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Title Exploring The Phenotypic Effects of Toll Signaling Pathways In Tumor Progression in EGFR-driven Glioblastoma Models

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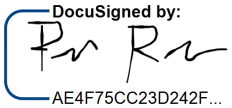

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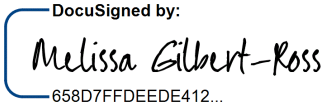

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Exploring The Phenotypic Effects of Toll Signaling Pathways In Tumor Progression in EGFR-driven Glioblastoma Models

By
Julia G. Saborio

B.S. Emory University, 2022

Advisor: Renee Read, PhD

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, 2023

Abstract

Exploring The Phenotypic Effects of Toll Signaling Pathways In Tumor Progression in EGFR-driven Glioblastoma Models

By
Julia G. Saborio

The treatment options for Glioblastoma (GBM) are currently limited to resection and chemotherapy approaches, but resistance to chemotherapy is common in patients. There is an urgent need for further understanding of the underlying biology of tumorigenesis in order to develop new treatment options. Previous genomic work on GBMs has shown that the epidermal growth factor receptor (EGFR) is frequently amplified and constitutively active in GBM tumors. To discover new factors that contribute to progression of EGFR-driven GBMs, we used our *Drosophila* GBM model in which GBM-like glial neoplasia can be induced by glial-specific overexpression of constitutively active forms of EGFR and dp110, a subunit of Phosphoinositide 3-kinase (PI3K), using the *UAS-Gal4* gene expression system. Using our *Drosophila* GBM model system, we performed a modifier screen in which we searched for genes and pathways that worsened or 'enhanced' glial neoplasia when specifically overexpressed or activated in neoplastic glia. We also performed a transcriptome analysis comparing wild-type larval brains to those expressing a *repo > dEGFR$^{\lambda}$;dp110^{CAAX}* genotype. From our screen and RNAseq data, we identified the Toll pathway, which in *Drosophila* activates an NF-KB signaling pathway. Furthermore, the Toll pathway normally functions in innate immune and injury responses in the brain as well as in development and cell proliferation. Using our *Drosophila* GBM model, we are studying how the Toll signaling pathway and its effectors, including NF- KB, contribute to enhanced growth of neoplastic glial cells. We will use genetic manipulation approaches such as RNAi constructs under the *UAS-Gal4* system to manipulate expression of Toll components in glia, and then evaluate phenotypic changes through volumetric analysis, cell counting, confocal microscopy, and immunohistochemical staining.

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Introduction and Background

Glioblastoma presentation in the clinic and epidemiology

Glioblastoma is the most common primary malignant brain tumor in adults[1]. It arises from neoplastic transformation of glial stem or progenitor cells in the brain[1]. Glioblastomas are highly aggressive tumors with an age-adjusted incidence of 3.22/100,000 persons and a poor prognosis[1]. Disease free survival after 5 years is only 6.8%, and the average survival after diagnosis is 15-18 months[1].

After diagnosis, the current standard of care is surgical resection followed by radiotherapy and chemotherapy with Temozolomide, a DNA alkylating agent[2]. However, due to the unique tumor microenvironment, tumor cell heterogeneity, and location of the tumor, most tumors progress after treatment[1]. The recurrence rate of glioblastoma in patients is about 90%[2]. Although targeted therapies and immunotherapies have been successful in other solid tumor types, the highly immunosuppressive microenvironment of glioblastoma have made treatment unsuccessful, especially in recurrent tumors[2]. Thus, there is an urgent need for a deeper understanding of the biology of glioblastoma tumorigenesis in order to uncover new therapeutic targets.

EGFR-driven Glioblastoma biology

Glioblastomas are high-grade gliomas (HGG), and are histologically characterized through pseudopalisading necrosis and microvascular proliferation[3]. There is a high amount of genetic and molecular heterogeneity between patients and within each individual tumor, which

makes glioblastomas particularly aggressive and resistant to therapy[1]. This also highlights the importance of characterizing oncogenic signaling pathways in these tumors that could be exploited in combination therapies. Furthermore, the tumor microenvironment consists of infiltrating and resident immune cells, such as tumor-associated macrophages, and other non-neoplastic glial cell types [3]. These cells are distinct from the neoplastic tumor cells, but contribute to tumorigenesis and have the ability to activate signaling pathways in tumor cells through extracellular signaling[3].

The Cancer Genome Atlas (TCGA) initiative has characterized the main genetic alterations driving glioblastoma as the dysregulation of receptor tyrosine kinase (RTK) signaling, activation of the Phosphoinositide 3-kinase (PI3K) pathway, and inactivation of the p53 tumor suppressor[4]. Glioblastomas show high Pi3K and Akt activity[5]. In tumor cells, Akt activation is dependent on Pi3K activity, as shown in Haas-Kogan et al[5], and requires phosphorylation on Serine 473 for oncogenic activity[6]. PTEN, a tumor suppressor, encodes a phosphatase that inactivates Pi3K activity through dephosphorylation of the kinase, and Akt signaling can be increased by PTEN mutation[5]. As this is a central regulator of cell proliferation, immune evasion, and tumor formation, the Pi3K-Akt pathway is key to tumorigenesis.

Glioblastomas are further subtyped by gene expression into Proneural, Mesenchymal, and Classical glioblastomas[4]. Verhaak et al. used profiling data from The Cancer Genome Atlas in order to subclassify glioblastoma by patterns of somatic mutations and DNA copy number[7]. They classified Mesenchymal subtype as having higher overall necrosis and inflammatory infiltrates, Proneural as having upregulation of proneural development genes like *SOX2,* and Classical as having neural precursor and stem cell markers such as EGFR mutations, CDKN2A

loss, and Notch upregulation[7]. Aberrations in EGFR, or Epidermal growth factor receptor, are associated with Classical glioblastoma, and EGFR-driven glioblastoma will be the focus of the work following[4]. The EGFR gene codes for a transmembrane protein that is part of the receptor tyrosine kinase family[8]. When activated by binding of a ligand, there is dimerization of the receptor, which leads to a downstream signaling cascade promoting cell proliferation[5]. EGFR signaling is frequently amplified in neoplastic glioblastoma cells[1]. Most commonly, EGFR signaling is constitutively active due to the presence of the variant III mutation, in which deletion of the extracellular domain creates a constitutively active EGFR mutant variant (EGFRvIII) [3]. This genetic alteration leads to the promotion of cell proliferation, evasion of growth suppressors, and resistance to apoptosis[3]. Chen *at al* was able to create mouse genetic models of Proneural glioblastoma by delivering RCAS-PDGB and RCAS-shRNAp53, Mesenchymal glioblastoma by delivering RCAS-shRNA-Nf1, RCAS-shRNAp53, and RCAS Cre, and Classical glioblastoma driven by EGFRvIII mutant, PTEN knockout, and Cdkn2a knockout[3]. All three murine models induced expression of specific markers associated with their respective subtype[3], and the Classical model was used in the work following.

Although EGFR has been targeted using tyrosine kinase inhibitors (TKIs), tumors typically progress past TKI treatment due to cellular and molecular heterogeneity of these tumors and activation of parallel and compensatory pathways that can rescue tumor cells from TKI treatment [9]. We hypothesize that there is crosstalk between EGFR and other signaling pathways active in the tumor cells, and that these other pathways are also dysregulated in neoplastic glia. Alternate pathways may be able to rescue the cells and drive tumor progression and treatment resistance. Therefore, we aim to find alternate pathways that promote neoplasia and may be

useful targets for future treatment options. Due to injury and innate immune signals in the tumor microenvironment in response to neoplasia, there can be cell-autonomous activation of innate immune and injury response signaling pathways in tumor cells[10]. We aim to examine the effects of glial cell-intrinsic innate immune and injury response signaling on EGFR-PI3K driven tumorigenesis.

Innate immune and injury response signaling in glial cells

Injury response signaling in the brain is activated by pattern recognition receptors that recognize microbes and signaling by damaged or dying cells[11]. Activation of injury response signaling leads to neuroinflammatory signaling in astrocytes, as well as proliferation and differentiation in neuro-glial stem cells (mNSCs)[12]. Toll like receptors (TLRs) are transmembrane pattern recognition receptors activated by extracellular signaling[11]. TLRs can be activated by damage-associated molecular patterns (DAMPs), developmental signaling ligands, and microbes[8]. They were first characterized as Toll-family receptors in *Drosophila*. In mammals, this receptor family regulates expression of proinflammatory cytokines and activation of innate immune processes[8].

In *Drosophila,* Toll activation leads to transcription of genes with sequence motifs related to NF-κB response elements in mammals[11]. Some of the targets of Dorsal, which is the NF-κB homolog and transcriptional effector of the Toll signaling pathway, include Drosomycin (Drs), dpp, twi and sog, which function as antimicrobial peptides, regulators of cell growth, and developmental transcription factors, respectively. It has been well established that, in *Drosophila melanogaster*, functioning Toll transmembrane receptors are required for resistance to pathogen infection[11]. The components of the pathway were first identified through genetic

screening in the *Drosophila* embryo[11]. Toll signaling is activated by the cleavage of the ligand Spatzle (spz), which leads to signal transduction through adaptor proteins dMyD88 and Tube[8]. These adaptor proteins are required for immune response, and mutants do not mount a comparable response[11]. Cactus is bound to Dorsal and Dorsal-related immunity factor (Dif) in the cytoplasm[8]. Cactus is a homolog of mammalian IκB, and is degraded upon activation, leading to nuclear localization of Dorsal and AMP transcription[13]. The Toll pathway has been shown to be activated in patterning during development, the antimicrobial response, and in injury response[8].

The Toll pathway is evolutionary conserved between *Drosophila* and mammals, including humans and mice, as shown in figure 1, which allows us to use our *Drosophila* EGFR-PI3K driven model of glioblastoma to study its function in neoplastic glia. In mammals, published work shows that TLR4, in particular, is expressed and promotes proliferation and survival in human neuro-glial stem cells, and inhibition of TLR4 promotes apoptosis[14]. Furthermore, enhanced TLR4 expression has been characterized in glial cells in inflamed CNS tissues in multiple sclerosis[15].

In the canonical signaling pathway in mammalian systems, when TLRs are activated through DAMP or PAMP recognition, MyD88 and IRAK kinases form a complex to trigger a signaling cascade[16]. IRAK family kinases are autophosphorylated and released from the complex, leading to activation of the IKK kinases[13]. This leads to degradation of the IκB kinase complex through IκB phosphorylation through IKK, and therefore the release of the NF-κB transcription factor[13]. NF-κB then localizes to the nucleus and begins transcription of proinflammatory genes[16]. However, the role of TLR signaling in glioblastoma is controversial. A

study showed increased expression of several TLRS, including TLR1, TLR2, TLR4, and TLR6, in human glioblastoma cells and in high grade astrocytoma tumor tissue specimens as compared to non-neoplastic tissue[17]. The same study showed a correlation between TLR4 expression and tumor grade, suggesting that TLR signaling is associated with poor prognosis[17]. In contrast, a different study showed that TLR4 downregulation in glioblastoma cancer stem cells (CSCs) aid in maintenance of the stem-like state to promote tumorigenesis[18]. Yet, based on our experimental results presented below, we hypothesize that the Toll/TLR signaling pathways contribute to glial neoplasia.

