

THE MICRORNA, MIR9-2, REGULATES PROGENITOR COMPETENCE AND MÜLLER  
GLIAL HOMEOSTASIS IN RETINAL DEVELOPMENT AND DISEASE

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**Abstract**

The microRNA, miR9-2, Regulates Progenitor Competence and Müller Glial Homeostasis in Retinal Development and Disease

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Proper retinal cell fate specification and regulation of homeostasis is essential to establishing a functional visual circuit. MicroRNAs (miRNAs) have been implicated in these processes, yet the individual miRNAs that contribute to this regulation have not been well-defined. The miR9-2 regulatory locus has been linked to retinal disease and related phenotypes and is active in human retinal progenitor cells (RPCs) and Müller glia (MG). To explore the function of miR9-2 in the developing and mature retina, I used transgenic miR9-2 knockout (KO) mouse models, single-nucleus RNA-sequencing, and histology. In germline miR9-2 KO retinas, I found that loss of miR9-2 in progenitors delayed the production of retinal cell types, led to MG disorganization, and generated hybrid neuronal-glial cells. Further, MG-specific KO of miR9-2 led to the rapid loss of recombined Müller cells, and disruptions to metabolic-, synaptic-, and cell-cell junction-related genes. I identified transcriptional targets of miR9-2 and found that miR9-2 can directly regulate several cell fate-determining transcription factor networks. Overall, these results indicate that miR9-2 is critical for controlling the timing of RPC competence and normal gliogenesis and that its dysregulation may contribute to retinal disease.

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## GLOSSARY

|                          |                                                              |
|--------------------------|--------------------------------------------------------------|
| AC                       | Amacrine cell                                                |
| AMD                      | Age-related macular degeneration                             |
| BC                       | Bipolar cell                                                 |
| $\beta$ -gal or beta-gal | Beta galactosidase                                           |
| bHLH                     | Basic-helix-loop-helix                                       |
| cKO                      | Conditional knockout (in reference to the MG-specific model) |
| CNS                      | Central nervous system                                       |
| DEG                      | Differentially expressed gene                                |
| DPI                      | Days post injection                                          |
| E16.5                    | Embryonic day 16.5                                           |
| GCL                      | Ganglion cell layer                                          |
| GOBP                     | Gene ontology biological processes                           |
| GRN                      | Gene regulatory network                                      |
| GSEA                     | Gene set enrichment analysis                                 |
| GWAS                     | Genome wide association studies                              |
| HC                       | Horizontal cell                                              |
| IHC                      | Immunohistochemistry                                         |
| INL                      | Inner nuclear layer                                          |
| IPL                      | Inner plexiform layer                                        |
| IRD                      | Inherited retinal disease                                    |
| KO                       | Knockout                                                     |

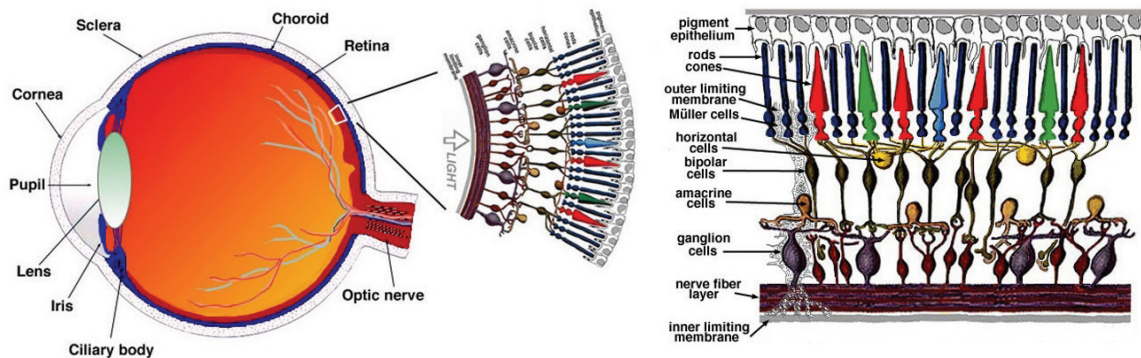
|            |                                                                 |
|------------|-----------------------------------------------------------------|
| MacTel     | Macular telangiectasia type 2                                   |
| MCA        | Mouse cell atlas                                                |
| MRCA       | Mouse retinal cell atlas                                        |
| MG         | Müller glia                                                     |
| miRNA      | MicroRNA                                                        |
| mRNA       | Messenger RNA                                                   |
| NBL        | Neuroblastic layer                                              |
| NFI        | Nuclear factor I                                                |
| ONL        | Outer nuclear layer                                             |
| OPL        | Outer plexiform layer                                           |
| PR         | Photoreceptor                                                   |
| P0         | Postnatal day 0                                                 |
| QC         | Quality control                                                 |
| RGC        | Retinal ganglion cell                                           |
| RISC       | RNA-induced silencing complex                                   |
| RPC        | Retinal progenitor cell                                         |
| scRNA-seq  | Single-cell RNA-sequencing                                      |
| snATAC-seq | Single-nucleus assay for transposase-accessible chromatin       |
| snRNA-seq  | Single-nucleus RNA-sequencing                                   |
| TEM        | Transmission electron microscopy                                |
| TF         | Transcription factor                                            |
| TUNEL      | Terminal deoxynucleotidyl transferase dUTP Nick-End<br>Labeling |



|      |                                               |
|------|-----------------------------------------------|
| UMAP | Uniform manifold approximation and projection |
| UTR  | Untranslated region                           |
| WT   | Wildtype                                      |
| +    | Positive                                      |

## CHAPTER 1. INTRODUCTION

Vision is one of the critical senses used to interpret the world around us. It requires generation and organization of the neural retina, the light-sensing tissue found in the back of the eye. The retina is essential for converting physical photon input into electrical signals that will ultimately be relayed to the brain and used to generate our visual perception and experience. For visual information to be relayed, light (photons) enters through the pupil, is focused by the lens, and is then directed to the retina (Fig. 1.1). The ability of the retina to convert this physical signal into an electrical one requires the development of a complex functional circuit composed of photo-sensing photoreceptor cells (rods and cones), interneurons (amacrine, horizontal, and bipolar cells) which relay and integrate signals, and retinal ganglion cells which further distill signal and project directly to the brain (Kolb et al., 1995).



**Figure 1.1: Schematic of human eye and retina.** Cross-sectional schematic of human eye (left) and a cross-sectional enlargement of the retina, with major cell classes labelled (right). Images from WebVision (Kolb et al., 1995).

Supporting these neurons, Müller glial cells (MG) are the predominant glial cell of the neural retina, providing architectural support and maintaining retinal homeostasis through regulation of glucose metabolism, recycling of waste, and maintenance of blood-retina interactions (Bringmann et al., 2006; Pfeiffer et al., 2020). Despite their diverse roles, the seven

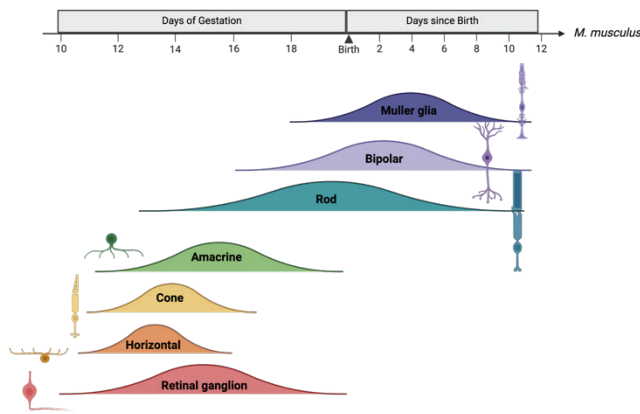
major cell classes arise from a single pool of multipotent retinal progenitors to generate this complex tissue. To create a properly formed retina, distinct transcription factor (TF) programs are modulated in retinal progenitor cells (RPCs) with a high degree of temporal precision to allow for unique cell types to form at the right time and place.

Regulation of retinal development and retinal homeostasis is essential to producing and maintaining a healthy retina, and disruption of these processes can lead to retinal disease and vision loss. Common retinal diseases, such as Age-Related Macular Degeneration (AMD), are estimated to affect 2% of the US population in their forties and up to 35% of the US population in their eighties (Rein et al., 2022). Many of the diseases that contribute to global vision loss are inherited, and many such inherited retinal diseases (IRDs) lack cures or treatments (Georgiou et al., 2021). Therefore, there is still a pressing need to understand how various disease pathologies originate and progress and what normal developmental or homeostatic mechanisms can be co-opted to develop treatments. Additionally, the regulatory mechanisms responsible for controlling retinal progenitor competence and homeostasis during development and in the healthy adult retina are still not fully understood; adding to our understanding of these processes can only improve our ability to maintain retinal health and treat retinal disease.

### *1.1 The mammalian retina is a highly stereotyped tissue with well-conserved mechanisms for cell type generation.*

The retina has been well-studied as a model for central nervous system (CNS) development. The structure of the retina is highly stereotyped, and the general function of individual cell classes and developmental trajectory of retinal progenitors is well-conserved across vertebrates, including the precise order that retinal cell classes are born and many of the transcription factors required to produce each distinct type. Retinal development is often

described using a model of progenitor competence, where multipotent progenitor cells become competent to produce each cell class in distinct but overlapping windows. As development progresses, progenitor cells can divide symmetrically or asymmetrically, with early symmetric divisions generating an increasing pool of progenitor cells, while late asymmetric and symmetric divisions can produce one progenitor cell and one post-mitotic precursor cell or two post-mitotic cells of the same or different classes. Cell class birth is split into two phases with retinal ganglion, horizontal, cone, and amacrine cells born early and rod, bipolar and Müller glial cells born late (Carter-Dawson and LaVail, 1979; Cepko et al., 1996; Livesey and Cepko, 2001).



**Figure 1.2: Schematic of retinal cell class birth order and timing.** Timeline shown corresponds to mouse (*Mus musculus*) retinal development. Figure created with BioRender.com.

of the early cell class birth will occur embryonically, in the approximately 9 days prior to birth, while the majority of late cell class birth occurs postnatally, completing around postnatal day (P)11 (Fig. 1.2). Even after all cells are born, post-mitotic cells will continue maturing until mice are roughly 3-5 weeks of age (Bright et al., 2025; Venugopalan et al., 2020).

Early-born cell classes are born from aptly named early RPCs, and as development continues, the progenitor population transitions and becomes dominated by fate-restricted, late RPCs which produce late-born cell classes. In mice, these phases are roughly split into embryonic and postnatal stages (Zhang et al., 2011). Most

More recent studies have found that retinal cell type birth is even further controlled, where individual cell types within each class are also born with a specific order and timing, especially for cell classes with high diversity, such as retinal ganglion, amacrine, and bipolar cells (Cherry et al., 2009; Nguyen-Ba-Charvet and Rebsam, 2020; Voinescu et al., 2009; West and Cepko, 2022). Subtypes, themselves, are still being defined, with newer classifications often stemming from single cell RNA-sequencing (scRNA-seq) clustering, and characterization of development at the cell subtype level is still being performed. As the field continues to elucidate individual cell types, further work is needed to understand the exact order and timing for the emergence of these cells. However, these precise, stereotyped patterns of development are what make the retina an especially tractable model for study.

### *1.2 Retinal progenitor competence is controlled by expression of distinct transcription factor networks.*

A substantial body of literature already exists that defines distinct retinal cell classes and the transcription factors required for producing the unique cell classes that compose the retina. Here, I give a brief overview of common transcriptional networks necessary to generating individual cell types.

#### *1.2.1 Proneural bHLH factors initiate early retinal cell differentiation.*

Neurogenesis of the retina begins with the production of retinal ganglion cells (RGCs), the earliest born cell class. The differentiation of RPCs into this early neural fate is initiated by the transcription factor, *Atoh7*, of the basic-helix-loop-helix (bHLH) class of TFs (Cepko, 1999; Perron and Harris\*, 2000). Classified as a bHLH activator, *Atoh7* promotes neurogenesis by priming RPCs for retinal ganglion cell fate, and this priming has been highly conserved across organisms with homologs formerly known as *Xath5*, *Math5*, and *Ath5* driving RGC fate in

*Xenopus*, mouse, zebrafish, and human (Hatakeyama and Kageyama, 2004; Kanekar et al., 1997; Ma et al., 2004; Wang et al., 2001). Following *Atoh7* expression, expression of additional downstream transcription factors is triggered, including *Pou4f1*, *Pou4f2*, and *Isl1* which are expressed independently or in various combinations to generate distinct subpopulations of retinal ganglion cells (Nguyen-Ba-Charvet and Rebsam, 2020). While markers for these subpopulations have been somewhat defined, clear molecular markers for each of the 33+ types of RGCs are still being elucidated, and currently, many of the RGC subtypes are still identified on a morphological or transcriptional clustering basis.

bHLH class transcription factors also help to define the amacrine cell (AC) class, through bHLH activating factors of the NeuroD family, including *Neurod4* (formerly *Math3*) and *NeuroD1* (Cherry et al., 2011; Inoue et al., 2002; Yan et al., 2005). Upstream of these factors, the expression of *Foxn4*, a forkhead/winged helix transcription factor, primes early progenitors to take on amacrine cell fate. Further, *Foxn4*<sup>+</sup> progenitors also produce horizontal cells (HCs) downstream, where this division of fates is dictated by the bHLH or other factors expressed later in these primed progenitors (Li et al., 2004).

#### *1.2.2. The Onecut family of transcription factors drives early-born cell fate.*

The Onecut family of transcription factors, composed of three paralogs (*Onecut1/2/3*), promotes the generation of early-born cell classes including retinal ganglion cells, horizontal cells, cone photoreceptors, and amacrine cells. The Onecut family is highly conserved in both mouse and zebrafish, and all three family members are expressed primarily in early retinal progenitors and in developing retinal ganglion cells and horizontal cells (Emerson et al., 2013; Vassalli et al., 2024; Wu et al., 2012). Loss of Onecut TFs in the mouse retina impairs normal development of retinal ganglion cells and cone photoreceptors and results in complete loss of

horizontal cells and the earliest-born amacrine cell type, cholinergic starburst amacrine cells (Emerson et al., 2013; Goetz et al., 2014; Wu et al., 2013). Onecut TFs promote these early cell fates by working in concert with a variety of factors including *Otx2* (cone photoreceptors), *Atoh7/Isl1/Pou4f2* (retinal ganglion cells), and *Ptf1a* (amacrine cells). Onecut TFs are also generally important to establishing horizontal cell identity and become restricted to this cell type in the adult retina.

In addition to promoting early cell fates, Onecut transcription factors also inhibit late-born cell fates, specifically the generation of rod photoreceptors. In a chick model of the developing retina, knockdown of *Onecut1* was sufficient to cause an increase in the number of rod photoreceptors (Emerson et al., 2013). Therefore, Onecut TFs play an essential role in early retinal neurogenesis, promoting the fate of early-born neurons.

### *1.2.3 The role of Notch signaling dynamics in retinal development.*

Notch signaling plays various roles across retinal development. In progenitor cells, high levels of Notch signaling promote the continued proliferation of progenitor cells while delaying neuronal differentiation (Bosze et al., 2023; Chen and Emerson, 2021; Jadhav et al., 2006; Maurer et al., 2014). Inhibition of Notch in early progenitors, through inhibition of Notch effectors like *Hes5*, creates a permissive state for the generation of fate-restricted progenitor cells that will eventually become horizontal cells or cone photoreceptors (Chen and Emerson, 2021). Further, constitutive knockout of various Notch pathway signaling proteins can lead to excess photoreceptors, especially cones (Bosze et al., 2023; Chen and Emerson, 2021). To generate these effects, Notch has been found, in part, to inhibit bHLH factors, *Atoh7* and *Neurog*, which are often expressed in progenitors primed for neural fates (Maurer et al., 2014). Later, in postnatal retinal development, Notch signaling promotes Müller glia cell fate and plays a role in

Müller cell maturation (Georgi and Reh, 2011; Jadhav et al., 2006; Nelson et al., 2011). Notch expression is also maintained at low levels in mature Müller glia and has been proposed to play a role in suppressing the neurogenic potential of glial cells (Le et al., 2024; Liao et al., 2026). However, while Notch has diverse roles throughout the course of retinal development and maturity, rather than strictly promoting any one fate, it often works in tandem with other factors to bring about these fates.

#### *1.2.4 Photoreceptor and bipolar cell development in the mammalian retina.*

Generation of late-born neurons in the retina, including rod photoreceptors and bipolar cells, is characterized by expression of several distinct transcription factors. Rapid upregulation of *Otx2* in cells exiting the cell cycle primes precursor cells for photoreceptor or bipolar cell fate (Brzezinski and Reh, 2015; Ghinia Tegla et al., 2020). Downstream of *Otx2*, upregulation of the transcription factor, *Vsx2*, represses photoreceptor genes and promotes bipolar cell fate (Brzezinski and Reh, 2015). As they become committed to photoreceptor cell fate, precursor cells upregulate expression of *Prdm1* and *Crx* (Brzezinski et al., 2013). Later, photoreceptor precursors upregulate *Nrl* which promotes rod differentiation and *Nr2e3* which promotes rod fate by suppressing cone genes (Chen et al., 2005; Mears et al., 2001). Conversely, cones are produced through progenitor upregulation of TFs such as *Onecut1*, and post-mitotic upregulation of genes such as *Thrb* or *Rxrg* (Brzezinski and Reh, 2015; Emerson et al., 2013; Roberts et al., 2005; Weir et al., 2026).

#### *1.2.5 Generating the primary glial cell of the retina, Müller glia.*

The primary glial cells of the retina, Müller glia, are the latest-born cell class. As discussed above, Notch signaling plays a critical role in regulating MG generation by downregulating neurogenic genes and preserving the progenitor-like qualities that characterize

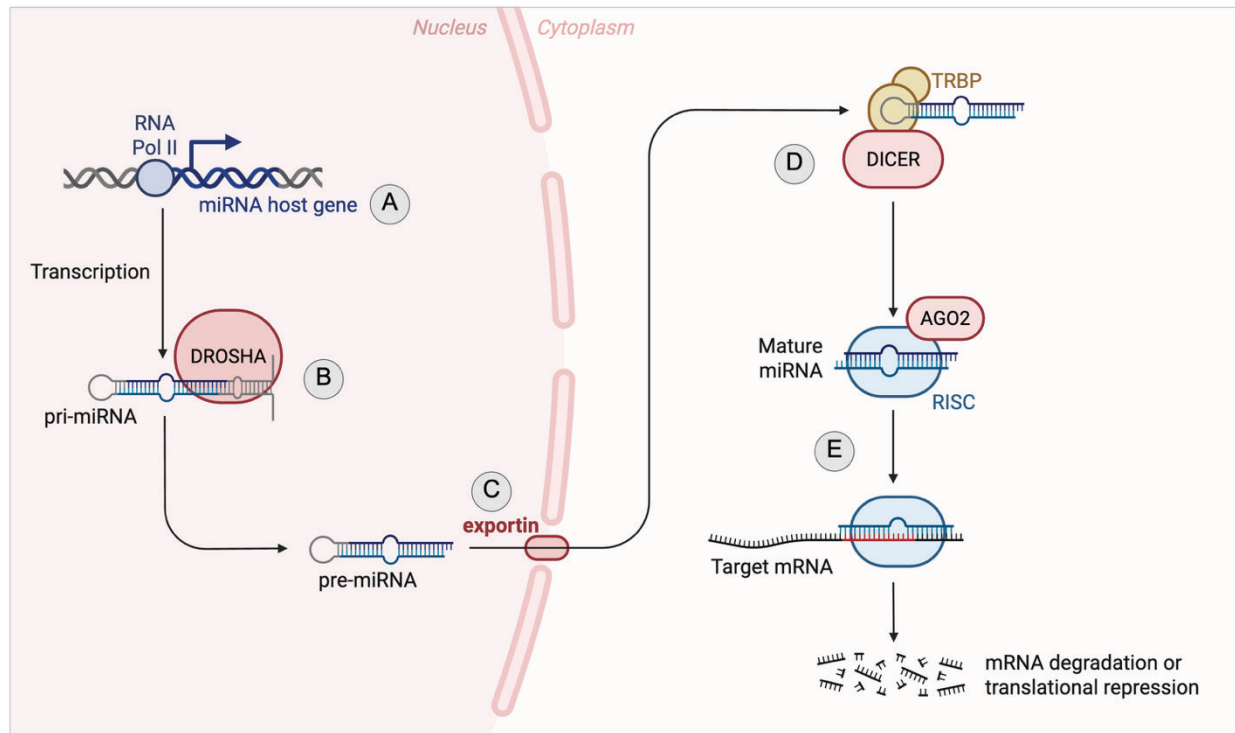


Müller glia. In addition to Notch signaling proteins, other transcription factors like *Sox9*, *Rax*, and *Lhx2* are found to promote Müller fate (Furukawa et al., 2000; Tworig and Feller, 2022). Mature Müller glia, in regeneration-competent organisms or when artificially induced, also retain the ability to de-differentiate and produce neuronal cell types, a quality which has made them a promising target for mammalian retinal regeneration.

While significant work has gone into defining the transcription factors required to drive particular cell fates, it is still unclear how these networks are regulated to allow different cell classes to emerge at distinct times. As progenitor cells progress through various competence states, how do they know when to become an early- or late-born cell type? How are various transcription factor programs activated with such precision that retinal cell types are generated with a distinct order and timing? Recent research has indicated that microRNAs (miRNAs) play an important role in regulating this developmental timing.

### *1.3 MicroRNAs are regulatory molecules that can control a diverse range of RNA targets.*

MicroRNAs are a class of single-stranded RNAs roughly 20 nucleotides in length. Functionally, they act as transcriptional repressors, targeting specific “seed” sequences, with perfect or imperfect complementarity, in the 3' UTR of target mRNA transcripts. Binding of the microRNA at the 3' UTR leads to transcript degradation or translational block, allowing miRNAs to modulate the levels of protein expression. Whether a target is degraded or blocked is thought to be determined by the complementarity of the miRNA and seed sequence. Higher complementarity allows miRNAs to mark transcripts for degradation, while lower complementarity favors translational repression, and various computational models have been developed to predict the numerous miRNA targets (Agarwal et al., 2015; Bartel, 2018, 2004).



**Figure 1.3: Schematic of microRNA processing steps.** *A. MicroRNAs are encoded within a larger host gene. B. Once transcribed, the pri-miRNA transcript hairpin loop is cleaved from the host gene by the activity of the enzyme, Drosha. C. The cleaved pre-miRNA is exported out of the nucleus by exportin transporters. D. The loop at the end of pre-miRNA hairpin is removed by the enzyme, Dicer, generating a double stranded RNA molecule. E. The mature miRNA forms a complex with Argonaute in a structure known as the RNA induced silencing complex (RISC). F. In the RISC, the fully mature, single-stranded miRNA in the RISC complex binds to target mRNA transcripts, leading to degradation or translational repression of that RNA. This schematic was modified from a template and created with BioRender.com.*

To become active, miRNAs are processed through a series of steps. The initial form of a microRNA is known as the pri-miRNA which takes the form of a hairpin loop residing within the miRNA host gene (Fig. 1.3A). miRNA processing begins by cleavage of the hairpin loop from the host transcript through the activity of an enzyme known as Drosha (Fig. 1.3B). This pre-miRNA hairpin loop is transported out of the nucleus of the cell (Fig. 1.3C), and an enzyme known as Dicer cleaves off the loop region leaving a double-stranded RNA molecule (Fig. 1.3D). The double-stranded RNA is separated, and one of the two single stranded fragments will be

loaded into the protein, Argonaute (Fig. 1.3E), forming a complex known as the RNA-induced silencing complex (RISC). Each miRNA has a 5p or 3p strand based upon the respective 5' and 3' ends of the original miRNA hairpin. In mammals, it is thought that the 3p strand is preferentially active; however, both strands of the miRNA can still have activity in the same cell (Bartel, 2018, 2004; Shang et al., 2023). Once the miRNA is loaded into Argonaute/RISC, it is in its final active form.

Some microRNAs have been classified into families of paralogs, with each microRNA being produced from distinct host genes but targeting the same seed sequence in their final processed form. This allows microRNAs to have tissue- and cell type-specific expression based upon the expression and regulation of the miRNA host gene. With some exceptions, miRNAs are extremely stable due to their positioning within the RISC, which protects them from normal degradation processes, and can remain in cells for days to weeks (Bartel, 2018). Therefore, miRNAs are particularly intriguing candidates as regulators of developmental timing and tissue homeostasis as an individual miRNA can affect many processes.

#### *1.4 MicroRNAs are implicated in retinal development.*

Much of the work studying miRNAs in the context of retinal development and neurogenesis has been done by studying knockouts of all miRNAs at once, or knockouts of complete miRNA families.

##### *1.4.1 Loss of all microRNAs perturbs normal retinal development.*

To understand what role microRNAs play in retinal development, several studies have sought to perturb function of all microRNAs through knockout of the miRNA processing enzyme, Dicer. Targeted disruption of all miRNAs in mouse retinal progenitor cells through Dicer knockout has been found to generate an overabundance of early-born cell classes

(including retinal ganglion, cone, amacrine, and horizontal cells) at the expense of late-born classes which are generally not produced (Georgi and Reh, 2010; Torre et al., 2013). Late progenitor-specific knockout of Dicer has also been found to result in immature late-born neurons, especially bipolar and glia cells, with corresponding increases in neurogenic and photoreceptor precursor cells and amacrine cells (Kang et al., 2025). As the loss of miRNAs biases retinas towards early-born cell class production and immature neurons, these data suggest that microRNAs play an essential role in regulating the balance of the early- and late-born cell fates. Additionally, an increase in apoptosis has also been reported in mouse retinas with Dicer-deficient retinas (Georgi and Reh, 2010; Iida et al., 2011), suggesting that miRNAs are important for the survival of progenitors and differentiating neurons as well.

Targeted knockout of microRNAs in mouse Müller glial cells also perturbs normal retinal structure and function. Loss of Dicer in Müller glial cells at either developing or adult timepoints results in disruption of Müller organization, with mis-localization of Müller nuclei, breakdown of the inner and outer limiting membranes, and/or incomplete formation of Müller processes (Kang et al., 2025; Larbi et al., 2025; Wohl et al., 2017). This perturbation of Müller cells also leads to eventual degradation of photoreceptors (both rods and cones) and, ultimately, impairment of visual function. Thus, miRNAs have a crucial role in ensuring that retinas can develop, mature, and function properly.

#### *1.4.2 The microRNA, miR9 regulates central nervous system development.*

While the impacts of miRNA loss are clear, the specific miRNAs that contribute most to these phenotypes have not yet been fully elucidated. One miRNA that has become of particular interest is the microRNA, miR9. As a highly conserved family of microRNAs, miR9 is found in a wide range of organisms including fruit flies, frogs, mice, and humans, with each species

containing several paralogs (Coolen et al., 2013). In both humans and mice, there are three miR9 paralogs, miR9-1, -2, and -3, and in the context of the nervous system, miR9 as a family has been implicated in many developmental processes.

The miR9 family of microRNAs has previously been implicated in neural development in several model organisms of varying complexity (Coolen et al., 2013, 2012). Early studies of miR9 in *Drosophila* found that loss of miR9 leads to excess production of neuronal precursors (Li et al., 2006), while subsequent studies found that modulation of miR9 in cell culture can modulate *Hes1*, and thereby Notch signaling, to dynamically control proliferation versus cell cycle exit (Bonev et al., 2012; Coolen et al., 2013, 2012; Roese-Koerner et al., 2017). In the developing zebrafish brain and retina, miR9 has been found to regulate *Onecut* and *Tlx* TFs, where dysregulation of these targets through loss of miR9 causes abnormal vascularization of these tissues (Coolen et al., 2012; Madelaine et al., 2018, 2017) and delayed cell cycle exit when miR9 is transiently disrupted (Coolen et al., 2012). Additionally, in the previously discussed retinal progenitor-specific Dicer knockout mice, addition of a miR9 mimic, in combination with other miRNA mimics, was able to partially rescue the ability of Dicer KO progenitor cells to produce late-born cell types (Torre et al., 2013). In contrast, overexpression of miR9 in the neonatal mouse retina leads to an excess of neurons and a concordant decrease in retinal Müller glia cells (Suzuki et al., 2020). However, while miR9 has been studied as a regulator of neurogenesis, most of these studies have focused on perturbing all microRNAs at once or disrupting the miR9 family as a whole. Further, while some mechanisms for miR9 activity have been proposed, the exact transcriptional targets it regulates to control the switch from proliferation to neurogenesis have not been clearly defined.

### *1.5 miR9-2, a member of the miR9 family of microRNAs, is implicated in retinal disease.*

Of three miR9 paralogs found in humans, miR9-2 has become particularly notable in the context of retinal disease, and genome wide association studies (GWAS) have increasingly associated it with various retinal diseases and disease phenotypes. Variants in the 5q14.3 locus, which contains the miR9-2 host gene, have been linked to the retinal degenerative disorders, Macular Telangiectasia Type 2 (MacTel) and Age-Related Macular Degeneration (AMD) (Bonelli et al., 2021b; Fritsche et al., 2016; Gorman et al., 2024; Scerri et al., 2017). In particular, the lead risk variant associated with MacTel lies in a putative enhancer region, recently investigated by our lab, which has been found to target the miR9-2 host gene (Thomas et al., 2022). Additional GWAS studies have also linked this locus to disease-related phenotypes, including changes in retinal vasculature – width and tortuosity of arterioles and veins – and alterations in macular thickness (X. R. Gao et al., 2019; Ikram et al., 2010; Jackson et al., 2025). Therefore, miR9-2 is a particularly compelling microRNA for investigation, as an understanding of its function may also offer insight into the mechanisms of retinal disease.

### *1.6 The retina as a model for studying retinal development and disease.*

The retina is a particularly approachable model for studying neurodevelopment and neurodegenerative diseases. The normal patterns of development and function are so well-characterized that the subtle changes or perturbations are more easily detectable. Further, as a tissue that is highly conserved across vertebrate systems, many of the studies done with *in vitro* cultures or *in vivo* animal models are more easily translatable to human developmental and disease contexts.

Here, I have provided a broad overview of what is known about retinal development, including the defined order and timing of retinal cell class birth and several of the conserved

transcriptional programs that generate these various cell types. While the progression of retinal development is well-defined, it is still not fully understood how this progression is regulated with temporal precision. I have presented several of the studies that propose mechanisms for this regulation, especially in regard to the role microRNAs may be playing in these processes. Additionally, I have identified a particular microRNA paralog, miR9-2, that has been highly associated with both retinal development and retinal disease.

Thus, in my thesis work, I sought to uncover some of the regulatory mechanisms that are responsible for governing proper cell fate specification and maintaining the health of the retina. I identified a particular regulatory locus, containing the microRNA, miR9-2, that has been implicated in retinal disease and development, specifically through its activity in human progenitor and Müller glia cells. Using a combination of transgenic mouse models, immunohistochemistry, electron microscopy, and single-nucleus transcriptional profiling, I have explored how miR9-2 controls the proper development of the retina and how disruption of miR9-2 contributes to retinal disease phenotypes. This work provides novel insight into the regulation of retinal development and homeostasis and provides a promising foundation for future investigations into retinal regeneration and treatment of retinal disease.

## CHAPTER 2. CONTROL OF RETINAL PROGENITOR COMPETENCE AND MÜLLER GLIAL IDENTITY BY MIR9-2 DURING RETINAL DEVELOPMENT

Chapter 2 is adapted with minimal modification from:

Callies, L. K., Jain, A., Xu, S., Thomas, E. D., and Cherry, T. J. Disease-associated microRNA, miR9-2, regulates timing of retinal progenitor cell competence and maintenance of Müller glial identity. *bioRxiv* 2026.03.27.714903; doi: 10.64898/2026.03.27.714903

### 2.1 Abstract

Development of the visual system is dependent upon precise regulation of cell fate specification. In the mammalian retina, a single pool of multipotent progenitor cells becomes competent to produce the seven major retinal cell classes in distinct but overlapping windows. MicroRNAs (miRNAs) have been implicated in controlling retinal progenitor competence and risk for retinal disease, but the specific contribution of individual miRNAs and how they are regulated is still unclear. Here we characterize a deeply conserved gene regulatory unit that includes the miRNA, miR9-2, and a retinal disease-associated enhancer that controls its expression. Loss of miR9-2, one of three mammalian miR9 paralogs, delays the emergence of late-born retinal cell classes and leads to misspecification of Müller glial cells to a hybrid neuronal-glial fate. Further, we identify transcription factors and gene regulatory networks directly controlled by miR9-2 during retinal development. Lastly, we provide evidence of a negative feedback loop through which miR9-2 regulates itself. Altogether, this study provides insight into mechanisms that regulate the timing of retinal progenitor competence and glial cell identity, and how this gene regulatory unit may contribute to retinal disease.



## 2.2 Introduction

Precise regulation of cell fate specification is essential for generating a functional central nervous system (CNS). In the mammalian retina, a single pool of retinal progenitor cells (RPCs) gives rise to all seven major retinal cell classes with a precise order and timing. These RPCs become competent to produce different cell classes in distinct but overlapping windows. In the retina, cell class birth is often split into two phases: early-born neurons – retinal ganglion (RGCs), cone photoreceptor, and horizontal cells (HCs) – produced first, followed by the late-born neurons – rod photoreceptor and bipolar cells (BCs) – and the primary glial cell class of the retina, Müller glia (MG). The amacrine cell class (ACs) spans these two developmental epochs with early-born ACs, like cholinergic starburst amacrine cells, and later-born ACs, such as the glycinergic amacrine cell types.

The genetic programs that influence retinal cell class specification are well studied. Transcription factors (TFs) have documented roles in modulating progenitor cell competence. Among them, basic helix-loop-helix proteins (bHLHs), homeodomain-containing TFs, Notch signaling proteins, and Nuclear Factor I (NFI) family members have been found to play roles in regulating cell proliferation, cell cycle exit, and cell class specification in the retina. TFs, such as the bHLH and *Onecut* genes, have documented roles in promoting specification of early-born cell types, including RGC fate (*Atoh7*) or horizontal and cone cell fate (*Onecuts*) (Emerson et al., 2013; Gao et al., 2014; Sapkota et al., 2014; Wang et al., 2001; Wu et al., 2013). Conversely, Notch signaling proteins and NFI factors maintain progenitor identity. Notch pathway signaling early in development, through effectors such as *Hes1* and *Hes5*, maintain RPCs in a proliferative state (Bosze et al., 2023; Chen and Emerson, 2021; Clements et al., 2011; Mills and Goldman, 2017; Perron and Harris\*, 2000), while recent work on NFI factors found that they regulate

specification of late-born cell fates, including bipolar and MG cells (Clark et al., 2019). While the roles of these key TFs are well understood, how the factors themselves are regulated across development allowing for robust transitions in cell fate specification is not fully understood.

MicroRNAs (miRNAs) have been implicated in controlling the timing of progenitor cell competence in the CNS. miRNAs are a class of small, single-stranded RNAs around 20 nucleotides in length and act as translational repressors, binding in a complementary fashion to target seed sequences in the 3' UTR of mRNAs which leads to repression and degradation of the transcript (Bartel, 2018; Coolen et al., 2013). Many miRNAs arose from ancient gene duplication events and are classified into families composed of miRNA paralogs which target the same seed sequences in their processed form but originate from different host genes and evolved to have different patterns of expression in distinct cell types (Bartel, 2018). In the mammalian retina, the importance of miRNAs in regulating retinal progenitor competence and homeostasis is clear, as deletion of Dicer, an enzyme important to processing miRNAs into their mature form, leads to a delay in the production of late-born cell types (Georgi and Reh, 2011, 2010; Kang et al., 2025; Torre et al., 2013). Further, the miR9 family, among others, was identified as a potential regulator of the shift in competence from early- to late-born cell types due to its increased expression when RPCs undergo this transition.

The highly-conserved miR9 family of microRNAs has previously been implicated in neural development in diverse model organisms (Coolen et al., 2013, 2012). Studies investigating neuronal miR9 function in *Drosophila*, zebrafish and mouse telencephalon, have found that loss of this miRNA can lead to increased progenitor proliferation and excess neuronal precursors, delayed cell cycle exit, and/or abnormal vascularization of CNS tissues, including the retina (Coolen et al., 2013, 2012; Li et al., 2006; Madelaine et al., 2017; Shibata et al., 2011).

Mechanistically, these studies have proposed candidate miR9 targets including *Hes1*, thereby influencing Notch signaling, as well as *Onecut* and *Tlx* TFs (Bonev et al., 2012; Coolen et al., 2013, 2012; Madelaine et al., 2017; Roese-Koerner et al., 2017). In the mammalian retina, overexpression of miR9 biases cells towards neuronal rather than glial fates (Suzuki et al., 2020). While these studies have proposed many functions for this microRNA family, there is still a significant gap in knowledge about the roles of individual miR9 family members, especially as each paralog displays distinct tissue and cell type-specific expression. In mice and humans, there are three miR9 paralogs, known as miR9-1, -2, and -3 (Coolen et al., 2013). Though prior studies implicate miR9 as a regulator of neurogenesis, the specific targets of the microRNA are not well defined and approaches thus far have more broadly disrupted miRNA function rather than focusing on the behavior of specific miR9 family members in distinct cell types.

Genome wide association studies (GWAS) have increasingly linked the miR9-2 host gene locus to retinal diseases, such as Macular Telangiectasia Type II (MacTel) and Age-Related Macular Degeneration (AMD), and disease phenotypes, including altered retinal vasculature caliber and altered retinal thickness (Bonelli et al., 2021b; X. R. Gao et al., 2019; Han et al., 2020; Ikram et al., 2010; Jackson et al., 2025; Scerri et al., 2017). More specifically, an enhancer for miR9-2, recently investigated by our lab, has been implicated in these GWAS (Fig. 2.1A). Using single-nucleus Assay for Transposase-Accessible Chromatin sequencing (snATAC-seq), we found that the miR9-2 enhancer is accessible in RPCs and MG of the human retina. Further, we determined that, in human retinal organoids, knockout of this enhancer leads to a decrease in miR9-2 host gene expression and aberrant developmental phenotypes, including altered cell class proportions indicative of a delay in the emergence of various cell classes (Thomas et al., 2022). These data suggest that miR9-2, independent of miR9-1 and miR9-3, plays an important

regulatory role in the retina. This work improved our understanding of the functional significance of miR9-2 *in vitro* in developing human tissue, but there are still many questions remaining surrounding how miR9-2 functions *in vivo* to regulate retinal development and disease. Our work on the miR9-2 enhancer, alongside pre-existing studies on the miR9 family, indicates that miR9-2 may be important in RPCs for properly specifying cell types and controlling the relative abundance of types in the mature retina. Additionally, the strong linkage of miR9-2 with retinal disease and macular degenerative disorders suggests that miR9-2 is important to maintaining glial homeostasis and preventing the MG cell loss that is frequently found in retinal degenerative diseases. These outstanding questions required an *in vivo* system to study miR9-2 in the developing and mature retina.

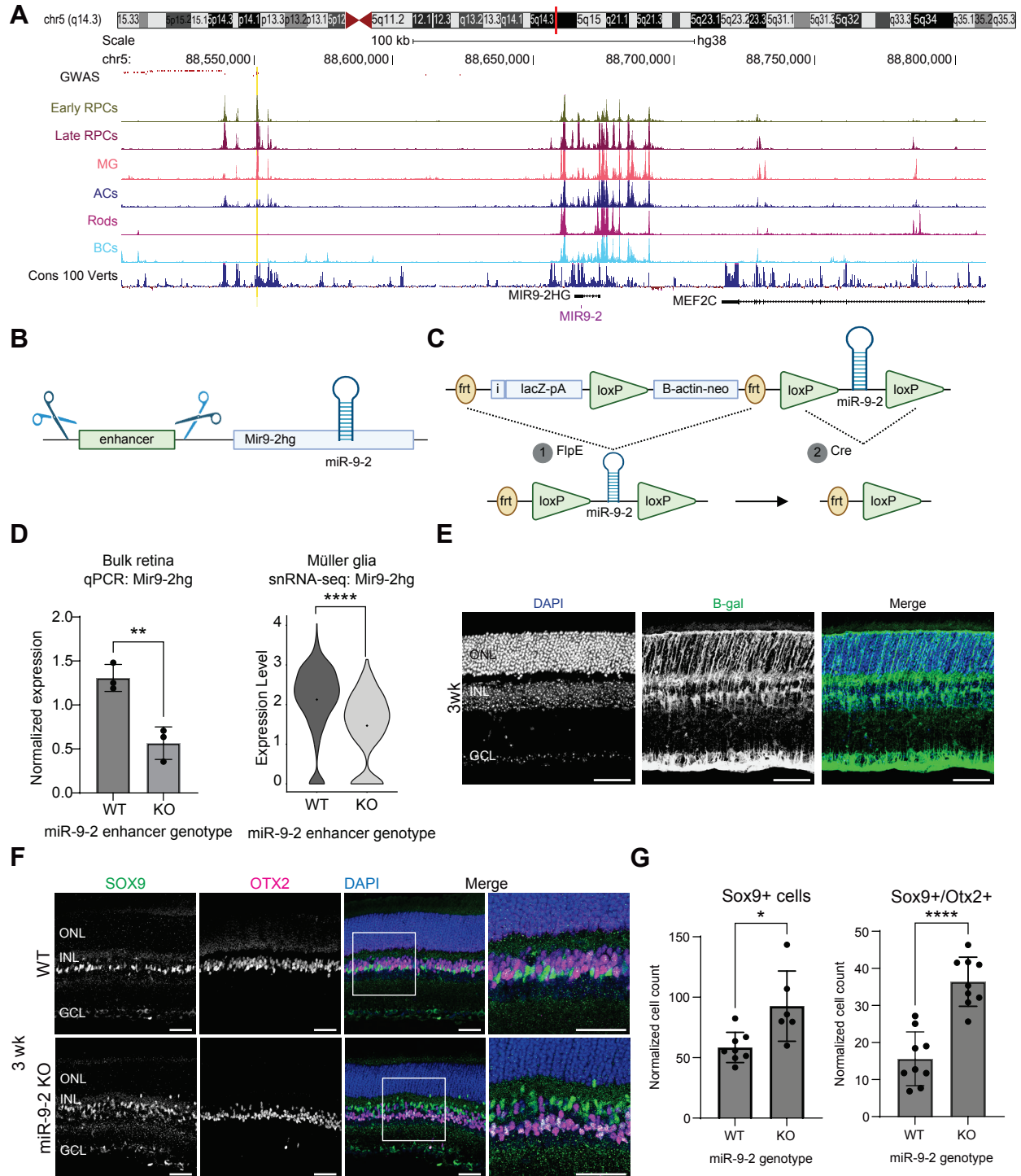
In this study, we sought to investigate the regulatory role of miR9-2 in the specification and maintenance of retinal cell identity. Using novel enhancer knockout and miR9-2 germline knockout (KO) mouse models, immunohistochemistry (IHC), and single-nucleus RNA-sequencing (snRNA-seq), we found that miR9-2 regulates the timing of retinal cell fate emergence. Developing miR9-2 KO mouse retinas contain larger proportions of early-born cell classes compared to sex-matched, littermate controls. We also found that miR9-2 is necessary at the end of development for MG to adopt and maintain their mature glial cell fate. In enhancer and miR9-2 knockout retinas, MG are mis-specified adopting hybrid neuronal- and glial-like cell fates. Further, we identify direct targets of miR9-2 in developing and mature cell populations and the downstream gene regulatory networks (GRNs) that influence cell fate. This work expands our knowledge of how the temporal dynamics of retinal cell class specification are controlled during development and adds to our understanding of miR9-2 in retinal disease risk.

## 2.3 Results

### 2.3.1 Conserved regulation of a retinal disease-associated microRNA.

To examine miR9-2 regulation and function in vivo, we generated a germline, enhancer knockout mouse model by CRISPR targeting a conserved ~300bp region corresponding to accessibility (snATAC-seq data) in the putative enhancer region (Fig. 2.1A-B; gRNAs in Table S2.7). Retinas from adult animals were used for qPCR analysis on bulk retinal tissue (n=3) targeting the miR9-2 host gene (*Mir9-2hg*). Consistent with our human organoid studies, we found a significant decrease of the *Mir9-2hg* expression in enhancer knockout animals compared to WT (Fig. 2.1D). Using single-nuclei RNA-sequencing (snRNA-seq; n=3), we also profiled WT and enhancer knockout retinas from mature animals to understand enhancer activity in a cell-specific manner (Fig. S2.1). We found that MG from enhancer knockout animals had a significant decrease in *Mir9-2hg* expression compared to WT (Fig. 2.1D; Fig. S2.1E). We also identified other significant differentially expressed genes (DEGs), particularly in MG cells, several of which are computationally predicted to be direct miR9 targets like *Foxp2* (Fig. S2.1E). These results demonstrate that this 5q14.3 enhancer has a conserved role in regulating the miR9-2 host gene, and thereby miR9-2, across humans and mice.

