Chapter 3: Downstream Signaling of Pgrn-a and Generation of Transgenic Reporter and Mutant Zebrafish Lines

ABSTRACT

In addition to the studies presented in chapter 2, I also completed multiple additional experiments as part of my dissertation research; some of them were successful, some of them were not, and some will serve as preliminary data for ongoing studies in the lab. In this chapter, I present preliminary data on the downstream signaling mechanisms of Pgrn-a. I also summarize the rationale behind and progress made towards generating 1) a BAC transgenic line to use as a Pgrn-a reporter and to study the function of microglia in the zebrafish retina, and 2) a CRISPR mutant line to study the function of *pgrn-a* in retinal development and regeneration.

INTRODUCTION

During zebrafish development, microglia are required for normal retinal neurogenesis (Chapter 1). Lack of microglia in the developing retina causes a significant delay in neurogenesis – retinal progenitors remain in the cell cycle, failing to exit the cell cycle at the appropriate time in development, and those that do exit the cell cycle fail to acquire markers of neuronal differentiation. Further, Pgrn-a acts to both attract microglial precursors into the developing brain and retina and regulate the rate at which retinal progenitors progress through the cell cycle. Knockdown of Pgrn-a results in a significant decrease in retinal microglia and significant increase in the duration of G1-, G2-, and M-phase of the cell cycle, and therefore an overall

lengthening of the total cell cycle. Quantification of genes that are more proximal regulators of the cell cycle machinery and presumptively downstream of Pgrn-a found diminished Pgrn-a results in a significant increase in genes that promote cell cycle progression, and a significant decrease in genes that promote cell cycle exit. To further elucidate the mechanistic details by which secreted Pgrn-a regulates microglial precursor migration and retinal progenitor cell cycle kinetics during zebrafish retinal development, characterization of receptors and signaling pathways downstream of Pgrn-a is necessary.

There are two putative receptors for PGRN: sortilin and tumor necrosis factor receptor (TNFR). Sortilin, also known as neurotensin receptor-3, is a member of the vacuolar protein sorting 10 protein (Vps10p)-domain containing type-1 transmembrane receptor family (Hermey, 2009). In embryonic mice, sortilin is expressed predominately by neurons in the cerebral cortex and throughout the neural retina (Hermans-Borgmeyer et al., 1999). In the adult rat brain, sortilin is expressed by cortical neurons and oligodendrocytes (Sarret et al., 2003). In normal adult human and mouse frontal cortex, sortilin is expressed by neurons (Hu et al., 2010). Sortilin was identified through unbiased ligand binding assays and expression cloning as a neuron-specific PGRN receptor (Hu et al., 2010). As an endocytic and intracellular sorting receptor, sortilin is a known mediator of protein trafficking between the cell surface and various intracellular compartments (Hermey, 2009). Sortilin-mediated endocytosis and lysosomal targeting of PGRN is thought to regulate extracellular and intracellular PGRN levels (Hu et al., 2010). *In vitro*, in HeLa cells, knockdown of sortilin significantly increased endogenous PGRN

expression, and overexpression of sortilin significantly decreased PGRN levels in the conditioned media (Carrasquillo et al., 2010). *In vivo*, serum PGRN levels are significantly increased in sortilin knockout mice (*Sort1*$^{-/-}$), and knockdown of sortilin in heterozygous *Grn* mutant mice (*Sort1*$^{-/-}$*Grn*$^{+/-}$) restores serum PGRN to WT levels (Hu et al., 2010). Further, in iPSCs from FTLD patients, inhibition of sortilin-PGRN interaction restores extracellular PGRN to normal levels (Lee et al., 2013).

Sortilin can also form receptor complexes on the cell membrane with the p75 neurotrophin receptor (p75^{NTR}) and tyrosine kinase receptors (TrkRs); and together with p75^{NTR} or TrkRs, bind neuropeptides, neurotrophic factors, and growth factors, such as unprocessed nerve growth factor (proNGF) and brain derived growth factor (proBDNF), and regulate signal transduction pathways involved in neuronal viability/survival and programmed cell death (Nykjaer et al., 2004; Jansen et al., 2007; Hermey, 2009; Willnow et al., 2010; Nykjaer and Willnow, 2012). Sortilin is expressed by numerous cancer cell lines and mediates tumor cell growth, migration, and invasiveness (Dal Farra et al., 2001; Roselli et al., 2015). Interestingly, Sortilin is endogenously expressed by the immortalized human microglial cell line, C13NJ (Hu et al., 2010), and is required for chemotaxis of C13NJ cells in a phosphatidylinositol 3 kinase (PI3K)- and mitogen-activated protein kinase (MAPK)-dependent manor (Martin et al., 2003). The immortalized murine microglial cell line, N11, also endogenously expresses sortilin (Dicou et al., 2004). Recent findings demonstrated that the Sortilin receptor is expressed by activated microglia in brain tissue from patients with multiple sclerosis (Reuter et al., 2015). Zebrafish have two sortilin genes (*sort1a* and *sort1b*), which are co-orthologues to the human sortilin gene

(*SORT1*). However, the expression and function of sortilin in the developing zebrafish retina remains unknown.

Notwithstanding all the evidence indicating sortilin is a neuronal receptor for PGRN, PGRN was found to bind to $Sort^{-/-}$ neurons, although at significantly reduced levels compared to WT (Hu et al., 2010). Other studies demonstrate the neuroprotective effects of PGRN do not require binding to sortilin (De Muynck et al., 2013). Further, *in vitro*, PGRN-mediated neurite outgrowth and branching occurs in $Sort^{-/-}$ neurons (Gass et al., 2012). These data suggest sortilin is not the only neuronal receptor for PGRN, and that the neurotropic properties of PGRN are mediated by receptors other than sortilin. A study using yeast two-hybrid screening identified direct binding between PGRN and TNFR1 and TNFR2 (Tang et al., 2011). In fact, recombinant human PGRN (rhPGRN) reportedly binds to TNFR with higher affinity than TNFα (Tang et al., 2011). Further, PGRN antagonizes TNFα signaling and results in anti-inflammatory effects in a mouse model of arthritis (Tang et al., 2011). The PGRN-TNFR interaction was disputed (Chen et al., 2013; Etemadi et al., 2013), but has since been resolved and validated (Jian et al., 2013; Uddin et al., 2014; Wang et al., 2015). Zebrafish possess numerous genes that are members of the TNFR superfamily, however, *tnfrsf1a* and *tnfrsf1b* mediate Tnfα signaling (Wiens and Glenney, 2011; Espín et al., 2013). The expression and function of *tnfrsf1a* and *tnfrsf1b* in the developing zebrafish retina remains to be determined.

Wnt signaling is an important regulator of vertebrate retinal development (Ciani and Salinas, 2005; Matsui et al., 2005; Lad et al., 2009) and cell cycle progression (Sommer, 2004; Sánchez-Sánchez et al., 2010). Wnt signaling includes

the canonical or β-catenin-dependent and noncanonical or β-catenin-independent pathways. In the zebrafish retina, canonical Wnt signaling is sufficient to maintain retinal progenitors in a proliferative state both during development and regeneration (Kubo, 2003; Kubo et al., 2005; Yamaguchi, 2005; Kubo and Nakagawa, 2008; Meyers et al., 2012). Noncanonical Wnt signaling regulates cell migration during early embryogenesis and CNS development (Veeman et al., 2003; Hardy et al., 2008; Komiya and Habas, 2008; Schambony and Wedlich, 2013). There is an inverse association between both canonical and noncanonical Wnt signaling and PGRN expression. In *GRN*-inactivated human neural stem cells, postmortem brain tissues from patients with FTLD/*GRN* mutations, and $Grn^{-/-}$ mice, genes that activate canonical Wnt signaling, including *WNT1*, *FZD2* and *APC2*, are upregulated and genes that inhibit canonical Wnt signaling, such as *GSK3β*, are downregulated (Rosen et al., 2011). In PGRN-deficient lymphoblasts, noncanonical wingless-type 5a (Wnt5a) signaling is overactivated (Alquezar et al., 2014). Interestingly, PGRN binding to TNFR negatively regulates *Wnt5a* expression (Alquézar et al., 2015). *Wnt5a* expression is positively correlated with cell proliferation, which is increased in lymphoblasts from FTLD/*GRN* mutation patients and *GRN* knockdown neuroblastoma cells (Alquézar et al., 2015). Therefore, Wnt signaling may be one of the downstream mechanisms by which Pgrn-a regulates cell proliferation in the developing retina.

In the vertebrate retina, the Notch signaling pathway regulates retinal progenitor cell proliferation and differentiation, and neurogenic versus gliogenic cell fate determination (Perron and Harris, 2000; Jadhav et al., 2006; Uribe et al., 2012).

In zebrafish, increasing Notch signaling by overexpression of a constitutively activated Notch receptor inhibits neuronal differentiation by promoting retinal progenitors to either become Müller glia or remain in an undifferentiated state and eventually undergo apoptosis (Scheer et al., 2001). Pharmacological or genetic inhibition of Notch signaling promotes neuronal differentiation, causing retinal progenitors to prematurely exit the cell cycle and Müller glia to fail to differentiate (Bernardos et al., 2005). Therefore, Notch normally functions to maintain retinal progenitors in a proliferative state by inhibiting neuronal differentiation and promoting Müller glial differentiation, perhaps as a downstream target of Pgrn-a during retinogenesis.

The utility of zebrafish as a genetic model for developmental and regenerative studies is widely reported (Glass and Dahm, 2004; Fadool and Dowling, 2008; Gemberling et al., 2013). Zebrafish can be manipulated using a wide range of forward and reverse genetic tools, making them a powerful model organism for studying both gene expression and function and the function of specific cell types during retinal development and regeneration. Due to the paucity of antibodies that label Pgrn-a in zebrafish tissue sections, a stable transgenic reporter line would utilize embryo transparency and enable the direct visualization of *pgrn-a* gene expression *in vivo* and *in situ* (Williams and Wong, 2004). However, identifying promoter fragments that mediate endogenous expression patterns and levels is challenging, and regulatory elements can be located both upstream and downstream of the coding sequence for a given gene (Zhang et al., 2007). Compared to promoter fragment approaches, Tol2-mediated, bacterial artificial chromosome (BAC)

transgenesis is more efficient and recapitulates endogenous gene expression patterns with high fidelity (Yang et al., 2006; Kawakami, 2007; Bussmann and Schulte-Merker, 2011; Suster et al., 2011). BAC cloning vectors hold large fragments of genomic DNA, and therefore can contain an entire gene and the proximal and distal regulatory elements that control the precise temporal and spatial expression patterns of that gene (Giraldo and Montoliu, 2001). The Tol2 element is a DNA-type transposon with a large cargo capacity and high rate of genomic integration, capable of efficiently delivering single-copy BAC transgenes to the zebrafish genome (Suster et al., 2009). Incorporating the nitroreductase (NTR)/metronidazole (Met) cell ablation system into the reporter line would render cells expressing *pgrn-a* susceptible to chemically inducible, temporally controlled genetic ablation (Curado et al., 2007; Pisharath et al., 2007; Curado et al., 2008; Montgomery et al., 2010). The NTR enzyme, encoded by the *Escherichia coli* (*E. coli*) *nfsB* gene, converts the prodrug Met into a cytotoxin. *pgrn-a* expression is initially ubiquitous in all neural progenitor cells, but becomes microglia-specific during early zebrafish development (Figure 2.5), an expression pattern that persists into adulthood (Craig et al., 2008). Therefore, a transgenic reporter line that expresses *nfsB* fused to a fluorescent protein under the control of the *pgrn-a* promoter and regulatory elements would allow us to specifically ablate microglial cells during retinal development or regeneration. By removing microglia from either developing or regenerating retinal tissues, we can then infer about the function of microglia during retinal development and regeneration.

There are multiple established reverse genetics approaches to manipulate genes of interest and facilitate the study of gene function during zebrafish development and regeneration. We previously used morpholino oligonucleotides (MOs; (Nasevicius and Ekker, 2000) to study the function of *pgrn-a* in the developing zebrafish retina (Chapter 2). However, knockdown experiments using MOs only temporarily block gene splicing or translation, and this technology can suffer from potentially confounding, non-specific effects (Ekker and Larson, 2001; Morcos, 2007; Eisen and Smith, 2008), though these off-target effects can be rigorously controlled for (Robu et al., 2007; Bill et al., 2009; Bedell et al., 2011). Nevertheless, it is no longer sufficient to use gene knockdown technology as a stand-alone approach for studying gene function. Members of the zebrafish community have recently advocated for confirming morphant phenotypes by comparison to a genetic mutant as criterion for substantiating the use of MOs (Schulte-Merker and Stainier, 2014; Kok et al., 2015). Therefore, generating a stable *pgrn-a* mutant line is an important and necessary next step towards validating the *pgrn-a* morphant phenotype and further elucidating signaling mechanisms downstream of Pgrn-a.

Recent major advancements in genome editing technology have established the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated 9 (Cas9) system as an exceptionally efficient tool for generating targeted mutations in the zebrafish genome (Hwang et al., 2013). Unlike the zinc finger nuclease (ZFN) and transcription activator-like effector nuclease (TALEN) genome engineering techniques that target DNA with specific DNA-binding nucleases individually synthesized for each target site, the relatively simple and adaptable

CRISPR/Cas9 system is a RNA-guided endonuclease technology (Lawson and Wolfe, 2011; Heidenreich and Zhang, 2016). In this two part system, a single synthetic guide RNA (sgRNA) binds to complementary genomic DNA and localizes Cas9 endonuclease to the target sequence upstream of a Protospacer Adjacent Motif (PAM). The PAM sequence is required for Cas9 binding (Sternberg et al., 2014). When bound, Cas9 creates a double stranded break (DSB). The DSB is repaired by either nonhomologous end joining (NHEJ), resulting in insertions or deletions (indels), or homology-directed repair when exogenous, homologous DNA template provided is inserted at the DSB. sgRNAs are easily generated to target any gene of interest. CRISPR/Cas9 effects genomic DNA, not RNA transcripts, and the correlation between the genotype and phenotype can be determined at the level of a single embryo.