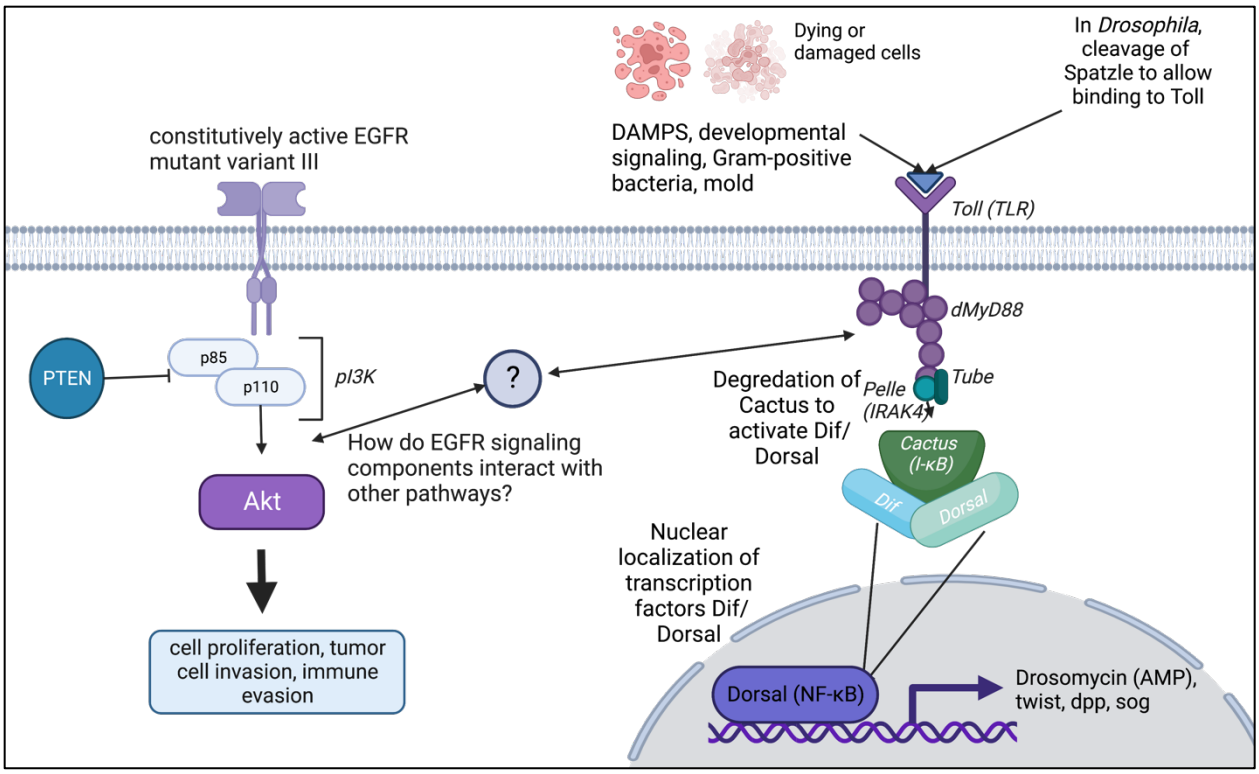

Figure 1. The EGFR and Toll signaling pathways. In human EGFRvIII mutant cells, the EGF receptor gene has a deletion of exons 2-7 of the coding sequence, which leads to shortening of the extracellular domain and a glycine residue[19]. This prevents any ligand from binding and results in constitutive activation of EGFR[19]. Signaling leads to cell proliferation, tumor invasion, and immune evasion through the phosphatidylinositol 3-kinase (Pi3K) signal transduction pathway and PTEN, a tumor suppressor, antagonizes the pathway[5]. Our *Drosophila* model is driven by dEFGR$^{\lambda}$, a constitutively active form of *Drosophila* EGFR[20], which also drives malignant cell proliferation and invasion[21]. Toll receptors respond to extracellular signaling to activate the Dorsal/Dif transcription factor in *Drosophila*, and this pathway is conserved in mammals as the canonical NF-κB pathway[11].

Scope of the thesis

The EGFR and Toll pathways are both implicated in glioblastoma tumorigenesis. We aim to find how these interact in models of EGFR-PI3K driven glioblastoma. Firstly, I will use our *Drosophila melanogaster* glioblastoma model to identify key players of Toll signaling in tumorigenesis driven by constitutive EGFR and PI3K activation. In part, this experimental work was done by a R. Gulbronson[22], who performed a phenotypic modifier screen in our *Drosophila* model and collected RNA for sequencing and comparison of control and tumor brains. I aim to inhibit or activate the pathway by transgene overexpression or RNAi using the Gal4-UAS system and then to evaluate the effects on glial neoplasia. Quantification of phenotypic changes will be done through confocal imaging for immunofluorescence staining followed by volumetric analysis of the brain lobes.

I will also use mammalian models in order to verify observations and further explore the effects of innate immune signaling activation in neuro-glial progenitor cells *in vitro*. Using a set of litter matched primary neural stem cell cultures, I will compare TLR signaling in cells with EGFRvIII mutation and PTEN knockout with matched controls. I will evaluate the effects of inhibition of both pathways.

Experimental methods

Phenotypic characterization of *Drosophila melanogaster* 3rd instar larval brains

The complete protocol for stable stock establishment and phenotypic characterization can be found in "A protocol to use *Drosophila melanogaster* larvae to model human glioblastoma" by Saborio et al[23]**.** *Drosophila melanogaster* were maintained at room temperature without controlled humidity. Virgin flies were collected from the F2 stock shown in Figure 2, since *Drosophila* can store sperm from previous mates for future fertilization of progeny. As controls, *repo>CD8 GFP Drosophila* virgins were also collected. Crosses were set up using 20-24 females and 6-10 males per vial, and a sprinkle of yeast was added to encourage egg lay. Crosses were then incubated at 25°C for 72 hours without disruption. After incubation, vials were flipped without anesthetizing once per day for 4 days, and flies remained in each vial for 24 hours. The vials containing eggs were kept in a 25°C incubator for 5 more days.

At day 5 after egg lay, 3rd instar wondering larvae of non-Tb phenotype, shown in Figure 2, were collected. Age matching is crucial to the model, as larvae experience a sharp increase in brain size between 2nd and 3rd instar stages. To confirm the desired phenotype from the cross, larvae were checked for glial cell bodies expressing GFP under the scope. Larvae were first sexed by the presence of gonads, as shown in Figure 2, and then whole brains were dissected in 1X PBS. In a 96 well plate separated by genotype and sex, brains were fixed in 1X PBS + 4% paraformaldehyde for one hour. The brains were then washed with PBS + 0.3% Triton and stored with 0.1% sodium azide for preservation.

Whole larval brains were immunohistochemically stained to prepare for confocal imaging. Repo, Cactus, or Dorsal antibodies (Developmental Studies Hybridoma Bank) were

used with an Alexa 555 secondary antibody (Jackson Immunolabs), and HRP was used to label neuropil. Stained brains were mounted in Vectashield and imaged at 40X on a LSM 700 confocal microscope (Zeiss). For volumetric analysis, 10X images were taken and volume of the brain lobes was calculated using Imaris imaging software.

RNA sequencing

RNA for sequencing was collected by R. Gulbronson and sequenced by Admera. R. Gulbronson sexed and age matched 3rd instar larvae from wild-type and *repo>dEGFR$^{\lambda}$;dp110^{CAAX}* flies staged according to procedures described in R. Gulbronson[22] and Saborio et al[23]. The ventral nerve cord was removed and only the brain lobes were used for RNA preparation. At least 3 brains were used for sample, duplicate total RNA samples were run for each genotype and sex, messenger RNA was prepared by polyA isolation, and RNA was sequenced at 40 millions reads per sample (20 million in each direction) for all replicates. After sequencing by Admera, raw read data was then acquired and processed using Alex's Lemonade Stand Childhood Cancer Data Lab bulk RNAseq analysis resources[24].

Mammalian *in vitro* transformed cultures derived from a mouse model

Chen et al. developed a genetically engineering mouse model for glioblastoma driven by EGFRvIII mutant, PTEN knockout, and Cdkn2a knockout[3]. For this model, commercially available mouse lines *Ntv-a, Cdkn2a$^{-/-}$, EGFRvIII $^{fl\text{-}stop\text{-}fl}$, and Pten$^{fl/fl}$* were crossbred to generate quadruple transgenic mice with the genotype *Ntv-a;Cdkn2a$^{-/-}$;EGFRvIII $^{fl\text{-}stop\text{-}fl}$;Pten$^{fl/fl}$* [3]. From the transgenic mice, we created a set of matched control murine neuro-glial stem cell (mNSC) cultures and transformed EGFR-Pi3K mNSC cultures. The *in vitro* work using primary mNSC cultures is from one litter of pups in order to control for genetic background. Furthermore, male and female pups were used in order to stratify for sex.

To create the cultures and their matched controls, mouse pups were euthanized at day 8 after birth, and their brains were immediately dissected. The dissected tissue was then dissociated and the neuro-glial progenitor cells cultured in DMEM/F12 medium supplemented with B27 with Vitamin A, 20 ng/ml bFGF, 50 ng/mL EGF, penicillin/streptomycin, and 5ug/mL heparin. Half of the media was replaced every 3 days following. Once the cultures were established, the EGF concentration was reduced to 20 ng/mL.

$2x10^6$ cells from each established culture were transformed to express EGFRvIII mutant and PTEN knockout. The mNSC cultures were infected with an adenovirus vector encoding GFP tagged Cre recombinase in order to flox in the EGFRvIII and flox out the PTEN. Two adenovirus infections were performed one week apart in order to achieve sustained Cre-GFP expression. In the days following adenovirus infection, infected target cells were FACS sorted for Cre-GFP expression through Emory University's Flow Cytometry Core in order to isolate potentially transformed cells. The cultures were then maintained and used for *in vitro* assays.

***In vitro* assays**

Cells were maintained in DMEM/F12 medium supplemented with B27 with Vitamin A, 20 ng/ml bFGF, 20 ng/mL EGF, penicillin/streptomycin, and 5ug/mL heparin. Each culture was passaged with Accutase dissociation and plated at a density of $1x10^5$ cells per 10cm dish in 7mLs of media every 6 days. Cultures with a passage number larger than 6 were not used for data collection. For evaluating pathway activation in *Ntv-a;Cdkn2*$^{-/-}$*,EGFRvIII,Pten*$^{-/-}$ transformed cells, they cells were depleted of EGF previous to harvesting for Western blot. Control and transformed cells were dissociated, counted, then plated at a concentration of $3x10^5$ cells per 3mLs of low growth factor media. Low growth factor media was made by diluting DMEM/F12 medium supplemented with B27 with Vitamin A, 20 ng/ml bFGF, 20 ng/mL EGF, penicillin/streptomycin, and 5ug/mL heparin at 1:50 with unsupplemented DMEM/F12, making a 2% growth factor media. Cells were maintained for 24 hours, then harvested and lysed using RIPA buffer in order to extract protein for Western blotting. Antibodies from Cell Signaling Technology were used for Western blot as follows: anti-Akt Rabbit mAb (#9271), anti-Phospho-Akt(Ser473)(D9E) Rabbit mAb (#4060), anti-EGF Receptor(D38B1) Rabbit mAb (#4267), anti-Phospho-EGF Receptor (Tyr1068) Antibody (#2234), anti-PTEN(138G6) Rabbit mAb (#9559), anti-NF-kappaB p65(D14E12) Rabbit mAb (#8242), anti-Phospho-NF-kappaB p65(S536)(93H1) Rabbit mAb (#3033S), anti-TBK1/NAK(D1B4) Rabbit mAb (#3504), anti-Phospho-TBK1/NAL(S172)(D52C2) Rabbit mAb (#5483S), anti-IκBα (44D4) Rabbit mAb (#4812), anti-Phospho-IκBα (Ser32) (14D4) Rabbit mAb (#2859), IKKα (2G12) Mouse mAb (#2682), IKKβ (D30C6) Rabbit mAb (#8943), and Phospho-IKKα/β (Ser176/180) (16A6) Rabbit mAb (#2697).

Anti-Actin (JLA20) mouse mAb from the Developmental Studies Hybridoma bank was used as a loading control.