Our previous work demonstrated that knockout of the miR9-2 enhancer in retinal organoids led to defects in retinal development (Thomas et al., 2022). To determine whether the miR9-2 enhancer has a similar role in mice, we analyzed cell class percentages from the snRNA-seq data and did not observe any significant differences (Fig. S2.1D). To validate this and determine if there were more subtle morphological differences, we performed immunolabelling for major markers of retinal cell classes in the mature enhancer KO and WT retinas. We did not observe any significant changes in cell class numbers (Fig. S2.1D & 1F; Fig. S2.2). However,



**Figure 2.1: Disruption to disease-associated miR9-2 regulatory locus leads to glial misspecification in developing mouse retina.** *A.* UCSC human genome browser tracks (hg38) of the genomic location of the microRNA, miR9-2 and its host gene. GWAS variants associated with Macular Telangiectasia Type 2 (MacTel) are depicted as red lines with the lead risk variant highlighted in yellow (Scerri et al., 2017). Tracks show accessibility from single-nucleus ATAC-

sequencing of human retina (all scaled 0-55) with bottom track showing regions conserved across vertebrate species (Scerri et al., 2017). **B.** Schematic of enhancer deletion by CRISPR targeting to generate miR9-2 enhancer knockout mouse. **C.** Schematic of generation of germline miR9-2 KO mice. **D.** qPCR of bulk retinal tissue (n=3) and snRNA-seq of MG (n=3) comparing mean Mir9-2-hg expression between miR9-2 enhancer knockout and WT retinas. qPCR: mean  $\pm$  1SD, student's t-test,  $**p \leq 0.01$ . snRNA-seq: mean represented as point, Wilcoxon rank sum test with Benjamini-Hochberg correction,  $****p \leq 0.0001$ . **E.** Immunolabeling of  $\beta$ -galactosidase (green) to visualize expression of lacZ as proxy for miR9-2 expression in 3-week mice containing floxed miR9-2 hairpin and  $\beta$ -gal reporter cassette. **F.** Immunolabeling for MG (SOX9, green) and BCs (OTX2, magenta) in cross-sections of WT and KO retinas. Cells co-expressing OTX2 and SOX9 indicated by white arrowheads. **G.** Cell count values normalized to retinal area for SOX9+ (WT, n=7; KO, n=6) and SOX9+/OTX2+ (WT, n=9; KO, n=9) cells in KO and WT retinas. Counts are average of two images of the same retina in corresponding regions. Values reported as mean  $\pm$  1SD. Mann Whitney test,  $*p \leq 0.05$ ,  $****p \leq 0.0001$ . All scale bars = 50 microns. Panels B and C were created with BioRender.com.

we did observe a change in the positioning of SOX9 positive (+) cells, where SOX9+ KO cells appeared to have migrated towards the choroidal side of the retina and were more interspersed with OTX2+ BCs (Fig. S2.2A).

To more directly dissect the role of miR9-2 in retinal development, we developed a germline miR9-2 hairpin KO mouse model. First, taking advantage of a previously published strain of MiR9-2<sup>tm1Mtm</sup> mice (JAX:036062-JAX) containing a knocked-in floxed allele of the miR9-2 hairpin loop and an upstream lacZ reporter cassette surrounded by Frt sites (Park et al., 2012), we assessed lacZ expression as a proxy for miR9-2 transcript expression. By immunolabelling  $\beta$ -galactosidase ( $\beta$ -gal) in 5-week-old mouse retinas, we visualized which cell classes expressed the reporter as proxy for miR9-2. In alignment with our previously reported accessibility and expression of the MIR9-2HG in human retina and human retinal organoids (Thomas et al., 2022), we confirmed MG-specific expression of miR9-2 in the adult mouse retina.  $\beta$ -gal+ cells had morphological features of MG cells, with long projections extending apically and basally through the retina and nuclei positioned within the inner nuclear layer (INL;



Fig. 2.1E). There did not appear to be other cell classes with reporter expression in the mature retina.

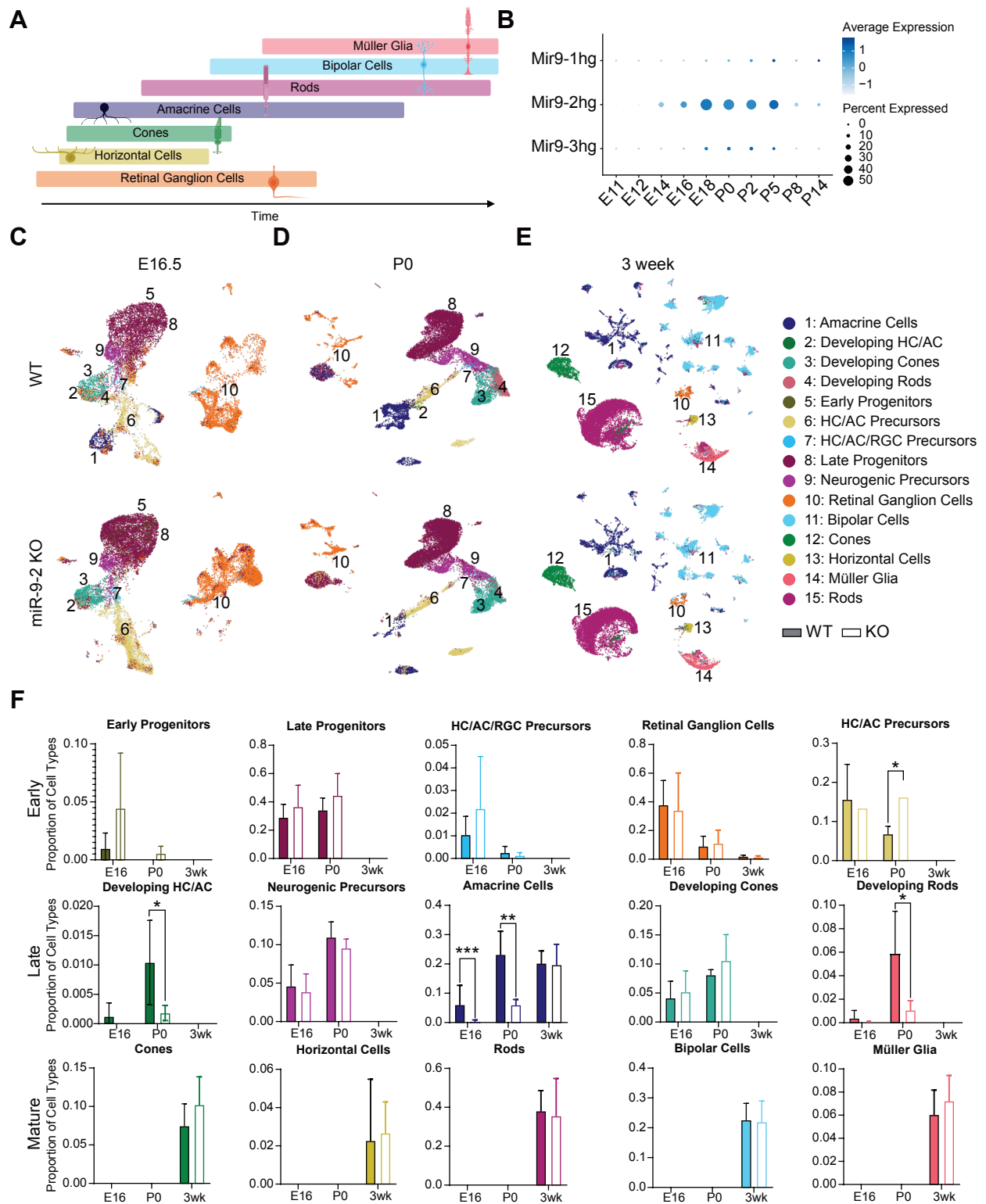
To generate the constitutive miR9-2 knockout (KO), we crossed *Mir9-2<sup>tm1Mtm</sup>* mice to transgenic *Rosa26::FLPE* knock-in mice (JAX:009086) to remove the lacZ reporter cassette and crossed the resulting progeny to *E2a-cre* mice (JAX:003724), which express germline Cre, to remove miR9-2 from all cells (Farley et al., 2000; Lakso et al., 1996). Notably, only the miR9-2 hairpin loop and nearby flanking sequence are deleted, while the remainder of the host gene is intact and able to be transcribed. We were able to confirm this directly by snRNA-seq, where we see that the host gene continues to be expressed even in KO retinas (Fig. S2.3).

To determine if cell fate specification was altered in miR9-2 KO animals, we performed immunolabeling for common markers of the major cell classes of the retina in 3-week WT and KO animals. Notably, in KO retinas, we found an increase in SOX9<sup>+</sup> cells, a marker for retinal Müller cells (Fig. 2.1F-G). Reminiscent of the enhancer knockout retinas, SOX9<sup>+</sup> cells also no longer maintained normal positioning within the retina. In the wildtype INL, while the nuclei of SOX9<sup>+</sup> MG typically reside below the nuclei of OTX2<sup>+</sup> BCs (vitreal), in the KO retinas, many of the SOX9<sup>+</sup> cells appeared to have migrated above the OTX2<sup>+</sup> BCs (choroidal). Several of the KO MG cells also entered the outer nuclear layer (ONL) which typically contains exclusively photoreceptor nuclei (Fig. 2.1F). Surprisingly, many of the knockout SOX9<sup>+</sup> cells co-expressed OTX2, a BC marker (Fig. 2.1F-G). These data indicate altered gliogenesis in miR9-2 KO retinas, leading to non-canonical marker expression and misspecification.

### *2.3.2 Delayed cell class emergence in miR9-2 KO retinas revealed by snRNA-Seq.*

To understand the origins of glial mispositioning and misspecification in the mature retina, we took advantage of snRNA-seq profiling and immunofluorescence. Normal retinal





**Figure 2.2: Loss of miR9-2 disrupts retinal cell class proportions during retinal development.**  
**A.** Schematic of overlapping windows of retinal cell class birth. **B.** Dot plot of average expression of miR9 host gene paralogs in mouse retina over time, from published retinal single-

cell RNA-sequencing atlas (Clark et al., 2019). **C-E.** UMAPs of annotated cell classes in miR9-2 WT and KO mice at E16.5 (C), P0 (D), and 3-weeks (E). **F.** Cell class proportions comparing WT (solid bar) and miR9-2 KO (empty bar) retinas for individual cell classes over time. Mean  $\pm$  1SD. Significance of WT to KO comparison was determined using EdgeR generalized linear model with Benjamini-Hochberg correction (Chen et al., 2025; Robinson et al., 2010). \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ . Panel A created with BioRender.com.

development occurs in a highly stereotyped manner, with a well-defined order and timing for the emergence of different cell classes (Fig. 2.2A). Prior literature suggests an important role for microRNAs during the transition from early- to late-born cell classes (Torre et al., 2013). To determine the timing of miR9 family member expression, we used a published single-cell atlas of mouse retinal development (Clark et al., 2019) and found that expression of miR9 host genes begins increasing at E16.5, peaking from E18.5-P5 (Fig. 2.2B). Further, *Mir9-2hg* appears to be the predominant miR9 family member during the entire time-course of retinal development, emphasizing its unique importance.

Next, we performed snRNA-seq on miR9-2 KO and sex-matched littermate control retinas at key timepoints during retinal development. We profiled WT and miR9-2 KO retinas at E16.5 to capture the early-to-late transitional period, P0 to profile during the window of peak late-born cell birth, and adult retinas at 3-weeks to assess the end of development (E16.5 & P0,  $n=4$ ; 3-weeks,  $n=5$ ; per genotype). At all timepoints, we identified and annotated the developmentally expected cell classes (Fig. S2.4). We then assessed the relative proportions of cell classes between WT and KO retinas to determine whether cell classes emerge normally in the absence of miR9-2. We found that direct loss of miR9-2 *in vivo* perturbs normal cell class proportions (Table S2.1). In developing retinas, the proportions of differentiated ACs (E16.5, P0) and developing HC/ACs (P0 only) were significantly decreased in KO retinas compared to WT (Fig. 2.2C-D, 2F; Fig. S2.5A). Correspondingly, there was a significant increase in the proportions of HC/AC Precursors (P0 only) in the KO compared to WT (Fig. 2.2D, 2.2F). This

increase in the precursor populations and decrease in developing and mature cells indicates that loss of miR9-2 leads to a more immature amacrine population.

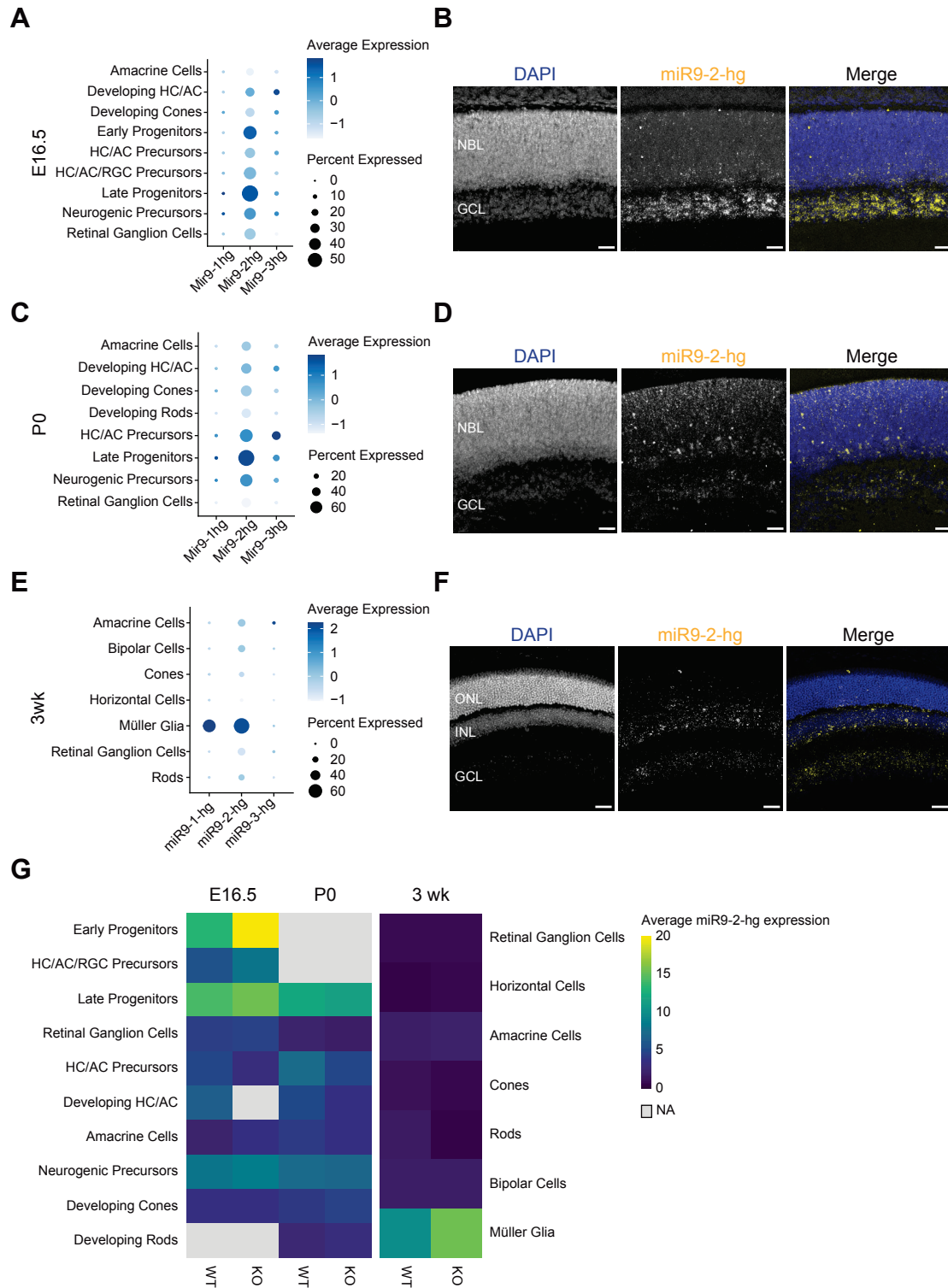
Dramatically, the emergence of rod photoreceptors, the most abundant cell class, was also disrupted. At P0, there was a significantly smaller proportion of developing rods in miR9-2 KO retinas compared to the WT. Newborn miR9-2 KO retinas appeared to almost completely lack rods (Fig. 2.2D, 2.2F). To determine whether this lack of photoreceptors could be attributed to cell death, we used Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling (TUNEL) and immunolabeling for Phospho-Caspase 3 but found no differences between WT and KO animals (Fig. S2.6). As rods are the dominant late-born cell type (Fig. 2.2A), this suggests that rods are not yet born in miR9-2 KO retinas, rather than dying. This phenotype is similar to prior studies which found that loss of Dicer in the retina leads to an inability of RPCs to produce late-born cell types (Georgi and Reh, 2011, 2010; Torre et al., 2013).

Despite these developmental differences, by 3-weeks, there were no significant differences in cell class proportions between WT and miR9-2 KO retinas (Fig. 2.2E-F). While proportions no longer differed, we know MG still developed abnormal phenotypes, including migration towards the ONL and mis-expression of bipolar markers (Fig. 2.1E-F). This suggests that the major cell classes or unique cell types of the retina still have altered transcriptional profiles, even as cell proportions are unchanged. Overall, these preliminary snRNA-seq analyses suggest that differences between WT and KO retinas during development are due to a delay in cell class production rather than a loss of late-born cells, like rods. Therefore, while it seems that miR9-2 is important for the proper timing of cell class emergence, retinas lacking miR9-2 still retain the ability to produce the major cell types, albeit with disrupted Müller glia.

### *2.3.3 miR9-2 is dynamically expressed in retinal progenitor cells and Müller glia.*

To understand more fully how loss of miR9-2 alters the timing of cell class emergence and cell identity, we sought to identify which cell classes miR9 is expressed in. Prior snATAC-seq data from our lab (Thomas et al., 2022) found that the enhancer of *MIR9-2HG* was most accessible in RPCs and MG in the developing and mature human retina, and at corresponding timepoints in human retinal organoids (Fig. 2.1A, previously published work (Thomas et al., 2022)). To determine whether this cell type-specific activity was recapitulated in the mouse retina, we used our snRNA-seq dataset to examine the expression levels of *Mir9-2hg* across cell types at each profiled timepoint. Using expression profiles from our WT mice, we found that at both E16.5 and P0, early and late RPCs had the highest *Mir9-2hg* expression, followed by several populations of precursor cells. In the mature, 3-week-old mouse retina, *Mir9-2hg* was predominantly expressed in MG. Further, as there are three paralogs of miR9 in the mouse, we checked the expression of the miR9-1 and miR9-3 host genes; at developmental timepoints, *Mir9-2hg* was the most predominantly expressed miR9 host gene in RPCs (Fig. 2.3A, 3C; Fig. S2.7). At 3-weeks, the expression of miR9 paralogs was highly specific to MG cells. While *Mir9-2hg* was still the most predominant paralog expressed, *Mir9-1hg* expression was also detectable (Fig. 2.3E; Fig. S2.7).

We aimed to validate the miR9-2 expression patterns using fluorescent *in situ* hybridization. We collected wildtype C57Bl6/J retinal tissue at timepoints aligning with our snRNA-seq data and used RNAscope Plus to probe for *Mir9-2hg* transcripts. At E16.5 and P0, the expression of the transcript was dispersed across the neuroblastic layer (NBL) of the retina (Fig. 2.3B, 2.3D). The positioning of these transcripts is reflective of the migratory movement of RPCs during development as they migrate apically and basally during division and proliferation



**Figure 2.3: *Mir9-2hg* is enriched in retinal progenitor cells and Müller glia.** Dot plots of *miR9* paralog expression in WT cells of major cell classes from snRNA-seq at E16.5 (**A**), P0 (**C**), and 3-weeks (**E**). In situ RNA hybridization images of *Mir9-2hg* (yellow) in retinal cross sections of WT C57Bl6/J animals at E16.5 (**B**), P0 (**D**), and 3-weeks (**F**). Scale bars = 50 microns. **G**. Single-nucleus RNA-seq heatmap of average expression of *Mir9-2hg* in WT and *miR9-2* KO mice

*in each cell class. Gray boxes indicate cell classes in negligible numbers/not present. Scaled (0-20) across all timepoints.*

(Baye and Link, 2008; Norden, 2017). Additionally, at E16.5 there appeared to be strong labeling in the developing ganglion cell layer (GCL), potentially corresponding to cytoplasmic accumulation in post-mitotic precursor cells (Fig. 2.3B). At 3-weeks, the expression of the transcript became more concentrated in the inner nuclear layer (Fig. 2.3E), consistent with the MG-specific expression seen by snRNA-seq and lacZ reporter mouse expression (Fig. 2.1E).

We next sought to understand how this expression was affected in the miR9-2 KO and whether other miR9 paralogs would be able compensate for loss of miR9-2. Using our snRNA-seq dataset, we found that *Mir9-2hg* remained the predominant paralog expressed in the mouse retina across all time points in WT and KO animals (Fig. 2.3G; Fig. S2.7). Unexpectedly, expression of *Mir9-2hg* appeared to be increased in KO retinas, particularly in early progenitors at E16.5 and MG in the mature retina (Fig. 2.3G). This implies a potential autoregulatory role of miR9, where disruption of an autoinhibitory signal from mature miR9 causes an increase in *Mir9-2hg* expression. Such a negative feedback loop for miR9 has been suggested in other systems but not directly demonstrated in mammals.

#### *2.3.4 Loss of miR9-2 leads to direct and indirect target gene disruption during the transition from early-to-late born retinal cell classes.*

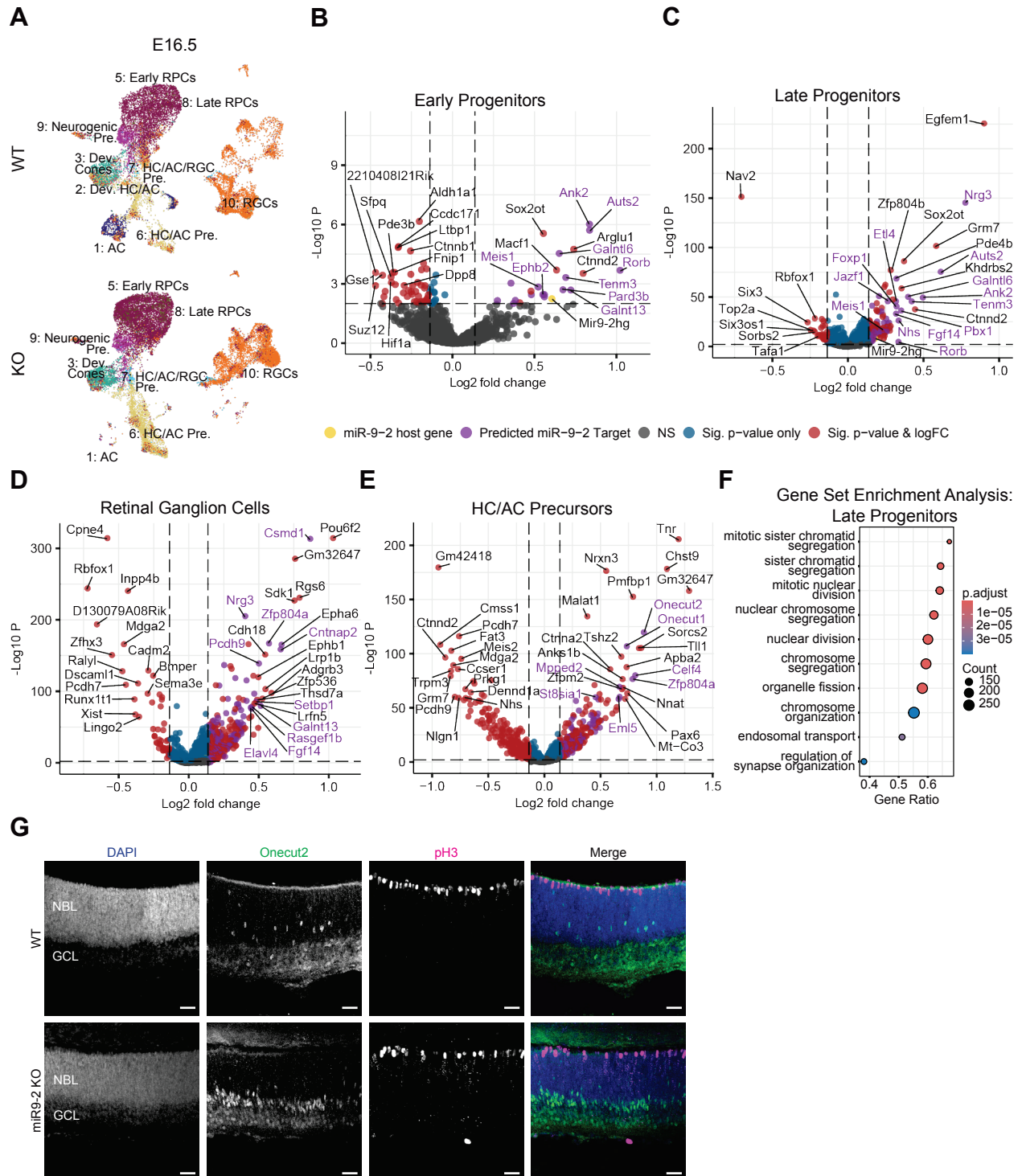
After identifying the major cell types that express miR9-2, we sought to characterize how each developmental period was affected by miR9-2 loss. To do so, we performed differential expression analyses using Presto (Korsunsky et al., 2022) to compare WT and miR9-2 KO transcriptional profiles of each cell class (Table S2.3). As the primary function of a miRNA is inhibiting translation of mRNAs through degradation or translational block of target transcripts with perfect or imperfect complementarity (Bartel, 2018), we expected that loss of a miR9-2

would disrupt a large set of putative target genes. To distinguish between direct and indirect targets of miR9-2, we first assembled computationally predicted targets of miR9 (both 3p and 5p targets) from the TargetScanMouse 8.0 database (Agarwal et al., 2015; McGeary et al., 2019). Next, we determined which of these predicted targets were expressed in WT and/or miR9-2 KO cells. For direct targets, we expected that loss of the miRNA would lead to de-repression and thereby upregulation of target genes in miR9-2 KO cells compared to WT. Concordant with these expectations, we found de-repression of specific target genes and widespread dysregulation of other indirect targets across timepoints and cell classes.

At E16.5, miR9-2 expression begins to rapidly increase during mouse retinal development (Fig. 2.2B; Torre et al., 2013), especially within RPCs. In early RPCs, some of the top upregulated genes included the TFs *Rorb* and *Meis1* (Fig. 2.4B) which are predicted to be direct miR9 targets. Furthermore, late RPCs have significant upregulation of additional putative miR9 target TFs including *Foxp1* and *Pbx1*, and early upregulation of Onecut family of TFs (*Onecut1/2/3*; not shown,  $p \leq 0.05$ ; Fig. 2.4C). Many of these TFs already have well documented roles in regulating neurogenesis, in the CNS and retina specifically. Outside of TFs, some of the predicted miR9 targets that were highly upregulated include *Auts2* and *Ank2*, which is consistent across early and late RPCs. These genes have been implicated in neurodevelopment and have documented roles in neuronal migration and synaptic formation and function (Hori et al., 2020; Yoon and Penzes, 2025).

Since loss of miR9-2 has wide-ranging effects beyond direct target de-repression, rather than focusing on individual genes, we performed pathway analyses for each cell class to give a broader sense of which processes are affected in miR9-2 KO retinas. We performed a Gene Set





**Figure 2.4: Transcriptional profiles of cell classes in miR9-2 KO retinas are disrupted at E16.5.** **A.** UMAP of cell classes in miR9-2 KO and WT retinas at E16.5. **B-E.** Volcano plots of differentially expressed genes in individual cell classes at E16.5 including early RPCs (B), late RPCs (C), RGCs (D), and HC/AC Precursors (E). Thresholds (dashed lines) correspond to  $p \leq 0.01$  and  $FC \geq 10\%$ . Wilcoxon rank sum test, Benjamini-Hochberg correction. **F.** GSEA of the



*top 10 enriched terms in E16.5 late RPCs. Adaptive multi-level split Monte-Carlo (Yu et al., 2012). G. Immunolabeling of ONECUT2+ (green) and pH3+ (magenta) cells of E16.5 KO and WT retinas in cross-section. All scale bars = 50 microns.*

Enrichment Analysis (GSEA) using the ClusterProfiler package (Yu et al., 2012) with the Gene Ontology Biological Processes (GOBP) database of pathways for reference (Ashburner et al., 2000; The Gene Ontology Consortium, 2026). Of the top 10 enriched pathways for late RPCs, most terms were associated with mitosis including “mitotic sister chromatid segregation” and “mitotic division” (Fig. 2.4F). This enrichment of mitotic pathway terms in KO retinas suggests increased proliferation, delay of cell cycle exit, or a difference in cell cycle length which would be in alignment with a more immature state. Using pH3 as a marker of dividing cells, we immunolabeled developing E16.5 retinas and found that KO retinas appeared to have a sparser distribution of dividing cells on the apical (choroidal) side of the neuroblastic layer, but an increased number of cells containing condensing chromatin dispersed throughout the neuroblastic layer (puncta; Fig. 2.4G). To investigate further, using Seurat, we performed cell cycle phase analysis, which classifies cells into G1, G2/M, or S phases, and found only one cell class, Developing Cones, that differed in G2M phase proportions between WT and KO retinas (Fig. S2.5; Table S2.2). However, for cones, especially as miR9-2 expressing RPCs aren’t affected, it is likely that this result is related to the overall alteration in cell class production rather than direct cell cycle modulation. These data suggest, therefore, that miR9-2 is controlling cell class emergence independently of cell cycling states.

While changes to RPCs are clear, we also wanted to understand how these alterations affect production of retinal cells following specification of retinal cell types. As *Mir9-2hg* expression is highest in RPCs and upregulated genes in early RPCs are significantly enriched for computationally predicted miR9-2 targets (Table S2.6), we expect later cell type alterations to be

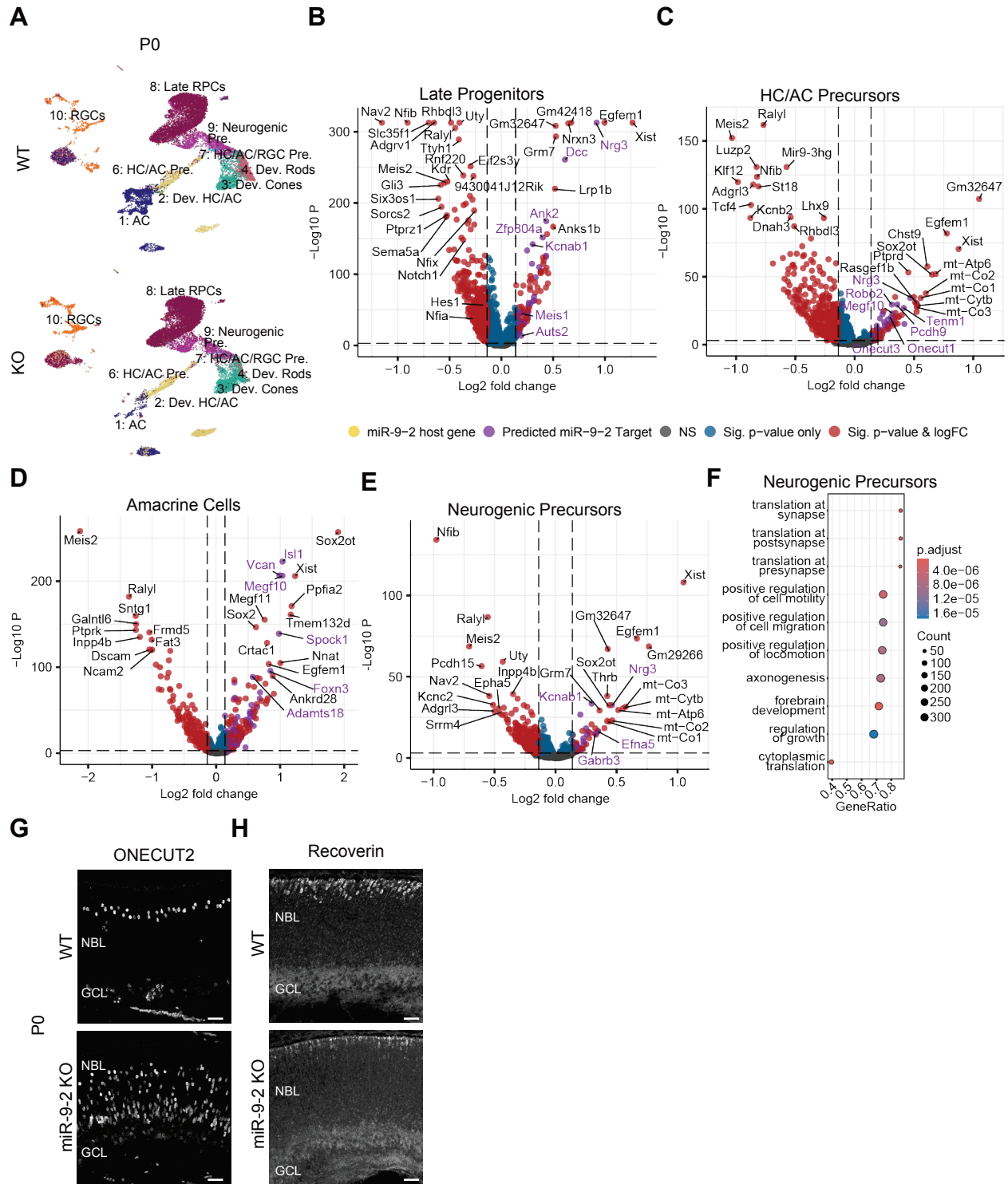
the result of upstream progenitor changes. Retinal ganglion cells (RGCs) are the earliest born cell type of the retina, and by E16.5, the majority of RGCs have been born. Between miR9-2 KO and WT retinas, there is a clear segregation of RGC populations visible on the E16.5 UMAPs (Fig. 2.4A). To understand why these populations are distinct, we compared the transcriptional profiles of KO and WT RGCs (Fig. 2.4D). The top upregulated gene in KO RGCs is *Pou6f2*, a ganglion cell marker that is expressed in immature RGCs and ACs during development and only develops a restricted expression pattern to RGC subtypes in adult retinas (Lyu and Mu, 2021; Zhou et al., 1996). Within individual cell classes, cell types are also born with a distinct order and timing; therefore, these data suggest that not only is the overall retina more immature, but also that individual cell types are immature as well.

As the AC population is decreased in E16.5 miR9-2 KO retinas, we focused on horizontal and amacrine precursor cells (HC/AC Precursors) to understand what leads to this alteration. Many of the top upregulated genes in these precursors persist from early and late RPCs and include the TFs, *Onecut1* and *Onecut2* (Fig. 2.4E). Onecut TFs are among the top computationally predicted targets of miR9 and have been experimentally confirmed to be direct targets of miR9 in the CNS (Agarwal et al., 2015; Madelaine et al., 2017; McGeary et al., 2019). To validate this finding, we immunolabeled E16.5 retinas for ONECUT2 and found a significant increase in ONECUT2+ cells in KO retinas compared to WT (Fig. 2.4G). In the context of retinal cell fate specification, Onecut TFs have well-characterized roles in specifying early-born cell classes, including ACs (Emerson et al., 2013; Sapkota et al., 2014; Wu et al., 2013). Altogether, these data support the hypothesis that mir9-2 KO causes a developmental delay in retinal cell class specification and differentiation.

### 2.3.5 Loss of miR9-2 leads to dysregulation of late-born cell classes in the developing mouse retina.

To see how the development of the retina is affected during the peak of late-born cell birth, we profiled WT and miR9-2 KO retinas at P0. By this stage, RPCs are primarily giving rise to post-mitotic neurons, such as rods, bipolars, and late-born amacrine, and later, MG (Cepko, 2014; Cepko et al., 1996). This timepoint is also during a plateau of high *Mir9-2hg* expression in the developing retina. We again compared the transcriptional profiles of WT and KO cells with a focus on RPCs and precursors that are implicated in the production of late-born cell types (Table S2.4). While several of the genes upregulated in E16.5 late RPCs remain persistently upregulated, like *Meis1*, *Ank2*, and *Auts2*, at P0, *Nfib* becomes one of the top downregulated genes in late RPCs. While more moderate, there is also a significant downregulation of other NFI factors, *Nfia* and *Nfix* (Fig. 2.5B). NFI factors, while not predicted to be direct miR9 targets, are found to have late RPC-specific expression and have been implicated in specification of late-born cell classes, such as BCs and MG (Clark et al., 2019). Additionally, other genes with documented roles in regulating neural progenitor proliferation and differentiation, including *Notch1*, *Hes1*, *Rbpj*, and *Notch2* (*Rbpj*, *Notch2*; not shown,  $p \leq 0.05$ ) (Bosze et al., 2023; Chen and Emerson, 2021; Mills and Goldman, 2017; Perron and Harris\*, 2000), are also significantly decreased in P0 late RPCs (Fig. 2.5B). Disruptions of downstream gene networks regulated by these factors likely contributes to further alterations in RPCs and the post-mitotic neurons they produce.

Among post-mitotic cells, developing HC/ACs and distinct ACs emerged by P0 in both WT and KO retinas (Fig. 2.5A). However, knockout ACs and their precursors still appear biased towards earlier born HC/AC types compared to their WT counterparts. In HC/AC precursors, as



**Figure 2.5: Transcriptional profiles of cell classes in miR9-2 KO retinas are disrupted at P0.** **A.** UMAPs of cell classes in P0 miR9-2 KO and WT retinas. **B-E.** Volcano plots of differentially expressed genes in individual cell classes at P0 including late RPCs (B), HC/AC precursors (C), ACs (D), and neurogenic precursors (E). Thresholds (dashed lines) correspond to  $p \leq 0.01$  and  $FC \geq 10\%$ . Wilcoxon rank sum test, Benjamini-Hochberg correction. **F.** GSEA of the top 10

*enriched terms in neurogenic precursors. Adaptive multi-level split Monte-Carlo (Yu, 2024; Yu et al., 2012). G. Immunolabeling of ONECUT2+ cells in P0 KO and WT retinas (WT: n=5; KO: n=6) in cross-section. Mean +/- 1SD. Wilcoxon rank sum test, \*\* $p \leq 0.01$ . H. Immunolabeling of Recoverin+ cells (green) in P0 KO and WT retinas. All scale bars = 50 microns.*

at E16.5, the Onecut TFs are still significantly upregulated (Fig. 2.5C, 2.5G). In KO retinas, we saw a significant increase in ONECUT2+ cells compared to WT. Further, while ONECUT2+ cells in WT retinas are organized in two distinct layers corresponding to HCs and ACs, in KO retinas, ONECUT2+ cells are more widely distributed. This disorganized state is characteristic of immature cells, as RPCs and precursors migrate apically and basally until they are fully matured. The ACs in KO retinas also have increased expression of early-born AC markers, and the top upregulated genes include *Isl1*, *Sox2*, *Megf10*, and *Megf11* which are specifically expressed in cholinergic starburst ACs, an early-born AC type (Cherry et al., 2009; Elshatory et al., 2007; Kay et al., 2012; Voinescu et al., 2009). Like the more immature RGCs at E16.5, these data indicate that ACs in KO retinas are dominated by earlier AC types.

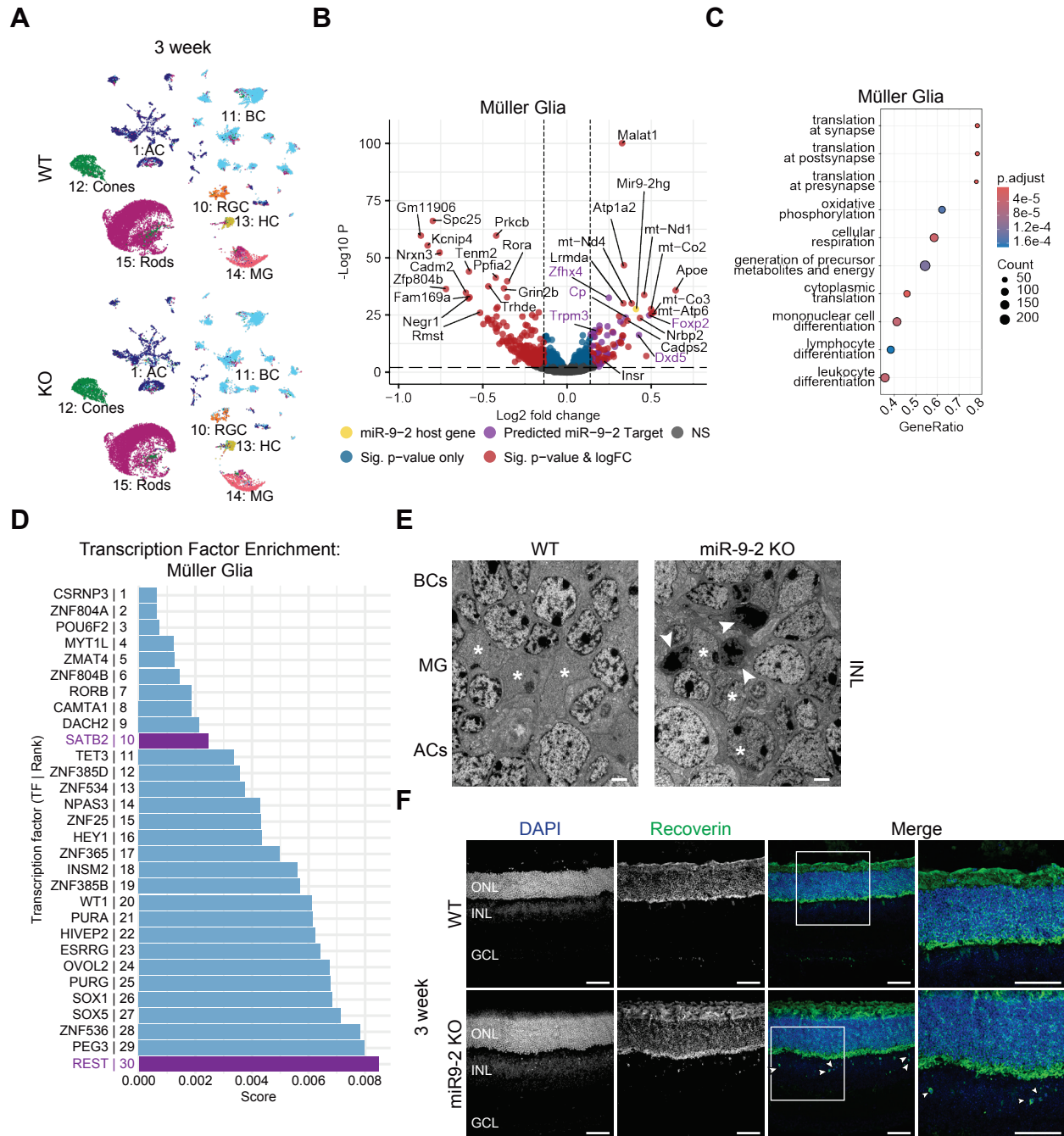
Outside of these progenitor and amacrine cell alterations, the most striking effect at P0 is the near absence of developing rods in miR9-2 KO retinas (Fig. 2.5A). To elucidate the cause, we compared neurogenic precursor cells, a population that can adopt photoreceptor fate at P0, between WT and KO retinas. *Thrb*, a marker of cones, is one of the top upregulated genes in KO neurogenic precursor cells, suggesting that these cells are more primed to adopt a cone fate, which is an earlier-born photoreceptor cell class compared to rods (Fig. 2.5E). When we performed a GSEA on these precursors, we found that some of the top enriched terms included “translation at the synapse”, “positive regulation of cell motility and migration”, and “axonogenesis” (Fig. 2.5F). These terms are indicative of processes related to neuronal maturation, as differentiating neurons need to migrate to the correct spatial location, locate synaptic partners and form functional circuits. Lastly, to validate changes to photoreceptors in

KO retinas, we performed immunolabeling for recoverin, a marker of retinal photoreceptors (Fig. 2.5H). In alignment with our snRNA-seq data, we find a decrease in the number of recoverin-positive cells in the KO compared to WT. This supports the observation that loss of miR9-2 delays formation of rods. Altogether, these data collected at the peak of late neurogenesis suggest that miR9 is important for both controlling cell class emergence and controlling the timing of cell class subtype specification.