As a first step in testing the hypothesis that Pgrn-a regulates microglial precursor migration and/or retinal progenitor cell cycle kinetics via the sortilin receptor we used RT-PCR and *in situ* hybridization to determine the temporal and spatial expression patterns of *sort1a* and *sort1b* in the zebrafish retina. The results show that *sort1a* and *sort1b* are expressed in 24hpf embryos, 48 and 72hpf embryo heads, and the adult retina. To determine whether Pgrn-a knockdown alters Wnt and Notch signaling during retinal development, we utilized qRTPCR, Western blot, and *in situ* hybridization to assay expression levels of genes in the Wnt and Notch signaling pathways. The results show that following Pgrn-a knockdown, canonical Wnt signaling is upregulated, and at 30hpf, the Notch target gene, *her4*, is significantly decreased, but at 72hpf, the expression of *notch1a* receptor and *her4*

are both significantly increased. Further studies on signaling downstream of Pgrn-a were suspended until stable transgenic and mutant zebrafish lines were generated. To further study the expression of Pgrn-a and the function of microglial cells in the developing and adult/regenerating retina, BAC recombineering was used to generate iTol2-containing BAC constructs encoding a NTR-fluorescent reporter fusion protein under the control of the *pgrn-a* promoter and regulatory elements. These constructs can be used to establish stable transgenic zebrafish lines that will serve as tools to study Pgrn-a and enable conditional targeted ablation of microglial cells. To further study the function of *pgrn-a* in the developing and adult/regenerating retina, CRISPR/Cas9 genome engineering technology was used to mutate *pgrn-a*. A viable *pgrn-a* mutant line will be a valuable resource for our lab and others.

MATERIALS AND METHODS

Animals

Adult AB wild type (WT) zebrafish (*Danio rerio*; ZIRC, University of Oregon, Eugene, OR, USA) were maintained at 28.5°C on a 14/10-h light/dark cycle. Embryos were collected immediately following natural spawns, incubated at 28.5°C on a 14/10-h light/dark cycle and staged by hours post fertilization (hpf). The Institutional Animal Care and Use Committee at the University of Michigan approved all protocols and procedures.

Immunohistochemistry

IHC was performed as previously described (Luo et al., 2012). Briefly, embryos were fixed overnight in 4% paraformaldehyde (PFA) in 100mM phosphate buffer at 4°C, cryoprotected with 20% sucrose in 100mM phosphate buffer, and embedded in

frozen Tissue-Tek optical cutting temperature (OCT; Sakura Finetek USA Inc., Torrance, CA, USA) compound. Sections (10μm thick) were mounted on slides, washed, incubated in heat inactivated normal sheep serum (NSS), and incubated overnight at 4˚C with primary antibodies. The following day, sections were washed and incubated in secondary antibodies. Nuclei were stained with DAPI. Antibodies are listed in Table 3.1.

S-phase labels

Cells in S-phase of the cell cycle were labeled with 5-ethynyl-2'deoxyuridine (EdU; Invitrogen, Carlsbad, CA, USA) as previously described (Ochocinska and Hitchcock, 2007; Luo et al., 2012; Chapter 2). Briefly, dechorionated embryos at 72hpf were incubated at room temperature (RT) for 20 minutes in 1.5mM EdU dissolved in E3 containing 15% DMSO. Following the labeling step, embryos/larvae were returned to E3 for 10 minutes prior to fixation. EdU was visualized using the Click-iT EdU Alexa Fluor 555 Imaging kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Western Blot

Western blot analysis was performed as previously described (Gramage et al., 2015). Briefly, for each condition, protein was isolated from 50 embryo heads and separated by gel electrophoresis. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (GenHunter Corp., Nashville, TN, USA) and incubated with rabbit anti-Cyclin D1 (1:1000; Anaspec #55399, Freemont, CA, USA) or rabbit anti-non-phospho (active) β-Catenin antibodies (1:1000; Cell Signaling #8814S; Danvers, MA, USA). Immunolabeled proteins were detected using enhanced

chemiluminescence assay (ECL detection system, Amersham Biosciences, Arlington Heights, IL, USA). Anti-actin (1:1000, Calbiochem, EMD Millipore, Billerica, MA, USA) was used as a loading control.

In situ hybridization

In situ hybridization (ISH) on retinal sections and whole mount *in situ* hybridization (wmISH) were performed as previously described (Hitchcock and Kakuk-Atkins, 2004; Ochocinska and Hitchcock, 2007; Craig et al., 2008; Luo et al., 2012). Approximately 1Kb of *sort1a* or *sort1b* was PCR amplified from cDNA (primer pairs listed in Table 3.3) and subcloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). Sense and antisense DIG-labeled riboprobes (Table 3.2) for *sort1a* and *sort1b* were synthesized from these constructs using *in vitro* transcription (Roche Diagnostic Corp., Indianapolis, IN, USA). Sense and antisense digoxigenin (DIG)-labeled riboprobes (Table 3.2) for *axin2* were synthesized from a full-length cDNA clone (Clone ID: 9038740; Thermo Scientific, Waltham, MA, USA) using *in vitro* transcription (Roche Diagnostic Corp., Indianapolis, IN, USA). Embryos were incubated with hybridization solution containing approximately 100ng of probe.

BAC recombineering to generate transgenic Tg(*pgrna*:*nfsB-EGFP*) and Tg(*pgrna*:*nfsB-mCherry*) lines

To create a *pgrn-a* reporter line that also expresses *nfsB* in microglia, transposon-mediated, bacterial artificial chromosome (BAC) transgenesis was performed as previously described (Suster et al., 2011). Briefly, the *pgrn-a* gene was identified in the ENSEMBL database (ENSDARG00000004954), and BAC clones encompassing the entire *pgrn-a* gene sequence were screened. Of the clones available, CH73-287L22 and CH73-47I9 from the CHORI-73 zebrafish genomic BAC library

(http://bacpac.chori.org/library.php?id=281) contained sufficient (≥20Kbp), yet different portions of 5' and 3' flanking sequence. Once obtained from the BACPAC Resources Center, CH73-287L22 and CH73-47I9 were modified and tested in parallel to determine the ideal clone for stable transgenesis. CH73-287L22 and CH73-47I9 sequences were verified using pgrn-a_ATG, UP10K and DOWN10K primer pairs. BAC plasmid DNA was electroporated (voltage: 1700V, resistor: 200Ω, capacitor: 25μF) into SW105 or SW106 *E. coli* cells that contain heat-inducible recombinase functions and can be used for *galK* positive/negative selection. To facilitate integration of the BAC construct into the zebrafish genome, the *iTol2-amp* cassette was PCR amplified from the pCR8GW-iTol2-amp plasmid (from Kawakami Lab) using the ptarbac_iTol2 primer pair and Expand high fidelity PCR system (Roche, Indianapolis, IN, USA) and recombineered into the BACs. Incorporation of the *iTol2-amp* cassette into the BACs was confirmed with the iTol2_screen primer pair. Both *nfsB-EGFP-SV40pA* and *nfsB-mCh-SV40pA* were PCR amplified from T2KXIG-*zop*:*nfsB-EGFP* (from the Hyde Lab; Montgomery et al., 2010) and T2KIXG-*ins*:*nfsB-mCherry* (from Mike Parsons; Pisharath et al., 2007) expression constructs, respectively, using NTR30 primer pairs and PfuUltra DNA polymerase (Agilent Technologies, Santa Clara, CA, USA), and subcloned into a *FRT-Kan-FRT* plasmid (PL451; from Dave Olson) with HindIII-HF and XhoI restriction enzymes (New England Biolabs, Ipswich, MA, USA). Reporter gene casettes containing *nfsB-EGFP-pA-FRT-Kan-FRT* or *nfsB-mCh-pA-FRT-Kan-FRT* with 50-bp homologies to the first and second coding exons in *pgrn-a* were PCR amplified using pgrnaNTR_F and pgrnaFRTKanFRT_R primers and the Expand high fidelity PCR system (Roche,

Indianapolis, IN, USA). Purified reporter gene casettes (5-10ug) were individually inserted into the *pgrn-a* start site in Tol2-containing BACs. BAC constructs were confirmed by PCR and sequencing of pgrn-a_ATG primer PCR products with pA, GFP or mCh and NTR primer pairs (University of Michigan DNA Sequencing Core). Primer sequences are listed in Table 3.4. The modified BAC DNA was prepared using the Nucleobond BAC 100 kit according to the manufacturer's instructions (Macherey-Nagel, Bethlehem, PA, USA). Transposase mRNA was synthesized from NotI-linearized pCS2-zT2TP using the mMessage mMachine SP6 kit according to the manufacturer's instructions (Ambion, ThermoFisher Scientific, Waltham, MA, USA). Modified BAC DNA (1ug) was co-injected with transposase mRNA (1ug) into the cytoplasm of one-cell stage embryos. Embryos were screened for GFP or mCherry expression between 24-96hpf.

CRIPSR-Cas9 Targeted Mutation of *pgrn-a*

To induce mutations at the *pgrn-a* locus, the CRISPR-Cas9 system was used as previously described (Hwang et al., 2013; Taylor et al., 2015). Briefly, *Cas9* (pCS2nCas9n Addgene Plasmid #46929) mRNA was made using the mMessage mMachine SP6 *in vitro* transcription kit (Ambion, ThermoFisher Scientific, Waltham, MA, USA), followed by RNA cleanup with the RNeasy mini kit (Qiagen, Valencia, CA, USA). 1nL containing *Cas9* mRNA (150pg/nL) and sgRNA (100pg/nL; Sigma, St. Louis, MO, USA) diluted in 1X Danieux buffer (Nasevicius and Ekker, 2000) and 2.5% phenol red was co-injected into the cell of a one-cell stage embryo. sgRNA target site sequences are listed in Table 3.5. Genomic DNA from AB WT breeding pairs used for this experiment was PCR amplified with the pgrna_CRISPR_screen

primer pair (Table 3.6) and sequenced to confirm the presence of sgRNA target sequences. At 72hpf, embryos were treated with EdU and collected: heads were processed for histology as described above, and tails were processed for genotyping described below. Total DNA was extracted from individual tails using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA). To detect CRISPR-induced insertions or deletions, the T7 endonuclease assay was performed as previously described (www.crisprflydesign.org/t7-endo-i-assay). Briefly, the *pgrn-a* sgRNA target site was PCR amplified using Platinum Taq HF (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) and the pgrna_CRISPR_screen primer pair. PCR product clean up was done using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). Purified PCR products (200ng) were annealed and digested with 10U T7 endonuclease (New England Biolabs, Ipswich, MA, USA), which recognizes and cleaves heteroduplex DNA, then separated by gel electrophoresis. Embryos with CRISPR-induced indels, indicated by a double band, were considered T7-positive. Purified PCR products (701bp) of T7-positive embryos were ligated into the pGEM-T Easy Vector (Promega, Fitchburg, WI, USA) using the manufacturer's protocol, and transformed into JM109 competent cells (Promega, Fitchburg, WI, USA). DNA from single colonies (n=9/embryo) was isolated using the QIAprep spin mini prep kit (Qiagen, Valencia, CA, USA), and sequenced (University of Michigan DNA Sequencing Core). Analysis of the mutation and functional consequences was completed using the Expasy translate tool (www.expasy.org).

Imaging

All sectioned material was sealed with glass coverslips and mounting media (Electron Microscopy Sciences, Hatfield, PA, USA). Fluorescence images were captured using a Leica TCS SP5 confocal microscope (Leica, Wetzler, Germany).

RESULTS

sortilin-1a and *sortilin-1b* are expressed in the developing embryo and adult retina

Sortilin is a relatively simple, single-pass type I transmembrane receptor made up of one Vps10p extracellular domain and a shorter intracellular cytoplasmic domain (Figure 3.1 A). The C-terminal tail of PGRN binds with high affinity to the beta-propeller region of the Vps10p extracellular domain of sortilin (Zheng et al., 2011); Figure 3.1 A). Zebrafish have two sortilin genes, *sort1a* and *sort1b*, likely due to a pan-genomic duplication event (Taylor et al., 2003). Sequence homology between *sort1a*, *sort1b*, and *SORT1* was determined using the Clustal Omega alignment program (http://www.ebi.ac.uk/Tools/msa/clustalo/). *sort1a* and *sort1b* share approximately 60% sequence homology (Figure 3.1 B), and are at least 58% and 55% homologous to *SORT1*, respectively. During embryonic development in mice, sortilin is highly expressed by neurons in the cerebral cortex and within the neural retina, suggesting sortilin may play a regulatory role in the developing brain and retina (Hermans-Borgmeyer et al., 1999). *In vitro*, sortilin is expressed by human and murine microglia, and is required for human microglial cell migration (Martin et al., 2005). Therefore, as a first step in testing the hypothesis that Pgrn-a regulates retinal progenitor cell cycle and/or microglial precursor migration through the sortilin

receptor, we first established the expression pattern of *sort1a* and *sort1b* in the developing and adult retina using RT-PCR. At 24hpf, *sort1a* and *sort1b* are expressed in the developing embryo; and at 48 and 72hpf, *sort1a* and *sort1b* are expressed in embryo heads (Figure 3.1 C). *sort1a* and *sort1b* are also expressed in the adult retina (Figure 3.1 C). At 24 and 48hpf, *sort1a* is more highly expressed compared to *sort1b*.

