Inflammation and Matrix Metalloproteinase 9 (Mmp-9) Function in the Zebrafish Retina

by

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I dedicate this to my family

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ABSTRACT

Inflammation in the central nervous system (CNS) activates a complex network of signaling molecules in dying neurons, surviving neurons, and glia. In contrast to mammals, inflammatory signals in zebrafish are required for injury-induced neuronal regeneration in the forebrain. In the retina of zebrafish, Müller glia serve as the resident stem cell and the cellular origin for regenerative neurogenesis. Our lab previously identified the expression of an inflammatory protease known as matrix metalloproteinase 9 (*mmp-9)* following photoreceptor death (Calinescu et al., 2009). MMP-9 has many functions during CNS development and following injury, however, the function of Mmp-9 during zebrafish development and photoreceptor regeneration is unknown. The purpose of my dissertation was to use the zebrafish model to (1) determine how inflammation regulates Müller glia-derived photoreceptor regeneration in adults, (2) investigate the role of Mmp-9 as a component of the inflammatory response, and (3) determine potential developmental phenotypes in the absence of *mmp-9*. My results show that genes encoding inflammatory molecules are strongly induced by photoreceptor death, and anti-inflammatory treatment suppresses both the number of injury-induced progenitors and regenerated photoreceptors. Following photoreceptor injury and death, *mmp-9* is expressed in Müller glia, the intrinsic retinal stem cell, and Müller glia-derived photoreceptor progenitors. Deleting *mmp-9* results in an over production of injury-induced progenitors and regenerated photoreceptors, but

compromises the maturation and survival of regenerated cones. These data provide a link between injury-induced inflammation in the vertebrate CNS and Mmp-9 function during photoreceptor regeneration.

Chapter one begins with a brief overview of our current understanding of regulators that govern Müller glia entry into the cell cycle and progenitor proliferation. Next, I discuss inflammatory mechanisms that govern neurogenesis in vertebrates, and finally I review the key literature about the inflammatory protease, Mmp-9. In chapter two, I present data supporting the role for injury-induced inflammation and Mmp-9 function governing photoreceptor regeneration.

In chapter three, I characterize additional *mmp-9* mutants and developmental phenotypes associated with the two mutants used in studies from chapter two. Finally, I conclude with a brief discussion of future experiments based on my dissertation work. Together, my work provides a link between injury-induced inflammation in the vertebrate CNS and Mmp-9 function during photoreceptor regeneration.

Chapter 1

Introduction

The Visual System

Arguably the most important of our senses (vision, hearing, smell, touch, taste, balance, and proprioception), vision makes up about 40% of our sensory input and 50% of the brain is used to process visual information (Dowling and Dowling 2016). The retina is well-characterized, and the cytoarchitecture and cellular function are evolutionarily conserved among all vertebrates, allowing researchers to study its function for potential therapeutic interventions for retinal diseases.

Basic Retinal Anatomy and Function

In the late 19th century, Ramón y Cajal first described the anatomy of the retina using Golgi stain and compared retinal histology across vertebrate species (Cajal 1972; Piccolino, Strettoi, and Laurenzi 1989). To this day, his detailed account of retinal anatomy serves as a foundation of retinal cellular biology. The retina is composed of five major neuronal cell types: photoreceptors, bipolar cells, horizontal cells, amacrine cells, and ganglion cells. Their nuclei are organized into three distinct nuclear layers. The outer nuclear layer (ONL) contains the nuclei of photoreceptors – rods and cones. The inner nuclear layer (INL) contains bipolar, horizontal, and amacrine cells, as well as

the non-neuronal Müller glia. The ganglion cell layer (GCL) contains ganglion cell bodies. Synaptic zones referred to as the outer plexiform layer (OPL) and the inner plexiform layer (IPL) separate the GCL, INL, and ONL.

Vision requires energy, in the form of light, to be transformed into biochemical and electrical signals that are then transmitted from the retina to the brain. Vision processing begins when photoreceptors absorb photons of light. Rods are sensitive in dim light and mediate night vision, whereas cones function in bright light and mediate daytime color vision. Light travels through the GCL and INL before reaching the photoreceptors in the ONL. The signal is transduced back through the retina: photoreceptors synapse onto bipolar and horizontal cell processes in the OPL, and bipolar and amacrine cells synapse with ganglion cell processes in the IPL. Amacrine and horizontal cells mediate interactions between and among bipolar, amacrine, and ganglion cells. The axons of ganglion cells collect to form the optic nerve and carry the visual information to the rest of the brain. Interestingly, a subset of retinal ganglion cells are intrinsically photosensitive and responsible for non-image-forming vision, such as measuring ambient light for entraining circadian rhythms or mediating pupil size (Do and Yau 2010; Fu et al., 2005). In mammals, most of the electrical signals from the retina first enter the lateral geniculate nucleus (LGN) of the thalamus, as well as the superior colliculus, before terminating in the primary visual cortex of the occipital lobe. In the cortex, conscious visual information is first analyzed in the visual area 1 (V1), then V2-5. In non-mammalian vertebrates such as fish, visual information travels from the retina to the tectum in the midbrain for sensory processing.

Zebrafish as a model organism for retinal regeneration

The zebrafish (*Danio rerio*) is a freshwater teleost fish native to tropical ponds, lakes, and rivers in South Asia. In the 1980s, George Streisinger and colleagues at the University of Oregon established zebrafish as a research model (Streisinger et al. 1981). There are numerous advantages to the zebrafish that make it a powerful model organism: optimal breeders produce ~100-300 embryos, embryos are fertilized externally, transparency during early development, suitable for live imaging, efficient genetic techniques, and amenability to large-scale pharmacological screens (Holtzman et al. 2016; Lieschke and Currie 2007). The zebrafish genome has been fully sequenced and annotated, and notably it has at least one identified orthologue for 70% of the human genome (Howe et al. 2013). Additionally, many human diseases have been modeled in zebrafish (e.g., Fadool and Dowling, 2008).

Müller glia in response to retinal injury

In the vertebrate retina, Müller glia serve the important function of homeostatic, metabolic, structural, and functional support to retinal neurons (Reichenbach and Bringmann 2013). Mammalian Müller glia respond to retinal injury similar to astrocytes in an injured brain by a process known as reactive gliosis (Bringmann et al. 2009). Reactive Müller glia become hypertrophic and increase the expression of intermediate filament genes, *Vimentin*, *Nestin*, and *Glial fibrillary acidic protein* (*Gfap*) (Bringmann et al. 2009; Reichenbach and Bringmann 2013). In the acute phase, reactive gliosis has neuroprotective effects through the release of antioxidants, neurotrophic factors, and the re-uptake of excess glutamate, which can be excitotoxic to neuronal cells

(Bringmann et al. 2009). In the chronic phase, Müller glia release pro-inflammatory cytokines resulting in limited proliferation and the formation of a glial scar, both of which prevent neuronal regeneration (Bringmann et al. 2009). The proliferative response in Müller glia and consequences following retinal injury greatly differs among vertebrate species.

Müller Glia is the Source of Retinal Regeneration in Teleost Fish

Investigators have long known that the adult teleost fish can regenerate retinal tissue, but the source of regeneration had not always been well understood (Hitchcock and Raymond, 1992). Initial studies in goldfish and trout showed that, following the removal of retinal quadrants or injection with the cytotoxin, ouabain, mitotically active cells can be found in the ONL and ciliary marginal zone (CMZ), a site that provides neuronal progenitors throughout the animal's life (Lombardo et al. 1968; Lombardo et al. 1972, Maier and Wolburg 1979; Kurz-Isler and Wolburg 1982; Johns and Easter 1977; Meyer 1978). Lombardo and Wolburg hypothesized, from this, that retinal regeneration occurred via retinal progenitors at the lesioned edge or from CMZ progenitors that migrated into the ONL. This hypothesis was subsequently disproved by Raymond et al., (1988); however, they made the observation that, following ouabain-induced damage, elongated, clusters of dividing cells were present in the INL (Raymond, Reifler, and Rivlin 1988). In subsequent studies, Raymond et al. showed that Müller glia would undergo proliferation and nuclear migration from the INL to the ONL following laser ablation of photoreceptors (Braisted, Essman, and Raymond 1994; Wu et al. 2001). All in all, the evidence converged to argue against CMZ progenitors being primarily

involved in retinal regeneration. Attention then shifted toward cells that inhabit the INL, specifically, Müller glia.

To explore the possibility that Müller glia are the resident stem cells in teleost fish, investigators began using zebrafish as the model organism due to their transgenic efficiency and the ability to undertake a form of lineage tracing studies (Holtzman et al. 2016). The first published report to suggest Müller glia are involved in retinal regeneration used the α1 tubulin (α1T) transgenic zebrafish line, which showed induced GFP expression following a stab lesion to the retina (Fausett and Goldman 2006). In this study, GFP+ cells colocalized with the proliferation marker, BrdU, and two Müller glia markers, glutamine synthetase (GS) and glial fibrillary acidic protein (GFAP). By 7-11 days post-injury (dpi), these GFP+ cells were found to express neuronal markers (ganglion cells Zn5, and amacrine HuC/D), demonstrating that Müller glia-derived progenitors could differentiate into retinal neurons (Fausett and Goldman 2006). Müller glia were definitively identified using two zebrafish transgenic lines – Tg(*gfap:EGFP*)*mi2002* and Tg(*gfap:nGFP*)*mi2004* – that established Müller glia were indeed the primary progenitors for retinal regeneration (Bernardos et al. 2007). These two transgenic lines express either cytoplasmic [Tg(*gfap:EGFP*)*mi2002*] or injury-induced nuclear GFP (nGFP) [Tg(*gfap:nGFP*)*mi2004*], driven by the glia-specific GFAP promotor (Bernardos et al. 2007). Following photoreceptor death, nGFP+ Müller glia-derived progenitors expressed the multipotent progenitor marker Pax6 (paired box gene 6) at 3-4 dpl; expressed the photoreceptor marker Crx+ (cone-rod homeobox) at 4-5 dpl; and finally expressed the cone marker, zpr1 at 5-6 dpl (Bernardos et al. 2007). Moreover, during development, Müller glia proliferated slowly resulting in rod-restricted

progenitors in uninjured zebrafish retina (Bernardos et al. 2007) (Fig 1.1). To this date, investigators are still attempting to elucidate the cellular and molecular signaling mechanisms underlying zebrafish retinal regeneration as it relates to Müller glia.

Regulators of Müller glia and derived progenitors following injury

Unbiased transcriptional and proteomic approaches have allowed investigators to identify key regulators of Müller glia and derived-progenitors following injury (Craig, Calinescu, and Hitchcock 2008; Ramachandran, Zhao, and Goldman 2012; Sifuentes et al. 2016; Nelson, Ackerman, and O'Hayer 2013; Qin, Barthel, and Raymond 2009; Kassen et al. 2007). The factors identified include: intracellular signal transducers, transcriptional regulators, and secreted factors (Lenkowski et al. 2013; Gorsuch and Hyde 2014; Wan and Goldman 2017). Interestingly, these regulators can be expressed by dying photoreceptors, microglia, and the Müller glia themselves. It has been hypothesized that Müller glia respond to dying cells through autocrine signaling, which then triggers the necessary intracellular signaling cascades to promote transcriptional changes for Müller glia to enter the cell cycle and generate progenitors for retinal regeneration. Summarized below are a few critical studies that led to our current understanding of the signaling mechanisms contributing to Müller glia-derived retinal regeneration in vertebrate species (Fig 1.2).

Müller Glial Entry into the Cell Cycle (Activation)

Following photolytic lesion, the secreted cytokine, tumor necrosis factor-alpha (TNF-α), was identified from a proteomic analysis of lesioned retinal homogenates

(Nelson et al. 2013). It was shown to be produced by dying photoreceptors, starting at 16 hpl, and then by Müller glia at 36 hpl. Morpholino knockdown of TNF-α suppressed Müller glial entry into the cell cycle and resulted in shorter outer segments in the regenerated photoreceptors at 14 dpl. Of note, though expressed by dying photoreceptors, photoreceptor death was not affected by TNF-α knockdown. Furthermore, TNF-α knockdown suppressed known regulators of Müller glial entry into the cell cycle, including *lin28a, acsl1, and stat3.* These findings suggest that TNF-α is expressed by dying photoreceptors and plays a pivotal role in the initiation of Müller glial entry into the cell cycle.

To identify DNA elements involved in retinal regeneration in zebrafish, Fausett and Goldman (2006) generated transgenic lines harboring different deletions in the alpha-1-tubulin (α1T) promoter driving GFP expression and screened for transgene expression in Müller glia following retinal injury (Fausett, Gumerson, and Goldman 2008). An E-box in the α1T promoter was identified and found to mediate the transactivation of achaete-scute complex-like 1a (*ascl1a*). Ascl1a is a proneural basic helix-loop-helix (bHLH) transcription factor induced in zebrafish Müller glia following needle stab injury (Fausett, Gumerson, and Goldman 2008). Knockdown of Ascl1a prevented *α1T* and *pax6* expression, resulting in the suppression of Müller glia entry into the cell cycle and fewer Müller glia-derived progenitors (Fausett, Gumerson, and Goldman 2008). Ascl1a was also later found to be required for the expression of *lin-28* (Ramachandran, Fausett, and Goldman 2010), a pluripotency factor that inhibits the miRNA, *let-7*, whose role is to negatively regulate genes associated with the de-differentiation of Müller glia, such as *ascl1a, hspd1, lin-28a, pax6b, c-myc*, and *oct4*

(Ramachandran, Fausett, and Goldman 2010). These studies lend support to the idea that *ascl1a* is a key transcription factor required for Müller glial entry into the cell cycle.

Midkine is a heparin-binding growth factor that is expressed in the developing nervous system, regulates cell cycle kinetics, and its zebrafish paralogues, *midkine a (mdka)* and *midkine b (mdkb)* were identified in the injury-induced retina (Calinescu et al. 2009; Gramage, Li, and Hitchcock 2014; Winkler and Yao 2014). Though *mdka and mdkb* have unique expression patterns during retinal development, following photoreceptor death, both are expressed by horizontal cells, Müller glia, and photoreceptor progenitors (Calinescu et al. 2009). Follow up studies on *mdka* showed that Mdka protein is localized with Müller glia end feet and each progenitor nuclei in the ONL and INL (Gramage et al. 2015). In the same study, Mdka knockdown following photoreceptor lesion decreased Müller glia-derived progenitors at 3 dpl, and reduced number of regenerated rod photoreceptors at 6 dpl. The authors suggest that decreased Mdka protein slows down cell cycle kinetics in Müller glia leading to a reduced number of progenitors and regenerated rods.

Heparin-binding epidermal-like growth factor (HB-EGF) is a transmembrane protein, proteolytically cleaved and released in soluble form, which signals as a mitogen activator (Oyagi and Hara 2012). In the zebrafish, HB-EGF was shown to be necessary and sufficient to induce Müller glial entry into the cell cycle in the injured and uninjured retina (Wan, Ramachandran, and Goldman 2012). *hb-egf* is rapidly induced in the injured retina as early as 1 hpl and remains detectable by 7 dpl. HB-EGF was shown to bind to the EGF receptor, activating mitogen-activated protein kinase (MAPK) to induce the expression of *ascl1a*. Pharmacological inhibition of the EGF receptor or MAPK

signaling suppressed Müller glial entry into the cell cycle, whereas intraocular injection of HB-EGF increased Müller glia-derived progenitors in the uninjured retina (Wan, Ramachandran, and Goldman 2012). This study nicely demonstrated that the secreted HB-EGF signals in an autocrine manner to induce Müller glial entry into the cell cycle to produce Müller glia-derived progenitors.

Müller Glia-Derived Progenitor Proliferation

A microarray study using FACS-sorted Müller glia, harvested from the injured retina, identified the transcriptional repressor, *insm1a*, as an essential regulator for the formation of Müller glia-derived progenitors (Ramachandran, Zhao, and Goldman 2012). Initially expressed at 6 hpl, *insm1a* became restricted to Müller glia-derived progenitors at 4 dpl. Morpholino knockdown of Insm1a decreased Müller glia-derived progenitors by 80% but increased *ascl1* and *insm1a* expression, suggesting a negative feedback loop at play. Further support of this feedback regulation came from the identification of Ascl1a and Insm1 putative binding sites within the promoter region of *insm1a*. In addition, Insm1a also repressed dickkopf (dkk), a wnt inhibitor that promotes Müller glial entry into the cell cycle. Of note, the Insm1a binding site was found in the *hb-egfa* promoter region, implicating a form of feedback regulation by other secreted growth factors. To summarize, *insm1a* transcriptionally suppressed *ascl1a* and *dkk* for the formation of Müller glia-derived progenitors in the injured retina.

The transcription factor, *stat3* (signal transducer and activator of transcription 3), was identified in a microarray study following photolytic lesion of zebrafish retina as a regulator of Müller glia-derived progenitors (Kassen et al. 2007; Nelson et al. 2012). In

response to growth factors and cytokines, STAT3 becomes phosphorylated by janus kinases (JAK), resulting in its dimerization and translation into the nucleus to initiate transcription (O'Shea, Gadina, and Schreiber 2002). After photolytic lesion, Stat3 is expressed in all Müller glia, while Ascl1a is only expressed by Müller glia in the cell cycle. Morpholino knockdown of Ascl1a or Lin28a decreased Müller glial entry into the cell cycle, while Stat3 knockdown decreased the maximal number of Müller glia-derived progenitors (Nelson et al. 2012). It was hypothesized that the pluripotent factor, *lin-28a,* induced *ascl1a,* which in turn drove *stat3* expression in Müller glia for maximal number of progenitors (Nelson et al. 2012).

Transcriptional repressors of TGF-β signaling were identified through microarray analysis of Müller glia isolates following photolytic lesion of zebrafish (Qin, Barthel, and Raymond 2009). Specifically, two corepressors TGF-β-induced factor homebox 1 (*tgif1)* and SIX homobox 3 (*six3b)* – were induced in proliferating Müller glia-derived progenitors (Lenkowski et al., 2013). Tgif1 helps maintain a stem cell's identity, and Six3b binds to Geminin, a DNA replication inhibitor, to promote retinal progenitor amplification through the inhibition of TGF-β signaling (Singh and Tsonis 2010). In *tgif1* mutants, the number of Müller glia-derived progenitors is decreased and the proneural transcription factor, *ascl1a* is increased, suggesting that *tgif1* maintains a proliferative state in progenitors (Lenkowski et al. 2013). Additionally, cone regeneration is significantly impaired in *tgif1*- and *six3*-double mutants, as compared to single mutants. Alternative studies in the zebrafish showed that pharmacological inhibition of the TGF type I receptor following rod photoreceptor death increased Müller glia-derived progenitors (Tappeiner et al. 2016). In conclusion, *tgif1* and *six3* corepressors inhibit

TGF-β signaling in Müller glia-derived progenitors, and thereafter TGF-β induction promotes differentiation.

NeuroD is a proneural basic helix-loop-helix (bHLH) transcription factor that was found to be expressed in photoreceptor progenitors following photolytic lesion (Thomas et al. 2012). *neuroD* + photoreceptor progenitors expressed *pax6a*, *nr2e3*, and *crx* following injury, however *neurod* and *nr2e3* expression became restricted to differentiated rods as they regenerate (Taylor et al., 2015). In the same study, NeuroD knockdown resulted in a higher number of progenitors and fewer regenerated rods, while cones were not affected. Furthermore, NeuroD was found to function through Delta-notch signaling, where inhibition of gamma secretase by DAPT rescued the proliferation and regenerated rod phenotype (Taylor et al. 2015). Together these results suggest that NeuroD governs cell cycle exit among Müller glia-derived photoreceptor progenitors and rod maturation.

A microarray study using laser-captured microdissection of the ONL following photolytic lesion identified the extracellular signaling molecule, galectin-1-like 2 (Drgal1-L2) (Craig, Calinescu, and Hitchcock 2008). Galectin-3 is a secreted protein that belongs to the β-galactoside-specific lectins, binds to cell-surface glycoproteins to mediate cell-cell interaction and a regulator of inflammation (Henderson and Sethi 2009; Yang, Rabinovich, and Liu 2008). Following a photolytic lesion, Drgal1-L2 is induced in microglia, Müller glia, and Müller glia-derived progenitors (Craig, Calinescu, and Hitchcock 2008). Knockdown of Drgal1-L2 had no effect on Müller glial entry into the cell cycle, however, it diminished the regeneration of rods, but not cones (Craig et al. 2010). Thus, Drgal1-L2 was thought to anchor rod-specific extracellular matrix growth

factors to promote regeneration. Drgal1-L2 regulates rod regeneration, but is solely injury-induced and not expressed otherwise or in the developing zebrafish retina. In retrospect, Drgal1-L2 also provided the initial hint regarding the potential involvement of the injury-induced inflammatory response during photoreceptor regeneration

Ciliary neurotrophic factor (CNTF) is a member of the IL-6-like cytokine family with neuroprotective effects in the retina (Fischer et al. 2004; Peterson et al. 2000). In zebrafish, however, even though CNTF has been shown to decrease photoreceptor cell death following photolytic lesion when injected intraocularly, its expression is not induced after injury (Kassen et al. 2009). In fact, the *cntf* gene remains to be identified in zebrafish. Nevertheless, *cntf* and *il-6* share a receptor subunit, *gp130,* which is expressed by Müller glia following a stab lesion (Zhao et al. 2014). Morpholino knockdown of Gp130 decreased Müller glia-derived progenitors, while intraocular injection of CNTF and IL-6 drives their proliferation via *jak/stat* induced expression of *ascl1a*. The addition of leptin, which is an energy expenditure hormone, combined with IL-6 oddly increases signaling of *jak/stat* and *ascl1a*, suggesting a synergistic relationship with the IL-6-like cytokine family members to induce Müller glia-derived progenitor proliferation.

Similarities and Differences in Vertebrate Müller glia following Retinal Injury

Given everything we know about retinal regeneration in the zebrafish, warm-blooded vertebrate studies have leveraged this information to induce Müller glia entry into the cell cycle to produce retinal neurons. Studies by Wan et al., (2012) in the zebrafish retina demonstrate that intraocular injections of HB-EGF induce Müller glial entry into

the cell cycle and the formation of Müller glia-derived progenitors in the uninjured retina. HB-EGF intraocular injections in the chick and mouse retina does not induce the formation of Müller glia-derived progenitors in the uninjured retina (Todd et al., 2015). In the same study, HB-EGF intraocular injections paired with NMDA-damage leads to the formation of Müller glia-derived progenitors in both chick and mice. Interestingly, following retinal injury paired with HB-EGF intraocular injections, MAPK/pERK signaling in Müller glia is conserved in the zebrafish, chick, and mouse (Todd et al., 2015; Wan et al., 2012). Similar to zebrafish, following retinal injury in the chick, JAK/STAT signaling is required for the formation of Müller glia-derived progenitors, while intraocular injection of growth factors, FGF2 and CNTF, induced JAK/STAT signaling in the absence of injury (Todd et al. 2016; Nelson et al. 2012; Wan et al., 2014). Moreover, TGF-β signaling is well conserved in zebrafish, rodent, and chick Müller glia, where signaling is inhibited during the formation of Müller glia-derived progenitors, but increases as the progenitors differentiate (Close, Gumuscu, and Reh 2005; Lenkowski et al. 2013; Tappeiner et al. 2016). Yet, a key difference among vertebrate species is the activation of the proneural transcription factor, *ascl1*. Ascl1 is expressed in the zebrafish and chick retina following injury, but not expressed in mice and rats (Wilken and Reh 2017). Forced expression of Ascl1a in mammalian Müller glia allows these cells to spontaneously enter the cell cycle following retinal injury and generate progenitors, that at a low efficiency differentiate into bipolar and amacrine cells (Elsaeidi et al. 2018; Ueki et al. 2015; Jorstad et al. 2017). These studies illustrate that vertebrate Müller glia share similarities and differences between species, which can provide potential mechanisms for Müller glia-derived retinal regeneration.

Acute Inflammation in Vertebrates

Inflammation is the body's response to injury, infection, or disease, resulting in the activation of the immune system to defend and heal the affected tissue (Barton 2008). Three inflammatory phases occur following tissue injury, initiation, acute inflammation, and resolution (Kizil, Kyritsis, and Brand 2015). The initiation phase occurs when tissue is damaged. Acute inflammation follows tissue damage and occurs within seconds by the release of chemokines, which serve as chemoattractants to immune cells, microglia in the CNS, and macrophages, neutrophils, basophils, and eosinophils, in the periphery (Allan and Rothwell 2003; Gadani et al. 2015). Once recruited to the affected tissue, leukocytes and microglia phagocytose debris and release cytokines (Li and Barres 2018; Muller 2013), which are secreted pro-inflammatory (e.g. NfkB, TNF-α, IL-1β, IL-6) or anti-inflammatory (e.g. IL-10 and TGF-β) proteins that can modulate immune and non-immune functions (Deverman and Patterson, 2009; Gadani et al., 2015; Szelényi, 2001). By binding to their respective receptors and activating specific signaling pathways, cytokines regulate a variety of cellular responses (Gadani et al., 2015). In healthy tissue, the resolution phase terminates the acute phase by release of anti-inflammatory cytokines such as, interleukin 10 (IL-10) and TGF-β (Nathan 2002; Gadani et al. 2015). Failure to terminate the acute phase can lead to chronic inflammation and progression of a variety of disease states. Of note, the two branches of the immune system: innate (non-specific) and adaptive (specific) are evolutionarily conserved across all vertebrates, including teleost fish (Meeker and Trede 2008) (Table 1.1). Additionally,

events following CNS injury are conserved between mammals and zebrafish (Table 1.2).

Inflammation Regulates Neurogenesis in Vertebrates

Inflammation in the vertebrate CNS has both beneficial and detrimental outcomes on neurogenesis depending on duration, cell type, extent of the insult, and species. (Kizil, Kyritsis, and Brand 2015; Borsini et al. 2015; Bosak et al. 2018). For example, injection of lipopolysaccharide (LPS), an inflammatory activator, into rats decreased hippocampal neurogenesis, which was reversed by anti-inflammatory treatment (Monje, Toda, and Palmer 2003; Ekdahl et al. 2003). In the same study by Monje et al., (2003), LPS stimulated microglia to release IL-6, a pro-inflammatory cytokine, which then inhibited neuronal differentiation *in vitro* and *in vivo* (Monje, Toda, and Palmer 2003). In contrast, however, Kim et al., (2009) demonstrated that suppression of inflammation following ischemic injury reduced activated microglia and neurogenesis in rodents, which suggests that inflammation may be necessary for neurogenesis after ischemic injury (Kim et al. 2009). This is bolstered by another study showing that activated microglia following ischemic injury induced proliferation and differentiation of cultured SVZ neurospheres (Deierborg et al. 2010). Together, the above studies demonstrate that inflammation is context dependent with both beneficial and detrimental effects in mammals.

In zebrafish, inflammation is necessary and sufficient for neurogenesis (Kyritsis et al. 2012; Kyritsis, Kizil, and Brand 2014). Stab lesion or injection of the yeast particle, Zymosan A, into the zebrafish telencephalon induces radial glia to produce neuronal progenitors for complete brain regeneration (Kyritsis et al. 2012). However, treatment

after injury by the anti-inflammatory steroid, dexamethasone, decreases inflammatory cytokines, microglia, and radial glial proliferation, as well as the successive neurogenesis. The same study also identified, using transcriptome analysis, the potent inflammatory mediator, cysteinyl leukotriene receptor 1 (cysltr1), being expressed by radial glia during regeneration. Fascinatingly, the cyslt1 ligand is expressed by activated microglia in rodents during the phagocytosis of injured cells (Chen et al. 2015; Yu et al. 2014; Wang Xiaorong et al. 2018). Whether zebrafish microglia express the cyslt1 ligand for binding to cysltr1 on radial glia during neurogenesis, however, remains to be determined.