For measuring gene expression through RT-qPCR, control and transformed cells were dissociated, counted, then plated at a concentration of $3x10^5$ cells per 3mLs of low growth factor media. Low growth factor media was made in DMEM/F12 medium supplemented with B27 with Vitamin A, 0.4 ng/ml bFGF, 0.4 ng/mL EGF, penicillin/streptomycin, and 0.1ug/mL heparin as per a 1:50 dilution from the media used for maintenance. Cells were maintained for 24 hours, then snap frozen in Trizol until RNA extraction. Once RNA was collected, it was DNase treated using the DNase I RNase free kit from BioRad (#970-4324). iScript Advanced cDNA Synthesis Kit for RT-qPCR from BioRad (#1725038) was used to make cDNA, and SsoAdvanced SYBR Green Supermix for Real-Time PCR (#1725270) was used to run RT-qPCR. All kits were used according to manufacturers instructions.

For drug treatment assays, cells were depleted of growth factors as described above for 12 or 24 hours and then treated with Erlotinib, an EGFR inhibitor, at a concentration of 5uM for 12, 24, or 48 hours prior to harvest. As a control, DMSO was used in the same concentration. To inhibit non-specific apoptosis, the zVAD caspase inhibitor was added to both DMSO and erlotinib treated cultures at a concentration of 10 uM.

Results

RNA seq gene expression profiling in a *Drosophila* model of EGFR-Pi3K driven glioblastoma shows enrichment of innate immune pathways

Read et al. developed a *Drosophila melanogaster* model for EGFR-PI3K driven glioblastoma in order to explore tumorigenesis[21]. This model was chosen due to the ease of genetic manipulation, short life span, and large number of progeny, along with the conserved functional homology between the genes of interest. The *Drosophila* CNS is composed of a central nerve cord and two brain hemispheres composed of glia and neurons, which allows for effective exploration of glial neoplasia and tumorigenesis[25]. The cortex contains all neuronal cell bodies, and the neuropil contains synapses[19]. Different classifications of glia within these structures include ensheathing glia and astrocytes in the neuropil, cortex glia, and surface glia[26]. Surface glia form layers of cells, astrocytes project into the neuropil to regulate synpases, while cortex glia form a honeycomb network throughout the brain. All CNS glia contain the transcription factor Repo, which allows for glial specific genetic manipulation, which is taken advantage of in the model[19].

The model uses a UAS-GAL4 system to allow for cell-type specific tissue manipulation. The model expresses the constitutively active forms of EGFR ($dEGFR^{\lambda}$) and dp110 ($dp110^{CAAX}$), which is the catalytic subunit of PI3K, which together drive glial neoplasia[25]. This is shown in Figure 1C. $dEGFR^{\lambda}$ encodes the *Drosophila* ortholog of the EGF receptor where a constitutive Lambda dimerization domain replaces the extracellular domain to create a constitutively active receptor variant[19]. As described in published work[23], we first generate a stable stock with UAS-

dEGFR$^{\lambda}$ and UAS-dp110^{CAAX} recombined on the X chromosome and *repo-Gal4* and *UAS-CD8 GFP* over a TM6B Tb balancer[23], as seen in Figure 1A. The former drives glial specific expression of the UAS components, and the latter marks glia with GFP[21].

The model system displays the hallmarks of cancer, including sustained proliferative signaling, evasion of growth suppressors, resistance to cell death, and tumor promoting inflammation[21]. Read *et al* showed coactivation of EGFR and Pi3K increases glial neoplasia 50 fold, and causes glia to express proteins related to cell cycle entry and inhibit cell cycle exit, which demonstrates sustained proliferation and resistance to cell death[21]. However, constitutive EGFR signaling and activation of the Pi3K are necessary for this phenotype[19]. The transformed glia also expressed an active form of the matrix metalloprotease dMMP1, which suggests invasion potential of the neoplastic cells[21]. This model can therefore be used to explore the interaction between RTK-PI3K signaling driving neoplasia and other pathways, such as innate immune signaling, in the tissue resident cell populations.

We used this *Drosophila* model for EGFR-PI3K driven glioblastoma to find other pathways involved in tumorigenesis. To do this, we collected transcriptomic data from our *repo>dEGFR$^{\lambda}$;dp110^{CAAX}* model and compared it to wild-type controls. Total RNA was collected from age matched whole 3rd instar larval brains in the tumor model and in wildtype flies. PolyA messenger RNA was sequenced by Admera and processed with resources from the Childhood Cancer Data Lab[24] to find differentially expressed gene sets in our tumor model when compared to control. We ran a Gene Set Enrichment Analysis[27],[28] using pathway targeting *Drosophila* specific gene sets from Cheng et al[29].

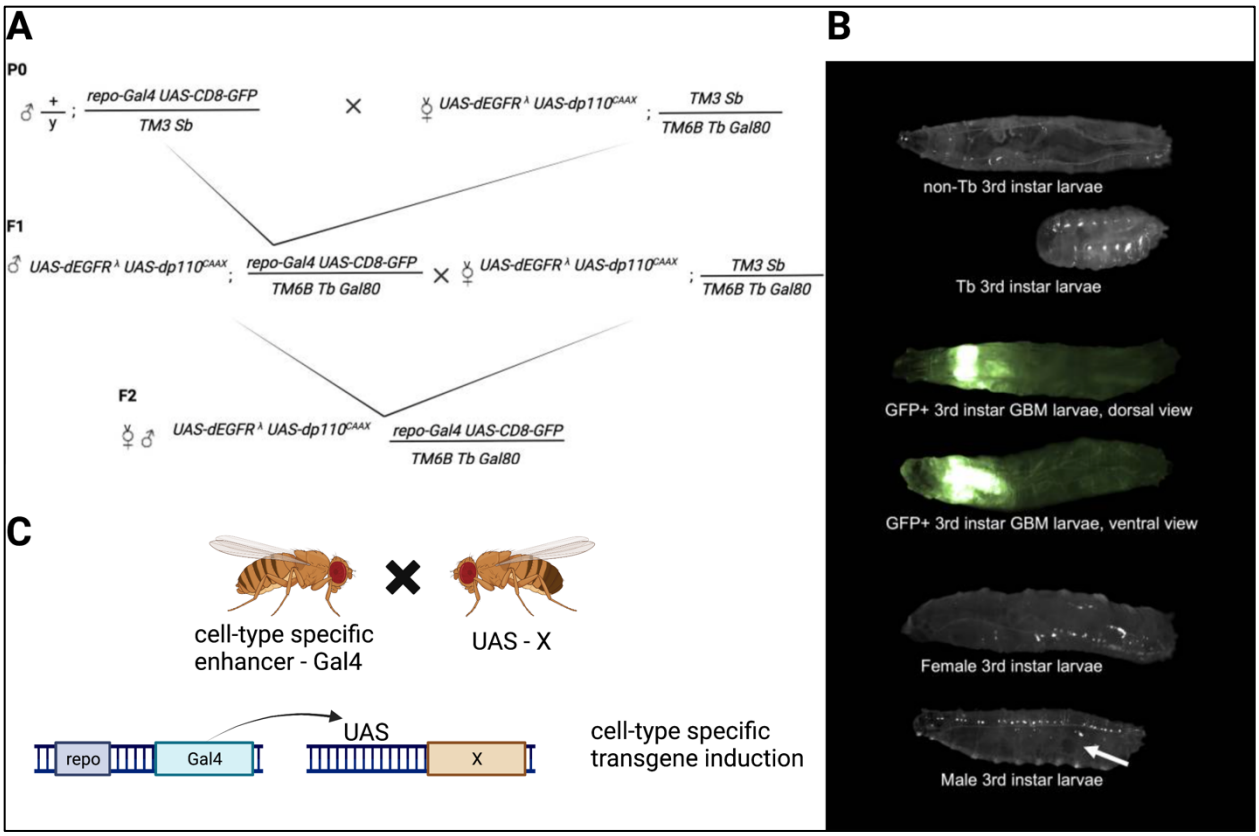

Figure 2. A) *Drosophila* crossing scheme used to create stable stocks for virgination. B) Depiction of experimental set up using the UAS-Gal4 system to perform a genetic screen for genes involved in tumorigenesis. C) Larval images showing the Tb balancer phenotype from the crossing scheme, glial expression of GFP driven by membrane bound CD8-GFP, and sexing technique of 3rd instar larvae.

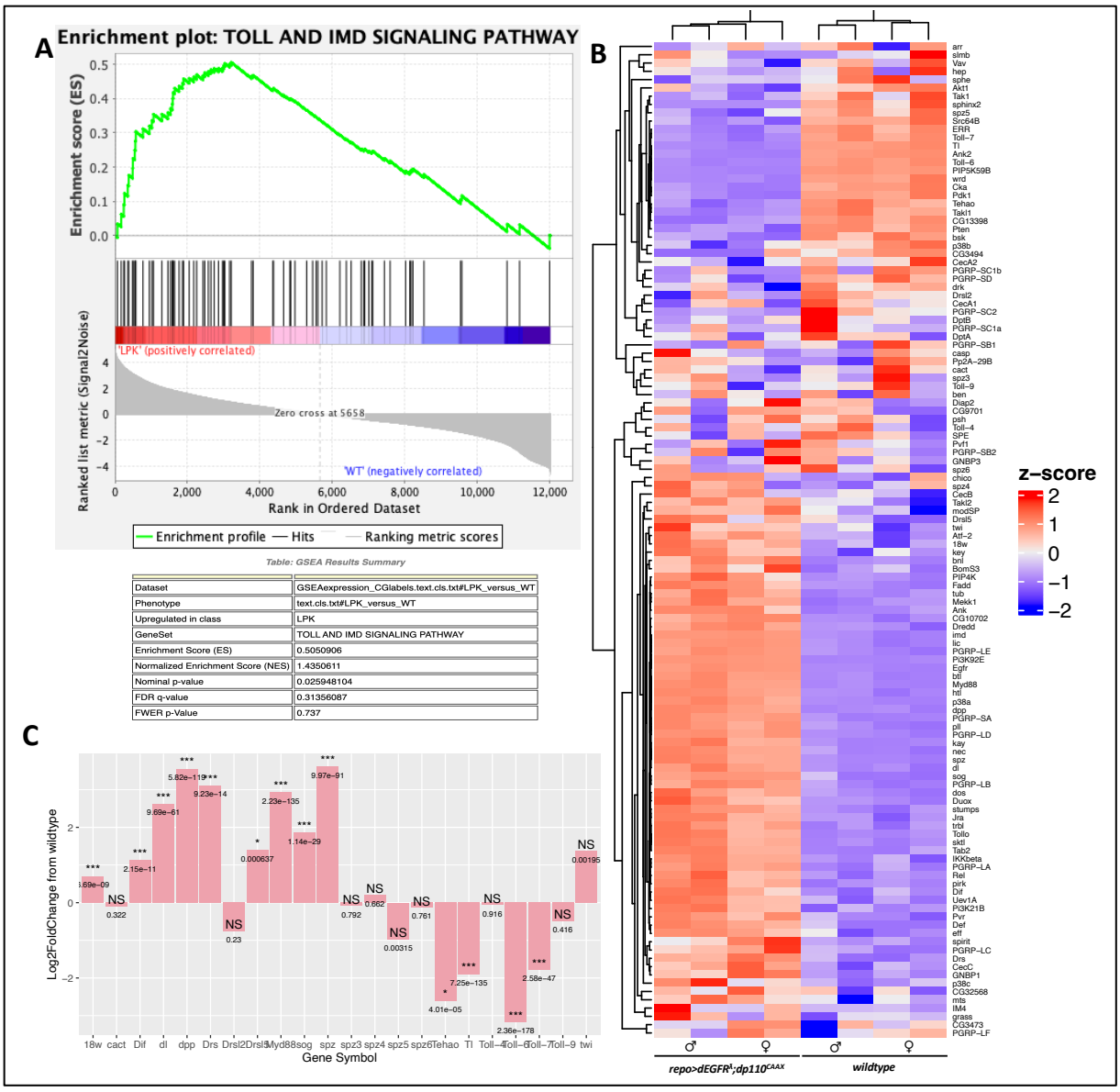

Figure 3. A) Enrichment plot of the gene set Toll and Imd signaling pathways from Chen et al. The results had an enrichment score of 1.435 and a p-value of 0.026. B) Heatmap showing the pathway expression profile of tumor model and wildtype brains. Genes listed are from the Toll and Imd gene set and Pi3K gene set. C) Log fold changes in mRNA expression were calculated by comparing expression of Toll receptors, target genes, and pathway components between tumor brains and wild-type brains. Adjusted p-values are included.