Canonical Wnt signaling is upregulated following Pgrn-a knockdown

Canonical Wnt signaling begins when Wnt ligands bind to the Frizzled receptor on the cell surface, which causes β-Catenin to be stabilized and translocate from the cytoplasm to the nucleus. Once in the nucleus, β-Catenin forms a complex with the ternary complex factor (TCF) and/or lymphoid enhancer-binding factor (LEF) transcription factors and stimulates transcription of Wnt target genes, such as *cyclinD1* and *axin2* (Denayer et al., 2008; Ciani and Salinas, 2005; Bikkavilli and Malbon, 2009; Ha et all., 2012). In humans and mice, canonical Wnt signaling is upregulated in PGRN-deficient neurons (Rosen et al., 2011). Previous studies showed in zebrafish, at 72hpf, cyclin-dependent kinase activator *cyclinD1* (*ccnd1*) mRNA is significantly increased following Pgrn-a knockdown (Figure 2.12 G). To test our hypothesis that Pgrn-a regulates retinal progenitor cell cycle through the canonical Wnt signaling pathway, Western blot, *in situ* hybridization and qRTPCR assays were used to determine whether knockdown of Pgrn-a alters the expression of genes in the canonical Wnt signaling pathway. At 48hpf, the stabilized (Active) form of β-Catenin, the key downstream effector of the canonical Wnt signaling

pathway, is undetectable in uninjected WT and MM MO-injected embryos (Figure 3.2 A+B). However, compared to uninjected and MM MO-injected controls, Active β-Catenin is increased in pgrn-a morphants (Figure 3.2 A+B). Similarly, Western blot analysis showed that compared to uninjected and MM MO-injected embryos, CyclinD1, a target gene of the canonical Wnt signaling pathway, is upregulated in *pgrn-a* morphant embryos (Figure 3.2 A+B). In uninjected WT and MM MO-injected retinas at 48hpf, *axin2* mRNA expression is restricted to the ciliary marginal zone (CMZ) at the edge of the retina (Figure 3.2 C). However, in *pgrn-a* morphants, *axin2* mRNA is expressed ubiquitously throughout the retina (Figure 3.2 C). qRTPCR was performed at 30 and 72hpf to measure the expression levels of *axin2* mRNA. Compared to uninjected and MM MO-injected controls, the expression of *axin2* at 30hpf is significantly increased in *pgrn-a* morphants, whereas at 72hpf the expression of *axin2* is significantly decreased following Pgrn-a knockdown (Figure 3.2 D).

Pgrn-a knockdown alters Notch signaling pathway gene expression

Notch signaling mediates cell fate via cell-to-cell signaling (Artavanis-Tsakonas et al., 1999; Hori et al., 2013). Notch ligands, Delta and Jagged, are membrane bound. Notch receptors, such as Notch1, are transmembrane proteins located at the cell surface with both extracellular and intracellular domains. When Delta or Jagged bind to the extracellular domain of Notch1, the Notch intracellular domain (NICD) is proteolytically cleaved by γ-secretase and translocates to the nucleus to activate transcription of Notch target genes, such as hairy/enhancer-of-

split (*hes*) or *hes*-related (*her*). Notch signaling represses cyclin-dependent kinase inhibitor 1B ($p27^{kip}$) expression (Hristova et al., 2013; Del Debbio et al., 2016). $p27^{kip}$ promotes cell cycle exit by inhibiting cell cycle re-entry (Dyer and Cepko, 2001a). In mice, over expression of $p27^{kip}$ causes retinal progenitors to prematurely exit the cell cycle, whereas, p27 deficiency leads to an increased proportion of mitotically active cells in the retina (Dyer and Cepko, 2001b). Previous studies showed in zebrafish, at 30 and 72hpf, *p27kip* mRNA is significantly decreased following Pgrn-a knockdown (Figure 2.12 F+G). To test the hypothesis that Delta-Notch signaling is upregulated in *pgrn-a* morphants, qRTPCR was performed at 30 and 72hpf to measure the expression levels of genes in the Notch signaling pathway. At 30hpf, there is no significant difference between the expression of *notch1a* in morphants compared to uninjected and MM MO-injected controls (Figure 3.3 A). However, compared to uninjected and MM MO-injected controls, at 30hpf, *her4* mRNA is significantly decreased in *pgrn-a* morphants (Figure 3.3 A). At 72hpf, compared to uninjected and MM MO-injected controls, the expression of *notch1a* and *her4* mRNA is significantly increased in *pgrn-a* morphants (Figure 3.3 B).

BAC recombineering

To further study *pgrn-a* expression and regulation in the developing and adult/regenerating retina, we sought to generate a transgenic reporter line. Previous attempts in the lab to generate a *pgrn-a* reporter line using 4Kb upstream of the *pgrn-a* ATG to drive EGFP expression were unsuccessful. Therefore, for our second attempt, we chose to use the iTol2-mediated, BAC transgenesis technique. Both

CH73-287L22 and CH73-47I9 BACs hold approximately 110Kb of genomic DNA, which includes the complete *pgrn-a* gene and, theoretically, distant 5' and 3' *cis*-regulatory elements required for the precise temporal and cell type specific expression of *pgrn-a*. The iTol2 cassette was inserted into the CH73-287L22 and CH73-47I9 BAC plasmids at a loxP site present in the pTARBAC2.1 backbone by recombineering. To incorporate the NTR/Met cell ablation system into the reporter line, we utilized kanamycin (kan) antibiotic selection to insert a reporter gene cassette in which *nfsB* was fused to either enhanced green fluorescent protein (EGFP) or mCherry fluorescent protein (mCh) into the iTol2-containing BAC plasmids at the first coding exon of *pgrn-a*. Ultimately, four BAC constructs were generated: Tol2-CH73-287L22 *pgrna*:*nfsB-EGFP* (Figure 3.4 A), Tol2-CH73-287L22 *pgrna*:*nfsB-mCh* (Figure 3.4 B), Tol2-CH73-47I9 *pgrna*:*nfsB-EGFP* (Figure 3.4 C), Tol2-CH73-47I9 *pgrna*:*nfsB-mCh* (Figure 3.4 D). Two or three kan-positive recombinant colonies of each BAC construct were screened by PCR to confirm homologous recombination and verify presence of the reporter gene cassette (Figure 3.5 A). The reporter cassette was present in Tol2-CH73-287L22 *pgrna*:*nfsB-mCh* colony #1 and #3, Tol2-CH73-287L22 *pgrna*:*nfsB-EGFP* colony #2, Tol2-CH73-47I9 *pgrna*:*nfsB-EGFP* colony #1 and #2, and Tol2-CH73-47I9 *pgrna*:*nfsB-mCh* colony #1 and #2. These seven colonies were further screened by PCR to individually confirm the presence of the *iTol2*, *nfsB*, and *EGFP* or *mCh* (Figure 3.5 B). The reporter gene cassette from each of the four recombineered BAC constructs was PCR amplified, gel purified, and confirmed with sequencing (Figure 3.5 C).

Generation of Tg(*pgrna*:*nfsB-EGFP*) and Tg(*pgrna*:*nfsB-mCh*) lines

To integrate the BAC construct into the zebrafish genome, the Tol2-CH73-47I9 *pgrna*:*nfsB-EGFP* #1 construct was co-injected with transposase mRNA into the cytoplasm of AB WT embryos at the one-cell stage. A BAC construct previously confirmed to result in GFP transgene expression in a distinct subset of cells in the tail (generated by Kenichi Iwasaki in the Kuwada Lab) was processed in parallel and used as a positive control for the DNA prep and microinjections. Starting at 24hpf, embryos injected with either construct were dechorionated and screened for GFP expression under a dissection microscope. At 24hpf, in embryos injected with the positive control BAC construct, GFP-positive cells were observed in the tails. However, at 24hpf, no transgene expression was observed in embryos injected with the Tol2-CH73-47I9 *pgrna*:*nfsB-EGFP* BAC construct. Between 48 and 72hpf, very faint and punctate GFP expression was observed in the heads and tails of a small subset (~10%) of embryos injected with the Tol2-CH73-47I9 *pgrna*:*nfsB-EGFP* BAC construct, but the expression was not robust, difficult to visualize, and representative images were inadequate. Due to the lack of robust transgene expression, no embryos were raised to identify germline carriers.

F0 *pgrn-a* CRISPR mosaic mutant retinal phenotype

To validate our *pgrn-a* morphant data and further investigate the function of *pgrn-a* in regulating microglial precursor migration and retinal neurogenesis, we used the CRISPR-Cas genome editing system to create a *pgrn-a* mutant zebrafish line. Four sgRNAs, each targeting slightly different regions of the first coding exon

(exon 3) of *pgrn-a*, 3' of the first ATG (see Figure 3.6 A and Figure 3.7 A), were individually co-injected with *cas9* mRNA into the cytoplasm of one-cell stage embryos (Figure 3.6 B). At 72hpf, ten embryos (F0 generation) from each of the four injection groups were collected: tails for genotyping and heads for immunohistochemistry. The remaining F0 embryos were raised. To identify F0 embryos carrying *pgrn-a* indel mutations and determine genome-targeting efficiency of individual sgRNAs, a T7 endonuclease assay was performed. The frequency of altered alleles was 0% for NM001001949reg1_0_60 (60)-injected embryos, 50% for NM001001949reg1_0_87 (87)-injected embryos, 80% for NM001001949reg1_0_90 (90)-injected embryos, and 90% for NM001001949reg1_0_94 (94)-injected embryos. Sequencing of T7-positive PCR products revealed indels ranging from 4-19bp insertions or 8-20bp deletions within or encompassing the sgRNA target sequence. The vast majority of indels created a frame shift and introduced a premature stop codon (Figure 3.7 B). Sequencing of T7-negative PCR products verified that T7-negative embryos possessed no mutated alleles, only wild type *pgrn-a*.

To determine the retinal phenotype of mosaic F0 embryos with observed indels, the corresponding heads were sectioned and immunolabeled, and qualitative measures of cell proliferation, neuronal differentiation, and retinal size were compared in retinas of T7-negative and T7-positive embryos. At 72hpf, T7-negative embryos have WT retinas, EdU-positive progenitors are few in number and restricted to the CMZ (brackets, Figure 3.7 C1). Further, the retina is fully laminated, and mature photoreceptors are present in the ONL (Figure 3.7 C1). The retinal phenotype of T7-positive embryos at 72hpf ranged from no apparent change to more

severe phenotypes, which are shown in Figure 3.7 (C2-C6). Approximately 15% of T7-positive F0 embryos screened had retinal phenotypes that recapitulated aspects of the *pgrn-a* morphant phenotype, including microphthalmia (Figure 3.7 C3), an increased proportion of retinal progenitors labeled with EdU and an absence of markers of differentiated, mature neurons, such as ZPR1 (Figure 3.7 C2, C4-C6). However, we used caution in analyzing phenotype data at this stage due to the fact that the F0 embryos are mosaic, and not all cells carry a mutation in *pgrn-a*. Therefore, the F0 phenotype is generally less severe than *pgrn-a* morphants.

Generation of *pgrn-a* mutant lines and characterization of *pgrn-a*$^{-/-}$ retinal phenotype

Yielding the highest frequency of altered alleles, two lines were propagated from both the sgRNA 90- and 94-injected groups (see Figure 3.6 B). F0 male founders were outcrossed with wild type females to generate a F1 colony. F1 fish were genotyped, and if a heterozygous male and a female possessed the same mutation they were incrossed. The F2 offspring from the incross were genotyped, and raised separately in wild type, heterozygous and homozygous groups. F2 homozygous male and female pairs were incrossed to generate a F3 homozygous colony. However, if the same mutation was not identified in a F1 heterozygous male and female pair, then a heterozygous male was outcrossed with a wild type female. Then, F2 offspring from the outcross were genotyped, heterozygous male and female pairs were incrossed, the F3 offspring from the incross will be genotyped and raised separately in wild type, heterozygous and homozygous groups, and F3

homozygous male and female pairs will be incrossed to generate a F4 homozygous colony. The status of lines being propagated is detailed in Table 3.6.

One of the four lines being propagated, the *pgrna*$^{-/-}$_90_5M_2F7M line reached the F3 homozygous colony stage. This mutant line possesses a 10bp deletion within the sgRNA target sequence (Figure 3.8 A), which is predicted to cause a frame shift and introduce a premature stop codon in the Pgrn-a sequence (Figure 3.8 B). *pgrna*$^{-/-}$_90_5M_2F7M embryos are viable, though initial clutches grew more slowly and were smaller than wild type age-matched controls. However, subsequent generations appeared normal. At 24hpf, Pgrn-a was detected by Western blot in de-yolked *pgrna*$^{-/-}$_90_5M_2F7M embryos (Figure 3.8 C). At 33hpf, Pgrn-a expression in *pgrna*$^{-/-}$_90_5M_2F7M embryos was reduced compared to the 24hpf time point (Figure 3.8 C), consistent with wild type Pgrn-a expression that is ubiquitously expressed in all neural progenitors at 24hpf, but then becomes microglia-specific and therefore restricted to a small subset of cells in the head. At 48 and 72hpf, retinas were sectioned and immunolabeled, and qualitative measures of cell proliferation, neuronal differentiation, and retinal size were compared in *pgrna*$^{-/-}$_90_5M_2F7M mutants and wild type controls. At 48 and 72hpf, *pgrna*$^{-/-}$_90_5M_2F7M embryos appear like WT retinas (Figure 3.8 D).

DISCUSSION

During embryonic development, sortilin is highly expressed by neurons in the cerebral cortex and in the neural retina; therefore suggesting sortilin may play a regulatory role in the developing retina (Hermans-Borgmeyer et al., 1999). Interestingly, sortilin is also expressed by microglial cell lines, and regulates

microglial chemotaxis (Martin et al., 2003; Hu et al., 2010). Histological and expression studies in mouse spinal cord suggest PGRN expressed and secreted by microglia interacts in trans with sortilin on motor neurons (Hu et al., 2010). Both *sort1a* and *sort1b* transcripts are expressed in the developing zebrafish embryo and adult retina. Further studies are needed to determine the specific temporal and spatial expression patterns of Sort1a and Sort1b in the developing zebrafish embryo. Whether *sort1a* and *sort1b* are expressed by microglial precursors and/or neural progenitors remains unknown. Although, *in vivo*, the neuroprotective effects of PGRN following subarachnoid hemorrhage injury in rats are dependent on sortilin (Li et al., 2015), other evidence indicates sortilin may not be required for the neurotrophic effects of PGRN (De Muynck et al., 2013). *In vitro*, PGRN regulation of neurite outgrowth and branching in mouse hippocampal neurons is independent of sortilin, therefore implicating other receptors (Gass et al., 2012). The expression and function of TNFR in the developing zebrafish retina remains to be determined. To elucidate the mechanism by which Pgrn-a attracts microglial precursors to migrate into the CNS and regulates retinal progenitor cell cycle, determining which Pgrn-a receptors are differentially expressed by early macrophages and neural progenitors, respectively, is key. Further, components of both the Wnt and Notch signaling pathways are altered following Pgrn-a knockdown. Whether these changes in Wnt and Notch signaling are a cause or read out of Pgrn-a knockdown phenotype remains to be determined.