Injury-induced inflammation in the zebrafish is also required for rod regeneration in larvae. Similar to what happens to zebrafish radial glia, following rod ablation, anti-inflammatory treatment suppresses Müller glia proliferation, neuronal progenitors, and the subsequent rod regeneration (White et al. 2017). In the same studies, co-ablation of microglia and rod photoreceptors decreases the proliferation of Müller glia and retinal progenitors, indicating that microglia regulate rod regeneration in the zebrafish larvae. Though anti-inflammatory treatment suppresses neurogenesis in the zebrafish brain and retina, the lesion paradigm (stab vs. genetic ablation), and succeeding inflammatory response greatly differs. For instance, stab lesion to the brain breaks down the blood brain barrier (BBB) allowing for the infiltration of peripheral leukocytes, whereas nitroreductase-induced rod death does not break down the BBB, and thus limits the injury response to retinal microglia. Moreover, White et al., (2017) showed that neutrophils do not respond to rod cell loss however, peripheral macrophages respond by morphological transitions and increased migration, but fail to enter the retina.

Therefore, results are solely ascribed to the potential inflammatory contributions of retinal microglia and injured tissues, but not peripheral leukocytes. As it is known, zebrafish Müller glia are regulated by inflammatory cytokines, *tnf-a, il-6,* and *tgf-β* during proliferation and regeneration (Nelson, Ackerman, and O'Hayer 2013; Zhao et al. 2014; Lenkowski et al. 2013). Together, these studies further serve to highlight the crucial role of inflammation in zebrafish neurogenesis.

Matrix Metalloproteinase 9 (Mmp-9)

Matrix metalloproteinase 9 (MMP-9) belongs to a large family of Zn^{2+}-dependent proteases that target a variety of substrates, such as extracellular matrix (ECM), growth factors, chemokines, and cytokines. It serves diverse functions among vertebrates (Pedersen et al. 2015; Page-McCaw, Ewald, and Werb 2007; Vandooren, Van den Steen, and Opdenakker 2013; Iyer et al. 2012). There are 23 unique MMPs in humans, 24 in mice, and 26 in zebrafish (Pedersen et al. 2015). Unlike other MMPs, Mmp-9 is uniquely conserved across all vertebrate species. Classically, all MMPs are known to degrade the ECM, a function identified from the first MMP, MMP-1 (Nagai, Lapiere, and Gross 1966), purified from the tadpole tail during metamorphic ECM degradation. Later, MMP-9 was purified from human leukocytes following stimulation by inflammatory cytokines (Masure et al. 1991; Nagai, Lapiere, and Gross 1966). Mmp-9 was previously named gelatinase B because its substrate, shared with Mmp-2, is denatured type-1 collagen (gelatin). MMPs are classified into six groups: gelatinases, collagenases, MT-MMPs, stromelysins, and matrilysins, based on their structure and specific substrates (Vandooren, Van den Steen, and Opdenakker 2013). MMP-9 (Gelatinase B, 92 kDa) and MMP-2 (Gelatinase A, 72 kDa) are secreted proteases that belong to the group of

gelatinases. All MMPs have unique sequence domains, but share three common domains: propeptide, catalytic, and hemopexin domain (Fig 1.3) (Vandooren, Van den Steen, and Opdenakker 2013). Additionally, Mmp-9 contains an O-glycosylated domain for post-translational modifications, fibronectin domain, and hemopexin domain, all of which facilitate enzymatic substrate binding (Vandooren, Van den Steen, and Opdenakker 2013; Page-McCaw, Ewald, and Werb 2007). All MMPs are synthesized as an inactive zymogen and require processing to be catalytically activity (See Mmp-9 Activation and Regulation) (Page-McCaw, Ewald, and Werb 2007; Vandooren, Van den Steen, and Opdenakker 2013). Since its identification, MMP-9 has been the most studied MMP, as researchers continue to discover novel roles of this secreted protease.

Mmp-9 Activation and Regulation

Decades of work have detailed the transcriptional activation and protein regulation of MMP-9 (Vandooren, Van den Steen, and Opdenakker 2013). It is well established that inflammatory cytokines can induce the expression of MMPs, including MMP-9 (Vandooren, Van den Steen, and Opdenakker 2013; Nagase, Visse, and Murphy 2006). In fact, MMP-9 was first purified from neutrophils and monocytes following stimulation by *IL-8* and *IL-1β,* implicating its role as an inflammatory protease (Masure et al. 1991; Opdenakker, Masure, Proost, et al. 1991). Several studies support the initial purification findings that cytokines induce MMP-9 expression through mitogen-activated protein kinases (MAPKs), STATS, and SMAD signaling (Wu et al. 2009; Vandooren, Van den Steen, and Opdenakker 2013; Opdenakker et al. 2001; Salo et al. 1991; Schröer et al. 2011). Transcription factors that regulate *Mmp-9* converge and bind to two common

promoter sites, AP-1 and NF-kβ (Vandooren, Van den Steen, and Opdenakker 2013). Once translated into a zymogen, MMP-9 must undergo a conformational change for proteolytic function, resulting in a “cysteine switch” to expose its active site (Vandooren, Van den Steen, and Opdenakker 2013). The cysteine switch requires that cysteine-99 break its interaction with the Zn^{2+} ion (Rosenblum, Meroueh, et al. 2007; Rosenblum, Van den Steen, et al. 2007), which may be achieved by NO, ROS, OA-NO2, plasmin/plasminogen binding to the thiol moiety at cysteine-99, or cleavage of the pro-domain (Vandooren, Van den Steen, and Opdenakker 2013; Philippe E. Van den Steen et al. 2002). Interestingly, MMP-3 can activate MMP-9 *in vivo,* either directly via cleavage (Ogata, Enghild, and Nagase 1992) or indirectly, along with MMP-1, by inducing TNF-α expression (Steenport et al. 2009). MMPs are inactivated by a group of tissue inhibitors of metalloproteinases (TIMPs) (TIMP-1-4) (Clark et al. 2008; Bauer, Eisen, and Jeffrey 1972). Although TIMP-1 has a greater affinity for MMP-9 than MMP-2, most TIMPs can bind to more than one Mmp (Olson et al. 1997). The C-terminus of TIMP-1 binds to the hemopexin domain of MMP-9 to inhibit its function (O’Connell et al. 1994; Goldberg et al. 1992). The N-termini of TIMP-1 and TIMP-2 interact directly with the catalytic domain of MMP-9, rendering it inactive (Goldberg et al. 1992). As described, MMP-9 is highly regulated in its activation and function, giving rise to functional complexity in a diversity of tissues.

Mmp-9 in Neuronal and Non-Neuronal Tissues during Development

During vertebrate development, MMP-9 is expressed and tightly regulated in neurons, osteoclasts, leukocytes, and hematopoietic stem cells (Ayoub et al. 2005); (Bednarek et al. 2009; Ulrich et al. 2005; Vu et al. 1998; Ando et al. 2017; Vandooren, Van den

Steen, and Opdenakker 2013). MMP-9 expression has been observed in many regions of the mammalian brain during development, such as the hippocampus, brainstem, cerebellum, and corpus callosum (Vaillant et al. 2003; Aujla and Huntley 2014; Oliveira-Silva et al. 2007). In the mouse corpus callosum, MMP-9 expression coincides with oligodendrocyte myelination, and knockout mice fail to myelinate axons (Uhm et al. 1998). Additionally, postnatal migration of mouse cerebellar granule cells into the granular layer is delayed by genetic or pharmacological inhibition of MMP-9, resulting in cell death (Vaillant et al. 2003). Notably, administration of an MMP-9 inhibitor does not affect the migration of cultured astrocytes, suggesting that, in mammals, MMP-9 affects migratory functions specific to neurons (Ogier et al. 2006). In developing zebrafish, *mmp-9* is expressed in the notochord between 12-14 hours post fertilization (hpf), and in circulating leukocytes by 24 hpf (Yoong et al. 2007). However, *mmp-9* is undetectable in the zebrafish CNS during development (Yoong et al., 2007; Zhang et al. 2017). Mmp-9 expression in the developing CNS is specific to mammals.

MMP-9 is also critical for bone growth and hematopoietic stem cell (HSC) development (Ando et al. 2017; Vu et al. 1998; Theodore et al. 2017). The first MMP-9 null mice had severe ossification defects (Vu et al. 1998). Although these null mutants were viable, by 4 weeks of age, vascularization and ossification at the growth plate became abnormal, and hypertrophic chondrocytes failed to go through apoptosis necessary for normal bone development (Vu et al. 1998). This defect was mediated by failure of MMP-9 to cleave and inactivate galectin 3, a lectin with anti-apoptotic activity (Colnot et al. 2001). The function of MMP-9 in bone development seems to be conserved across all vertebrates. Zebrafish osteoblast progenitor cells require Mmp-9

during transition from mesenchymal precursors to osteoclasts (Ando et al. 2017). With regard to Mmp-9 function in hematopoiesis, morpholino knockdown of Mmp-9 inhibits HSC migration and colonization into secondary niches during zebrafish embryo development (Theodore et al. 2017). This migratory defect is thought to arise from the inability of Mmp-9 to cleave a chemokine substrate, *cxcl12* (Theodore et al. 2017).

Mmp-9 Function following Tissue Injury

Although MMP-9 is expressed during development, it is downregulated in adulthood and induced by injury or in disease states (Vandooren, Van den Steen, and Opdenakker 2013). Cortical injury in mice increases MMP-9 catalytic activity in parallel with motor deficits, which are attenuated in MMP-9 knockouts (Wang et al. 2000). Similarly, spinal cord injury in mice induces MMP-9 activity in the spinal microvascular endothelium and is associated with elevated TNF-α expression (Hansen et al. 2013). Deletion of MMP-9 in knockout mice restores TNF-α expression to homeostatic levels, attenuates remote microglia activation, and improves locomotion recovery after insult (Hansen et al. 2013). The cause of motor deficits following cortical or spinal injury is thought to be neuronal cell death. A potent inducer of neuronal cell death, nitric oxide (NO), activates MMP-9 expression in mouse models of stroke (McCarthy et al. 2008; Iadecola 1997; Kurzepa et al. 2014). For example, transient focal cerebral ischemia in mice increases NO activated MMP-9 and drives the cleavage of laminin, a major component of the basal lamina (Gu et al. 2005, 2002). In the mouse retina, NMDA-induced ganglion cell death stems from NO-mediated MMP-9 expression and is reversible by nNOS mutation or inhibition (Manabe, Gu, and Lipton 2005). In the corneal epithelium, MMP-9 increases SMAD-2 signaling after injury to inhibit proliferation during regeneration (Mohan et al. 2002). In

MMP-9 mutant mice, epithelial proliferation is enhanced, SMAD-2 signaling is delayed, and IL-1α is elevated (Mohan et al. 2002). Likewise, Schwann cell proliferation following sciatic nerve injury is inhibited by MMP-9 cleavage of IGF-1 to activate MEK/ERK signaling, whereas inhibition of MMP-9 increases proliferation of Schwann cells (Chattopadhyay and Shubayev 2009; Y. Kim et al. 2012).

Induction of zebrafish Mmp-9 following injury is necessary for proper fin, retina, and heart regeneration (Xu et al. 2018; LeBert et al. 2015; Lemmens et al. 2016; Kaur et al. 2018a). The first study to show *mmp-9* expression after tail clip also showed that neutrophils were recruited to the lesion site (Yoong et al. 2007). In addition, a zebrafish mutant with an insertion error in the hepatocyte growth factor activator-inhibitor gene 1 (*hai1/spint1*) showed elevated *mmp-9* levels (Mathias et al. 2007; LeBert et al. 2015). Following caudal fin amputation, epithelial cells increase the expression of *mmp-9* and recruit leukocytes to promote proper ECM reorganization of collagen fibers during regeneration (LeBert et al. 2015). Osteoblast progenitor cells (OPCs) also increase *mmp-9* expression following zebrafish fin-ray amputation, and ablation of OPC-*mmp-9*+ cells reduces bone calcification during regeneration (Ando et al. 2017). Recently, Kaur et al. showed similar *mmp-9* expression patterns in Müller glia and Müller glia-derived progenitors in the zebrafish retina following needle stab injury (Kaur et al. 2018). Pharmacological or morpholino knockdown of Mmp-9 after injury decreases the production of Müller glia progenitors (Kaur et al. 2018). Optic nerve crush (ONC) in the zebrafish also increases Mmp-9 expression in the inner plexiform layer, a location dense with synaptic connections (Lemmens et al. 2016), implicating the role of Mmp-9 in synaptic remodeling. In mammals, stimulation of the serotonin receptor, 5-HT7R,

increases MMP-9 activity through cleavage of CD44 to regulate long-term potentiation-induced synaptogenesis (Bijata et al. 2017). In the heart, *mmp-9* and *mmp-13* regulate cardiocyte regeneration through activation of *il-8,* an inflammatory chemokine required for leukocyte recruitment of (Xu et al. 2018). Studies have yet to determine if these two Mmps can function independently to recapitulate the phenotype, however.

Mmp-9 and Inflammation

MMP-9 is a marker for many inflammatory diseases such as rheumatoid arthritis (RA), multiple sclerosis (MS), and age-related macular degeneration (AMD) (Vandooren, Van Damme, and Opdenakker 2014; Vandooren, Van den Steen, and Opdenakker 2013; Gijbels et al. 1992). The first human inflammatory disease associated with MMP-9 was RA, which had elevated levels of MMP-9 protein in synovial fluid (Opdenakker, Masure, Grillet, et al. 1991). In a RA mouse model, MMP-9 mutants showed reduced cartilage degradation and proteoglycan cleavage in joints, as well as an overall milder form of the disease (Itoh et al. 2002). It was hypothesized that MMP-9 may promote ECM degradation or increase inflammatory factors for leukocyte recruitment (Ahrens et al. 1996; Jovanovic et al. 2000).

In MS, MMP-9 is detected in the cerebrospinal fluid (CSF) and is expressed by endothelial cells and leukocytes in blood vessels at the sites of demyelinating lesions (Cossins et al. 1997; Lindberg et al. 2001; Gijbels et al. 1992). It is known that MS patients have disruptions in the blood-brain barrier (BBB) and leukocyte infiltration (Minagar and Steven Alexander 2003; Kermode et al. 1990). Experimental autoimmune encephalomyelitis (EAE) is a mouse model of MS with T-cell-mediated inflammation and breakdown of the BBB (Constantinescu et al. 2011). In this model, young MMP-9

knockout mice are partially resistant to the development of EAE (Dubois et al. 1999), whereas MMP-2 and MMP-9 double-knockout mice are completely resistant (Agrawal et al. 2006). T-cells that secrete TNF-α and interferon-Y (IFN-Y) are known to induce MMP-9 permeabilization of the BBB for leukocyte infiltration (Song et al. 2015). Therefore, MMP-9 may serve as a key effector of BBB breakdown and leukocyte infiltration into the CNS during MS pathogenesis. Of note, human MMP-9 can cleave myelin basic protein (MBP) into fragments, suggesting a direct role in demyelination (Opdenakker and Van Damme 1994).

AMD is the leading cause of blindness among human retinal diseases, and it results from photoreceptor degeneration (Kauppinen et al. 2016; Hernández-Zimbrón et al. 2018). AMD patients have evaluated levels of MMP-9 in plasma samples; however, a subset of the population exhibit single nucleotide polymorphism (SNP) variant in the *MMP-9* gene (Chau et al. 2007; Liutkeviciene et al. 2015; Fritsche et al. 2016). Pathogenesis of AMD is thought to be caused by oxidative stress, autophagy dysfunction, and inflammation targeting the photoreceptors, RPE, and Bruch's membrane (Kauppinen et al. 2016). In a subset of AMD patients, MMP-9 catalytic activity is reduced within the Bruch's membrane, suggesting that MMP-9 may be a contributor to ECM impairment during AMD pathogenesis in patients with SNPs (Hussain et al. 2011; Hussain et al. 2014; Kumar et al. 2010). MMP-9 levels are also increased in a rodent AMD model, and pharmacological inhibition of MMP-9 enhances rod survival (Shin et al. 2016). Collectively, MMP-9 function is context dependent in various disease states, but now serves potential therapeutic targets for RA, MS, and AMD.

During leukocyte recruitment, MMP-9 cleaves chemokines to modulate their functional activity (Parks, Wilson, and López-Boado 2004). Leukocyte recruitment to the injured or diseased tissue is critical for neutralizing inflammatory signaling, phagocytosis of necrotic debris, and ECM modeling (Petri, Phillipson, and Kubes 2008). MMP-9 can cleave IL-8, thereby increasing its binding to the CXC chemokine receptor-1 (CXCR1) in a positive feedback loop of leukocyte recruitment (Van Den Steen et al. 2003). On the other hand, MMP-9 can also cleave CXCL5 and CXCL6, and in doing so, inactivate their chemotactic activity (Van den Steen et al. 2000). *In vivo* studies have demonstrated that MMP-9 knockout mice showed decreased leukocyte migration towards a CXCL6 gradient produced by granulocytes (D'Haese et al. 2000). In an allergic lung inflammation model, MMP-9 null mice showed decreased leukocyte egression driven by CCL11, CCL7, and CCL17 (Corry et al. 2004). The zebrafish Mmp-9 functions in a similar manner, as mentioned previously (see, Mmp-9 Function Following Tissue Injury). Together, these studies suggest that MMP-9 interacts with different chemokines to create an environment attractive for leukocyte recruitment.

MMP-9 can also cleave the inflammatory cytokines, TGF-β and IL-1β (Yu and Stamenkovic 2000; Schönbeck, Mach, and Libby 1998). In keratinocytes, CD44 is a surface antigen glycoprotein cleaved by MMP-9, which in turn cleaves latent TGF-β during ECM degradation and tumor growth (Yu and Stamenkovic 2000). MMP-9 also cleaves the precursor, IL-1β, into its mature form to increase its functional activity (Schönbeck, Mach, and Libby 1998). Of note, cultured astrocytes and microglia stimulated by IL-1β induce MMP-9 expression, suggesting a positive feedback signaling cascade similar to that found in leukocyte recruitment (Gottschall and Yu 2002).

Retinal regeneration in the zebrafish has been studied for decades with a recent focus on inflammatory mechanisms. Though inflammation is necessary and sufficient for neuronal regeneration in the zebrafish forebrain, its role in adult photoreceptor regeneration and the function of matrix metalloprotease 9 (mmp-9) is unknown. Mmp-9 is a multifunctional inflammatory protease expressed during vertebrate development and induced by injury in adulthood. However, its role in the zebrafish retina during photoreceptor regeneration has yet to be investigated. In the subsequent chapters, I present data on the role of inflammation and Mmp-9 during regeneration in the zebrafish retina. Lastly, I present a combination of developmental characterization of Mmp-9 loss-of-function mutants and future tools for a transgenic line.

Figures

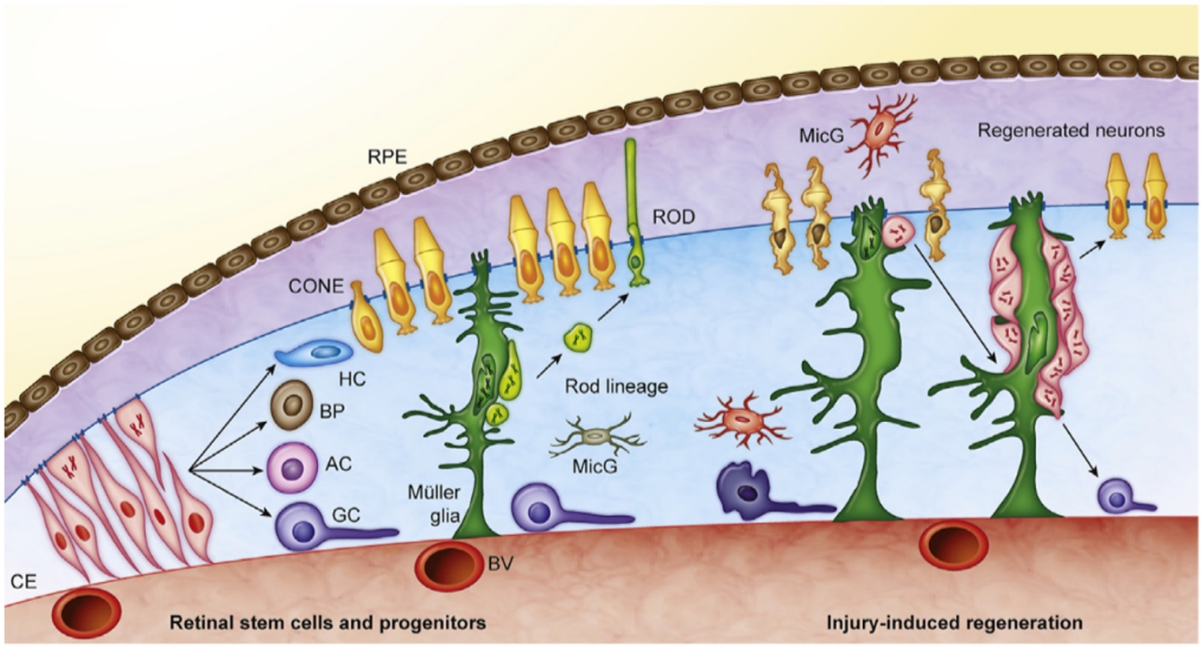

Figure: 1.1. Neurogenesis in the adult zebrafish retina during growth and regeneration (Lenkowski and Raymond, 2014).

Neurogenesis in the adult teleost fish retina during growth and regeneration. Germinal zone with multipotent retinal stem cells and committed retinal progenitors (magenta) at the junction between the neural retina and the ciliary epithelium (CE). New retinal neurons are generated sequentially: first retinal ganglion cells (GC), then interneurons (amacrine cells, AC; bipolar cells, BP; horizontal cells, HC), then cone photoreceptors. The apical surface of the neural retina faces the retinal pigmented epithelium (RPE) at the back of the eye; the basal surface of the neural retina is vascularized (blood vessels, BV). Radial processes of Müller glial cells (green) span the apical-basal extent of the retina and lateral processes enwrap the neurons. Müller glia are somatic retinal stem cells supporting the rod lineage: they divide infrequently, with an asymmetric, self-renewing di- vision, to give rise to proliferating, committed progenitors that migrate to the apical surface to generate rod photoreceptors within the differentiated retina. When retinal neurons are destroyed, microglia (MicG) are activated and remove cellular debris. The nuclei of Müller glia translocate to the apical surface, divide asymmetrically to give rise to proliferating, multipotent retinal progenitors that accumulate around the radial glial fiber and migrate to the appropriate retinal laminae to regenerate neurons (e.g., cone photoreceptors or retinal ganglion cells).

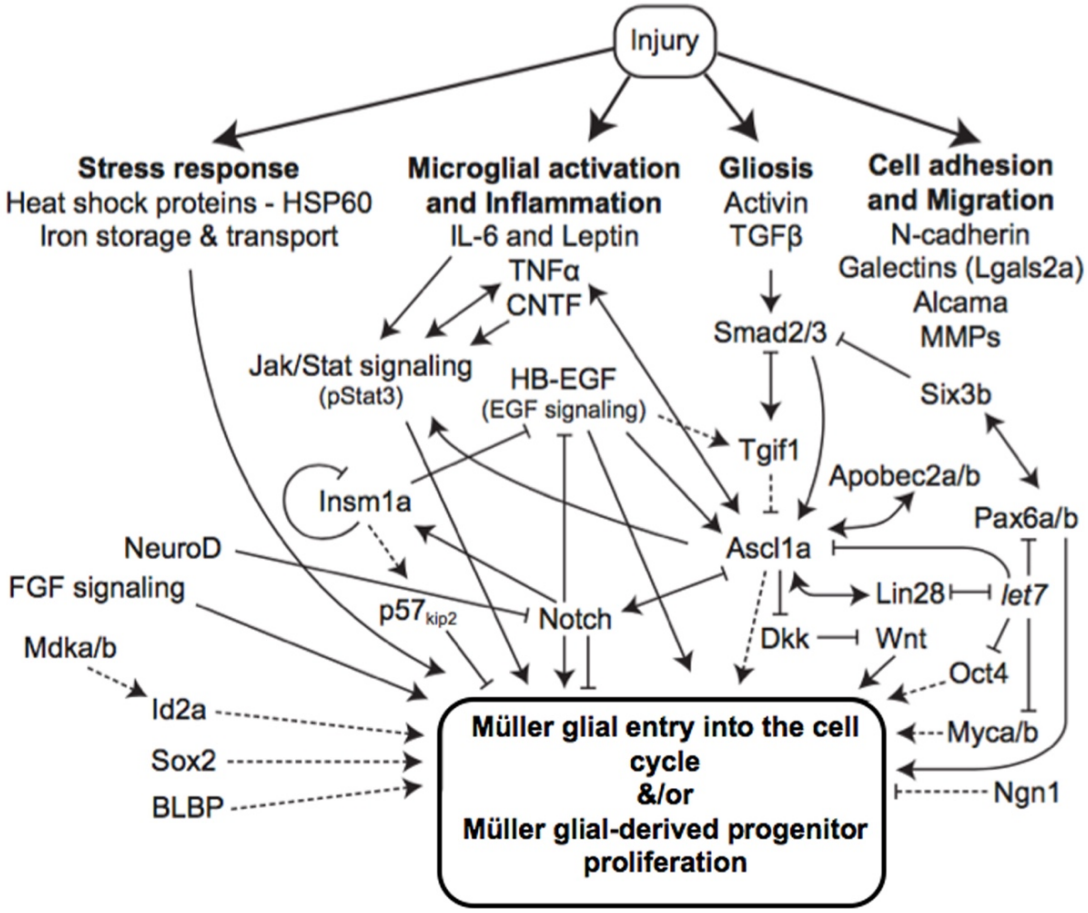

Figure: 1.2. An overview of signaling pathways that regulate Müller glia entry into the cell cycle and Müller glia-derived progenitors following injury in adult zebrafish, adapted from (Lenkowski and Raymond, 2014).

Following retinal injury, expression of factors associated with a stress response, inflammation, gliosis, and cell adhesion and migration are modified to regulate cell proliferation leading to regeneration of retinal neurons. Solid lines indicate regulatory interactions that have been described in the literature; dashed lines indicate suggested or indirect interactions.

