Factors Regulating Initiation of Retina Regeneration in Zebrafish

By

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DEDICATION

To my grandfather Robert Anderson,

and

my loving family

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CHAPTER I

INTRODUCTION

Zebrafish Retina Regeneration

Retinal degeneration is the main cause of blindness in humans. A potential strategy for treating retina damage is to induce regeneration. However, humans, and mammals in general, are incapable of naturally regenerating the retina. Zebrafish are an ideal system to study retina regeneration because they are capable of endogenous retina regeneration and the structure and function of the retina are largely conserved between mammals and zebrafish (Stenkamp, 2007; Fadool and Dowling, 2008; Lamba et al., 2008; Gallina et al., 2014). It is therefore important to understand the various mechanisms by which zebrafish are able to regenerate the retina to develop potential treatments for humans.

Retina Structure

The vertebrate retina is a light-sensitive tissue in the posterior of the eye that converts photons to electrical signals that are then relayed to the visual cortex of the brain for further processing (Bassett and Wallace, 2012). The retina develops as part of the central nervous system (CNS) and forms three distinct cellular layers (Figure 1) (Otteson et al., 2001; Chuang and Raymond, 2002; Stenkamp, 2007).

The outer nuclear layer (ONL) is the layer furthest from the lens and consists of rod and cone photoreceptors. The inner nuclear layer (INL) is made up of interneurons including horizontal cells,

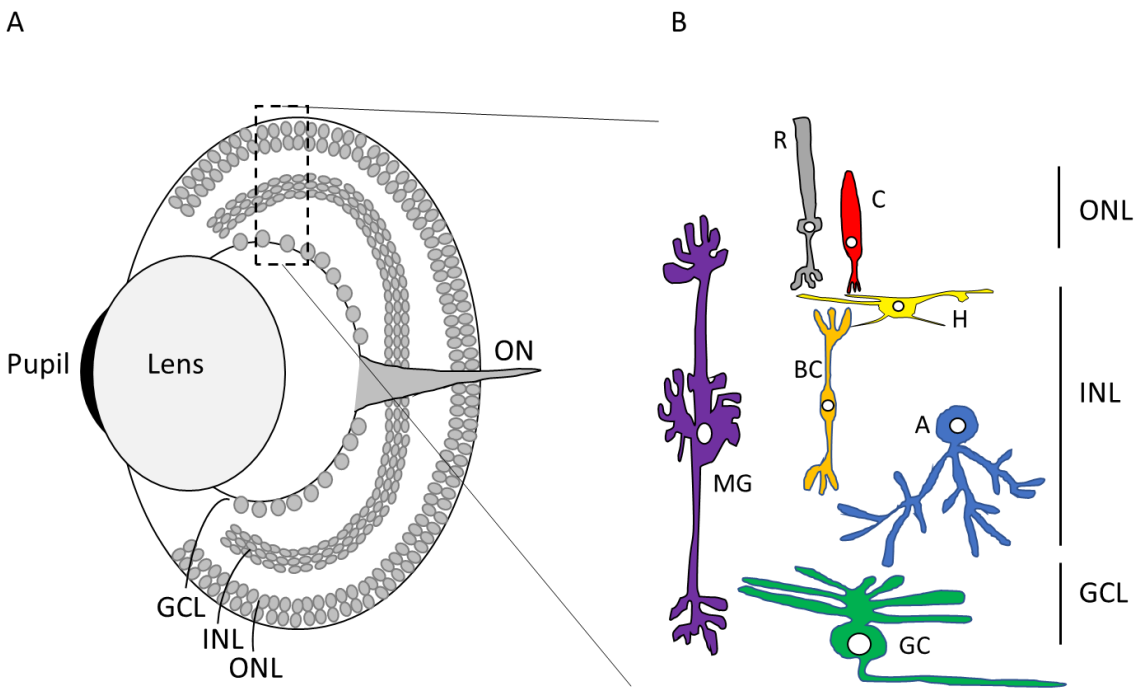

Figure 1. Vertebrate retina

(A) A diagram of the vertebrate eye with the retina lining the posterior of the eye. (B) A schematic of the layers of the retina and the cell types of each layer. The outer nuclear layer (ONL) consists of rods (R, gray) and cones (C, red). The inner nuclear layer (INL) contains bipolar cells (BP, orange), horizontal cells (H, yellow), amacrine cells (A, blue), and Müller glia (MG, purple). The ganglion cell layer (GCL) contains ganglion cells (GC, green).

bipolar cells, and amacrine cells, as well as a type of glial cell, Müller glia (MG). The third layer, the ganglion cell layer (GCL), is closest to the lens and consists entirely of ganglion cells. There are two additional layers of synaptic connections. The outer plexiform layer (OPL) separates the ONL and INL, and the inner plexiform layer (IPL) separates the INL and GCL.