### *2.3.6 Loss of miR9-2 leads to dysregulation of MG cells in the mature mouse retina.*

Despite the striking developmental delay in miR9-2 KO developing retinas, by 3-weeks, the proportions of major cell classes are not significantly different (Fig. 2.2, Fig. 2.6A). Paradoxically, this result appears to be in direct conflict with our KO retinal histology, where we find an increase in SOX9<sup>+</sup> cells suggesting an increase in MG. To resolve this incongruency, we first compared the transcriptional profiles of WT and KO Müller glia (Fig. 2.6B; Table S2.5). Among the top upregulated genes, we found several predicted miR9 targets including *Cp*, *Trpm3*, *Foxp2*, and *Dxd5*, among other genes including *Apoe*, *Nrbp2*, *Cadbps2*, and *Lrmda*. Top downregulated genes included *Prkcb*, *Rora*, *Nrxn3*, and *Spc25*. We performed a GSEA, to understand what processes were disrupted in these cells. Some of the top enriched terms included “translation at synapse”, “cellular respiration”, and “mononuclear cell differentiation” (Fig. 2.6C). Disruptions to these pathways suggest a disruption to Müller identity, where enrichment of synapse formation and changes to metabolic processes could indicate that KO Müllers have more neuronal attributes. We also performed a TF enrichment analysis using CHEA3 (Keenan et al., 2019) and found that among the top predicted TFs, there are putative miR9 targets, including *Satb2* and *Rest*, which have documented roles in chromatin remodeling (Fig. 2.6D) (Gyorgy et al., 2008; Hwang and Zukin, 2018; Wahl et al., 2024). Taken together,





**Figure 2.6: 3-week mature MG cells are disrupted in miR9-2 KO retinas.** **A.** UMAPs of cell classes in 3-week miR9-2 KO and WT retinas. **B.** Volcano plot of differentially expressed genes in 3-week MG. Thresholds (dashed lines) correspond to  $p \leq 0.01$  and  $FC \geq 10\%$ . **C.** GSEA of the top 10 enriched terms in 3-week MG. **D.** Top 30 ranked genes from TF enrichment analysis (rCHEA3) for MG. Low-scoring TFs contribute most. Over-representation analysis, Fisher's Exact Test. TFs that are upregulated in KO and are computationally predicted miR9 targets in purple. **E.** Transmission electron microscopy images of INL of WT and KO retinas. MG cells (white asterisks), ACs, and BCs are visible. Rods indicated by white arrowheads. Scale bars = 2

*microns. F. Immunolabelling of recoverin<sup>+</sup> (green) photoreceptors in KO and WT retinas in cross-section. Rods in INL marked by white arrowheads. Lower expressing recoverin<sup>+</sup> BCs are seen in both WT and KO. Scale bars = 50 microns.*

these data provide further evidence for the altered state of KO MG and is in alignment with our early findings that KO MG co-express both Müller markers and neuronal (BC) markers (Fig. 2.1E-F).

To further probe the alterations to 3-week retinas, we used transmission electron microscopy (TEM) to investigate MG morphology. We harvested mature retinas from 3-week-old mice and assessed for gross morphological differences between WT and miR9-2 KO animals. Focusing on MG, we found that the nuclei of KO cells appeared altered. MG nuclei are characterized by their polygonal shape and linear positioning in the middle of the INL, with their heterochromatin normally appearing uniformly diffuse across the nucleus (Fig. 2.6E, asterisks, WT). However, in KO retinas, MG chromatin looks like the surrounding interneuron nuclei (ACs, BCs), where chromatin has multiple focally condensed chromocenters within each nucleus (Fig. 2.6E, asterisks, KO). KO retinas also appeared disorganized, with infiltration of rods into the INL even though these nuclei would normally only reside in the ONL. Rod nuclei have a distinctive ring-like appearance as rod heterochromatin is condensed to a large central mass (Fig. 2.6E, arrowheads) (Solovei et al., 2009). To further validate our findings, we performed immunolabelling for markers of rods in 3-week KO and WT animals. As in the TEM, we found recoverin<sup>+</sup> cells abnormally localized in the INL (Fig. 6F, arrowheads). Like our snRNA-seq data, these data indicate a disorganization of normal retinal structure and point to potential alterations in cell class identity, especially as mature MG cells no longer display their characteristic chromatin patterning, instead taking on a more neuronal appearance. Further, as MG often play a structural support role in the retina, this disruption to the glia may contribute to



rod mis-localization and disruption of retinal architecture. Dysregulation of glial identity and disruption of critical pathways such as oxidative phosphorylation, cellular respiration, along with de-repression of neuronal programs in miR9-2 KO MG may shed light on mechanisms of retinal disease risk associated with miR9-2 and its conserved enhancer.

## 2.4 Discussion

The importance of microRNAs in generating a fully functional retina has been well established, but it is not fully known which miRNAs contribute most to developmental regulation and which aspects of development they regulate. In this work, we developed an *in vivo* germline knockout mouse model of miR9-2 function and found that disruption of miR9-2 delays the timing in which retinal cell classes emerge. Surprisingly, we find this delay occurs not just for the major cell classes, but also for cell types within those classes, such as cholinergic and glycinergic ACs. Additionally, in the absence of miR9-2, we determined that, while all retinal cell classes will eventually form, the MG of miR9-2 KO animals appear to be mis-specified and adopt a hybrid neuronal- and Müller-like cell fate. This suggests that miR9-2 sits at a critical intersection of GRNs to control retinal progenitor competence with a high degree of temporal precision.

We identified several key TFs that miR9-2 directly regulates to control these cell fates, including the *Onecut*, *Rorb*, and *Foxp* families. Of these key regulators, all are computationally predicted to be direct target genes of miR9-2 and are de-repressed in miR9-2 KO RPCs, the cell type with highest miR9-2 expression in development. Several putative miR9-2 targets included TFs that are known to promote progenitor proliferation, such as *Rorb*, or TFs that promote early-born cell fates, like *Foxp1* and the *Onecut* TFs (including *Onecut1/2/3*) (Emerson et al., 2013; Huot et al., 2014; Sapkota et al., 2014; Vassalli et al., 2024; Wu et al., 2013, 2012; Zhang et al.,

2022). In addition to interneurons, Onecut TFs and *Rorb* have documented roles in regulating photoreceptor fates, including promotion of cone cell fate (former) and maintenance of the balance between cone and rod fates (latter) (Emerson et al., 2013; Jia et al., 2009; Sapkota et al., 2014; Srinivas et al., 2006; Vassalli et al., 2024). With the loss of miR9-2, these TFs were downregulated, likely contributing to the increased proportions of early-born cell classes and the overall immature phenotype of the KO retinas. Therefore, the direct interactions of miR9-2 with these crucial TFs seem to contribute to the ability of RPCs to transition from producing early- to late-born cell fates at the proper developmental time.

More broadly, the loss of miR9-2 appeared to target genes and pathways that are found to be highly involved in retinal cell fate specification. Specifically, expression of Notch signaling effectors was downregulated in miR9-2 KO late RPCs, including a large (25%) decrease in *Notch1* with more subtle, yet still significant decreases in expression of *Hes1*, *Notch2*, and *Rbpj* (Fig. 5B). Notch expression can promote continued proliferation of RPCs and regulate the formation of early-born cell types like RGCs and cones (Bansod et al., 2017; Bosze et al., 2023; Chen and Emerson, 2021; Coolen et al., 2012; Mills and Goldman, 2017; Perron and Harris\*, 2000). Late RPCs also had a substantial downregulation of NFI factors. This may be driven, in part, by the upregulation of the TF, *Foxp1*, in E16.5 late RPCs (Fig. 2.5B), as its overexpression has been found to inhibit *Nfib* (J. Zhang et al., 2023). Recent single cell studies found that NFI factors are restricted to late RPCs during retinal development and promote MG and BC fates (Clark et al., 2019). Therefore, in addition to directly regulating key drivers of early-born cell fate, miR9-2 seems to indirectly regulate the proper balance of TFs and signaling pathways that are important for promoting late-born fate.

By the end of development, while all retinal cell classes eventually formed, the presence of mis-specified MG cells in the mature miR9-2 KO retina was distinct. We found mis-expression of OTX2, choroidal migration of SOX9<sup>+</sup> MG to the ONL, and qualitative changes in the chromatin structure of these cells. Altogether, these data suggest that KO MG have a neuronal-glial hybrid identity and develop a more neuronal fate. While our immunofluorescence results suggested that the number of SOX9<sup>+</sup> Müller glia cells were increased (Fig. 1E-F), the hybrid state of MG may contribute to our inability to see these cell proportion changes in our snRNA-seq dataset; it is probable that hybrid cells segregate into MG and BC clusters. Therefore, this presumed increase in the number of MG could result from an extended window of proliferation in progenitor cells, whose transcriptional profile is most similar to Müller glia, resulting in the conversion of extra RPCs into extra glial cells at the end of development. This hypothesis is supported by our gene set enrichment analysis which suggests that mitotic processes in RPCs are upregulated, but future experiments investigating postnatal retinal development are needed to resolve this question. Transcriptionally, of the DEGs identified in MG of miR9-2 KO and miR9-2 enhancer knockout mice, there are only four upregulated putative miR9 targets shared between models, including *Trpm3* (addressed below), *Foxp2*, *Insr*, and *Cdh4*. *Insr*, *Cdh4*, and *Foxp2* have all been implicated in synapse formation in the CNS (Babb et al., 2005; Olguin et al., 2025; Sánchez-Cruz et al., 2022; Vernes et al., 2011) while *Foxp2*, in particular, is a TF that has also been linked to speech and motor circuit formation and neurogenesis (Tsui et al., 2013).

In regenerative animals such as zebrafish, MG act as latent RPCs, and in response to injury can de-differentiate to become neurons. While mammalian MG do not retain this native ability, they are known to have progenitor-like expression profiles and have some neurogenic

potential when artificially induced to differentiate. Therefore, miR9-2 may have a role in maintaining the progenitor-like identity of these MG, and its loss may be permissive for differentiation into other cell fates. The MG-restricted expression of miR9-2 in the mature retina also suggests that miR9-2 is important for the maintenance and homeostasis of these cells. Further work is required to explore the potential of miR9-2-related modifications to MG identity and determine whether miR9 depletion can be used regeneratively, to promote Müller glia reprogramming. As this paper models the disruption of miR9-2 in all tissues, from conception, it is possible that more targeted disruptions to miR9 function, occurring at later timepoints or targeting specific cell types (such as Müller glia) could provide great value in future study.

Lastly, this mouse model of miR9-2 loss serves as a starting point for understanding retinal disease risk. The miR9-2 locus has been increasingly linked to Macular Telangiectasia Type II (MacTel), a rare age-related degenerative disease that leads to central vision loss. In MacTel, early-stage patients appear to lose MG cells prior to photoreceptor and subsequent vision loss while also experiencing metabolic defects (Gantner et al., 2019; Powner et al., 2010; Scerri et al., 2017). In this study, we found several genes upregulated in miR9-2 KO MG that could contribute to this disease, including *Cp*, a copper transporter and iron oxidoreductase (García-Castiñeiras, 2010), and *Trpm3*, a Ca<sup>2+</sup> channel that can be activated by sphingosine (Hoffmann et al., 2010). In the context of MacTel, dysregulation of *Cp*, and thereby iron homeostasis, could lead to inflammation and/or ferroptosis (García-Castiñeiras, 2010), while *Trpm3* over-activation by sphingolipids could cause eventual loss of MG in MacTel patients (Gantner et al., 2019). *Trpm3* is also the host gene of another miRNA, miR204. As *Trpm3* is upregulated in KO retinas, we looked for repression of its predicted targets and found that significantly downregulated genes in KO MG were enriched for predicted miR204 targets (Table

S2.6). While this was an unexpected finding, it solidifies the concept that miR9-2 sits at a vital intersection of GRNs which allows it to have these wide-ranging effects. This study highlights how a single individual miRNA can have a significant impact on proper retinal development and its risk for retinal disease.

#### *2.4.1 Limitations of the study*

In this study, we find new insight into the function of an individual miRNA, miR9-2, and its effects on retinal development and glial identity. However, whole body disruption of miR9-2 led to additional unexpected phenotypes in the brain and affected animal survival (see Fregoso *et al.*, 2025) which made it difficult to capture much of the postnatal development of these mice and led to small sample size. While we were able to capture 3-week retinas for use in single-nucleus profiling, our ability to obtain these samples was exceedingly rare. This also only allowed us to study retinas from development to early maturity, even though many retinal diseases are late onset. Similarly, this made it difficult for us to parse if miR9-2 has distinct roles in development versus glial homeostasis. Future studies would benefit from targeted disruptions of miR9-2 function and investigation into miR9-2 function in aging retinas.

### *2.5 Methods*

#### *2.5.1 Mice*

*Generation of miR9-2 enhancer knockout animals:* Mice lacking the miR9-2 enhancer were generated by CRISPR targeting a ~410bp region (chr13: 83,872,798-83,873,238; mm10) containing the putative miR9-2 enhancer and corresponding to chromatin accessibility determined by our previously published ATAC-sequencing of mouse retina (Cherry *et al.*, 2020). Briefly, two sets of CRISPR guide pairs were designed to target the putative enhancer region and

were co-injected with Cas9 into fertilized eggs from C57BL/6J mice to generate knockout animals (performed by Cyagen). This resulted in animals with deletion of the putative miR9-2 enhancer region in all cells (see Table S2.7 for guides and genotyping primers).

*Generation of miR9-2 knockout animals:* miR9-2<sup>tm1Mtm</sup> mice, containing a beta-galactosidase (beta-gal) reporter for miR9-2, a neomycin selection cassette, and the floxed miR9-2 hairpin loop were obtained from Jackson Laboratories (Park et al., 2012). To remove the selection cassette and beta-gal reporter which are flanked by Frt sites, we first crossed miR9-2<sup>tm1Mtm</sup> mice with ROSA26::FLPe knock in mice (Farley et al., 2000); subsequently, to knockout miR9-2, we crossed the resulting progeny with E2a-cre mice (Lakso et al., 1996). This resulted in a germline miR9-2 knockout (KO) strain with constitutive loss of miR9-2 in all cells (see Table S2.7 for genotyping primers for all lines). To ensure uniform C57Bl6/J background, heterozygous animals were backcrossed to C57Bl6/J breeders for at least 10 generations.

*Sample harvest:* To obtain retinas from embryonic timepoints, breeding animals were paired and plug checks performed daily. Once plugs were confirmed, females were separated from males and housed individually. Females were monitored to confirm pregnancy before embryos were extracted at embryonic age (E), E16.5. To obtain postnatal timepoints, breeders were monitored for birth daily, and pups were collected at relevant postnatal (P) timepoints, P0 and 3-weeks (P21-P25). For immunofluorescence assays, P0 and E16.5 whole heads were harvested, or 3-week eyes were enucleated and immediately fixed post-harvest. For all single-cell samples, eyes were enucleated, retinas dissected out, and tissue immediately flash frozen in liquid nitrogen and stored at -80C or in liquid nitrogen until use. For RNAscope samples, we used C57Bl6/J timed pregnant dams from JAX (JAX:000664) to harvest wildtype retinas at E16.5, P0, and 3-week

timepoints. Retinas from these animals were collected and harvested for immunohistochemistry as described below.

### *2.5.2 Immunohistochemistry*

*Fixation and cryosectioning:* Fixation: For E16.5 and P0 whole head samples, tissue was fixed for 24 hours at 4C. For 3-week samples, enucleated mouse eyes were fixed for 1 hour at room temperature with cornea opened to expose tissues. All samples were fixed in a solution of 4% PFA in 1X PBS. After fixing, samples were washed 3 times with 1X PBS. Cryosectioning: For enucleated eyes, cornea and lens were dissected off. Retinas and whole heads were then washed through a sucrose gradient of 5%, 15%, and 30% sucrose in 1X PBS until tissues sank in solution. Tissue was embedded in 100% O.C.T., frozen on dry ice, and stored at -80C. Cryosectioning of 40-micron sections was performed at -20C, and sections were mounted on TruBond Gold slides, dried on a 37C slide warmer for 1 hour, and stored at -20 to -80C until use.

*Antibody staining:* Upon removal from storage, before staining, slides were dried for 3 hours at 60C to maximize tissue adherence. To remove O.C.T., tissue was rinsed for 15 minutes in 1X PBS. To promote antigen retrieval, slides were steamed for 10 minutes in sodium citrate buffer (10mM Citric Acid, 0.05% Tween 20, pH 6.0) and washed with 1X PBS to prepare for antibodies. For primary antibody (see table for antibodies and dilutions), antibodies were diluted into blocking solution (1% BSA, 0.05% SDS, 0.05% Triton in 1X PBS) and left on slides at 4C overnight in a humidified chamber. Primary antibody was removed by washing slides 3 times with 1X PBS. Secondary antibody was diluted (1:750) in blocking solution and left on tissue for 2-3 hours at room temperature (RT). Antibody was removed by washing 3 times with 1X PBS and slides were cover-slipped using Prolong Gold Antifade (Invitrogen).

*TUNEL staining for cell death:* TUNEL assays were performed using the *In Situ* Cell Death Detection Kit, Fluorescein (Roche). Experimental slides prepped for immunohistochemistry along with two control (positive and negative) slides were used for each experiment. The positive control tissue was treated with DNase I recombinant, grade I at a concentration of 300U/mL in 50mM Tris-HCl, pH 7.5, 10mM MgCl<sub>2</sub>, 1mg/mL BSA for 10 minutes at 37C to induce DNA strand breaks prior to labeling. All slides were washed 30 minutes with 1X PBS then permeabilized in 0.2% Triton X-100 in fresh 0.1% sodium citrate solution at RT for 30 minutes. Slides were rinsed twice with 1X PBS and then excess liquid removed. In a humidified chamber, 150µL of TUNEL reaction mixture (1:9 ratio of Enzyme to Label solution) was applied to each slide. The negative control was treated only with the Label solution. Slides were then incubated for 60 minutes at 37C, washed 3 times with 1X PBS, then stained with DAPI antibody at a 1:1000 concentration for 10 minutes. After a 1X PBS wash, slides were cover-slipped using Prolong Gold Antifade (Invitrogen).

### *2.5.3 In Situ Hybridization*

*In situ* hybridization was performed using RNAscope Plus smRNA-RNA HD Reagent Kit (ACD). Tissue was fixed as described previously, cryosectioned into 20-micron sections at -20C, and then mounted on Trubond Gold slides. Slides were air-dried for 60 minutes at RT, then washed for 5 minutes with 1X PBS before baking for 60 minutes at 60C in a hybridization oven. Baked slides then underwent post-fixation in 4% PFA in 1X PBS for 15 minutes at 4°C. To dehydrate tissue, slides were put through an ethanol gradient (50%, 70%, 100% EtOH) for 5 minutes each at RT before air-drying for 5 minutes at RT. Tissue was post-fixed in 4% PFA in 1X PBS overnight at 4°C. Slides were then treated with RNAscope Hydrogen Peroxide for 10 minutes at RT and washed twice in distilled water. To perform target retrieval, slides were first



immersed in prewarmed distilled water for 10 seconds to acclimate to temperature before being immersed in prewarmed 1X Target Retrieval Reagent and steamed for 10 minutes. They were promptly removed and rinsed in distilled water for 15 seconds, immersed in 100% EtOH for 3 minutes, then air-dried at RT for 5 minutes. RNAscope Protease III was applied to each slide for 10 min at 40°C in a hybridization oven, then rinsed twice with distilled water. Advanced Cell Diagnostics (ACD) Bio performed probe design with the S1 probe targeting the miR9-2 host gene. Slides were covered in the probe solution, allowed to hybridize for 2 hours at 40C, then rinsed twice in 1X Wash Buffer. For amplification, RNAscope Plus smRNA-RNA HD AMP1 was added to slides for 30 minutes at 40C, then washed twice in 1X Wash Buffer. This step was repeated for AMP2 then AMP3, however AMP3 was only incubated for 15 minutes. To develop the signal, RNAscope Plus smRNA-RNA HRP-S1 was applied for 15 minutes at 40C and washed twice in 1X Wash Buffer. To conjugate the fluorophore to probe, the slide was incubated with TSA Vivid Fluorophore 520 dye (1:500, in TSA buffer) for 30 minutes at 40C. Slides were rinsed twice in 1X Wash buffer, incubated in HRP Blocker for 15 minutes at 40C, and rinsed twice again with 1X Wash Buffer. After the final rinse, DAPI was added to each slide for 30 seconds at RT then cover-slipped using Prolong Gold Antifade (Invitrogen). Coverslips were dried at RT overnight and protected from light before imaging or storage at -20C.

#### *2.5.4 Electron Microscopy*

Eyes were enucleated from 3-week miR9-2 knockout and wildtype littermate pairs and fixed in 4% glutaraldehyde in 0.1M cacodylate and then washed 4 times in 0.1M sodium cacodylate buffer. Samples were post-fixed in 2% osmium tetroxide, buffered, followed by 1% uranyl acetate in water, and then washed 4 times with water. Tissue was dehydrated in a series of ethanol washes from 30-100% ethanol and then given two changes of propylene oxide. Tissue

was embedded in Epon Araldite 502 and cured for 24 hours at 60C. A JEOL JEM 1200EXII electron microscope at 80kV and a spot size of three was used to image 80nm sections.

#### *2.5.5 Confocal Microscopy and Image Processing*

*Microscopy:* All slides were imaged using the Leica Sp5 or Zeiss LSM 900 or 980 confocal microscopes. Images were taken using a 20X air or 40X oil immersion lenses, with Z stacks taken at 2-micron increments, spanning the entire thickness of the section.

Image processing: Images were processed using open-source FIJI processing software (Schindelin et al., 2012). For cell counting of SOX9 and OTX2 and for images of co-staining, a single slice from the middle of the collected Z-stacks was chosen to prevent false overlap of cells in different planes. For all other cell counting and images, max intensity Z-projections were created using the full Z stack.

*Cell Counting:* Cell counting was performed on cryosectioned, immunostained, and imaged slides. All counted images were taken at 20X or 40X magnification and investigators were masked to the sample condition in all cases. Two images taken at the same magnification were counted for each sample and were independently counted by at least two investigators to ensure consistency. Images chosen for counting were taken in roughly analogous regions of the central retina, identified by the presence of the optic nerve, and taken in proximal sections on each side of the nerve. Counts per sample were taken as the average of the two images. All sample counts were normalized to the area of the retina, defined by the DAPI-positive area encompassing the outer nuclear layer, inner nuclear layer, and ganglion cell layer.

#### *2.5.6 qPCR analysis*

*RNA Purification and cDNA Purification:* Eyes from litter- and sex- matched pairs were harvested from miR9-2 enhancer knockout animals (n=3) and retinas were dissected out. Tissue was immediately placed in 500  $\mu$ L of Trizol and RNA was extracted according to standard Trizol kit protocol. Briefly, samples were incubated for 5 minutes in solution and then homogenized before chloroform extraction. To separate phases, 0.1 mL of chloroform was added to each sample, incubated 3 minutes, and centrifuged at 12000g at 4C for 15 minutes. The aqueous phase was collected and remainder of sample discarded. Following RNA extraction, 100% ethanol was added at 1:1 concentration with extraction solution. Samples were then purified using the Zymo RNA Clean and Concentrator Kit with DNase treatment according to protocol. Samples were immediately stored at -80C until use. When ready to generate cDNA, reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit and samples were processed according to standard protocol. Samples were stored at -80C until use.

*qPCR:* cDNA from bulk retinal tissue generated from litter- and sex-matched miR9-2 enhancer knockout and wildtype pairs was collected (n=3). Primers were designed for *Tuba1a* (reference gene) and *Mir9-2hg* to confirm whether the putative enhancer targets miR9-2 (Table S2.7). qPCR was performed using SYBR Green Master Mix according to standard protocol.

#### *2.5.7 Single Nuclei Library Generation and Analyses*

*Tissue dissociation:* Retinas were dissociated using a modified version of the 10X Genomics protocol for Nuclei Isolation from Embryonic Mouse Brain Tissue for Single Cell Multiome ATAC + Gene Expression Sequencing. Briefly, flash frozen retina tissue was submerged in lysis buffer (10mM Tris 7.4, 10mM NaCl, 3 mM MgCl<sub>2</sub>, 1% BSA, 0.1% Tween 20, 0.1% IGEPAL CA-630, 1U/ $\mu$ L RNase inhibitor, and 1mM DTT, if for multiome), immediately without

thawing. Tissue was transferred to and broken up by glass dounce (10X dounce A, then 10X dounce B) before being transferred to clean tubes. Tissue was incubated in dissociation buffer for a total of 10 minutes (and agitated halfway through). Lysis was immediately neutralized in wash buffer (10mM Tris 7.4, 10mM NaCl, 3 mM MgCl<sub>2</sub>, 1% BSA, 0.1% Tween 20, 1U/μL RNase inhibitor) following incubation, and nuclei were passed through a 70-micron filter and subsequent 40-micron filter (BelArt H13680-0070; BelArt H13680-0040) to clear debris. Nuclei were pelleted and washed with wash buffer twice to clear ambient RNA before being counted using Acridine Orange/Propidium Iodine Cell Stain on the ThermoFisher Automated Countess II automated cell counter. Nuclei were spun and resuspended in 1 mL of nuclei buffer (1% BSA, 1U/μL RNase inhibitor in 1X PBS) to reach target nuclei concentration plus 10% for 10,000 cells for input into 10X Multiome or 10X 3' GEX expression, based on 10X guidelines. To verify the final concentration, resuspended nuclei were recounted using the Countess before proceeding with 10X library generation.

*Library preparation:* For E16.5 and P0 samples, libraries were generated following the protocols for the 10X Single Cell Chromium NextGEM 3' v3.1 kit. Equal numbers of littermate, sex-matched male and female pairs (n=2) were used to generate individual libraries. For 3-week samples, libraries were generated for sequencing following the protocols for the 10X Chromium Single Cell Multiome ATAC + Gene Expression kit. Littermate, sex-matched male and female pairs were used to generate individual libraries (n=3); due to poor sample quality, one male pair was discarded leaving three female pairs, and two male pairs for analysis.

*Sequencing:* All sequencing was performed by the Northwest Genomics Center at the University of Washington. Samples were sequenced using the NovaSeq SP100 flow cell, with parameters

for all sequencing runs following 10X Genomics recommendations for cycles and sequencing depth. All data was returned in FastQ file format.

*Single Cell Alignment:* All single cell data was demultiplexed and aligned using the 10X Genomics Cellranger v9.0 Count pipeline. Following alignment, raw output matrices from Cellranger were input into the Cellbender v0.3.2 remove-background pipeline to filter data for real cells and to remove ambient RNA reads (Fleming et al., 2023). Cellbender output was assessed using provided output report, and all samples that did not pass initial Cellbender quality control (QC) using default settings were run through the pipeline again using the recommended modified learning rate of 5e-5. Filtered Cellbender output matrices that passed the provided QC metrics were used for all downstream processing.

*Processing, clustering, and cell type identification of single cell datasets:* Seurat v5 was used to perform analyses of individual and aggregated samples (Hao et al., 2024). The filtered Cellbender output matrices were used to generate Seurat objects. Quality control was performed on individual samples and cells were filtered on several metrics, including mitochondrial percentage (<5%), number of UMIs, and number of genes. Individual samples of each timepoint were merged for analysis, and relevant metadata (genotype, sex, batch, sample) were annotated. Each timepoint was analyzed as its own dataset. Merged objects were normalized and scaled using SCTransform (Choudhary and Satija, 2022; Hafemeister and Satija, 2019). Dimensional reduction was performed, and the Harmony package (Korsunsky et al., 2019) was used to integrate samples before generating Uniform Manifold Approximation and Projections (UMAPs) and clustering. Merged objects were assessed for presence of doublets using scDoubletFinder (McGinnis et al., 2019). As very low numbers of doublets were found and not concentrated to any one cluster, objects were not filtered. To annotate mature retinal cell types, the 3-week

dataset was annotated using the Mouse Cell Atlas (MCA) with automated scMCA pipeline for mouse cell class identification (Sun et al., 2019). For any specific cell types called by scMCA, cell identification was simplified to the major cell class containing that cell type. To annotate developmental retinal cell types, the previously published mouse atlas of retinal development (Clark et al., 2019) was used as a reference, and cell class identities were mapped to the datasets using the Seurat FindTransferAnchors function. All automated cell type identification was validated using prior knowledge of expected cell type-specific gene expression.

*Differential gene expression analyses:* To perform differential gene expression analysis, the RNA assay of annotated Seurat objects was normalized using Seurat NormalizeData and filtered using the EdgeR (Lund et al., 2012; Robinson et al., 2010) filterbyExp (with default parameters) to remove genes with inadequate expression for statistical power. Presto (Korsunsky et al., 2022) was used to determine differentially expressed genes between wildtype and knockout samples for each cell type of interest. Predicted miR9 targets in the differentially expressed genes were identified using TargetScan8.0 (Agarwal et al., 2015; McGeary et al., 2019). Differentially expressed genes for each sample and relevant cell types were plotted as volcano plots using the EnhancedVolcano (Blighe et al., 2026) R package.

*Pathway Analysis:* To perform gene set enrichment analysis, the unfiltered list of differentially expressed genes from Presto was ranked according to the Area Under the Curve (AUC) metric multiplied by the sign of logFC. We used gseGO (default settings) from ClusterProfiler (Yu et al., 2012) to identify enriched pathways from the list of Gene Ontology Biological Processes database (Ashburner et al., 2000; The Gene Ontology Consortium, 2026) and plotted the top enriched pathways using the ClusterProfiler dotplot function.

### *2.5.8 Quantification and Statistical Analyses*

To determine enrichment of miR9-2 targets in differentially expressed genes at timepoints and cell types of interest, predicted miR9 targets were identified using TargetScan8.0 (Agarwal et al., 2015; McGeary et al., 2019). To check for enrichment, hypergeometric tests were performed using base R. For bar graphs, all plots were created and analyzed with GraphPad and are shown with error bars +/- 1SD. Mann Whitney U tests were performed to determine whether cell counts were significantly different. For all statistical analyses,  $p \leq 0.05$  were considered statistically significant.

### *2.5.9 Data Availability*

All snRNA-seq files available at GEO with accession number GSE325932. All unique code generated in this study is available at [github.com/TCherryLab/miR9-2-KO-2026](https://github.com/TCherryLab/miR9-2-KO-2026).

## *2.6 Acknowledgements*

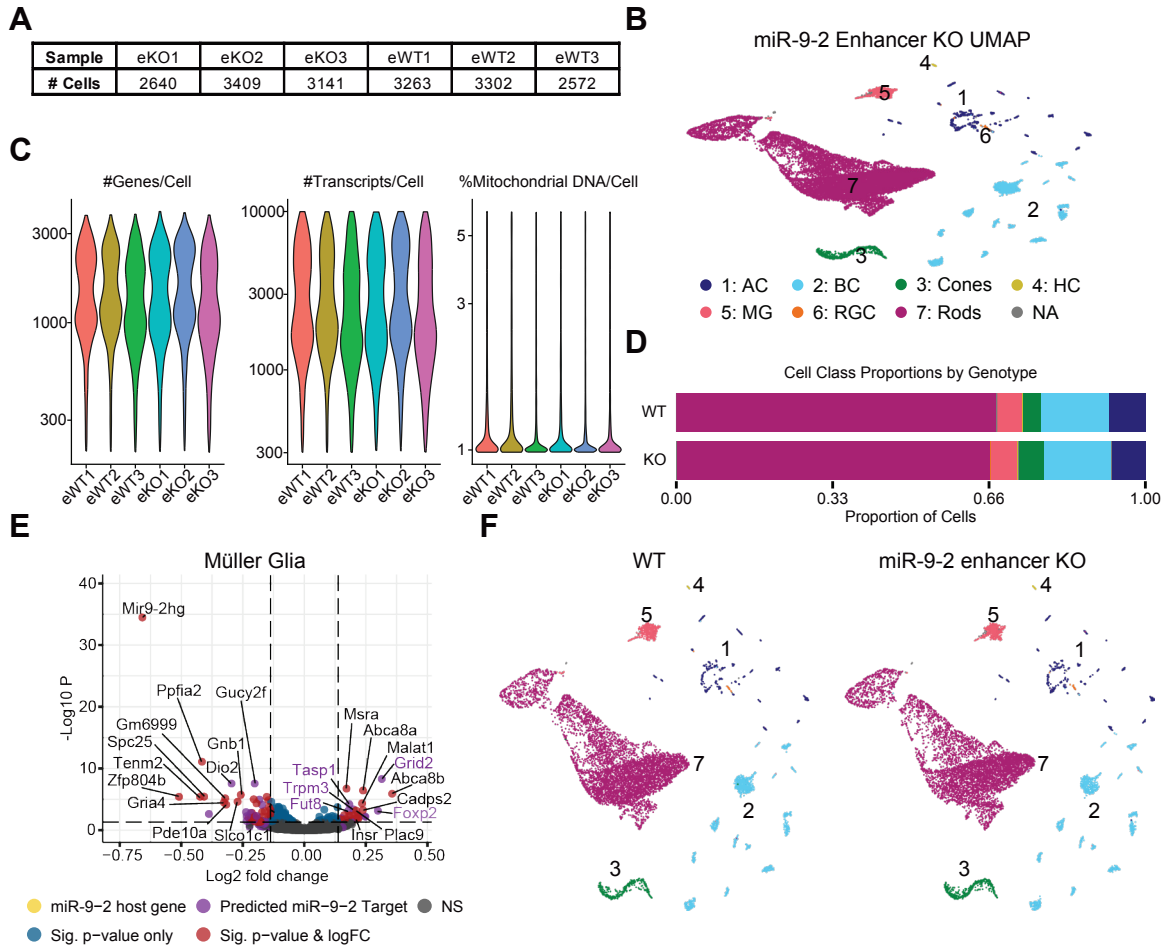
We would like to thank all members of the Cherry Lab, CDBRM, the Lowy Medical Research Institute (LMRI), as well as the LMRI Board of Scientific Governors, for helpful discussions about this project. We thank the animal care team at Seattle Children's Research Institute for their hard work and daily maintenance of our animal colony. We would also like to thank the University of Washington Vision Core and Dr. Abbi Engel for their assistance with the TEM experiments. This work was supported by grants from the NIH National Eye Institute (R01EY028584 & R01EY033364) and the Lowy Medical Research Institute to T.J.C., an NIH P30 grant from the NIH National Eye Institute (P30EY001730) to the UW Vision Core, and an NIH T32 award (EY007031) and an NIH F31 grant (EY035932) to L.K.C.

## *2.7 Author Contributions*

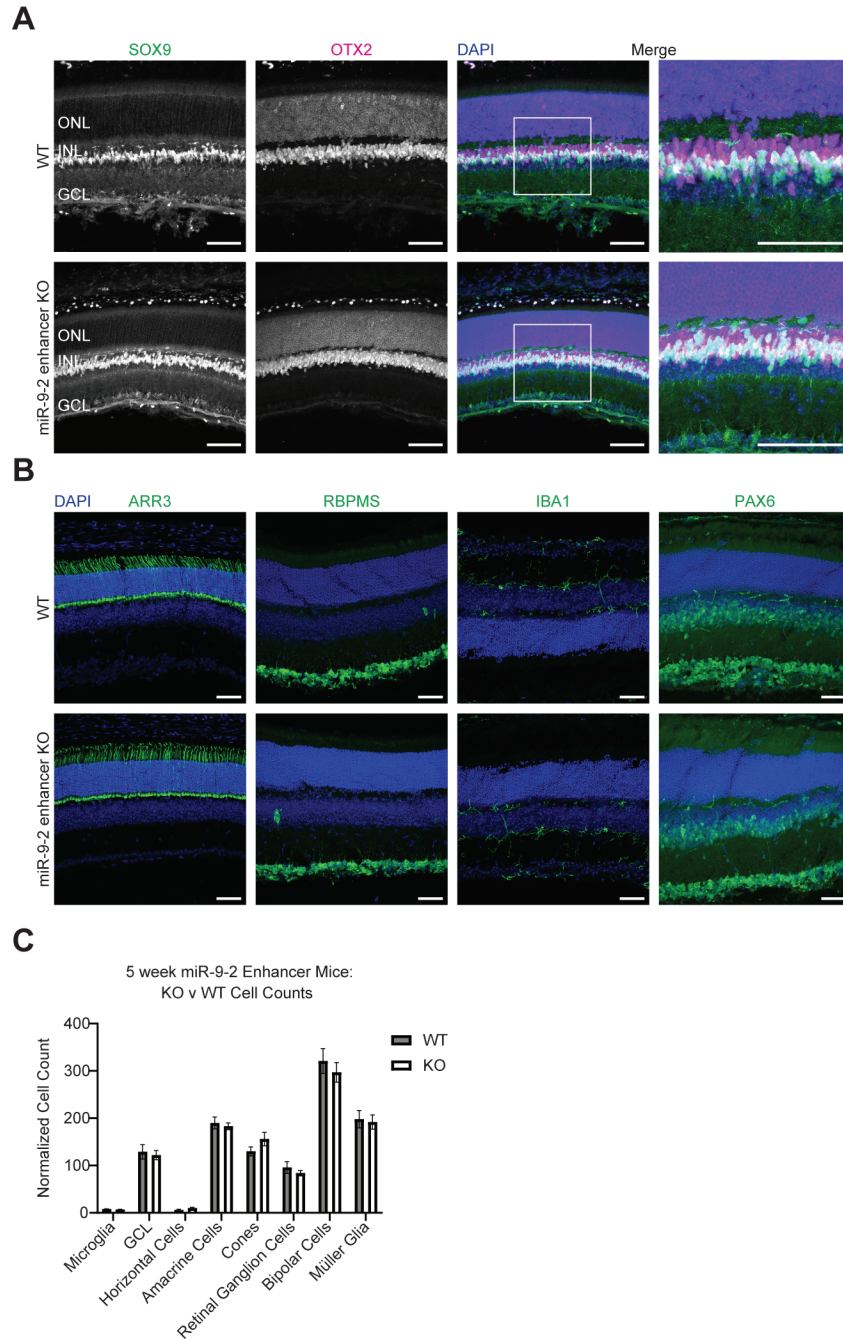
This work was performed with contributions from myself (L.K.C), Aarushi Jain (A.J.), Stella Xu (S.X.), Emma D. Thomas (E.D.T), and Timothy J. Cherry (T.J.C). L.K.C., A.J., S.X., E.D.T, and T.J.C. designed and performed all experiments and analyses associated with this work. L.K.C generated single nuclei RNA-sequencing datasets for the miR9-2 knockout mouse model and completed all bioinformatic analyses. L.K.C, A.J., and S.X. performed animal management and histology. E.D.T. generated the raw single-nuclei RNA-sequencing data from the miR9-2 enhancer knockout model. S.X. performed qPCR and histological analysis of the miR9-2 enhancer knockout. L.K.C and T.J.C. conceptualized study design and wrote the pre-print manuscript with input and feedback from all co-authors.



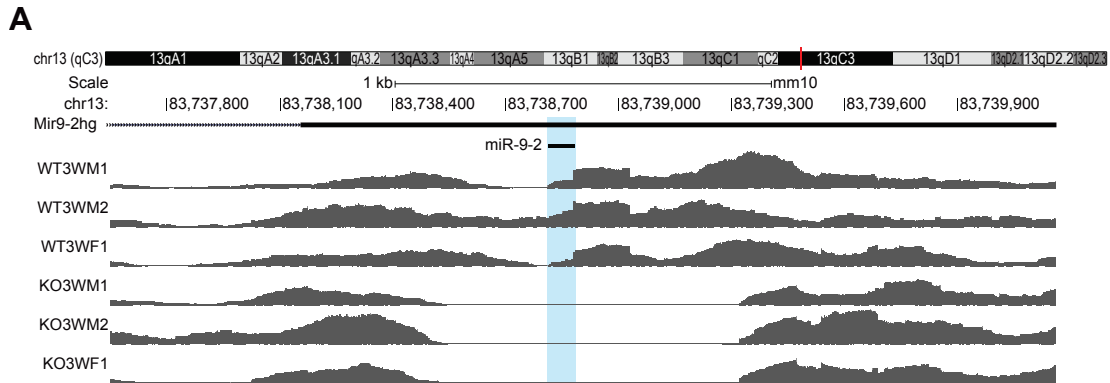
## 2.8 Supplemental Data



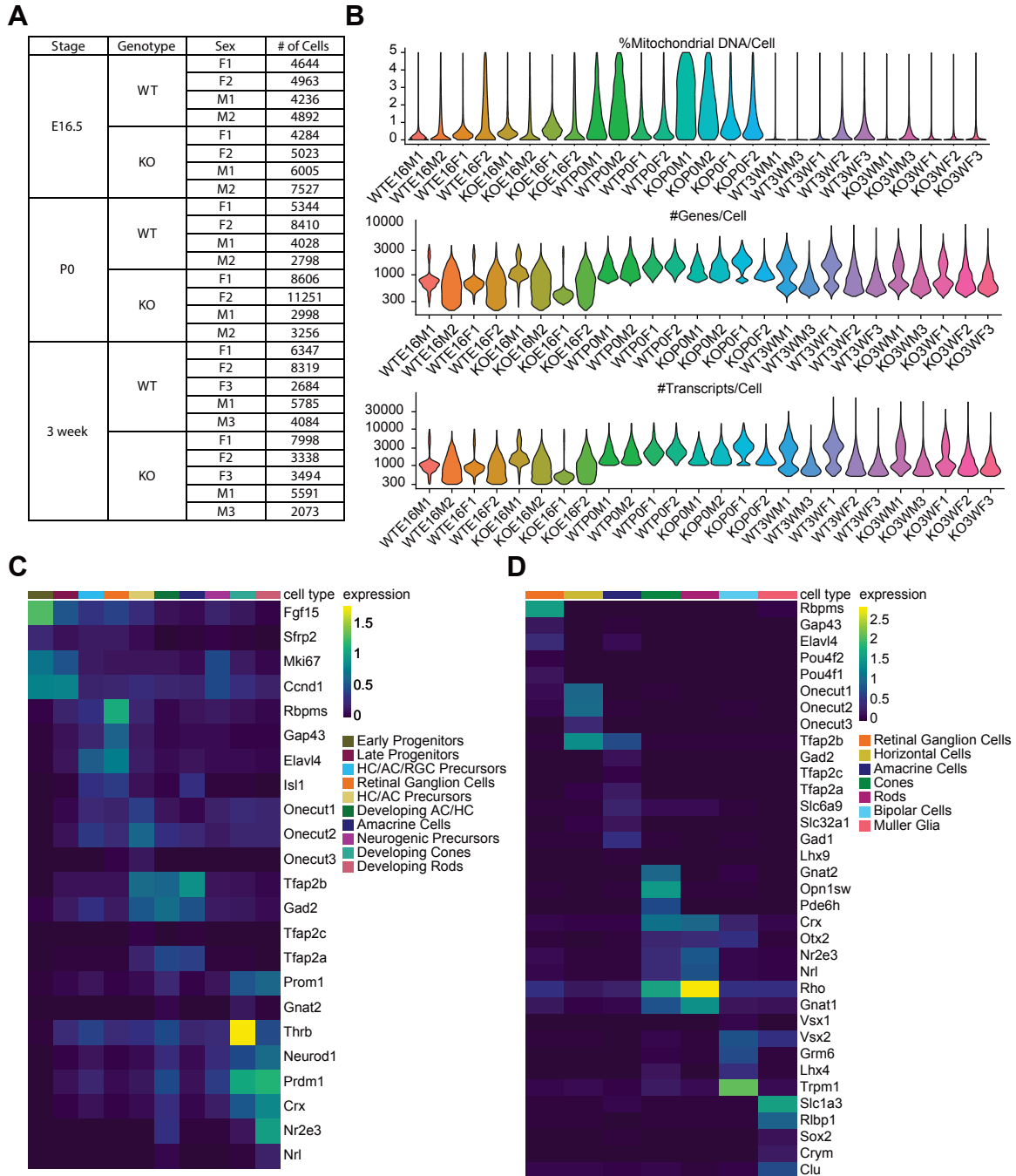
**Figure S2.1: Quality control metrics, UMAP, and differential expression analysis for snRNA-sequencing of retinas from 5-week miR9-2 enhancer knockout and littermate control mice (related to Fig. 2.1).** **A.** Cell counts of high-quality cells from each WT and miR9-2 enhancer KO sample for snRNA-seq. **B.** UMAPs of all enhancer knockout and WT pairs, annotated by cell class. **C.** Quality control metrics for individual snRNA-seq samples. **D.** Stacked bar plots of proportions of individual cell classes in WT and miR9-2 enhancer knockout mice. **E.** Volcano plot of differentially expressed genes comparing KO and WT MG. Thresholds (dashed lines) correspond to  $p \leq 0.01$  and  $FC \geq 10\%$ . Wilcoxon rank sum test, Benjamini-Hochberg correction. **F.** UMAPs, split by genotype, for WT and miR9-2 enhancer knockout retinas, annotated by cell type.