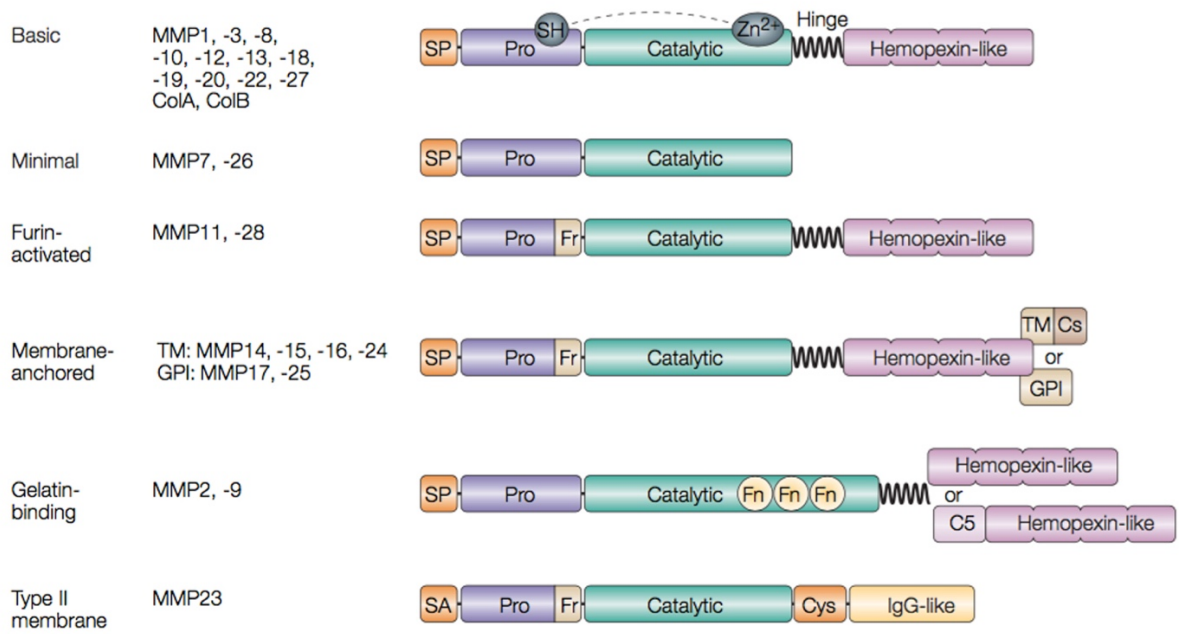

Fig: 1.3. Domain structure of the mammalian MMP family (Parks et al., 2014)

The important features of matrix metalloproteinases (MMPs) are illustrated, showing the minimal domain structures. Although MMPs are often subdivided into groups on the basis of differences in domain composition (shown here), there is little consensus in the field about how such subdivisions should be assigned. Domain structure alone does not predict function. One clear division is between MMPs that are secreted and those that are anchored to the cell surface by an intrinsic motif: namely, a transmembrane (TM) domain (MMP14, -15, -16 and -24), a glycosylphosphatidylinositol (GPI) anchor (MMP17 and MMP25) or an amino (N)-terminal signal anchor (SA) (MMP23). Both the TM domains and GPI anchors are attached to the hemopexin-like domain by a short linker. As discussed in the text, the secreted MMPs might still be confined to the cell surface through interactions with specific accessory macromolecules. Because the mechanisms that control activation (that is, conversion of proMMP to active MMP) are key steps in the regulation of proteolysis, another grouping of the MMPs can be made on the basis of intracellular activation by furin proteinases. Nine MMPs, including all of the membrane-anchored enzymes, have a furin-recognition domain. C5, type-V-collagen-like domain; Col; collagenase-like protein; Cs, cytosolic; Cys, cysteine array; Fn, fibronectin repeat; Fr, furin-cleavage site; Pro, pro-domain; SH, thiol group; SP, signal peptide; Zn, zinc.

Tables

Table: 1.1. An overview of the zebrafish immune system compared to humans, adapted from (Meeker and Trede, 2008)

	Human	Zebrafish
General		
Sites of early hematopoiesis	Yolk sac	ICM
	AGM	Dorsal aorta
	Fetal liver	CHT
Site of adult hematopoiesis	Bone marrow	Kidney marrow
Lymphatic system	ZF has no lymph nodes but does have putative lymphatic vessels including a thoracic duct	
Complement system	Well developed in ZF with shared human elements	
Inflammatory proteins	Well conserved between human and ZF, i.e., TNFα, NF-κB, COX2, IL-1, IL-8 and other C-C and C-X-C chemokines	
MHC	Class I, II, III all present in ZF	
Myeloid		
Neutrophils		
Nuclear lobes	4–5	2–3
Cytoplasm	Heterophilic with azurophilic and non-azurophilic granules similar to human	
Motility	+	+
Phagocytic activity	+	+
Myeloperoxidase	+	+
Acid phosphatase	+	+
Periodic acid Schiff	+	–
Respiratory burst	+	+
Eosinophils		
Morphology	Morphologically distinct from human counterpart	
Monocytes/macrophages		
Motility	+	+
Phagocytic activity	+	+
Respiratory burst	+	+
Ability to activate T/B cells	+	+
Mast cells and basophils	Currently not characterized in ZF	
Lymphoid		
Thymus		
Anatomy	Multilobulated midline structure	Bilateral single-lobed twin structures which remain attached to pharyngeal endoderm
Histology	Demarcated cortex and medulla in human and ZF	
T cells		
Site of initial development	Bone marrow	Kidney marrow
Educated in thymus	+	+
TCR	ZF TCRα, β, δ, γ chains identified [http://www.ncbi.nlm.nih.gov/Genbank/index.html]	
B cells		
Embryonic development	Initially develop in ZF pancreas	
Ig subtypes	A,D,G,E, M	D, M, Z
Rag-dependent V(D)J	+	+
immunization		
NK cells		
Receptor	Classical NK receptor	Putative NITRs

Table: 1.2. A comparison of the events following an acute CNS injury between mammals and zebrafish, adapted from (Kyritsis, Kizil, and Brand 2014)

Event after CNS injury	Mammals	Zebrafish
Cell death	+++	+
Microglial activation	+++	+++
Leukocyte invasion	+++	+
Reactive proliferation	+++	+++
Regenerative neurogenesis	+	+++
Neuronal survival/integration	–	+++

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Chapter 2

Inflammation and Matrix Metalloproteinase 9 (Mmp-9) Function in the Zebrafish Retina

ABSTRACT

Brain injury activates a complex network of inflammatory signals in dying neurons, surviving neurons, and glia. In zebrafish, inflammatory signals are required for injury-induced neuronal regeneration in the forebrain. Here, I establish that in zebrafish inflammation also regulates photoreceptor regeneration and determine the cellular expression and function of the inflammatory protease, matrix metalloproteinase 9 (Mmp-9), during this process. Genes encoding inflammatory molecules are strongly induced by photoreceptor death, and anti-inflammatory treatment suppresses both the number of injury-induced progenitors and regenerated photoreceptors. Following photoreceptor injury and death, *mmp-9* is expressed in Müller glia, the intrinsic retinal stem cell, and Müller glia-derived photoreceptor progenitors. Deleting *mmp-9* results in an over production of injury-induced progenitors and regenerated photoreceptors, but compromises the maturation and survival of regenerated cones. These data provide a link between injury-induced inflammation in the vertebrate CNS and Mmp-9 function during photoreceptor regeneration.

Introduction

Unlike mammals, zebrafish have an astonishing ability for neuronal regeneration following brain injury, which is sustained by radial glia that serve also as intrinsic stem cells (Lenkowski and Raymond, 2014; Goldman, 2014; Gorsuch and Hyde, 2014, Kyritsis et al., 2012). In all vertebrate retinas, Müller glia serve as specialized radial glia that provide homeostatic, metabolic, structural, and functional support to retinal neurons (Bringmann et al., 2009; Reichenbach and Bringmann, 2013). In response to retinal injury, Müller glia in mammals undergo reactive gliosis with initial neuroprotective effects that become pathological during chronic states (Bringmann et al., 2009; Reichenbach and Bringmann, 2013). In contrast, Müller glia in zebrafish also serve as intrinsic stem-cells, supporting the ability to regenerate all types of retinal neurons (Fausett and Goldman, 2006; Bernardos et al., 2007; Powell et al., 2016). In response to neuronal injury and death in the retina, Müller glia enter the cell-cycle divide once to produce multipotent progenitors, which proliferate, migrate to sites of neuronal death, exit the cell cycle, and differentiate, mature and integrate into existing synaptic circuits (Gorsuch and Hyde, 2014; Nagashima et al., 2013). A distinguishing feature of the Müller glia in mammals compared to fish is the repertoire of injury-induced genes that allow for retinal regeneration in fish. For example, the proneural transcription factor, *ascl1a,* is expressed by Müller glia in zebrafish and is required for retinal regeneration (Fausett et al., 2008). This gene is not expressed by mammalian Müller glia, however, forced expression of Ascl1 in mammalian Müller glia allows these cells to spontaneously enter the cell cycle following a retinal injury and generate progenitors, which at low efficiency differentiate into bipolar and amacrine cells (Ueki et al., 2015; see also Yao et al., 2018). Therefore, determining the genetic regulators of retinal regeneration in zebrafish

may provide for novel therapeutic approaches for restoring retinal neurons and restoring vision in in mammals, including humans.

Inflammatory cytokines are small secreted proteins that modulate immune and nonimmune functions during development, injury, and disease (Szelényi, 2001; Deverman and Patterson, 2009). In mammals, inflammatory cytokines can promote or prevent neurogenesis (Bringmann et al., 2009; Kyritsis et al., 2014; Kizil et al., 2015; Borsini et al., 2015). *In vitro* application of the pro-inflammatory cytokine, TNF-α, signaling through NfkB induces proliferation in mouse hippocampal neurospheres (Widera et al., 2006). In contrast, *in vivo* systemic injections of the inflammatory activator, lipopolysaccharide (LPS), into rats increases IL-6 expression, but results in decreased hippocampal neurogenesis, which can be restored by anti-inflammatory treatment (Monje, 2003). In the zebrafish inflammation is both necessary and sufficient to induce neuronal regeneration (Kyritsis et al., 2012). Anti-inflammatory treatment suppresses the production of glial-derived progenitors after injury to the forebrain, an injection of immunogenic Zymosan A is sufficient to induce neuronal regeneration in the absence of an injury (Kyritsis et al., 2012). In the zebrafish retina, inflammatory cytokines, *tnf-α, il-6,* and *tgf-β* regulate aspects injury-induced proliferation (Nelson et al., 2013; Zhao et al., 2014; Lenkowski et al., 2013).

I sought to determine a general role for injury-induced inflammation during the selective regeneration of photoreceptors and to identify the functional role of the Matrix metalloproteinase 9 (Mmp-9). Mmp-9 is a secreted protease that is expressed during CNS development and plays a prominent role in tissue remodeling (Vandooren et al., 2014). In contrast, Mmp-9 is downregulated during adulthood, but strongly induced

following tissue injury or disease (Bonnans et al., 2014; Vandooren et al., 2014, 2013). Secreted Mmp-9 cleaves a variety of extracellular molecules, including adhesion molecules, growth factors, and inflammatory cytokines (Bonnans et al., 2014; Manicone and McGuire, 2008; Parks et al., 2004; Le et al., 2007; Vandooren et al., 2013b). Cortical injuries in mice increase MMP-9 catalytic activity in parallel with motor deficits, which are attenuated in *mmp-9* knock-outs (Wang et al., 2000). In developing zebrafish, *mmp-9* is expressed in the notochord between 12-14 hours post fertilization (hpf), but is undetectable in the brain or retina (Yoong et al., 2007). However, in adult zebrafish, *mmp-9* is strongly induced by injury to the retina, independent of the nature of the wound (Calinescu et al., 2009b; Lemmens et al., 2016; Kaur et al., 2018). Interestingly, inflammatory cytokines *tnf-a*, *il-1b*, and *il-8* induce the expression of Mmp-9 demonstrating its role as an inflammatory protease (Vandooren et al., 2014). Moreover, patients with retinal diseases, such as wet age-related macular degeneration (AMD) have mutational variants in inflammatory regulators, TGFBR1, TNFRSF10A, and MMP-9 (Fritsche et al., 2016).

In the current study, I used adult zebrafish to evaluate the general role of inflammation and the specific function of *mmp-9* during photoreceptor regeneration. The data show that inflammatory genes are rapidly induced by photoreceptor-specific death and display dynamic expression patterns that correspond to the various phases of photoreceptor regeneration. Anti-inflammatory treatment suppresses the expression of these genes and diminishes the number of Müller-derived progenitors and regenerated photoreceptors. During photoreceptor injury and death, catalytically active Mmp-9 is expressed by Müller glia, anticipating re-entry into the cell cycle, and Müller glia-derived

progenitors. Additionally, *mmp-9* expression is regulated by the proinflammatory cytokine, TNF-α. In animals homozygous for non-functional *mmp-9* alleles, photoreceptor injury results in the overproduction of both Müller glia-derived progenitors and regenerated photoreceptors. However, subsequent to their regeneration, cone photoreceptors selectively fail to mature or survive. Together, these data show that in zebrafish inflammation positively regulates photoreceptor regeneration. Additionally, the inflammatory protease, Mmp-9, governs the early proliferation of injury-induced photoreceptor progenitors and is required for the subsequent maturation and survival of regenerated cones.

MATERIALS AND METHODS

Animals

AB wild-type zebrafish (Danio rerio; ZIRC, University of Oregon, Eugene, Oregon) were propagated, maintained, and housed in recirculating habitats at 28.5°C and on a 14/10-h light/dark cycle. Embryos were collected immediately after spawning and incubated at 28.5°C on a 14/10-h light/dark cycle. Adults were between 6 and12 months of age. The transgenic reporter line, *Tg[gfap:EGFP]mi2002,* was used to identify Müller glia in retinal sections (Bernardos and Raymond, 2006). All experimental protocols were approved by the University of Michigan's Committee for the Use and Care of Animals.

Light Lesions

To kill photoreceptors, fish were dark adapted, then exposed to high intensity light (ca. 100,000 lux) for 30 minutes, followed by exposure to constant light (ca. 30.000 lux) for 72 hours (Taylor et al., 2015). After 72 hours fish were returned to the recirculating habitats and normal light/dark cycle.

RNA extraction and Quantitative Real-Time PCR (qPCR)

Total RNA was extracted from whole dissected retinas using TRIzol (Invitrogen, Carlsbad, CA). For each time point, three independent biological replicates were collected, and each replicate contained six homogenized retinas from three fish (18 retinas/time point). Reverse transcription to cDNA was performed using 1 µg RNA (Qiagen QuantiTect Reverse Transcription kit; Venlo, Netherlands). For qPCR, each biological replicate was run in triplicate with 6 ng cDNA and Bio-Rad IQ SYBR Green Supermix (Bio-Rad CFX384 Touch Real Time PCR Detection System; Hercules, CA). Data represented as Log2 fold change was calculated using DDC_T method and normalized to the housekeeping gene, *gpia*. Primers used are listed in Table 2.1.

Anti-inflammatory treatment

A previously described protocol was used suppress injury-induced inflammation (Kyritsis et al., 2012). Briefly, fish were housed in system water with 15 mg/ L dexamethasone (Sigma, Ca. No: D1756) diluted in 0.1% MetOH for 14 days, which included the 72hrs of light exposure (Kyritsis et al., 2012). Dexamethasone was changed daily and fish were fed brine shrimp every other day. Controls animals were housed in system water containing the vehicle solution.

Immunohistochemistry

Whole larvae (48-72 hpf) and dissected eyecups (adults) were fixed overnight at 4°C in phosphate buffered 4% paraformaldehyde, cryoprotected with 20% sucrose, and embedded in optical cutting temperature (OCT) medium (Sakura Finetek USA, Torrance, CA). Immunohistochemistry (IHC) was performed as previously described (Taylor et al., 2015). 10-µm-thick sections, collected through the optic nerve head from both embryos and eye cups, were mounted on glass slides. Sections were washed in phosphate buffer saline with 0.5 % Triton-x (PBST) and incubated in 20% heat

inactivated normal sheep serum for 2 hours (NSS; Sigma-Aldrich Corp., St Louis MO). Primary antibodies were applied overnight at 4°C. Sections were then washed with PBST and incubated in secondary antibodies for 1 hour at room temperature. Prior to IHC for BrdU, sections were immersed in 100°C sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) for 30 minutes cooled at room temperature for 20 minutes. Sections were then subjected to IHC as described above. IHC performed on whole retinas was conducted as described previously (Nagashima et al., 2017). Prior to IHC, retinas were dissected from dark-adapted animals, flattened with radial relaxing cuts, fixed at 4 C overnight in 0.1M phosphate buffer 4% paraformaldehyde with 5% sucrose. The antibodies used here and their concentrations are listed in Table 2.2.

Labeling dividing cells

Dividing cells were labeled with either EdU (larvae) or BrdU (adults). Larvae were incubated for 20 minutes in ice-cold (48 hpf) or room-temperature (72 hpf) EdU (1.5 mM), dissolved in embryo rearing solution containing 15% dimethylsulfoxide (DMSO). EdU labelled cells were visualized using the Click-it Assay kit (Invitrogen, Carlsbad, CA, USA; Walsh and Hitchcock, 2017). Dividing cells in adults were labeled by housing animals in system water containing 5 mM BrdU for 24 hours (Gramage et al., 2015).

***In situ* Hybridization**

In situ hybridizations were performed as previously described (Luo et al., 2012). Digoxigenin (DIG)-labeled riboprobes for *rhodopsin* and *pde6c* were generated from full-length cDNA clones (Ochocinska and Hitchcock, 2007), whereas riboprobe for *mmp-9* was generated by PCR amplification using primers containing the T3 or T7 promoter sequences (David and Wedlich, 2001). *mmp-9* (F): 5’

TAATACGACTCACTATAGGGGATTCTTCTACTTCTGCCGGG 3' *mmp-9* (R): 5' AATTAACCCTCACTAAAGGGCTTAATAAATTTGTAAACAAG 3'.

Briefly, 10-µm-thick sections were hybridized with riboprobes at 55°C, incubated with an alkaline-phosphatase-conjugated anti-DIG antibody and visualized using Fast Red TR/Naphthol AS-MX (SIGMA*FAST*) as the enzymatic substrate. When *in situ* hybridizations were combined with BrdU IHC, sections were removed from the fast red solutions, rinsed and post-fixed in buffered 4% paraformaldehyde for 10 minutes then processed for BrdU IHC as described above.

Mmp-9 antibodies

Antibodies specific to zebrafish Mmp-9 were generated by Pocono Rabbit Farm & Laboratory (PRF&L) as previously described (Calinescu et al., 2009a). A 24 amino acid peptide was used as the immunogen CDIDGIQYLYGPRTGPEPTAPQPR; NCBI: AY151254. Polyclonal antibodies were affinity purified and confirmed by ELISA (data not shown). Western blots performed with retinal proteins using pre and post-immune sera confirmed the post-immune serum detected a 76 kDa band, the predicted size of Mmp-9.

Western Blot Analysis

Protein samples were obtained from whole retinas homogenized in RIPA lysis buffer (ThermoFisher Scienific, Waltham, MA) containing protease and phosphatase inhibitor cocktail (5872S; Cell Signaling Technology, Danvers, MA, USA). Each sample contained six pooled retinas from three adult fish. Proteins were separated in a 12% Mini-PROTEAN TGX Precast gel (BioRad; Hercules, CA) and transferred to a polyvinylidene difluoride (PVDF) membrane (GenHunter Corp., Nashville, TN). To block non-specific protein, the membrane was incubated in 5% nonfat dry milk in Tris buffered

saline containing 0.3% Tween-20 (TBST) for 2 hours. Membranes were incubated with the antibodies-containing solution overnight at 4°C. Blots were then washed in TBST and incubated with horseradish peroxidase-conjugated secondary IgG (1:1000) for 1 hour at room-temperature. Antibody-bound protein was visualized using Pierce ECL Western blotting substrate (32106; ThermoFisher Scienific, Waltham, MA). To visualize loading controls, blots were also probed with antibodies against actin. Images were captured using the Azure C500 (Azure Biosystems). Densitometric analysis of protein bands was performed with ImageJ software (https://imagej.nih.gov/ij/).

Zymogram Analysis

Protein samples were prepared as described for Western blot analysis. Proteins were separated on 10% Zymogram Plus (Gelatin) Protein Gels, (ThermoFisher Scientific; Waltham, MA). Following electrophoresis, proteins were renatured in 1X renaturing buffer (ThermoFisher Scientific; Cat#LC2670) for 30 mins at room temperature, then incubated in 1X developing buffer (ThermoFisher Scientific; Cat#LC2671) overnight at 37º. Gels were rinsed in deionized water and stained with SimplyBlue SafeStain (ThermoFisher Scientific; Cat#LC6060). Gels were imaged with long-wave ultraviolet light using the Azure C500 (Azure Biosystems; Dublin, CA). Active recombinant human MMP-9 (Calbiochem; PF140) was used as a positive control. Densitometric analysis of the digested bands was performed with ImageJ software.

Purification and intraocular injection of TNF-α

To express the soluble form of zebrafish Tnf-α, the pQE30 plasmid containing zebrafish Tnf-α cDNA was transfected into M15 cells (QIAGEN, Germantown, MD) as described previously (Conner et al., 2014). IPTG (final concentration: 1 mM, isopropylthio-β-galactoside, Life Technologies; Carlsbad, CA) was used to induce Tnf-α protein

expression. An empty vector was used as the control. Control and Tnf-α samples were subjected to the same purification methods. Purified Tnf-α was diluted to 1 mg/ml. 0.5 µl of either control lysate or recombinant Tnf-α was intravitreally injected using a Hamilton syringe. Intravitreal injections were repeated every 24 hours, and total RNA was collected at 72 hours post injection (hpi).

CRISPR-mediated gene mutation

mmp-9 mutants were generated according to previously described methods (Hwang et al., 2013). ZiFiT software (available in the public domain at zifit.partners.org) was used to identify the 19 bp sgRNA target sequence for *mmp-9* (GGCTGCTTCATGGCATCAA). Target sequence Oligo1-TAGGCTGCTTCATGGCATCAA and Oligo2-AAACTTGATGCCATGAAGCAG were annealed and subcloned into the pT7 gRNA vector (Addgene ID: 46759). The pCS2 nCas9n vector (Addgene ID: 46929) was used for Cas9 synthesis. To produce RNAs, the MEGAshortscript T7 kit, (Ambion: AM1354) and mirVana miRNA Isolation kit (Ambion: AM 1560) was used for the gRNA, whereas, mMesage mMACHINE SP6 kit (Ambion: AM1340) and RNeasy mini Kit from (Qiagen: 74104) was used for Cas9 mRNA. Single cell-stage embryos were injected with 2 nL solution containing 100 pg/nl sgRNA and 150 pg/nl Cas9 mRNA. Founders (F0) were outcrossed to AB wild-type fish. Mutations were identified using screening primers (F: 5′- AAGTCTGCAACTACATATCAGC -3′, R: 5′-GTACACACTGTAGATGCTGATAAG-3’) that flanked the *mmp-9* sgRNA target site. Standard protocols for PCR used Platinum Taq-HF polymerase (Invitrogen; city) with genomic DNA as the template. The purified PCR product was subcloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) for Sanger sequencing. Mutant and wild-type sequences were aligned using pairwise blast (National Center for Biotechnology Information [NCBI], Bethesda, MD,

USA). Premature stop codons were identified by comparing predicted amino acid sequences for wild-type and mutants using the ExPASy translate tool (available in the public domain at (www.expasy.org). F1s carrying a *mmp-9* mutation were in-crossed and homozygous F2 mutants were identified by a combination of Sanger sequencing and T7 endonuclease assays (New England Biolabs, Ipswich, MA, USA) as previously described (www.crisprflydesign.org).

Imaging and Statistical Analysis

Fluorescence images of retinal sections were captured using the Leica TCS SP5 confocal microscope (Wetzlar, Germany). Cell counts were conducted using z-stack images and analyzed with Imaris software (Bitplane, South Windsor, CT, USA). Regenerated photoreceptors were identified by the colocalization of DAPI, BrdU, and the ISH markers *pde6c* (cones) or *rho* (rods). Cone photoreceptors were counted in Xcontrol and Xexperimental whole-mount retinas. For each, five separate regions, totaling 5625^2 μm/retina, were sampled. Statistical comparisons between control and experimental retinas were performed using a Student's t-test by GraphPad Prism 5 (La Jolla, CA, USA). ANOVA was performed using JMP 9.0 (SAS Institute, Inc). A p-value less than 0.05 was considered a statistically significant difference.

RESULTS

Inflammatory genes are strongly induced during photoreceptor regeneration

To characterize the inflammatory response during photoreceptor regeneration, qPCR was used to quantify the expression of a subset of inflammatory genes, *mmp-9, tnf-a*, *tnf-β, il-8, nfkb1,* and *nfkb2*, previously shown to regulate various forms of tissue regeneration in zebrafish (LeBert et al., 2015; Nelson et al., 2013; de Oliveira et al., 2013; Karra et al., 2015). The time course of gene expression follows closely the well-established time course for the injury response in Müller glia, the appearance of the Müller glia-derived progenitors, and the subsequent differentiation of regenerated photoreceptors (Figure 2.1A; see Gorsuch and Hyde, 2014). Upregulation of gene expression is detectable by 8 hours post lesion (hpl), and the rising phase in expression corresponds to the interval where Müller glia prepare to enter the cell cycle. Expression levels then slowly decline as the Müller glia-derived photoreceptors progenitors divide, migrate to the outer nuclear layer and differentiate into regenerated photoreceptors (168 hpl; Figure 2.1A). Interestingly, among these genes, *mmp-9* shows the highest levels expression, which is maximal at 48 hpl, and by 168 hpl *mmp-9* levels are reduced to only about 50% of their starting values (ANOVA F-ratio = 22.23, p= .0001; Figure 2.1A). The pro-inflammatory cytokine, Tnf-a, has been posited to be responsible for initiating photoreceptor regeneration in zebrafish (Nelson et al., 2013), but at all time points in this assay, the levels of *tnf-a* expression were not significantly different from controls. These data show that in zebrafish a photolytic lesion, which leads to photoreceptor death, induces rapid expression of inflammatory genes that follows a time course reflecting the well-described events that underlie photoreceptor regeneration. Further,

the expression of *mmp-9* remains significantly elevated as photoreceptor differentiation commences.

Inflammation regulates injury-induced proliferation and photoreceptor regeneration

To determine if inflammation regulates aspects of photoreceptor regeneration, the glucocorticoid steroid, dexamethasone (Dex), was used to suppress the inflammatory response (see, Kyritsis et al., 2012). To validate Dex treatment, the expression levels of inflammatory genes were quantified in control and Dex-treated groups. At 72 hpl, the expression of *mmp-9*, *il-6*, and *nfkb1* were significantly decreased following dexamethasone treatment (p values = .0001, .0061, .0003, respectively; Figure 2.1—figure supplemental 2.1A). Dex treatment did not suppress the expression of *tnf-a*, which was expected, given this gene was not significantly induced in the original assays. The failure to suppress *il-8* expression by Dex treatment is unresolved.

Based on previous results (Kyritsis et al., 2012), assays of cell proliferation and photoreceptor regeneration were performed for control animals and animals treated with Dex. Here, dividing cells were labelled with BrdU between 24 and 48 hpl to mark those progenitors that will give rise to regenerated photoreceptors, and regenerated photoreceptors were identified as BrdU-labeled nuclei in the outer nuclear layer surrounded by *in situ* hybridization signal for either *rho* (rods) or *pde6c* (cones). At 72 hpl (Figure 2.1B, Methods), there was significantly less BrdU-labeled cells in Dex-treated retinas compared to controls (p= 0.0079; Figure 2.1C, D). Similarly, at 7 days post lesion (dpl) there were significantly less regenerated rods and cones, in Dex-treated groups compared to controls (Figure 2.2B, C; p=0.0012). These results indicate that, consistent with data showing the role of inflammation in neuronal regeneration in

the forebrain of zebrafish (Kyritsis et al., 2012), inflammatory mechanisms also govern photoreceptor regeneration in the retina. Further, inflammation in the zebrafish central nervous system positively regulates proliferative events that underlie neuronal regeneration, the key step in quantitatively determining the number of regenerated neurons.

Müller glia and Müller glia-derived progenitors express *mmp-9*

Due to the variable roles played by *mmp-9* in injured/regenerating tissues (Vandooren et al., 2014**),** and its persistent expression throughout the time-course of photoreceptor regeneration (Figure 2.1A), the specific function of Mmp-9 was investigated further. *In situ* hybridization was performed first, to identify the cellular patterns of expression of *mmp-9*. This evaluation of cellular gene expression was combined with the Müller glia reporter line, *Tg[gfap:EGFP]mi2002,* and BrdU labeling 24 hrs prior to sacrifice to identify dividing cells. There is no detectable expression of *mmp-9* in unlesioned retinas. In contrast, by 24 hpl, *mmp-9* is expressed exclusively in Müller glia*,* but at a time-point prior to their entry into S-phase of the cell cycle (Figure 2.3A). By 48 hpl, ~97% of BrdU-labeled Müller glia express *mmp-9* (Figure 2.3A, B, C). At 72 hpl, the expression of *mmp-9* decreases (Figure 2.3C), and the cellular expression shifts to the Müller glia-derived progenitors, as evidenced by BrdU-labeled cells that express *mmp-9* but lack eGFP (Figure 2.3A). These data suggest that *mmp-9* expression identifies the subset of Müller glia that will enter the cell cycle in response to photolytic lesion, and the strict association of *mmp-9* expression in dividing cells suggests that Mmp-9 may govern aspects of cell division within Müller glia, Müller glia-derived progenitors or both.