Our data analysis, as shown in Figure 3, showed enrichment of the innate immune and inflammatory Toll and Imd pathways, which were upregulated in our tumor model as compared to our wild-type controls, and the findings had an enrichment score of 1.435 and a p-value of 0.026, demonstrating significance. Within the Toll-IMD pathway gene set defined by Cheng at al[29], we found that Toll target genes dpp, Drs, Drsl5, and sog were significantly upregulated 2-8 fold in *repo>dEGFR$^{\gamma}$;dp110^{CAAX}* larval brains compared to controls, shown in Figure 3C. Furthermore, downstream pathway components such as Dif, dl, and Myd88 are also upregulated in *repo>dEGFR$^{\gamma}$;dp110^{CAAX}* larval brains. The log fold change values are statistically significant with an adjusted p-value <0.05. In contrast, we found that there was significant downregulation of the Toll family receptors Tehao, Tl, Toll-6, Toll-7, and 18w in *repo>dEGFR$^{\gamma}$;dp110^{CAAX}* larval brains compared to controls. All had negative log fold change values and were significant. However, in our analysis of single cell RNA sequencing data from the *Drosophila* larval brain from Janssens *et al*[30] on the Scope interface developed by the Aerts lab[30], we found that Tehao and Toll-6 are mostly expressed in normal neurons, and that Tl, Toll-7, and 18w are expressed in both normal glia and neurons. Thus, an increase in glial cell number in the neoplastic brains could account for the change in bulk mRNA expression.

In order to determine expression in glial cells, we performed IHC in *repo>dEGFR$^{\gamma}$;dp110^{CAAX}* larval brains to visualize localization of key effector proteins in the Toll pathway, including the transcription factors Dorsal and Relish and their regulator and transcriptional target Cactus, shown in figure 4. We observed some nuclear localization of Dorsal (Figure 4A) and Relish (Figure 4C) in neoplastic glia, suggesting pathway activation in tumor cells.

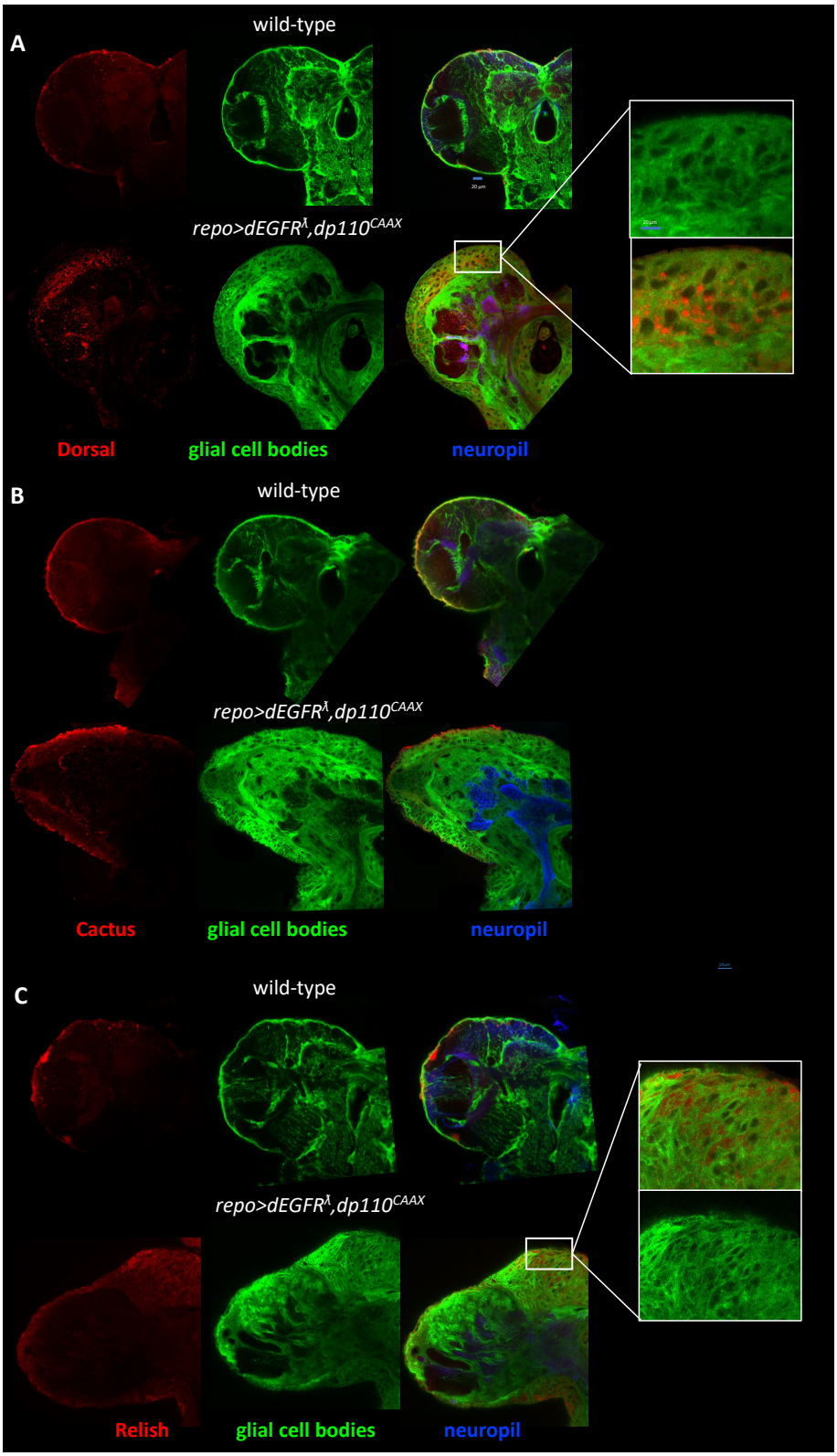

Figure 4. Optical sections (2 µm) of 3rd instar brain hemispheres. *repo>CD8-GFP* labels glia; HRP (blue) labels neuropil; red labels (A) Dorsal transcription factor, (B) Cactus, which is a pathway inhibitor, or (C) Relish transcription factor. Protein Expression is visualized in neoplastic glia.

Toll pathway activation leads to an enhanced *dEGFR$^{\lambda}$;dp110^{CAAX}* phenotype

To determine if function of the Toll pathway contributes to EGFR-PI3K driven tumorigenesis, we tested the effects of glial-cell specific genetic manipulation of Toll pathway components for their ability to enhance or suppress of the *dEGFR$^{\lambda}$;dp110^{CAAX}* larval brain phenotype. We found that activation of the pathway through glial-cell specific overexpression construct of an active version Spatzle (Spz), which is a ligand for Toll, Tollo, 18w, Tehao, Toll-4, Toll-7, Toll-6, and Toll-9[31], significantly increased brain lobe overgrowth in *repo>dEGFR$^{\lambda}$;dp110^{CAAX}* larvae (Figure 5B). In addition, Spz overexpression also caused enlargement of the ventral nerve cord (Figure 5B). Furthermore, there is an increase in the amount of glial cells present in *repo>Spz^{act}; dEGFR$^{\lambda}$;dp110^{CAAX}* larval brains compared to *repo>dEGFR$^{\lambda}$;dp110^{CAAX}* controls, as shown by Repo labeling of glial cell nuclei in Figure 5A.

We repeated the experiments using a Cactus RNAi construct in order to drive pathway activation. Inhibition of transcription and translation of the Cact gene will lead to more DIF and Dorsal in the nucleus, as Cactus inhibits nuclear localization and therefore transcription factor activity. Cact knockdown increased brain volume in 3rd instar larvae and we observed significant changes in both male and female larvae compared to *repo>dEGFR$^{\lambda}$;dp110^{CAAX}* controls, shown in Figure 5C. Through IHC and 40X imaging, we saw glial cell bodies in *repo>dEGFR$^{\lambda}$;dp110^{CAAX}* brains are more compact, and there are more glial cell nuclei labeled with Repo, and this is further enhanced with Toll pathway activation, as shown in Figure 5A. In order to further evaluate phenotypic changes with Toll pathway manipulation, experiments are in progress to examine cell cycle, apoptosis, and invasion in glia when Toll pathway function is increased or decreased in neoplastic glia in our *repo>dEGFR$^{\lambda}$;dp110^{CAAX}* glioblastoma model system.

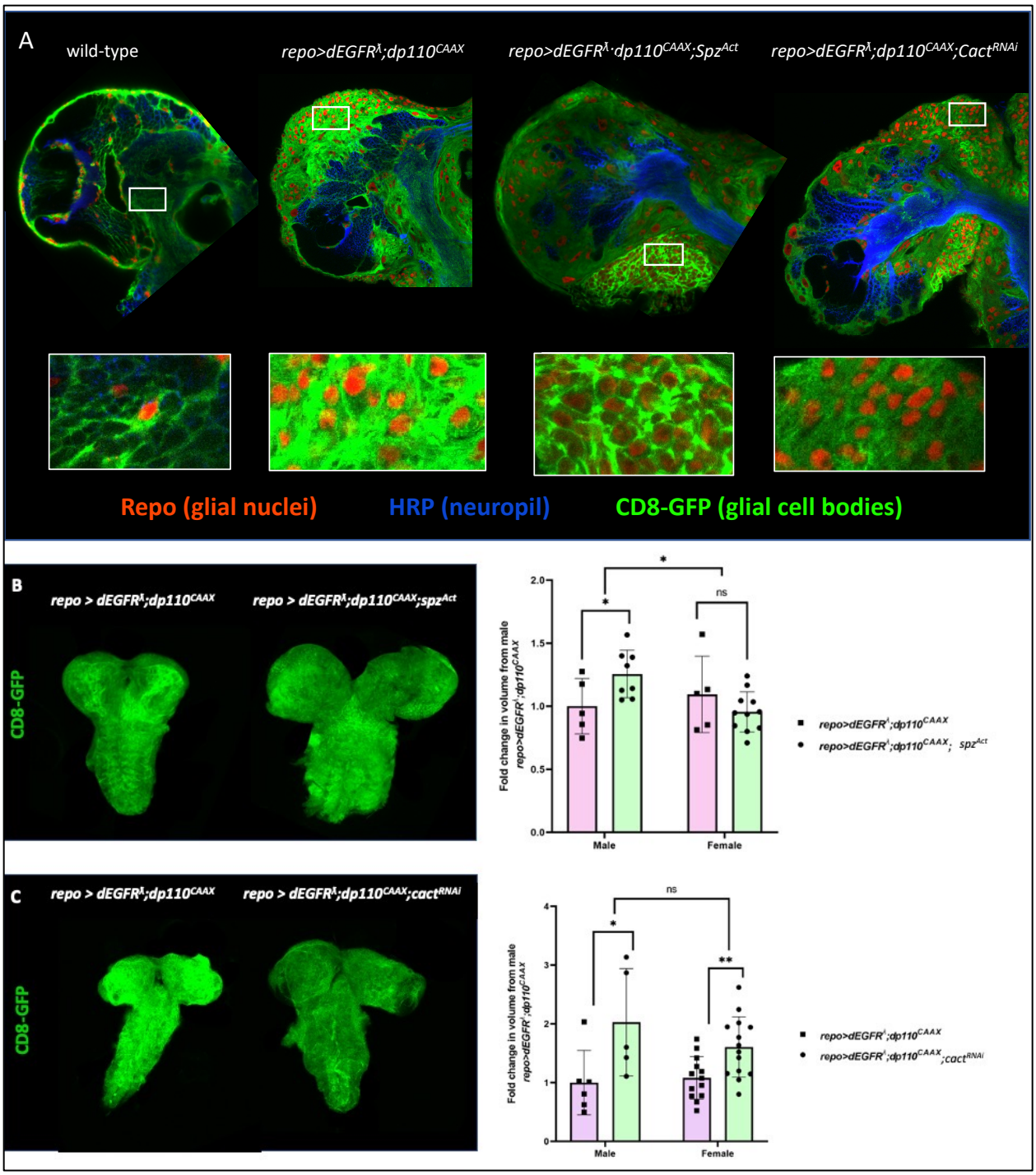

Figure 5. A) Optical sections (2 µm) of 3rd instar brain hemispheres. *repo>CD8-GFP* labels glia; HRP (blue) labels neuropil; Repo labels glial cell nuclei (red). Scale bar indicates 50µM. B, C) Optical projections of whole brain-nerve cord complexes from 3rd instar larvae approximately 5 days old, imaged with a 10× objective on a Zeiss 700 confocal microscope. Dorsal view facing forward; anterior up. CD8-GFP (green) expressed by *repo-Gal4* labels glial cell bodies. Scale bar indicates 100 µm. Volumetric analysis of brain lobe size shows an increase in volume with both

Spz^{Act} and $cact^{RNAi}$, with p-values < 0.05 denoted with *. This was quantified through measuring the brain lobe volume using Imaris image analysis software on our z-stacks of brain projections.