The data presented here provide insight into the molecular mechanisms downstream of Pgrn-a during zebrafish retinal development. Transgenic and reverse

genetics approaches were employed to create a *pgrn-a* reporter line incorporating the NTR/Met conditional targeted cell ablation system and a *pgrn-a* mutant line. Tol2-BAC constructs were made and confirmed by PCR and sequencing, and now can be used to create Tg(*pgrna*:*nfsB-EGFP*) and Tg(*pgrna*:*nfsB-mCh*) transgenic reporter lines. Taking advantage of the NHEJ repair mechanism, the CRISPR/Cas9 system generated indels in the *pgrn-a* gene, and multiple mutant lines are being propagated for phenotype screening. The *pgrna*$^{-/-}$_90_5M_2F7M line has no retinal phenotype and expresses normal levels of Pgrn-a. Therefore, the 10bp deletion was not sufficient to knock out *pgrn-a*. However, additional lines with different indel mutations may yield different results. A stable *pgrn-a* reporter/NTR line and validated *pgrna*$^{-/-}$ line will become valuable resources for our lab and others, and will serve as the basis for future studies on the role of microglia in the developing and adult/regenerating retina and the function of Pgrn-a during retinal development and regeneration. The future studies proposed using these lines are detailed in the final chapter of this thesis.

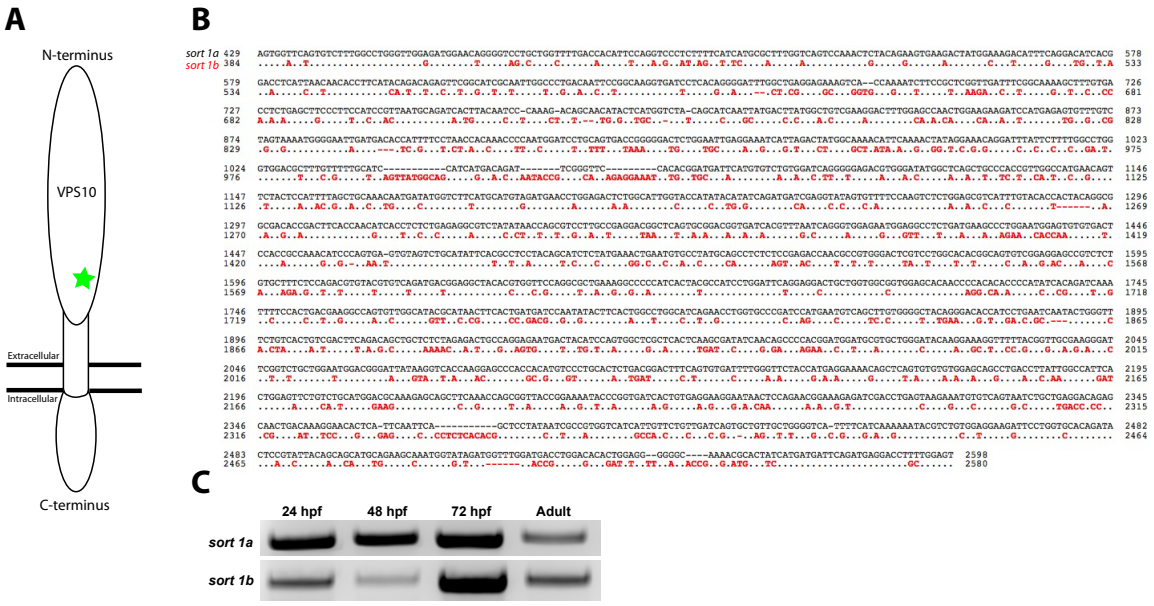


Figure 3.1: Zebrafish sortilin receptors. (A) Diagram of Sortilin receptor structure. PGRN binding site represented as green star. **(B)** Zebrafish *sort1a* and *sort1b* sequence alignment generated by NCBI Nucleotide BLAST. **(C)** RT-PCR of *sort1a* and *sort1b* expression in 24hpf de-yolked embryos, 48 and 72hpf embryo heads, and adult retinas.

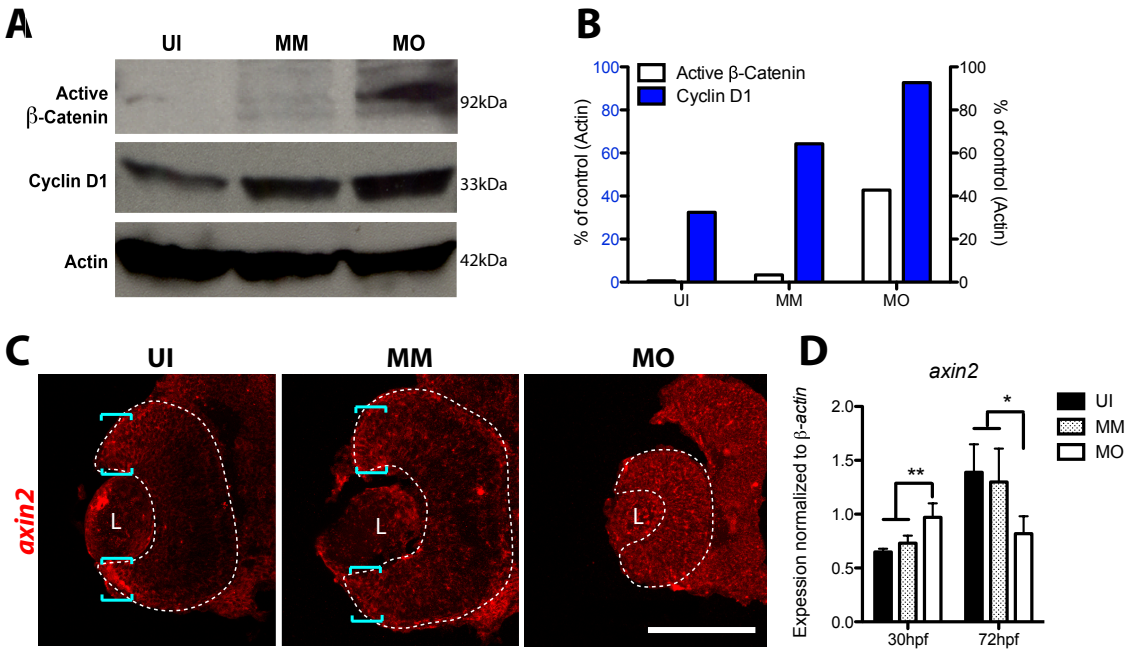


Figure 3.2: Canonical Wnt signaling is upregulated following Pgrn-a knockdown. (A) Western blot of Active β-catenin, Cyclin D1, and Actin in 48hpf uninjected (UI), mismatch morpholino-injected (MM), and 5'UTR morpholino-injected (MO) embryos. **(B)** Histogram showing quantification of Active β-catenin in UI (0.59%), MM (3.39%), and MO (42.76%), and Cyclin D1 in UI (32.45%), MM (64.30%), and MO (92.72%). Quantitative data from one biological replicate is normalized to Actin. **(C)** *axin2 in situ* hybridization in 48hpf UI, MM, and MO embryo retinas (dotted outline). Lens (L); ciliary marginal zone (CMZ, brackets); scale bar equals 75μm. **(D)** Histogram showing relative *axin2* mRNA expression normalized to *beta-actin* at 30 and 72hpf; *p≤0.05, **p≤0.01. Quantitative data are represented as mean; error bars represent the standard deviation.

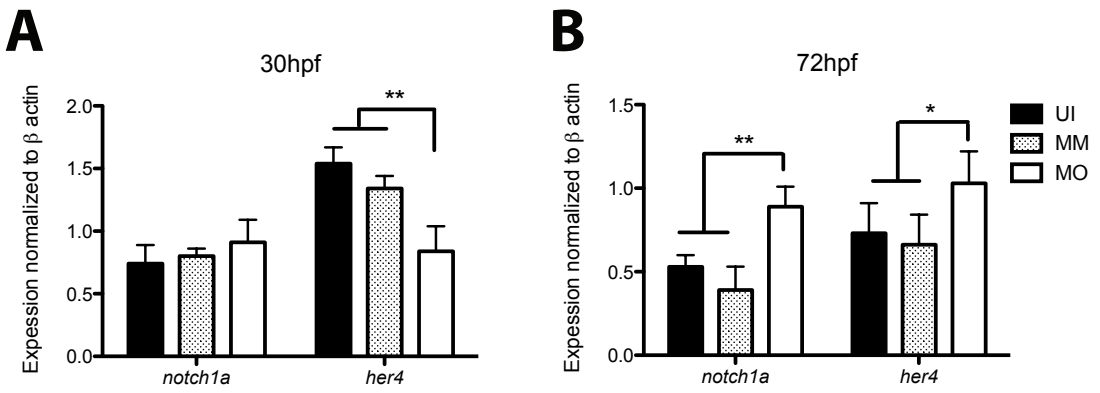


Figure 3.3: Notch signaling pathway gene expression is altered following Pgrn-a knockdown. (A) Histogram showing relative *notch1a* and *her4* mRNA expression normalized to *beta-actin* at 30hpf. **(B)** Histogram showing relative *notch1a* and *her4* mRNA expression normalized to *beta-actin* at 72hpf. Uninjected (UI), mismatch morpholino-injected (MM), 5'UTR morpholino-injected (MO); *p≤0.05, **p≤0.01. Quantitative data are represented as mean; error bars represent the standard deviation.

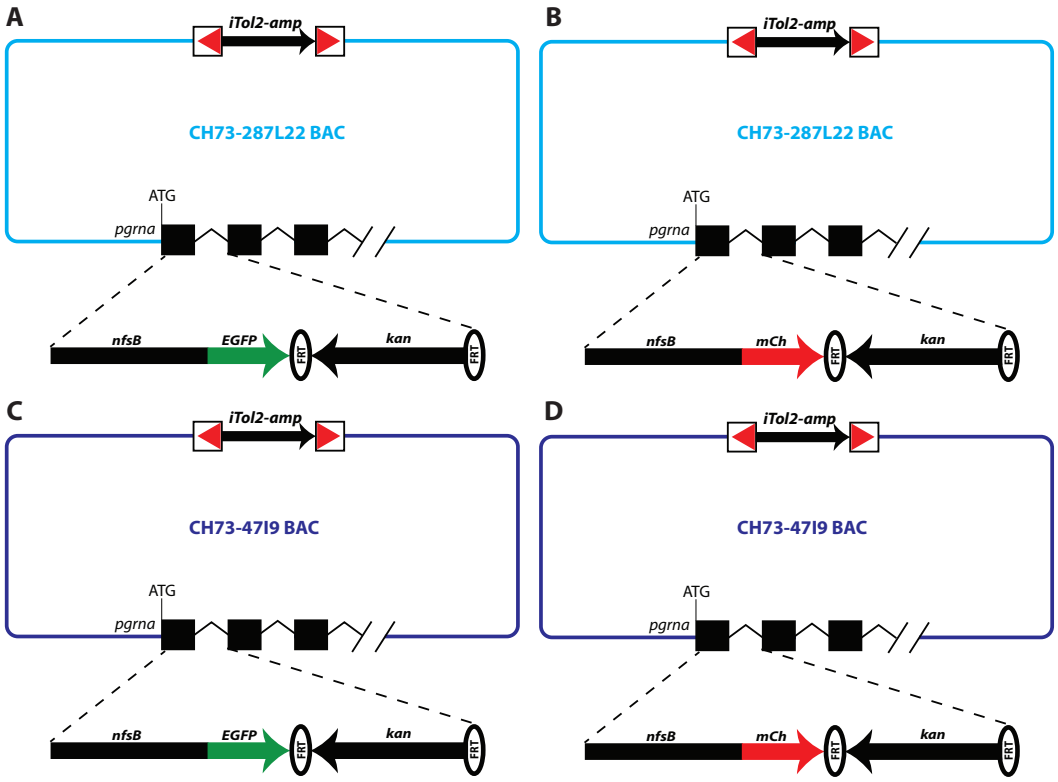
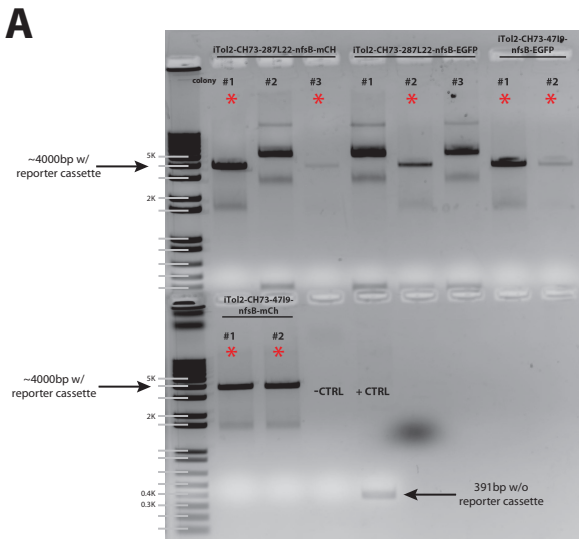
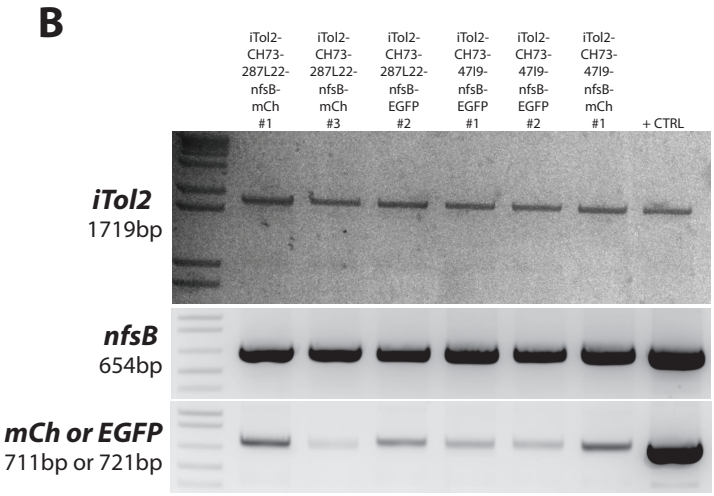


Figure 3.4: Representative maps of recombineered *pgrna*:*nfsB-EGFP* and *pgrna*:*nfsB-mCh* BAC constructs. (A) iTol2 cassette-containing CH73-287L22 BAC with *pgrna*:*nfsB-EGFP* reporter cassette inserted into the *pgrn-a* start site. **(B)** iTol2 cassette-containing CH73-287L22 BAC with *pgrna*:*nfsB-mCh* reporter cassette inserted into the *pgrn-a* start site. **(C)** iTol2 cassette-containing CH73-47I9 BAC with *pgrna*:*nfsB-EGFP* reporter cassette inserted into the *pgrn-a* start site. **(D)** iTol2 cassette-containing CH73-47I9 BAC with *pgrna*:*nfsB-mCh* reporter cassette inserted into the *pgrn-a* start site.