Mmp-9 protein is present and catalytically active following photoreceptor death

In tissues, Mmp-9 is synthesized and secreted in a proform, as a zymogen, then converted into an active protease (Vandooren et al., 2013b). Western blot analysis was used to characterize the induction of protein synthesis and zymogram assays were used to determine the time course of protein synthesis and catalytic activity for Mmp-9 during photoreceptor regeneration (Vandooren et al., 2013a, 2013b). Mmp-9 is not detected in unlesioned retinas, consistent with the qPCR and *in situ* hybridization data (Figure 2.1A, 2.3A, 2.4A). At 16 hpl, the first time point sampled, the un-cleaved, pro-form of Mmp-9 is detected as an upper band in Western blots (Figure 2.4A). Mmp-9 levels peak by 24 hpl, and both forms of Mmp-9 are detected. Mmp-9 levels decrease between 24 and 72 hpl, and during this interval the protein mostly appears in a slightly lower band, consistent with the active form of the protein. Mmp-9 levels are undetectable by Western blot analysis at 5 dpl (ANOVA F-ratio = 8.377, p =.0013) (Figure 2.4 A, B). To evaluate the catalytic activity of Mmp-9, the same protein samples as were used for the Western blot analysis were used for the zymogram analysis (Vandooren et al., 2013a; Chadzinska et al 2008). Recombinant, active human MMP-9 was used as a positive control and shows strong catalytic activity as evidenced by the large negatively stained band. Unlesioned retinas contain no Mmp-9 catalytic activity. In contrast, and as would be predicted, the catalytic activity parallels the data from the Western blot analysis (ANOVA F-ratio = 11.870, p = .0003) (Figure 2.4C, D). Together, results from the Western blot and zymogram analyses show that catalytically-active Mmp-9 is induced by a lesion that selectively injures photoreceptors, and the time course of protein synthesis and catalytic activity parallels the proliferative phases of photoreceptor regeneration.

Tnf-α is sufficient to induce *mmp-9* expression in unlesioned retinas

Tnf-α induces *mmp-9* expression in a variety of tissues (Vandooren et al., 2014). In zebrafish, this cytokine is induced by photoreceptor injury and death, and it is required for Müller glia to enter the cell cycle (Nelson et al., 2013). Therefore, the ability of Tnf-α to induce *mmp-9* expression in the zebrafish retina was tested. Tnf-α was injected into the vitreous space of unlesioned eyes, and *mmp-9* expression was quantified by qPCR (Figure 2.5A). There was no induction of *mmp-9* in eyes that were uninjected or eyes that received injections of elution buffer only. In contrast to these controls, *mmp-9* was strongly induced in the retinas of eyes that received intraocular injections of Tnf-α ($p = 0.0229$) (Figure 2.5B). *Stat-3*, which serves as a positive control for injury-induced proliferation in the retina (Nelson et al., 2012) was also strongly induced by Tnf-α injections ($p = 0.0344$) (Figure 2.5C). These results indicate that Mmp-9 functions downstream of Tnf-α and signaling molecule, potentially works upstream to induce *mmp-9* expression following photoreceptor death.

CRISPR mutants lack Mmp-9 protein and catalytic activity

To investigate the function of *mmp-9* during photoreceptor regeneration, mutants were generated using CRISPR-Cas9 (Hwang et al., 2013), targeted to the *mmp-9* catalytic domain in exon 2 (Figure 2.6A). The 19bp sgRNA produced several alleles, two of which were bred to homozygosity and characterized further (Figure 2.6B). Individuals from the two mutant lines were crossed to create compound heterozygotes to evaluate the potential effects off target effects in the two independent lines (see next section). The two *mmp-9* lines carry mutations that result in a frameshift that gives rise to predicted premature stop codons (Figure 2.6—figure supplemental 2.6A). Retinas from mutants were characterized by Western blot analysis and zymogram assays.

Unlesioned retinas had no detectable Mmp-9 or catalytic activity (Figure 2.6C, D). As expected, following a photolytic lesion, catalytically-active Mmp-9 is present wild-type retinas. In contrast, there was only trace Mmp-9 and catalytic activity in the retinas of mutants carrying the 8 bp insertion, and no detectable Mmp-9 or catalytic activity in the retinas carrying the 23 bp deletion (Figure 2.6D). Based on these results, all subsequent experiments were conducting using the 23bp deletion mutant, though the basic observations reported here were confirmed in the 8bp insertional mutant (data not shown) and the compound heterozygotes (see below).

Photoreceptor death results in overproduction of photoreceptor progenitors

Given that injury-induced proliferation was assayed in mutants (see below), the number of Müller glia and the number of mitotic progenitors that support intrinsic retinal neurogenesis, a feature of all teleost retinas (see Raymond et al., 2006), were also compared in wild-type and mutant retinas. This showed that in unlesioned retinas there were no differences between wild-type and mutants in the number of Müller glia the level of intrinsic cell proliferation (Figure 2.7—figure supplemental 2.7D). To determine the consequences of Mmp-9 loss-of-function, wild-type and mutant animals received photolytic lesions, Müller glia-derived photoreceptor progenitors were labeled with BrdU between 48-72 hpl, and BrdU-labeled cells were counted at 72 hpl. Relative to wild-type animals, mutants had significantly more BrdU-labeled progenitors at 72 hpl ($p = .0186$) (Figure 2.7A, B). This over production of injury-induced progenitors was also observed in the 8bp insertional mutant (data not shown) and the compound heterozygotes (Figure 2.7—figure supplemental 2.7C), confirming that the hyperproliferation can be ascribed solely Mmp-9 loss-of-function.

It is well established that Mmp-9 can modulate cell migration (Parks et al., 2014). To determine if Mmp-9 loss-of-function alters migration of photoreceptor progenitors from the inner nuclear layer, where Müller glia reside to the outer nuclear layer, to the outer nuclear layer, which exclusively contains photoreceptor nuclei, animals were subjected to photolytic lesions, exposed to BrdU between 48 and 72 hpl and allowed to survive to 7 days post lesion (dpl), a time point where proliferation and migration are complete and photoreceptor differentiation has commenced. Qualitative inspection shows that Mmp-9 loss-of-function does not alter the migration of photoreceptor progenitors (Figure 2.7A, C). Further, cell counts showed that the over production of photoreceptor progenitors observed in mutants at 72 hpl resulted in a significantly greater number of BrdU-labeled cells within the ONL at 7 dpl ($p = .0001$) (Figure 7B, D), and a significantly greater number of regenerated rod and cone photoreceptors ($p = .0301$ and $p = .0005$, respectively) (Figure 2.7 E-G). Consistent with the established patterns of expression (see above), these data show that, in the absence of an injury, Mmp-9 loss-of-function has no impact on early retinal development, the proliferative potential of the adult retina or migration of progenitor cells through across retinal and synaptic layers. However, the hyperproliferation of injury-induced progenitors in mutants shows that Mmp-9 functions to negatively regulate proliferation of Müller glia-derived progenitors, though the mechanisms through which this is accomplished are not yet known (see Discussion).

Mmp-9 governs maturation and survival of regenerated cone photoreceptors

The qPCR data showed that *mmp-9* levels remain significantly elevated at the time injury-induced photoreceptor progenitors exit the cell cycle and begin differentiating into mature photoreceptors. This suggested that Mmp-9 may have functional roles beyond

governing proliferation during the initial stages of photoreceptor regeneration. Therefore, regenerated rod and cone photoreceptors were qualitatively and quantitatively compared in wild-type and mutants at 21 dpl, a time point where regeneration is complete (Powell et al., 2016). For both groups, retinal sections were labeled with rod- and cone-specific antibodies and regenerated photoreceptors were counted in sections and whole mount preparations. These analyses showed there were no qualitative differences between wild-type and mutants in the appearance of regenerated rod photoreceptors (Figure 2.8A), and the initial over production of rod photoreceptors observed at 7 dpl was present at 21 dpl (Figure 2.8B, C).

At 21 dpl, the maturation and survival of cones in mutant retinas were clearly compromised. Relative to controls, regenerated cones in mutants have shorter outer segments and appear to be fewer in number (Figure 2.8A, inset 8B). Counts of regenerated cones in tissues sections show the initial over production of cones, evident at 7 dpl, is absent (2.8C). Based on their immature appearance and the downward trend in the number of regenerated cones photoreceptors between 7 and 21dpl, cones were counted in whole mount retinas (Figure 2.9). In the whole mounts, cones were identified by their prominent profiles in optical sections taken through the inner segments, visible when retinas are stained with an antibody against the tight junction protein, ZO-1 (Nagashima et al., 2017). In both wild-type and mutant retinas, cone photoreceptors in unlesioned retinas are characterized by the very precise lattice mosaic, characteristic of cone photoreceptors in teleost fish (Nagashima et al., 2017; Figure 2.9A). Following photoreceptor regeneration, areas of the retina that contain regenerated photoreceptors are readily identifiable by the marked spatially degraded of the mosaic, though

individual cone photoreceptors are readily identifiable (Nagashima et al., 2017). Counts of regenerated cones in whole mounts show that mutants have significantly less regenerated cones than wildtype animals ($p = .0229$) (Figure 2.9A, B). Finally, as an independent measure of the maturation of cone photoreceptors, Western blot analysis was performed using an antibody against the cone-specific transducin protein, Gnat-2 (Figure 2.9C; Lagman et al., 2015). As expected, Gnat-2 levels in unlesioned retinas are comparable in wild-type and mutant animals. In lesioned retinas, Gnat-2 levels begin to recover in wild-type animals by 14 dpl, and by 21 dpl values in wild-type animals are nearly at the levels found in unlesioned retinas. In contrast, in mutants Gnat-2 levels lag behind wild-type values, and at 21 dpl Gnat-2 levels in mutant animals is significantly less than in wild-types ($p = .0014$) (Figure 2.9D). Comparable measures specific to rod photoreceptors show there were no differences between wild-type and mutant animals (data not shown). Together, these results show that Mmp-9 also has a functional role during photoreceptor regeneration well after the phase covering injury-induced proliferation and the initial differentiation of regenerated photoreceptors. Further, the function of Mmp-9 is specific to cone photoreceptors and governs aspects of the maturation and survival of these cells.

DISCUSSION

In this study I characterized the roles of inflammation and the matrix metalloproteinase 9 (Mmp-9) during photoreceptor regeneration in the adult zebrafish. Inflammatory genes, *tnf-a*, *tnf-β, il-8, nfkb1, nfkb2,* including *mmp-9* are expressed during injury-induced Müller glia proliferation, production of progenitors, and photoreceptor regeneration. Anti-inflammatory treatment suppresses, inflammatory gene expression, the production of progenitors, and photoreceptor regeneration. Interestingly, *mmp-9* is expressed in Müller glia as they enter the cell cycle and progenitors as they proliferate. Mmp-9 expression is catalytically active as Müller glia produce progenitors for photoreceptor regeneration. The pro-inflammatory cytokine, Tnf-α signals upstream of *mmp-9* and sufficient to stimulate its expression in an unlesioned retina. Our generated *mmp-9* CRISPR mutants respond to the photolytic lesion by hyper-proliferation and an initial increase in photoreceptor regeneration. However, subsequent to their genesis cone maturation and survival is compromised. The elements of these results are captured in the model summarized in Figure 10. Our data provides a link between inflammation, Mmp-9 function, and photoreceptor regeneration in the adult zebrafish.

In the zebrafish, anti-inflammatory treatment by Dexamethasone (Dex) has varying effects on neuronal progenitors that is dependent on the time of treatment (White et al., 2017). Zebrafish larvae that receive genetic ablation of rod photoreceptors combined with pretreatment of Dex, that is continued during regeneration results in a decrease in microglia migration and rod regeneration (White et al., 2017). However, in the same study, Dex treatment one day following rod ablation results in accelerated rod regeneration. Our prolonged exposure to Dex following specific rod and cone photolytic lesion in adult zebrafish results in a reduction of Müller glia-derived progenitors and

regenerated photoreceptors, consistent with pretreatment larval rod ablation studies, but shows a reduction for both regenerated photoreceptors. Moreover, our data parallels studies conducted in the zebrafish brain and retina of the chick (Gallina et al., 2014; Kyritsis et al., 2012), which showed that Dex treatment suppressed microglia activation and neuronal progenitors. While our study did not focus on the inflammatory contributions of microglia, I cannot exclude their roles as previously demonstrated in the zebrafish brain and retina during regeneration (Kyritsis et al., 2012; White et al., 2017). Collectively, these results demonstrate that components of the inflammatory response regulate the ability of progenitors to proliferate and can differ depending on time of treatment in the zebrafish.

I characterized the expression of *mmp-9* during photoreceptor regeneration and demonstrate that expression is restricted to Müller glia and its progenitors. Recently, Kaur et al., (2018) showed that zebrafish Müller glia and derived progenitors express *mmp-9* following needle poke. Pharmacological or morpholino inhibition of *mmp-9* following needle poke lead to a decrease in the production of Müller glia progenitors (Kaur et al., 2018). However, our studies show that following photolytic lesion, Mmp-9 loss-of-function mutants have an increase number of progenitors and regenerated photoreceptors. The difference in results may account for a number of reasons. Morpholinos provide temporary inhibition and have been shown to have potential off-target effects as an experimental tool (Kok et al., 2015). The pharmacological inhibitors used in Kaur et al., (2018) studies are not specific and target *mmp-2* as well (Lin et al., 2007; Tao et al., 2010). Additionally, it is possible that *mmp-9* mutants have off-target effects that cannot be detected. Though, our compound heterozygotes address this

potential issue and respond to lesion with a similar phenotype to individual *mmp-9* mutants. In the rat, MMP-9 is expressed in Schwann cell and NG2+ progenitors during injury-induced proliferation (Chattopadhyay and Shubayev, 2009; Liu and Shubayev, 2011). Genetic or pharmacological inhibition of MMP-9 following CNS injury in rodents increases the number of progenitors (Chattopadhyay and Shubayev, 2009; Liu and Shubayev, 2011; Liu et al., 2010; Kim et al., 2012). Our data parallels mammalian studies and suggest a conserved mechanism by which *mmp-9* negatively regulates injury-induced proliferation.

MMP-9 was first purified from human leukocytes following stimulation by cytokines and is now recognized as an inflammatory proteinase (Masure et al., 1991; Opdenakker, Masure, Grillet et al., 1991; Opdenakker, Masure, Proost et al., 1991). Under injury and disease, Mmp-9 is secreted to mediate inflammation by the direct cleavage of cytokines and growth factors for their activation or degradation (Bonnans et al., 2014; Manicone and McGuire, 2008; Parks et al., 2004; Le et al., 2007; Vandooren et al., 2013). It is well established that inflammation is a key factor resulting in the pathogenesis of retinal diseases including, but not limited to retinitis pigmentosa and age-related macular degeneration (AMD), resulting in the degeneration of photoreceptors (Kauppinen et al., 2016; Wang et al., 2011; Whitcup et al., 2013; Yoshida et al., 2013; Chen and Xu, 2012). I hypothesize that in the absence of *mmp-9,* injury-induced inflammatory cytokines fail to be degraded and this contributes an unresolved chronic inflammatory state similar to AMD and retinitis pigmentosa. The observed hyperproliferation phenotype in *mmp-9* mutants following photoreceptor lesion is directly opposite to anti-inflammatory treatment by Dex, suggesting that injury-

induced inflammation regulates the extent proliferation. Interestingly, anti-inflammatory treatment is commonly used to treat patients with retinal diseases associated with inflammation (Abadia et al., 2016; Wang et al., 2011). AMD or macular edema patients that received dexamethasone intravitreal implants have improved vision, associated with a decrease in intraretinal fluid and vein occlusions, respectively (Calvo et al., 2015; Haller et al., 2010).

Recently, mutational variants in inflammatory regulators, *TIMP-3*, *TGFB*, *TNFα,* and *MMP-9* were identified in Human patients of AMD (Fritsche et al., 2016). The decrease in regenerated cones observed in our *mmp-9* mutants phenocopies Human retinal disease states, suggesting an exacerbated inflammatory state contributes to photoreceptor degeneration. However, the difference we observe in photoreceptor survival sheds light on the specific sources of regenerated photoreceptors (Hitchcock and Raymond 2004; Raymond et al., 2006). It is established that an alternative source of rod precursors located in the ONL of teleost fish provides lineage-restricted rod progenitors throughout development (Otteson, D'Costa, and Hitchcock, 2001; Raymond et al., 2006). Therefore, in addition to Müller glia-derived photoreceptor progenitors, rod restricted progenitors may provide an additional source to counteract rod death, contributing to no difference in *mmp-9* mutants.

Moving forward, the identification of Mmp-9 substrates following photolytic lesion that signal to promote retinal regeneration in zebrafish will be critical to further investigate this signaling mechanism. MMP-9 has over 100 substrates adding to its functional complexity as a secreted protease (Vandooren et al., 2014). A hand full of inflammatory cytokines *Il-8*, *il-1b, tgf-beta,* and *galectin-1* have been identified by *in vitro*

cell culture and proteomic studies as direct Mmp-9 substrates (Ito et al., 1996; Prudova et al., 2010; Yu and Stamenkovic, 2000). It is suggested that the growth factor, heparin-binding epidermal-like growth factor (HB-EGF) may serve as a potential substrate for Mmp-9 and necessary for Müller glia proliferation (Wan et al., 2012). Future studies should consider the identified inflammatory cytokines and potential growth as Mmp-9 substrates involved in zebrafish retinal regeneration. A challenge will be to create zebrafish specific antibodies that will recognize Mmp-9 substrates that govern tissue and context dependent phenotypes. Moreover, we must understand the inflammatory conditions that contribute to the negative effects seen in mammalian studies, but positive regenerative roles in non-mammalian vertebrates. Our studies and previous have begun to shed light on Mmp-9 with a promising link between inflammation regulating proliferation and photoreceptor regeneration in the zebrafish retina.

Figures

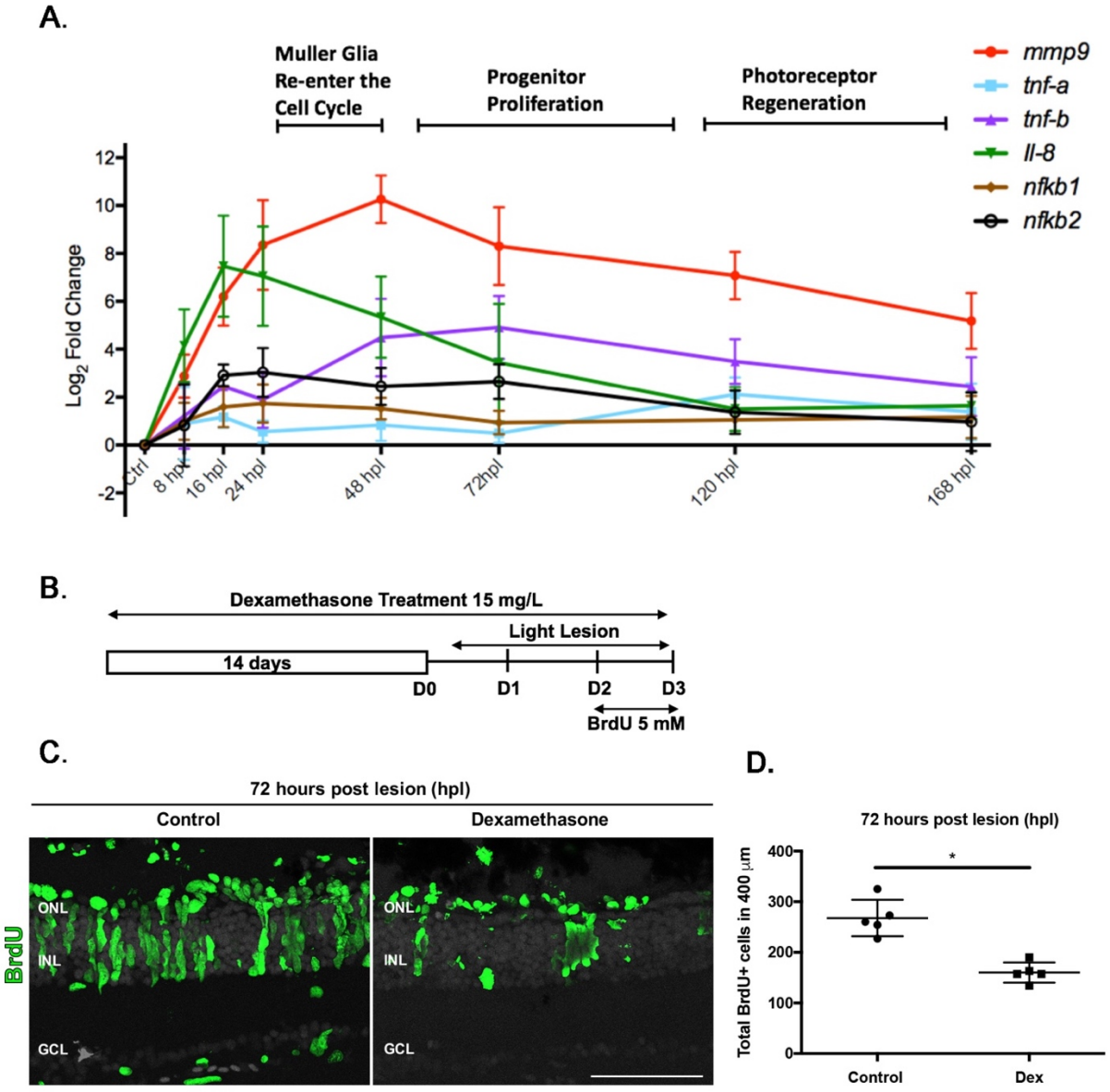
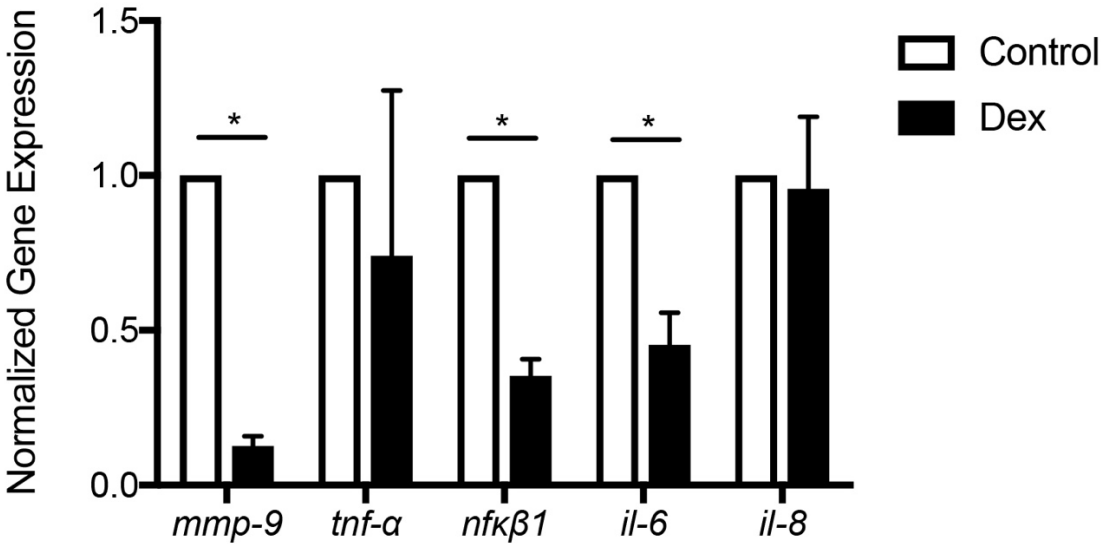


Figure 2.1: Inflammation is induced following photoreceptor death and regulates reactive-proliferation

(A) qPCR time-course of inflammatory genes from 8 to 168 hours post lesion (hpl) relative to uninjured controls. Data represented as fold change calculated using DDC_T method (n=3; 6 retinas per sample). **(B)** Experimental schematic for anti-inflammatory treatment by Dex and proliferation analysis with BrdU. **(C)** BrdU immunostaining (green) from untreated controls and Dex treatment at 72 hpl. **(D)** Quantification of BrdU+ cells from untreated controls (268 ± 36.1 cells; *n*=5) compared to Dex treatment (160.2 ± 20.02 cells; *n*=5) at 72 hpl; p=0.0079. Quantitative data are represented as mean; error bars represent the standard deviation. ONL- outer nuclear layer; INL- inner nuclear layer; GCL- ganglion cell layer. Scale bar equals 50 μm.

A.



Supplemental Fig 2.1 Dex treatment suppresses inflammation following photoreceptor injury.
(A) qPCR for inflammatory genes *mmp-9, tnf-a, nfkb1, il-8,* and *il-6* following Dex treatment compared to untreated controls at 72 hpl. Data calculated using DDC_T method and represented as normalized data to untreated controls (n=3; 6 retinas per sample). Quantitative data are represented as mean; error bars represent the standard deviation. *p≤0.05.

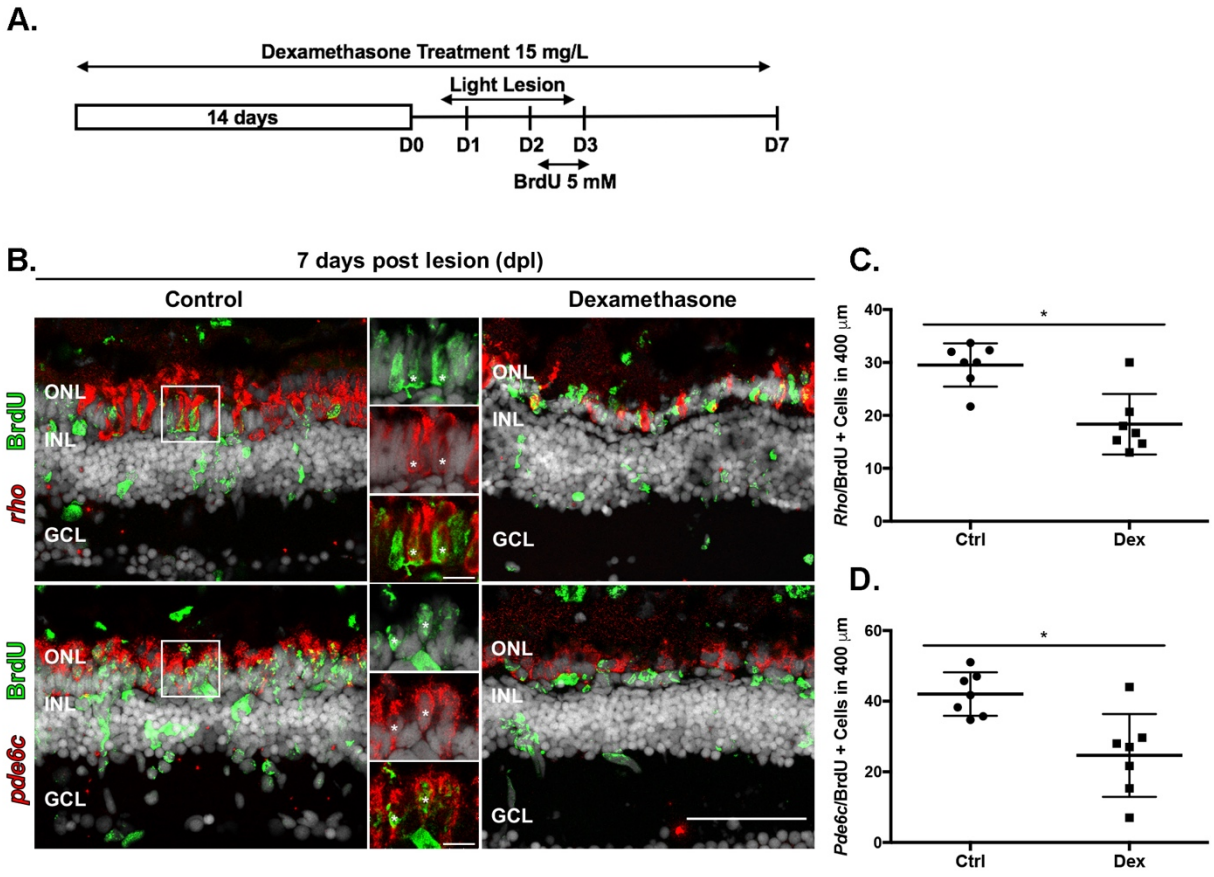


Figure 2.2: Anti-inflammatory treatment by Dexamethasone prevents photoreceptor regeneration.
(A) Experimental schematic of anti-inflammatory treatment for photoreceptor regeneration. **(B)** *in situ* hybridization for *pde6c* (cones) and *rhodopsin* (rods) combined with BrdU immunostaining (green). The high magnification panel indicates the colocalization of regenerated photoreceptors in the ONL are indicated by asterisk (*). **(C)** Quantification of regenerating cone photoreceptors in the ONL from untreated controls (42 ± 6.1 cells; *n*=7) compared to Dex treatment (25 ± 11.72 cells; *n*=7) at 168 hpl; p=0.0047. **(D)** Quantification of regenerating rod photoreceptors in the ONL from untreated controls (29.52 ± 4.1 cells; *n*=7) compared to Dex treatment (18.33 ± 5.71 cells; *n*=7) at 168 hpl; p=0.0012. Quantitative data are represented as mean; error bars represent the standard deviation. ONL- outer nuclear layer; INL- inner nuclear layer; GCL- ganglion cell layer. Scale bar equals 50 μm.