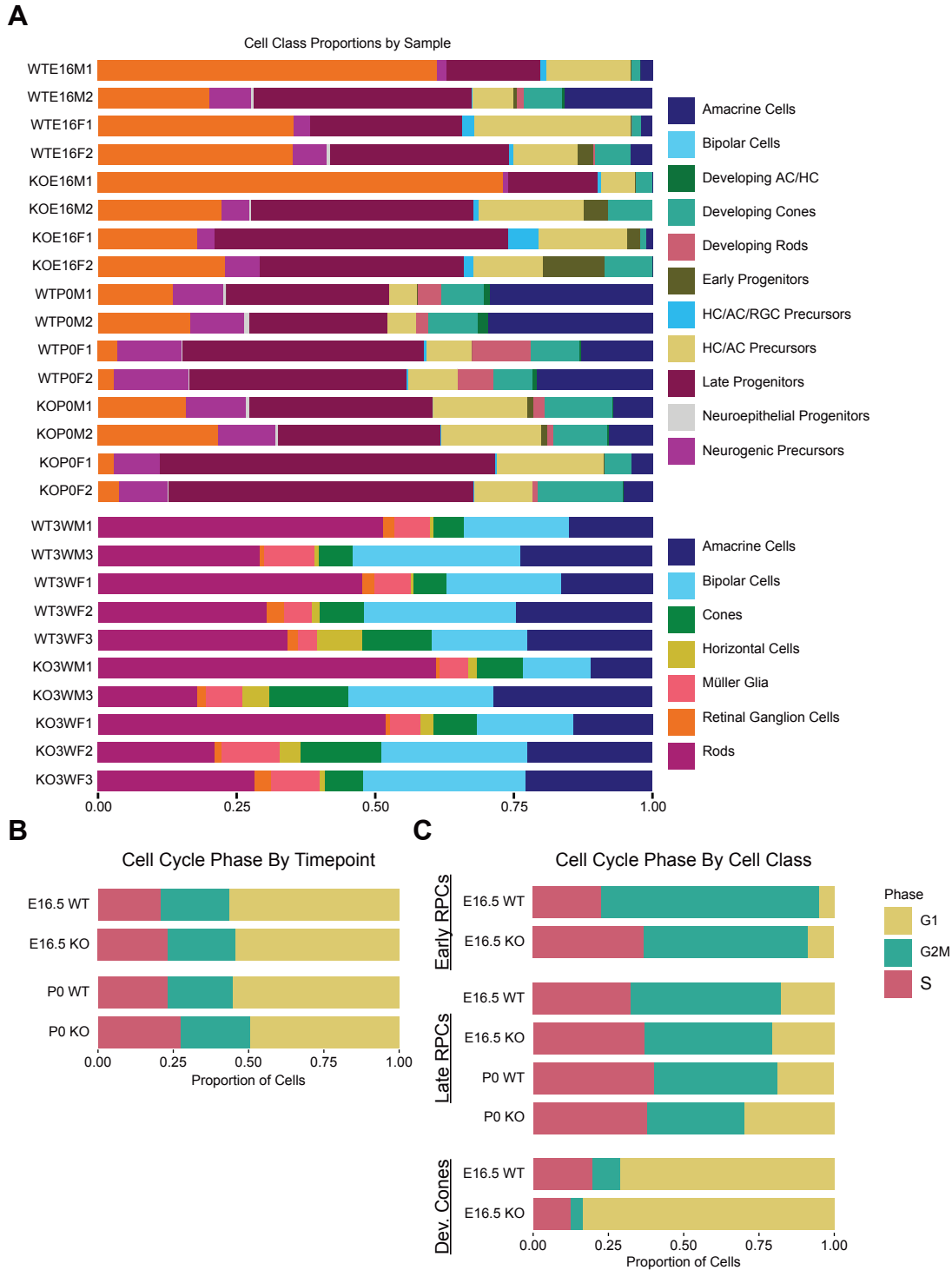
**Figure S2.2: Immunofluorescent analysis of cell classes in miR9-2 enhancer knockout and WT mice (related to Fig. 2.1).** *A.* Immunolabeling of SOX9 (green) and OTX2 (magenta) to label MG and bipolar cells, respectively, in WT and miR9-2 enhancer knockout retinas from 5-week-old mice. *B.* Representative images of immunofluorescence for individual cell classes (green), including cones (ARR3), RGCs (RBPMS; PAX6), microglia (IBA1), and ACs (PAX6). All scale bars = 50 microns. *C.* Cell count values for individual cell types in miR9-2 enhancer KO and WT retinas, including microglia, cells in GCL, horizontal cells, ACs, cones, RGCs, bipolar cells, and MG. Counts normalized to retinal area. Values reported as mean  $\pm$  1SD. ANOVA, post hoc *t*-test,  $p > 0.05$  for all.



**Figure S2.3: UCSC Genome Browser tracks of sequencing reads from miR9-2 knockout and wildtype animals (related to Fig. 2.1).** A. Representative UCSC genome browser tracks at genomic locus of miR9-2 host gene in mouse. Blue highlight indicates miR9 hairpin. Tracks show number of sequencing reads corresponding to each genomic position.

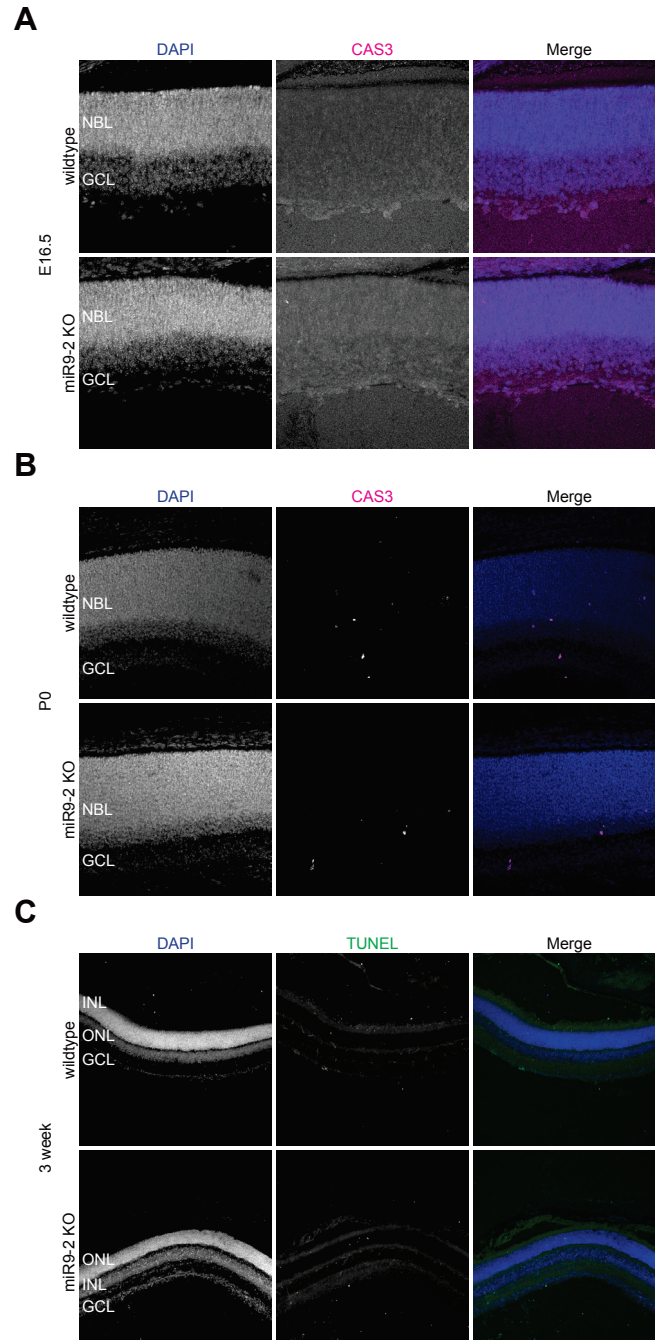


**Figure S2.4: Quality control metrics and cell class markers for single-nucleus RNA-sequencing of miR9-2 KO and WT animals (related to Fig. 2.2).** **A.** Table of cell counts of high-quality cells for individual WT and miR9-2 KO samples at each profiled timepoint. **B.** Quality control metrics from snRNA-sequencing for each sample. **C.** Heatmap of annotated cell types and corresponding cell class marker expression for all E16.5 and P0 combined samples. **D.** Heatmap of annotated cell types and corresponding cell class marker expression for all 3-week samples.

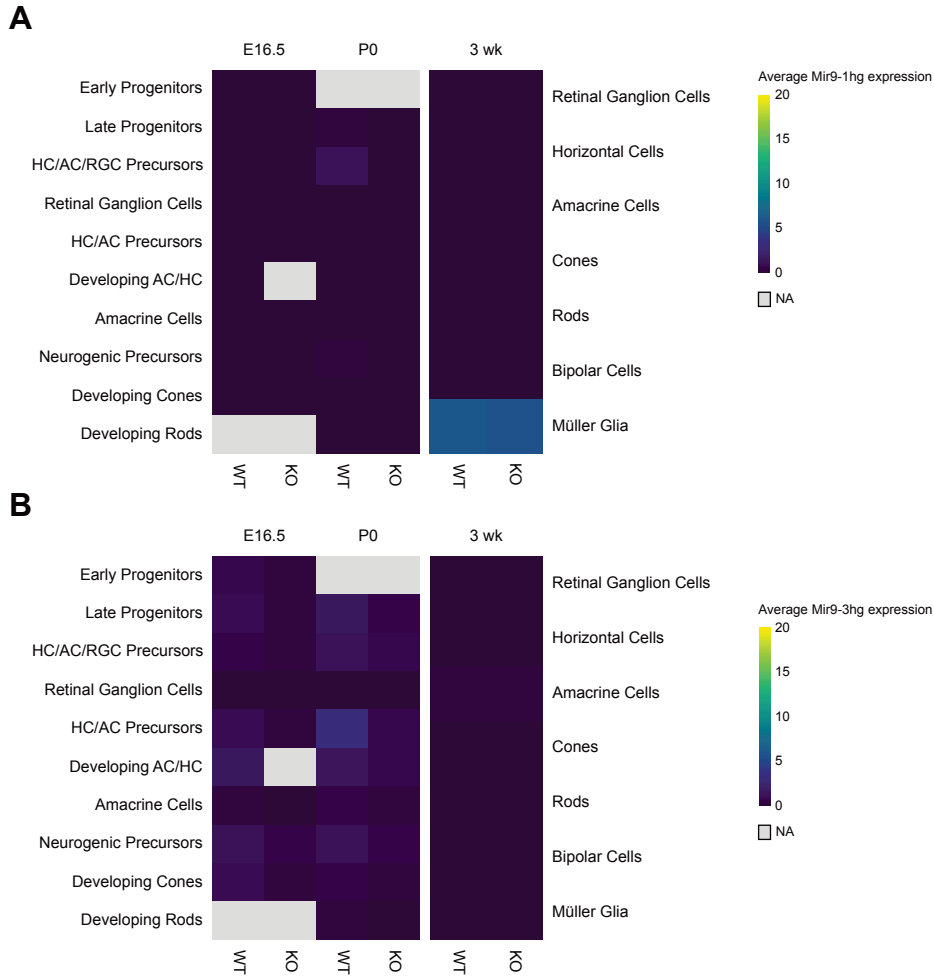


**Figure S2.5: snRNA-seq analysis of proportions for cell classes and cell cycle phases of *miR9-2* KO and WT retinas (related to Fig. 2.2).** *A.* Stacked bar plots representing proportions of cell types for each sample at E16.5, P0, and 3-week timepoints. *B.* Stacked bar plots of cell cycle phase proportions for WT and KO samples at each developmental timepoint. *C.* Stacked bar plots of cell cycle phases for early and late RPCs at E16.5 and P0 and developing cones at

*E16.5. Data fit using EdgeR generalized linear model with Benjamini-Hochberg correction (Chen et al., 2025; Robinson et al., 2010), ns for all cell classes except E16.5 Developing Cones, G2M,  $p \leq 0.05$*



**Figure S2.6: Markers of cell death in miR9-2 KO and WT retinas (related to Fig. 2.2; Fig. 2.4-2.6).** Immunolabelling of apoptotic cells with Cas3 (magenta) in miR9-2 KO and WT retinas at E16.5 (**A**) and (**B**) P0. **C.** Images of TUNEL assay (green) to label apoptotic cells, in miR9-2 KO and WT retinas at 3-weeks.



**Figure S2.7 Expression of paralogous miR9 host genes in individual cell classes of miR9-2 KO and WT retinas (related to Fig. 2.3):** snRNA-seq heatmaps of average expression for miR9 host genes, miR9-1hg (A) and miR9-3hg (B), in miR9-2 KO and WT cells of each cell class. Gray boxes indicate that the cell class was only present in negligible numbers/not present. Scale (0-20) is consistent across all heatmaps and in corresponding Figure 2.3F.



**Table S2.1. Differential abundance of cell types**

**E16.5 Differential Abundance of Cell Types**

| <b>Cell Type</b>      | <b>logFC</b> | <b>logCPM</b> | <b>F</b> | <b>PValue</b> | <b>FDR</b> |
|-----------------------|--------------|---------------|----------|---------------|------------|
| Amacrine Cells        | -4.4115      | 15.52663      | 16.97575 | 0.000806      | 0.005643   |
| Early Progenitors     | 1.916568     | 15.1692       | 3.293212 | 0.089895      | 0.314631   |
| HC/AC/RGC Precursors  | 0.74572      | 14.62653      | 0.828488 | 0.377328      | 0.666467   |
| HC/AC Precursors      | -0.40935     | 17.88558      | 0.389238 | 0.542068      | 0.666467   |
| Neurogenic Precursors | -0.36423     | 15.94909      | 0.319263 | 0.580393      | 0.666467   |
| Developing Cones      | 0.415808     | 16.19644      | 0.312284 | 0.584772      | 0.666467   |
| Late Progenitors      | 0.26194      | 18.96957      | 0.192684 | 0.666467      | 0.666467   |

**P0 Differential Abundance of Cell Types**

| <b>Cell Type</b>            | <b>logFC</b> | <b>logCPM</b> | <b>F</b> | <b>PValue</b> | <b>FDR</b> |
|-----------------------------|--------------|---------------|----------|---------------|------------|
| Amacrine Cells              | -1.96435     | 17.1562       | 24.78963 | 0.000596      | 0.007157   |
| Developing AC/HC            | -2.7723      | 12.63844      | 14.61208 | 0.00305       | 0.015729   |
| Developing Rods             | -2.62893     | 15.09864      | 13.07892 | 0.005165      | 0.015729   |
| HC AC Precursors            | 1.243622     | 16.8149       | 12.78777 | 0.005243      | 0.015729   |
| Early Progenitors           | 3.857765     | 11.60389      | 4.76068  | 0.048038      | 0.115292   |
| Microglia                   | -2.14212     | 8.957226      | 1.424527 | 0.257124      | 0.509721   |
| Late Progenitors            | 0.380593     | 18.58593      | 1.148568 | 0.309633      | 0.509721   |
| Developing Cones            | 0.380879     | 16.51406      | 0.993827 | 0.342883      | 0.509721   |
| HC/AC/RGC Precursors        | -0.73534     | 11.18896      | 0.731719 | 0.411873      | 0.509721   |
| Neuroepithelial Progenitors | -0.71579     | 12.00721      | 0.694188 | 0.424767      | 0.509721   |

**3-Week Differential Abundance of Cell Types**

| <b>Cell Type</b>       | <b>logFC</b> | <b>logCPM</b> | <b>F</b> | <b>PValue</b> | <b>FDR</b> |
|------------------------|--------------|---------------|----------|---------------|------------|
| Retinal Ganglion Cells | -0.83547     | 14.08346      | 3.13309  | 0.097478      | 0.781119   |
| Cones                  | 0.406629     | 16.4393       | 2.109844 | 0.173582      | 0.781119   |
| Bipolar Cells          | -0.22487     | 17.7693       | 0.915124 | 0.358645      | 0.804438   |
| Amacrine Cells         | -0.22207     | 17.61066      | 0.830883 | 0.380932      | 0.804438   |
| Horizontal Cells       | 0.534959     | 14.6046       | 0.470902 | 0.504541      | 0.804438   |
| Müller Glia            | 0.149446     | 16.02648      | 0.275964 | 0.609558      | 0.804438   |
| Rods                   | 0.131753     | 18.49265      | 0.201805 | 0.661699      | 0.804438   |
| Rgs16                  | -0.26105     | 13.12049      | 0.137187 | 0.715056      | 0.804438   |
| Astrocytes             | -0.15576     | 11.64168      | 0.022005 | 0.883674      | 0.883674   |



**Table S2.2. Differential abundance of cell cycle phases**

**E16.5 Differential Abundance of Cell Cycle Phases**

| Cell Type              | Phase | logFC    | logCPM   | F        | P-Value  | FDR      |
|------------------------|-------|----------|----------|----------|----------|----------|
| Bulk                   | S     | 0.170609 | 17.76068 | 0.680579 | 0.430725 | 0.919256 |
| Bulk                   | G1    | -0.04967 | 19.08933 | 0.079391 | 0.784501 | 0.919256 |
| Bulk                   | G2M   | -0.0459  | 17.75074 | 0.010869 | 0.919256 | 0.919256 |
| Amacrine Cells         | S     | 1.019454 | 17.34773 | 0.854493 | 0.376946 | 0.598356 |
| Amacrine Cells         | G1    | -0.18279 | 19.58102 | 0.313857 | 0.586339 | 0.598356 |
| Amacrine Cells         | G2M   | 0.828249 | 15.92189 | 0.299284 | 0.598356 | 0.598356 |
| Developing Cones       | G2M   | -1.08275 | 16.11751 | 8.695405 | 0.008589 | 0.025767 |
| Developing Cones       | S     | -0.61806 | 17.1539  | 3.622992 | 0.073102 | 0.109653 |
| Developing Cones       | G1    | 0.162154 | 19.60834 | 0.808012 | 0.380576 | 0.380576 |
| Early Progenitors      | S     | 0.43565  | 18.2369  | 0.242294 | 0.629843 | 0.847182 |
| Early Progenitors      | G2M   | -0.2635  | 18.88363 | 0.098579 | 0.758016 | 0.847182 |
| Early Progenitors      | G1    | 0.378635 | 17.72326 | 0.038531 | 0.847182 | 0.847182 |
| HC/AC Precursors       | G2M   | -0.82207 | 16.2147  | 1.590259 | 0.242648 | 0.391894 |
| HC/AC Precursors       | S     | -0.67417 | 17.10157 | 1.458862 | 0.261263 | 0.391894 |
| HC/AC Precursors       | G1    | 0.186828 | 19.58553 | 0.714572 | 0.422153 | 0.422153 |
| HC/AC/RGC Precursors   | S     | -0.17999 | 17.59292 | 0.189534 | 0.670363 | 0.782577 |
| HC/AC/RGC Precursors   | G1    | 0.058525 | 19.42862 | 0.111591 | 0.744013 | 0.782577 |
| HC/AC/RGC Precursors   | G2M   | 0.087272 | 17.04157 | 0.079145 | 0.782577 | 0.782577 |
| Late Progenitors       | S     | 0.179067 | 18.42698 | 0.18899  | 0.671926 | 0.975105 |
| Late Progenitors       | G2M   | -0.12765 | 18.67359 | 0.048025 | 0.830998 | 0.975105 |
| Late Progenitors       | G1    | -0.03927 | 17.81457 | 0.001031 | 0.975105 | 0.975105 |
| Neurogenic Precursors  | S     | 0.113722 | 17.83512 | 0.282147 | 0.609728 | 0.875764 |
| Neurogenic Precursors  | G2M   | -0.10641 | 18.87982 | 0.051333 | 0.826467 | 0.875764 |
| Neurogenic Precursors  | G1    | 0.12088  | 18.14987 | 0.026063 | 0.875764 | 0.875764 |
| Retinal Ganglion Cells | G2M   | -0.23102 | 15.59741 | 0.018648 | 0.892896 | 0.996206 |
| Retinal Ganglion Cells | G1    | 0.013261 | 19.66404 | 6.29E-05 | 0.99376  | 0.996206 |
| Retinal Ganglion Cells | S     | -0.00809 | 16.88191 | 2.32E-05 | 0.996206 | 0.996206 |

**P0 Differential Abundance of Cell Cycle Phases**

| Cell Type        | Phase | logFC    | logCPM   | F        | P-Value  | FDR      |
|------------------|-------|----------|----------|----------|----------|----------|
| Bulk             | S     | 0.186462 | 17.76343 | 0.309896 | 0.584599 | 0.685318 |
| Bulk             | G1    | -0.14491 | 19.00322 | 0.190513 | 0.66768  | 0.685318 |
| Bulk             | G2M   | 0.138133 | 17.94558 | 0.169614 | 0.685318 | 0.685318 |
| Amacrine Cells   | S     | 0.26337  | 16.8915  | 0.136394 | 0.716201 | 0.94779  |
| Amacrine Cells   | G2M   | 0.186861 | 15.78291 | 0.061856 | 0.806401 | 0.94779  |
| Amacrine Cells   | G1    | -0.04552 | 19.65501 | 0.004409 | 0.94779  | 0.94779  |
| Developing Cones | G2M   | -0.53711 | 17.60704 | 0.398444 | 0.536447 | 0.875939 |
| Developing Cones | S     | 0.261803 | 17.41459 | 0.164648 | 0.690068 | 0.875939 |
| Developing Cones | G1    | 0.096617 | 19.26054 | 0.025132 | 0.875939 | 0.875939 |
| Developing Rods  | S     | 0.511037 | 16.79744 | 0.398403 | 0.535849 | 0.843685 |
| Developing Rods  | G2M   | 0.6221   | 15.39539 | 0.348222 | 0.562457 | 0.843685 |
| Developing Rods  | G1    | -0.05099 | 19.68924 | 0.006115 | 0.938534 | 0.938534 |

|                        |     |          |          |          |          |          |
|------------------------|-----|----------|----------|----------|----------|----------|
| Developing AC/HC       | G2M | -1.90425 | 17.18725 | 1.879304 | 0.187266 | 0.561797 |
| Developing AC/HC       | G1  | 0.17062  | 19.49791 | 0.131101 | 0.721509 | 0.979318 |
| Developing AC/HC       | S   | 0.022937 | 17.53316 | 0.000691 | 0.979318 | 0.979318 |
| HC/AC Precursors       | S   | 0.062501 | 17.08873 | 0.015267 | 0.904716 | 0.983592 |
| HC/AC Precursors       | G2M | 0.024621 | 16.12835 | 0.000969 | 0.97591  | 0.983592 |
| HC/AC Precursors       | G1  | -0.00466 | 19.59652 | 0.00045  | 0.983592 | 0.983592 |
| Late Progenitors       | G1  | 0.556014 | 17.29696 | 0.20431  | 0.663081 | 0.828333 |
| Late Progenitors       | G2M | -0.1326  | 18.91059 | 0.071551 | 0.795769 | 0.828333 |
| Late Progenitors       | S   | -0.06938 | 18.40594 | 0.050155 | 0.828333 | 0.828333 |
| Neurogenic Precursors  | G2M | 0.187585 | 18.58777 | 0.119042 | 0.738964 | 0.885445 |
| Neurogenic Precursors  | G1  | -0.2161  | 18.48396 | 0.112824 | 0.745585 | 0.885445 |
| Neurogenic Precursors  | S   | 0.033374 | 17.88913 | 0.022123 | 0.885445 | 0.885445 |
| Retinal Ganglion Cells | S   | 1.20975  | 15.72274 | 1.31289  | 0.266878 | 0.541064 |
| Retinal Ganglion Cells | G2M | 0.996581 | 15.46408 | 0.879663 | 0.360709 | 0.541064 |
| Retinal Ganglion Cells | G1  | -0.10714 | 19.786   | 0.013563 | 0.908578 | 0.908578 |

**Table S2.6. miRNA predicted target enrichment.**

**Enrichment of all DEGs for computationally predicted miR9 targets**

| <b>Timepoint</b> | <b>Cell Type</b>       | <b>sig<br/>Predicted</b> | <b>sigNot<br/>Predicted</b> | <b>ns<br/>Predicted</b> | <b>nsNot<br/>Predicted</b> | <b>Pval</b> | <b>CI<br/>low</b> | <b>CI<br/>high</b> | <b>Odds<br/>Ratio</b> |
|------------------|------------------------|--------------------------|-----------------------------|-------------------------|----------------------------|-------------|-------------------|--------------------|-----------------------|
| E16.5            | Amacrine Cells         | 200                      | 230                         | 1905                    | 3494                       | 2.717E-06   | 1.344             | Inf                | 1.595                 |
| E16.5            | Developing Cones       | 274                      | 513                         | 2806                    | 6456                       | 4.983E-03   | 1.077             | Inf                | 1.229                 |
| E16.5            | HC AC Precursors       | 1520                     | 3016                        | 1942                    | 5389                       | 2.385E-16   | 1.306             | Inf                | 1.398                 |
| E16.5            | HC AC RGC Precursors   | 50                       | 53                          | 1826                    | 2970                       | 2.060E-02   | 1.083             | Inf                | 1.534                 |
| E16.5            | Early Progenitors      | 77                       | 87                          | 1736                    | 3444                       | 3.096E-04   | 1.334             | Inf                | 1.756                 |
| E16.5            | Retinal Ganglion Cells | 2445                     | 4834                        | 1386                    | 5830                       | 7.912E-87   | 1.994             | Inf                | 2.127                 |
| E16.5            | Neurogenic Precursors  | 66                       | 92                          | 2677                    | 5637                       | 7.732E-03   | 1.138             | Inf                | 1.511                 |
| E16.5            | Late Progenitors       | 1326                     | 2547                        | 2398                    | 7296                       | 1.286E-28   | 1.479             | Inf                | 1.584                 |
| P0               | Amacrine Cells         | 683                      | 1269                        | 2883                    | 7899                       | 1.317E-13   | 1.351             | Inf                | 1.475                 |
| P0               | Developing Cones       | 1675                     | 3565                        | 1871                    | 5444                       | 3.148E-15   | 1.279             | Inf                | 1.367                 |
| P0               | Developing Rods        | 774                      | 1220                        | 2119                    | 5156                       | 2.048E-16   | 1.413             | Inf                | 1.544                 |
| P0               | HC AC Precursors       | 1890                     | 3817                        | 1656                    | 5049                       | 2.919E-25   | 1.413             | Inf                | 1.510                 |
| P0               | Developing AC HC       | 10                       | 19                          | 1421                    | 2261                       | 7.372E-01   | 0.398             | Inf                | 0.838                 |
| P0               | Retinal Ganglion Cells | 1924                     | 4182                        | 1669                    | 5243                       | 4.204E-21   | 1.354             | Inf                | 1.445                 |
| P0               | Neurogenic Precursors  | 1261                     | 2197                        | 2213                    | 6302                       | 7.619E-30   | 1.521             | Inf                | 1.634                 |
| P0               | Late Progenitors       | 2828                     | 6929                        | 1053                    | 4085                       | 3.763E-30   | 1.479             | Inf                | 1.583                 |
| 3wk              | Amacrine Cells         | 1561                     | 3669                        | 2301                    | 7780                       | 2.971E-21   | 1.349             | Inf                | 1.439                 |
| 3wk              | Cones                  | 377                      | 653                         | 3100                    | 8554                       | 1.160E-11   | 1.421             | Inf                | 1.593                 |
| 3wk              | Rods                   | 2055                     | 5139                        | 1772                    | 6022                       | 1.709E-16   | 1.277             | Inf                | 1.359                 |
| 3wk              | Horizontal Cells       | 947                      | 1426                        | 1885                    | 4750                       | 9.999E-25   | 1.539             | Inf                | 1.673                 |
| 3wk              | Müller Glia            | 577                      | 1010                        | 2911                    | 7687                       | 4.723E-13   | 1.372             | Inf                | 1.509                 |
| 3wk              | Retinal Ganglion Cells | 69                       | 76                          | 3263                    | 7780                       | 4.238E-06   | 1.619             | Inf                | 2.165                 |
| 3wk              | Bipolar Cells          | 559                      | 1138                        | 3250                    | 10041                      | 9.641E-14   | 1.383             | Inf                | 1.518                 |

### Enrichment of upregulated DEGs for computationally predicted miR9 targets

| Timepoint | Cell Type              | Up Predicted | UpNot Predicted | Down Predicted | DownNot Predicted | Pval      | CI low | CI high | Odds Ratio |
|-----------|------------------------|--------------|-----------------|----------------|-------------------|-----------|--------|---------|------------|
| E16.5     | Amacrine Cells         | 67           | 71              | 133            | 159               | 3.157E-01 | 0.786  | Inf     | 1.128      |
| E16.5     | Developing Cones       | 123          | 168             | 151            | 345               | 5.383E-04 | 1.284  | Inf     | 1.672      |
| E16.5     | HC AC Precursors       | 937          | 2121            | 583            | 895               | 1.000E+00 | 0.607  | Inf     | 0.678      |
| E16.5     | HC AC RGC Precursors   | 1            | 3               | 49             | 50                | 9.338E-01 | 0.013  | Inf     | 0.343      |
| E16.5     | Early Progenitors      | 22           | 20              | 55             | 67                | 2.615E-01 | 0.699  | Inf     | 1.338      |
| E16.5     | Retinal Ganglion Cells | 2328         | 4610            | 117            | 224               | 6.378E-01 | 0.793  | Inf     | 0.967      |
| E16.5     | Neurogenic Precursors  | 47           | 57              | 19             | 35                | 1.492E-01 | 0.815  | Inf     | 1.515      |
| E16.5     | Late Progenitors       | 1091         | 2070            | 235            | 477               | 2.353E-01 | 0.922  | Inf     | 1.070      |
| P0        | Amacrine Cells         | 2206         | 5569            | 1360           | 3599              | 1.268E-01 | 0.980  | Inf     | 1.048      |
| P0        | Developing Cones       | 925          | 2433            | 2621           | 6576              | 8.581E-01 | 0.885  | Inf     | 0.954      |
| P0        | Developing Rods        | 691          | 2051            | 2202           | 4325              | 1.000E+00 | 0.607  | Inf     | 0.662      |
| P0        | HC AC Precursors       | 936          | 2723            | 2610           | 6143              | 1.000E+00 | 0.751  | Inf     | 0.809      |
| P0        | Developing AC HC       | 910          | 1547            | 521            | 733               | 9.965E-01 | 0.735  | Inf     | 0.828      |
| P0        | Retinal Ganglion Cells | 1050         | 2950            | 2543           | 6475              | 9.899E-01 | 0.844  | Inf     | 0.906      |
| P0        | Neurogenic Precursors  | 939          | 2728            | 2535           | 5771              | 1.000E+00 | 0.727  | Inf     | 0.784      |
| P0        | Late Progenitors       | 1041         | 2817            | 2840           | 8197              | 6.670E-02 | 0.994  | Inf     | 1.067      |
| 3wk       | Amacrine Cells         | 2631         | 7735            | 1231           | 3714              | 2.648E-01 | 0.960  | Inf     | 1.026      |
| 3wk       | Cones                  | 1653         | 4158            | 1824           | 5049              | 8.693E-03 | 1.030  | Inf     | 1.100      |
| 3wk       | Rods                   | 1095         | 2809            | 2732           | 8352              | 1.716E-05 | 1.111  | Inf     | 1.192      |
| 3wk       | Horizontal Cells       | 2214         | 4373            | 618            | 1803              | 7.284E-14 | 1.351  | Inf     | 1.477      |
| 3wk       | Müller Glia            | 1585         | 4347            | 1903           | 4350              | 1.000E+00 | 0.780  | Inf     | 0.833      |

|     |                        |      |      |      |      |           |       |     |       |
|-----|------------------------|------|------|------|------|-----------|-------|-----|-------|
| 3wk | Retinal Ganglion Cells | 1264 | 3592 | 2068 | 4264 | 1.000E+00 | 0.676 | Inf | 0.726 |
| 3wk | Bipolar Cells          | 2051 | 6113 | 1758 | 5066 | 8.198E-01 | 0.908 | Inf | 0.967 |

### Enrichment of downregulated DEGs for computationally predicted miR204 targets

| Timepoint | Cell Type              | Down Predicted | DownNot Predicted | Up Predicted | UpNotPredicted | Pval                 | CI low | CI high | Odds Ratio |
|-----------|------------------------|----------------|-------------------|--------------|----------------|----------------------|--------|---------|------------|
| 3wk       | Amacrine Cells         | 2348           | 12963             | 1128         | 9238           | 4.201E-01            | 0.942  | Inf     | 1.009      |
| 3wk       | Cones                  | 1390           | 11294             | 1682         | 4129           | 2.411E-01            | 0.962  | Inf     | 1.031      |
| 3wk       | Rods                   | 861            | 14127             | 2516         | 1388           | 2.099E-01            | 0.963  | Inf     | 1.038      |
| 3wk       | Horizontal Cells       | 1873           | 7135              | 607          | 5980           | 9.993E-01            | 0.769  | Inf     | 0.842      |
| 3wk       | Müller Glia            | 1427           | 10758             | 1659         | 4273           | 9.018E-04            | 1.063  | Inf     | 1.140      |
| 3wk       | Retinal Ganglion Cells | 1184           | 10004             | 1769         | 3087           | 1.24530528616848e-05 | 1.118  | Inf     | 1.202      |
| 3wk       | Bipolar cells          | 1857           | 13131             | 1552         | 6612           | 5.094E-01            | 0.937  | Inf     | 1.000      |

**Table S2.7. Oligos generated/used.**

CRISPR gRNA target sequences for generation of miR9-2 enhancer KO mice, Figure 1.

| CRISPR Guide Pair      | Forward                  | Reverse                  |
|------------------------|--------------------------|--------------------------|
| miR9-2 enhancer pair 1 | CTAAGAGACAGAAAAGTTGT-TGG | ATTAAGCTCTAACTATGCTC-TGG |
| miR9-2 enhancer pair 2 | TAAATAGATATTTTGTGCTG-AGG | AGTGAATGCAAGTGTTAACA-AGG |

Primers for qPCR, Related to STAR Methods, Figure 1

| Gene           | Forward Primer         | Reverse Primer        |
|----------------|------------------------|-----------------------|
| Tuba1a control | ggcagtgttcgtagacctggaa | ctccttgccaatgggttagtg |
| miR9-2-hg      | TGGAGTTCAGCCAGAGGAAG   | TCGGTGACCTTGAAGGAGTT  |

Primers for genotyping, Related to STAR Methods, Figure 1

| Gene               | Forward Primer               | Reverse Primer          |
|--------------------|------------------------------|-------------------------|
| miR9-2-lacZ-fl-neo | GAGGGGCTTGTACAACTGGA         | CCCTTTCCCTGCTAGCTCT     |
| miR9-2 KO          | ACATCTCAGTAGAGGAAGGC         | CGTGGGTTGTGGCGTAAA      |
|                    | GGGTGACCACTGATGCATAT         |                         |
| miR9-2 Enh KO      | CAATTGAAGGAGGAGTGTGCTTTA     | GACAGTTGTCATGGACATCACTG |
|                    | GAGCAGAGTTAATCTGGGAC         |                         |
| Jarid1c/d          | CTGAAGCTTTTGGCTTTGAG         | CCACTGCCAAATTCTTTGG     |
| FlpE               | GCTGAACCTAACCTATTTATGTTGGATG | GCGTCTTGTATTTAACTGGAGTG |
|                    | CACGTGGGCTCCAGCATT           | TCACCAGTCATTTCTGCCTTTG  |

## CHAPTER 3. MIR9-2 REGULATES MÜLLER GLIA HOMEOSTASIS IN THE ADULT MOUSE RETINA

### *3.1 Introduction*

Macular disease causes vision loss for millions of people globally and is characterized by progressive degeneration of the macula, leading to loss of central vision and visual acuity. In the developed world, Age-related Macular Degeneration (AMD) is the leading cause of vision loss and is characterized by the presence of deposits, called drusen, behind the retina (dry AMD) and/or by increased angiogenesis in the macula and infiltration of immune cells (wet AMD) (Han et al., 2020). Macular Telangiectasia Type 2 (MacTel) is a rarer form of macular degeneration and is characterized by neovascularization and macular thinning (Kedariseti et al., 2022). Patients with macular degeneration often do not experience perceivable changes in vision until their fifties, sixties, or seventies, and manifestation of disease can have both environmental and genetic contributions. Existing studies have sought to understand the origins, progression, and mechanisms of these diseases to slow or treat these disorders, and much work is being done to understand the cell types affected in these diseases.

While the loss of photoreceptors directly contributes to the loss of central vision, it is thought that perturbations of surrounding cell types, such as Müller glia, influence the disease pathologies in macular degeneration. Müller glia are the primary glial cells of the retina and are the support cells for surrounding neurons, playing an important role in maintaining retinal homeostasis. Müller cells stretch across the entire retina, with cell bodies positioned in the inner nuclear layer (INL), basal processes terminating in endfeet that form the internal limiting membrane (ILM) and apical processes extending through the photoreceptor layer to form the outer limiting membrane (OLM). This radial structure allows Müller glia to provide architectural support for the retina by supporting surrounding neurons and interacting with retinal vasculature.

Further, glia are responsible for regulating the metabolic environment of the retina and recycling waste, functions which are crucial to protecting the health of retinal neurons like photoreceptors (Kobat and Turgut, 2020; Navneet et al., 2024).

Unsurprisingly then, Müller glia are often disrupted in retinal disease contexts. In AMD, Müller cells can undergo gliosis, a stress response that when overactivated can increase immune infiltration and photoreceptor cell loss (Navneet et al., 2024). While it is still unclear whether Müller glia are disrupted before or after photoreceptor loss, this Müller stress response could contribute to further macular damage. In MacTel, there is evidence that Müller dysfunction does occur prior to photoreceptor loss. An analysis of MacTel in human patients found loss of macular Müller glia cells even while the corresponding photoreceptors were still present (Powner et al., 2010). This suggests that Müller dysfunction can serve as an origin point in macular degeneration. Thus, an understanding of how Müller cells become disrupted initially may elucidate some of the causal mechanisms that lead to macular degeneration. Macular disease can stem from both environmental and genetic factors; while the environmental factors that influence retinal health are documented, much of the genetic contribution to these diseases is still unexplained.

Genome wide association studies (GWAS) have been conducted to identify loci that contribute to specific retinal diseases, including AMD and MacTel, as well as related retinal disease phenotypes, including changes to retinal vasculature caliber and retinal thickness (Bonelli et al., 2021b; Fritsche et al., 2016; X. R. Gao et al., 2019; Gorman et al., 2024; Han et al., 2020; Ikram et al., 2010; Jackson et al., 2025; Scerri et al., 2017). Of the genomic regions identified by these GWAS, few are located directly within the coding region of genes. Instead, many risk variants have been found in noncoding regions of the genome, and ongoing research is



being conducted to understand how variants in these regions can alter gene expression and contribute to disease. The noncoding genome includes regulatory elements such as enhancers and microRNAs (miRNAs). Enhancers generally function by recruiting transcription factors and transcriptional machinery and looping to contact the promoter of their target gene to increase expression of that target. MiRNAs are single-stranded RNAs roughly 20 nucleotides in length and function to regulate gene expression through repression of target transcripts. By binding to target mRNA transcripts with perfect or imperfect complementarity, miRNAs inhibit gene expression through translational block or transcript degradation, and individual miRNAs can regulate many target transcripts (Bartel, 2018). In prior work done by our lab, we found that the region containing the top risk variants associated with MacTel contains an enhancer and host gene for the miRNA, miR9-2 (Fig. 3.1A; Thomas, et al. 2022). Further, disruption of this enhancer-microRNA regulatory unit has been found to perturb normal retinal development in both human retinal organoids and mouse retina (Callies et al., 2026; Thomas et al., 2022). miR9-2 is highly expressed in retinal progenitor cells of the developing retina, which likely explains the developmental perturbations when miR9-2 expression is disrupted (Callies et al., 2026; Torre et al., 2013). In the adult retina of mice and humans, the activity of the miR9-2 enhancer and expression of the miR9-2 host gene become highly specific to Müller glial cells (Fig. 3.1A). However, it is not yet understood exactly what role miR9-2 plays in regulating adult Müller cells.

There has been some preliminary work to understand the importance of miRNAs and miR9-2 in Müller glial cells. Disruptions of Dicer, and thereby all miRNAs, in adult Müller glia cells of the mouse retina have been found to lead to large aggregations of Müller glial cells, resulting in disorganization of retinal structure and corresponding decreases in visual acuity (Wohl et al., 2017). Further studies of this Dicer disruption in Müller glia also found thinning of

the retina, loss of cone function, and subsequent loss of rod function (Larbi et al., 2025). In neonatal Dicer knockout and germline miR9-2 knockout in the developing mouse retina, loss of miRNAs has been found to perturb the Müller glia populations that form in adulthood. In the neonatal Dicer knockout animals, Müller glia were found in reduced numbers (Larbi et al., 2025) while specific germline knockout of miR9-2 resulted in an increased population of Müller glia-like cells and misspecification of these cells to a hybrid glial-neuronal identity (Callies et al., 2026). While these studies demonstrate a range of phenotypes caused by microRNA disruption, they highlight an important gap in knowledge in understanding which miRNAs regulate Müller glial function and how the function of miRNAs, such as miR9-2, may differ with respect to development versus Müller glial homeostasis. While the Dicer model examines adult Müller glia-specific function, it does not distinguish which individual miRNAs are important for regulation. On the other hand, while the germline miR9-2 knockout influences one miRNA directly, the scope of the experiments does not directly parse the developmental role of miR9-2 in producing Müller glia from the regulation of Müller function and homeostasis in the adult retina. Therefore, there is still a pressing need to develop a Müller glia-specific model of miR9-2 function in the adult retina, to understand how disruption of this singular miRNA may influence Müller glia homeostasis and contribute to macular disease.

In this study, we sought to investigate the role of miR9-2 in retinal disease pathologies and Müller glia maintenance. Using a novel conditional knockout mouse model of miR9-2 function, immunohistochemistry, and single-nuclei RNA-sequencing, we found that miR9-2 is critical for maintaining the health and survival of Müller glial cells in adult mouse retinas. By inducing Müller glia-specific miR9-2 loss in animals at 5 weeks of age or later, we found significant loss of recombined cells in miR9-2 conditional knockout animals in as few as 3 days.

Further, we determined that this Müller cell loss does not show classical hallmarks of apoptosis or gliosis, suggesting that miR9-2 represses pathways that result in other forms of cell death. Finally, we identified direct targets regulated by miR9-2 in adult Müller glial cells and characterized how disruption of these Müller cells affected other cell populations of the retina. This study expands our knowledge of how homeostasis is maintained in healthy Müller cells and increases our understanding of how disruptions to Müller glial cells contribute to retinal disease risk.

### 3.2 Results

#### 3.2.1 Conditional knockout of miR9-2 in adult Müller glial cells leads to abnormal Müller morphology and Müller cell loss.