We then evaluated whether pathway inhibition could suppress glial neoplasia. We found that reducing pathway activation with a Dorsal RNAi lead to suppression of the *dEGFR$^{\lambda}$;dp110^{CAAX}* brain overgrowth phenotype: brain lobe volume was decreased in male 3rd instar larvae (Figure 6B). Ongoing work in the lab includes incorporating a UAS-dcr genotype into the Dorsal RNAi flies, in order to enhance RNAi mediated *dorsal* knockdown.

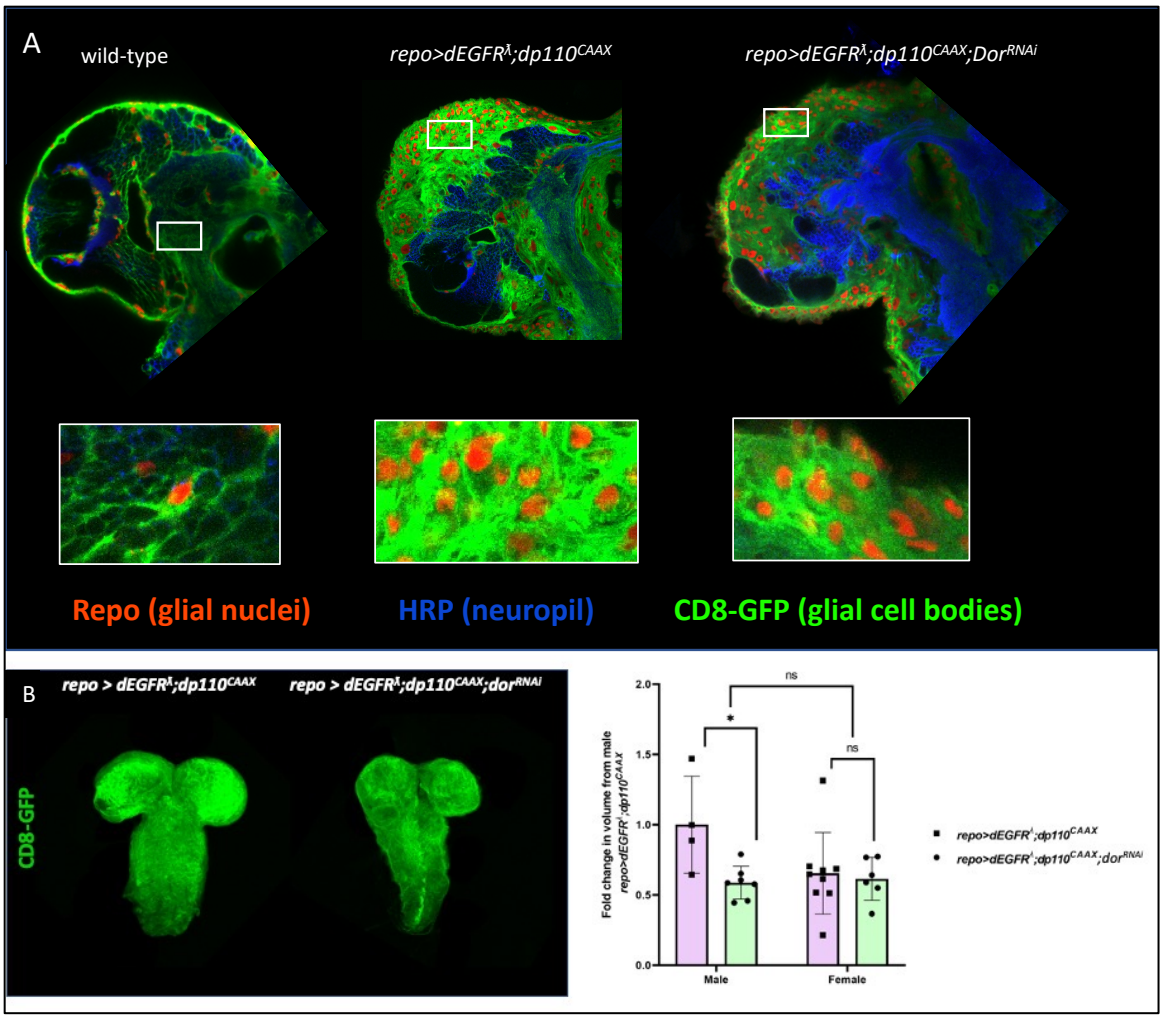

Figure 6. A) Optical sections (2 µm) of 3rd instar brain hemispheres. *repo>CD8-GFP* labels glia; HRP (blue) labels neuropil; Repo labels glial cell nuclei (red). Scale bar indicates 50µM. B) Optical projections of whole brain-nerve cord complexes from 3rd instar larvae approximately 5 days old, imaged with a 10× objective on a Zeiss 700 confocal microscope. Dorsal view facing forward; anterior up. CD8-GFP (green) expressed by *repo-Gal4* labels glial cell bodies. Scale bar indicates 100 µm. Volumetric analysis of brain lobe size shows a decrease in volume with dor^{RNAi}, with p-values < 0.05 denoted with *.

***Ntv-a;Cdkn2a$^{-/-}$;EGFRvIII $^{fl\text{-}stop\text{-}fl}$;Pten$^{fl/fl}$* mammalian model of EGFR-Pi3K driven glioblastoma**

Following our findings in our *Drosophila* EGFR driven model of glioblastoma, we aimed to find whether these would apply in a mammalian model system as well. Previous work in the lab has shown that in a modifier screen, modifier RNAi constructs tested for the ability to alter the *dEGFR$^{\lambda}$;dp110^{CAAX}* phenotype had mirrored genetic interactions with *Drosophila* engineered human EGFRvIII in *repo>hEGFR^{vIII};dp110^{CAAX}* animals[32]. This indicates that interactions seen in our *repo>dEGFR$^{\lambda}$;dp110^{CAAX}* model may be common to neoplastic phenotypes in *Drosophila* and human EGFR[32].

Chen et al. developed a genetically engineered mouse model of Classical glioblastoma by introducing the expression of 4 transgenes, each with commercially available breeders[3]. Chen crossbred four individual mouse lines: *Ntv-a, Cdkn2a$^{-/-}$, EGFRvIII$^{fl\text{-}stop\text{-}fl}$* [8], and *PTEN$^{fl/fl}$* in order to create a model for glioblastoma. All were from a C57BL/6 background. EGFRvIII expression is not enough to effectively capture the glioblastoma phenotype, and deletion of the p53 or PTEN tumor suppressors is needed[8]. *In vivo*, mice injected with RCAS-Cre had nearly 100% tumor penetrance, and their tumors were classified as grade IV glioblastoma[3].

For the scope of this thesis, this model was used to derive litter matched primary murine neuro-glial stem cell (mNSC) cultures. Litter matching the cultures controls for genetic variation. The cultures were treated *in vitro* using an adenovirus vector encoding GFP tagged Cre recombinase, therefore creating mNSC untransformed cultures of genotype *Ntv-a;Cdkn2a$^{-/-}$;EGFRvIII $^{fl\text{-}stop\text{-}fl}$;Pten$^{fl/fl}$* each with a matched culture expressing *Ntv-a;Cdkn2a$^{-/-}$;EGFRvIII;Pten$^{-/-}$* (Figure 7A,C). Transformed neuro-glial stem cells were isolated through flow cytometry based on Cre- GFP expression after adenovirus treatment (Figure 7B).

These cultures were used in order to evaluate the effect of constitutive oncogenic signaling from EGFRvIII and PTEN knockout on innate immune TLR signaling.

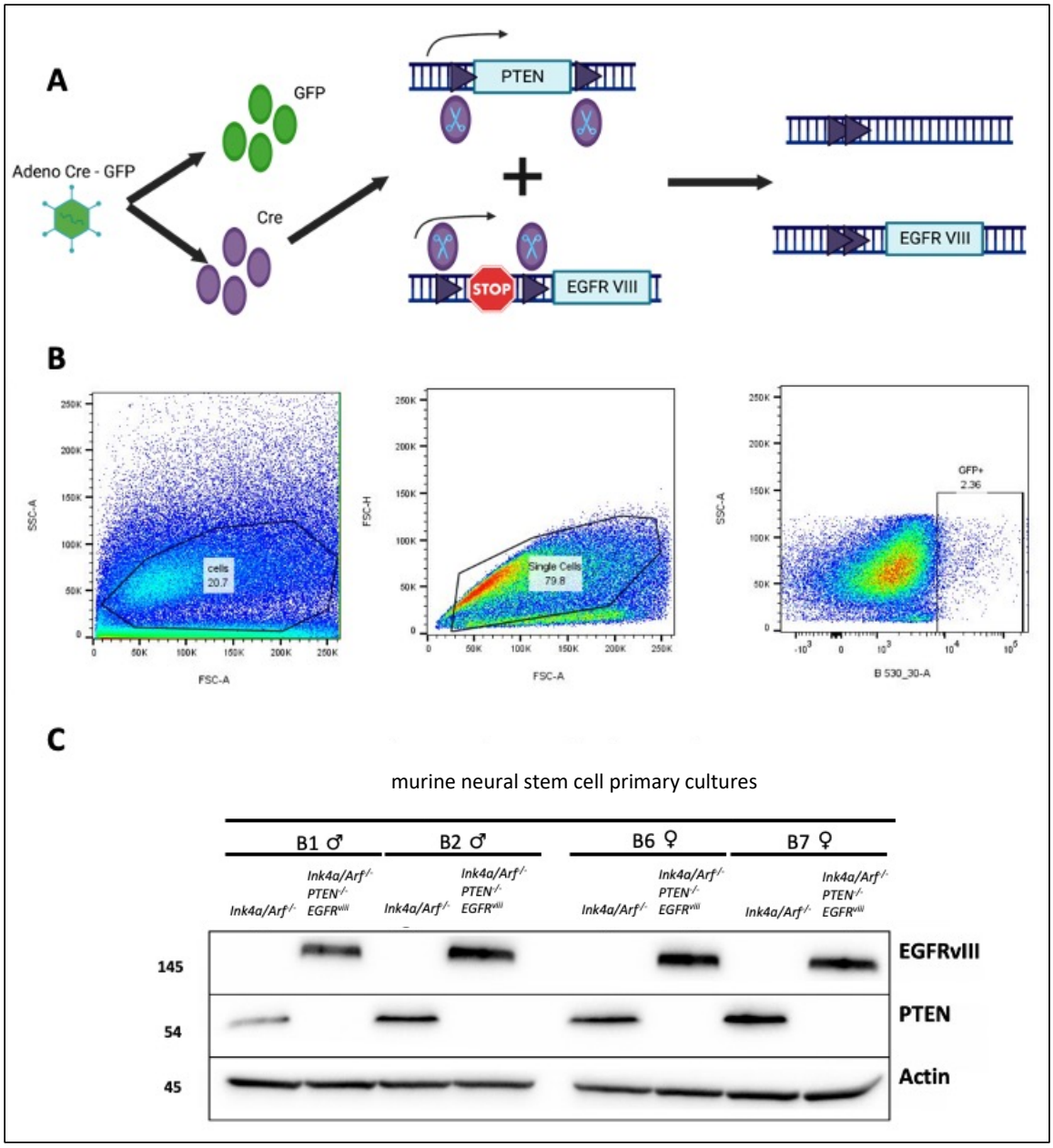

Figure 7. A) Flox-Cre system used to transform mNSCs into EGFRvIII, PTEN knockout transformed cells. B) Flow cytometry read used to sort by GFP expression. C) Western blot showing successful transformation of each *C57BL/6* cell line, (B1, B2, B6 and B7) and its matched control.