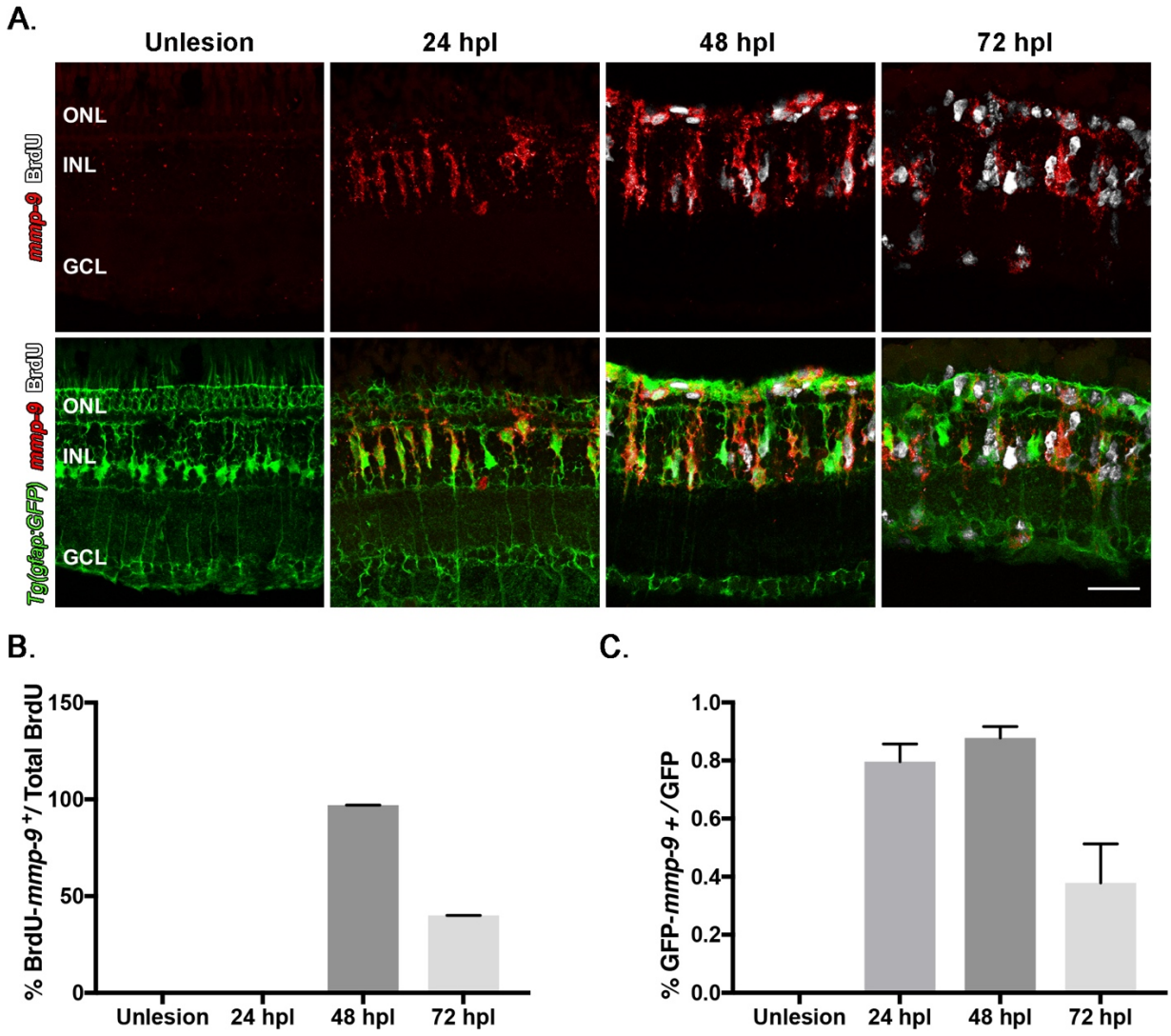


Figure 2.3: Müller glia and Müller glia-derived progenitors express injury-induced *mmp-9*.
(A) ISH time-course for *mmp-9* performed on Müller glia transgenic line *Tg[gfap:EGFP]mi2002* followed by BrdU and GFP immunostaining at 24, 48, and 72 hpl. Arrowheads indicate *mmp-9*+, GFP+, and/or BrdU+ **(B)** Quantification of BrdU+ cells expressing *mmp-9* at 24, 48, and 72 hpl (n=4). **(C)** Quantification of GFP+ Müller glia colocalized to *mmp-9* expression (fast red) at 24, 48, and 72 hpl (n=4). Quantitative data are represented as mean; error bars represent the standard deviation. ONL- outer nuclear layer; INL- inner nuclear layer; GCL- ganglion cell layer. Scale bar equals 25 μm.

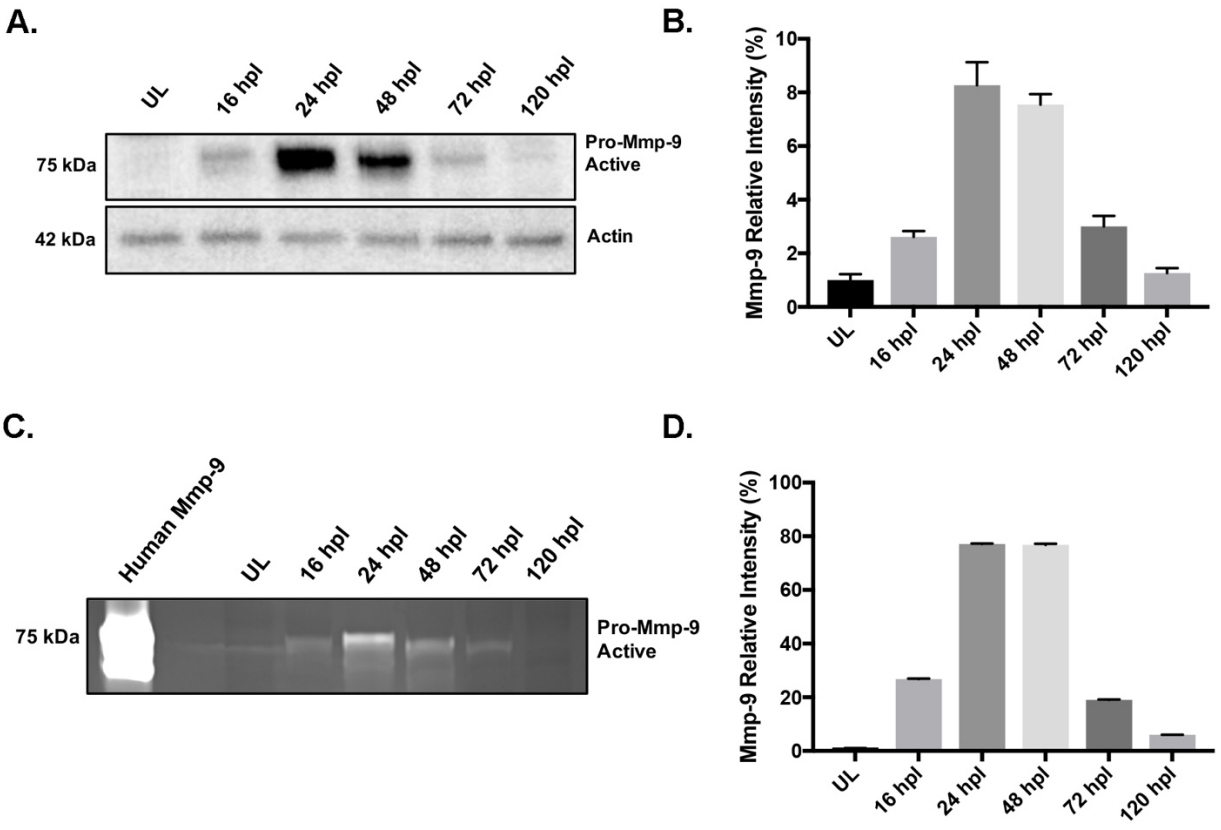


Figure 2.4: Mmp-9 is expression and catalytically active following photoreceptor death.
(A) Western blot time-course of retinal protein extracts from unlesion (controls), 16 hpl, 24 hpl, 48 hpl, 72 hpl, and 120 hpl probed with anti-Mmp-9 antibody and anti-actin as a loading control (n=3; 6 retinas per sample). **(B)** Densitometric quantification of Mmp-9 protein from 16-120 hpl relative to uninjured controls and normalized to actin. **(C)** Zymographic analysis of Mmp-9 catalytic activity from wild-type uninjured controls and 24-120 hpl (n=6; retinas per sample, n=3; biological replicates). **(D)** Densitometric quantification of Mmp-9 activity from uninjured controls compared to 24-120 hpl and normalized to recombinant-active human MMP-9 (positive control). Quantitative data are represented as mean; error bars represent the standard deviation.

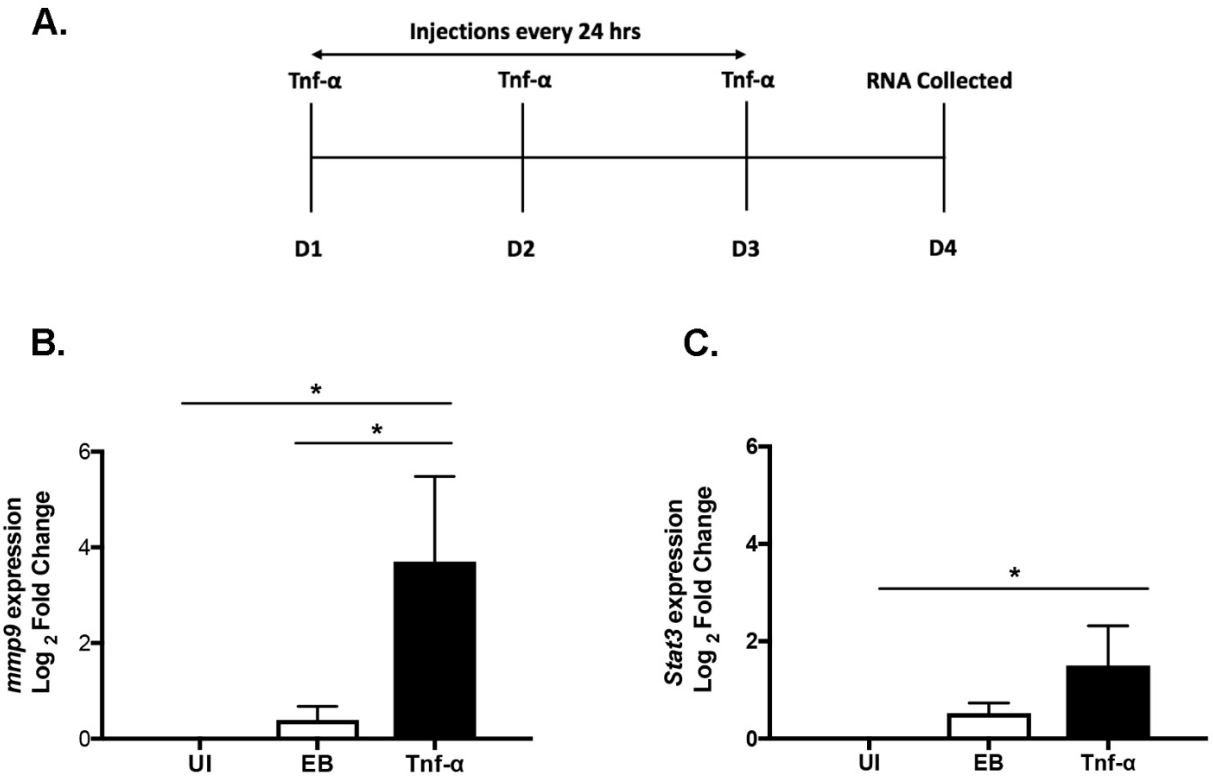


Figure 2.5: *mmp-9* is a component of the inflammatory response.
(A) Experimental schematic for Tnf-a intraocular injections into unlesioned retinas every 24 hours and RNA collected at 4 days post injection (hpi). **(B, C)** qPCR for *mmp-9* and *stat-3* gene expression at 4 dpi. Data calculated using DDC_T method and represented as normalized data to untreated controls (n=3; 6 retinas per sample). Quantitative data are represented as mean; error bars represent the standard deviation. *p≤0.05.

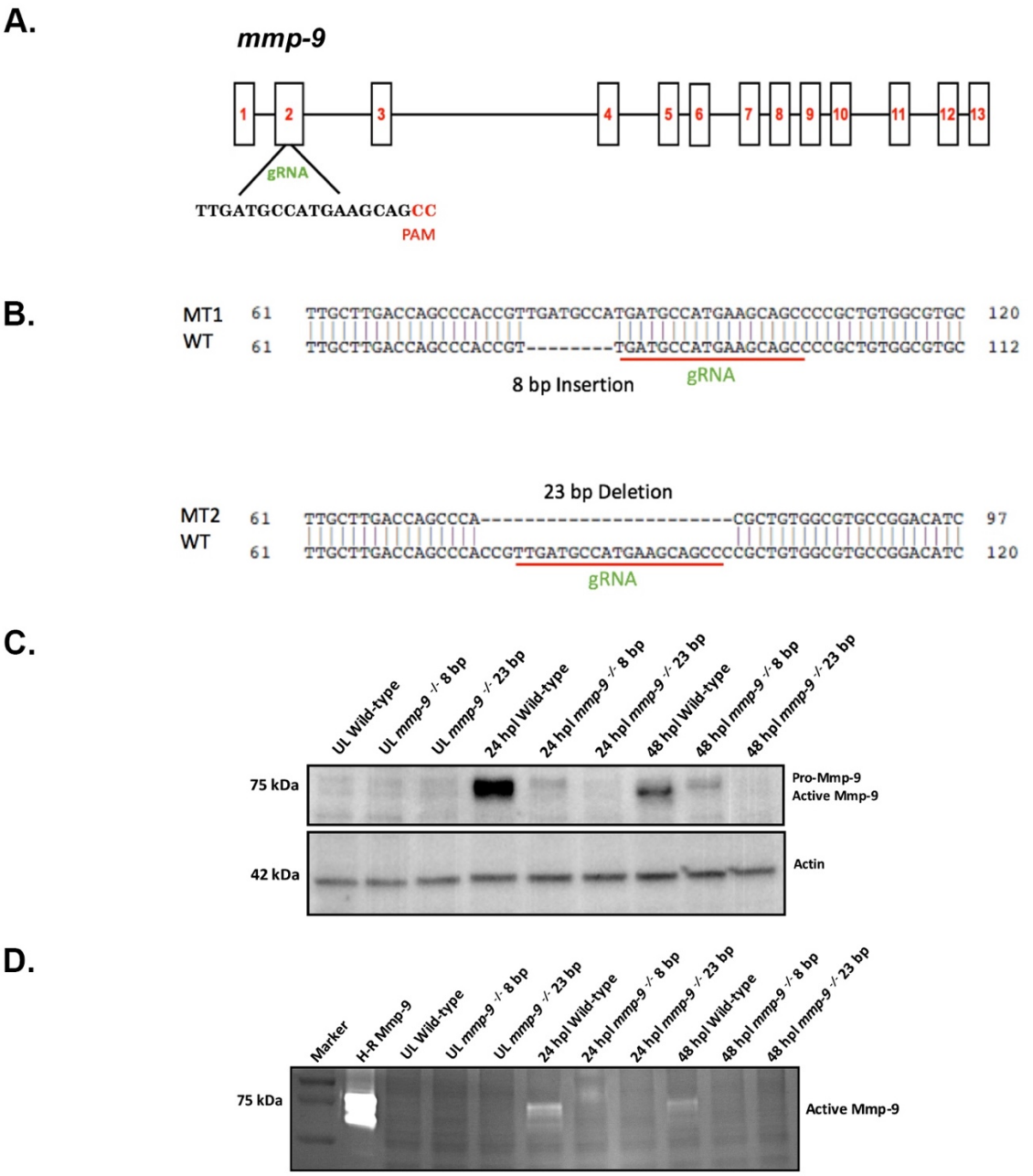


Figure 2.6: CRISPR mutants lack the expression of Mmp-9 and its catalytic activity.
(A) Genomic structure of *mmp-9* and gRNA target sequence. **(B)** Sequence alignment identifies two indel mutations; 8 bp insertion and 23 bp deletion from *mmp-9* $^{-/-}$ mutants compared to wild-types. Red underline indicates 19 bp gRNA targeting sequence. **(C)** Mmp-9 western blot from wild-type and *mmp-9* $^{-/-}$ mutants at 24 and 48 hpl. Anti-actin as a loading control. (n=3; 6 retinas per sample). **(D)** Zymographic analysis of Mmp-9 catalytic activity from wild-type and *mmp-9* $^{-/-}$ mutants at 24-120 hpl. Purified human recombinant protein as positive control (n=3; 6 retinas per sample). Quantitative data are represented as mean; error bars represent the standard deviation.

A.

Supplemental Fig 2.6. *mmp-9* INDELS produce premature stop codons.
(A) The predicted amino acid sequence for wild-type and *mmp-9* $^{-/-}$ mutants. Black box denotes the gRNA target.

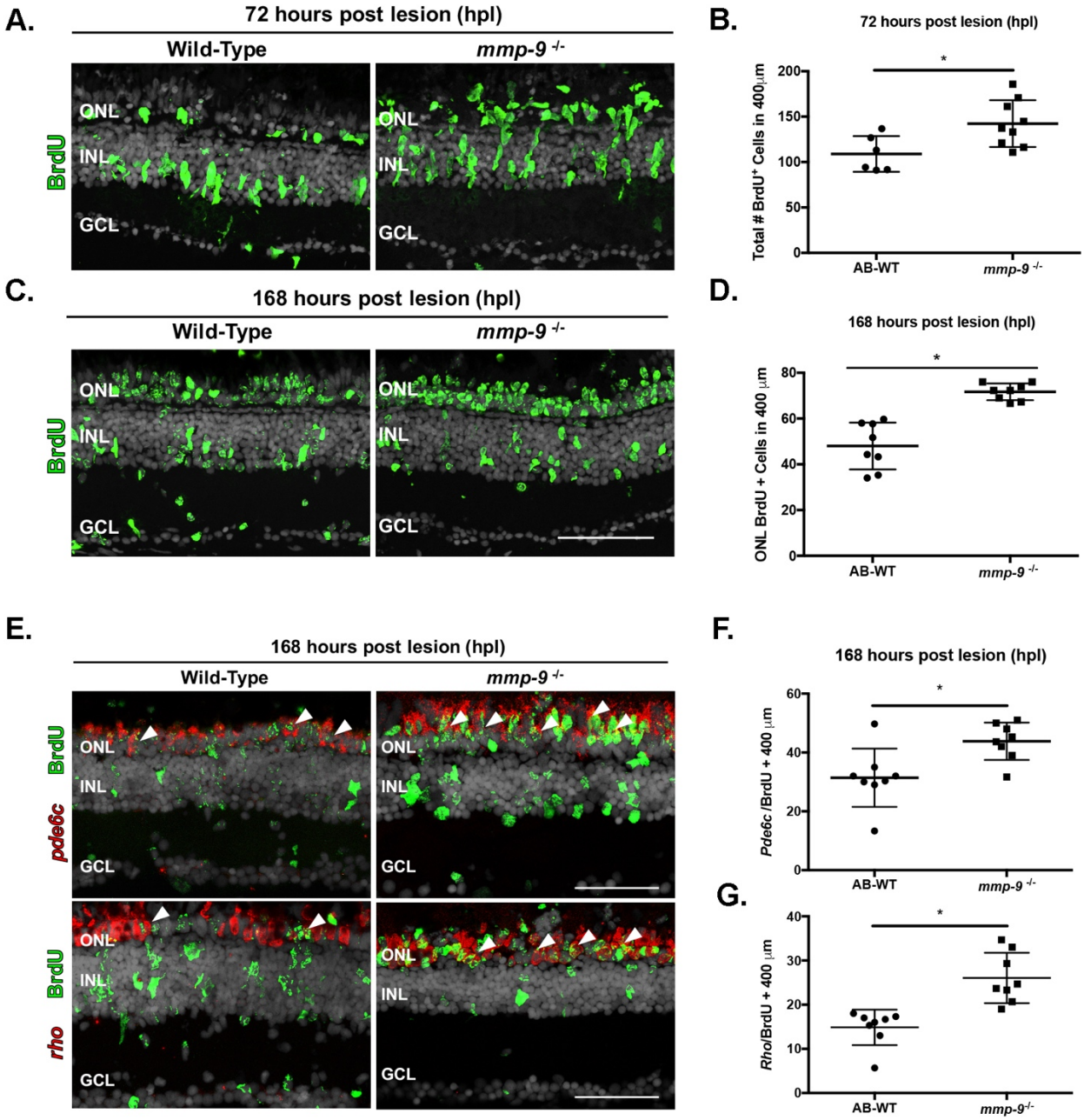
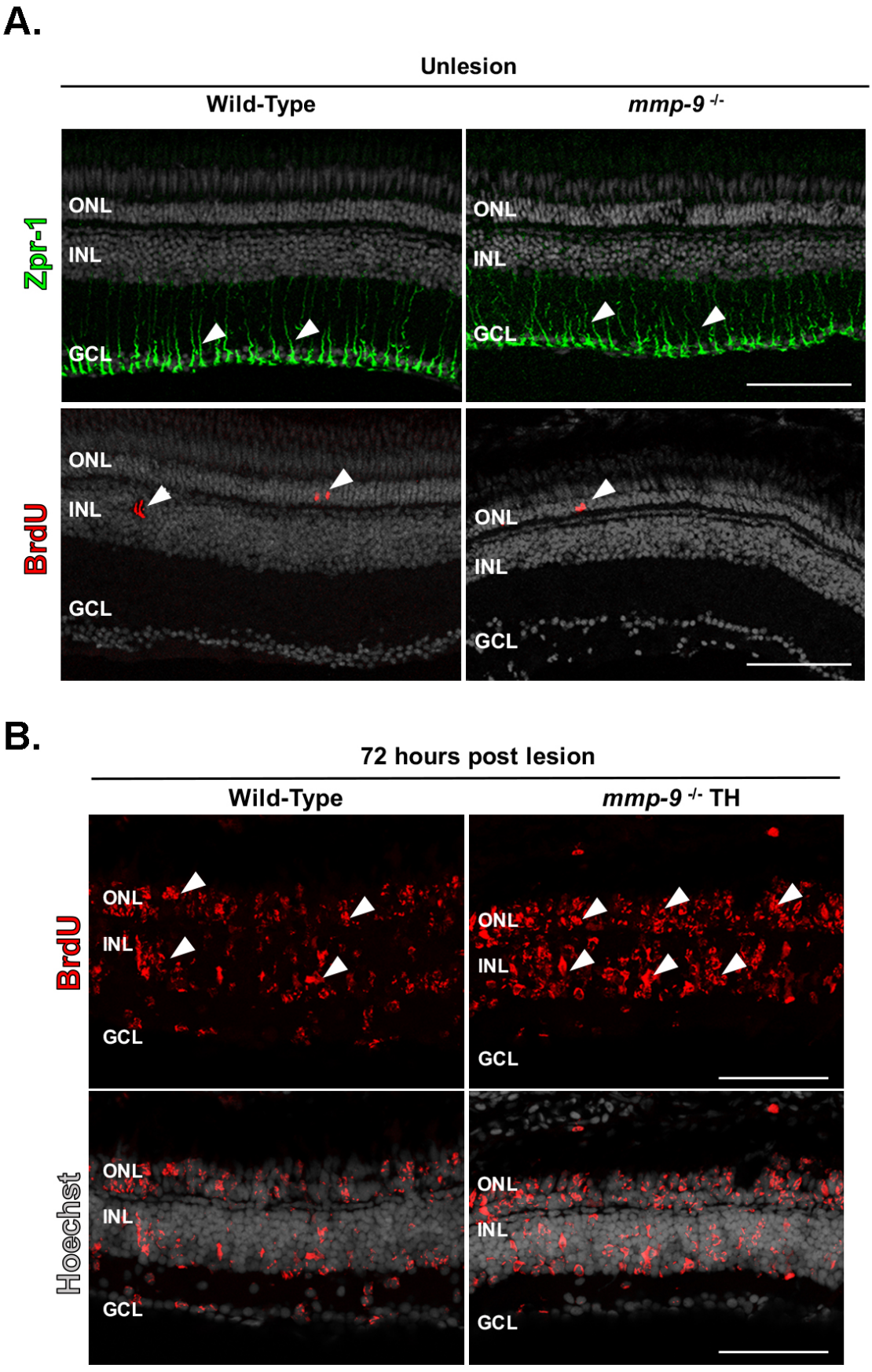

A.
72 hours post lesion (hpl)
Wild-Type
mmp-9 -/-
BrdU
ONL
INL
GCL
B.
72 hours post lesion (hpl)
Total # BrdU+ Cells in 400μm
AB-WT
mmp-9 -/-
C.
168 hours post lesion (hpl)
D.
ONL BrdU + Cells in 400 μm
E.
pde6c
rho
F.
Pde6c /BrdU + 400 μm
G.
Rho/BrdU + 400 μm

Figure 2.7: Deleting *mmp-9* results in an over production of injury-induced progenitors and regenerated photoreceptors
(A) BrdU immunostaining (green) was used to quantify Müller glia derived progenitors in wild-type and *mmp-9* $^{-/-}$ mutants labelled between 48-72 hpl. **(B)** Proliferation quantification of BrdU+ cells from wild-types (109 ± 19.66 cells; *n*=6) compared to *mmp-9* $^{-/-}$ mutants (142.3 ± 25.72 cells; *n*=9) at 72 hpl; p=0.0186. **(C)** BrdU immunostaining (green) was used to quantify Müller glia-derived progenitors labelled between 48-72 hpl and located in the ONL 168 hpl. **(D)** Quantification of BrdU+ cells located in the ONL for controls (48 ± 10.24 cells; *n*=8) compared to *mmp-9* $^{-/-}$ mutants (71.71 ± 3.7 cells; *n*=8) at 168 hpl; p=0.0001. **(E)** *rhodopsin* (rods) and *pde6c* (cones) mRNA was detected by *in situ* hybridization (ISH) (fast red) and combined with BrdU immunostaining (green) to determine regenerated photoreceptors in the ONL as indicated by arrowheads at 168 hpl from wild-type and *mmp-9* $^{-/-}$ mutants. **(F)** Quantification of regenerated cone photoreceptors in wild-type (31.42 ± 9.88 cells; *n*=8) compared to *mmp-9* $^{-/-}$ mutants (43.83 ± 10.68 cells; *n*=8) at 168 hpl; p=0.0301. **(G)** Quantification of regenerated rod photoreceptors in wild-type (14.88 ± 4.02 cells; *n*=8) compared to *mmp-9* $^{-/-}$ mutants (26.04 ± 5.69 cells; *n*=8) at 168 hpl; p=0.0005. Quantitative data are represented as mean; error bars represent the standard deviation. *p≤0.05. ONL- outer nuclear layer; INL- inner nuclear layer; GCL- ganglion cell layer. Scale bar equals 50 μm.