To examine miR9-2 function in the maintenance and survival of adult Müller glia cells *in vivo*, we generated a Müller-specific inducible knockout mouse model of miR9-2. We crossed mice containing a floxed miR9-2 hairpin loop (Park, et al. 2012) with either Ai14 mice containing a tdTomato Cre reporter allele (Madisen et al., 2010) or with *Glast*-creERT2 mice containing an allele for a tamoxifen-inducible Cre under the control of the *Glast* promoter. The resulting progeny of these two crosses were crossed with each other to generate animals expressing tamoxifen-inducible *Glast*-CreERT2, the tdTomato Cre reporter, and either a floxed (miR9-2 cKO) or wildtype (control) miR9-2 hairpin loop (Fig. 3.1B; full breeding schematic in Fig. S3.1A). To understand the mature, Müller glia-specific role of miR9-2, Cre-recombination was initiated by a single intraperitoneal injection of tamoxifen (2 mg in corn oil) in WT animals that were at least 5 weeks of age, a timepoint when the retina is fully mature. Subsequently,



region, with accessibility specific to human Müller glia and progenitor cells. All tracks scaled 0 to 55. Conservation of region across vertebrates shown at bottom. **B.** Schematics of tamoxifen injection paradigm for retinas harvested for single nuclei and histology (left) and of the floxed *miR9-2*, inducible *CreERT2*, and *tdTomato* reporter alleles in inducible MG-specific *miR9-2* knockout mice. **C.** Cross-sectional images of immunohistochemistry for markers of Müller glia (*Sox9*, green) and recombined cells (*tdTomato*, red). **D.** Cross-sectional images of control and MG-specific *miR9-2* knockout retinas 7 days following a single tamoxifen injection, with nuclear staining (DAPI, blue) and recombined cells (*tdTomato*, red). **E.** Bar graph quantifying the number of *tdTomato*<sup>+</sup> cells found in *miR9-2* cKO and WT retinas. Cell counts normalized by area of retina counted. Bars shown as  $\pm$  1SD. \* $p < 0.05$ . Panel B created with BioRender.com.

retinas were harvested 7 days post injection (dpi) for histological analysis (Fig. 3.1B). To confirm the specificity of the knockout, we visualized the morphology of *tdTomato* positive (+) cells in WT animals at 7dpi and co-stained for SOX9, a common nuclear marker of Müller glia. As expected, WT *tdTomato*<sup>+</sup> cells had clear morphological features of Müller glia including long projections extending apically and basally into the outer nuclear and ganglion cell layers. Further, WT *tdTomato*<sup>+</sup> nuclei were positioned within the inner nuclear layer and co-expressed SOX9 (Fig. 3.1C).

Next, we sought to understand how loss of *miR9-2* affected mature Müller glia cells. We compared littermate-matched *miR9-2* knockout and WT animals by inducing Cre recombination in adult animals, and retinas were harvested at 3 or 7dpi. Strikingly, when we compared both cross-sectional and *en face* flat-mounted wildtype and *miR9-2* knockout retinas, we found a significant decrease in the number of *tdTomato*<sup>+</sup> Müller glia cells in *miR9-2* knockout retinas compared to wildtype. Müller cell loss occurred rapidly as we saw this decrease in as few as 3dpi (7dpi: Fig. 3.1D-E; 3dpi: Fig. S3.1B). In the remaining *tdTomato*<sup>+</sup> Müller glia observed in cross-section, the morphology of these cells appeared abnormal with notable swelling in the apical projections of the glia and disorganization of the inner and outer limiting membranes formed by Müller termini (Fig. 3.1D, arrows). Similarly, we visualized these cells *en face*, in flat-mounted *miR9-2* knockout retinas, and MG cell bodies also appeared larger and more “swollen” and

seemed to cluster together compared to the more evenly distributed Müller glia of wildtype retinas (Fig. 3.2A). Altogether, these data suggest the importance of miR9-2 in maintaining normal Müller glia homeostasis, as miR9 loss rapidly impacts Müller morphology and number within a short timeframe.

### *3.2.2 Early Müller glial cell loss does not have markers of apoptosis or reactive gliosis.*

Next, we sought to investigate the origin of Müller glial morphology changes and cell loss. As the simplest explanation for the decrease in tdTomato<sup>+</sup> cells is death of cells, we first looked for standard markers of cell death. To understand whether apoptosis is a potential contributor to these morphological changes and rapid decreases in number of miR9-2 knockout MG, we compared markers of apoptosis in miR9-2 knockout and wildtype retinas. We immunolabelled retinas for phospho-Caspase 3 as a marker of apoptotic cell death but found no notable differences (Fig. 3.2B). While these data suggests that Müller glia cells are not being lost by apoptosis, it is still possible that cells are lost through alternative forms of cell death, including ferroptosis and pyroptosis.

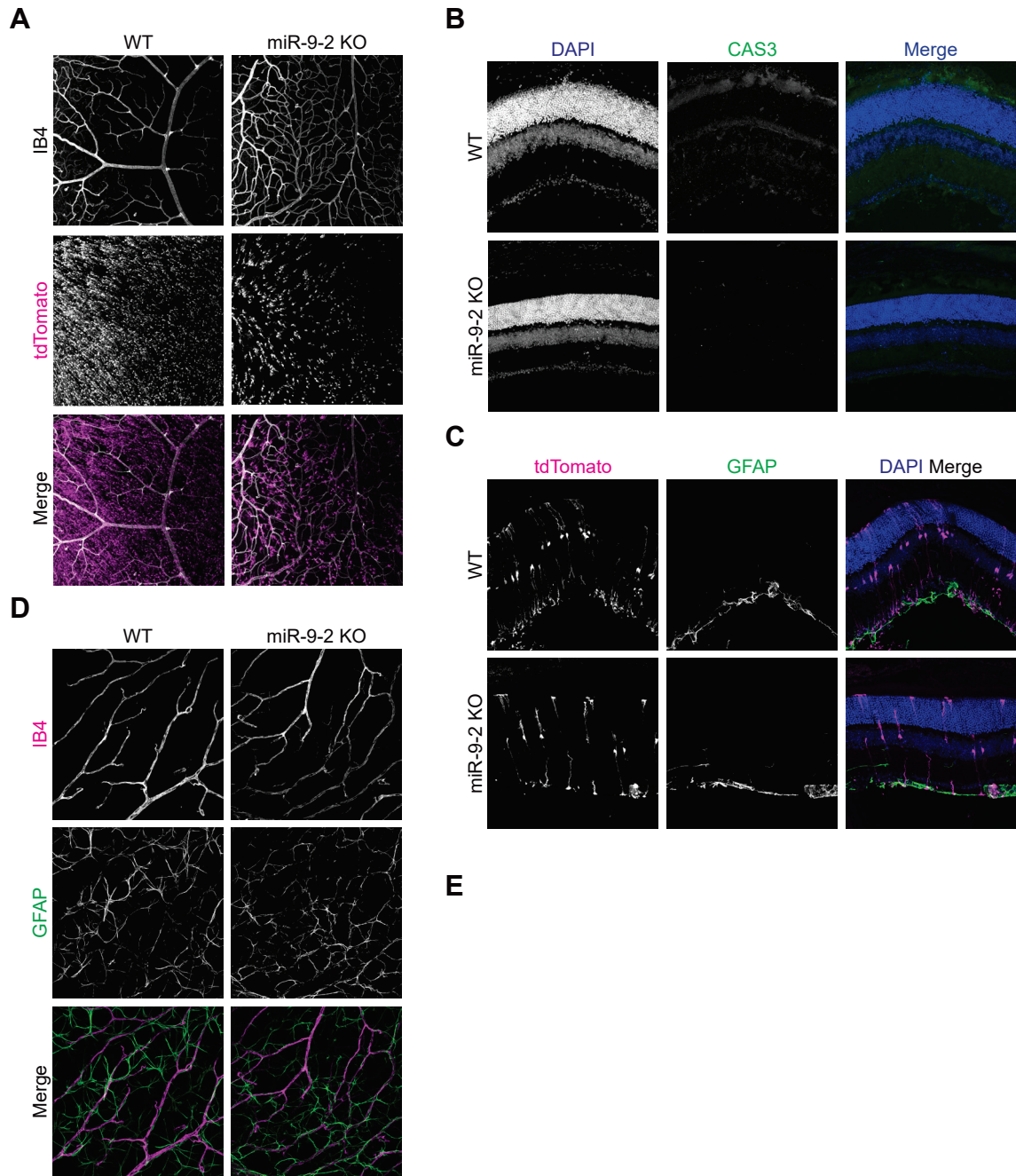
Müller glia can also undergo physical, morphological changes as an early response to cell stress or injury, in a process known as reactive gliosis. Glial cells undergoing reactive gliosis are also characterized by hypertrophy, an increase in proliferative markers, and an increase in the expression of intermediate filament proteins, including GFAP (Graca et al., 2018; Yoo et al., 2021). While activation of gliosis can protect against tissue damage, if overactivated, reactive gliosis can also lead to cytotoxicity in the retina and contribute to further tissue damage and cell loss. To determine if the changes to miR9-2 knockout glia were a consequence of reactive gliosis, we immunolabelled retinas for GFAP. When we compared miR9-2 knockout and wildtype retinas, we found no notable upregulation of GFAP in tdTomato<sup>+</sup> cells (Fig. 3.2C).

GFAP is also a standard marker of astrocytes, a rarer retinal glial cell, which also undergo a reactive response to retinal stress and upregulate GFAP upon injury. Therefore, to check if there were any other indicators of gliosis in the retina, we also compared astrocyte morphology between miR9-2 KO and wildtype retinas. There were no notable differences in the morphology or distribution of GFAP<sup>+</sup> astrocytes between miR9-2 KO and wildtype retinas (Fig. 3.2D). Therefore, it appears that loss of miR9-2 is not causing massive upregulation of gliotic responses which suggests that Müller glial alterations can be attributed to other causes.

### *3.2.3 The transcriptional profiles of miR9-2 knockout Müller glial cells are disrupted.*

To more directly identify the cause of morphological changes in Müller cells and Müller cell loss, we sought to understand whether there were transcriptional changes in miR9-2 knockout Müller glia. To capture Müller cells early in their response to miR9-2 loss, we used 10X Genomics single-nuclei RNA-sequencing (snRNA-seq) to profile retinas from sex- and littermate-matched adult retinas harvested 3 days after induction of miR9-2 loss. Following sequencing, we were able to find and annotate all major cell classes in wildtype and knockout animals using the scMCA (Sun et al., 2019) automated pipeline for cell cluster identification (Fig. 3.3A, Fig S3.2). To assess whether there were any changes to retinal cell class composition due to Müller dysfunction, we compared cell class proportions between miR9-2 knockout and wildtype retinas but found no significant differences for any cell class, including Müllers (Fig. S3.2D). While these results appear to contradict our histological observations, mice for these experiments only received a single tamoxifen injection which leads to sparse targeting of Müller cells. Even with the observed loss of tdTomato<sup>+</sup> Müller glia in miR9-2 knockout retinas, it's likely that there were still populations of non-recombined, wildtype Müller glia that masked the loss of directly affected Müllers.



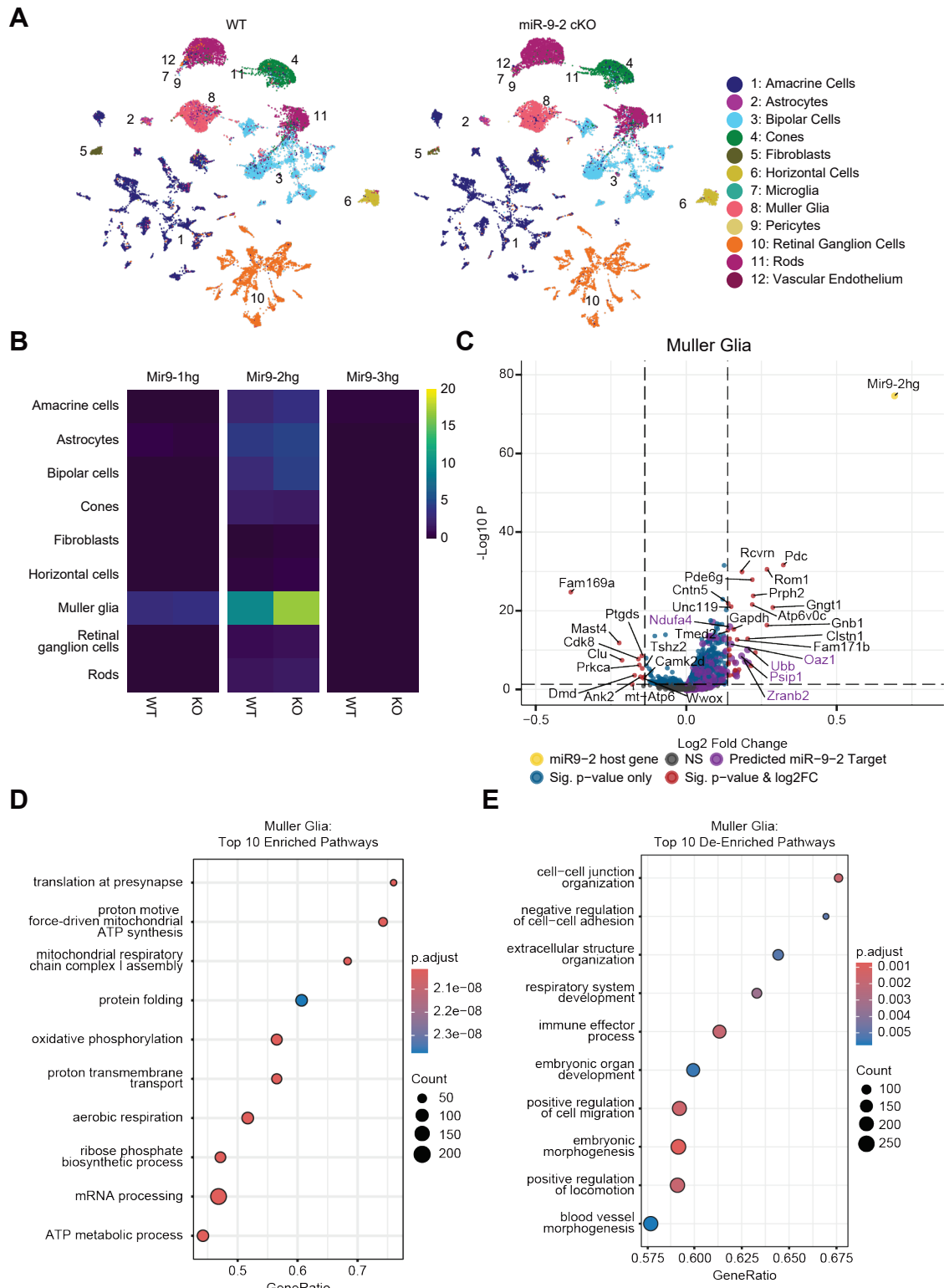


**Figure 3.2: miR9-2 knockout retinas lack markers of apoptosis and reactive gliosis.** Comparisons of miR9-2 knockout and WT control retinas depicting: **A.** En face flat-mounted retinas with markers of retinal vasculature (IB4, gray) and tdTomato<sup>+</sup> recombined cells (magenta). **B.** Cross-sectional images of markers of apoptotic cell death (CAS3, green) and nuclei (DAPI, blue). **C.** Cross-sectional images with markers of reactive gliosis (GFAP, green) and tdTomato<sup>+</sup> recombined cells (magenta). **D.** En face flat-mounted retinas with markers of reactive gliosis and astrocytes (GFAP, green) and retinal vasculature (IB4, magenta).



However, while we could not see changes to Müller cell proportions in our snRNA-seq dataset, we still expected to capture recombined Müller cells in our transcriptional profiling, and further, we anticipated that non-targeted retinal cell classes would still be transcriptionally affected by the dysregulation and/or loss of neighboring glial cells. Therefore, we used Presto (Korsunsky et al., 2022) to perform differential expression analyses for each cell type. As miRNAs block translation through transcriptional degradation or translational block, we expected direct targets of miR9-2 to be de-repressed (upregulated) in our miR9-2 conditional knockout retinas. To identify direct targets, we used TargetScan8.0 (Agarwal et al., 2015) as a reference for computationally predicted targets of miR9-2. As we specifically targeted Müller glial cells, we expected MG to be the cells most directly affected. However, as Müller glia are a crucial support cell for maintaining retinal health and architecture, we still expected other cell types to undergo changes in response to impaired Müller glia function and Müller cell loss. In alignment with these expectations, we found that transcriptional profiles were widely altered across many of the cell types in miR9-2 knockout retinas.

To begin to understand these alterations, we first compared the transcriptional profiles of miR9-2 KO and WT Müller glia. Previously, in our miR9-2 germline knockout mouse model, we found that the *Mir9-2hg* was the predominant form of miR9 in the mature retina and was specific to Müller glial cells. Further, we saw that germline loss of miR9-2 did not result in compensatory upregulation of other miR9-2 host genes during development or in the mature retina (Callies et al., 2026). In our conditional knockout model, we found this same predominance of miR9-2, specifically in Müller glial cells, and saw that miR9-2 loss did not lead to any notable upregulation of the other miR9 family members (Fig. 3.3B). Instead, we found that MG-specific loss of miR9-2 led to dramatic upregulation of its host gene, *Mir9-2hg*, in these cells (Fig. 3.3C).



**Figure 3.3: miR9-2 knockout Müller glia have disrupted transcriptional profiles.** *A.* UMAPs wildtype and miR9-2 conditional knockout retinas depicting the major cell classes of the retina. *B.* Heatmaps of the expression of the three miR9 host gene paralogs split by cell class and genotype. Heatmaps all scaled 0 to 20. *C.* Volcano plot depicting the differentially expressed

genes found in comparisons of miR9-2 cKO and WT retinas. **D.** Dot plot depicting the top 10 enriched pathways in miR92 cKO Müller glia, determined by gene set enrichment analysis. **E.** Dot plot depicting the top 10 de-enriched pathways in miR92 cKO Müller glia, determined by gene set enrichment analysis.

This pattern is similar to the large upregulation of *Mir9-2hg*, and proposed autoregulatory activity, that we previously found in the developing retinas of germline miR9-2 knockout mice, suggesting that miR9-2 has an autoregulatory role in Müller glia of the mature retina as it seems to have in development.

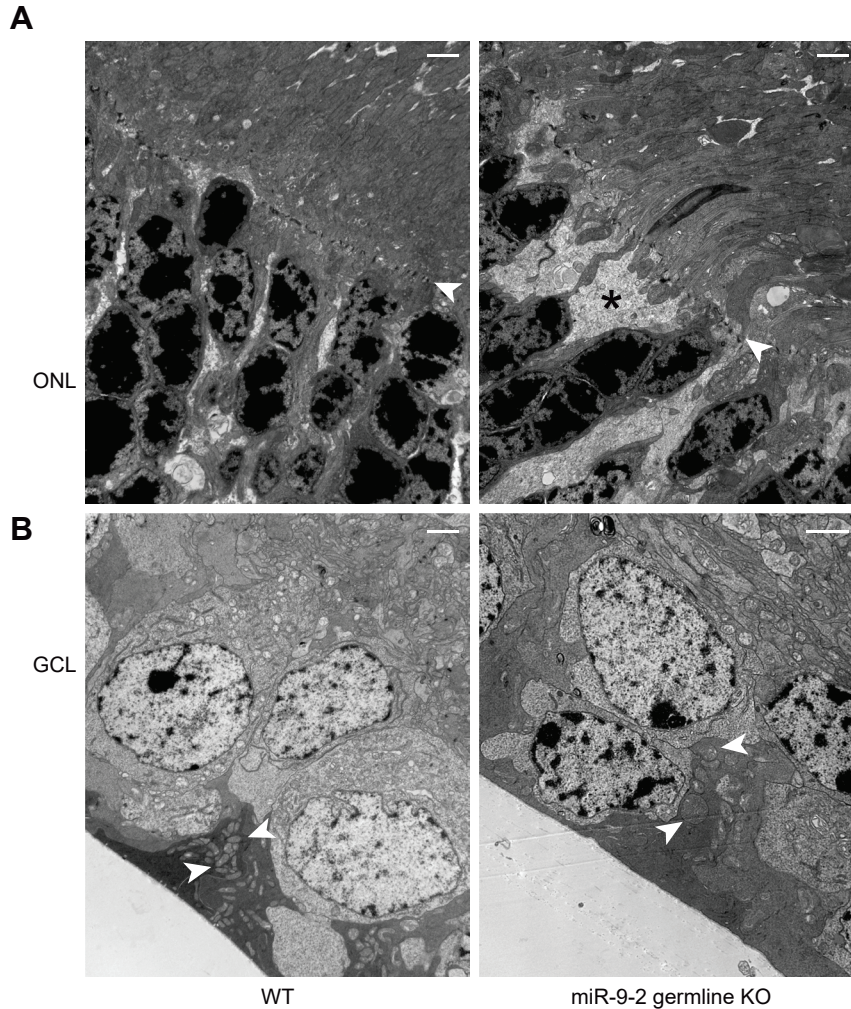
In addition to upregulation of the miR9-2 host gene, some of the top upregulated genes in Müller glia were predicted to be miR9 targets including *Ndufa4*, *Oaz1*, *Ubb*, and *Psip1* (Fig. 3.3C), and these genes have been implicated in a range of processes related to retinal homeostasis, including regulation of mitochondrial metabolism, polyamine synthesis, protein degradation, and stress responses (Palanimurugan et al., 2004; Sagar et al., 2021; Shinohara et al., 2002; Zhou et al., 2024). The top downregulated genes included the Müller glia marker, *Clu*, among others such as *Fam169a*, *Mast4*, *Ptgds*, and *Cdk8* (Fig. 3.3C). Intriguingly, these downregulated genes have been linked to disease conditions including photoreceptor and ganglion cell degeneration, ciliopathies, and mitochondrial dysfunction (Mustafi et al., 2016; Sakaji et al., 2023). These associations are likely attributed, in part, to the homeostatic functions of some of these genes as *Ptgds* is found to regulate prostanoids, which can act as signaling molecules for inflammation and immune responses, while *Cdk8* is essential to cell cycle regulation and modulation of RNA polymerase activity (Liao et al., 2024; Ward et al., 2026).

Knockout Müller glia also appeared to upregulate neuronal markers. As in our miR9-2 germline knockout models, where MG appeared to have hybrid neuronal-glia traits, MG from the conditional knockout retinas upregulated genes that are normally expressed in neurons, including *Rcvrn*, *Prph2*, and *Gngt1*, which are well-established photoreceptor genes. Further,

there was also significant downregulation of normal Müller glia markers, including the gene, *Clu*. Therefore, miR9-2 seems to be important for maintaining glial identity in the mature retina, with loss of miR9-2 leading to the development of neuronal traits.

However, as the expression of many genes was altered, we performed a gene set enrichment analysis to understand what functional processes are most affected in miR9-2 knockout Müller glia. Many of the top enriched pathways are associated with metabolism, including things like “mitochondrial ATP synthesis”, “oxidative phosphorylation” and “ribose phosphate biosynthetic process” (Fig. 3.3D). Therefore, Müller cells are rapidly upregulating many of their metabolic pathways, which would be consistent with an injury or damage response. Some of the top de-enriched pathways include “cell-cell junction organization”, “negative regulation of cell-cell adhesion” and “extracellular structure” organization (Fig. 3.3E). This downregulation of cell-cell interactions aligns with our immunohistochemistry experiments that shows disorganization of the inner and outer limiting membranes, suggesting that Müllers are somehow losing the ability to maintain these interactions.

Interestingly, some of our previously collected histological data from miR9-2 germline knockout retinas seem to align with this miR9-2 cKO pathway enrichment analysis. In germline knockouts, we utilized transmission electron microscopy (TEM) analysis to compare the morphologies of miR9-2 germline knockout and wildtype retinas. When we examined germline KO Müller glial morphology, we found potential evidence of mitochondrial dysfunction, where mitochondrial cells present in the endfeet of miR9-2 germline knockout Müller cells appeared to have a more “swollen” phenotype (Fig. 3.4B). Further, when we examined the apical projects of germline knockout Müller glia, we also found striking disorganization of the outer limiting



**Figure 3.4: Apical and basal termini of Müller glia are disrupted.** *A. Transmission electron microscopy (TEM) images of the outer nuclear layer of miR9-2 germline knockout and WT retinas. Outer limiting membrane (OLM) formed by Müller glia apical projections form a diagonal line across image as denoted by white arrowhead. Asterisk indicates gaps in cell bodies and disorganization of OLM. B. TEM images of the ganglion cell layer or miR9-2 germline knockout and WT retinas. Triangular darker colored structures are Müller endfeet and contain mitochondria with a few highlighted by white arrowheads. For all images, scale bar = 20 microns.*

membrane compared to wildtype (Fig 3.4A). Altogether, loss of miR9-2 triggers an injury- or stress-like response in Müller glia, with associated changes in metabolism and retinal architecture, suggesting that miR9-2 is essential for maintaining a stable homeostatic state in Müller cells.

### 3.2.4 *The loss of miR9-2 in retinal Müller glial cells alters the transcriptional profiles of surrounding neurons.*

While loss of miR9-2 led to substantial alterations in Müller glial cells, we also sought to understand how these changes affected the surrounding neuronal cell types of the retina. As a support cell, Müller cells contact and interact with all neuronal cell types of the retina.

Additionally, in the case of retinal diseases like MacTel, Müller cell loss precedes the loss of photoreceptor cells, and eventually vision (Powner et al., 2010). As discussed previously, we found no significant alterations in cell class proportions between miR9-2 knockout and wildtype retinas (Fig. S3.2). However, as retinal dysfunction such as that found in macular disease is often late-onset and progresses at varying speeds, it is likely that Müller cell disruption has not yet led to neuronal cell loss – especially as our single cell profiling occurred just three days following induction of miR9-2 loss.

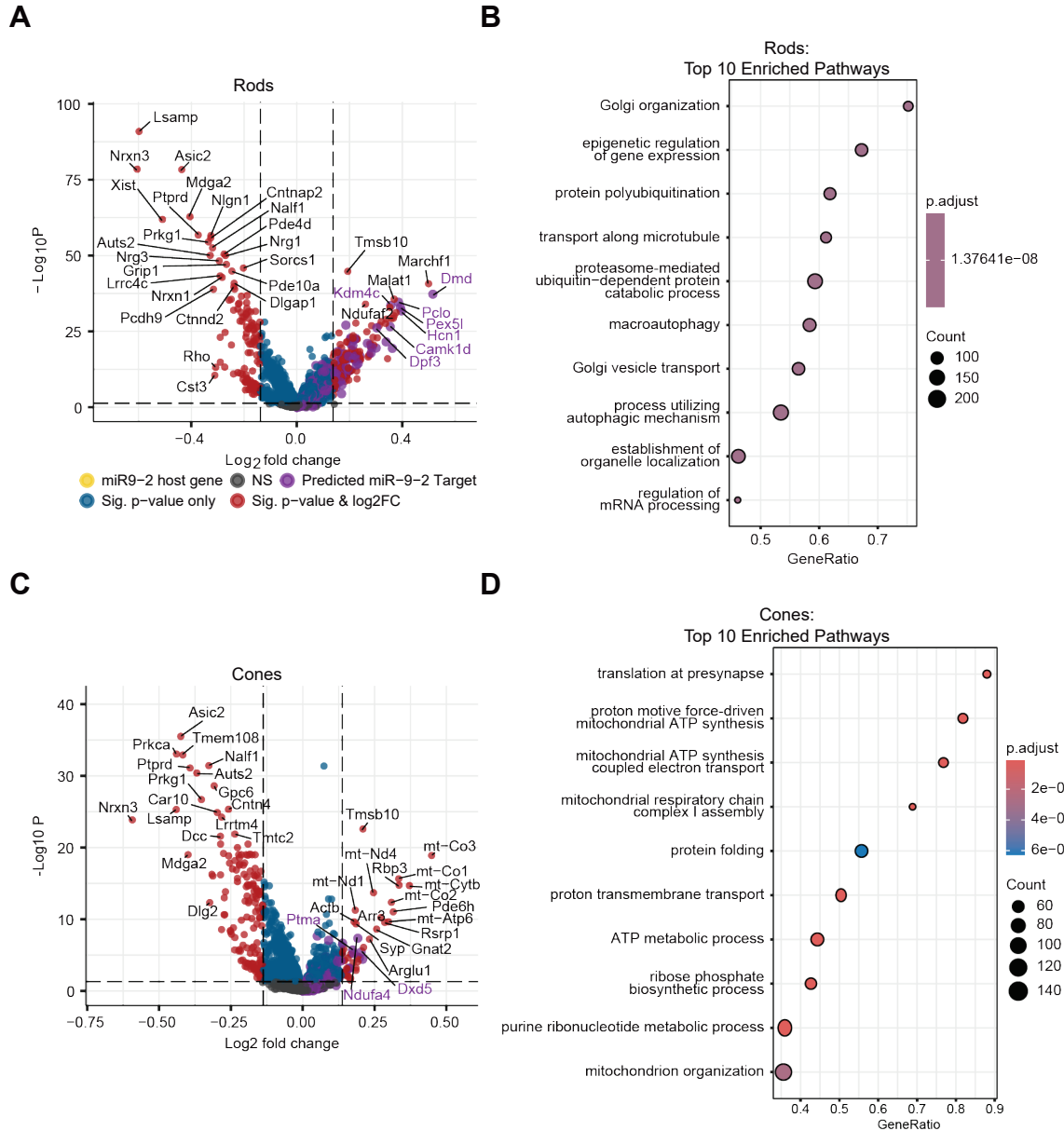
However, by capturing an early time window following Müller disruption, we were able to determine how neurons, such as photoreceptors, respond to the loss of miR9-2 and subsequent dysfunction of Müller glia. Rods are the most abundant cell class in the mouse retina, and are often significantly affected in disease contexts, so we first compared the transcriptional profiles of rod cells originating from MG-specific miR9-2 knockout retinas with control rods. Within rods, we saw many more downregulated genes, including *Lsamp*, *Nrxn3*, *Asic2*, and *Ptprd* (Fig 3.5A). *Lsamp*, *Ptprd*, and *Nrxn3* all have documented roles in promoting neural adhesion and synaptic development. As a result of their role in synapse formation, all three genes have been implicated in neurodevelopmental and neuropsychiatric disorders as dysregulation of synaptic connections can lead to disrupted circuit formation and possible subsequent loss of neurons (Bregin et al., 2020; Kunze et al., 2024; Sinopole et al., 2024; Uhl and Martinez, 2019; R. Zhang



et al., 2023). Further, *Asic2* is an acid-sensing ion channel that is important for regulating photosensitivity of photoreceptors, and loss of *Asic2* can sensitize photoreceptors to light, increasing susceptibility for light-induced damage (Ettaiche et al., 2004).

Further, when we determined which pathways were most enriched in rods from cKO retinas, we found several processes related to “protein polyubiquitination”, “macroautophagy”, and “Golgi organization” (Fig. 3.5B). These pathways are suggestive of photoreceptor stress, as all are linked to protein degradation and responses to it. Therefore, although miR9-2 expression is not enriched in rods as it is in Müller glia, Müller glial dysfunction may be contributing to an immediate stress response of rod photoreceptors, a phenotype which could ultimately lead to photoreceptor loss and disease pathologies as a long-term consequence.

Similarly, as loss of photoreceptors is what most directly leads to perceptible visual changes, we also investigated the effects of Müller glial miR9-2 loss on cone photoreceptor cells. When we compared the transcriptional profiles of cone photoreceptors in miR9-2 knockout and wildtype retinas, we again found downregulation of many genes including *Asic2*, *Tmem108*, *Prkca*, *Ptprd*, and *Auts2* (Fig. 3.5C). As in rods, the downregulation of *Asic2* may make cone photoreceptors more sensitive to light-induced damage. Further, genes, including *Tmem108*, *Ptprd*, and *Prkca*, which are associated with synapse formation and signal transduction, are downregulated just as they are in rods (Ettaiche et al., 2004; Liu et al., 2007; Ruether et al., 2010; Sarin et al., 2018; Uhl and Martinez, 2019; Wakeham et al., 2019). Lastly, in cones, there is significant downregulation of the *Auts2* gene which, while typically implicated in autism and other neurodevelopmental disorders, is also found to be a transcriptional activator and regulator of RNA metabolism (Biel et al., 2022; Hori et al., 2020). Disruption of so many crucial pathways and disease-associated genes is likely to lead to eventual cone dysfunction in these cells. Further,



**Figure 3.5: Transcriptional profiles of photoreceptors are disrupted in retinas perturbed by loss of Müller glial miR9-2 expression.** **A.** Volcano plot of differentially expressed genes for miR9-2 cKO and wildtype rod photoreceptors. **B.** Dot plot showing the top 10 enriched pathways in miR9-2 cKO rod photoreceptors, determined by gene set enrichment analysis (GSEA). **C.** Volcano plot of differentially expressed genes comparing miR9-2 cKO and wildtype cone photoreceptors. **D.** Dot plot showing the top 10 enriched pathways in miR9-2 cKO cone photoreceptors, determined by GSEA.

when we looked at which pathways were most enriched in cone cells, like in glia, we found an upregulation of mitochondrial- and ATP synthesis-related processes (Fig. 3.5D). Cone (and rod)



photoreceptors require a large amount of energy to function and participate in phototransduction, making them particularly susceptible to metabolic perturbation.

Therefore, even as miR9-2 loss only directly affects Müller glial cells, even this sparse perturbation of MG disrupts photoreceptor functions. These disruptions are a plausible cause for such a strong linkage of the miR9-2 genomic locus to retinal disease, as disruption of Müller cell function can have wide-ranging effects on the whole retina.

### *3.3 Discussion*

While Müller glia-specific disruption of miRNAs has been found to disrupt glial function, and subsequently overall retinal health, the exact miRNAs that regulate glial function have not been fully defined. Here, we generated an inducible, Müller-glia specific knockout mouse model for miR9-2 function. Strikingly, we found that induction of miR9-2 knockout in adult retinas led to rapid loss of recombined cells, within as few as three days. While we were able to assess knockout MG for markers of apoptosis and gliosis, they did not show clear signs of either state, suggesting that alternative forms of cell death contribute to cell loss. One form of cell death that may be relevant for miR9-2 knockout Müller glia is ferroptosis. In our previous germline knockout model of miR9-2, we found that Müller glia seemed to have dysregulated iron metabolism (Callies et al., 2026), with substantial upregulation of *Cp*, or ceruloplasmin, which is important for regulating iron export (Harris et al., 1999). Further, when we performed pathway enrichment analysis comparing miR9-2 KO MG with WT, we found that mitochondrial function was highly dysregulated. Some studies suggest that, in certain contexts, mitochondria can participate in regulation of iron production, where an increase in oxidative phosphorylation can generate the lipid peroxides that drive ferroptosis-mediated cell death (Do and Xu, 2023; M. Gao et al., 2019). However, investigations assessing MG for markers of ferroptosis or other

forms of cell death will be required for the future to determine a clear mechanism for Müller cell loss.

Transcriptionally, when we compared differentially expressed genes in Müller glia of miR9-2 germline and conditional knockout retinas, we found that many of the genes that were de-repressed and predicted to be miR9 targets could be functionally grouped. Some of these genes, like *Srgap1*, *Cap2*, and *Rnfl65*, control neuronal migration and axonal growth (Ce et al., 2013; Cheheltani et al., 2025; Coutinho-Budd et al., 2012; Ip et al., 2011; Wong et al., 2001). Several other genes were linked to stress responses and retinal disease, including *Sdc4*, *Sl100a10*, and *Lamp1*, which have been implicated in pathological angiogenesis, regulation of neuroinflammation, and regulation of lysosome biogenesis and phagocytosis, respectively (De Rossi et al., 2021; de Souza Ferreira and Saito Regatieri, 2026; Grishchuk et al., 2016; Huynh et al., 2007). To understand how this transcriptional upregulation translates to changes in Müller function, future assessments looking at markers of neuroinflammation or lysosomal function, among others, will help elucidate these changes. Additionally, the identification of a pro-angiogenic gene like *Sdc4* has interesting implications for the association of the miR9-2 locus with MacTel and AMD which both can manifest vascular phenotypes. While we wouldn't necessarily expect any vascular phenotypes to manifest within the one-week timeframe we assessed, especially with our sparse recombination paradigm, it would be interesting to investigate the vascular development of these animals after a more extended period following induction of miR9-2 disruption.

In the context of specific disease phenotypes, one gene of particular interest, *Sptssa*, was a shared de-repressed gene between miR9-2 germline and conditional knockout models and is a serine palmitoyltransferase which initiates sphingolipid synthesis (Kuo and Hla, 2024).

Dysregulation of sphingolipids has been increasingly associated with MacTel (Gantner et al., 2019), and more generally, sphingolipid accumulation has been found in cases of neurodevelopmental and/or neurodegenerative disease (Kuo and Hla, 2024; Srivastava et al., 2023). While Müller cells of germline knockout animals were also likely affected by developmental defects, the genes shared between germline and conditional knockout retinas indicate distinct biological processes of adult Müller glia that were disrupted by loss of miR9-2 in both models. Therefore, outside of development, miR9-2 may mediate the proper balance of various Müller homeostatic functions, where dysregulation of these processes may be enough to push retinas towards a more vulnerable or diseased state.

The role of miR9-2 in maintaining glial identity also seemed to be conserved across both the germline and conditional miR9-2 knockout models. We previously found that germline knockout MG appear to adopt a hybrid neuronal-glial fate, co-expressing bipolar and glial markers – OTX2 and SOX9, respectively – and upregulating neuronal processes, like the formation of synaptic connections (Callies et al., 2026). Similarly, MG from conditional knockout retinas appeared to upregulate neuronal, photoreceptor markers (*Rcvrn*, *Prph2*, *Gngt1*) while also downregulating normal Müller genes, including *Clu*. Therefore, miR9-2 seems necessary for establishing and maintaining Müller glial identity, during both retinal development and in mature adult retinas.

Outside of Müller glial cells, we assessed how photoreceptors, which are vulnerable in retinal disease, were affected by the MG-specific loss of miR9-2. We saw distinct responses between the photoreceptor classes, with the rod response characterized by upregulation of autophagy and protein degradation-based processes while cones dramatically upregulated their metabolic responses. While both responses are indicative of stress, variation in response could

reflect differences in the vulnerabilities of each photoreceptor type. Metabolically, cones are found to have higher energy consumption than rods and also prioritize different metabolic pathways (Ingram et al., 2020; Li et al., 2020). Recent Dicer knockout studies looking at the effects of MG-specific miRNA disruption on photoreceptors found that loss of all miRNAs appeared to perturb normal cone function earlier than rod function (Larbi et al., 2025). As the human macula is a particularly cone-dense region of the eye, increased sensitivity of cones to glial perturbations and differing metabolic needs could, in part, explain why some retinal degenerative disorders, like MacTel and AMD, originate at the macula.

This study provides insight into why variation at the miR9-2 genomic locus may confer retinal disease risk and why this locus has become so highly associated with macular degeneration. The data presented here also elucidate a potential mechanism for the regulation of normal glial functions and how disruptions to glia could be causal for photoreceptor loss in retinal diseases such as Macular Telangiectasia Type 2. Further work is required to continue elucidating the precise mechanisms for miR9-2 in regulating Müller function. However, this early research provides a promising target for treatment of retinal disease, while also revealing how an individual microRNA is able regulate the wide range of biological processes necessary for maintaining retinal homeostasis.

### *3.4 Methods*

#### *3.4.1 Mice*

*Generation of tamoxifen-inducible, miR9-2 conditional knockout animals.* miR9-2<sup>tm1Mtm</sup> mice containing a floxed miR9-2 hairpin loop followed by a beta-galactosidase reporter and a neomycin selection cassette (Park et al., 2012) were obtained from Jackson Laboratories (RRID:

MMRRC\_036062-JAX). To remove the beta-gal reporter and selection cassette, we crossed animals with ROSA26:FLPe knock in mice (RRID: IMSR\_JAX:009086) (Farley et al., 2000), generating mice containing just the floxed miR9-2 hairpin loop (hereby referred to as miR9-2 floxed animals). Following the FLPe cross, miR9-2 floxed animals homozygous for the floxed hairpin loop (miR9-2 fl/fl) were crossed to a strain containing Glast-creERT2 (RRID: IMSR\_JAX:012586) which allowed for tamoxifen-inducible Cre recombination specifically within Müller glial cells. Separately, to visualize cells where Cre recombination occurred, miR9-2 fl/fl animals were also crossed to an Ai14 reporter strain (Ai14; RRID: IMSR\_JAX:007914) containing a floxed STOP codon upstream of a CAG-tdTomato reporter. Progeny resulting from each of the two crosses that were heterozygous for the miR9-2 floxed hairpin loop (miR9-2 fl/wt) and either homozygous for the Ai14 allele (Ai14 fl/fl) or heterozygous for the tamoxifen-inducible Glast-cre (GlastCreERT2/wt) were then bred to produce animals used in subsequent experiments. To account for any off-target Cre or tamoxifen effects, we specifically selected animals that were heterozygous for Glast-creERT2, heterozygous for the Ai14 allele, and homozygous for either the wildtype or floxed miR9-2 alleles to be used in all experimental comparisons (miR9-2 wt/wt; GlastCreERT2/wt; Ai14 fl/wt versus miR9-2 fl/fl; GlastCreERT2/wt; Ai14 fl/wt).

*Induction of Cre recombination:* To induce Cre recombination in experimental animals (as described above), we introduced 2mg of tamoxifen dissolved in corn oil to adult animals (>5 weeks) via intraperitoneal injection. Animals were monitored daily for 3 days following injection to ensure that they remained healthy.

*Sample harvest:* Following injection, animals were harvested either 3 days (immunohistochemistry; single-nuclei RNA-sequencing) or 7 days (immunohistochemistry)

later. For IHC, eyes were enucleated and fixed directly post-harvest. For single-nuclei experiments, eyes were enucleated, retinas dissected out, flash frozen using liquid nitrogen, and stored at -80C or in liquid nitrogen until use.

### *3.4.2 Immunohistochemistry*

*Fixation and cryosectioning:* After making a hole in the cornea to expose tissues, eyes were fixed in 4% PFA in 1X PBS for 1 hour at room temperature. Tissue was subsequently washed 3X with 1X PBS and then prepared for cross sectioning or flatmounting. For tissue used in cross-sections, cornea and lens were dissected off and then the eye cup was put through a sucrose gradient of 5%, 15%, and 30% sucrose until tissues sank in solution. The eye cup was then embedded in 100% OCT, frozen on dry ice, and stored at -80C until sectioning. Cryosectioning was performed at -20C using a cryostat to generate 40-micron sections mounted on TruBond Gold slides. Slides were baked for 1 hour at 37C and then stored at -20 to -80C until use. For tissue used in flatmounting, retinas were dissected out and stored in 1X PBS at 4C for  $\leq 2$  weeks, until use.

*Antibody staining:* For cross-sections: To ensure tissue adherence prior to staining, slides were baked at 37C for up to 3 hours. To remove OCT, slides were washed 3X with 1X PBS. For primary antibody (see table for antibodies and dilutions), antibodies were diluted in blocking buffer (1% BSA, 0.05% SDS, 0.05% Triton in 1X PBS) and left on sections at 4C overnight. The primary antibody solution was removed by washing slides 3X with 1X PBS. The secondary antibody solution was diluted 1:750 in blocking buffer and left to incubate on sections for up to 4 hours at room temperature. To remove secondary antibodies, slides were washed 3X with 1X PBS. Slides were dried and then cover-slipped using Prolong Gold Antifade (Invitrogen). For flat mounting: Primary antibody was diluted in blocking solution and left on tissue at 4C for 3 nights.

Retinas were washed 3X with 1X PBS. Secondary antibody was diluted in blocking solution and left on tissue at RT for 4 hours. Tissue was washed 3X with 1X PBS and cut at several points around the periphery to allow tissue to lie flat before being mounted on TruBond Gold slides and cover-slipped using Prolong Gold Antifade (Invitrogen).

*Microscopy:* All slides were imaged using the Leica Sp5 or Zeiss LSM 900 or 980. Images were taken using a 20X air or 40X oil immersion lens. For flat mounted tissue, retinas were generally imaged near the periphery and z-stacks were taken in two-micron increments, with stacks spanning the region from the outermost layer of vasculature near the outer nuclear layer to the ganglion cell layer. For cross-sectioned tissue, z-stacks were taken in two-micron increments spanning the entire thickness of the retina.

*Image processing:* Images were processed using open-source FIJI processing software (Schindelin et al., 2012). For counted images and images of co-staining, a single slice from the middle of the collected Z-stacks was chosen to prevent false overlap of cells in different planes. For all other images, max intensity Z-projections of the entire Z-stack were created.

*Cell Counting:* Cell counting was performed on cryosectioned, immunostained, and imaged slides. All counted images were taken at 20X or 40X magnification and the sample conditions were masked to investigators during counting. Two images were counted for each sample and were independently counted by at least two investigators to ensure consistency. Cross-sectional images chosen for counting were taken in roughly analogous regions of the central retina, identified by the presence of a clear optic nerve, and taken in proximal sections on each side of the nerve. Counts per sample were taken as the average of the two images. All sample counts

were normalized to the area of the retina, spanning the region defined by the outermost edges of the DAPI-positive outer nuclear layer and DAPI-positive ganglion cell layer.