Transformed murine neuro-glial stem cells expressing EGFRvIII and PTEN knockout show TLR-NF-κB pathway activation *in vitro*

The transformed *Ntv-a;Cdkn2a*$^{-/-}$*;EGFRvIII;Pten*$^{-/-}$ mNSCs, shown in Figure 8A, were compared to their matched control *Ntv-a;Cdkn2a*$^{-/-}$*;EGFRvIII* $^{fl\text{-}stop\text{-}fl}$*;Pten*$^{fl/fl}$ mNSCs. The cells are cultured in neural stem cell media containing EGF and other growth factors, as detailed in the methods. To ensure that EGFR-PI3K signaling in the transformed mNSCs is dependent on the oncogenic mutations in EGFR and PTEN and not confounded by signaling from exogenous growth factors in the media, we depleted all cultures of growth factors for 24 hours prior to harvest for protein and mRNA.

Previous work in the lab showed that EGFR-PI3K mediated transformation of both *Drosophila* and mammalian neoplastic cells relies on mTorC2 dependent phosphorylation of Akt[32]. Therefore, in order to ensure we were accurately modeling disease in our experiments, we examined levels of Akt phosphorylation on Serine-473. As expected, transformed *Ntv-a;Cdkn2a*$^{-/-}$*;EGFRvIII;Pten*$^{-/-}$ cultures showed increased phospho-Akt with growth factor depletion. This suggests the transformed neuro-glial progenitor cells are relying on EGFRvIII-PI3K signaling, and this is shown in figure 8B.

In order to evaluate the effects of constitutive EGFRvIII-Pi3K signaling on mNSC cell cultures, we depleted the cells of exogenous growth factors and evaluated the state of pathway activation through immunoblotting. We found that transformation of *Ntv-a;Cdkn2a*$^{-/-}$*;EGFRvIII* $^{fl\text{-}stop\text{-}fl}$*;Pten*$^{fl/fl}$ cells into *Ntv-a;Cdkn2a*$^{-/-}$*;EGFRvIII;Pten*$^{-/-}$ leads to differential activation of innate immune signaling pathways. We used total and phosphorylated NF-κB as readouts of the canonical NF-κB signaling pathway, which can be seen in Figure 1. NF-κB is a transcription factor

that localizes to the nucleus on activation by TLR signaling and regulates gene transcription[16]. We found that neoplastic transformation of cells through EGFRvIII-Pi3K signaling leads to increased activation of phospho-NF-κB, but no difference in total NF-κB protein, shown in Figure 8C. This suggests innate-immune pathway activation in *Ntv-a;Cdkn2a*$^{-/-}$*;EGFRvIII;Pten*$^{-/-}$ cells.

Next, we aimed to characterize the upstream components leading to differential NF-κB activation in our *Ntv-a;Cdkn2a*$^{-/-}$*;EGFRvIII* $^{fl\text{-}stop\text{-}fl}$*;Pten*$^{fl/fl}$ and *Ntv-a;Cdkn2a*$^{-/-}$*;EGFRvIII;Pten*$^{-/-}$ cells. We hypothesized the canonical NF-κB signaling pathway was driving activation. The canonical NF-κB signaling pathway signals through effector kinases MyD88 and Irak4, which activate the IKK complex, made up of IKKα, IKKβ, and IKKγ[33]. Activation of the IKK complex leads to phosphorylation of serines 32 and 36 of the IκBα protein, followed by polyubiquitination and degradation[33]. Once IκBα is degraded, NF-κB is able to localize to the nucleus of the cell[27].

To analyze the association of phospho-NF-κB with the canonical signaling pathway, we looked at expression of the protein components of the pathway through immunoblotting. We found that transformed EGFRvIII-Pi3K neoplastic mNSCs had higher expression of phospho-IKKα/β, shown in Figure 8D. Phosphorylation is necessary for a conformational change of the IKK complex and therefore activation of IKK[34]. There was no change in expression of total IKKα or IKKβ. Neoplastic *Ntv-a;Cdkn2a*$^{-/-}$*;EGFRvIII;Pten*$^{-/-}$ mNSCs showed higher expression of IκB. Although our analysis suggests there in an association between phospho-NF-κB and the components of the canonical signaling pathway, more work is needed to determine whether phospho-NF-κB is dependent on the canonical pathway components. This will be done through

evaluating total and phospho-NF-κB in the cells with genetic or pharmacologic inhibition of the upstream pathway components.

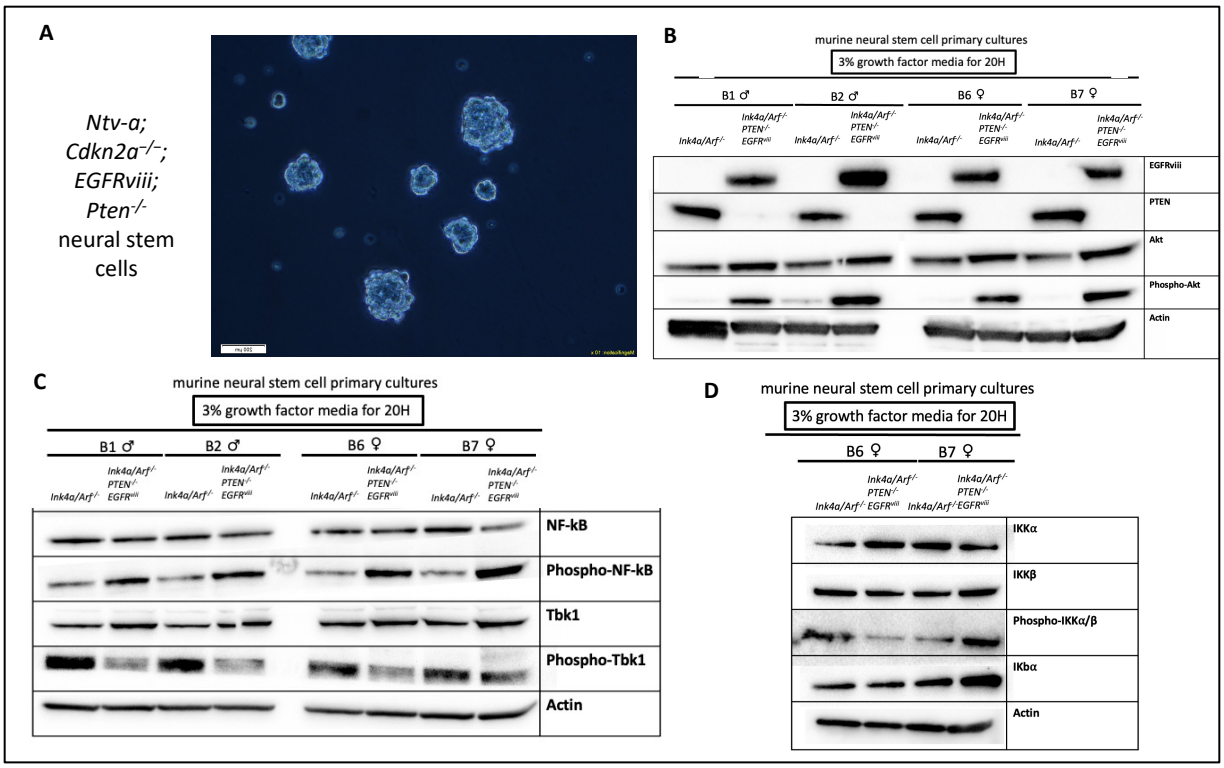

Figure 8. A) *Ntv-a;Cdkn2a*$^{-/-}$*;EGFRvIII;Pten*$^{-/-}$ neurospheres grown in standard DMEM/F12 medium supplemented with B27 with Vitamin A, 20 ng/ml bFGF, 20 ng/mL EGF, penicillin/streptomycin, and 5ug/mL heparin. Imaged at 10X. B, C) Immunoblot showing (B) mutant EGFRvIII, PTEN, total Akt, phospho-Akt, Actin, (C) total NF-κB, phospho-NF-κB, total Tbk1, phospho-Tbk1, and Actin expression in cells incubated at a concentration of $3x10^5$ cells per 3mLs DMEM/F12 medium supplemented with B27 with Vitamin A, 0.4 ng/ml bFGF, 0.4 ng/mL EGF, penicillin/streptomycin, and 0.1ug/mL heparin, which is a 1:50 dilution from maintenance media. 4 transformed cultures and their matched controls were tested. Two are female and two are male. D) Immunoblot showing IKKα, IKKβ, phospho-IKKα/β, Ikbα, and Actin expression in cells incubated in 1:50 diluted media, as mentioned in (B,C). Two cultures and their matched controls were tested.

EGFR Tyrosine Kinase Inhibitor Erlotinib is associated with canonical NF-κB pathway components in *Ntv-a;Cdkn2a$^{-/-}$;EGFRvIII;Pten$^{-/-}$* murine neural stem cells

In our lab, it has been previously observed that treatment of high grade glioma cells transformed by with MET RTK fusions upregulate innate immune signaling pathways in response to treatment with tyrosine kinase inhibitors (TKIs)[35]. We aimed to find whether this is true in our EGFRvIII-Pi3K driven mammalian model as well, which could suggest innate immune signaling pathways are turned on in tumor cells to compensate for EGFRvIII-Pi3K inhibition. We used Erlotinib, an inhibitor of EGFR. Erlotinib is a small molecule inhibitor of the receptor that functions by reversible competitive inhibition of phosphorylation of the intracellular domain of EGFR[36], shown in Figure 9B.

Since Akt activity is required for oncogenic EGFRvIII-Pi3K signaling in our *Drosophila repo>dEGFR$^{\lambda}$;dp110^{CAAX}* model and in the mammalian *Ntv-a;Cdkn2a$^{-/-}$;EGFRvIII;Pten$^{-/-}$* model, as mentioned previously, we used phospho-Akt to read out the extent of inhibition of the EGFR-Pi3K pathway[32]. As shown in figure 9A, erlotinib treatment decreased EGFR-Pi3K signaling in *Ntv-a;Cdkn2a$^{-/-}$;EGFRvIII$^{fl\text{-}stop\text{-}fl}$;Pten$^{fl/fl}$* and *Ntv-a;Cdkn2a$^{-/-}$;EGFRvIII;Pten$^{-/-}$* cells. We tested different treatment times, including different incubation times in low growth factor media. We found that, at a concentration of 5μM for Erlotinib, the optimal drug treatment time was between 12 to 24 hours following 24 hours of growth factor depletion, shown in Figure 9A. Drug treatment for over 24 hours allowed for the effects of erlotinib to recede and EGFR-Pi3K signaling rebounded in *Ntv-a;Cdkn2a$^{-/-}$;EGFRvIII$^{fl\text{-}stop\text{-}fl}$;Pten$^{fl/fl}$* and *Ntv-a;Cdkn2a$^{-/-}$;EGFRvIII;Pten$^{-/-}$* mNSCs cells.