A.
Unlesion
Wild-Type
mmp-9 -/-
Zpr-1
BrdU
ONL
INL
GCL
B.
72 hours post lesion
Wild-Type
mmp-9 -/- TH
BrdU
Hoechst
ONL
INL
GCL

Supplemental Fig 2.7. The absence of *mmp-9* does not affect endogenous proliferation or Müller glia numbers.
(A) Immunostaining for BrdU+ (red) cells in wild-type and transheterozygous (*mmp-9* $^{-/-}$ 8 bp x *mmp-9* $^{-/-}$ 23 bp) mutants as indicated by arrowheads. **(B)** Immunostaining labels Müller glia processes (zrf-1) as a readout of Müller glia number in unlesion wild-type and mutants as indicated by arrowheads. BrdU+ (red) cells in unlesion wild-type and mutants as indicated by arrowheads. Quantitative data are represented as mean; error bars represent the standard deviation. ONL- outer nuclear layer; INL- inner nuclear layer; GCL- ganglion cell layer. Scale bar equals 25 μm.

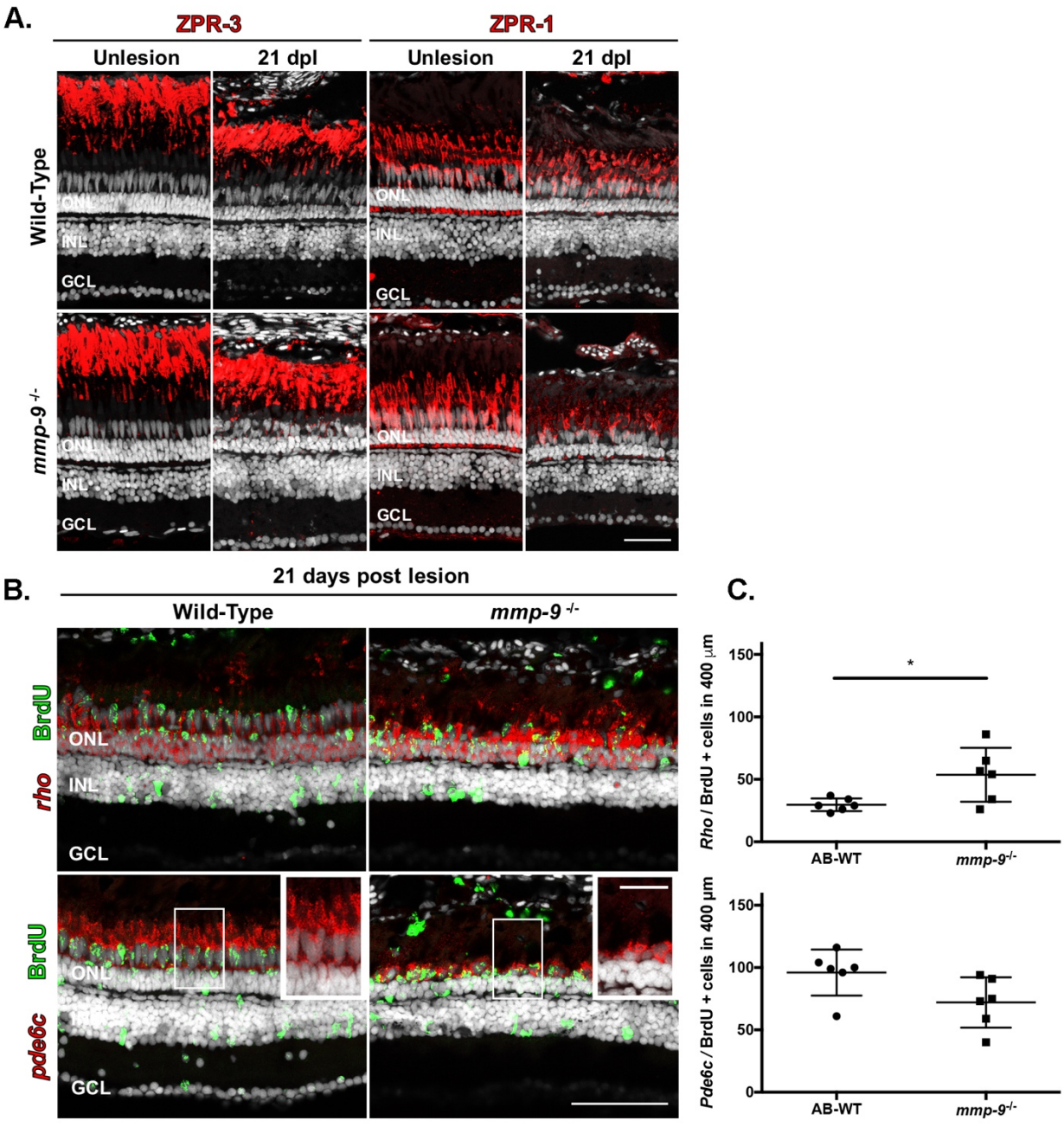

A.
ZPR-3
ZPR-1
Unlesion
21 dpl
Unlesion
21 dpl
Wild-Type
mmp-9 -/-
ONL
INL
GCL
B.
21 days post lesion
Wild-Type
mmp-9 -/-
BrdU
rho
pde6c
C.
Rho / BrdU + cells in 400 µm
Pde6c / BrdU + cells in 400 µm
AB-WT
mmp-9-/-
*

Fig 2.8. Mmp-9 regulates the maturation of regenerated photoreceptors.
(A) Immunostaining for red-green double cones marker ZPR-1 and ZPR-3 (rods) in wild-type and mutants in unlesion and 21 dpl. **(B)** *pde6c* (cones) and *rhodopsin* (rods) expression detected by *in situ* hybridization (ISH) (fast red) and combined with BrdU immunostaining (green) to determine regenerated photoreceptors in the ONL at 21 days post lesion (dpl) from wild-type and *mmp-9* $^{-/-}$ mutants. **(C)** Quantification of regenerated cone photoreceptors in wild-type (95.81 ± 18.49 cells; *n*=6) compared to *mmp-9* $^{-/-}$ mutants (71.89 ± 20.37 cells; *n*=6) at 21 dpl; p=0.059. Quantification of regenerated rods photoreceptors in wild-type (29.6 ± 4.94 cells; *n*=6) compared to *mmp-9* $^{-/-}$ mutants (53.39 ± 21.62 cells; *n*=6) at 21 dpl; p=0.0270. Quantitative data are represented as mean; error bars represent the standard deviation. ONL- outer nuclear layer; INL- inner nuclear layer; GCL- ganglion cell layer. Scale bar equals 50 μm.

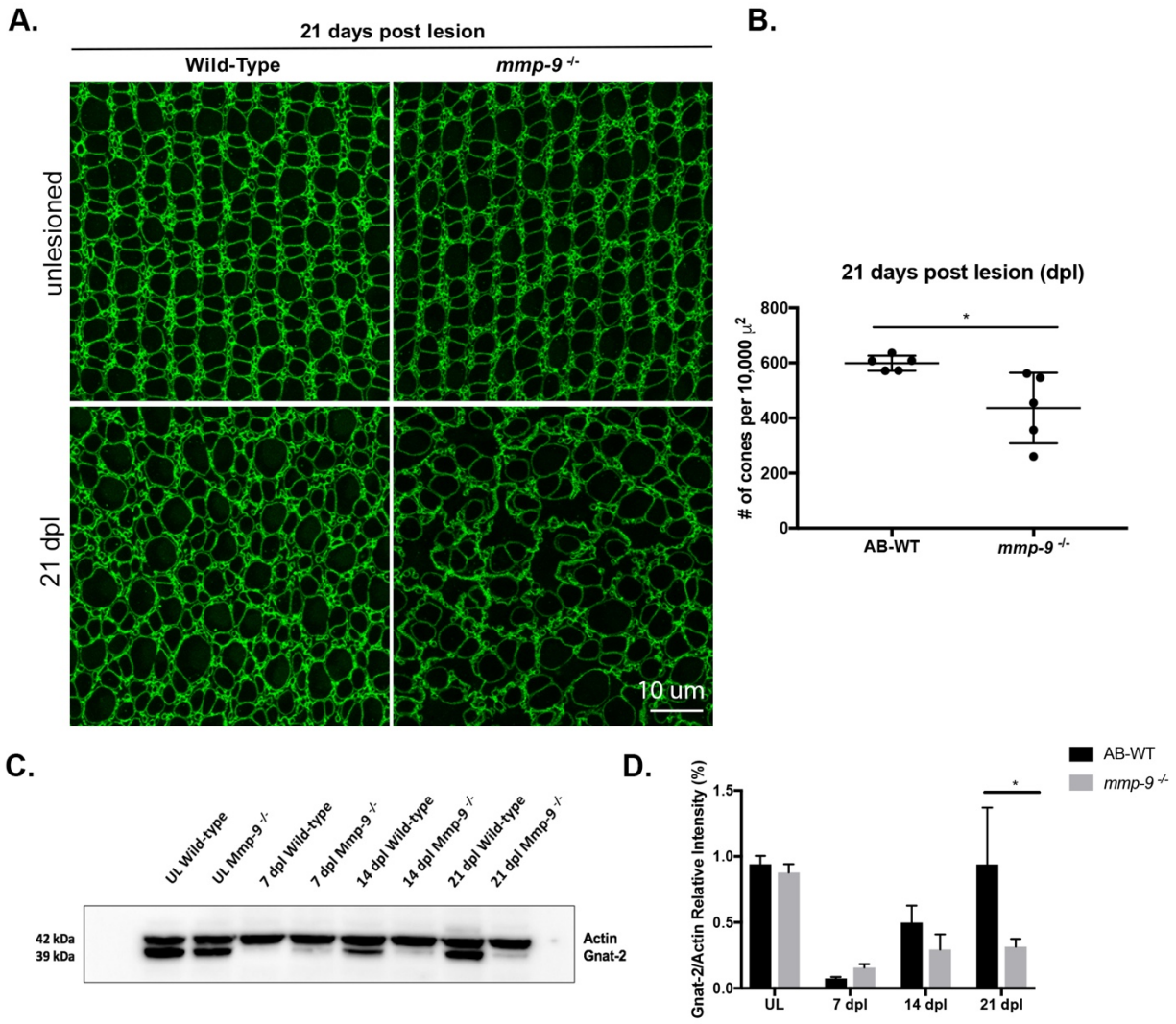


Fig 2.9. Mmp-9 is regulates for the survival of regenerated cone photoreceptors. **(A)** Flat-mount retinas from wild-type and *mmp-9* $^{-/-}$ mutants immunostained for ZO-1, labelling the apical cell prolife in unlesion and 21 dpl retinas. **(B)** Quantification of regenerated cones from wild-type (599.11 ± 27.42 cones; n=5) and *mmp-9* $^{-/-}$ mutants (436.09 ± 128.04 cones; n=5) at 21 dpl; p=.0238. **(C)** Western blot time-course for wild-type and *mmp-9* $^{-/-}$ mutants from unlesion (controls), 7 dpl, 14 dpl, and 21 dpl probed with anti-gnat-2 (cones) antibody and anti-actin as a loading control. **(D)** Densitometric quantification of GNAT-2 protein from the western blot time-course of unlesion (controls), 7 dpl, 14 dpl, and 21 dpl samples in wild-type and *mmp-9* $^{-/-}$ mutants. GNAT-2 protein levels in wild-type (.094 ± .43; n=3; 6 retinas per sample) and *mmp-9* $^{-/-}$ mutants (.32 ± .059; n=3; 6 retinas per sample) at 21 dpl; p=.0014. Compared groups mean using Sidak's multiple comparison test; two-way ANOVA.

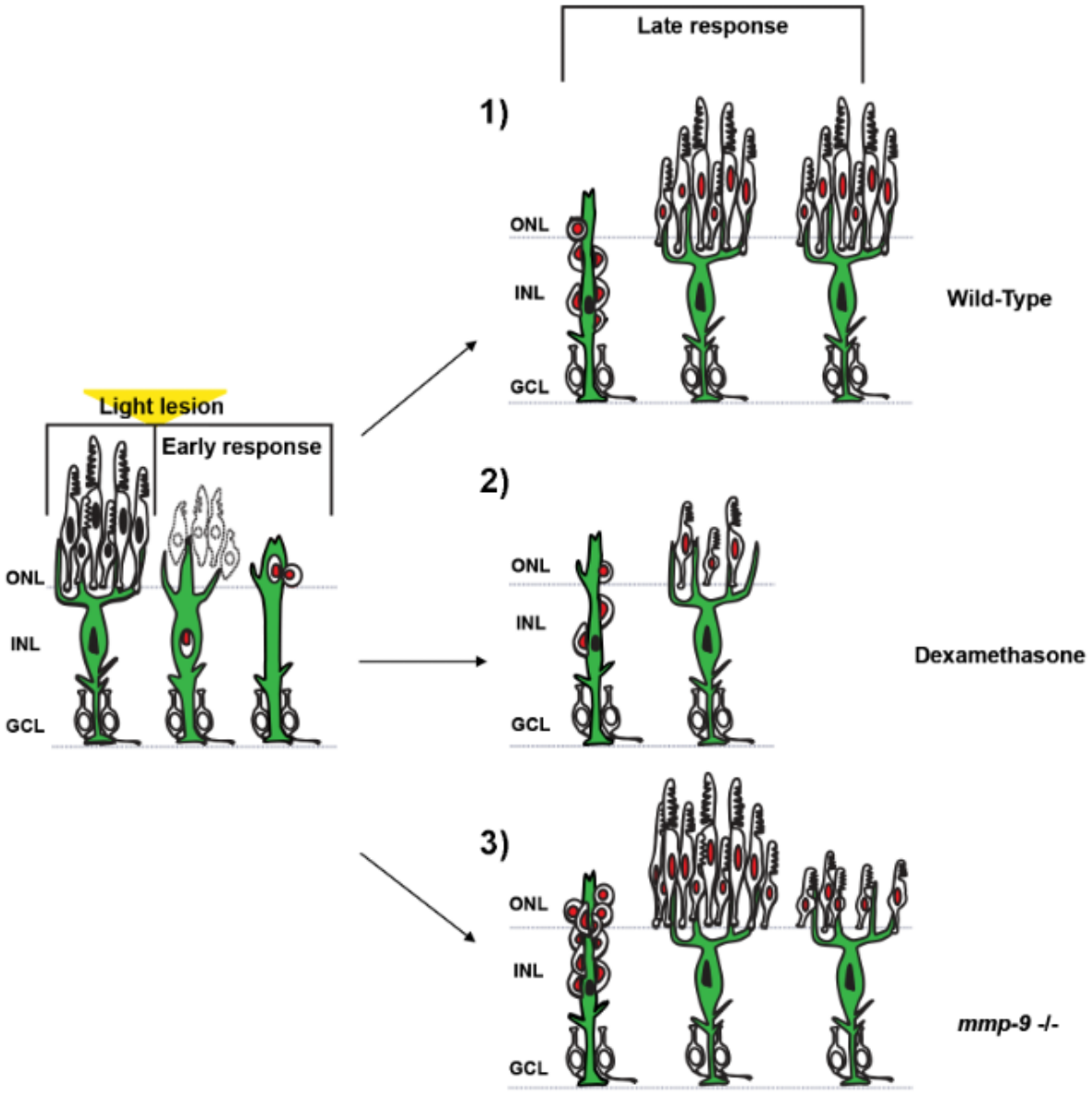


Fig 2.10. Inflammation and *mmp-9* regulates Müller glia-derived progenitors and cone photoreceptor regeneration following injury.
(A) Light-lesion induces Müller glia to activate, express *mmp-9*, undergo interkinetic nuclear migration, and produce a neuronal progenitor in the ONL. **1)** In normal conditions, the neuronal progenitor will continuously proliferate and form Müller glia progenitor cells. Müller glia progenitors will then migrate to the site of lesion and completely regenerate photoreceptors. **2)** In the condition that inflammation is suppressed by Dex, fewer Müller glia progenitors will be produced and; therefore, less regenerated photoreceptors. **3)** Absence of *mmp-9* produces excess Müller glia progenitors and regenerating photoreceptors; yet, cone survival is diminished. ONL- outer nuclear layer; INL- inner nuclear layer; GCL- ganglion cell layer.

Tables

Table 2.1: Primer Sequences

Name	Sequence
gpia	F) 5'- TCCAAGGAAACAAGCCAAGC-3' R) 5'- TTCCACATCACACCCTGCAC-3'
mmp-9	F) 5'- TGATGTGCTTGGACCACGTAA-3' R) 5'- ACAGGAGCACCTTGCCTTTTC-3'
tnf-α	F) 5'- GCGCTTTTCTGAATCCTACG-3' R) 5'- TGCCCAGTCTGTCTCCTTCT-3'
tnf-β	F) 5'- CCTCAGACCACGGAAAAGT-3' R) 5'- GCCCTGTTGGAATGCCTGAT-3'
il-6	F) 5'- TCTTTCCCTCTTTTCCTCCTG -3' R) 5'- TCAACTTCTCCAGCGTGATG -3'
il-8	F) 5'- GTCGCTGCATTGAAACAGAA -3' R) 5'- AGGGGTCCAGACAGATCTCC-3'
nfκb1	F) 5'- ACCAGACTGTGAGCGTGAAG -3' R) 5'- CGCAAGTCCTACCCACAAGT -3'
nfκb2	F) 5'- CATATGTCCCACACAATCAAGAC-3' R) 5'- AGCCACCATAATGATCTGGAA -3'
stat3	F) 5'- GAGGAGGCGTTTGGCAAA -3' R) 5'- TGTGTCAGGGAACTCAGTGTCTG -3'

Table 2.2: Antibody List

Primary Antibodies	Company	Dilution
Mouse Monoclonal anti-Zn5	ZIRC; zfin.org/ZDB-ATB-081002-19	1:200
Mouse Monoclonal anti-Zpr1 (anti-Arrestin-3)	ZIRC; zfin.org/ZDB-ATB-081002-43	1:200
Mouse Monoclonal anti-Zpr-3	ZIRC; zfin.org/ZDB-ATB-081002-45	1:200
Mouse anti-HPC1	ZIRC; zfin.org/ZDB-ATB 130225-1	1:200
Mouse anti-Zrf1	ZIRC; zfin.org/ZDB-ATB-081002-46	1:1000
Mouse Monoclonal ZO-1A-12	Invitrogen 33-9100	1:200
Mouse anti-BrdU	BD Biosciences 347580	1:100
Rat anti-BrdU	Abcam 6326	1:200
Polyclonal anti-GNAT2	MBL: PM075	1:1000
Mouse Monoclonal anti-1D1 (ZF Rhodoposin)	Gift from Dr. Jim Fadool (FSU)	1:1000
Secondary Antibodies		
Alexa Fluor goat anti-mouse 488, 555, or 647	Invitrogen	1:500
Alexa Fluor goat anti-rabbit 488, 555, or 647	Invitrogen	1:500
Alexa Fluor goat anti-rat 488, 555, or 647	Invitrogen	1:500

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Chapter 3

Developmental Phenotypes Associated with the *mmp-9* Zebrafish Mutants and Preliminary Data for CRISPR-Mediated *mmp-9*-P2A-EGFP Donor Plasmid

ABSTRACT

Zebrafish has been a powerful model for genetic manipulation. Recent advancements in gene editing techniques, particularly clustered regularly interspaced short palindromic repeats (CRISPR), has enabled efficient and inexpensive reverse genetic screens to study any gene of interest. Our lab previously identified matrix metalloproteinase 9 (Mmp-9), an inflammatory protease known to regulate inflammatory mediators, cell migration, proliferation, and apoptosis. In this chapter, I describe the developmental phenotypes from *mmp-9* CRISPR mutants I created as part of my thesis research. Early embryonic survival in *mmp-9* mutants is compromised compared to wild-types. Mutants that do survive develop somite segmentation defects, and abnormal tail development. Consistent with the embryonic patterns of *mmp-9* expression, retinal development is normal. Lastly, I present data on the generation of an *mmp-9*-P2A-EGFP donor plasmid to create a stable Tg(*mmp-9:EGFP)* transgenic line for *mmp-9* studies.

Introduction

Zebrafish are amenable to a variety of genetic and molecular approaches to study gene function (Holtzman et al. 2016). Knockdown methodologies are favorable and commonly used to investigate *in vivo* gene function. Antisense morpholinos (MOs) suppress the translation of specific mRNAs and were initially a widely-accepted approach (Ekker and Larson 2001). However, MOs are known to have off-target effects and induce p53-dependent apoptosis (Robu et al. 2005; Gerety and Wilkinson 2011). Additionally, MOs usage is limited in the adult retina, which requires electroporation that can damage cells by the application of the electric field (Teh, Parinov, and Korzh 2005). Fortunately, genome-editing approaches based on nuclease activity can efficiently induce DNA double-strand breaks (DSBs) in the targeted gene (Kawahara et al. 2016). In particular, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and recently, clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR associated endonuclease (Cas9) have transformed zebrafish gene editing research.

The CRISPR/ Cas9 system in zebrafish has proven to be straightforward, efficient, relatively quick, and reliable for generating INDELs (Sertori et al. 2016). This technology was first discovered as the adaptive immune response of bacteria and archaea to degrade foreign DNA (Deveau, Garneau, and Moineau 2010; Horvath and Barrangou 2010). The CRISPR/Cas9 system is composed of two components: a single guide RNA (sgRNA) that binds to targeted genomic DNA and an endonuclease Cas9 that cleaves double-strand DNA (Hwang et al. 2013). The protospacer adjacent motif (PAM) is a DNA sequence following the sgRNA target sequence and facilitates Cas9 binding for cleavage. Following DNA cleavage, endogenous mechanisms attempt to

repair the damaged DNA by non-homologous end joining (NHEJ). NHEJ introduces a random insertion or deletion into the targeted genome that can result in a frameshift inducing a premature stop codon and thus non-functional protein (Sertori et al. 2016). CRISPR/Cas9 technology is not limited to single gene targeting, but multiple sgRNAs can be injected to analyze more than one gene (Jao, Wente, and Chen 2013). Furthermore, site-specific integration of donor DNA into the genome can be used to generate reporter lines in the zebrafish by homologous recombination (HR) and HR-independent methods (Kimura et al. 2014; Li et al. 2015).

Matrix metalloproteinase 9 (MMP-9) belongs to a family of zinc-dependent endopeptidases classically known to regulate physiological and pathophysiological tissue remodeling (Page-McCaw, Ewald, and Werb 2007). MMP-9 is synthesized as a secreted zymogen and converted into active MMP-9 upon cleavage (Vandooren, Van den Steen, and Opdenakker 2013). In the developing zebrafish, *mmp-9* is expressed in the notochord at 12 hours post fertilization (hpf), anterior mesoderm from 14-16 hpf, tail at 19 hpl, and circulating leukocytes by 24 hpf (Yoong et al. 2007). As zebrafish develop into adults, *mmp-9* expression is downregulated, but injury-induced in various tissues (LeBert et al. 2015; Lemmens et al. 2016; Xu et al. 2018; Ando et al. 2017). We identified *mmp-9* from an unbiased microarray screen following photoreceptor death (Calinescu et al. 2009), however, its functional role during zebrafish development has yet to be investigated.

Here I report four *mmp-9* alleles induced by CRISPR/Cas9 technology, confirmed by Sanger sequencing, and T7 endonuclease assay. In addition to the characterization of the initial two mutants from chapter 2, I characterized another two by western blot

and zymogram analysis. The initial *mmp-9* mutants from chapter two were further characterized for potential developmental phenotypes. Starting at 24 hpf, mutants exhibit survival defects compared to wild-type. True to the expression pattern of *mmp-9* during development, mutants exhibit somite segmentation defects, an abnormal caudal fin, however retinal development is normal in larvae and adults. Furthermore, I present preliminary data for generating a CRISPR-mediated *mmp-9* transgenic reporter.

MATERIALS AND METHODS

Animals

AB wild-type zebrafish (Danio rerio; ZIRC, University of Oregon, Eugene, Oregon) were propagated, maintained, and housed in recirculating habitats at 28.5°C and on a 14/10-h light/dark cycle. Embryos were collected immediately after spawning and incubated at 28.5°C on a 14/10-h light/dark cycle. Adults were 6-12-months-old at the time of the experiment. All experimental protocols were approved by by the University of Michigan's Institutional Animal Care and Use Committee (IACUC).

Light Lesions

To kill photoreceptors, fish were dark adapted, then exposed to high intensity light (ca. 100,000 lux) for 30 minutes, followed by exposure to constant light (ca. 30.000 lux) for 72 hours (Taylor et al., 2015). After 72 hours fish were returned to the recirculating habitats and normal light/dark cycle.

CRISPR-mediated gene mutation

mmp-9 mutants were generated according to previously described methods (Hwang et al., 2013). ZiFiT software (available in the public domain at zifit.partners.org) was used to identify the 19 bp sgRNA target sequence for *mmp-9* (GGCTGCTTCATGGCATCAA). Target sequence Oligo1-TAGGCTGCTTCATGGCATCAA and Oligo2-AAACTTGATGCCATGAAGCAG were annealed and subcloned into the pT7 gRNA

vector (Addgene ID: 46759). The pCS2 nCas9n vector (Addgene ID: 46929) was used for Cas9 synthesis. To produce RNAs, the MEGAshortscript T7 kit, (Ambion: AM1354) and mirVana miRNA Isolation kit (Ambion: AM 1560) was used for the gRNA, whereas, mMesage mMACHINE SP6 kit (Ambion: AM1340) and RNeasy mini Kit from (Qiagen: 74104) was used for Cas9 mRNA. Single cell-stage embryos were injected with 2 nL solution containing 100 pg/nl sgRNA and 150 pg/nl Cas9 mRNA. Founders (F0) were outcrossed to AB wild-type fish. Mutations were identified using screening primers (F: 5′- AAGTCTGCAACTACATATCAGC -3′, R: 5′-GTACACACTGTAGATGCTGATAAG-3’) that flanked the *mmp-9* sgRNA target site. Standard protocols for PCR used Platinum Taq-HF polymerase (Invitrogen; city) with genomic DNA as the template. The purified PCR product was subcloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) for Sanger sequencing. Mutant and wild-type sequences were aligned using pairwise blast (National Center for Biotechnology Information [NCBI], Bethesda, MD, USA). Premature stop codons were identified by comparing predicted amino acid sequences for wild-type and mutants using the ExPASy translate tool (available in the public domain at (www.expasy.org). F1s carrying a *mmp-9* mutation were in-crossed and homozygous F2 mutants were identified by a combination of Sanger sequencing and T7 endonuclease assays (New England Biolabs, Ipswich, MA, USA) as previously described (www.crisprflydesign.org).