A
iTol2-CH73-287L22-nfsB-mCH
iTol2-CH73-287L22-nfsB-EGFP
iTol2-CH73-47I9-nfsB-EGFP
colony #1 #2 #3 #1 #2 #3 #1 #2
~4000bp w/ reporter cassette
5K
2K
iTol2-CH73-47I9-nfsB-mCh
#1 #2
~4000bp w/ reporter cassette
-CTRL
+ CTRL
0.4K
0.3K
391bp w/o reporter cassette


B
iTol2-CH73-287L22-nfsB-mCh #1
iTol2-CH73-287L22-nfsB-mCh #3
iTol2-CH73-287L22-nfsB-EGFP #2
iTol2-CH73-47I9-nfsB-EGFP #1
iTol2-CH73-47I9-nfsB-EGFP #2
iTol2-CH73-47I9-nfsB-mCh #1
+ CTRL
iTol2
1719bp
nfsB
654bp
mCh or EGFP
711bp or 721bp

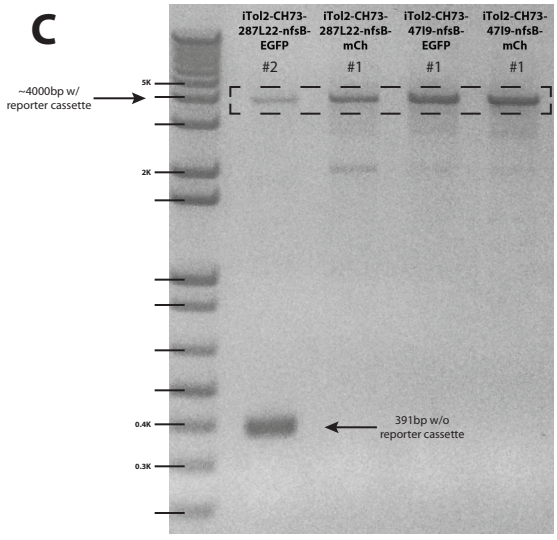

C
iTol2-CH73-287L22-nfsB-EGFP #2
iTol2-CH73-287L22-nfsB-mCh #1
iTol2-CH73-47I9-nfsB-EGFP #1
iTol2-CH73-47I9-nfsB-mCh #1
~4000bp w/ reporter cassette
5K
2K
0.4K
0.3K
391bp w/o reporter cassette

Figure 3.5: Confirmation of BAC constructs. (A) RT-PCR analysis of three kanamycin-positive colonies from iTol2-CH73-287L22 *pgrna*:*nfsB-mCh* and iTol2-CH73-287L22 *pgrna*:*nfsB-EGFP* BAC recombinants, and two kanamycin-positive colonies from iTol2-CH73-47I9 *pgrna*:*nfsB-EGFP* and iTol2-CH73-47I9 *pgrna*:*nfsB-mCh* BAC recombinants using the ATG primer set. BAC constructs containing the ~4000bp reporter cassette are denoted by a red asterisks (*). No template and the original CH73-47I9 BAC were used as negative and positive controls, respectively. **(B)** RT-PCR analysis of the seven BAC constructs containing the reporter gene cassette using iTol2, nfsB, mCh and EGFP primer sets. The pCR8GW-iTol2-amp plasmid, and T2KXIG-*zop*:*nfsB-EGFP* and T2KIXG-*ins*:*nfsB-mCherry* expression constructs were used as a positive controls. **(C)** RT-PCR analysis using ATG primer set. Amplified PCR products were analyzed by electrophoresis next to a 10Kb DNA ladder, and ~4Kb bands (outlined by the dotted line) were gel purified and sequenced.

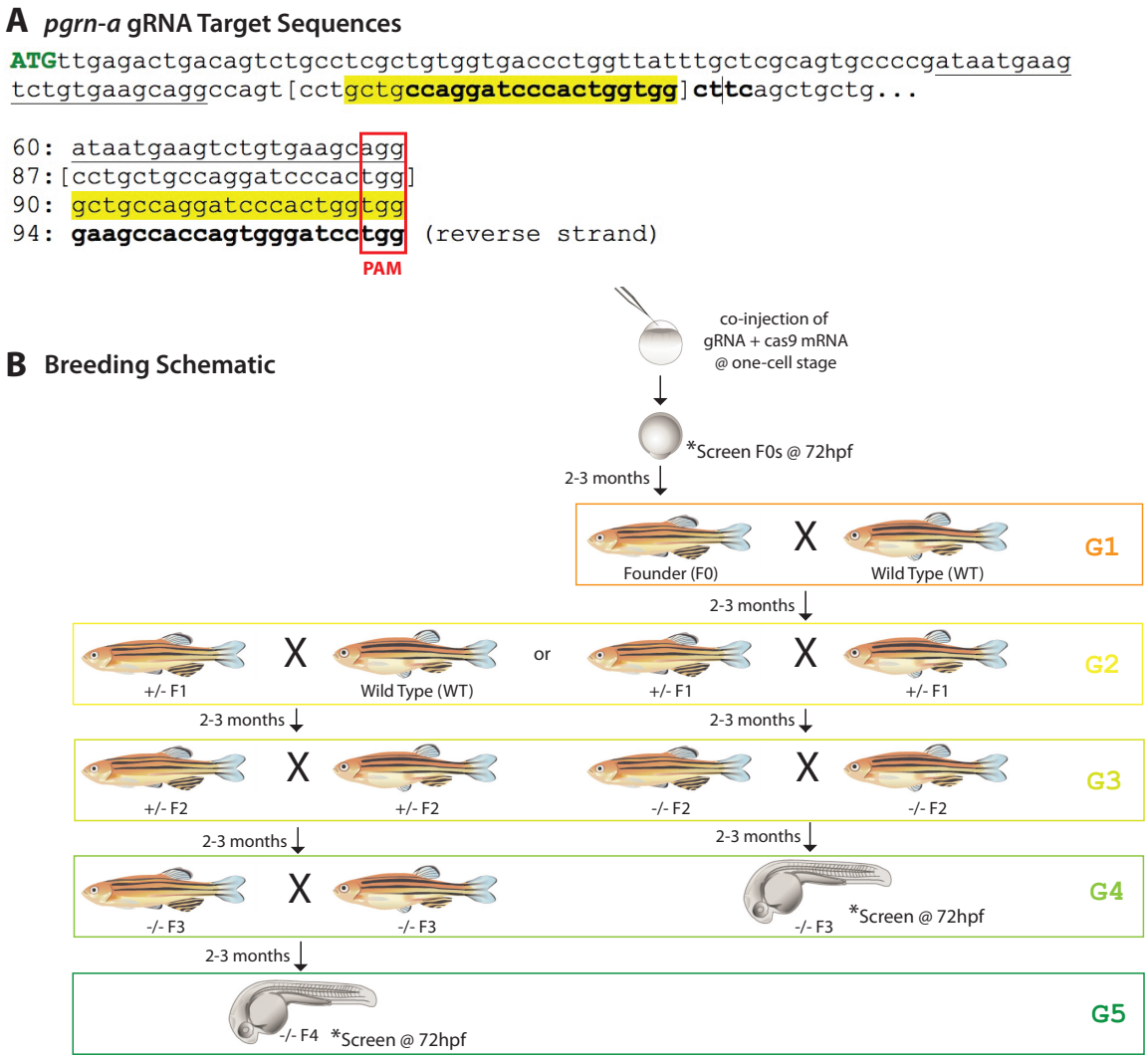


Figure 3.6: CRISPR/Cas-9 genome engineering system for *pgrn-a* mutant line. (A) Four individual *pgrn-a* gRNA target sites and sequences; different target sites are distinguished by underline, brackets, highlighting in yellow, or bold font. Numbers (60, 87, 90, 94) denote the target ID given by Sigma-Aldrich. The PAM sequence for each gRNA is outlined by the red box. **(B)** Injection and breeding schematic to generate stable *pgrn-a* mutant line (adapted from Kawakami, 2007). Generation 1 (G1); Generation 2 (G2); Generation 3 (G3); Generation 4 (G4); Generation 5 (G5).

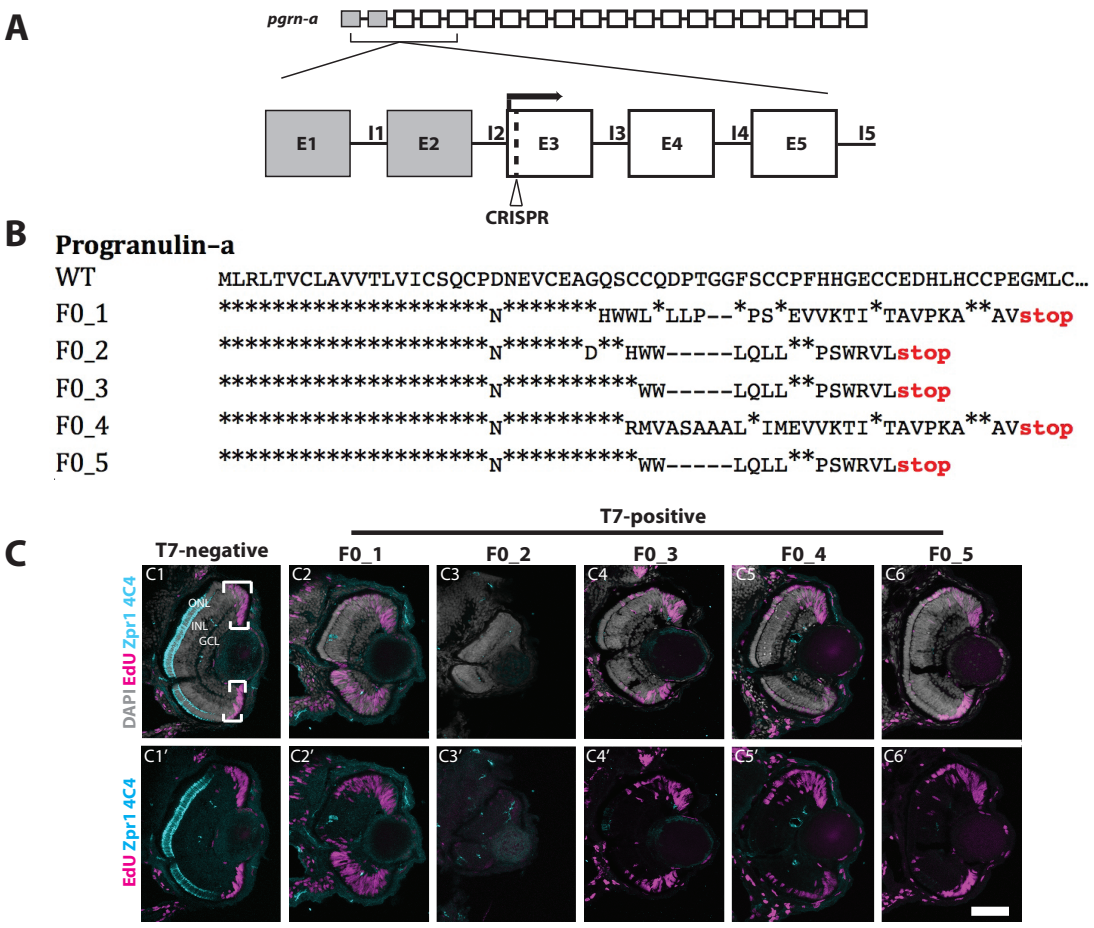


Figure 3.7: Characterization of F0 *pgrn-a* mosaic mutants. (A) A diagram of *pgrn-a* gene structure denoting the approximate location of RNA target site on the first coding exon 3. **(B)** Predicted amino acid sequence of WT and five F0 *pgrn-a* mosaic mutant embryos. **(C)** Traverse sections through central retina of T7-negative/WT and five T7-positive/F0 mosaic mutant embryos at 72hpf. Sections are immunolabeled with cell-specific antibody markers (cyan) for red-green double cone photoreceptors (ZPR1), microglia (4C4), EdU (fusia), and DAPI (gray). Scale bar equals 50μm.