### *3.4.3 Single Nuclei Library Generation and Analyses*

*Tissue dissociation:* Retinas were dissociated using a modified version of the 10X Genomics protocol for Nuclei Isolation from Embryonic Mouse Brain Tissue for Single Cell Multiome ATAC + Gene Expression Sequencing. Briefly, flash frozen retina tissue was submerged in lysis buffer (10mM Tris 7.4, 10mM NaCl, 3 mM MgCl<sub>2</sub>, 1% BSA, 0.1% Tween 20, 0.1% IGEPAL CA-630, 1U/uL RNase inhibitor, and 1mM DTT if for multiome), immediately without thawing. Tissue was transferred to and broken up by glass dounce (10X dounce A, then 10X dounce B) before being transferred to clean tubes. Tissue was incubated in dissociation buffer for a total of 10 minutes (and agitated halfway through). Lysis was immediately neutralized in wash buffer (10mM Tris 7.4, 10mM NaCl, 3 mM MgCl<sub>2</sub>, 1% BSA, 0.1% Tween 20, 1U/uL RNase inhibitor) following incubation, and nuclei were passed through a 70-micron filter and subsequent 40-micron filter (BelArt H13680-0070; BelArt H13680-0040) to clear debris. Nuclei were pelleted and washed with wash buffer twice to clear ambient RNA before being counted using Acridine Orange/Propidium Iodine Cell Stain on the ThermoFisher Automated Countess II automated cell counter. Nuclei were spun and resuspended in 1 mL of nuclei buffer (1% BSA, 1U/uL RNase inhibitor in 1X PBS) to reach target nuclei concentration plus 10% for 10,000 cells for input into the 10X Single Cell Chromium NextGEM 3' v3.1 kit, based on 10X guidelines. To verify the final concentration, resuspended nuclei were recounted using the Countess before proceeding with 10X library generation.



*Library preparation:* Libraries were generated following the protocols for the 10X Single Cell Chromium NextGEM 3' v3.1 kit. Equal numbers of littermate, sex-matched male and female pairs (n=3) were used to generate individual libraries. Due to poor sample quality, one conditional knockout male sample was discarded leaving three female cKO/WT pairs, two male cKO/WT pairs, and one individual wildtype male for analysis.

*Sequencing:* Sequencing was performed by the Northwest Genomics Center at the University of Washington or by the Fred Hutchinson Cancer Center Genomics Core. Samples were sequenced using the Illumina NovaSeq SP6000, the Element AVITI™ Cloudbreak Freestyle High Output, or the NovaSeqX with parameters for all sequencing runs following 10X Genomics recommendations for cycles and sequencing depth. All data was returned in FastQ file format.

*Sequencing Alignment:* Single cell data was demultiplexed and aligned using the 10X Genomics Cellranger v9.0 Count pipeline. Following alignment, raw output matrices from Cellranger were input into the Cellbender v0.3.2 remove-background pipeline to filter data for real nuclei and to remove cell-free droplets and ambient RNA reads. Cellbender output was assessed using the provided output report, and all samples that did not pass initial Cellbender quality control (QC) using default settings were run through the pipeline again using the recommended modified learning rate of 5e-5. Filtered Cellbender output matrices that passed the provided QC metrics were used, in addition to Cellranger filtered feature matrices for two samples that did not pass Cellbender QC.

*Processing, clustering, and cell type identification of single cell datasets:* Seurat v5 was used to perform analyses of individual and aggregated samples (Hao et al., 2024). Seurat objects were generated from input matrices and quality control assessment was performed on individual

samples. scDoubletFinder (McGinnis et al., 2019) was run on individual samples, and then samples were filtered to remove identified doublets. Subsequently, cells were filtered by additional QC metrics, including mitochondrial percentage ( $<5\%$ ), number of UMIs and number of genes. Individual samples were merged for analysis and relevant metadata (genotype, sex, batch, sample) were annotated. Merged objects were normalized and scaled using SCTransform (Choudhary and Satija, 2022; Hafemeister and Satija, 2019). Dimensional reduction was performed, and the Harmony package (Korsunsky et al., 2019) was used to integrate samples before generating Uniform Manifold Approximation and Projections (UMAPs) and clustering. To annotate mature retinal cell types, we identified cell types using two distinct mouse atlases. We called cell class identities from two pipelines: first, we used the Mouse Cell Atlas (MCA) automated scMCA pipeline (Sun et al., 2019) for calling cell class identities, and secondly, we also used a separate Mouse Retina Cell Atlas (MRCA) (Li et al., 2024), to map cell class identities to our data using the Seurat FindTransferAnchors function. There was high concordance between the two methods of cell class identification, and we used the scMCA cell class IDs for almost all cells and only utilized MRCA cell class identities for several hundred cells that scMCA failed to identify. All cell type identification was validated using prior knowledge of expected cell type-specific gene expression.

*Differential gene expression analyses:* To perform differential gene expression analysis, the RNA assay of annotated Seurat objects was normalized using Seurat NormalizeData and filtered using the EdgeR (Lund et al., 2012; Robinson et al., 2010) filterbyExp (with default parameters) to remove genes with inadequate expression for statistical power. Presto (Korsunsky et al., 2022) was used to determine differentially expressed genes between wildtype and knockout samples for each cell type of interest. Predicted miR9 targets in the differentially expressed genes were

identified using TargetScan8.0 (Agarwal et al., 2015; McGeary et al., 2019). Differentially expressed genes for relevant cell types were plotted as volcano plots using the EnhancedVolcano R package (Blighe et al., 2026) .

*Pathway Analysis:* To perform gene set enrichment analysis, the unfiltered list of differentially expressed genes from Presto was ranked according to the Area Under the Curve (AUC) metric multiplied by the sign of logFC. We used gseGO (default settings) from ClusterProfiler (Yu et al., 2012) to identify enriched pathways from the list of Gene Ontology Biological Processes database (Ashburner et al., 2000; The Gene Ontology Consortium, 2026) and plotted the top enriched and/or de-enriched pathways using the ClusterProfiler dotplot function.

#### *3.4.4 Quantification and Statistical Analysis*

For cell counting results, all bar graphs were created and analyzed with GraphPad Prism and are shown with error bars +/- 1SD. Mann Whitney U tests were performed to determine whether cell counts were significantly different. For all statistical analyses,  $p \leq 0.05$  were considered statistically significant.

#### *3.4.5 Data Availability*

All snRNA-seq files will be made available at GEO. All unique code generated in this study will be made available at [github.com/TCherryLab](https://github.com/TCherryLab).

#### *3.5 Acknowledgements*

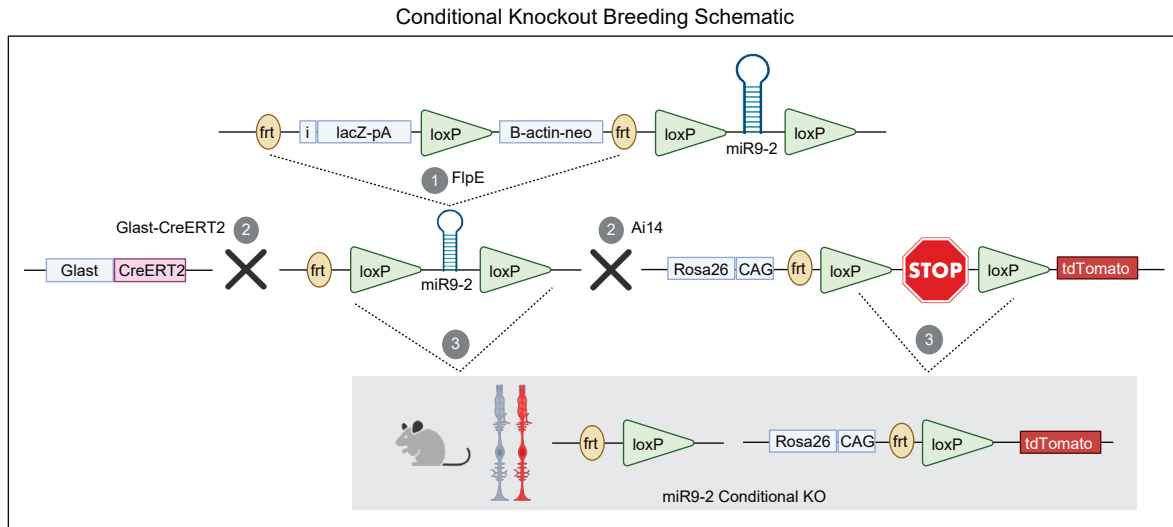
I would like to thank all members of the Cherry Lab, CDBRM, the Lowy Medical Research Institute (LMRI), as well as the LMRI Board of Scientific Governors, for helpful discussions about this project. I thank the animal care team at Seattle Children's Research Institute for their hard work and daily maintenance of our animal colony. I would also like to thank the University of Washington Vision Core and Dr. Abbi Engel for their assistance with the TEM experiments. This work was supported by grants from the NIH National Eye Institute (R01EY028584 & R01EY033364) and the Lowy Medical Research Institute to T.J.C., an NIH P30 grant from the NIH National Eye Institute (P30EY001730) to the UW Vision Core, and an NIH T32 award (EY007031) and an NIH F31 grant (EY035932) to L.K.C.

### *3.6 Author Contributions*

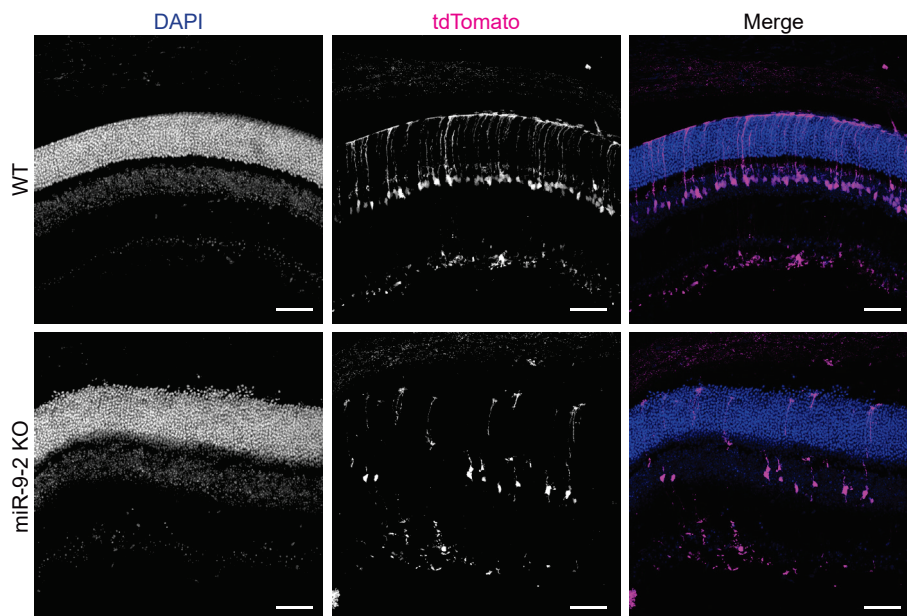
This work was performed with contributions from myself (L.K.C), Aarushi Jain (A.J.), and Timothy J. Cherry (T.J.C). L.K.C., A.J., and T.J.C. designed and performed all experiments and analyses associated with this work. L.K.C generated single nuclei sequencing datasets and performed all bioinformatic analyses. L.K.C. generated the Müller glia-specific miR9-2 knockout mouse model and completed all sample harvests. L.K.C. and A.J. performed animal management and histology. L.K.C and T.J.C. conceptualized study design. L.K.C wrote this chapter.

### 3.7 Supplemental Information

**A**

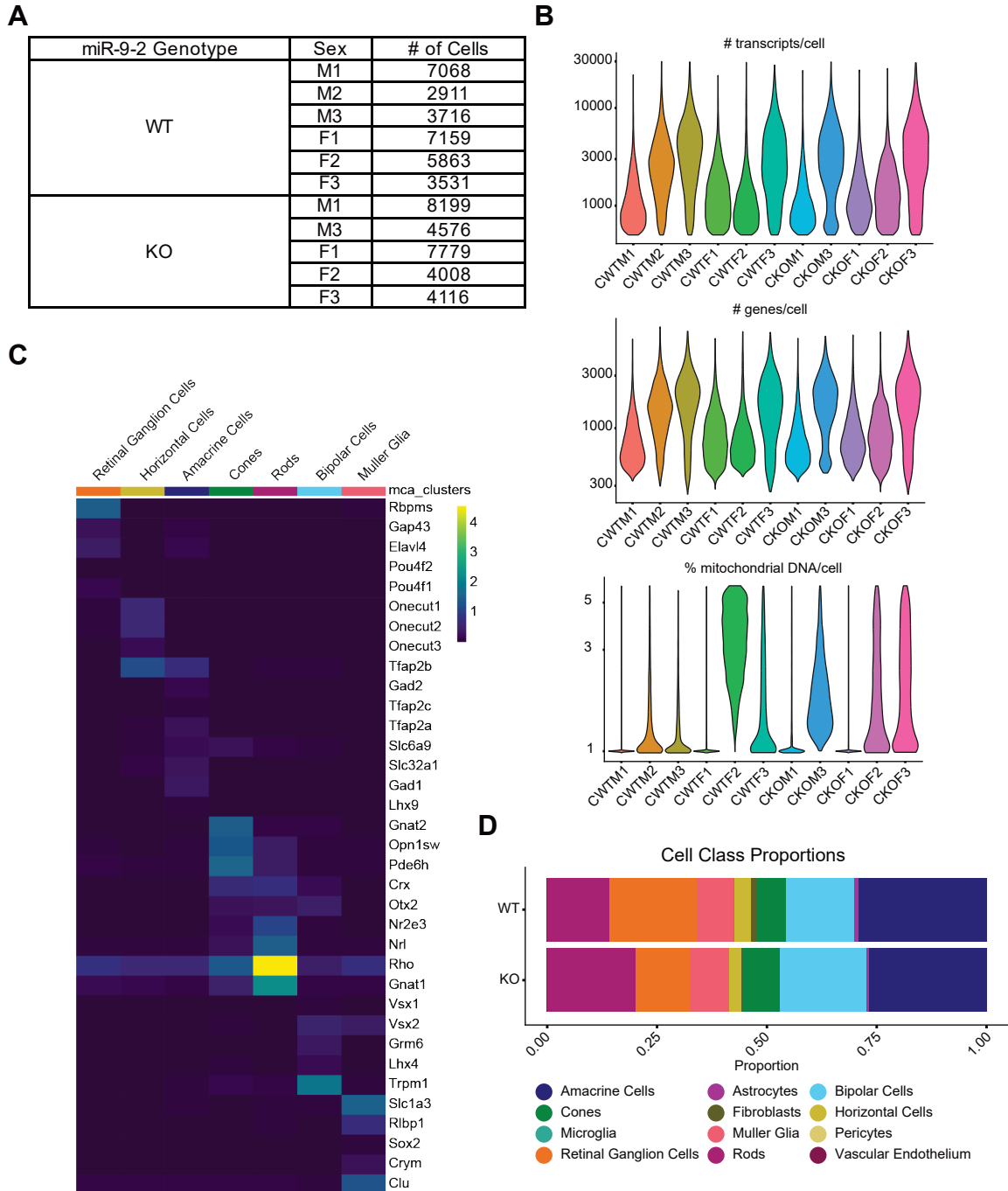


**B**



**Figure S3.1 Breeding and comparison of *tdTomato*-positive recombined cells for Müller glia-specific *miR9-2* knockout retinas.** **A.** Breeding schematic showing the crosses required to generated MG-specific *miR9-2* cKO and control animals with the first cross (1) removing the *lacZ* reporter cassette, followed by (2) generation of animals containing the alleles for the non-recombined floxed *miR9-2* hairpin loop and either the inducible *CreERT2* under the control of the *Glast* promoter or the *Ai14* *tdTomato* reporter allele for *Cre* activity. Lastly, (3) progeny from the two previous crosses shown in (2) were bred together to generate *miR9-2* cKO and control animals. **B.** Cross-sectional images of comparison of *miR9-2* cKO and wildtype retinas 3 days

*following a single tamoxifen injection showing tdTomato<sup>+</sup> recombined cells (red), and nuclei (DAPI, blue). Panel A created with BioRender.com.*



**Figure S3.2 Quality control metrics for single-nuclei sequencing of miR9-2 conditional knockout and wildtype retinas.** *A.* Number of cells that passed standard QC metrics for the individual retinas used in snRNA-seq experiments. *B.* Violin plots of standard QC metrics split by sample for the number of transcripts per cell, the number of genes per cell, and the percent mitochondrial DNA per cell. *C.* Heatmap of all cells used in snRNA-seq experiments, showing standard markers of each retinal cell class and split by individual cell types. *D.* Stacked bar plot

*showing the averaged cell class proportions for miR9-2 cKO and WT retinas based on snRNA-seq data.*



## CHAPTER 4. DISCUSSION

In this dissertation, I have provided an overview of my investigation into mammalian retinal development and the role of the microRNA, miR9-2, in its regulation. I have sought to elucidate the role miR9-2 plays in regulating retinal progenitor competence and Müller glial cell homeostasis. Further, in this work, I aimed to identify gene regulatory networks directly and indirectly controlled by this microRNA to develop a clearer mechanism of how miR9-2 exerts its effects.

### *4.1 An overview of my studies into miR9-2 function.*

I sought to understand how the miR9-2 regulatory locus controls the timing of retinal cell fate specification. Using a germline miR9-2 knockout mouse model, I generated a single-nucleus RNA-sequencing dataset, capturing the period of retinal development where progenitor cell competence transitions from production of early- to late-born cell fates. Additionally, I analyzed an unpublished snRNA-seq dataset of retinas from a miR9-2 enhancer knockout mouse model. Using these datasets, RNAscope *in situ* hybridization, and immunohistochemistry (IHC), I found that miR9-2 expression is highest in retinal progenitor cells during development, and that by adulthood, its expression is restricted to Müller glial cells. Further, I saw that germline disruption of miR9-2 in progenitor cells delays the production of late-born cell classes, and later-born cell types within each cell class, although ultimately, all major cell classes of the retina were produced. However, by the end of development, germline loss of miR9-2 resulted in misspecification of Müller glial cells to a hybrid neuronal-glial identity. In both miR9-2 enhancer knockout and germline miR9-2 knockout retinas, I also found that retinas have disorganization of Müller nuclei within and/or outside of the inner nuclear layer. Overall, these results indicate that miR9-2, an individual member of the larger miR9 family of microRNAs, and its regulatory

locus, alone, are critical for controlling the proper transition of progenitor cell competence from early to late cell fates.

In addition to its expression in retinal progenitor cells, by adulthood, miR9-2 expression becomes highly restricted to Müller glia cells. Therefore, I sought to understand if miR9-2 has a distinct role in modulating normal Müller cell homeostasis. To directly understand its mature role, I specifically disrupted miR9-2 in adult Müller glia (MG) by taking advantage of the Cre-lox system to generate an inducible, MG-specific knockout mouse model. I induced miR9-2 knockout when animals were at least 5 weeks of age, when the retina is fully mature, and collected retinas three to seven days following induction. Using a combination of IHC and snRNA-seq, I profiled retinas of mice containing the wildtype or floxed miR9-2 knockout allele. I found that loss of miR9-2 in Müller glia led to rapid loss of Müller cells, loss of normal Müller glia identity, and disruption of normal Müller cell morphology, with a visible swelling phenotype of Müller endfeet and Müller projections. Further, I found that this loss of miR9-2 led to transcriptional changes in not only Müller cells but also rod and cone photoreceptors. Most notably, I found significant enrichment of metabolic pathways among disrupted genes in Müller and photoreceptor cells.

#### *4.2 How do cell class proportions in miR9-2 knockout retinas “catch-up”?*

One of the surprising findings that we revealed during our characterization of the miR9-2 germline knockout retinas, is that, despite developmental delays, normal cell class proportions are restored by three weeks. However, this begs the question, how is cell class birth actually delayed? Is cell class proliferation prolonged or cell cycle length altered? Do knockout retinas produce normal or increased numbers of cells? Do changes in proportions cause a change in developmental cell death? Due to unexpected brain neurodevelopmental phenotypes in our

germline knockout mice (see Appendix A), we were unable to reliably harvest postnatal retinal samples of the miR9-2 knockout retinas. Therefore, we were unable to determine how normal retinal development proceeded between postnatal day 0 and adulthood, even though retinal cell birth will continue until around P12 and maturation completes roughly 3-5 weeks post-birth.

Mouse models specifically targeting retinal progenitor cells have successfully been utilized to understand Dicer and miRNA function by driving Cre-recombinase expression under the control of early progenitor-specific promoters such as *Chx10* (Damiani et al., 2008). To avoid postnatal mortality originating from hydrocephalus phenotypes, further studies would benefit from generation of an inducible progenitor-specific miR9-2 knockout model, such as one using *Chx10*-CreERT2. Using this model, firstly, we would be able to assess how postnatal development progresses, as we were unable to assess any of the development occurring between P0 and three weeks. While there are some indicators of alterations to cell class proliferation, the substantial presence of dividing progenitors before birth makes it difficult to detect if there are more subtle alterations. Postnatally, proliferation will eventually decline, allowing for easier detection of persisting proliferation. Secondly, if the birth of various cell types is delayed, does the retina overproduce early born cell classes? If so, what happens to those cell classes? Retinas normally experience a large wave of programmed cell death in the first 1-2 weeks following birth. Immunostaining for markers of proliferation and cell death in the postnatal retina may give us a much clearer picture of what happens to cells in the miR9-2 knockout retinas.

To further expand on this model of miR9-2 function, it would be interesting to employ lineage tracing techniques to define how exactly the production of cell types is changed. Continuous improvements have been made to Brainbow technologies over time, and inducible Cre-driven tools currently exist that have increasing numbers of distinct colors to use for lineage

tracing (Cai et al., 2013). Labeling individual progenitor cells and tracking the subsequent clones would allow us to understand whether miR9-2 knockout retinas have increased proliferation and whether individual clones grow in size. Further, although the retina does eventually catch up, it would be interesting to see if miR9-2 knockout clones become more biased toward earlier cell fates.

#### *4.3 Are miR9-2 knockout retinas functional?*

Due to postnatal lethality, another aspect of retinal development we were unable to assess was whether miR9-2 knockout retinas are truly functional, as these tests typically are done once the retina is fully mature. While all the cells of retina do develop, are they still able to properly carry out their functions? Could specific cell types be more susceptible to miR9 loss than others? Visual acuity can be measured in mice through behavioral assessments such as the optomotor test which measures how long a mouse can track a rotating screen of finer and finer gratings until they no longer can perceive its movement. Neuronal function can also be directly assessed through electrophysiology experiments like an electroretinogram (ERG) which directly measures neuronal action potentials in response to light (Leinonen and Tanila, 2018). Administering tests such as these to progenitor-specific miR9-2 knockout and wildtype mice, at 5 weeks or later when retinas should be fully matured, would allow us to understand if knockout mice can form functional retinas. It would also be particularly interesting to assess the specific function of the hybrid neuronal-glial cells of miR9-2 knockout animals. Electrophysiological experiments on these hybrid cells would allow us to understand if they have functional neuronal properties, including the ability to generate action potentials, or if they are more glia-like and retain only more passive membrane properties.

Lastly, many of the developing cell types in the miR9-2 germline knockout retinas had upregulation of genes related to synapse formation and maturation, which was also evident in the pathway enrichment analysis. Further, many of these genes were predicted to be direct targets of miR9 suggesting that this microRNA plays an important role in regulating synaptic function. While these results could simply be reflective of the relative immaturity of knockout retinas, it would also be interesting to assess whether synaptic development is occurring normally. Electrophysiology measurements of mature miR9-2 knockout and wildtype retinas would also allow for measurement of neuronal response to stimuli to assess synaptic functionality. Additionally, more TEM experiments focusing on the synaptic layers of the retina would also allow us to visualize whether synaptic interactions of KO retinas are normal.

#### *4.4 Does miR9-2 play a role in regulating Müller cell metabolism?*

In the mature retina, while I was able to determine that Müller homeostasis was significantly disrupted, the exact cause of these changes was unclear. Further, due to technical challenges with immunohistochemistry and the extensive time required to collect littermate-matched pairs of knockout and wildtypes retinas, I was not able to elucidate the cause of Müller cell loss. Retinas lacked hallmarks of apoptosis or reactive gliosis, leaving many lingering questions related to the function of miR9-2 in mature MG. How do Müller cells disappear? Further, what causes these rapid Müller disruptions? One finding that was particularly notable was the metabolic, and specifically mitochondrial, disruption within Müller glial cells. Not only were induced miR9-2 knockout MG cells enriched for mitochondrial metabolic terms, but also, in our model of germline miR9-2 knockout retinas, preliminary electron microscopy analysis showed the possible presence of swollen mitochondria residing in the Müller endfeet.

In the future, using the MG-specific inducible knockout model, measurement of retinal metabolism could help elucidate metabolic changes in Müller cells and provide insight into the role of miR9-2 in regulating homeostatic functions like metabolism. *Ex vivo* retinal explant culture would allow for comparisons of metabolic activity in conditional knockout and wildtype animals, including measurements of glycolysis and mitochondrial respiration through Seahorse metabolic assays. By taking measurements at varying timepoints following induction, we may be able to distinguish between changes in Müller metabolism which we would expect to happen more rapidly, and later changes in photoreceptor metabolism which we might expect to take longer to occur. Further, metabolomics profiling and/or metabolite isotope tracing to compare wildtype and miR9-2 conditional knockouts may elucidate specific metabolites or enzymes that are directly impacted. It would be interesting to see if any particular metabolites are depleted or accumulated. As Müller cells, and retinas in general, typically utilize aerobic glycolysis (Hass et al., 2023; Winkler et al., 2000), it is surprising to see such dramatic upregulation of mitochondrial processes and oxidative phosphorylation. Further, retinal diseases, such as Macular Telangiectasia, are associated with retinal and systemic dysregulation of serine/glycine ratios and generation of toxic deoxysphingolipids (Bonelli et al., 2021a; Lim et al., 2024). Therefore, metabolic studies may also inform whether disease-associated linkages of miR9-2 contribute to metabolic dysfunction.

#### *4.5 What are the dynamics of miR9 targeting, translational repression, and transcriptional degradation in the context of the retina?*

From our snRNA-seq data, we identified computationally predicted direct and indirect targets of miR9 that were significantly affected by knockout of miR9-2, including Onecut family TFs, *Rorb*, and *Foxp1*. However, due to the complex dynamics of miRNAs, it is still difficult to

verify which targets directly interact with miR9-2 and which have been affected by the complex interplay of miR9 regulation. One complementary technique to understanding miRNA-mRNA interactions is through the usage of proteomics. As miRNAs work through transcriptional degradation or translational repression, while snRNA-seq may identify mRNA interactions that result in degraded transcript, parallel techniques such as immunohistochemistry or proteomics are required to fully capture the spectrum of miRNA targets. Targeted proteomics analysis of tdTomato+ glial cells or bulk retinal tissue may allow us to construct a clearer network of miRNA-mRNA-protein interactions. Similarly, we could also perform spatial transcriptomics on internally controlled, sparsely labeled miR9-2 conditional knockout retinas. Platforms like 10X Visium have assays that are designed for the detection of RNA and proteins in the same tissue, which would allow us to compare the levels of predicted targets in both induced miR9-2 knockout and wildtype glia.

Further, in our early characterization of miR9-2, we were able to perform *in situ* hybridization for the miR9-2 host gene as proxy for expression of miR9-2 activity. We also attempted to perform miRNAscope, an assay designed for *in situ* detection of active, processed microRNAs. However, technical limitations with tissue preparation and protocol optimization prevented us from successfully performing this assay to detect miR9 in the retina. In contrast, in studies of miR9-2 brain function conducted in the lab, miRNAscope performed on brain tissue showed localization of miR9 to cells that were not thought to express miR9. Existing studies have profiled extracellular vesicles from serum and vitreous fluid of the eye, and identified many miRNAs that are able to leave the cells they were produced in (Wooff et al., 2020); further, a single study exists profiling vitreous fluid of patients with diabetic retinopathy that suggests that miR9-3p has been found in exosomes outside of the Müller glial cells it is thought to be normally

produced in. To fully understand miR9 activity, it would be prudent to investigate whether miR9 is commonly exported outside of its originating cell. The most approachable next step would be to determine whether processed miR9 can be found outside of cells we expect it to be in by further optimizing miRNAscope or using other methods like *in situ* Hybridization Chain Reaction which has been used successfully to detect retinal miRNAs (Zhuang et al., 2020). If activity of miR9 is detected outside of the expected host cells, spatial transcriptomics, like the experiments described above, may also let us spatially compare the profiles of neurons that are proximal to WT, miR9-2 expressing- or miR9-2 knockout Müller glia.

#### *4.6 Concluding thoughts regarding miR9-2 in retinal development and disease*

Over the last few decades, the significance of microRNAs in the regulation of normal development and biological processes has been increasingly established and acknowledged. Most recently, this recognition has culminated in the awarding of the Nobel Prize for the discovery of microRNAs and their role in post-transcriptional regulation (Lee et al., 1993; Wightman et al., 1993). Further, as the body of knowledge surrounding miRNAs has rapidly advanced, there has been increasing need to continue defining the diverse functions of miRNAs in distinct organisms, tissues, and cell types.

Over the course of my PhD, I have investigated the role of one individual microRNA, miR9-2, from the perspectives of retinal development, homeostasis, and degenerative disease. I have generated novel insights into how miR9-2 regulates the timing and progression of retinal progenitor cell competence, through its regulation of distinct gene regulatory networks. By sitting at a crucial intersection of TF networks with well-defined roles in generating distinct cell types, I have discovered that miR9-2 can directly target TFs that promote early-born cell fates and can repress TFs that generate late-born cell classes. While disruption of miR9-2 alone does



not completely prevent the production of late-born cell classes, miR9-2 is still essential to ensuring the proper fate specification and repression of neuronal attributes in developing Müller glia, in particular. Moreover, miR9-2 is able to regulate cell fates at both the cell class and cell type levels, acting with a high level of specificity and temporal precision during the establishment of cell fate. In the adult retina, through its restricted expression in mature Müller glia cells, miR9-2 is also important for Müller cell survival and maintenance of proper retinal homeostasis. By regulating the balance of metabolic pathway activity and cell-cell interactions, including formation of cell-cell junctions and synaptic communication, miR9-2 is necessary to establishing a healthy retinal environment.

By developing an understanding of these normal developmental and cellular processes, I have also sought to understand how dysregulation of development and/or Müller glial cell function may contribute to increased retinal vulnerability to diseases like macular degeneration. Risk for retinal degenerative disease and retinal disease phenotypes have been associated with the miR9-2 regulatory locus, and it is likely that perturbations of miR9-2 activity or function create this increased susceptibility to neuronal degeneration and/or vascular dysfunction. Through my dissertation work described here, I have identified several important functions of miR9-2 while generating new, open questions regarding the mechanisms and regulation of miR9-2.

Future analyses of miR9-2 will benefit from exploring the role of miR9-2 during postnatal retinal development, following the early- to late-born cell class transition, and determining whether miR9-2 is critical for generation of a functional retina. Further, while miR9-2 has clear effects on mature Müller glial homeostasis, precisely defining the activity and consequences of normal and perturbed miR9-2 expression at a more mechanistic level will

expand our knowledge of its role in healthy Müller glia function. As a regulatory molecule, the elucidation of the distinct mechanisms for miR9-2-mRNA target interactions and regulation of miR9-2 expression itself will further enhance our understanding of retinal development and disease. In conclusion, I have established here that miR9-2 is a critical regulatory element for controlling progenitor competence, neurogenesis and gliogenesis. It is my hope that this knowledge can provide a foundation for future investigations into miR9-2 regulation and that future work describing the exact mechanisms of miR9-2 may ultimately allow us to develop a causal understanding of retinal disease and find a path for treatment or prevention.

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APPENDIX A. LOSS OF MIR9-2 LEADS TO HYDROCEPHALUS AND DISRUPTION OF  
CELL TYPE-SPECIFIC DEVELOPMENTAL GENE NETWORKS.

Work presented here comes from the preprint manuscript:

Fregoso, S. P., Attapattu, M., Callies, L.K., Monet, D., Leonardson, A., Clark, L., Xu, S., and Cherry, T. J. Loss of miR9-2 causes cerebral hemorrhage and hydrocephalus by widespread disruption of cell-type-specific neurodevelopmental gene networks. bioRxiv 2025.07.31.668014; <https://doi.org/10.1101/2025.07.31.668014>

For this work, I contributed to animal care, data analysis including preliminary snRNA-seq processing and pathway analysis, monitoring and analysis for survival of germline miR9-2 knockout animals at birth and weaning, tracking incidence of hydrocephalus, and commentary and review of written manuscript.

# Loss of *miR-9-2* Causes Cerebral Hemorrhage and Hydrocephalus by Widespread Disruption of Cell-Type-Specific Neurodevelopmental Gene Networks.

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## ABSTRACT

MicroRNAs exert significant post-transcriptional regulation on neural gene networks. *MiR-9-2* is highly and widely expressed in the developing brain and its gene locus can be disrupted in neurological diseases including 5q14.3 Microdeletion Syndrome. However, the contribution of *miR-9-2* disruption to these neurodevelopmental diseases remains insufficiently explored. Here, we investigate the unique role of *miR-9-2* in mammalian brain development. We find that *miR-9-2* deletion causes embryonic cerebral hemorrhages and ventriculomegaly followed by post-natal hydrocephalus. Single-cell transcriptomic analysis of *miR-9-2* knockout cerebral cortex revealed dysregulation of neural, vascular and choroid plexus gene networks pointing towards defects in cell adhesion, vascular development and choroid plexus ciliogenesis. Altogether, this demonstrates that *miR-9-2* is a critical post-transcriptional regulator of both neural and non-neural gene networks and plays an important role in maintaining vascular and cerebrospinal fluid homeostasis. This suggests that *miR-9-2* loss contributes functionally to conditions such as 5q14.3 Microdeletion Syndrome.

## INTRODUCTION

Neural cell fate and function in the developing central nervous system (CNS) is driven by genetic programs regulated post-transcriptionally by microRNAs (miRNAs)<sup>(1,2)</sup>. In mammals, the *miR-9* family of miRNAs, comprised of *miR-9-1*, *-2* and *-3*, plays a prominent role in early brain development through regulation of neural proliferation, differentiation and maturation<sup>(2,3)</sup>. Although identical in sequence, each *miR-9* paralog has a distinct spatiotemporal expression pattern during embryonic brain development, with *miR-9-2* displaying the highest and broadest expression levels, with peak expression occurring during the neurogenic period of cortical development between embryonic days (E) 13.5-16.5<sup>(3)</sup>. The variable expression patterns of each *miR-9* transcript have likely diversified their cell type-specific roles in shaping gene networks. However,

how each *miR-9* paralog contributes to neural development or disease in mammals remains poorly understood.

In humans, *MIR-9-2* has been associated with autism spectrum disorder risk<sup>(4,5)</sup> and the *MIR-9-2* locus is frequently lost in 5q14.3 Microdeletion Syndrome<sup>(6-8)</sup> (Fig. 1A). Patients with 5q14.3 Microdeletion Syndrome often develop neurological conditions such as seizures, corpus callosum agenesis, and lack of speech development as well as vascular disorders, such as capillary and arteriovenous malformations<sup>(9,10)</sup> with frequent incidence of ventriculomegaly and prominent perivascular spaces<sup>(9,11)</sup>. The features of 5q14.3 Microdeletion Syndrome are typically attributed to loss of protein-coding genes at this locus, including *MEF2C*<sup>(6,12-14)</sup>, *TMEM161B*<sup>(15)</sup> or *RASA1*<sup>(10,12)</sup>. However, the *MIR-9-2* host gene (*MIR9-2HG*) is highly and broadly expressed among neural cells in the developing human brain, particularly during the 1<sup>st</sup>-2<sup>nd</sup> trimesters of fetal development<sup>(16)</sup> (Fig. 1B-C), indicating that this specific *MIR-9* family member may be an overlooked and significant contributor to 5q14.3 Microdeletion Syndrome.

In this study, we investigate the specific role of *miR-9-2* in murine brain development through histological characterization of pre- and post-natal development and by whole transcriptome sequencing at early and mid- neurogenic time points to define the gene networks disrupted by loss of *miR-9-2* expression. We find that both heterozygous and homozygous *miR-9-2* deletion results in embryonic ventriculomegaly and postnatal hydrocephalus, i.e. accumulation of cerebrospinal fluid (CSF), coupled with widespread cerebral microhemorrhages. Furthermore, our transcriptional analyses revealed significant disruption to neural, vascular and choroid plexus developmental gene networks. Our findings uncover an unexpected role for *miR-9-2* in the regulation of cerebral vascular and choroid plexus development, critical for brain CSF homeostasis, which may point to unappreciated contributions of *MIR-9-2* disruption in human diseases such as 5q14.3 Microdeletion Syndrome.

## RESULTS

### Loss of *miR-9-2* results in embryonic ventriculomegaly and postnatal hydrocephalus risk

In mice, *miR-9-2hg* lies in a region syntenic to the 5q14.3 human locus where *miR-9-2* is positioned between *Mef2c* and *Tmem161b* (Fig. 1A & 2A). To assess the role of *miR-9-2* in brain development and disease, we generated germline *miR-9-2* knock-out (KO) mice using the *Mir9-2<sup>tm1Mtm</sup>* conditional KO line<sup>(17)</sup> (Fig. 2B). We observed that *miR-9-2* heterozygous KO (HET) mice develop domed heads (Fig. 2C), consistent with hydrocephalus by approximately 3 weeks of age (Fig. 2D) at a frequency of 27 and 32% in males and females, respectively (Fig. 2E). Although *miR-9-2* homozygous KO mice are born alive and at expected Mendelian ratios, 98% of *miR-9-2* KO pups die within 1-2 days of birth (Fig. 2F). Those that survive up to 3 weeks of age are distinguished by their small body size relative to wild-type (WT) littermates (Fig. 2C). To determine if hydrocephalus develops embryonically, we assessed *miR-9-2* WT, HET, and KO littermates histologically from E12.5, E16.5 and postnatal day (P)0 (Fig. 2G) coincident with the timing of peak *miR-9-2* expression<sup>(3)</sup>. Embryonic brain development appears normal among *miR-9-2* mutants (HETs and KOs) at E12.5. However, by E16.5, enlargement of the third ventricle is frequently observed in *miR-9-2* mutant embryos. By P0, enlargement of lateral and third ventricles is evident in 100% of *miR-9-2* heterozygous and homozygous mutants and is particularly prominent in *miR-9-2* KOs (Fig. 2G).

In mice, congenital hydrocephalus is most commonly caused by loss of ventricular ependymal cells<sup>(18,19)</sup>, blockade of the cerebral aqueduct between the 3<sup>rd</sup> and 4<sup>th</sup> ventricles<sup>(19)</sup>, or defective ependymal motile cilia that fail to efficiently circulate the CSF<sup>(20)</sup>. However, histological

examination of these structures revealed no discernible defects (S.Fig. 1A-C). While we cannot rule out ciliary dysfunction from histology, this is unlikely to initiate ventriculomegaly in *miR-9-2* mutant embryos as ependymal cilia do not form until the first postnatal week<sup>(21)</sup> whereas ventriculomegaly is evident by E16.5 (Fig. 2G). *Mir-9-2* mutants do, however, often present with cerebral hemorrhages, a known cause of hydrocephalus<sup>(22)</sup>, as early as E12.5 with higher prevalence among *miR-9-2* mutants (Fig. 2H).

### **Transcriptional analysis of *miR-9-2* mutants reveals dysregulation of neural gene networks**

To define the cellular and molecular consequences of *miR-9-2* loss in the developing brain, we performed single-nucleus RNA-sequencing (snRNA-seq) on *miR-9-2* WT and KO dorsal forebrains (Fig. 3A; see S.Fig. 2A,B for sample details) at stages prior to and coincident with the ventriculomegaly phenotype (E12.5 and E16.5, respectively). Profiled cells were annotated using canonical cell type markers (S.Fig. 2C,D) and revealed proportional capture of neural and non-neural cell classes characteristic of the sampled stages (Fig. 3B-D)<sup>(23,24)</sup> We further confirmed that, as in humans, *miR-9-2hg* is more highly and broadly expressed in the developing mouse forebrain relative to other 5q14.3 Microdeletion Syndrome associated genes: *Mef2c*, *Tmem161b* and *Rasa1* (Fig. 3E). The miR-9 family has been shown to regulate cell cycle progression and therefore influence cell class proportions<sup>(2,3)</sup>. However, analysis of cell class (Fig. 3F) and cell cycle proportions (Fig. 3G) revealed no significant differences between *miR-9-2* WT and KO cortices (S. Table 1), potentially due to partial redundancy with the other miR-9 family members. We therefore hypothesized that loss of *miR-9-2* drives cerebral hemorrhages and enlarged ventricles by disrupting cell-type-specific transcriptional programs. We expected vascular endothelial cells, which form the developing vasculature, and choroid plexus cells, which secrete and regulate CSF, to be most strongly affected. Indeed, both these cell classes showed significantly affected gene expression in KO brains (Fig. 3H). However, we also observed significant dysregulation of transcriptional programs across many cell classes, suggesting that loss of *miR-9-2* has widespread consequences on the developing brain (Fig. 3H).

At E12.5, before the peak of *miR-9-2* expression in the brain, the transcriptional consequences of *miR-9-2* loss are subtle. We saw de-repression of computationally predicted *miR-9-2* targets<sup>(25,26)</sup> in early born cell types, including Cajal-Retzius cells, cortical hem cells and newborn neurons (S. Fig. 3A-C). These included transcription factors like *Pbx3* in Cajal-Retzius Cells and newborn neurons, *Cntn1* and *Lmo3* in Cortical Hem cells, and *Zfhx4* and *Foxp2* in newborn neurons (S. Fig. 3A-C; S. Table 2). Only modest dysregulation of gene expression is observed among other cell classes at this timepoint (S. Fig. 3D-F; S. Table 2).

By E16.5, after the peak of *miR-9-2* expression, the consequences of *miR-9-2* loss were more dramatic and widespread (Fig. 3H). Among neural cell types, the largest number of de-repressed predicted *miR-9-2* targets occurred within intermediate progenitor cells, corticocortical projection neurons and interneurons. These included de-repression of cell adhesion-mediating genes *Tspan9* and *Ptprt* in intermediate progenitor cells and transcription factors *Scrt2* in corticocortical projection neurons and interneurons and *Bcl11a* and *Zbtb16* in interneurons, among many other genes (Fig. 4A,C,E; S. Table 2). Interestingly, Fast Gene Set Enrichment Analysis (FGSEA) of differentially expressed genes (DEGs) using Gene Ontology-Biological Process (GO-BP) terms revealed prevalent enrichment of cell adhesion pathways among many affected neural cell types (Fig. 4B,D,F). Transcriptional alterations to many additional neural cell types were more modest but reflect the widespread effects of *miR-9-2* post-transcriptional regulation of the developing brain (S. Figs. 4-5; S. Tables 2-3).

## Vascular endothelial cells show transcriptional de-repression of vascular integrity genes

*MiR-9-2* KO embryos frequently present with superficial cerebral hemorrhages at E12.5 (Fig. 2H) with subsequent ventriculomegaly by E16.5 (Fig. 2G), which could indicate defects in vascular development. Cerebral hemorrhaging is known to contribute to hydrocephalus, specifically through the secondary activation of choroid plexus CSF hypersecretion<sup>(22)</sup>. Thus, to gain a more comprehensive understanding on the incidence of hemorrhages in *miR-9-2* mutants, we assessed the frequency and distribution of hemorrhages in brain sections at E12.5 and E16.5 using a combination of vascular (IB4) and red blood cell (TER-119) immunofluorescence. While hemorrhages in E12.5 *miR-9-2* mutants appear to be intraventricular or subdural (Fig. 2H), we did not observe parenchymal hemorrhages at E12.5. However, E16.5 mutants display prevalent deep, parenchymal microhemorrhages (Fig. 5A,B); *miR-9-2* KOs show the highest incidence of microhemorrhages (Fig. 5C) which span brain regions and are highly variable in frequency and spatial distribution (Fig. 5D) suggesting widespread and stochastic vascular destabilization. We further assessed the active or passive state of observed hemorrhage events in E16.5 *miR-9-2* mutants by fluorescent vascular tracer analysis and indeed detected sites of compromised blood vessels and active hemorrhage (S.Fig. 6A).