Western Blot Analysis

Protein samples were obtained from whole retinas homogenized in RIPA lysis buffer (ThermoFisher Scienific, Waltham, MA) containing protease and phosphatase inhibitor cocktail (5872S; Cell Signaling Technology, Danvers, MA, USA). Each sample contained six pooled retinas from three adult fish. Proteins were separated in a 12%

Mini-PROTEAN TGX Precast gel (BioRad; Hercules, CA) and transferred to a polyvinylidene difluoride (PVDF) membrane (GenHunter Corp., Nashville, TN). To block non-specific protein, the membrane was incubated in 5% nonfat dry milk in Tris buffered saline containing 0.3% Tween-20 (TBST) for 2 hours. Membranes were incubated with the antibodies-containing solution overnight at 4°C. Blots were then washed in TBST and incubated with horseradish peroxidase-conjugated secondary IgG (1:1000) for 1 hour at room-temperature. Antibody-bound protein was visualized using Pierce ECL Western blotting substrate (32106; ThermoFisher Scienific, Waltham, MA). To visualize loading controls, blots were also probed with antibodies against actin. Images were captured using the Azure C500 (Azure Biosystems). Densitometric analysis of protein bands was performed with ImageJ software (https://imagej.nih.gov/ij/).

Zymogram Analysis

Protein samples were prepared as described for Western blot analysis. Proteins were separated on 10% Zymogram Plus (Gelatin) Protein Gels, (ThermoFisher Scientific; Waltham, MA). Following electrophoresis, proteins were renatured in 1X renaturing buffer (ThermoFisher Scientific; Cat#LC2670) for 30 mins at room temperature, then incubated in 1X developing buffer (ThermoFisher Scientific; Cat#LC2671) overnight at 37º. Gels were rinsed in deionized water and stained with SimplyBlue SafeStain (ThermoFisher Scientific; Cat#LC6060). Gels were imaged with long-wave ultraviolet light using the Azure C500 (Azure Biosystems; Dublin, CA). Active recombinant human MMP-9 (Calbiochem; PF140) was used as a positive control. Densitometric analysis of the digested bands was performed with ImageJ software.

Immunohistochemistry

Whole larvae (48-72 hpf) were fixed overnight at 4°C in phosphate buffered 4% paraformaldehyde, cryoprotected with 20% sucrose, and embedded in optical cutting temperature (OCT) medium (Sakura Finetek USA, Torrance, CA). Immunohistochemistry (IHC) was performed as previously described (Taylor et al., 2015). 10-µm-thick sections were collected through the optic nerve head and mounted on glass slides. Sections were washed in phosphate buffer saline with 0.5 % Triton-x (PBST) and incubated in 20% heat inactivated normal sheep serum for 2 hours (NSS; Sigma-Aldrich Corp., St Louis MO). Primary antibodies were applied overnight at 4°C. Sections were then washed with PBST and incubated in secondary antibodies for 1 hour at room temperature. The antibodies used here and their concentrations are listed in Table 3.1.

Labeling dividing cells

Dividing cells were labeled with EdU. Larvae were incubated for 20 minutes in ice-cold (48 hpf) or room-temperature (72 hpf) EdU (1.5 mM), dissolved in embryo rearing solution containing 15% dimethylsulfoxide (DMSO). EdU labelled cells were visualized using the Click-it Assay kit (Invitrogen, Carlsbad, CA, USA; Walsh and Hitchcock, 2017).

JB-4 plastic sections and Lee's Stain

Eye cups were fixed overnight at 4°C in 2% PFA and 2% glutaraldehyde then embedded the following day using the JB-4 Embedding Kit (Electron Microscopy Sciences). Lee's stain was performed with .13% methylene Blue and .13% Basic Fuchsin on 4-µm-thick cross-sections at the optic nerve. Bright field images were captured with a Leica DM600 microscope (Wetzlar, Germany).

Generating the *mmp-9-EGFP* donor plasmid

To generate the *mmp-9*-P2A-EGFP donor plasmid, we modified the *gfap-P2A-EGFP* plasmid (Addgene: 65564) (Li et al. 2015). ZiFiT software (available in the public domain at zifit.partners.org) was used to identify the 20 bp sgRNA target sequence for *mmp-9* (GGCATCAGAGATGTTATTGC). Target sequence Oligo1-TAGGCATCAGAGATGTTATTGC and Oligo2-AAACCGCAATAACATCTCTGATG were annealed and subcloned into the pT7 gRNA vector (Addgene ID: 46759). To generate the donor plasmid, we first PCR-amplified the zebrafish *mmp-9* genome from the 3' end of intron 11 to the 3' UTR region, and subcloned into the pGEM-T easy vector (Promega: A1360). This plasmid serves as the template to amplify the right and left arms of the *mmp-9* gene. The commercially available plasmid *gfap*-P2A-EGFP (Addgene: 65564) was used as donor backbone. To remove the *gfap* DNA arms, we linearized the plasmid using EcoRI and BamHI (left arm) and AgeI and SalI (right arm) (New England Biolab, MA). The PCR-amplified *mmp-9* arms were then ligated to the donor backbone using standard ligation methods. The subcloning of *mmp-9* arms was confirmed by Sanger sequencing following ligand to the donor plasmid. Each embryo was injected with 2 nl of solution containing 200 ng/µl zCas9 mRNA, 80 ng/µl sgRNA, and 15 ng/µl donor plasmid. The presence of *gfp* expression was analyzed in 3 dpf injected embryos. All primers used for the generation of the *mmp-9*-P2A-EGFP plasmid are listed on Table 3.2.

RESULTS

Confirmed *mmp-9* mutants exhibit survival defects

To study the function of *mmp-9* during development and photoreceptor regeneration (Chapter Two), mutants were generated using the CRISPR-Cas9 system (Hwang et al. 2013). The sgRNA was targeted to the *mmp-9* catalytic domain in exon 2 and produced several alleles, four of which were confirmed by Sanger sequencing (Figure 3.1A). In addition to the characterization of the two mutants from chapter 2, we also determined the predicted amino acid sequence of the 13 bp insertion and 22 bp deletion *mmp-9* mutants. The respective INDELs resulted in a frameshift and premature stop codon (Supplemental Figure 3.1A). Next, we determined if the 13 bp and compound heterozygotes from chapter 2 produce functional Mmp-9 protein at 24 hpl. Interestingly, although both mutants produce Mmp-9 protein, it is not catalytically active (Supplemental Figure 3.1B).

To detect CRISPR-mediated double strand breaks (DSBs) in the DNA and identify homozygous mutants, we used the T7 endonuclease assay (Qiu et al. 2004). The T7 endonuclease assay recognizes and cleaves heteroduplex dsDNA formed by mismatched base pairs induced by mutagenesis (Gohlke et al. 1994). In the 1st round, we identified single and double bands (Figure 3.1B). Wild-types and homozygotes appear as single bands, because they have complementary double strand DNA that fail to form a heteroduplex dsDNA required for digestion. In contrast, heterozygotes appear as double bands. In the 2nd round, we added wild-type DNA to wild-types or homozygote samples, and wild-types appear as single bands again, but homozygotes now appear as a double band (Figure 3.1B). The identified homozygous mutants were fertile and bred to homozygosity. However, though wild-type and mutant survival is

comparable at 4 hpf for all groups, starting at 24 hpf, survival is significantly compromised in both mutants (Figure 3.1C). Interestingly, each allele mutant showed different survival rates at 24 hpf, *mmp-9* -/- 8 bp (~40%) and *mmp-9* 23 bp (~60%) (Figure 3.1C). Of note, by 24 hpf, survival is sustained, suggesting that *mmp-9* function is critical for the early stages of development.

Somite segmentation and caudal fin abnormally develop in *mmp-9* mutants

Due to the expression pattern of *mmp-9* during development as previously reported (Yoong et al. 2007), we qualitatively compared the wild-type and mutant caudal fins during development into adulthood. Unexpectedly, 24 hours post fertilization (hpf), we observed that somite segmentation is undefined in *mmp-9* mutants compared to wild-types as indicated by arrows (Figure 3.2A). While at 48 hpf, wild-type caudal fins appear normal compared to mutants (Figure 3.2B). Furthermore, the yolk sack of mutants appears larger in size (Figure 3.2B). To determine if the tail phenotype persist into adulthood, we compared wild-type and mutant caudal fins from adults. Compared to wild-types, mutant adults exhibit a severe form of the initial caudal fin phenotype observed during development (Figure 3.2C). Together, these results suggest that *mmp-9* function governs somite segmentation and tail development, which corresponds to the previously published expression pattern of *mmp-9* during zebrafish development.

Retinal development is normal in mutants

Retinal development in zebrafish is rapid (Dowling 2012) and larvae are hatched and become visually-active by 72 hpf (Schmitt and Dowling 1994; Malicki 1999; Easter and Malicki 2002). Therefore, retinas were evaluated for potential developmental defects at 72 hpf using cell type-specific markers and quantitative assays for eye size and cell proliferation (see, (Walsh and Hitchcock 2017)). Comparing wild-type and mutant

retinas showed there were no differences in the labeling patterns for ganglion cells, amacrine cells or rod photoreceptors (Figure 3.2A). Two additional measurements were used, eye size and endogenous proliferation in the ciliary marginal zone (CMZ). The CMZ serves as a retinal stem cell niche that provides new neurons as teleost fish and amphibians grow throughout life (Johns 1977; Straznicky and Gaze 1971). The eye size and CMZ proliferative zone showed no difference between wild-type and mutants at 72 hpf (Figure 3.2B,C). To determine the retinal cytoarchitecture in wild-type and *mmp-9* mutants, we performed a standard histological stain using Lee's stain on plastic sections from adult tissue. Comparing wild-type and *mmp-9* mutants, we observe no detectable differences in lamination and developed photoreceptors (Figure 3.2D). Therefore, results indicate that there is no difference between viable wild-type and mutants in retinal cell types, eye size, and endogenous proliferation during development, and adult retinal anatomy is comparable for both groups.

Preliminary Results for the Generation of a *mmp-9*-P2A-eGFP donor plasmid

To further study the role of *mmp-9* during development and retinal regeneration, we sought to generate a transgenic reporter line using the intron mediated CRISPR/Cas9 knockin system (Li et al. 2015). This technique permits the integration of eGFP into the 3' end of the *mmp-9* endogenous intron locus. The donor plasmid is composed of three parts: a left arm, a P2A-EGFP coding sequence, and a right arm. The left and right arms are identical to the endogenous regions of the *mmp-9* gene required for integration, whereas P2A-EGFP serves as a peptide linker for multicistronic expression of EGFP (Li et al. 2015; Kim et al. 2011). To retain the normal function of the *mmp-9* coding region, each arm contains full coding sequences; 5' side of the sgRNA targets intron 11 through E13 (left arm) and stop codon and 3' UTR (right arm). First we, subcloned a 3 kb region

from the 3’ end of the *mmp-9* gene into a pGEM-T easy vector to amplify the right and left arms of *mmp-9* (Figure 3.4A). To generate the donor plasmid, we used the *gfap*-P2A-EGFP plasmid as a backbone by removing the *gfap* DNA arms and subcloning *mmp-9* arms, which were confirmed by Sanger sequencing (Supplemental Figure 3.4A, B). The *mmp-9* sgRNA targets the donor plasmid sequence and the endogenous locus in intron 11 for recombination (Figure 3.4B). This donor plasmid serves as a reagent that can be used in future studies.

DISCUSSION

In this study, we characterize the developmental phenotypes associated with the newly generated *mmp-9* mutant zebrafish. Additionally, we provide preliminary data for the generation of an *mmp-9*-P2A-*EGFP* donor plasmid for future studies. Our data show that compared to wild-types, *mmp-9* mutant survival is compromised starting at 24 hours post fertilization (hpf). Viable mutants exhibit somite segmentation defects, abnormal caudal fins, but show no effect on retinal development. Though our *mmp-9*-P2A-EGFP donor plasmid failed to integrate into the genome for the generation of a stable Tg(*mmp-9*:EGFP) zebrafish transgenic line, future studies can optimize the plasmid.

Since MMPs, including MMP-9 are critical for tissue remodeling, their expression provides a window to when and where this process occurs during development (Page-McCaw, Ewald, and Werb 2007). During the early stages of zebrafish development, *mmp-9* is expressed in the anterior mesoderm, leukocytes, and notochord (Yoong et al. 2007). Because *mmp-9* is expressed in the notochord, we compared wild-type and mutant survival from 4 hpf to 72 hpf, and found that mutant survival is severely compromised. The notochord is necessary for neurulation, a process by which the neural plate receives signals from the notochord leading to invagination and formation of the neural tube (Compagnon and Heisenberg 2013). Failure in neurulation results in severe congenital malformations and premature death in most cases (Copp and Greene 2013). Therefore, Mmp-9 may function to cleave morphogens such as BMPs, Shh, and/or TGF-β required for vertebrate development (Vandooren, Van den Steen, and Opdenakker 2013). While MMP-9 knockout mice are viable, aspects of bone development are affected, such as apoptosis, vascularization, and ossification (Vu et al.

1998). A possible explanation for the difference in survival among vertebrates can be due to the fact that *Mmp-17* is expressed in the mouse notochord, but Mmp-9 has yet to be detected (Blanco et al. 2017; Cañete-Soler et al. 1995).

In vertebrates, somites are repeated segments developed from mesoderm that give rise to skeletal muscle, vertebrae, and dermis (Saga and Takeda 2001; Stickney, Barresi, and Devoto 2000). The dorsoventral patterning of somitic mesoderm is induced by the notochord (Pourquié et al. 1993), and *mmp-9* is expressed by the zebrafish notochord as previously mentioned. Interestingly, we found that *mmp-9* mutants exhibit somite segmentation defects, suggesting that in the absence of *mmp-9* expression from the notochord, somitic mesoderm patterning is defective. Alternatively, previous studies show that collagen II, a common substrate of MMP-9, is essential for mouse intervertebral discs' formation (Cheah et al. 1991; Vandooren, Van den Steen, and Opdenakker 2013). Therefore, we speculate that collagen II is not properly degraded by Mmp-9 in our mutants and may contribute to the observed segmentation defects.

In teleost fish, fins are composed of radially arranged osteoblasts that express *mmp-9* during development and caudal fin regeneration (Ando et al. 2017; Knopf et al. 2011; Tu and Johnson 2011). Consistent with its expression pattern and function, *mmp-9* mutants exhibit an abnormal tail phenotype during development that persists into adulthood. MMP expression in extremities during development is not specific to non-mammalian vertebrates. For instance, *Mmp-8* and *Mmp-13* are expressed in osteoblasts and chondrocytes during rat hind limb development (Sasano et al. 2002). Therefore, we believe that *mmp-9* mutants fail to develop proper osteoblasts necessary for caudal fin development. An alternative role for Mmp-9 contributing to proper caudal

fin development sheds light on ECM remodeling. Mmp-9 modulates collagen matrices during zebrafish caudal fin regeneration and inhibition leads to structural defects following regrowth (LeBert et al. 2015). Though the role of Mmp-9 during fin regeneration is well studied, it will be important to address its contributions during caudal fin development.

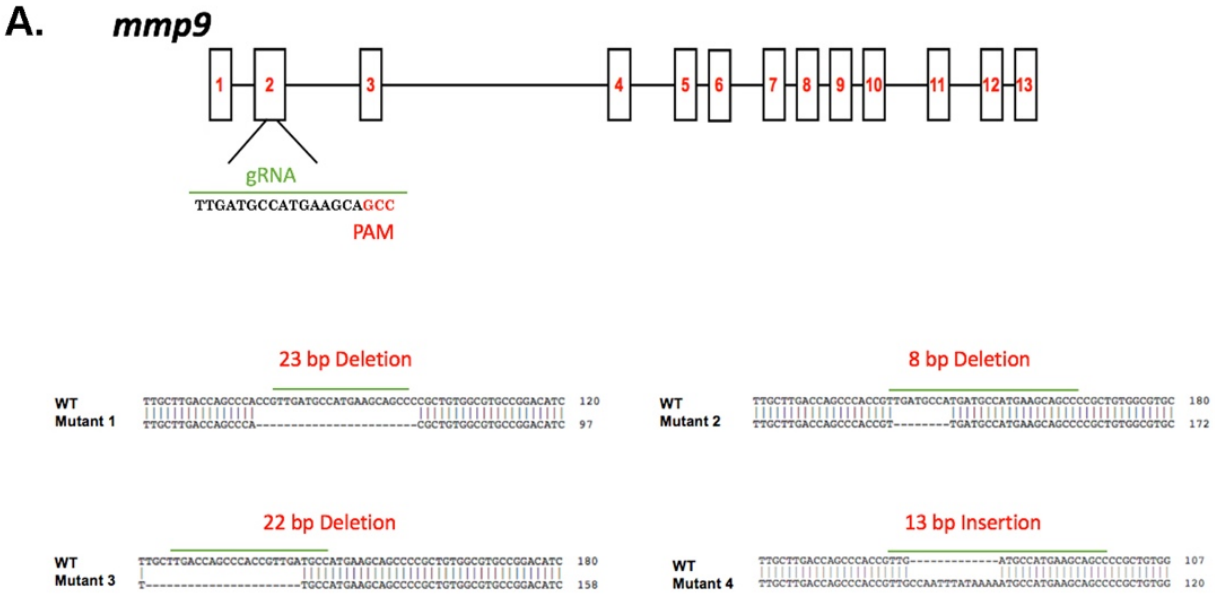
Our initial interest to generate the *mmp-9* mutants was due to our observation showing that *mmp-9* is expressed in the zebrafish retina following photoreceptor injury and death (Craig, Calinescu, and Hitchcock 2008). Accordingly, we characterized the retinal development to determine any potential phenotypes associated with the absence of *mmp-9*. We found that wild-type and both mutants develop retinal cell types at 72 hpf, suggesting that Mmp-9 does not govern their development. Similarity, retinal size and endogenous proliferation at the CMZ was not affected in all groups. Although, *mmp-9* is expressed during zebrafish development it is undetected in the central nervous system (CNS) (Yoong et al. 2007; Zhang et al. 2017), consistent with the absence of a detectable retinal phenotype. Of note, although *mmp-9* is undetected in the developing zebrafish retina, it is induced following CNS injury in Müller glia and derived progenitors (see Chapter Two).

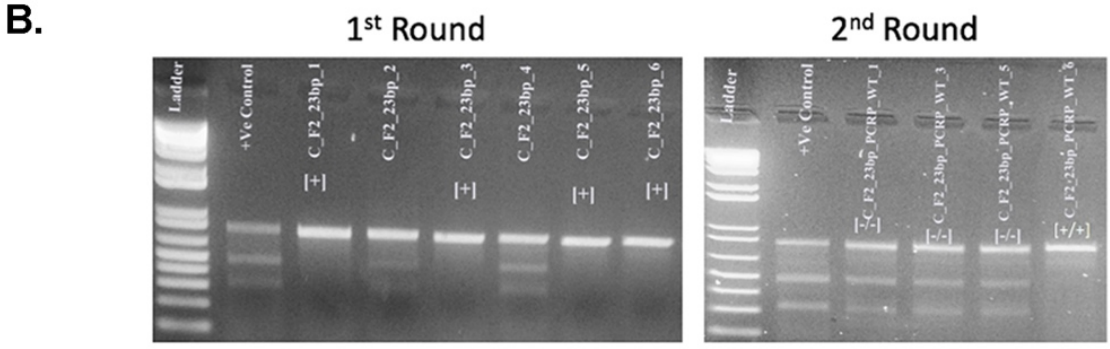
Transgenic reporter lines are an integral component to study the expression of a specific gene of interest. Tol2-mediated bacterial artificial chromosome (BAC) transgenesis recapitulates endogenous gene expression with high fidelity and is commonly used (Yang et al. 2006; Fuentes et al. 2016; Kawakami 2007). Yet, due to the recent advancements of CRISPR/Cas9 technology, we attempted to generate a stable transgenic line using the CRISPR-mediated knockin of EGFP (Li et al. 2015). Our

results indicate that subcloning and sequencing contains the correct right and left arms of *mmp-9* necessary for integration into the genome DNA. Yet, our sgRNA target failed to cleave genomic DNA as indicated by the negative T7 assay. Fortunately, two additional sgRNA targets were found in the left arms and serve as potential targets. Alternatively, we can remove the left arm and replace it with another containing a newly identified sgRNA. The *Tg*(*mmp-9:egfp*) transgenic reporter line was recently generated using traditional BAC transgenesis (Ando et al. 2017), and now provides the opportunity to study the expression of *mmp-9* in the zebrafish model.

Our study highlights the complexity of Mmp-9 function in multiple tissues during zebrafish development. The results are consistent with the location and timing of previously reported *mmp-9* expression patterns in zebrafish. Specifically, Mmp-9 functions in osteoblasts, chondrocytes, neural crest cells, and the notochord, all of which are vital during development as suggested by the data. Additionally, our *mmp-9* mutants show that Mmp-9 plays no functional role in the developing retina. Moving forward, it will be important to identify cellular signaling mechanisms contributing to the function of Mmp-9 during zebrafish development.

Figures


A.
mmp9
1
2
3
4
5
6
7
8
9
10
11
12
13
gRNA
TTGATGCCATGAAGCAGCC
PAM
23 bp Deletion
WT
Mutant 1
8 bp Deletion
WT
Mutant 2
22 bp Deletion
WT
Mutant 3
13 bp Insertion
WT
Mutant 4


B.
1st Round
2nd Round
Ladder
+Ve Control
C_F2_23bp_1
C_F2_23bp_2
C_F2_23bp_3
C_F2_23bp_4
C_F2_23bp_5
C_F2_23bp_6
[+]
Ladder
+Ve Control
C_F2_23bp_PCRP_WT_1
C_F2_23bp_PCRP_WT_3
C_F2_23bp_PCRP_WT_5
C_F2_23bp_PCRP_WT_6
[-/-]
[-/-]
[-/-]
[+/+]

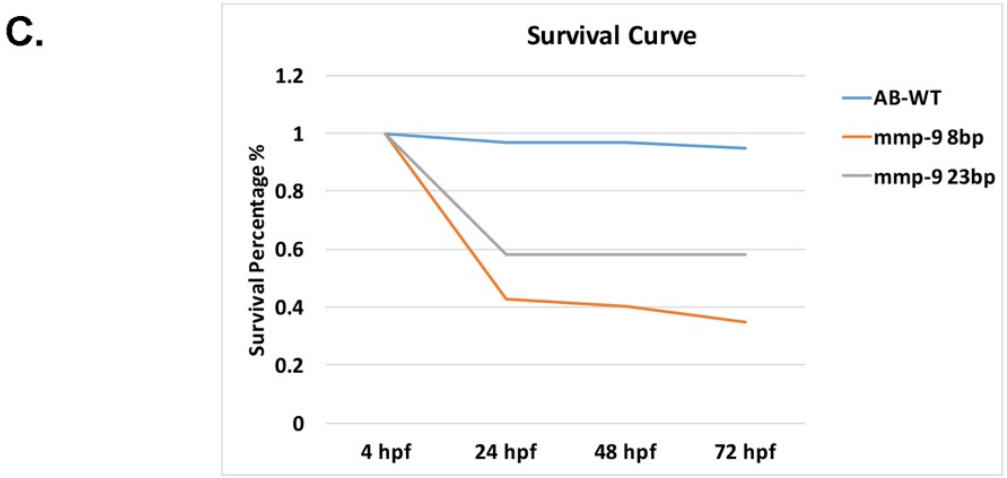
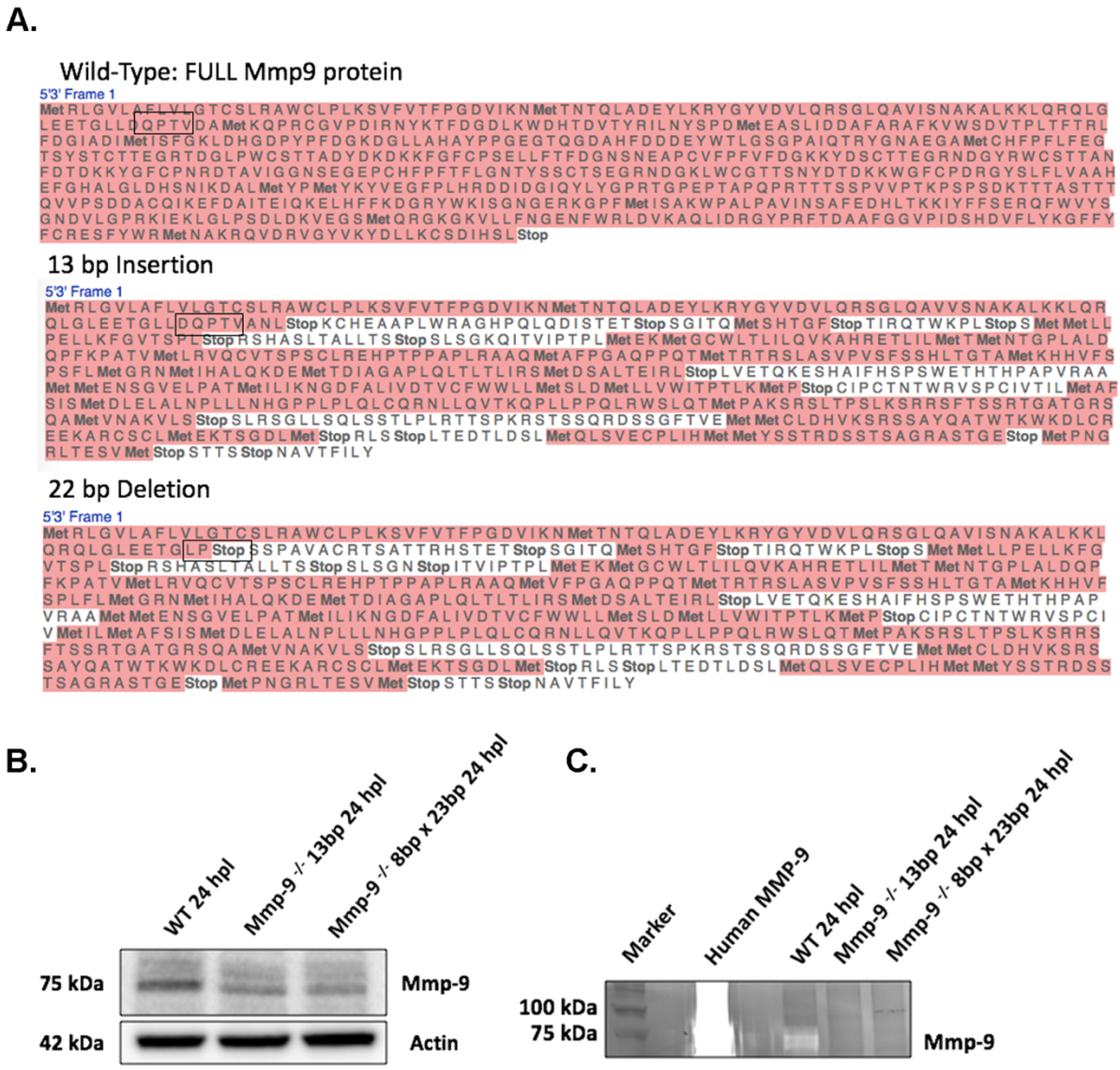

C.
Survival Curve
Survival Percentage %
1.2
1
0.8
0.6
0.4
0.2
0
4 hpf
24 hpf
48 hpf
72 hpf
AB-WT
mmp-9 8bp
mmp-9 23bp

Figure 3.1: Confirmed *mmp-9* mutants exhibit survival defects during development.
(A) Genomic structure of *mmp-9* and gRNA target sequence. Sequence alignment identifies two indel mutations; 8 bp insertion and 23 bp deletion from *mmp-9* $^{-/-}$ mutants compared to wild-types. Red underline indicates 19 bp gRNA targeting sequence. **(B)** T7 endonuclease assay confirms DSBs from PCR-amplified regions containing the gsRNA target site. 1st round identifies [+]; wild-type or homozygote. 2nd round contains added wild-type PCR-product to determine homozygotes [-/-]. **(C)** Survival curve of wild-types and *mmp-9* $^{-/-}$ mutants from 4 - 72 hours post fertilization (hpf).