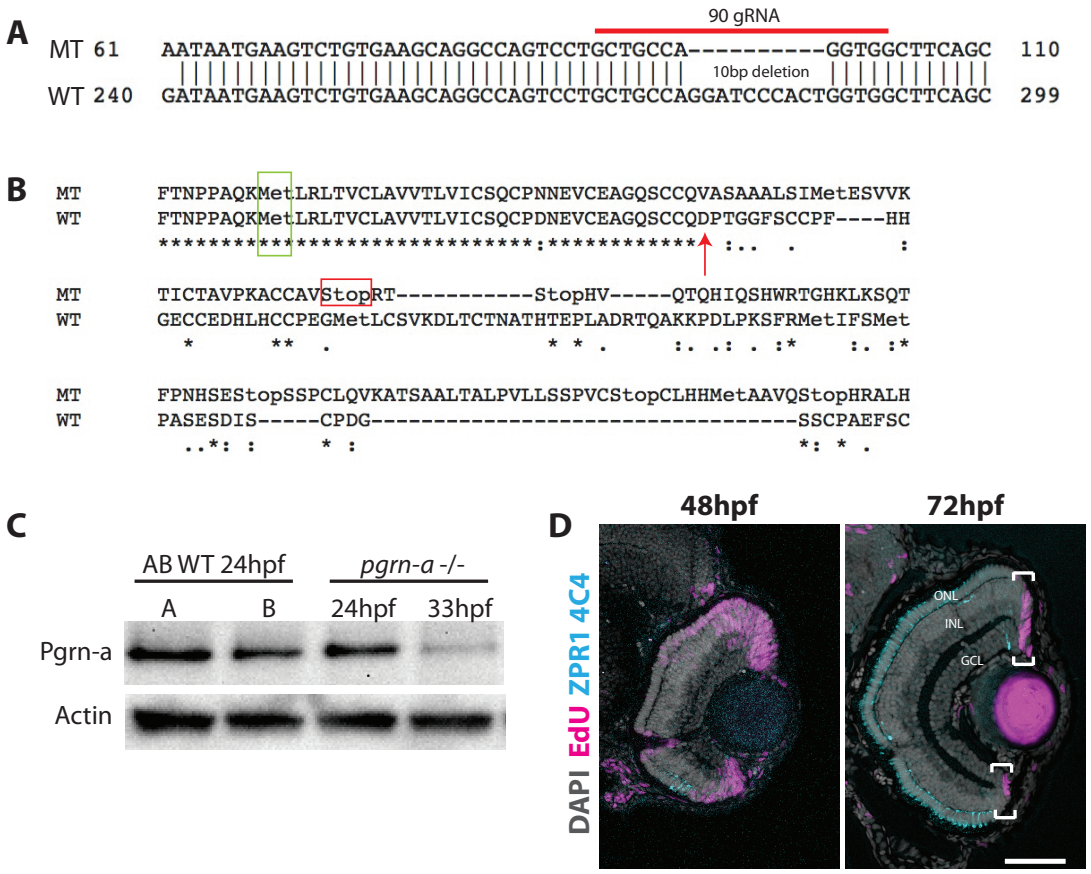


Figure 3.8: Characterization of F3 *pgrn-a* homozygous (*pgrna*$^{-/-}$) mutants. (A) The nucleic acid sequence of mutant (top) and WT (bottom) *pgrn-a*. sgRNA 90 target site is represented by a red line. **(B)** The predicted amino acid sequence of mutant (top) and WT (bottom) Pgrn-a. A green box outlines translation start site. A red arrow marks the reading frame shift caused by the 10bp deletion. A red box outlines the premature stop codon. **(C)** Western blot showing Pgrn-a and Actin expression in two biological replicates of 24hpf AB WT and 24 and 33hpf *pgrna*$^{-/-}$ embryos. **(D)** Traverse sections through central retina of 48 and 72hpf *pgrna*$^{-/-}$ embryos. Sections are immunolabeled with cell-specific antibody markers (cyan) for red-green double cone photoreceptors (ZPR1), EdU (fusia), and DAPI (gray). Scale bar equals 50μm.

Table 3.1: List of Antibodies

Primary Antibodies	Company	Dilution
Monoclonal anti-Zn5	ZIRC; zfin.org/ZDB-ATB-081002-19	1:200
Monoclonal anti-Zpr1 (anti-Arrestin)	ZIRC; zfin.org/ZDB-ATB-081002-43	1:200
Mouse anti-HPC1 (anti-Syntaxin)	Sigma S0664	1:200
Mouse anti-BrdU	BD Biosciences 347580	1:100
Secondary Antibodies		
Alexa Fluor goat anti-mouse 488/555	Invitrogen	1:500

Table 3.2: Restriction Enzymes and RNA Polymerases for *axin2* and *sort1a/b* Riboprobe Synthesis

Probe	Restriction Enzyme	RNA Polymerase
axin2 sense	NotI	T3
axin2 anti-sense	PmeI	T7
sort1a sense	SacI	T7
sort1a anti-sense	SacII	SP6
sort1b sense	SacII	SP6
sort1b anti-sense	SacI	T7

Table 3.3: Primer Sequences for *sort1a/b* Riboprobe Synthesis

Gene Name	Forward Primer	Reverse Primer
sort1a	GCTGTCGAAGGACTTTGGAG	AACCACGTGTAGCCTCCATC
sort1b	CCACCAATAGGAGGTGCTGT	AACGTCGAGAGCCAAG

Table 3.4: Primer Sequences for BAC Recombineering and Screening

Name	Forward Primer	Reverse Primer
pgrn-a_ATG	TTGGAGATCATCTGCCTCC	GCAGACCACTTCATCTGTG
UP10K	GCTGCTGCACTCAAGTTTGG	CAATACAGCCAACCAAGTG
DOWN10K	GCCCACTGGCTTAGATGTAAC	GTAGTGCGTTGAGTTTCAGG
ptarbac_iTol2	GCGTAAGCGGGGCACATTTC ATTACCTCTTTCTCCGCACCC GACATAGATCCCTGCTCGA	CGCGGGGCATGACTATTGGC GCGCCGGATCGATCCTTAATT AAGTCTACTAATTATGATC
iTol2_screen	CTGAATAAGTGATAATAAGCG	GATCCTCTCCCTATAGTGAGT CG
pA	GCGGCCGCCACCGCGGTGG AGCTC	GATCTAGAGGATCATAATCAG CCA
mCh	ATGGTGAGCAAGGGCGAGGA GGAT	TTACTTGTACAGCTCGTCCAT GCC
GFP	ATGGTGAGCAAGGGCGAGGA GCTG	TTACTTGTACAGCTCGTCCAT GCC
NTR	ATGGATATCATTTCTGTCGCC TTAAAGCG	TTACACTTCGGTTAAGGTGAT GTT
pgrnaNTR_F & pgrnaFRTKanFRT_R	GTTTGCATAAGAATTTTTTTCT GACATATTTCGACTTTCTTTTC ATCCAGATGGATATCATTTCT GTCGCCTTAAAGCGT	TCTGATCCACTTTCTACCACC TAACTGCTTATTATGTATGAGT TTCTCACATACGAAGTTATATT ATGTACCTGACTGAT
NTR30	CTCGAGCTCGAGCTCGAGCT CGAGCTCGAGATGGATATCAT TTCTGTCGCCTTA	AAGCTTAAGCTTAAGCTTAAG CTTAAGCTTGATCTAGA GGATCATAATCAGCCA

Table 3.5: *pgrn-a*-targeting sgRNA Sequences

gRNA ID	Concentration	Target Site
NM001001949reg1_0_60	200 ng/ul	ATAATGAAGTCTGTGAAGCAGG
NM001001949reg1_0_87	200 ng/ul	CCTGCTGCCAGGATCCCACTGG
NM001001949reg1_0_90	200 ng/ul	GCTGCCAGGATCCCACTGGTGG
NM001001949reg1_0_94	200 ng/ul	GAAGCCACCAGTGGGATCCTGG

Table 3.6: Primer Sequences for CRISPR/Cas-9 Genome Editing of *pgrn-a*

Name	Forward Primer	Reverse Primer
pgrna_CRISPR_screen	AATAAGGCAACAAGGCACTG	ATCAAGTTCTACATCTGGTC

Table 3.7: Status of *pgrn-a* mutant lines

Line	sgRNA	Name	Mutation/ InDel	Predicted AA Sequence	Breeding Stage	Homozygous Phenotype
1	90	*pgrna*$^{-/-}$_90_5M_2F7M	10bp deletion	Frame shift, premature stop codon	F3 homozygous embryos; 4th generation	Pgrn-a detectable by Western blot. No retinal phenotype.
2	*90*	*pgrna*$^{-/-}$_90_5M_4F	14bp deletion	Frame shift, premature stop codon	F2 heterozygous adults; 3rd generation	TDB
3	*94*	*pgrna*$^{-/-}$ _94_4M_1M5F11M	8bp deletion	Frame shift, premature stop codon	F2 homozygous adults; 3rd generation	TDB
		pgrna$^{-/-}$_94_4M_3M9F	14bp deletion	Frame shift, premature stop codon	F2 homozygous adults; 3rd generation	TDB
		pgrna$^{-/-}$_94_4M_2M7F	17bp deletion	Frame shift, premature stop codon	F2 homozygous adults; 3rd generation	TDB
4	*94*	*pgrna*$^{-/-}$ _94_5M_3M5M9M	5bp insertion	Frame shift, premature stop codon	F2 heterozygous adults; 3rd generation	TDB

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Chapter 4

Discussion and Future Directions

Summary of Results

Using zebrafish retinal development as a model, I tested the hypothesis that microglia and the factors they express and secrete play a fundamental role in vertebrate neurogenesis. I utilized genetic and pharmacological models that prevent macrophages/microglial precursors from colonizing neuroepithelial tissues as a means to test this hypothesis. I also used a reverse genetics approach to determine the function of the microglia-specific growth factor, Pgrn-a, in the developing zebrafish retina, and collect preliminary data regarding potential downstream signaling pathways. In addition, to enable further study of the function of microglia and Pgrn-a in the developing, adult, and regenerating retina, I 1) recombineered BAC constructs to generate transgenic *pgrn-a* reporter zebrafish lines that will enable conditional targeted genetic ablation of microglial cells, and 2) used genome engineering technology to establish a *pgrn-a* mutant zebrafish line.

In chapter 2, I presented data that demonstrate microglia govern early developmental neurogenesis in the retina. Both genetic and pharmacological approaches gave equivalent results: when microglia fail to colonize the retina, early neurogenesis is substantially altered. Retinal progenitors do not exit the cell

cycle at the appropriate developmental time, and both early retinal growth and neuronal differentiation is delayed. I also evaluated the expression and function of Pgrn-a during embryonic retinal neurogenesis. I showed there is a dynamic temporal and spatial pattern of *pgrn-a* expression in the developing zebrafish retina. At 24hpf, *pgrn-a* and Pgrn-a are ubiquitously expressed throughout the zebrafish forebrain and retina, but by 48hpf *pgrn-a* becomes exclusively expressed by microglia and their precursors. For loss-of-function experiments, I used morpholino oligonucleotides to knockdown Pgrn-a either by blocking protein translation or pre-mRNA splicing. I presented data demonstrating that compared to the genetic and pharmacological models that exclude microglia from the retina, knockdown of Pgrn-a resulted in similar, albeit more severe retinal phenotype. Blocking Pgrn-a translation diminished the number of microglia in the retina. Moreover, Pgrn-a knockdown caused a significant increase in the length of the cell cycle and a corresponding paucity of neuronal differentiation. Depleting of Pgrn-a increased the expression of genes that promote cell cycle progression and decreased the expression of genes that promote cell cycle exit. By extension, the more severe developmental defects observed following Pgrn-a knockdown were interpreted as a consequence of both the absence of retinal microglia and Pgrn-a.

From these data, we conclude that microglial colonization of embryonic neuroepithilial tissues is required for normal early neurogenic events in the developing retina. Further, Pgrn-a functions to recruit microglial precursors to the embryonic CNS, govern the rate at which retinal progenitors progress through

the cell cycle and time cell cycle exit and neuronal differentiation. Therefore, the physical presence of microglia in the embryonic retina and the presumptive regulatory molecules they secrete play fundamental roles in governing early developmental neurogenesis.

In chapter 3, I presented preliminary data on the downstream signaling mechanisms of Pgrn-a. I showed that neuronal Pgrn-a receptor transcripts, *sort1a* and *sort1b*, are expressed in the developing embryo and adult zebrafish retina. Further, following knockdown of Pgrn-a, canonical Wnt signaling is upregulated in the embryonic retina, and Notch signaling pathway genes are altered during embryogenesis. These studies provided insight into the molecular mechanisms downstream of Pgrn-a during zebrafish retinal development. I also used bacterial recombineering to create BAC constructs encoding a nitroreductase-fluorescent reporter fusion protein under the control of the *pgrn-a* promoter and regulatory elements. I presented data confirming the BAC constructs contain the iTol2 and reporter cassettes necessary to generate stable Tg(*pgrna*:*nfsB-EGFP*) and Tg(*pgrna*:*nfsB-mCh*) transgenic zebrafish lines. Finally, I utilized the CRISPR/Cas-9 genome engineering technology to create targeted changes in the *pgrn-a* loci of zebrafish. I presented data demonstrating one of the four lines propagated carrying a 10bp deletion in *pgrn-a* expressed normal levels of Pgrn-a and possessed no retinal phenotypes. Finally, I detailed the status of lines that remain to be screened for the presence or absence of Pgrn-a and retinal phenotype. Once established, these lines will be used to

enhance future studies on the expression and function of Pgrn-a and the function of microglial cells in the developing and adult/regenerating zebrafish retina.

Microglia and Pgrn-a Regulate Early Neurogenic Events in the Retina

Microglia are emerging as important regulators of neurogenic events in the developing CNS (Chapter 1). Moreover, it has been demonstrated that microglia govern neurogenesis in part via the molecules they express and secrete (Morgan et al., 2004; Walton et al., 2006; Antony et al., 2011; Nikolakopoulou et al., 2013). Neurogenesis, the process by which neurons are generated from neural progenitor cells, includes neural progenitor proliferation, cell division, cell cycle exit, terminal differentiation, survival, and cell migration. The precise role of microglia and/or their secreted factors in regulating neurogenesis in the developing retina is not completely understood. The data presented in chapter 2 demonstrate that when microglia and the microglia-specific protein, Pgrn-a, are excluded from the retina, retinal neurogenesis is significantly altered. Specifically, the length of retinal progenitor cell cycle is significantly increased and neuronal differentiation is delayed. In *pgrn-a* morphants, retinal progenitors take significantly longer to transit both G1- and G2-phase and undergo mitosis (Figures 2.12 and 4.1), which serves as a mechanistic explanation for the delay in retinal neurogenesis observed when microglia and the molecules they release are absent from the retina. The fact that Pgrn-a knockdown did not increase cell death in the developing retina suggests that Pgrn-a does not govern survival of retinal progenitor cells (Figure 2.10). Further, the recovery of retinal development

in *panther* mutants, PLX5622-treated embryos, and *pgrn-a* morphants suggests that microglia and Pgrn-a do not govern cell fate determination or cell migration or the developmental processes that regulate spatial cues, synaptogenesis and retinal organization that give rise to the laminar structure of the retina (Figures 2.3 and 2.9). Interestingly, at 8dpf, recovered *panther* mutant and *pgrn-a* morphant retinas are fully differentiated, but still lack WT levels of microglia. Therefore, the colonization of neuroepithelial tissues by microglia seems to be critical for only early neurogenic events. Microglia and the factors they secrete determine the timing of cells exiting the cell cycle, but other cellular and/or molecular mechanisms remain unaltered and drive retinal neurogenesis to completion later in development.