Indeed, our snRNA-seq analysis of DEGs in vascular endothelial cells revealed a number of de-repressed predicted *miR-9-2* targets that play roles in vascular development, including *Tspan18*, *Chd7*, *Myo10*, and *Lpp* (Fig. 5E). We therefore assessed vascular network development in *miR-9-2* mutants. However, we did not detect significant changes in vascular network organization at either E12.5 or E16.5 (S.Fig. 6B-F). Nevertheless, disruptions to physiological development of blood vessels, including vessel tone and integrity, could contribute to hemorrhage events. Interestingly, as in neurons, disrupted cell adhesion features prominently in our FGSEA GO-BP results for endothelial cells (Fig. 5F).

Although we expected to see altered gene expression among vascular cell types due to the occurrence of hemorrhages in *miR-9-2* mutants, the number of de-repressed *miR-9-2* target genes that emerged as DEGs was surprising as *miR-9-2hg* appears to show modest to low levels of expression among vascular cell types (Fig. 3D,E). Thus, to determine whether the de-repression of *miR-9-2* target genes in vascular endothelial cells could arise cell autonomously, we performed multiplexed *in situ* hybridization to assess both *miR-9-2hg* and mature *miR-9*(3P-strand) expression in E16.5 cortex sections and found robust expression of *miR-9(3p)/miR-9-2hg* in capillary-caliber blood vessels (Fig. 5G). We further sub-clustered endothelial cells from our snRNA-seq dataset and found that *miR-9-2hg* expression was enriched in a distinct subset of cells (Fig. 5H) that appear to be of a capillary-venule identity (S. Fig. 7), confirming *miR-9-2* expression among endothelial cells of distinct vascular zones.

## Choroid plexus cells show massive de-regulation of developmental transcriptome

The choroid plexus is a specialized structure in the brain known to be the primary site of CSF secretion. Consistent with our observed hydrocephalus phenotype among *miR-9-2* KOs, the choroid plexus displays the highest number of derepressed *miR-9-2* targets and DEGs (Fig. 3H and Fig. 6A; S. Table 2). Upregulated choroid plexus genes included several calcium-binding/signaling genes such as *Tmtc2*, and predicted *miR-9* target genes *Calml4* and *Cacnb2* (Fig. 6A) among others (S. Table 2), which may indicate indirect evidence of increased CSF secretion. Interestingly, several centriole/cilia assembly genes, including *Ulk4*, *Cfap221*, *Pacrg*, *Bbs9* and predicted *miR-9* target gene *Cep135* emerged as significant DEGs (Fig. 6A) and could point to choroid plexus ciliary signaling and/or motility defects that regulate CSF production. *Cep* genes encode cilia basal body structural proteins important for cilia function, and previous work



has shown that deletion of *Cep164* in multi-ciliated cells produces hydrocephalus<sup>(27)</sup>. Indeed, our FGSEA GO-BP results for choroid plexus revealed positive enrichment for cilia development and extracellular transport (Fig. 6B).

As with the vascular endothelial cells, we sought to determine whether the de-repression of *miR-9-2* target genes in the choroid plexus could arise cell autonomously. We assessed *miR-9-2hg* and mature *miR-9(3P)* expression in E16.5 cortex sections via multiplexed *in situ* hybridization and found the presence of *miR-9-2hg/miR-9(3P)* puncta within the choroid plexus (Fig. 6C). We further sub-clustered choroid plexus cells from our snRNA-seq data to more closely assess *miR-9-2hg* expression and we indeed found a subset of choroid plexus cells that express *miR-9-2hg* (Fig. 6D). Additional histological verification of *miR-9-2* expression from brain sections of non-recombined *Mir9-2<sup>tm1Mtm</sup>* mice driving  $\beta$ -galactosidase expression from the *miR-9-2hg*<sup>(17)</sup> revealed *miR-9-2hg* reporter expression among epithelial cells of the choroid plexus (Fig. 6D), the primary secretory cell type of the choroid plexus.

## Discussion

The results of this study reveal critical roles for *miR-9-2* that have immediate relevance for understanding human neurodevelopmental disorders. *MIR-9-2* is frequently deleted from human patients with 5q14.3 Microdeletion Syndrome, leading to *MIR-9-2* haploinsufficiency<sup>(6–8)</sup>. Interestingly, both HET and KO *miR-9-2* mouse mutants frequently display phenotypes associated with 5q14.3 Microdeletion Syndrome, including ventriculomegaly, vascular defects and aberrant neural development<sup>(11)</sup>. The neurological effects of 5q14.3 microdeletions have been largely attributed to loss of protein-coding genes at the locus, including *MEF2C*<sup>(6,12–14)</sup> or in some cases *TMEM161B*<sup>(15)</sup>. Our transcriptional analysis of *miR-9-2* KO mice, however, revealed widespread deregulation of gene networks within developing neural cell types, which displayed a large number of de-repressed predicted *miR-9* target transcripts. These included genes with their own widespread regulatory functions, such as transcription factors, which likely promote further secondary defects. Interestingly, homozygous loss of *miR-9-2* did not alter neuronal cell class proportions or progenitor cycling dynamics, as was reported to occur from dual loss of *miR-9-2/3* in mice<sup>(3)</sup>. Moreover, *miR-9-1* and *miR-9-3* KO mice survive for several weeks postnatally<sup>(39,40)</sup>, while *miR-9-2* KO mice in our study predominantly die at birth, strengthening the ideas that *miR-9* genes have distinct, complementary and dosage-dependent roles in neural development<sup>(2,3,28)</sup>. Loss of post-transcriptional repression by *miR-9-2* in the developing mouse brain resulted in prevalent deregulation of neural gene networks, revealed by pathway analyses to largely alter cell polarity and adhesion. In particular, we see significantly altered gene expression in neural progenitor and differentiating neuron populations that may alter cortical structural integrity, supporting newly proposed roles for these cell classes in the pathobiology of hydrocephalus<sup>(29–32)</sup>. These findings represent non-redundant roles for *miR-9-2* across various differentiating and maturing cortical cell types driven by the unique spatiotemporal expression dynamics of *miR-9-2* compared to other *miR-9* paralogs.

The vascular defects commonly associated with 5q14.3 Microdeletion Syndrome have been attributed to *RASA1* deletion<sup>(10,12)</sup>. However, deletion of *miR-9-2* also resulted in defects to blood vessel networks. Our histological and transcriptional analyses of *miR-9-2* mutants revealed widespread microhemorrhages in the developing cortex coupled with significant de-repression of vascular development gene networks, including *miR-9-2* target transcripts *Tspan18* and *Lpp*. *Tspan18* has not only been shown to play roles in regulating angiogenesis<sup>(33)</sup>, but also plays an important role in regulating endothelial  $\text{Ca}^{2+}$  signaling and Von Willebrand factor release<sup>(34)</sup>, which may play roles in regulating blood vessel tone and platelet clotting. *Tspan18* over-expression may thus increase blood vessel stiffness as well as hyperactivate platelet clotting leading to blood

vessel rupture. *Lpp* plays crucial roles in cell adhesion and mechanosensation and can contribute to increased blood pressure in human populations by increasing vascular tone<sup>(35)</sup>, which may further increase the likelihood of hemorrhage events. While not a predicted *miR-9-2* target, DEG *Plekha7* from our data modulates mechanical force loading in endothelial cells to regulate blood flow<sup>(36)</sup> and has recently been implicated as a hydrocephalus risk gene<sup>(37)</sup>. Thus, our transcriptomic results revealed molecular changes that implicate *miR-9* as a regulator of vascular development, a finding supported by previous work<sup>(38)</sup>.

Ventriculomegaly is also frequently observed in 5q14.3 Microdeletion Syndrome<sup>(9,11)</sup>. However, the cause for this phenotype has not been determined in *miR-9* KO mice or 5q14.3 Microdeletion patients. A possible cause for the hydrocephalus observed in our *miR-9-2* mutant mice lies in the occurrence of cerebral hemorrhages. Hydrocephalus frequently results from hemorrhage due to increased CSF production resulting from an upregulation of Na<sup>+</sup>/K<sup>+</sup>/Cl<sup>-</sup> ion channels as a response to ChP immune activation<sup>(41–43)</sup>. However, we did not detect ChP immune-response signatures in our snRNA-seq cohorts, (i.e. *Interleukin*, *Interferon*, *Cxcr* or *Tlr* genes). Our snRNA-seq data did, however, reveal misexpression of important cilia structural components within the ChP, including *miR-9* target transcript *Cep135*. These findings suggest direct disruption to transcriptional programs that regulate physiological functionality of the ChP, including sensing and production of CSF<sup>(44)</sup>. All *miR-9-2* mutants display ventriculomegaly while only roughly 30% develop hydrocephalus. Thus, we propose that CSF overproduction in *miR-9-2* mutants may begin with altered ChP development that is then exacerbated in a subset of animals that take on an excessive cerebral hemorrhage burden at postnatal ages. Co-incident disruptions to neural progenitor and neuronal cell polarity and adherence in *miR-9-2* mutants may further increase susceptibility to hydrocephalus.

Our results reveal a novel role for *miR-9-2* in transcriptional regulation not only within neural and vascular cell types, but also within the ChP. This work extends beyond previous studies by showing that, among other *miR-9* family members, *miR-9-2* is a unique, non-redundant regulator of cell-type-specific gene expression and is essential for the proper development of the forebrain. Furthermore, the evident dramatic phenotypes associated with *miR-9-2* haploinsufficiency further highlight the importance of *miR-9-2* dosage during forebrain development and may help explain genetic risk for disorders like 5q14.3 Microdeletion Syndrome or autism associated with a cortical enhancer of *MIR-9-2* expression<sup>(4,5)</sup>. Thus, *MIR-9-2* warrants closer attention as a potential contributor to human neurodevelopmental disorders and possibly as a therapeutic target for these intractable conditions.

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## METHODS

### Animals

Mice were housed in a specific pathogen-free facility approved by the AAALAC. All work with animals was compliant with the Cherry Lab's Seattle Children's Research Institute-approved IACUC protocol (IACUC00050). *Mir-9-2* KO mice were generated by crossing the *Mir-9-2<sup>tm1Mtm</sup>* conditional KO mice (RRID:MMRRC\_036062-JAX)<sup>(17)</sup> first to Rosa-FLP (RRID:IMSR\_JAX:009086)<sup>(45)</sup> then to E2a-Cre (RRID:IMSR\_JAX:003724)<sup>(46)</sup> mouse lines to create *miR-9-2* heterozygous animals. Subsequently, *miR-9-2* HET animals were back-crossed to C57BL/6 (Jackson Labs) animals for at least 10 generations to homogenize the strain background. The uniformity of the C57BL/6J genetic background was validated using the MiniMUGA genotyping array (NeoGen). *Mir-9-2* HET x HET crosses were then performed to generate WT, HET and KO littermate cohorts for experiments and analyses. Timed pregnant breeding was utilized to generate embryonic samples with day of copulation plug noted as E0.5. Equal numbers of male and female mice were used for all analyses, with the exception of E16.5 WT female samples, of which one was discarded due to poor quality of the sequencing library, reducing E16.5 WT representation to one female and two males (S.Fig. 2A).

### Immunohistochemistry and Imaging

All tissue for immunohistochemistry was collected and fixed in cold 4% paraformaldehyde overnight at 4°C and then cryoprotected in 30% sucrose in sterile 1X phosphate buffered saline (1X PBS) at 4°C for 2-4 days. Tissue was then embedded and frozen in TissueTek O.C.T. (Sakura #4583) and sectioned on a Leica CM 3050 S cryostat (40µm sections) and mounted on TruBond 380 glass microscope slides. Prior to staining, slides were incubated in a 60°C oven for up to 3 hours to enhance tissue adhesion. Slides were washed 3x in 1X PBS before and after incubation with primary and secondary antibodies. Prior to primary antibody incubation, sections were subjected to sodium citrate (0.01M Na-Citrate, pH 6.0) antigen retrieval in a pressure cooker for 8-10 minutes on the "high" setting. All antibodies were prepared in 1X PBS containing 5% bovine serum albumin (BSA) and 0.2% Triton X-100. DAPI was used to counterstain nuclei. The following antibodies and stains were used as follows: Rabbit anti-ZO-1 (1:250; Abcam Cat# ab221547, RRID:AB\_2892660); Mouse anti-Acetylated Tubulin (1:250; Sigma-Aldrich Cat# T6793, RRID:AB\_477585); Goat anti-Sox9 (1:250; R and D Systems Cat# AF3075, RRID:AB\_2194160); Rat anti-TER-119 (1:200; Thermo Fisher Cat# 14-5921-82, RRID:AB\_467727); Rabbit anti-B-galactosidase (1:200; Sigma-Aldrich Cat# AB1211, RRID:AB\_177320); Donkey anti-Rabbit Alexa Fluor 555 (1:500; Thermo Fisher Cat# A-31572, RRID:AB\_162543); Donkey anti-Mouse Alexa Fluor 555 (1:500; Thermo Fisher Cat# A-31570, RRID:AB\_2536180); Donkey anti-Goat Alexa Fluor 488 (1:500; Thermo Fisher Cat# A-11055, RRID:AB\_2534102); Donkey anti-Rat Alexa Fluor 488 (1:500; Thermo Fisher Cat# A-21208, RRID:AB\_141709); Isolectin GS-IB<sub>4</sub>-Alexa Fluor 647 conjugated (1:50; Thermo Fisher Cat# I32450).

All microscopic imaging was performed using either an Olympus APX100 fluorescence microscope or Zeiss LSM 900 or 980 confocal microscopes.

### Image Analysis

Cerebral hemorrhage counts were performed on E12.5 and E16.5 brain sections: n = 3 mice per genotype, n = 3 slides per animal containing 6-8 coronal sections each representing the full rostral-to-caudal axis. Absolute numbers of hemorrhages were counted in whole section slide scanner images using ImageJ/FIJI.

Vascular network analysis was performed using AngioTool<sup>(47)</sup> on isolectin-B4-stained E12.5 and E16.5 brain sections. Images were first cropped to cerebral cortex regions of interest (i.e. medial and lateral cortex) using ImageJ/FIJI then imported into AngioTool. IB4 labeled blood vessel images were then scaled, skeletonized, and measured for vessel length, vascular density, and junction density.

### **10X Genomics Single Nucleus Transcriptome Library Generation**

Cerebral cortices were harvested from E12.5 and E16.5 embryos (n = 2 WT or KO/sex; total n = 4/genotype) in ice cold sterile filtered 1X PBS and immediately flash frozen in liquid nitrogen in nuclease-free microcentrifuge tubes then stored in vapor phase liquid nitrogen until processing for snRNA-seq.

For nuclei isolation, samples were thawed on ice and processed via a modified 10X Genomics Nuclei Isolation from Mouse Brain Tissue protocol (CG000212 Rev. B). 500 µl of cold 0.1X Lysis Buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl<sub>2</sub>, 0.01% Tween-20, 0.01% IGEPAL CA-360, 1% BSA, 1 U/µl RNase Inhibitor (Roche #03335402001)) was added to each sample-containing tube and triturated 15-20 times. The suspensions were further homogenized using dounce homogenizers by applying 10 dounces each with an A then B pestle. Following transfer of each sample to a new tube, the samples were incubated on ice for 5 minutes. Sample suspensions were pipette-mixed 10 times, then incubated on ice for 10 minutes. 500 µl of cold Wash Buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl<sub>2</sub>, 1% BSA, 0.1% Tween-20, 1 U/µl RNase Inhibitor) was then added, and samples were pipette-mixed 5 times and then passed through a 40 µm Flowmi Cell Strainer into a fresh tube. Dissociated sample nuclei were then washed two times by centrifugation pelleting at 500 rcf in a 4°C swinging-bucket centrifuge and resuspension in 750 µl cold Wash Buffer. Sample nuclei concentrations were determined using propidium iodide and a Countess II FL Automated Cell Counter. Nuclei were then centrifuged at 500 rcf for 5 minutes at 4°C. The supernatant was discarded and the nuclei were resuspended in cold Nuclei Buffer (1X 10X Genomics Nuclei Buffer) to achieve a nuclei concentration range of 700-1200 nuclei/ µl, which is recommended for targeting 10,000 cells in the 10x Genomics snRNA-seq protocol. The final nuclei resuspension concentration was confirmed using the Countess II, followed by completion of the 10X Genomics Chromium Next GEM Single Cell 3' v3.1 snRNA-seq protocol.

### **Sequencing**

All sample transcriptome libraries were sequenced by the Northwest Genomics Center at the University of Washington. Samples were sequenced per 10X Genomics' recommended cycles and sequencing depth using either a NovaSeq SP or NovaSeq S1 100-cycle flow cell, according to the number of samples submitted for sequencing (SP for 4 or S1 for 5-9 samples). Data were formatted and returned for analysis as FASTQ files.

### **snRNA-seq and Statistical Analyses**

All data processing and statistical analysis was performed using R<sup>(48)</sup>. Sequencing reads were aligned to GRCm39/mm39 and UMI counts per gene and cell were quantified using Cellranger

7.2.0 (10X Genomics). Cellbender 0.3.0<sup>(49)</sup> was used to remove background UMI counts and DoubletFinder 2.0.6<sup>(50)</sup> was used to remove doublets. Seurat v5<sup>(51)</sup> was then used for quality control, filtering, normalization, ordination, integration, and cell cycle scoring. Cells were retained for analysis if they had between 1000 and 15,000 UMIs, at least 200 genes detected, and less than 5% of UMIs were mitochondrial, resulting in 84,128 cells. Genes were retained for analysis if they were detected in at least 20 cells. Seurat's 'NormalizeData' function was used for data normalization, and the 2000 most variable genes were used for principal components analysis. Based on preliminary clustering, integration was run across four batches of libraries, each containing equal representation of sex and genotype (1 WT: 1 KO each of male and female samples) per stage, which generated a set of reduced dimensions used for cell clustering and Uniform Manifold Approximation and Projection (UMAP). Samples were distributed by sex, stage, and timepoint across five batches, and integration was run across batches of libraries using the Reciprocal PCA method from the Seurat package, after which cells were re-clustered. Cell clusters were labeled and manually grouped using marker gene expression. Nebula<sup>(52)</sup> was then used for differential gene expression analysis within cell type and stage for genes that had a non-zero count in at least 10% of cells, comparing *miR-9-2* WT to KO cells using sample as a random effect with a Benjamini-Hochberg corrected p-value threshold of 0.05. Volcano plots were generated using the Enhanced Volcano R package<sup>(53)</sup> (version 1.4.0) to show differentially expressed genes per cell class and timepoint. The list of input genes and their associated log fold changes and false discovery rates (FDR) was generated from Nebula<sup>(52)</sup>. The significance threshold was set at  $-\log_{10} \text{FDR} \leq 0.05$ , and the significant  $\log_2$  fold change was set at  $\pm 0.5$ . Predicted *miR-9-2* target genes were identified using TargetScan 8.0<sup>(25,26)</sup>. The FGSEA R package<sup>(54)</sup> was used to test for enrichment of gene sets within the Gene Ontology (GO) biological processes gene sets. To rank genes for FGSEA within cell type and stage, the log-fold-change was multiplied by the negative log base 10 of the P-value<sup>(55)</sup>. Differential cell type abundance at each stage, between wild type and knockout cells, was determined by performing a quasi-likelihood F-test and fitting a negative binomial generalized linear model to the cell count data using EdgeR 4.4.2<sup>(56)</sup> and using a Benjamini-Hochberg corrected p-value threshold of 0.05. Cluster subsetting for vascular endothelial and choroid plexus cells was done by isolating the cluster of interest and using Seurat for ordination, integration, PCA, clustering, and UMAP, as described above. Raw sequencing files and metafiles are available through GEO (GSE304879).

### **Embryonic Cerebral Hemorrhage Detection via Fluorescent Dextran Perfusion**

Control and mutant embryos (E16.5) from timed-pregnant dams were perfused with fluorescent tracer dextran as previously described<sup>(57)</sup>. Briefly, E16.5 embryos were surgically exposed from their anesthetized mother's uterus, leaving the umbilical cord intact to continually supply blood to the embryos during the perfusion. Embryos were then injected with 5 $\mu$ l of lysine-fixable Texas Red dextran (70kD) (Thermo Fisher Cat# D1864) with a 300 $\mu$ l insulin syringe via their highly fenestrated livers and allowed to circulate through each embryo for 3 min. Embryo umbilical cords were then severed and embryo heads were drop fixed in cold 4% paraformaldehyde overnight at 4°C then processed for histological analysis as described above.

### **Quantifications, Statistics and Plots of Imaging Data**

Statistical analyses for image quantifications were performed using GraphPad Prism 10.1.1. Comparisons of absolute values between groups of three were performed using one-way ANOVA with Tukey's multiple comparisons test. Comparisons of trends between groups of three were performed using a two-way ANOVA. Statistical significance was set at adjusted  $p \leq 0.05$ .

### **FIGURE LEGENDS**

**Fig. 1. The *MIR-9-2* locus is frequently lost in 5q14.3 Microdeletion Syndrome and is highly expressed compared to nearby protein-coding genes.** **A.** UCSC Genome Browser track showing the human 5q14.3 locus containing *MIR-9-2*, its host gene *MIR9-2HG*, and representative human 5q14.3 microdeletions<sup>(6,8)</sup>. **B-C.** Fetal human brain scRNA-seq (B) UMAPs (top), feature plots (bottom) and (C) gene expression heatmap generated from data published in Wang *et al.*<sup>(16)</sup> highlighting 1<sup>st</sup>-3<sup>rd</sup> trimester cerebral cortex cell classes showing high expression of *MIR9-2HG* relative to other protein-coding 5q14.3 Microdeletion Syndrome associated genes *MEF2C*, *TMEM161B* and *RASA1* during early to mid-neural development.

**Fig. 2. Loss of *miR-9-2* causes ventriculomegaly/hydrocephalus and cerebral hemorrhages in the developing mouse brain.** **A.** UCSC Genome Browser track showing the homologous mouse 5q14.3 locus (13qC3). **B.** Schematic showing the breeding strategy for germline deletion of *miR-9-2*. **C.** Photographs of 3-week-old *miR-9-2* WT and HET mice showing doming of cranium in HETs consistent with hydrocephalus. *miR-9-2* homozygous KOs display a reduction in overall body size compared to WT littermates. **D.** DAPI-stained coronal brain sections of 3-week-old *miR-9-2* HETs, KOs and WT corroborate a range of hydrocephalic phenotypes. Scale bar = 1mm. **E.** Survival rates among *miR-9-2* HET male (73%) and female (68%) animals that do not develop severe hydrocephalus. Data comprises 511 animals from 166 litters. **F.** *miR-9-2* WT, HET and KO birth and survival rates. **G.** DAPI-stained coronal brain sections at E12.5, E16.5 and P0 show progression of ventriculomegaly phenotype, beginning with enlargement of third ventricle (red arrow heads) at E16.5 followed by enlargement of lateral ventricles (red arrows) by P0. Scale bars at: E12.5 = 250µm, E16.5 and P0 = 500µm. **H.** Photographs of E12.5 *miR-9-2* mutant embryos showing intraventricular (HET) and subdural (KO) hemorrhages. The incidence of hemorrhage is higher among *miR-9-2* HETs and KOs compared to WT, though not statistically significant (N=8 animals/genotype; One-way ANOVA: Kruskal-Wallis = 2.925, P = 0.2317).

**Fig. 3. Single-nucleus RNA-seq of *miR-9-2* KO and littermate control cortices reveals transcriptional de-repression among neural and non-neural cell classes.** **A.** Schematic showing snRNA-seq workflow. **B-D.** UMAP representations of harvested cortical cell types collected at (B) E12.5 and (C) E16.5 timepoints. **D.** Integrated *miR-9-2* WT and KO samples at both timepoints. **E.** Gene expression feature plots showing relative expression of *miR-9-2hg*, *Mef2c*, *Rasa1* and *Tmem161b* among all E12.5 and E16.5 WT cell populations. **F.** Stacked bar plots showing captured cell class proportions between E12.5 and E16.5 *miR-9-2* WT and KO samples. **G.** Stacked bar plots showing distribution of cycling cells by mitotic phase for E12.5 and E16.5 *miR-9-2* WT and KO RGCs and IPCs. **H.** Heat maps showing numbers of total differentially expressed genes ( $-0.5 \leq \log_2FC \leq 0.5$ ) and numbers of derepressed predicted *miR-9-2* target genes ( $\log_2FC \geq 0.5$ ) per cell class per timepoint between *miR-9-2* WT and KO littermates at FDR  $\leq 0.05$ . Black boxes indicate *no data*. RGCs = radial glial cells, IPCs = intermediate progenitor cells, CFPNs = corticofugal projection neurons, CPNs = cortical projection neurons, GPCs = glial precursor cells, CH = cortical hem, ChP = choroid plexus.

**Fig. 4. E16.5 *miR-9-2* KO developing cortical cells show de-repression of genes that promote neural differentiation.** **A,C,E.** Volcano plots showing differentially expressed genes at FDR  $\leq 0.05$  and  $-0.5 \leq \log_2FC \leq 0.5$  for E16.5 (A) IPCs, (C) CPNs and (E) interneurons. **B,D,F.** FGSEA results for enriched GO-BPs for E16.5 (B) IPCs, (D) CPNs and (F) interneurons showing top and bottom 14 most differentially enriched BPs. BPs with asterisks indicate pathways significant at FDR  $\leq 0.05$  and  $p \leq 0.05$ . All other pathways are significant at  $p \leq 0.05$ . FGSEA = fast gene set enrichment analysis, GO-BP = gene ontology biological processes, (Red = GO-BP



terms with plausible phenotypic relevance). IPCs = intermediate progenitor cells, CPNs = cortical projection neurons.

**Fig. 5. E16.5 *miR-9-2* KO brains show widespread hemorrhages and disruptions to gene networks in vascular endothelial cells that regulate angiogenesis and cell adhesion.** **A.** Immunohistology showing clusters of extravasated TER-119<sup>+</sup> red blood cells, indicative of microhemorrhages within the E16.5 KO brain parenchyma. **B.** High-magnification confocal images showing extravasated TER-119<sup>+</sup> red blood cells surrounding IB4-stained blood vessels in E16.5 *miR-9-2* mutants. Scale bar = 50µm. **C.** Total hemorrhage counts among E16.5 *miR-9-2* mutants and controls spanning rostral to caudal coronal brain sections. One-way ANOVA with Tukey's multiple comparisons test revealed significant differences among all groups: P = \*0.0337, \*\*0.0023, \*\*\*0.0002. **D.** Numbers of hemorrhages by rostral to caudal (R-C) position among E16.5 *miR-9-2* mutants and controls. Two-way ANOVA revealed no significant difference: P = 0.4179. (**C,D**: n = 3 animals per genotype, n = 3 slides per animal containing 6-8 coronal sections each). **E.** Volcano plots showing differentially expressed genes at FDR ≤ 0.05 and -0.5 ≤ log<sub>2</sub>FC ≥ 0.5 for E16.5 vascular endothelial cells. **F.** FGSEA results for enriched GO-BPs for E16.5 vascular endothelial cells showing top and bottom 14 most differentially enriched BPs. BPs with asterisks indicate pathways significant at FDR ≤ 0.05 and p ≤ 0.05. All other pathways are significant at p ≤ 0.05. (Red = GO-BP terms with plausible phenotypic relevance) **G.** *In situ* hybridization confocal images showing expression of both mature *miR-9(3P)* and *miR-9-2hg* transcripts in E16.5 IB4<sup>+</sup> capillary-caliber cortical blood vessel. The blood vessel was skeletonized and isolated to clarify *miR-9* puncta expression within IB4<sup>+</sup> signal. Scale bars = 10µm. **H.** UMAP of subclustered vascular endothelial cells from snRNA-seq dataset showing *miR-9-2hg* expression. Endothelial cell clusters are shown by stage, subcluster and cell type-specific marker expression of *Vegfr2*.

**Fig. 6. The choroid plexus reveals highest number of de-repressed *miR-9-2* target genes in KO forebrains.** **A.** Volcano plot showing differentially expressed genes at FDR ≤ 0.05 and -0.5 ≤ log<sub>2</sub>FC ≥ 0.5 for E16.5 choroid plexus. **B.** FGSEA results for enriched GO-BPs for E16.5 choroid plexus showing top and bottom 14 most differentially enriched BPs. BPs with asterisks indicate pathways significant at FDR ≤ 0.05 and p ≤ 0.05. All other pathways are significant at p ≤ 0.05. (Red = GO-BP terms with plausible phenotypic relevance). **C.** *In situ* hybridization confocal images showing expression of both mature *miR-9(3P)* and *miR-9-2hg* transcripts in E16.5 choroid plexus. Scale bars = 100 µm 1<sup>st</sup> panel, 25µm 2<sup>nd</sup> panel. **D.** UMAP of subclustered choroid plexus cells from snRNA-seq dataset showing *miR-9-2hg* expression. Cell clusters are shown by stage, subcluster and cell type-specific marker expression of *Ttr*. Immunofluorescence images show choroid plexus β-galactosidase expression in *miR-9-2hg* reporter mice at P0. FGSEA = fast gene set enrichment analysis, GO-BP = gene ontology biological processes.

**Supplemental Fig. 1. Cerebral structures commonly associated with embryonic hydrocephalus appear normal in *miR-9-2* HETs and KOs.** **A.** DAPI-stained P0 *miR-9-2* WT, HET and KO mouse brains showing no evidence of stenosis along the cerebral aqueduct. Scale bar = 100µm. **B.** Immunohistology of E16.5 *miR-9-2* WT, HET and KO mouse brains showing intact ZO-1<sup>+</sup> ependyma along lateral, 3<sup>rd</sup> and 4<sup>th</sup> ventricles. Scale bar = 100µm. **C.** Immunohistology of P7 *miR-9-2* WT and HET mouse brains showing normal-appearing Acetylated Tubulin<sup>+</sup> motile cilia on Sox9<sup>+</sup> ependymal cells. Scale bar = 20µm.

**Supplemental Fig. 2. Quality control metrics and cell cluster identification for *miR-9-2* WT and KO snRNA-seq samples.** **A.** Cell count summaries for E12.5 and E16.5 *miR-9-2* WT and KO snRNA-seq samples. **B.** Quality control metrics for all snRNA-seq samples, including numbers of genes per cell, numbers of transcripts per cell and percent mitochondrial DNA per cell. **C.** Gene

expression dot plot showing cell class markers that define captured cortical cell classes for E12.5 and E16.5 *miR-9-2* WT and KO samples. **D.** Gene expression feature plots showing spatial distribution of key cell class marker genes on combined E12.5 and E16.5 UMAPs. RGCs = radial glial cells, IPCs = intermediate progenitor cells, CFPNs = corticofugal projection neurons, CPNs = cortical projection neurons, GPCs = glial precursor cells.

**Supplemental Fig. 3. Volcano plots showing DEGs for E12.5 neural cell types. A-F.** Volcano plots showing differentially expressed genes at  $FDR \leq 0.05$  and  $-0.5 \leq \log_2FC \leq 0.5$  for E12.5 (A) Cajal-Retzius cells, (B) cortical hem, (C) newborn neurons, (D) RGCs, (E) IPCs, and (F) interneurons. RGCs = radial glial cells, IPCs = intermediate progenitor cells.

**Supplemental Fig. 4. Volcano plots showing DEGs for E16.5 neural cell types. A-F.** Volcano plots showing differentially expressed genes at  $FDR \leq 0.05$  and  $-0.5 \leq \log_2FC \leq 0.5$  for E16.5 (A) RGCs, (B) CFPNs/stellate neurons, (C) Cajal-Retzius cells, (D) cortical hem cells, (E) fibroblasts, and (F) immune cells. RGCs = radial glial cells, CFPNs = corticofugal projection neurons.

**Supplemental Fig. 5. Pathway analysis of genes differentially expressed in *miR-9-2* KO versus control neural cell types at E16.5. A-C.** FGSEA results for enriched GO-BPs for E16.5 (A) RGCs, (B) CFPNs/stellate neurons and (C) Cajal-Retzius cells, showing top and bottom 14 most differentially enriched BPs. BPs with asterisks indicate pathways significant at  $FDR \leq 0.05$  and  $p \leq 0.05$ . All other pathways are significant at  $p \leq 0.05$ . FGSEA = fast gene set enrichment analysis, GO-BP = gene ontology biological processes. (Red = GO-BP terms with plausible phenotypic relevance). IPCs = intermediate progenitor cells, CPNs = cortical projection neurons.

**Supplemental Fig. 6. Comparison of vascular integrity and patterning in *miR-9-2* mutant and control embryo brains. A.** Confocal images showing leakage of 70kD fluorescent dextran coincident with extravasated TER-119<sup>+</sup> red blood cells around IB4-stained blood vessels in E16.5 *miR-9-2* HETs. Scale bar = 50 $\mu$ m. **B-C.** Confocal images showing IB4-stained blood vessels in DAPI-counterstained cerebral cortices at (B) E12.5 and (C) E16.5 for WT, HET and KO *miR-9-2* embryos. Scale bar (B) = 100  $\mu$ m, (C) = 200 $\mu$ m. **D-F.** AngioTool Metrics showing quantifications for (D) average vessel length, (E) vascular density and (F) junctional density for IB4-stained blood vessel networks in E12.5 and E16.5 *miR-9-2* mutant cortices. Quantifications comprised n = 4-5 animals and n = 2-4 images per animal spanning rostral-caudal axis of cerebral cortex. One-way ANOVA with Tukey's multiple corrections tests revealed no significant differences between cohorts.

**Supplemental Fig. 7. Identification of vascular endothelial cell subtype expressing *miR-9-2*. A.** Gene expression dot plot from Fig. 5H subclustered endothelial cells showing expression of known vascular zonation (Capillary-Arteriole, Capillary, or Capillary-Venule) marker genes among brain endothelial cells. Marker genes curated from Chen *et al.*<sup>(58)</sup>. Endothelial subcluster 0 is enriched for *miR-9-2* expression.

**Table S1. snRNA-seq differential abundance analyses of cell cycle and cell class proportions for *miR-9-2* WT and KO cortical samples. A,B.** Differential abundance statistical analyses results for (A) RGC and IPC cell cycle phase abundance and (B) cell class abundance by sample. RGCs = radial glial cells, IPCs = intermediate progenitor cells.

**Table S2. Differential gene expression analyses from snRNA-seq by cell class for E12.5 and E16.5 *miR-9-2* WT and KO cortical samples.**

**Table S3. FGSEA GO-BP analyses from snRNA-seq by cell class for E12.5 and E16.5 *miR-9-2* WT and KO cortical samples.**

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Figure 1

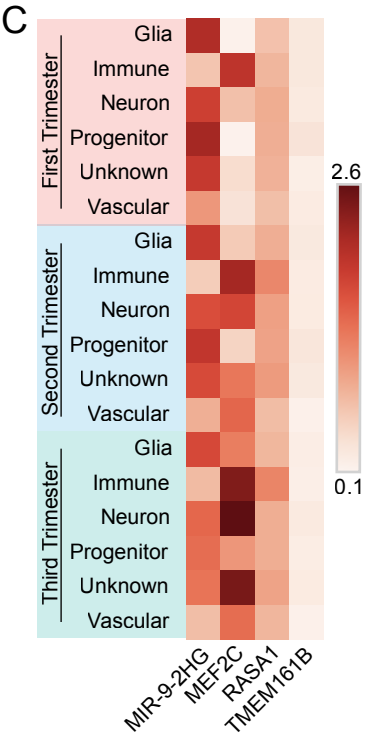
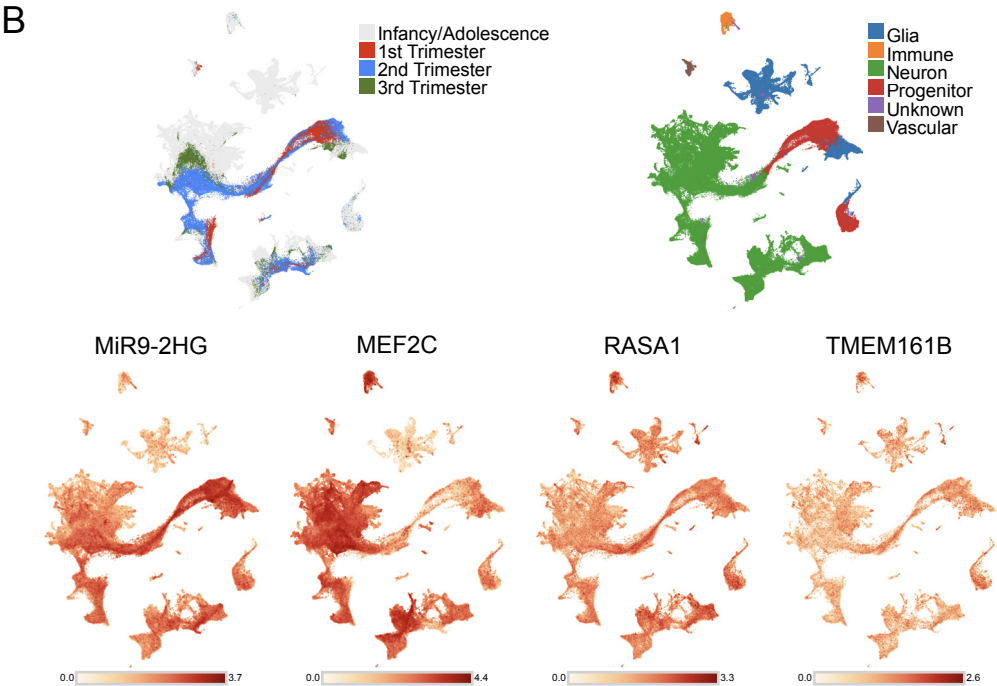
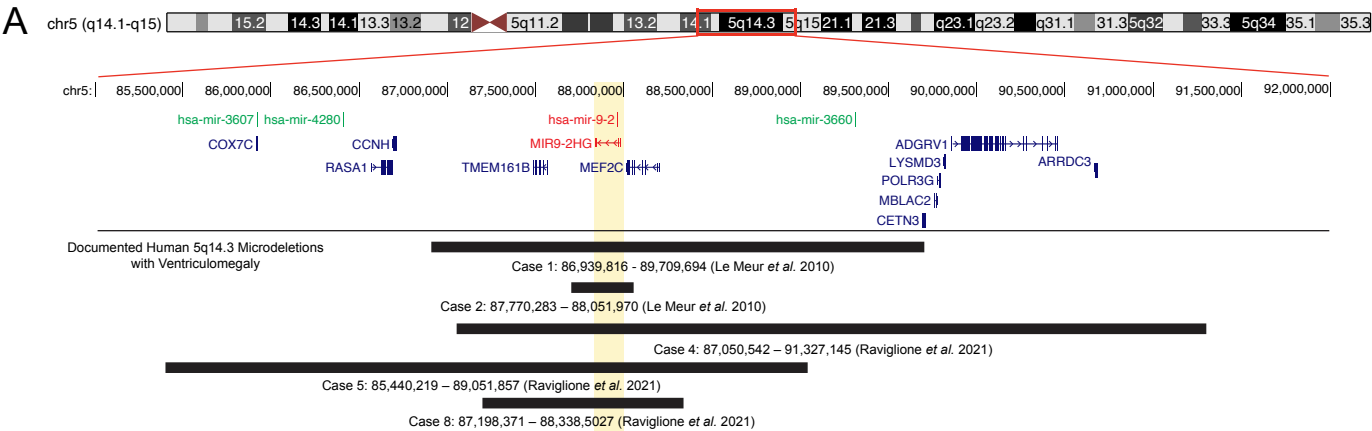