We aimed to then evaluate the effect of pharmacological EGFR inhibition with a TKI on innate immunity signaling pathways in *Ntv-a;Cdkn2a*$^{-/-}$*;EGFRvIII*$^{fl\text{-}stop\text{-}fl}$*;Pten*$^{fl/fl}$ and *Ntv-a;Cdkn2a*$^{-/-}$*;EGFRvIII;Pten*$^{-/-}$ cells. We found that, contrary to our findings in our pediatric high grade glioma model, innate immunity signaling was downregulated in mNSC cells treated with an EGFR TKI. In figure 9C, we show high expression of phospho-IKKα/β and IκBα only in *Ntv-a;Cdkn2a*$^{-/-}$*;EGFRvIII;Pten*$^{-/-}$ cells treated with control DMSO. *Ntv-a;Cdkn2a*$^{-/-}$*;EGFRvIII;Pten*$^{-/-}$ cells treated with a TKI and all control *Ntv-a;Cdkn2a*$^{-/-}$*;EGFRvIII*$^{fl\text{-}stop\text{-}fl}$*;Pten*$^{fl/fl}$ cells have low expression of phospho-IKKα/β and IκBα. This suggests a positive correlation between EGFR-Pi3K signaling and cell-intrinsic innate immune signaling.

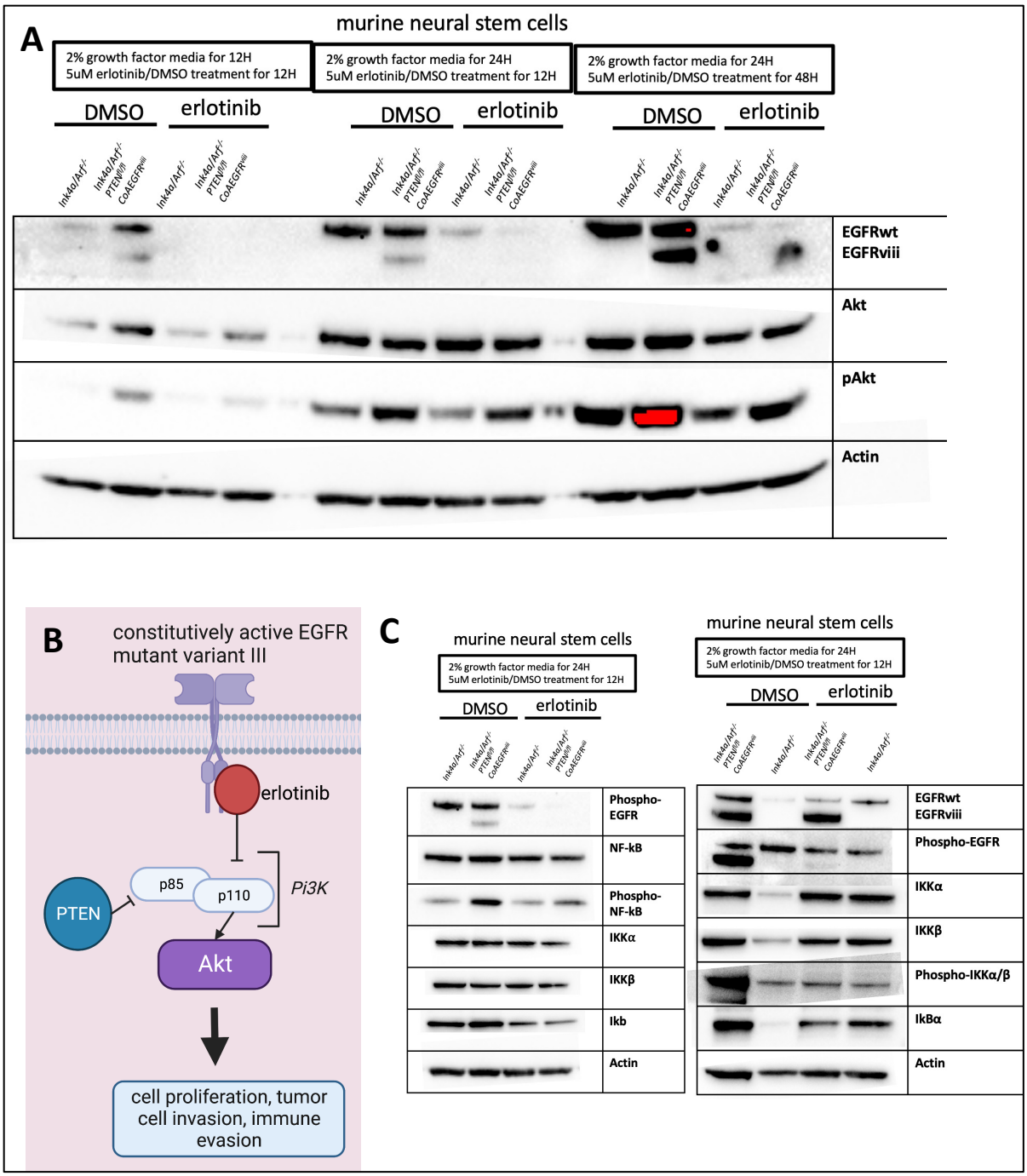

Figure 9. A) Western blot showing a time course experiment of Erlotinib treatment. Cells were plated at $3x10^5$ cells in 3mLs DMEM/F12 medium supplemented with B27 with Vitamin A, 0.4 ng/ml bFGF, 0.4 ng/mL EGF, penicillin/streptomycin, and 0.1ug/mL heparin, which is a 1:50 dilution from maintenance media, for 12 or 24 hours of growth factor depletion. Cells were then treated with 5uM Erlotinib, or an equal volume of DMSO, for 12 or 48 hours. Expression of wildtype and viii mutant EGFR, Akt, Phospho-Akt, and Actin is shown for each set. B) Mechanism of action of erlotinib, a small molecule inhibitor of EGFR that inhibits phosphorylation of the intracellular domain of EGFR[37]. C) Western blot expression of

phosphorylated wildtype and vIII mutant EGFR, NF-κB, phospho-NF-κB, IKKα, IKKβ, phospho-IKKα/β, Ikbα, and Actin. Cells were plated at $3x10^5$ cells in 3mLs DMEM/F12 medium supplemented with B27 with Vitamin A, 0.4 ng/ml bFGF, 0.4 ng/mL EGF, penicillin/streptomycin, and 0.1ug/mL heparin for 24 hours, then treated with 5uM Erlotinib, or an equal volume of DMSO, for 12 hours. Each blot is from a separate experimental replicate.

Discussion and Future Directions

Conclusions

In this work, we aimed to answer the question - what is the role of cell-intrinsic innate immune signaling in transformed neoplastic glia? We hypothesized an association between EGFRvIII-Pi3K transformation of glia and innate immune signaling through the Toll/TLR pathways. In our *Drosophila* model of EGFR-Pi3K driven glioblastoma, we established enrichment of the Toll and Imd Pathways gene set in *repo>dEGFR$^{\lambda}$;dp110^{CAAX}* third instar larval whole brains when compared to wild-type. To evaluate the involvement of cell-intrinsic innate immune signaling in tumorigenesis, we used our *repo>dEGFR$^{\lambda}$;dp110^{CAAX}* model to analyze if Toll pathway activation was a phenotypic modifier of our neoplastic EGFR-Pi3K phenotype. We found that Toll pathway activation, either by constitutive ligand overexpression and by RNA-inhibition of the negative regulator Cactus, enhanced the EGFR-Pi3K neoplastic phenotype. When we inhibited pathway activation with an RNAi construct for the Dorsal transcription factor, we observed suppression of the neoplastic phenotype.

This led us to explore the role of TLR signaling in tumor cells in a mammalian *in vitro* model of glioblastoma with the genotype *Ntv-a;Cdkn2a$^{-/-}$;EGFRvIII;Pten$^{-/-}$* . For this purpose, we compared activation of the NF-κB signaling pathway in cells with EGFRvIII and PTEN knockout to control cells with genotype *Ntv-a;Cdkn2a$^{-/-}$;EGFRvIII $^{fl\text{-}stop\text{-}fl}$;Pten$^{fl/fl}$,* and found an association between activation of oncogenic EGFRvIII-Pi3K signaling and the canonical NF-κB pathway. Activation of the NF-κB transcription factor and upstream canonical NF-κB kinases is higher in mNSCs transformed by EGFR-PI3K activity, and can be reduced with a tyrosine kinase inhibitor against EGFR. This work suggests there are interactions between constitutive EGFR-Pi3K

signaling and cell-intrinsic innate immune signaling that could be used to better understand glioblastoma tumorigenesis. Our exploration of the kinases upstream of NF-κB activation suggests the opportunity for us to test kinase inhibitors against innate immune pathways in transformed EGFRvIII-Pi3K tumor cells in order to evaluate the functional role of TLR signaling. This will be important in the field to characterize an oncogenic or suppressive role of innate immune signaling in neoplastic neuro-glial progenitor cells, and to gain further insight into alternative pathways that could be involved in tumorigenesis.

Future directions using our *Drosophila repo>dEGFR$^{\lambda}$;dp110^{CAAX}* model

In both our *Drosophila repo>dEGFR$^{\lambda}$;dp110^{CAAX}* model and preliminary data from our mammalian *in vitro* model, we see increased expression of effector and target genes of innate immune pathways. Interestingly, we also observed decreased expression of the Toll/TLR receptors. A possible explanation to this could be that neoplastic cells are downregulating Toll receptors in response to increased innate immune signaling from tumorigenesis, making the Toll family receptors the rate limiting step. We aim to evaluate whether manipulating other Toll family receptors in glia enhances the tumor phenotype more severely than manipulation of the downstream pathway components

We will compare, in a modifier experiment, changes in the *dEGFR$^{\lambda}$;dp110^{CAAX}* phenotype with a Cactus RNAi construct, which activates the Toll pathway through inhibition of a suppressor, to the phenotypic effect of a Toll receptor overexpression construct. If the Toll receptors are the rate limiting step of the primed Toll pathway, we would see a significant enhancement of the *dEGFR$^{\lambda}$;dp110^{CAAX}* phenotype in *repo>dEGFR$^{\lambda}$;dp110^{CAAX};Toll^{OE}* animals

when compared to *repo>dEGFR$^{\lambda}$;dp110^{CAAX};Cact^{RNAi}* animals. It is important to evaluate whether the receptors are the rate limiting step of Toll pathway activation in *Drosophila* neoplastic glia as we consider future experiments in the model. If our findings translate into a mammalian model, it is important to note this as the field considers therapies with TLR agonists or antagonists, as efficacy of such therapeutics may be dependent on expression of the receptors.

Future directions using our mammalian *Ntv-a;Cdkn2a$^{-/-}$;EGFRvIII;Pten$^{-/-}$* model of glioblastoma

Our findings show that activation of the canonical NF-κB signaling pathway is associated with constitutive EGFRvIII-Pi3K signaling. However, more work is needed on the functional role of cell-intrinsic innate immune signaling in transformed neuro-glial stem cells. Although we have established an association, we aim to explore the phenotypic effects of inhibitors that target kinases upstream of NF-κB in the *Ntv-a;Cdkn2a$^{-/-}$;EGFRvIII;Pten$^{-/-}$* mNSC cells. We will compare the effects of inhibiting innate immunity signaling pathways on oncogenic phenotypes, including neurosphere formation and cell survival. Further work could include evaluating the efficacy of RCAS-Cre infection in creating *in vivo* tumors when combined with RCAS-shRNA constructs of innate immune pathway components. This will be valuable work, as there is currently no consensus in the field on whether TLR activation is protective or damaging to tumor cells in the brain.