Signaling Downstream of Pgrn-a

In retinal progenitors, Pgrn-a promotes progression through and exit from the cell cycle by regulating the expression of cell cycle regulatory genes. Data presented in chapters 2 and 3 demonstrate that knockdown of Pgrn-a results in a significant increase in cyclin D1 mRNA and protein (Figures 2.12 and 3.2) and significant decrease in CDK inhibitor *p27* mRNA (Figure 2.12). Cyclin D/CDK complexes promote progression through G1 and the transition between G1 and S-phase of the cell cycle, whereas, p27 interacts with and inhibits Cyclin D1, preventing G1-phase progression and promoting cell cycle exit (Dyer and Cepko, 2001a; b). Cyclin D1 is a downstream target of the canonical Wnt signaling pathway (Denayer et al., 2008). As it might be predicted, knockdown of Pgrn-a results in a significant upregulation of canonical Wnt signaling (Rosen et al.,

2011) Figure 2.12 and 3.2). Notch signaling negatively regulates *p27* expression (Hristova et al., 2013; Del Debbio et al., 2016). Similarly, knockdown of Pgrn-a resulted in a significant upregulation of genes in the Notch signaling pathway at 72hpf (Figure 3.3). Based on these data, it appears that in the normal developing retina, Pgrn-a promotes withdrawal of retinal progenitors from the cell cycle and neuronal differentiation in part by negatively regulating both Wnt and Notch signaling. Decreased Wnt and Notch signaling leads to down regulation of Cyclin D1 and up regulation of p27, leading to retinal progenitor cell cycle exit and neuronal differentiation. Though, to establish whether Pgrn-a acts on cell cycle machinery via the Wnt and/or Notch signaling pathways requires a great deal of further investigation (detailed below).

Previous reports have demonstrated that in embryonic and adult epithelial cells, PGRN acts on the cell cycle both as a competence and progression factor, promoting mitosis by stimulating classic growth factor signal transduction cascades, such as mitogen-activated protein kinase (MAPK)/extracellular-signal-related kinase (ERK) and phosphatidylinositol 3-kinase (PI3K) pathways (Ong and Bateman, 2003; Bateman and Bennett, 2009). Cross-talk between the MAPK/ERK and canonical Wnt signaling pathways has been widely reported (Bikkavilli and Malbon, 2009). Further, *in vitro*, growth factor stimulation of MAPK signaling strongly induces histone deacetylase 1 (HDAC1) expression (Bartl et al., 1997; Hauser et al., 2002). In zebrafish, Hdac1 functions upstream of the cyclin D1/p27 interaction, and regulates retinal progenitor proliferation and neuronal differentiation by inhibiting both Wnt and Notch signaling (Yamaguchi et

al., 2005). It is therefore possible that Pgrn-a acts through the MAPK/ERK signaling pathways or *Hdac1* gene to regulate Wnt and Notch signaling.

To begin to elucidate the complex signaling downstream of Pgrn-a, an important next step is to determine which Pgrn-a receptors are differentially expressed by early macrophages and neural progenitors. There are two putative receptors for PGRN: Sortilin and tumor necrosis factor receptor (TNFR). Sortilin was identified as a neuron-specific PGRN receptor (Hu et al., 2010), though microglia-specific Sortilin expression has been demonstrated, both *in vitro* (Dicou et al., 2004; Hu et al., 2010) and *in vivo* (Reuter et al., 2015). PGRN also binds to TNFR with high affinity (Tang et al., 2011). Whether PGRN binds to TNFR expressed on neurons and/or microglia remains unknown. Pgrn-a may differentially regulate microglial precursor migration into the CNS and retinal progenitor cell cycle through the Sortilin, TNFR and/or other receptors not yet identified. Likewise, whether the receptors that mediate the function of Pgrn-a in the developing versus adult/regenerating retina are the same or different remains to be determined.

Evidence of the neurogenic role microglia play in the adult CNS is also increasing (Chapter 1). Previous studies in birds demonstrated that microglia are required for the injury-induced proliferative response of Müller glia-derived neural progenitor cells in the retina (Fischer et al., 2014). Unlike mammals, zebrafish possess the capacity to regenerate many tissues after injury, including fin, heart, brain and retina (Gemberling et al., 2003). Hence, the zebrafish has become a well-established model for studying both retinal development and regeneration

(Glass and Dahm, 2004; Hitchcock and Raymond, 2004; Raymond et al., 2006; Bernardos et al., 2007; Fadool and Dowling, 2008). In the adult zebrafish retina, following light damage, Tnfα is significantly increased in dying retinal neurons and is required for the proliferative response of Müller glial cells during retinal regeneration (Nelson et al., 2013). Similarly, *pgrn-a*, expressed exclusively by microglia in the adult zebrafish retina, is significantly upregulated in retinal microglia after light-induced photoreceptor cell death and during photoreceptor regeneration (Craig et al., 2008). When bound to TNFR, Pgrn-a antagonizes Tnfα signaling, promoting anti-inflammatory effects (Tang et al., 2011). Whether microglia and/or the factors they express or secrete, including the growth factor Pgrn-a, regulate neurogenesis in the regenerating zebrafish retina remains to be determined and is an important and exciting area of future research. Determining whether Pgrn-a regulates retinal regeneration by mediating Tnfα signaling via the TNFR is a potential topic for future study.

Pgrn-a Regulation

In addition to identifying the molecular targets (receptors and signaling pathways) downstream of Pgrn-a, it is also important to understand how Pgrn-a expression is regulated within the developing, adult and regenerating retina. Data presented in Chapter 2 demonstrate *pgrn-a* is expressed in a dynamic temporal and spatial pattern during retinal development. At 24hpf, *pgrn-a* is expressed by all neural progenitors, but beginning around 36hpf *pgrn-a* expression becomes limited to microglia and their precursors in the yolk sac, brain and retina. In the

adult retina, *pgrn-a* continues to be expressed exclusively by microglia, and is upregulated in microglia following retinal injury (Craig et al., 2008). Understanding how *pgrn-a* expression is regulated in space and time in both the normal and injured retina could provide further insight into the neural progenitor-specific versus microglia-specific functions of Pgrn-a during neurogenesis.

In the developing and adult mammalian CNS, PGRN is expressed by neurons and microglia (Daniel et al., 2000; Suzuki and Nishiahara, 2002; Daniel et al., 2003). Therefore, as an aside, the zebrafish retina provides a unique model to study the microglia-specific function of *pgrn-a*. Insights gained from the zebrafish retina on microglia-specific *pgrn-a* function may inform our limited understanding of the function of PGRN in the mammalian CNS (Eriksen, 2010). Extracellular and intracellular PGRN levels are known to be regulated by proteolysis and receptor-mediated endocytosis. Secreted PGRN can be proteolytically cleaved by emzymes, such as elastase, protease-3 and metalloproteinases, into smaller peptide fragments called granulins (Zhu et al., 2002; Kessenbrock et al., 2008; Suh et al., 2012). The secreted leukocyte protease inhibitor (SLPI) can bind to and inhibit PGRN proteolysis (Zhu et al., 2002). Sortilin, an endocytic and intracellular sorting receptor that mediates protein trafficking between the cell surface and intracellular compartments (Hermey, 2009), binds PGRN and is thought to regulate both exracellular and intracellular PGRN levels (Hu et al., 2010). Whether Pgrn-a expression in the zebrafish retina is regulated by similar mechanisms remains to be demonstrated.

The abovementioned studies provided insight into PGRN regulation at the post-translational level. However, little is known about the transcriptional and post-transcriptional regulation of the progranulin gene (*GRN*/*Grn*). One study identified microRNA-29b as a post-transcriptional regulator of PGRN expression (Jiao et al., 2010). Also, CNS injury and disease induces microglia-specific upregulation of progranulin message and protein expression (Craig et al., 2008; Moisse et al., 2009; Naphade et al., 2009; Philips et al., 2010). In fact, dysregulation of PGRN expression in the CNS is related to multiple brain pathologies. Within the CNS, increased PGRN expression is correlated with brain cancer malignancy (Liau et al., 2000; De Muynck and Van Damme, 2011; Wang et al., 2011), whereas decreased PGRN expression is associated with neurodegeneration and dementia (Baker et al., 2006; Cruts et al., 2006). Therefore, a fuller understanding of the regulation of PGRN expression may enhance strategies for therapeutic intervention for CNS disorders and diseases caused by aberrant PGRN levels.

In peripheral tissues and within the CNS, PGRN functions as both an autocrine and paracrine regulator of cell growth and cell migration (Chapter 1). Data presented in chapter 2 were the first to demonstrate that, in the developing zebrafish embryo, Pgrn-a is a non-cell autonomous regulator of microglial precursor migration to and colonization of neuroepithelial tissues (see also (Pickford et al., 2011). My data suggest that neural progenitors in the forebrain serve as a discrete source of Pgrn-a, establishing a morphogenic gradient between the brain and yolk sac that is sensed by microglial precursors. These

data also provide interesting insight into the dynamic cross talk or bidirectional signaling between neurons and microglia during developmental neurogenesis (Eyo and Wu, 2013). Still, an interesting question remains: if Pgrn-a is a chemoattractant to microglia, then why do microglia not aggregate when *pgrn-a* expression becomes microglia-specific? The most parsimonious explanation is dose-dependence. The attractiveness of Pgrn-a to microglia depends on concentration or the level of Pgrn-a expression. There is precedence for this in the neuroinflammatory literature regarding the acute migratory response of microglia to the site of an injury. Both nitric oxide and ATP stimulate concentration-dependent microglial migration (Chen et al., 2000; Aaron M Miller, 2009; Yuanli Duan, 2009; Miyake et al., 2015). Interestingly, in the injured adult zebrafish retina, *pgrn-a* is strongly upregulated specifically in microglia and there is a corresponding increase in microglial migration to the site of injury (Craig et al., 2008). One could imagine Pgrn-a acts as a chemoattractant in development when expressed by all neural progenitors, but this function is lost when expression becomes restricted to microglia, which are relatively few in number. However, following an injury, the significant increase in *pgrn-a* expression may elicit a chemotactic response in microglia, causing migration towards each other at the site of injury. It is possible, however, that *pgrn-a* expression by neural progenitors is not the driving force attracting primitive macrophages into the CNS, but instead happens to correlate in time. The absence of *pgrn-a* specifically in cells of the microglia lineage may result in a change in their migration. Therefore, an alternative interpretation is that the dependence on *pgrn-a* for

microglial precursor migration is cell-autonomous, whereby, *pgrn-a* expressed in microglial precursors is required for their migration and colonization of the developing CNS.

Limitations and Future Directions

There are several limitations to the methodology and models used for these studies, including 1) the lack of antibodies that label Pgrn-a in zebrafish tissue sections, 2) the limited number of markers of microglial precursors and mature microglia, and 3) the absence of precise spatial and temporal control when using morpholino oligonucleotides. These limitations are discussed below. And, possible future directions to address these limitations are proposed.

Currently, there are no commercially available antibodies that detect zebrafish Pgrn-a in tissue sections. Several individual labs have generated antibodies raised against synthetic Pgrn-a peptides (Chitramuthu et al., 2010; Li et al., 2010; Solchenberger et al., 2015). In general, these antibodies are used to detect denatured Pgrn-a, but are ineffective in labeling Pgrn-a in tissue sections. The polyclonal antibodies raised against residues 244-264 of Pgrn-a (Li et al., 2010), were used here for Western blot analysis (Figures 2.5 and 2.6). One study previously reported Pgrn-a immunostaining of primary motor neurons in whole mount zebrafish embryos using a polyclonal antibody raised against residues 242-256 of a synthetic zebrafish Pgrn-a peptide (Chitramuthu et al., 2010); though, the labeling appeared non-specific, and antibodies were not confirmed in knockdown studies. At 24hpf, the expression of *pgrn-a* was detected throughout the forebrain, and Western blot analysis showed that Pgrn-a is present at 24hpf

(Figure 2.5). Therefore, we inferred that Pgrn-a is reliably expressed where *pgrn-a* transcript is present. However, it is important to confirm localization of Pgrn-a in tissue sections. No *pgrn-a* transgenic reporter line exists, which is, in part, the motivation for creating a BAC transgenic line with a fluorescent reporter under the control of the *pgrn-a* promoter and regulatory elements. Such a line would enable both *in vivo* imaging and *in situ* labeling of Pgrn-a expression.

There are a limited number of markers that distinguish macrophages/microglial precursors from terminally differentiated, mature microglia in zebrafish. In zebrafish, macrophages/microglial precursors migrate from the yolk sac into the head mesenchyme between 22-40hpf, colonize neuroepithelial tissues, including the retina, between 30-48hpf, then undergo a phenotypic transformation around 60hpf to adopt their final microglial state (Herbomel et al., 1999; Herbomel, 2001). Since microglia are derived from mesodermal origins, they share aspects of myeloid cell phenotype and express markers of yolk-sack macrophages. The myeloid ETS transcription factor *pU.1* and macrophage-expressed gene 1 (*mpeg1*) are pan-macrophage or myeloid lineage markers. Transgenic zebrafish lines in which a fluorescent reporter is expressed under the control of the *pU.1* (Tg(*pU1*::*Gal4-UAS*::*eGFP*) and Tg(*pU1*::*Gal4-UAS*::*TagRFP*)) or *mpeg1* (Tg(*mpeg1*:*EGFP*)) promoter were generated and used to label early macrophages/microglial precursors and mature microglia in the brain (Peri and Nüsslein-Volhard, 2008; Ellett et al., 2011; Sieger et al., 2012; Shiau et al., 2013; Meireles et al., 2014; Rossi et al., 2015a). Similarly, in zebrafish, the actin binding protein gene that regulates macrophage

migration, *coronin1a* (*coro1a*), is expressed by leukocytes and myeloid cells, and transgenic reporter lines using the *coro1a* promoter region to drive EGFP have been used to label macrophages/microglial precursors (Li et al., 2012a) and microglia in the brain (Li et al., 2012b). In these transgenic reporter lines, microglia were distinguished from macrophages based on their location within the CNS parenchyma. However, screening of these lines in our lab revealed either minimal reporter expression in the retina at early developmental time points or not all retinal microglia were labeled by the fluorescent reporter. Antibodies that reliably label zebrafish pU.1, Mpeg1, and Coro1a in tissue sections are not currently available commercially.