Supplemental Figure 3.1: *mmp-9* INDELS produce a premature stop codon and lack catalytic activity

(A) The predicted amino acid sequence for wild-type and *mmp-9* $^{-/-}$ mutants. Black box denotes the gRNA target. **(B)** Mmp-9 western blot from wild-type and *mmp-9* $^{-/-}$ mutants at 24 hpl. Anti-actin as a loading control. (n=3; 6 retinas per sample). **(C)** Zymographic analysis of Mmp-9 catalytic activity from wild-type and *mmp-9* $^{-/-}$ mutants at 24 hpl. Purified human recombinant protein as positive control (n=3; 6 retinas per sample).

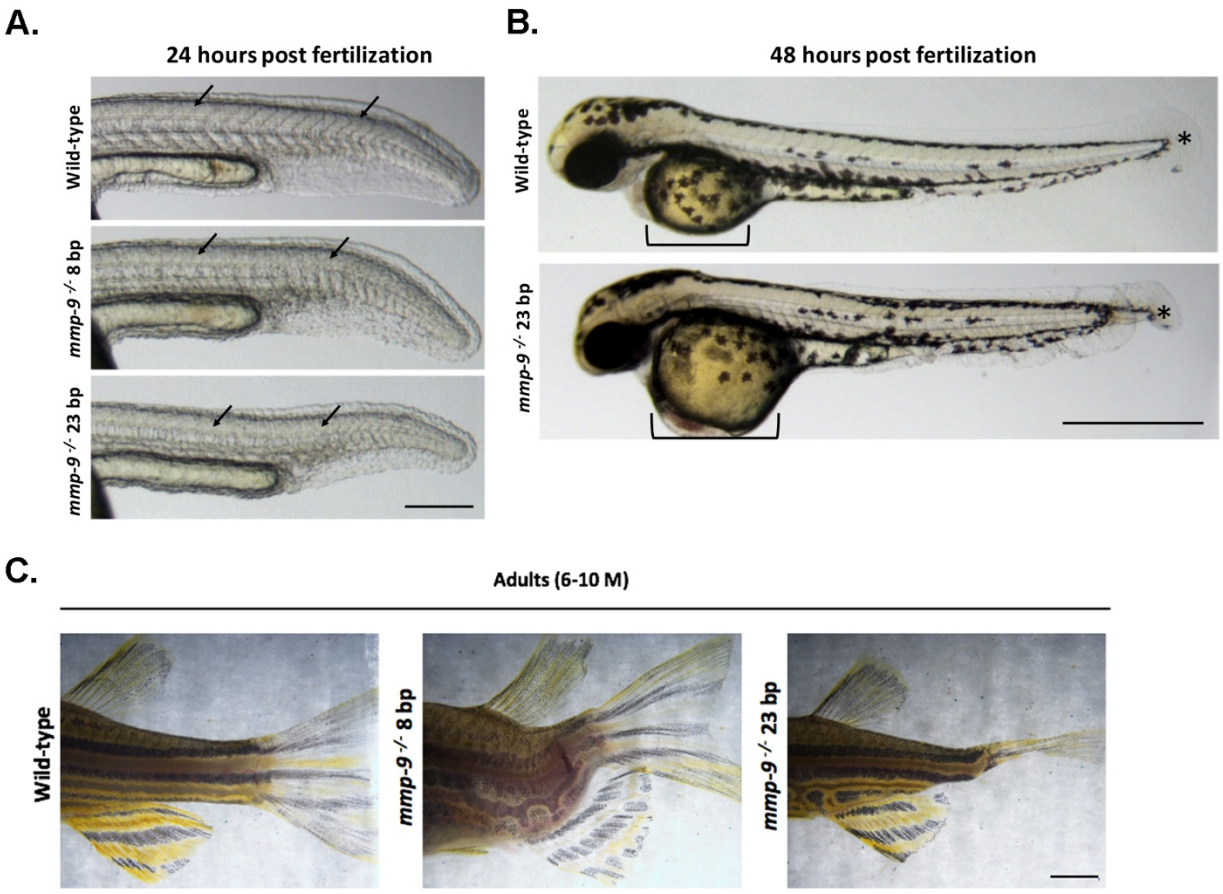


Figure 3.2: Somite segmentation and caudal tail development is abnormal in *mmp-9* mutants.
(A) Qualitative observation of wild-type and mutants somites at 24 hours post fertilization (hpf) as indicated by arrows. Scale bar equals 50 μm. **(B)** Wild-type and mutant caudal fins at 48 hpf indicated by asterisk. Brackets denote the yolk sack. Scale bar equals 200 μm. **(C)** Adult wild-type and mutant tails at 6-10 months. Scale bar equals 500 μm.

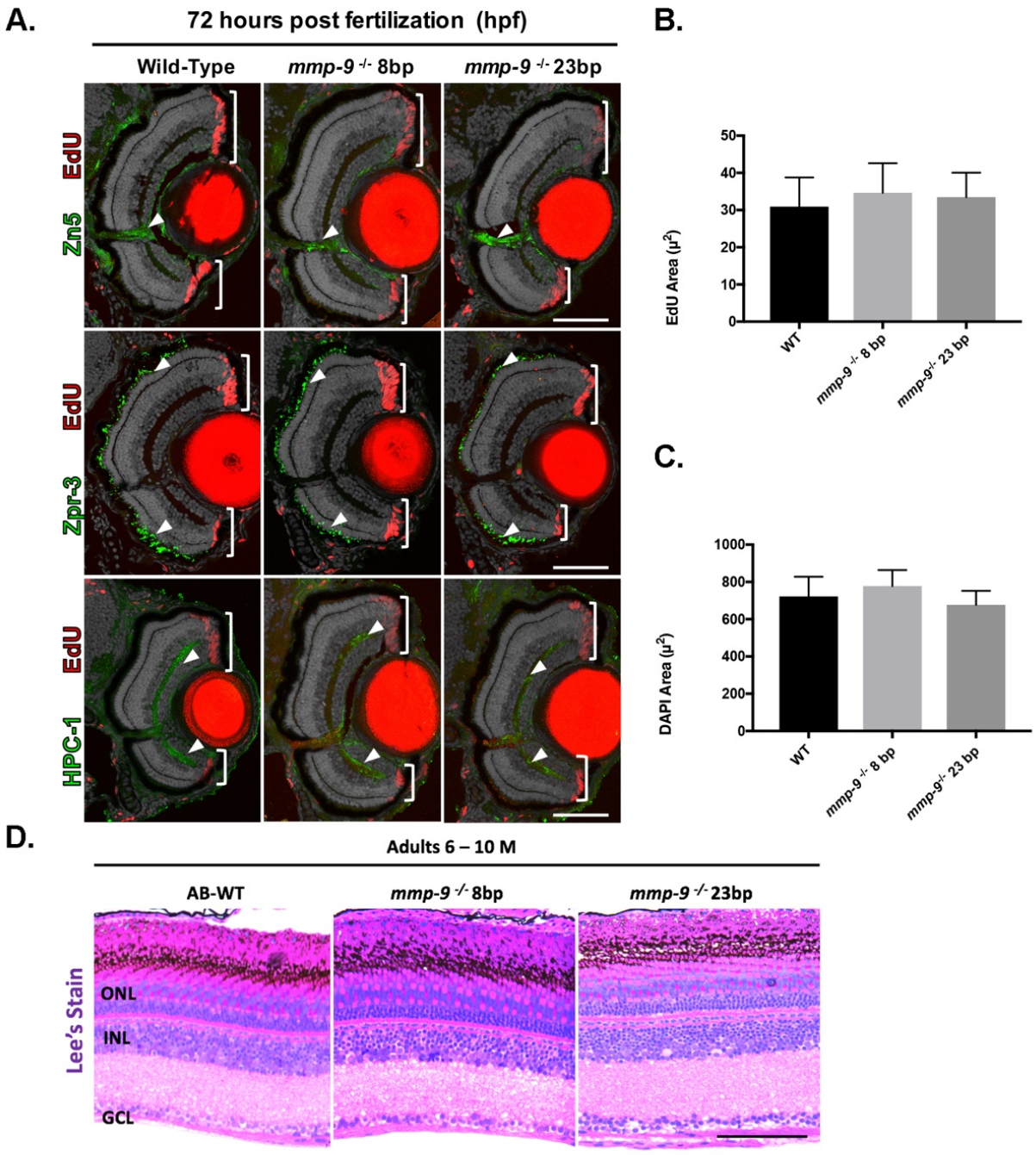


Figure 3.3: Retinal development in *mmp-9* mutants is normal.
(A) Immunostaining of retinal cell types showing ganglion cells (ZN5), rods (ZPR-3), and amacrine cells (HPC1) from wild-type and mutants at 72 hpf. Arrowheads indicate expression of retinal cell types. **(B)** Quantification of retinal area in wild-type (722.36 ± 105.39 μm; *n*=12) compared to *mmp9* $^{-/-}$ mutants (676.95 ± 75.05 μm; *n*=9) at 72 hpf. **(C)** Quantification of EdU (proliferation) area in wild-type (30 ± 7.84 μm; *n*=12) compared to *mmp9* $^{-/-}$ mutants (33.5 ± 6.59 μm; *n*=9) at 72 hpf. **(D)** Lee's Stain for wild-type and *mmp-9* mutant adults. Quantitative data are represented as mean; error bars represent the standard deviation. Scale bar equals 25 μm.

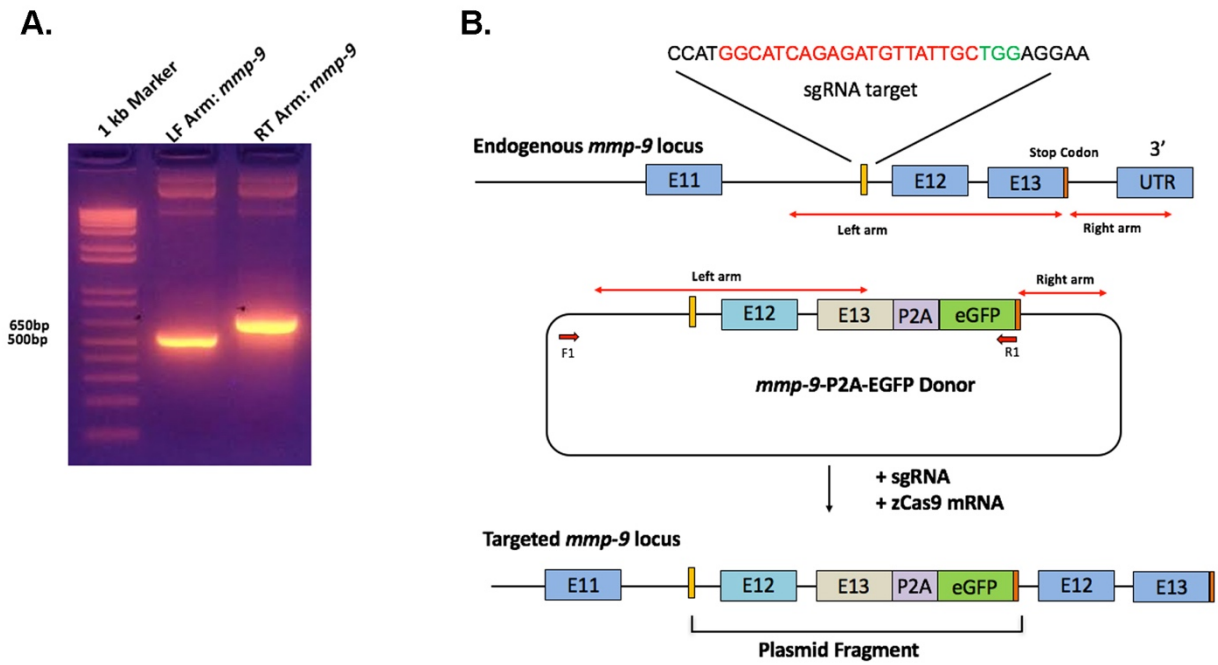
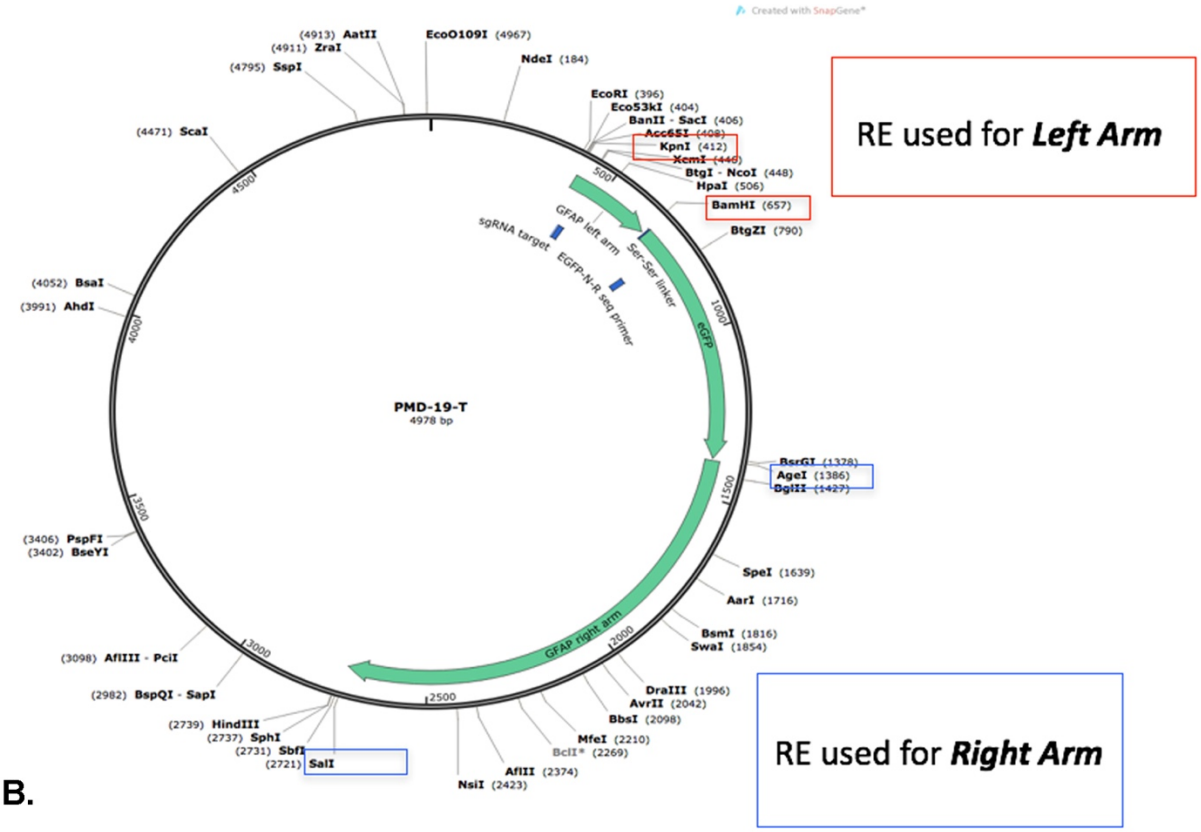


Figure 3.4: The generation of the *mmp-9*-P2A-EGFP donor plasmid.
(A) PCR amplification of the left and right arms of the zebrafish *mmp-9* gene used for subcloning into the backbone donor plasmid. The left arm is 498 bp and the right arm is 643 bp. **(B)** Schematic of the intron-mediated EGFP knockin using the CRISPR/Cas9 system. The sgRNA target sequence is in red and the protospacer adjacent motif (PAM) in green. The sRNA targets the endogenous *mmp-9* locus of intron 11 and the left arm of the *mmp*-9-P2A-EGFP donor plasmid. The linearized plasmid will integrate into the targeted mmp-9 locus following recombination.

A.



B.

Wild-type	184	AATAGTGACTTATCTCTTATATTTCCTCTCAGACTTGATGTAAAGGCTCAGCTGATTGAC	243
LF Arm: *mmp-9*	186	AATAGTGACTTATCTCTTATATTTCCTCTCAGACTTGATGTAAAGGCTCAGCTGATTGAC	245

Wild-type	180	ATAGGCACATGAGACGGGATGTTAGGCATATTTGTCCGTCAGCTTTACCATGGTCTTATA	239
RT Arm: *mmp-9*	183	ATAGGCACATGAGACGGGATGTTAGGCATATTTGTCCGTCAGCTTTACCATGGTCTTATA	242

Supplemental Figure 3.4: The *gfap*-P2A-EGFP donor plasmid map and confirmed Sanger sequencing of the *mmp-9* left and right arm of the *mmp-9*-P2A-EGFP donor plasmid.
(A) Map of the *gfap*-P2A-EGFP donor plasmid map used as the backbone for subcloning and restriction enzyme sites used for removing the *gfap* left and right arms. **(B)** Sanger sequencing alignment confirms subcloning of the *mmp-9* left and right arms into the generated *mmp-9*-P2A-EGFP donor plasmid.

Tables

Table 3.1: Antibody List

Primary Antibodies	Company	Dilution
Mouse Monoclonal anti-Zn5	ZIRC; zfin.org/ZDB-ATB-081002-19	1:200
Mouse Monoclonal anti-Zpr1 (anti-Arrestin-3)	ZIRC; zfin.org/ZDB-ATB-081002-43	1:200
Mouse Monoclonal anti-Zpr-3	ZIRC; zfin.org/ZDB-ATB-081002-45	1:200
Mouse anti-HPC1	ZIRC; zfin.org/ZDB-ATB 130225-1	1:200
Secondary Antibodies		
Alexa Fluor goat anti-mouse 488, 555, or 647	Invitrogen	1:500
Alexa Fluor goat anti-rabbit 488, 555, or 647	Invitrogen	1:500
Alexa Fluor goat anti-rat 488, 555, or 647	Invitrogen	1:500

Table 3.2: Primer Sequences

Name	Sequence
mmp-9 (pGEM-T easy primers)	F) 5'-ATCTCAGGCAATGGTGAACGCAAAG-3' R) 5'-ATAGCGCGTGGTGTAGCAATTAAG-3'
mmp-9 (Left Arm)	F) 5'-CCGGAATTCGAGTGAGCAGCACCTGTAACC- 3' R: 5'-CGCGGATCCTAGAGAATGAATGTCACTGCA- 3'
mmp-9 (Right Arm)	F: 5'-GGCCGACACCGGTTAGACCTTCGACATCCTAATC- 3' R: 5'-GGCCGACGTCGACTTAAAGGATCTTGTTTACAAA- 3'

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Chapter 4

Discussion

Summary

The zebrafish is a powerful model to study the cellular and molecular mechanisms governing retinal regeneration (Wan and Goldman 2017; Gorsuch and Hyde 2014; Lenkowski and Raymond 2014). Our lab previously identified matrix metalloproteinase 9 (*mmp-9)* from an unbiased microarray screen following photoreceptor death (Calinescu et al. 2009). I tested the hypothesis that in the zebrafish, *mmp-9* is a component of the inflammatory response and functions to regulate photoreceptor regeneration. To test my hypothesis, I used a combination of pharmacological and genetic approaches that suppress the inflammatory response and mutate the *mmp-9* gene, respectively. In addition, I characterized developmental phenotypes associated with the *mmp-9* mutants. Lastly, I provide preliminary data for the generation of a *mmp-9*-P2A-EGFP donor plasmid to create a stable transgenic line in the zebrafish.

In chapter one, I briefly summarized our current understanding of regulators that govern Müller glia entry into the cell cycle and progenitor proliferation. Among these regulators are intracellular transducers, transcriptional regulators, and secreted factors. Next, I discussed inflammatory mechanisms that govern neurogenesis in vertebrates, and finally I review the key literature about the inflammatory protease, Mmp-9.

In chapter 2, I present data demonstrating that inflammation and matrix metalloproteinase 9 (*mmp-9*) function to regulate photoreceptor regeneration. First, I characterized the expression of genes encoding inflammatory molecules induced by photoreceptor death and show that inflammation regulates the number of progenitors and regenerated photoreceptors. Following photoreceptor death, I found that Müller glia express *mmp-9* prior to reentry into the cell cycle, and their derived retinal progenitors during regeneration. Moreover, I show that Mmp-9 protein is expressed and catalytically active in the zebrafish retina following photoreceptor death. We found that the pro-inflammatory cytokine, Tnf-α signals upstream of *mmp-9* and is sufficient to induce its expression in the uninjured retina. To determine a functional role for Mmp-9, I generated *mmp-9* mutants in the zebrafish using the CRISPR/Cas9 system. *mmp-9* mutants respond to photoreceptor death by an increase in Müller glia-derived retinal progenitors and regenerated photoreceptors. However, in mutants subsequent to their genesis the maturation and survival of regenerated cones is compromised. These data provide a link between injury-induced inflammation in the vertebrate CNS and Mmp-9 function during photoreceptor regeneration. Specifically, inflammation and Mmp-9 function to regulate the number of Müller glia-derived retinal progenitors, but does not affect their differentiation into regenerated photoreceptors.

In chapter 3, I presented developmental data associated with the *mmp-9* mutants. I used the T7 endonuclease assay to confirm and identify homozygotes as well as Sanger sequencing, western blot, and zymogram analysis in chapter 2. I showed that compared to wild-types, *mmp-9* mutant survival is compromised, where by 24 hours post fertilization (hpf), nearly 40% of *mmp-9* 23 bp and 60% of *mmp-9* 8 bp mutant

embryos die. Viable mutants exhibit somite segmentation defects at 24 hpf and their caudal fins appear abnormal at 48 hpf. The caudal fin phenotype present in mutants during development became noticeably apparent in adulthood. However, I observe no apparent retinal phenotype between wild-type and mutants, as indicated by retinal cell types, ciliary marginal zone (CMZ), and normal size retina. Lastly, I presented preliminary data that confirmed the generation of a *mmp-9*-P2A-EGFP donor plasmid to create a stable Tg(*mmp-9-P2A-EGFP*) transgenic zebrafish line using the intron-mediated CRISPR/Cas9 system. Together these data have the potential to enhance our understanding of Mmp-9 function during development and regeneration in the zebrafish.

Future Directions

Identify potential Mmp-9 substrates

My results demonstrate that Mmp-9 regulates the initial events governing the number of Müller glia-derived progenitors, and later the maturation and survival of regenerated cones. I hypothesize that the two phenotypes from the initial and later stages reported in the *mmp-9* mutants are regulated by different substrates cleaved by Mmp-9. As described in chapter one, MMP-9 cleaves over one-hundred substrates, which include inflammatory cytokines and growth factors (Vandooren et al., 2014). However, the specific substrates cleaved by Mmp-9 to govern photoreceptor regeneration in the zebrafish are unknown.

To identify potential substrates cleaved by Mmp-9 to regulate the number of Müller glia-derived progenitors in the initial phase and cone survival in the later phase, I propose a proteomic approach. Fortunately, proteomic analysis in the zebrafish has

been previously validated in our photolytic lesion model (Nelson et al. 2013). Briefly, total protein will be isolated from either unlesioned or lesion retinas in both wild-type and *mmp-9* mutants at 3 and 21 days post lesion (dpl), when we observe the phenotypes. The protein homogenates will be separated by 2D gel electrophoresis and changes in protein levels will be quantified and detected by the fluorescence intensity of protein spots. Spots will be identified by mass spectrometry and analyzed by GPS and MASCOT software.

Due to the roles of MMP-9 as an inflammatory protease that resolves inflammation following injury, I predict that *mmp-9* mutants will have an increased level of inflammatory cytokines at 3 dpl. Though *mmp-9* mutants regenerate cone photoreceptors at 7 dpl, subsequent to their genesis maturation and survival is compromised, suggesting that a potential growth factor is absent at 21 dpl. Together, I reason that in the absence of functional Mmp-9, its respective substrates will not be degraded or activated during photoreceptor regeneration and thus contribute to the observed phenotypes.

Does inflammation contribute to the mmp-9 mutant phenotypes observed in the early and/or later stages of regeneration?

In *mmp-9* mutants, the increased number of Müller glia-derived progenitors at 3 dpl parallels the number of regenerated photoreceptors at 7 dpl, suggesting that Mmp-9 regulates the proliferative state of Müller glia-derived progenitors (see chapter two). Whether the hyperproliferation phenotype observed at 3 dpl is regulated by an unresolved inflammatory response in the absence of *mmp-9* is unknown. To test the hypothesis that *mmp-9* mutants have an exacerbated inflammatory response following photoreceptor death, I used Dexamethasone to suppress injury-induced inflammation in

mmp-9 mutants at 3 dpl. Briefly, I collected eyecups from wild-type and *mmp-9* mutants at 3 dpl treated with vehicle or Dexamethasone. Due to time constraints, I have not processed the collected tissue to determine if the hyperproliferation phenotype is rescued in mutants. However, I predict that if inflammation is driving the proliferation phenotype observed in mutants, then Dexamethasone should restore the appropriate number of Müller glia-derived progenitors.

Previous work has demonstrated a role for MMP-9 in age-related macular degeneration (AMD) (see chapter one), and we observe a decrease in regenerated cones in the absence of *mmp-9* (see chapter two). Furthermore, it is well established that inflammation is a hallmark of AMD and contributes to photoreceptor pathology (Copland et al. 2018). To test the hypothesis that chronic inflammation contributes to a decreased number of regenerated cones in mutants, we used Dexamethasone to suppress injury-induced inflammation and potentially rescue cone survival. Briefly, wild-type and mutants were treated with Dexamethasone from 3 - 14 dpl following the production of Müller glia-derived progenitors and collected at 21 dpl. I predict regenerated cone survival in mutants will be restored following Dexamethasone treatment if inflammation is contributing to the cone phenotype. Currently, we are processing the tissue to determine the state of regenerated cones.

Together these studies can potentially address the initial and/or later phenotypes observed from our mmp-9 mutants following photoreceptor death. If my hypotheses are incorrect, we can potentially exclude inflammatory contributions or identify an alternative approach.

How do microglia respond and function following photoreceptor death in mmp-9 mutants?

My preliminary studies indicate that following photoreceptor death, *mmp-9* is expressed in a limited number of microglia. In the zebrafish retina, microglia are key regulators that govern the regeneration of rod photoreceptors (White et al. 2017). Importantly, peripheral macrophages of MMP-9 null rats fail to be recruited to the lesioned spinal cord due to migration defects (Shubayev et al. 2006). The precise role of Mmp-9 function in zebrafish microglia during photoreceptor death is unknown. To determine if Mmp-9 regulates microglia migration to the damaged photoreceptors following photolytic lesion, I crossed our *mmp-9* mutants to the microglia reporter line, Tg(*mpeg1*:mcherry). The transgenic line is currently being propagated to homozygosity. Future studies should investigate the presence of microglia in the outer nuclear layer (ONL) at 24 hours post lesion, when microglia are present for phagocytosis of retinal debris (Mitchell, Lovel, and Stenkamp 2018). The number of microglia present in the ONL should be compared between wild-type and *mmp-9* mutants at 24 hpl. If Mmp-9 regulates the migration of microglia, I predict fewer in number in the ONL from *mmp-9* mutants compared to wild-type.

Final Conclusions

Inflammation in the central nervous system (CNS) has both beneficial and detrimental outcomes depending on the duration, cell type, extent of insult, and vertebrate species (Lucas et al., 2006; Kyritsis et al., 2014; Kizil et al., 2015; Bosak et al., 2018). Though inflammation impedes the regenerative capacity in mammals, our results and the work of others indicate that this limitation is species dependent. Thus,

zebrafish offers a unique opportunity to identify components of the inflammatory response, such as Mmp-9, and other key regulators for potential regenerative applications for mammals.

Over the past decade, zebrafish research has provided a wealth of contributions to the field of retinal regeneration. The discoveries have been a result of this animal's unique ability to regenerate combined with powerful research methodology including transgenic lines, genetic mutants, flow-cytometry, RNA sequencing methods, and proteomics. It will be critical to continue identifying novel regulators of retinal regeneration in the zebrafish, but importantly investigating their potential conservation in the mammalian retina. Utilizing both vertebrate species will provide the potential to discover mechanisms to restore vision.

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