous phase containing the dissociated brain tissue.
6. Remove the aqueous phase and transfer to a new 1.5 ml microcentrifuge tube. Add equal volume isopropanol alcohol and incorporate by inverting the microcentrifuge tube.
7. Incubate sample at 30°C for 10 minutes.
8. Spin sample (and appropriate balance) in prechilled centrifuge at 14,000 RPMs for 30 minutes or longer if necessary.
9. Decant off the supernatant, being careful not to disturb the RNA pellet.
10. Add 500µl 70% ethanol to the sample to rinse pellet.
11. Spin sample (and appropriate balance) in prechilled centrifuge at 14,000 RPMs for 10 minutes.
12. Decant off the supernatant and place microcentrifuge tube upside down on a paper towel to allow the remaining alcohol to evaporate.
    i. DO NOT let the pellet completely dry out or become clear in color.

ii. Use micropipette or forceps and paper towel to ensure all alcohol is removed from sample.

13. Resuspend RNA pellet in 20µl nuclease free water.
14. Incubate in heat block at 55°C for 10 minutes.
15. Store in -80°C freezer.