There are several other markers of microglia used in zebrafish. First is the *fms* gene that encodes the macrophage colony stimulating factor 1 receptor (Csf1r), which is expressed by cells in the macrophage lineage, including early microglia (Herbomel, 2001); see also Figure 2.5). However, the Csf1r antibodies available only detect denatured protein and are used explicitly for Western blot or ELISA assays. Leukocyte-specific plastin (L-plastin) is a cytosolic, actin-bundling protein expressed by the macrophage lineage, and therefore microglial precursors, that is down regulated in mature differentiated microglia in the brain and retina (Herbomel et al., 1999; Herbomel et al., 2001). L-plastin antibodies are used to label leukocytes, macrophages and microglia in zebrafish tissue sections (Herbomel and Levraud, 2005; see also Figure 2.5). Following down regulation of the *L-plastin* gene, mature differentiated microglia begin expressing apolipoprotein E (*apoE*), a neurotropic lipid carrier, after 72hpf (Herbomel et al.,

1999; Herbomel, 2001; Peri and Nüsslein-Volhard, 2008). To date, in zebrafish, *apoE* is the only known marker specific to mature differentiated microglia. To visualize mature microglia in the zebrafish brain, a transgenic reporter line was made using BAC homologous recombineering to insert the lynEGFPpA cassette into the *apoE* locus (Peri and Nüsslein-Volhard, 2008). However, when screening this line in our lab, we found that in the developing retina, both microglia and cells in the CMZ express EGFP. Finally, the 4C4 antibody detects an unknown antigen on CNS microglia (Becker and Becker, 2001; Raymond et al., 2006); see also Figure 2.5). Whether 4C4 labels all or a subset of microglia in the CNS remains unknown. Based on the temporal and spatial expression pattern (Figure 2.5), and previous reports demonstrating *pgrn-a* is expressed exclusively by microglia in the adult retina (Craig et al., 2008), *pgrn-a* can be added to this list of microglial markers. Therefore, generating a stable transgenic *pgrn-a* reporter line would enable visualization of microglia in the developing and adult retina.

An increased understanding of microglial development and maturation in zebrafish is emerging. Recent studies have begun to identify cell autonomous factors that are expressed during different stages of microglial development and regulate microglial precursor migration and differentiation, including *slc7a7* (Rossi et al., 2015b), *xpr1b* (Meireles et al., 2014), *nlrc3-like* (Shiau et al., 2013). Further, in zebrafish, microRNA 124 (*miR-124*) is exclusively expressed by microglia, distinguishing them from macrophages, and regulates microglial maturation (Svahn et al., 2015). Identifying additional distinctive markers of primitive macrophages, microglial precursors, and mature microglia is an active

and important area of research. Such work will provide insight into microglial heterogeneity between and within regions of the zebrafish CNS, apart from the functionally heterogeneous resting versus activated states distinguished by morphological and immunophenotypical dynamics of single cells. A recent study in zebrafish demonstrated embryonic/larval and adult microglia arise from distinct progenitor pools and display distinct temporospatial distribution in the brain and retina (Xu et al., 2015). These data, and the fact that the majority of zebrafish reporter lines used to visualize microglia in the brain lack reporter expression in the retina at early developmental time points, lead to the following testable hypothesis: 1) Different cues direct microglial precursors to colonize the retina versus the brain, 2) Microglial sub-populations differentially populate the retina versus the brain, and 3) Functional differences exist between embryonic/larval and adult microglial populations.

Although, the morpholino oligonucleotide technology provides a necessary and viable alternative to genetic methods (Morcos et al., 2015), and when used at limiting doses and rigorously controlled, has a place in the repertoire of zebrafish biologists, it has several limitations. For instance, the morpholino oligonucleotide-mediated knockdown technique lacks precise spatial and temporal control. This approach knocks down the target protein in every cell from the one cell stage. Therefore, parsing out the neural progenitor-specific versus microglial-specific function of Pgrn-a during early retinogenesis is difficult. Also, morpholino concentrations decrease with each cell division, and as a result, morpholino-dependent translation inhibition or splice blocking may diminish over

time, and is generally limited to the first 3 dpf (Nasevicius and Ekker, 2000; Bill et al., 2009). Further, the morpholino oligonucleotide knockdown technique is not complete; protein is still produced albeit at significantly reduced levels (see Figure 2.6), and the level of knockdown varies between embryos (Eisen and Smith, 2008). These limitations thwart the study of Pgrn-a function past 72hpf, and perhaps make changes in signaling molecules downstream of Pgrn-a difficult to decipher. Therefore, generating a *pgrn-a* knockout is necessary for further investigation of signaling downstream of Pgrn-a in the developing retina and the function of Pgrn-a in the injured/regenerating retina.

Using bacterial recombineering, we have generated iTol2-containing BAC constructs encoding a nitroreductase (NTR)-fluorescent reporter fusion protein under the control of the *pgrn-a* promoter and regulatory elements (Figure 3.4). These constructs can be used to establish stable transgenic zebrafish lines that would enable further study of the expression of Pgrn-a and the role of microglia in the developing and regenerating retina. The iTol2-CH73-47I9 *pgrna*:*nfsB-EGFP* #1 construct was co-injected with transposase mRNA, but did not result in transgene expression that recapitulated endogenous *pgrn-a* expression. Though target regions containing repetitive sequences can limit efficacy of BAC recombineering (Suster et al., 2011), PCR and sequencing analysis confirmed each construct contains the iTol2 cassette and nfsB-fluorescent reporter cassette at the *pgrn-a* translation start site (Figure 3.5). However, due to the fact that each BAC contains different amounts of 5' and 3' sequence flanking the *pgrn-a* gene, whether all of the regulatory elements that control the temporal and spatial

pattern of *pgrn-a* expression are encompassed within the BAC is unknown. Therefore, the other BAC constructs may yield different results and transgene expression that more reliably recapitulates endogenous Pgrn-a expression. To confirm that the transposon protein is properly excising and integrating the iTol2-BAC construct into the genome, a PCR excision assay can be performed (Suster et al., 2011).

If injecting the other confirmed constructs leads to Tg(*pgrna*:*nfsB-EGFP*) and Tg(*pgrna*:*nfsB-mCh*) lines that recapitulate endogenous *pgrn-a* expression patterns, then the lab will be well positioned to do the following future experiments. First, as a reporter of Pgrn-a expression, these lines would enable *in vivo* time-lapse imaging of Pgrn-a-expressing cells and therefore microglial migration and morphology. Further, utilizing fluorescence activated cell sorting (FACS), cells expressing *pgrn-a* could be isolated at different time points during development in order to perform microarray or proteomic analysis. Second, conditional targeted cell ablation using NTR/metronidazole would enable chemically inducible, temporally controlled genetic ablation specific to *pgrn-a*-expressing cells. Therefore, the Tg(*pgrna*:*nfsB-EGFP*) and Tg(*pgrna*:*nfsB-mCh*) lines would enable specific ablation of microglial cells from either developing or regenerating retinal tissues.

However, if none of the BAC constructs generate a reporter line, which recapitulates endogenous *pgrn-a* expression, then there are several potential alternative approaches available to study the function of microglia in the developing and adult zebrafish retina. First, is PLX5622, the selective small

molecule inhibitor of the colony-stimulating factor 1 receptor (CSF1R) from Plexxikon Inc. CSF1R is expressed by macrophages and microglia and required for microglial precursor migration into the brain and retina (Herbomel, 2001; Ginhoux et al., 2010; Erblich et al., 2011); Figure 2.1). In adult mice, inhibiting CSF1R with PLX5622 promotes microglial apoptosis (De et al., 2014; Elmore et al., 2014). Therefore, PLX5622 can potentially be used to ablate mature microglia in the larval (post 72hpf) and adult zebrafish retina. If swimming larvae in PLX5622-containing media or intraocular injections of PLX5622 in adults successfully kills microglia, then this model can be used in combination with models of retinal injury to determine whether the presence or absence of microglia regulate neurogenesis in the regenerating retina. Second, although transgenic reporter lines using the *mpeg1* promoter to drive reporter expression did not show expression in the retina at early developmental time points, these lines might prove to be valuable for studies in the larval (post 72hpf) or adult retina. We have crossed the Tg(mpeg1:Gal4) and Tg(UAS:nfsB-mCherry) lines and are raising a Tg(*mpeg1*:*Gal4*:*UAS*:*nfsB*-mCherry) colony. If mCherry co-localizes with microglial markers, such as 4C4, in the larval or adult retina, then this line can be used to ablate microglial cells in a temporally controlled manner. Finally, zebrafish mutant lines lacking CNS microglia, such as the *panther* mutant (Herbomel, 2001), offer another potential alternative approach to studying the function of microglia during retinal regeneration. Several other zebrafish mutants that lack microglia in the embryonic/larval brain and retina have recently been reported: *slc7a7* (Rossi et al., 2015b), *xpr1b* (Meireles et al., 2014), *nlrc3-like*

(Shiau et al., 2013). Determining whether the retinal phenotype of these embryos recapitulate the *panther* mutant, PLX5622-treated, and *pgrn-a* morphant phenotype would enhance the data set reported in Chapter 2 and bolster the conclusion that microglia are required for early neurogenic events in the developing retina. If these mutants also lack microglia in the adult retina, then they also can be used in combination with the photolytic lesioning model to determine whether the absence of microglia and/or the factors they express or secrete alters neurogenesis in the regenerating retina.

To further study the function of *pgrn-a* in the developing, adult and regenerating retina, a viable *pgrn-a* mutant zebrafish line is necessary. Using the CRISPR/Cas-9 genome technique, we mutated the *pgrn-a* gene and are in the process of screening for viable mutants. Embryos generated from the homozygous *pgrna*$^{-/-}$_90_5M_2F7M line possessed a 10bp deletion within the sgRNA target sequence. Yet, this mutation did not alter the expression of Pgrn-a (Figure 3.8), which explains the normal retinal phenotype observed in these embryos. Currently, additional lines possessing different indel mutations in the *pgrn-a* gene are being propagated to generate homozygous colonies. Homozygous embryos from these lines will be screened for the presence or absence of Pgrn-a at 24hpf. The retinal phenotype will be characterized at 48 and 72hpf in any line lacking Pgrn-a to determine whether some or all aspects of the *pgrn-a* morphant phenotype are recapitulated in the *pgrn-a* mutants. If a phenotype is observed in the *pgrn-a* mutants, confirming some or all aspects of the morphant phenotype (microphthalmia, lack of retinal microglia and/or delay in

early retinal neurogenesis), then the lab will be well positioned to do the following future experiments. First, building off the preliminary data presented in Chapter 2, *pgrn-a* mutant embryos will be used to further elucidate the downstream signaling mechanisms of Pgrn-a during retinal development. Specifically, the expression of a full complement of Wnt and Notch signaling pathway and target genes will be compared between mutants and wild type embryos at different developmental time points using qRTPCR, Western blot analysis and immunohistochemistry. Second, genetic and pharmacological models that inhibit Wnt or Notch signaling will be used to determine whether the retinal phenotype in *pgrn-a* mutants can be rescued. Third, RNA sequencing can be used to profile the transcriptome of wild type and *pgrn-a* mutant embryos and determine unknown gene targets of Pgrn-a. Finally, our lab previously reported that *pgrn-a* expression is significantly increased in retinal microglia following photoreceptor injury and during photoreceptor regeneration in the adult zebrafish retina (Craig et al., 2008). Based on this expression data and the known neurotrophic properties of progranulin (Van Damme et al., 2008; Ryan et al., 2009; De Muynck et al., 2013), we hypothesize that microglia and Pgrn-a play fundamental roles in both developmental and regenerative neurogenesis. Therefore, larvae and/or adult *pgrn-a* mutants will be used to determine the function of Pgrn-a during retinal regeneration. Specifically, retinal phenotype before and after light lesioning will be compared between *pgrn-a* mutant and wild type fish.

It is possible that the additional lines propagated will produce results similar to those reported for the *pgrna*$^{-/-}$_90_5M_2F7M line. If none of the lines

generated turn out to be usable mutants, then one alternative approach to mutate *pgrn-a* using the CRISPR/Cas-9 system would be to simultaneously inject multiple sgRNAs targeting different regions of the *pgrn-a* sequence. This would potentially result in larger indels, altering a greater portion of *pgrn-a* coding sequence, and increase the likelihood of disrupting Pgrn-a expression. Another possible outcome is that the retinal phenotype of viable *pgrn-a* mutants does not recapitulate the *pgrn-a* morphant phenotype. Multiple recent reports have demonstrated a poor correlation between morpholino-induced and mutant phenotypes (Kok et al., 2015). Though, these discrepancies may be due in part to differences in knock out versus knock down timing and site of action and issues with morpholino dosing and the lack of appropriate experimental controls (Morcos et al., 2015). An established potential outcome when generating zebrafish mutants is gene compensation (Rossi et al., 2015a). The fact that co-injection with *pgrn-b* mRNA was able to rescue aspects of the *pgrn-a* morphant phenotype (Figure 2.11) suggests Pgrn-b may function similarly to Pgrn-a. To preclude the potential compensatory effects of *pgrn-b*, a double mutant line can be generated. Another potential alternative approach to studying the function of Pgrn-a in the adult/regenerating retina is electroporatation of morpholino oligonucleotides in the adult zebrafish retina to knockdown Pgrn-a following photolytic lesion (Thummel et al., 2011).

Reports of stable single (*grna*$^{-/-}$; *grnb*$^{-/-}$) and double (*grna*$^{-/-}$*b*$^{-/-}$) progranulin mutant zebrafish lines created using zinc finger nucleases (ZFNs) emerged after we began using CRISPR/Cas-9 to target *pgrn-a* (Solchenberger et al., 2015).

These mutants did not recapitulate the axon outgrowth defect observed for spinal motor neurons previously reported for *pgrn-a* morphants (Chitramuthu et al., 2010; Laird et al., 2010), and reportedly lack any morphological, pathological, or biochemical phenotype. However, whether these mutants lack retinal microglia and/or display developmental defects in the CNS remains to be determined. Therefore, if the CRISPR/Cas-9 genome engineering technique does not yield a viable stable *pgrn-a* mutant line, then we can obtain these previously verified mutants and determine whether the embryos possess a retinal phenotype that recapitulates what we observed in *pgrn-a* morphants.

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