Lathia *et al*, for example, found that glioblastoma cancer stem cells downregulate TLR4 in order to evade innate immune suppression of self renewal, and TLR4 overexpression inhibits

proliferation and maintenance of CSCs[18]. Grasselli *et al*, on the other hand, found that TLR4 expression is downregulated in human neural stem cells upon differentiation, and cells treated with a TLR4 antagonist decreased proliferation[14]. This suggests TLR activation has a significantly different role in glioblastoma cancer stem cells than in healthy neural stem cells, and our work will aid in uncovering the effects of oncogenic signaling on TLR activation.

Furthermore, it is possible that the effect of TLRs on tumor progression may differ depending on tumor stage, and that this is where the discrepancies arise from. Cells of origin and other tumor initiating cells may downregulate the receptors in order to survive in a hostile environment and maintain a stem-like state. This would fit with findings that transformed tumor cells downregulate the Toll/TLR receptors. However, once a tumor microenvironment is established, tumor-cell intrinsic TLR-mediated activation of the NF-κB pathway may become advantageous for increased proliferation and survival, as we observe in our *Drosophila* model.

To evaluate this observation experimentally, we aim to use our mouse model. We will compare transcriptomic data across various stages of tumor initiation and progression. Using our *Ntv-a;Cdkn2a*$^{-/-}$*;EGFRvIII*$^{fl\text{-}stop\text{-}fl}$*;Pten*$^{fl/fl}$ mouse model, we can collect data and different time points after the primary Cre induction in the brain, and evaluate changes in TLR expression in emerging tumor cells over time, our cell population of interest. A limitation to this approach would be the difficulty of mimicking canonical tumor initiation in a small number of cells, so it is important to localize Cre induction when injecting RCAS-Cre. We may label these transformed cells with GFP using the RCAS-system so tumor initiation and progression may be tracked[38]. In this way, we could use immunohistochemistry and flow cytometry to isolate these cells at different time points in tumor progression and evaluate TLR expression in them. Uncovering

changes in pathway regulation with tumor progression would be an important step in understanding the contribution of TLR and NF-κB signaling in glioblastoma tumorigenesis.

Data from TGCA 2013 shows significant differences in survival in tumors with high vs. low TLR4 expression, defined at the top and bottom 10% of samples profiled (Figure S1)[39]. Lower TLR4 expression was associated with better survival[38]. However, it is important to note that along with tumor cells, TLRs are expressed in non-tumor myeloid and astrocyte cells[40], as well as microglia[41] and sensory neurons[42], so conclusions on tumor cell intrinsic innate immune signaling cannot be drawn from bulk sequencing. Single cell sequencing assays would be needed to specify cell type specific expression.

The role of NF-κB activation in glioma

Some phenotypic effects of NF-κB on glioblastoma stem cells have been characterized in recent years. NF-κB activation in tumor cells has been shown to create a mesenchymal like phenotype of glioma stem cells[43],[44]. Mesenchymal like tumors have been known to have worse prognosis, and the role of NF-κB may be important in the tumorigenesis of these tumors[45]. Kim et al. showed that mixed lineage kinase 4 (MLK4), directly phosphorylates the IKK complex, leading to NF-κB activation[44]. Inhibition of either player leads to the loss of the mesenchymal signature in patient derived glioma cultures[32]. If glioblastoma subtype can be driven by NF-κB activation, it is important to classify the functional role in a variety of models of glioblastoma, as has been done in this work.

NF-κB has been shown to have an association with other phenotypic changes in glioma cell lines. Rinkenbaugh *et al*, for example, showed that inhibiting either the canonical or

alternate NF-κB pathway in patient derived glioma stem cells impairs self-renewal, which is shown by reduced tumorsphere formation and GBM expansion in an *ex vivo* model[10]. Furthermore, Wang *et al* showed that suppression of the canonical NF-κB pathway through MiR-98 lead to apoptosis in U87 glioma cells[46]. NF-κB signaling clearly plays a central role in determining the phenotype of glioma stem cells, yet there is still work to be done to uncover the specific mechanisms by which these changes take place, and how TLR signaling pathways mediate NF-κB activation.

In summary, our work presented provides new insight into the interactions between constitutive EGFRvIII-Pi3K signaling and cell-intrinsic innate immune signaling. Moving forward, we must first establish the functional role of this innate immune signaling in transformed neoplastic glia in both our *Drosophila* and mammalian model. Further work, such as that described in this section, will provide us with a deeper understanding of glioblastoma tumorigenesis. It may also give us insight into novel targets that could be exploited for glioblastoma treatment.

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Appendices

Supplementary Figures

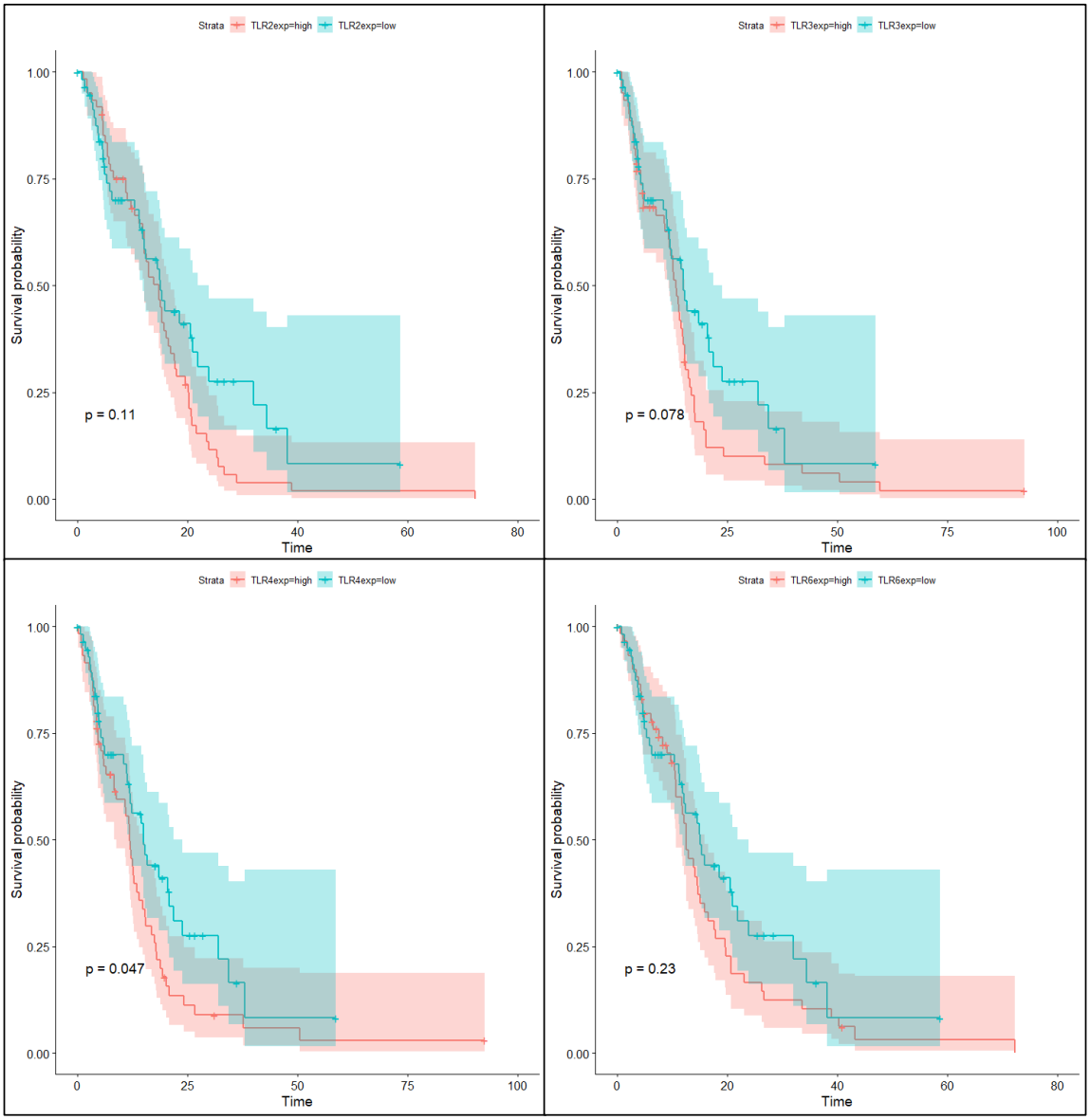

Figure S1. Overall patient survival data from glioblastoma samples profiled by The Cancer Genome Atlas in 2013[47]. Stratified by the top 10% of samples with lowest or highest TLR expression, as shown through mRNA expression.

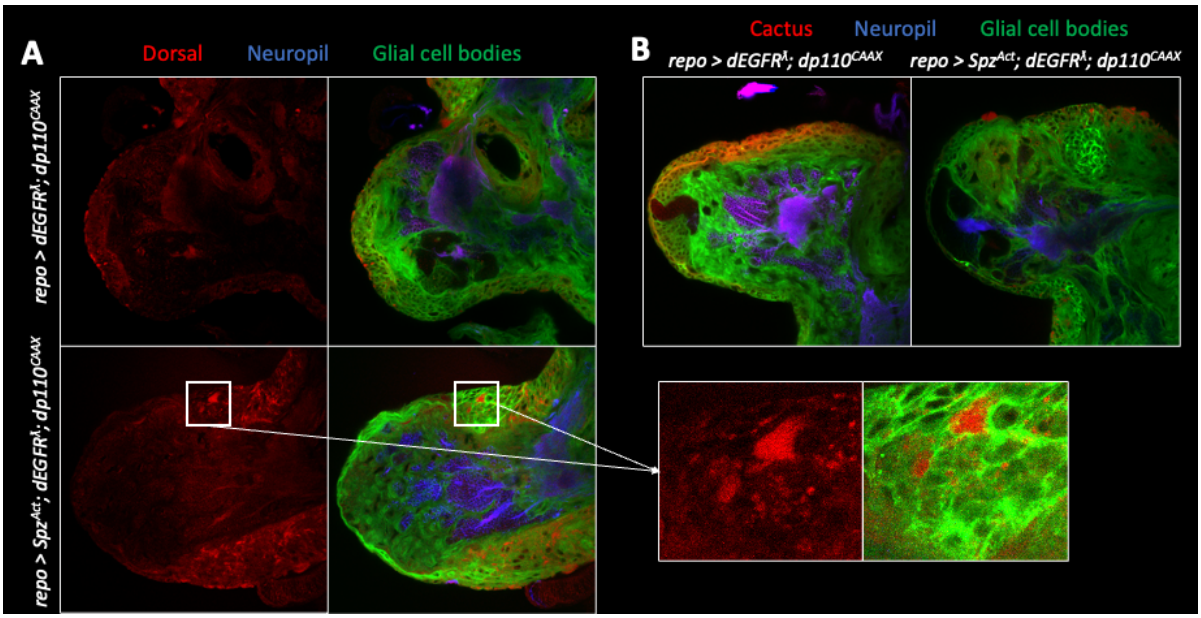

Figure S2. Optical sections (2 µm) of 3rd instar brain hemispheres show succesfull upregulation of Dorsal with *repo>Spz^{Act};dEGFR$^{\lambda}$;dp110^{CAAX}* when compared to *repo>dEGFR$^{\lambda}$;dp110^{CAAX}*. *repo>CD8-GFP* labels glia; HRP (blue) labels neuropil; Dorsal stain (red). Scale bar indicates 50µM.