Figure 2

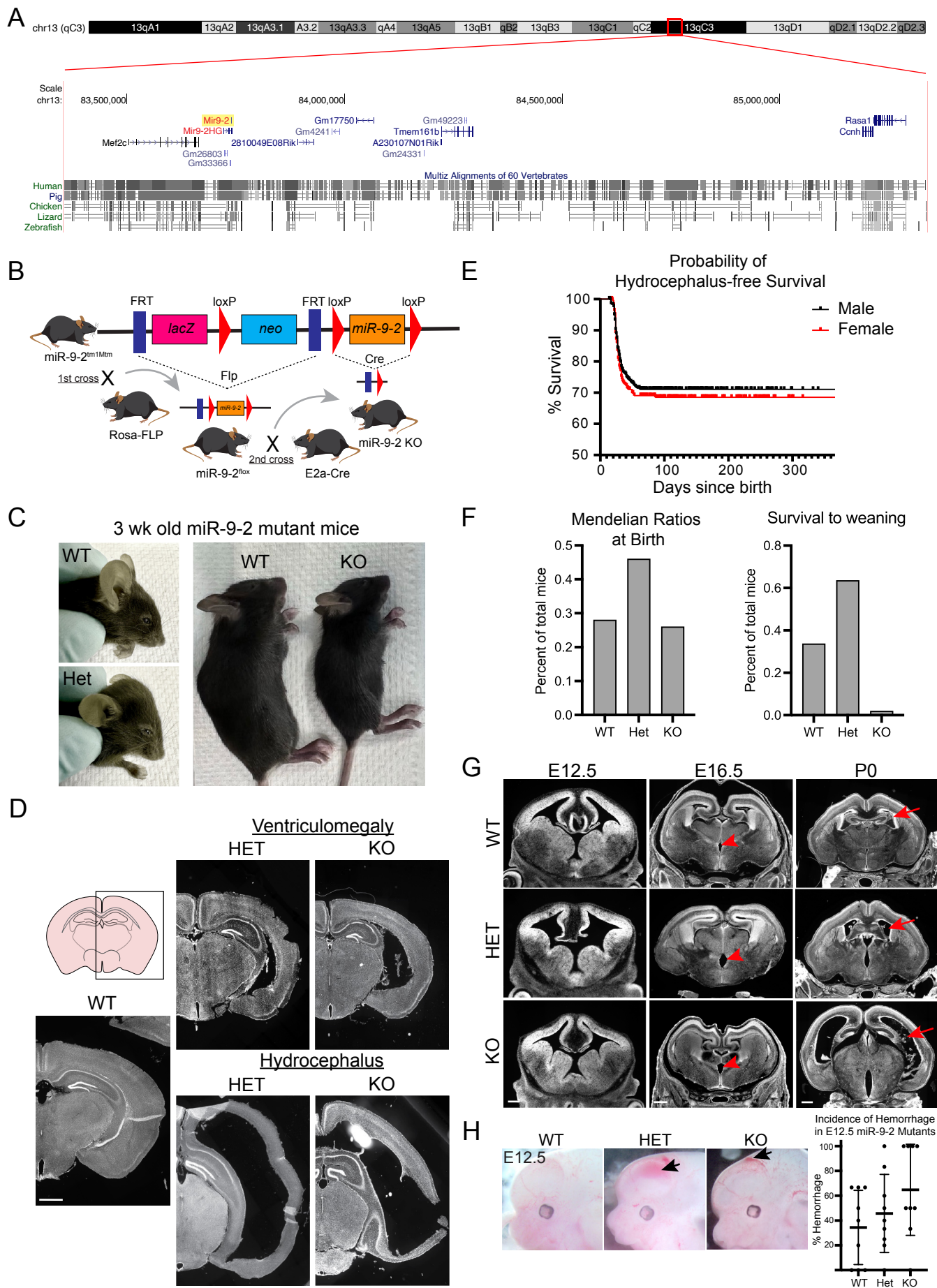


Figure 3

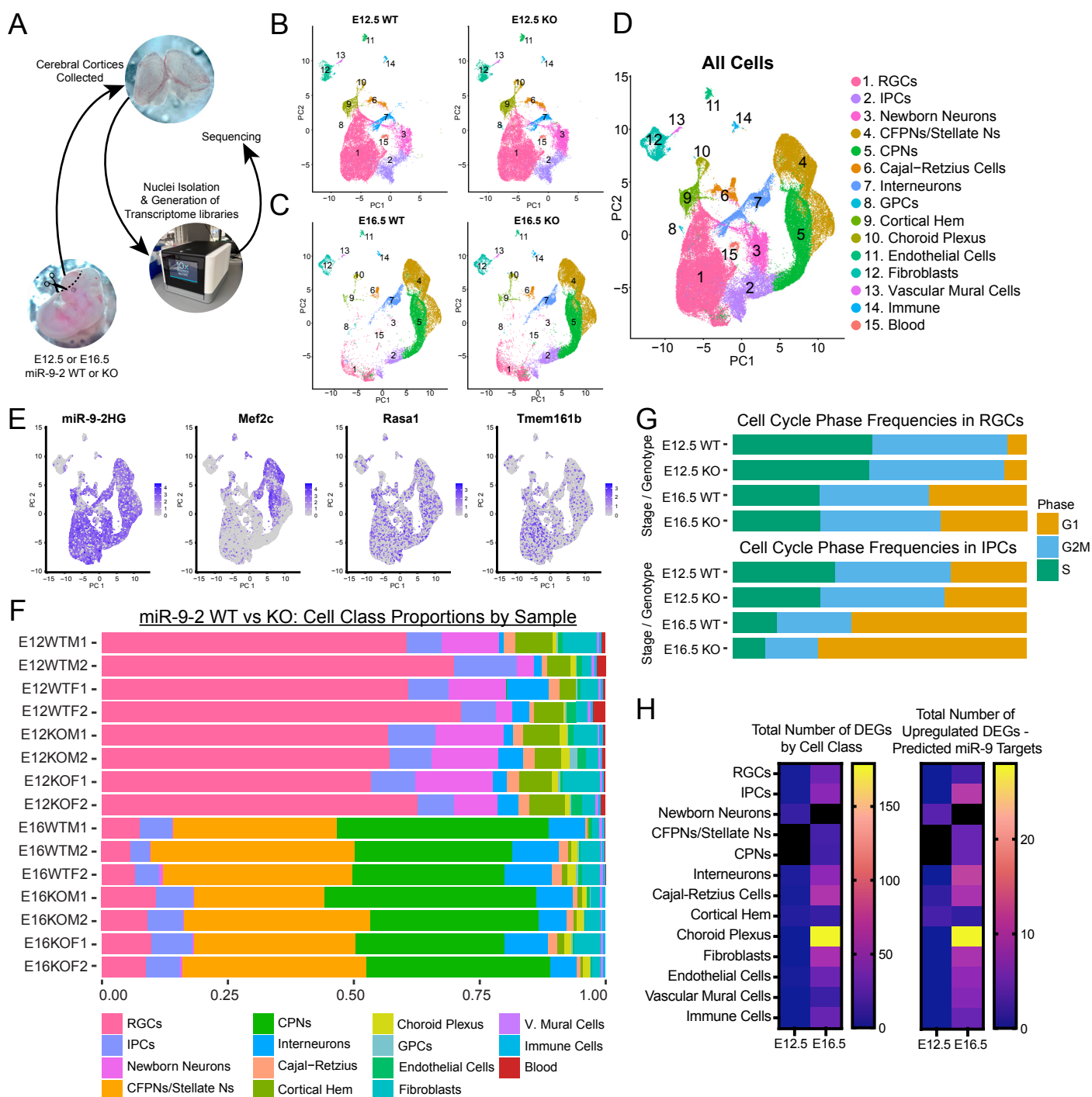




Figure 4

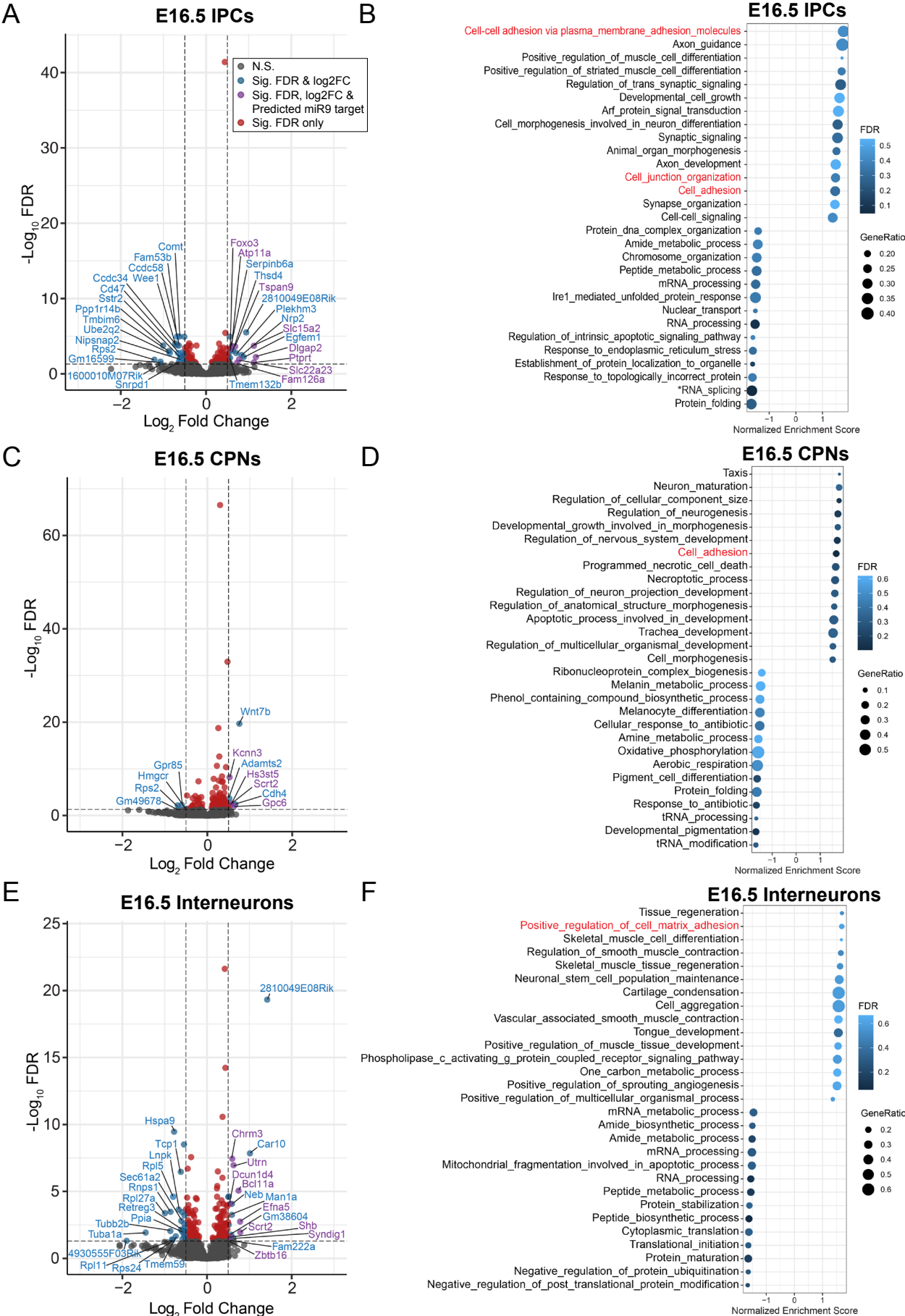


Figure 5

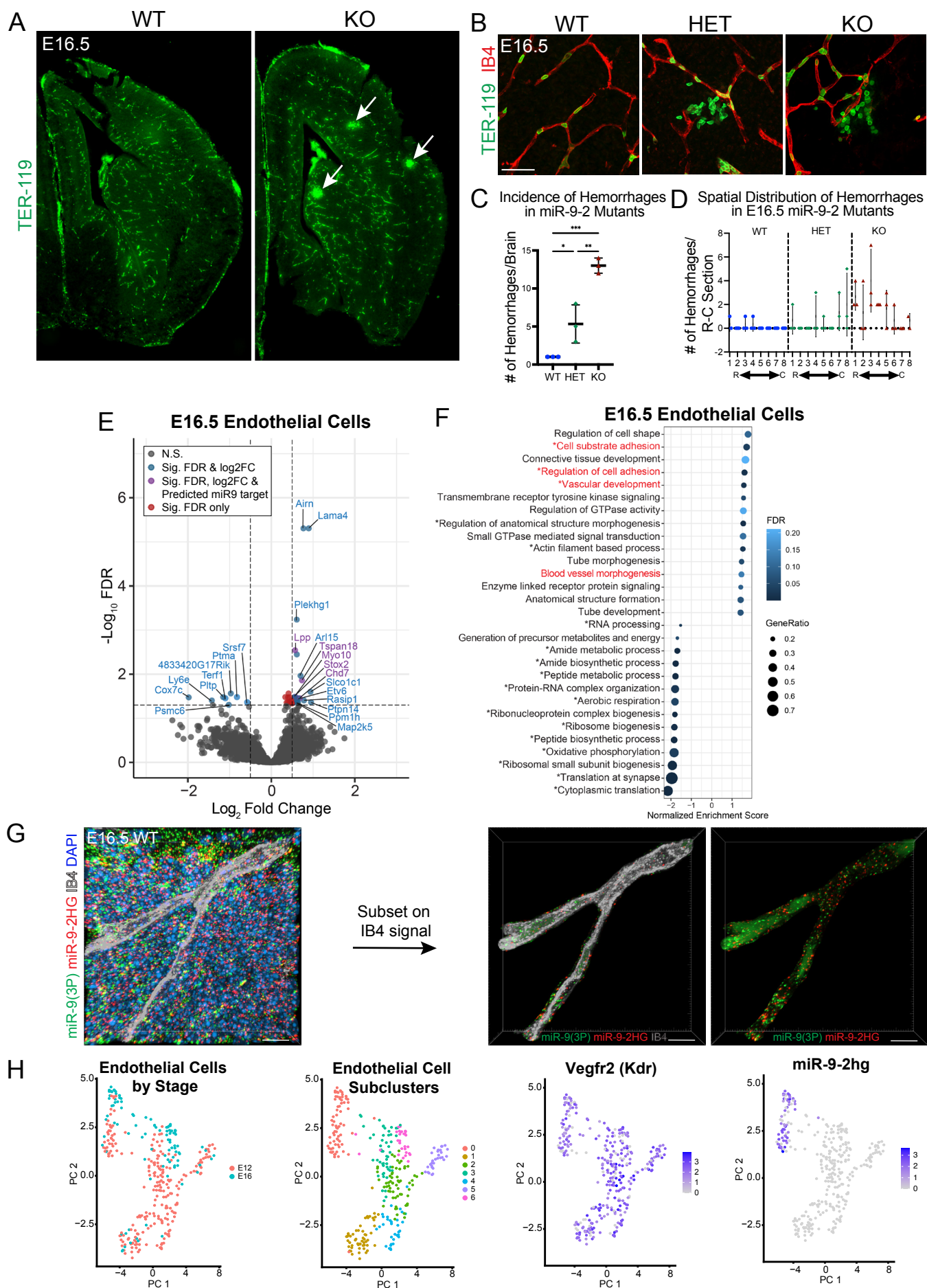
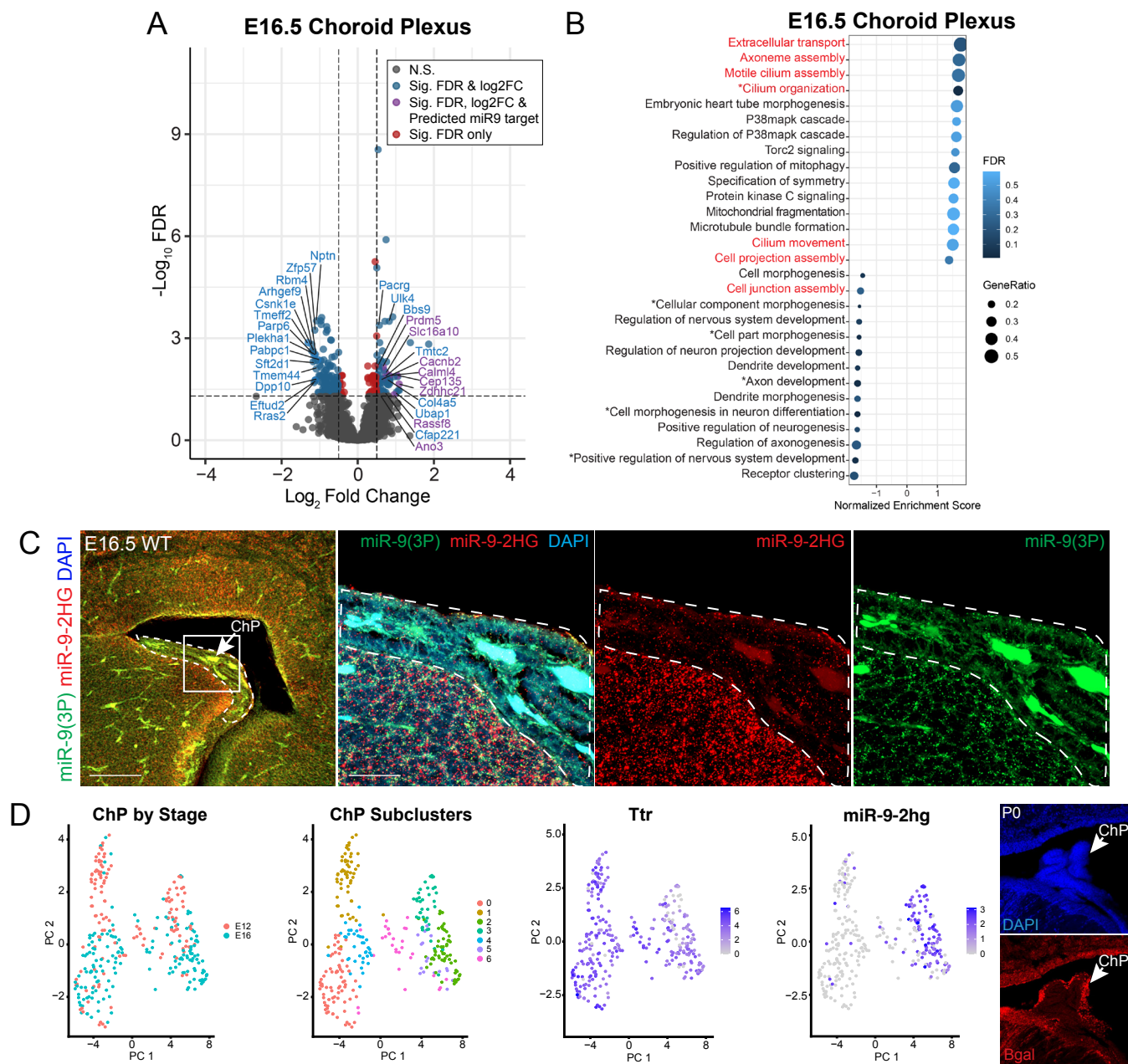
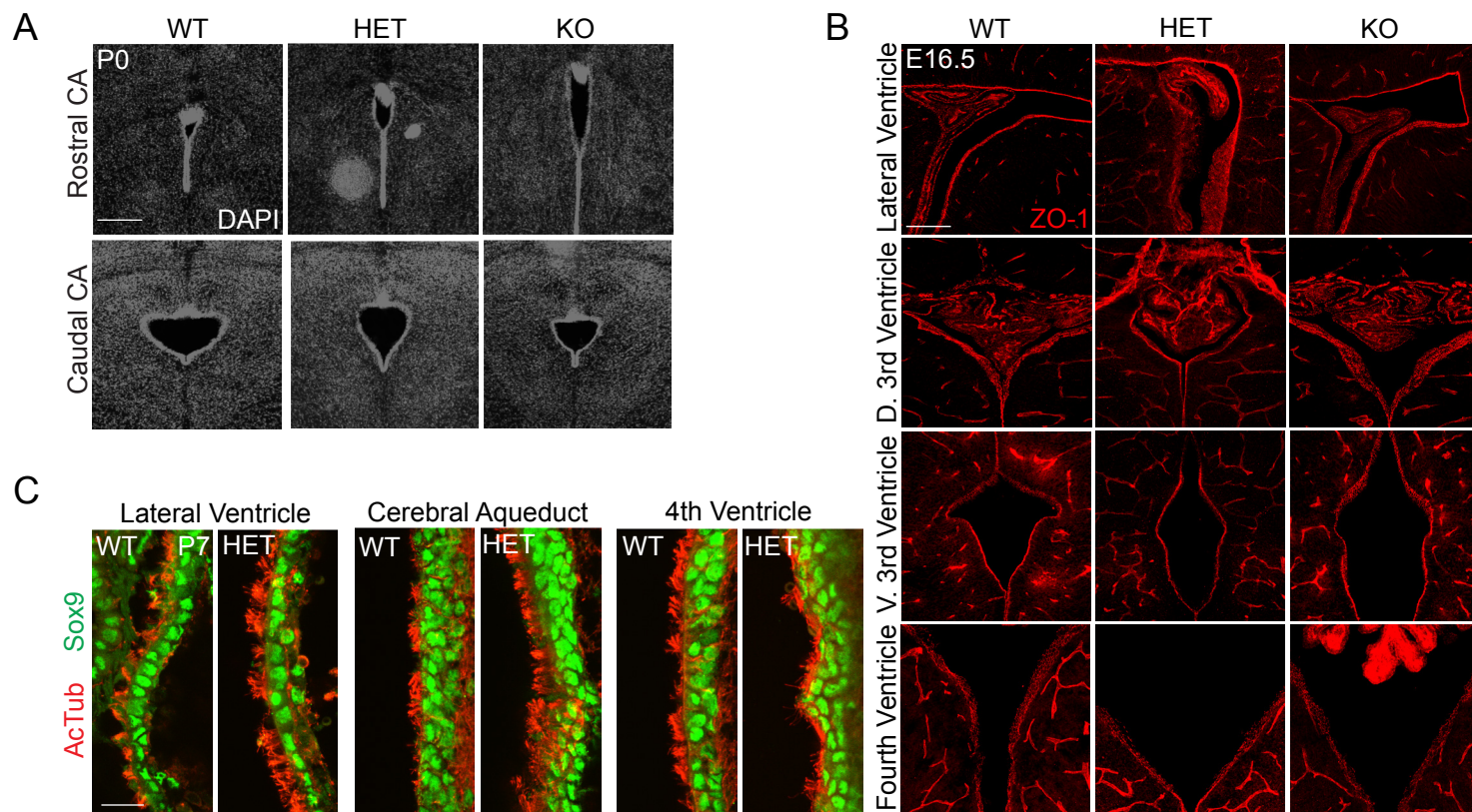


Figure 6

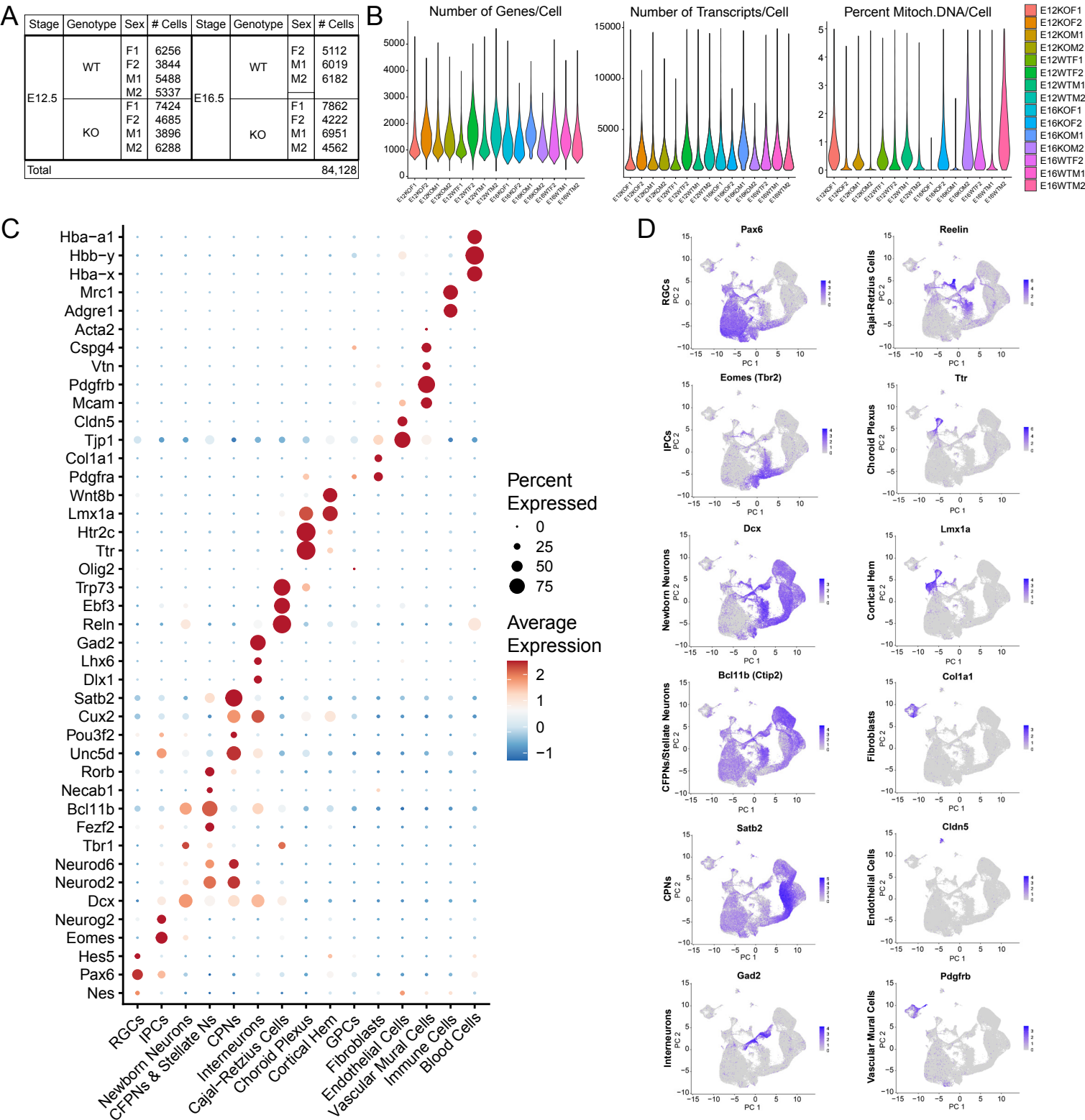




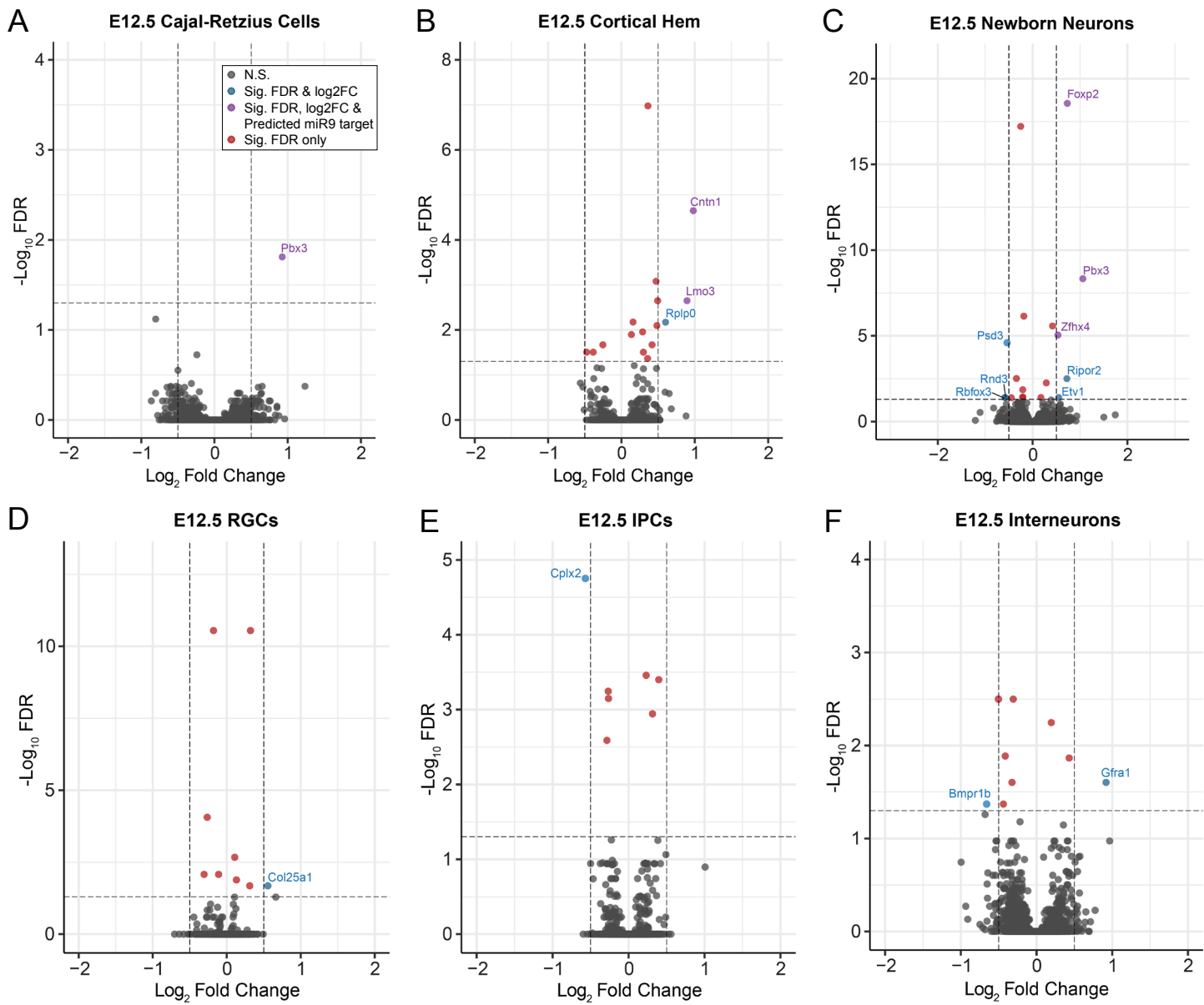
Supplemental Figure 1



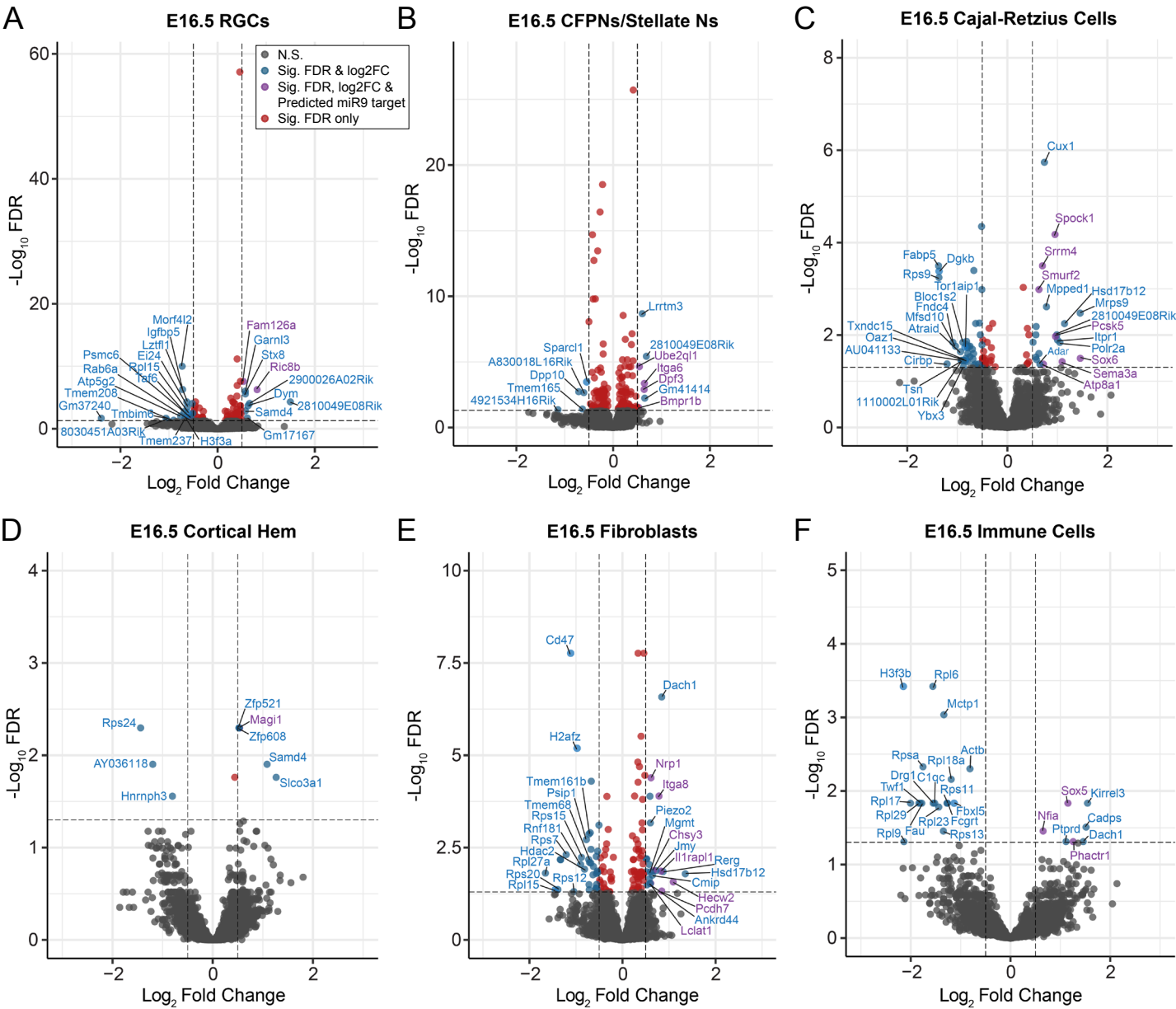
Supplemental Figure 2



Supplemental Figure 3

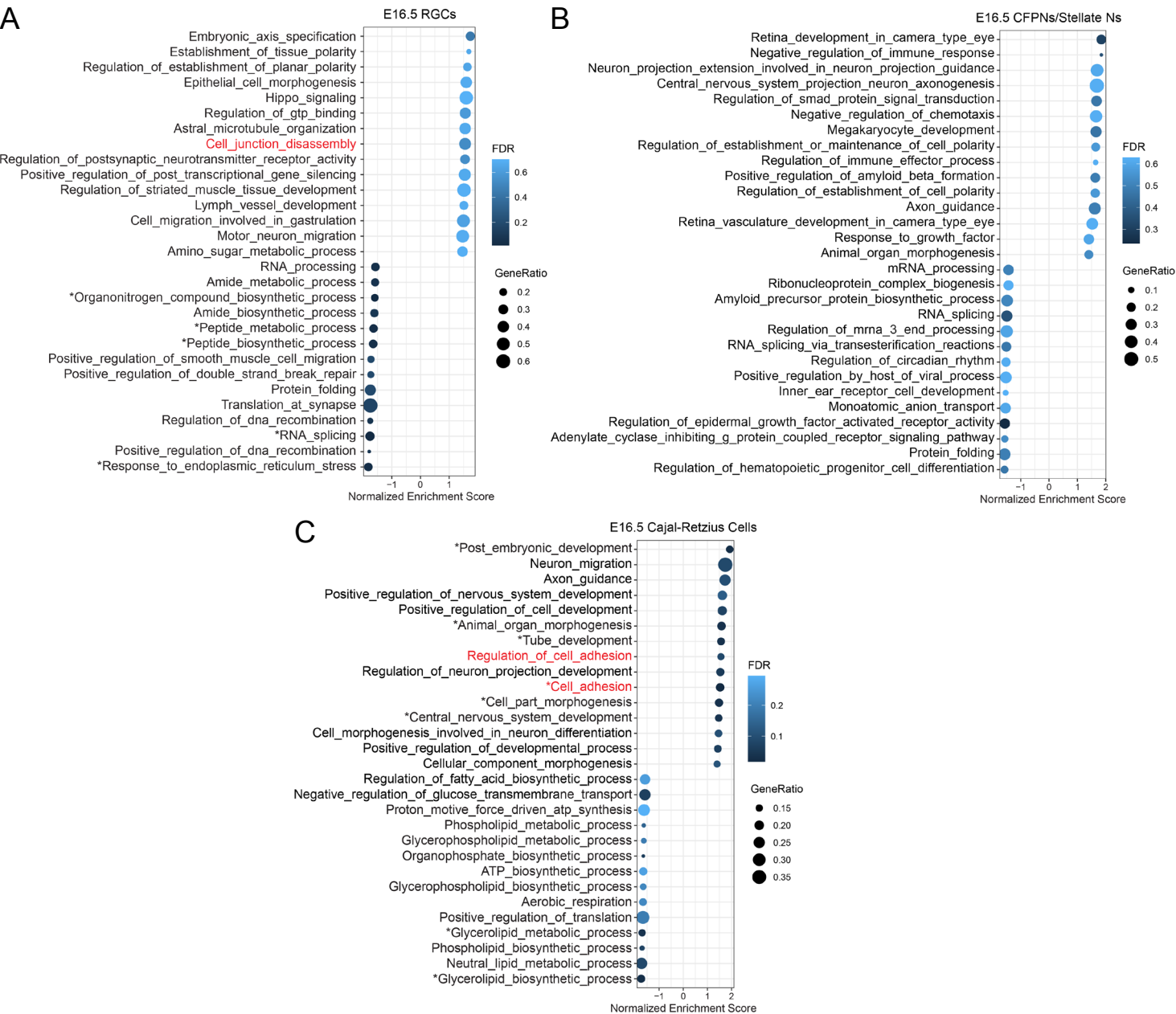


Supplemental Figure 4

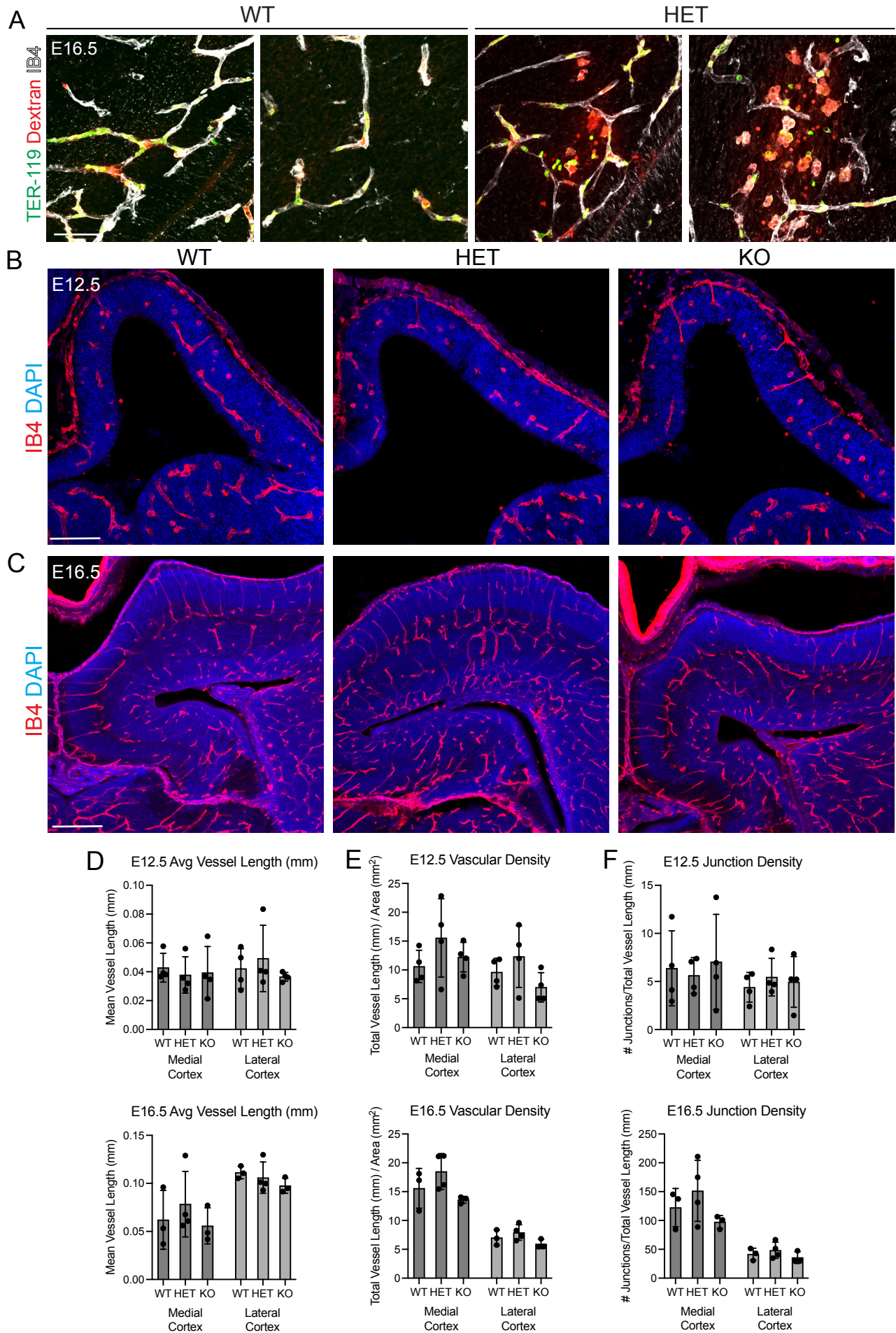




Supplemental Figure 5



# Supplemental Figure 6



Supplemental Figure 7

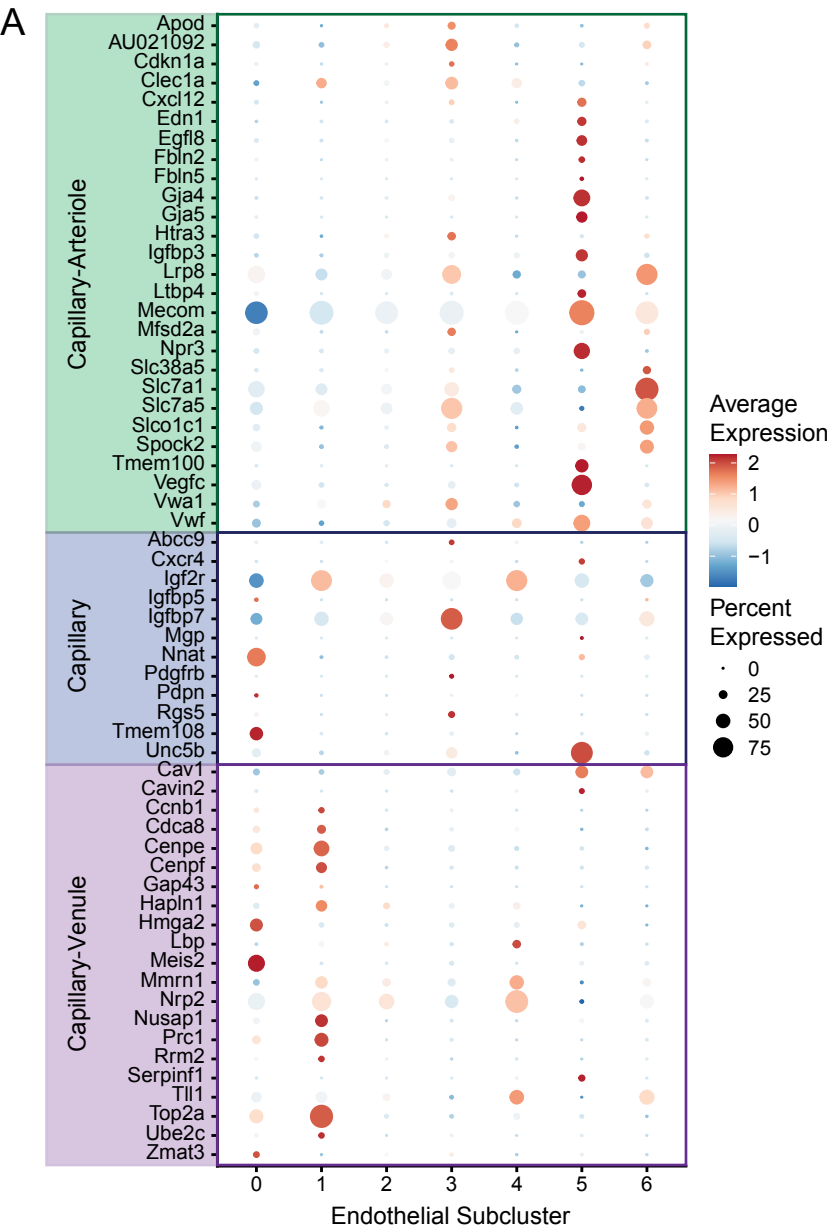


Table 1

A

| Cell Type | Stage | Phase | logFC  | logCPM | F     | PValue | FDR   |
|-----------|-------|-------|--------|--------|-------|--------|-------|
| RGCs      | E12   | G1    | 0.186  | 16.149 | 0.429 | 0.521  | 0.977 |
| RGCs      | E12   | S     | -0.035 | 18.842 | 0.031 | 0.863  | 0.977 |
| RGCs      | E12   | G2M   | 0.006  | 18.805 | 0.001 | 0.977  | 0.977 |
| RGCs      | E16   | G1    | -0.216 | 18.183 | 0.357 | 0.567  | 0.894 |
| RGCs      | E16   | G2M   | 0.138  | 18.656 | 0.142 | 0.716  | 0.894 |
| RGCs      | E16   | S     | 0.028  | 18.164 | 0.019 | 0.894  | 0.894 |
|           |       |       |        |        |       |        |       |
| Cell Type | Stage | Phase | logFC  | logCPM | F     | PValue | FDR   |
| IPCs      | E12   | S     | -0.165 | 18.308 | 0.709 | 0.414  | 0.819 |
| IPCs      | E12   | G1    | 0.109  | 18.061 | 0.178 | 0.680  | 0.819 |
| IPCs      | E12   | G2M   | 0.059  | 18.631 | 0.054 | 0.819  | 0.819 |
| IPCs      | E16   | S     | -0.405 | 17.000 | 6.170 | 0.042  | 0.125 |
| IPCs      | E16   | G1    | 0.244  | 19.290 | 2.097 | 0.191  | 0.286 |
| IPCs      | E16   | G2M   | -0.473 | 17.812 | 1.186 | 0.312  | 0.312 |

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| E12.5 Differential Abundance of Cell Types by Genotype |        |        |        |        |       |  |
|--------------------------------------------------------|--------|--------|--------|--------|-------|--|
| Cell Type                                              | logFC  | logCPM | F      | PValue | FDR   |  |
| RGCs                                                   | -0.192 | 19.232 | 1.193  | 0.305  | 0.522 |  |
| IPCs                                                   | -0.015 | 16.398 | 0.004  | 0.954  | 0.954 |  |
| Newborn Neurons                                        | 0.786  | 16.615 | 2.704  | 0.138  | 0.448 |  |
| Interneurons                                           | -0.054 | 15.074 | 0.006  | 0.939  | 0.954 |  |
| Cajal-Retzius Cells                                    | 0.456  | 14.323 | 1.880  | 0.206  | 0.518 |  |
| Cortical Hem                                           | 0.296  | 15.833 | 1.110  | 0.321  | 0.522 |  |
| Choroid Plexus                                         | 1.106  | 13.387 | 4.318  | 0.071  | 0.440 |  |
| GPCs                                                   | 1.491  | 12.038 | 3.394  | 0.101  | 0.440 |  |
| Endothelial Cells                                      | 0.294  | 13.725 | 0.346  | 0.572  | 0.827 |  |
| V. Mural Cells                                         | -0.421 | 12.139 | 1.608  | 0.239  | 0.518 |  |
| Fibroblasts                                            | 0.253  | 15.278 | 0.239  | 0.638  | 0.829 |  |
| Immune                                                 | 0.069  | 12.319 | 0.050  | 0.829  | 0.954 |  |
| Blood                                                  | -1.116 | 13.355 | 4.606  | 0.064  | 0.440 |  |
|                                                        |        |        |        |        |       |  |
| E16.5 Differential Abundance of Cell Types by Genotype |        |        |        |        |       |  |
| Cell Type                                              | logFC  | logCPM | F      | PValue | FDR   |  |
| RGCs                                                   | 0.552  | 16.349 | 12.180 | 0.009  | 0.107 |  |
| IPCs                                                   | 0.516  | 15.991 | 6.319  | 0.038  | 0.226 |  |
| CFPNs                                                  | -0.169 | 18.409 | 0.973  | 0.354  | 0.876 |  |
| CPNs                                                   | 0.033  | 18.425 | 0.032  | 0.863  | 0.941 |  |
| Interneurons                                           | -0.380 | 16.186 | 2.976  | 0.125  | 0.499 |  |
| Cajal-Retzius Cells                                    | -0.150 | 13.801 | 0.092  | 0.770  | 0.923 |  |
| Cortical Hem                                           | 0.424  | 12.947 | 0.855  | 0.384  | 0.876 |  |
| Choroid Plexus                                         | 0.336  | 13.597 | 0.214  | 0.657  | 0.876 |  |
| Endothelial Cells                                      | -0.236 | 12.457 | 0.444  | 0.525  | 0.876 |  |
| V. Mural Cells                                         | -0.187 | 12.024 | 0.301  | 0.599  | 0.876 |  |
| Fibroblasts                                            | 0.021  | 14.881 | 0.002  | 0.967  | 0.967 |  |
| Immune                                                 | 0.135  | 12.494 | 0.336  | 0.579  | 0.876 |  |