Retinal Cell Types

Cone photoreceptors, interneurons, and ganglion cells can all be further divided into subgroups based on function and morphology. At least 39 transcriptionally distinct subpopulations have been identified due to recent advances in single cell RNA sequencing (Masland, 2001; Masland, 2012). It is likely with further advances, more will be discovered as it is estimated that there are approximately 60 distinct subpopulations of neurons that make up the structure of the retina (Macosko et al., 2015).

Photoreceptors are the cells that sense photons entering the retina and mediate phototransduction. Rod photoreceptors are used for dim light vision and have low spatial acuity. Cones are active at higher intensity light, are responsible for color vision, and have a higher spatial acuity (Kolb et al., 2001) . Zebrafish have four different classes of cones (Raymond et al., 1993; Fadool and Dowling, 2008; Allison et al., 2010). Blue-sensitive cones are single long cones. Red and green-sensitive cones are paired as double long cones. The final class of cones are UV-sensitive cones, which are single short cones. These cones are arranged in alternating rows of UV/blue and red/green cones. This mosaic arrangement of cones is thought to be important for resolution of color vision (Solomon and Lennie, 2007).

Bipolar cells are interneurons responsible for transmitting signals from photoreceptors to ganglion cells. There are four main types of retinal bipolar cells: ON, OFF, sustained, and transient (Werblin and Dowling, 1969; Kaneko, 1970; Masland, 2012) . In humans, there are different

subpopulations of bipolar cells that synapse either with single cone photoreceptors or with small groups of rod photoreceptors, totaling 12 different subpopulations of bipolar cells based on synaptic connections (Masland, 2012). However, there are an estimated 33 different types of bipolar cells in zebrafish (Li et al., 2012). Only one of these types of bipolar cells was found to be restricted to a single cone type, green cones, while one of the rod-cone bipolar cells was found to receive input from all photoreceptors (Li et al., 2012).

Horizontal cells are an inhibitory class of interneurons that modulate the signal between photoreceptors and bipolar cells (Poché and Reese, 2009; Thoreson and Mangel, 2012). Horizontal cells are in the upper INL, and their processes are part of the OPL. In general, mammals have two different types of horizontal cells: A-cells, which are axonless with a large dendritic field; and B-cells, with smaller dendritic fields and with an axon with a terminal arbor extending from the cell body (Fisher and Boycott, 1974). Primates and rats differ in this. Primates have three types of horizontal cells, with the third type resembling teleost horizontal cells with a long axon with no terminal arborization (Ahnelt and Kolb, 1994; Dacey et al., 1996; Dacey et al., 2000). Rodents have only B-cell morphology (Peichl and Gonzalez-Soriano, 1994). Teleosts, such as zebrafish, have four different horizontal cells, all of which are axon-bearing: H1-H3 synapse with cone photoreceptors; H4 synapses only with rod photoreceptors (Stell, 1975; Stell and Lightfoot, 1975; Weiler, 1978; Song et al., 2008). Horizontal cells provide inhibitory feedback to rod and cone photoreceptors (Masland, 2012).

Amacrine cells are another type of inhibitory neurons, found in the lower parts of the INL. Amacrine cells are a highly diverse type of retinal neurons, with the different types varying greatly across different species: 43 in roach teleost fish (Wagner and Wagner, 1988), 22 in cats (Kolb et al., 1981), and approximately 30 in rabbits (Masland, 2012). In zebrafish, 28 different types have been observed in the larval stage (Jusuf and Harris, 2009), with many of these also present in adults (Marc and Cameron, 2001; Yazulla and Studholme, 2001; Connaughton et al., 2004; Arenzana et al., 2006; Yeo

et al., 2009; Jang et al., 2011). Amacrine cells can be light-sensitive, and are also largely responsible for modulating the signal between bipolar cells and ganglion cells and providing the majority of synaptic input to ganglion cells. Amacrine cells can also synapse with each other (de Vries et al., 2011; Eggers and Lukasiewicz, 2011; Masland, 2012; Zhang and McCall, 2012).

Müller glia (MG) are the major glial cells of the retina, comprising approximately 4-5% of all cells in the retina (Jadhav et al., 2009; Reichenbach and Bringmann, 2010a, 2013). While the cell bodies are found in the INL, MG have processes that extend through all three layers of the retina and provide physical support for the retina. They provide additional help by uptaking excess neurotransmitters, removing debris, regulating K+ ion concentration, and providing additional nutrients for the retina (Newman and Reichenbach, 1996; Bringmann et al., 2006a; Reichenbach and Bringmann, 2010b). In addition to this support, MG are crucial for the damage response, either gliosis in most vertebrates or regeneration in certain species such as zebrafish.

Ganglion cells receive input from various types of bipolar and amacrine cells and output these signals to the visual cortex of the brain for further processing (Lamba et al., 2008). Mammals generally have a greater number of types of ganglion cells (Kolb et al., 1981)(Masland, 2012) than zebrafish (Mangrum et al., 2002). In zebrafish, there are 11 different types based on dendritic extent and stratification patterns (Mangrum et al., 2002). This is because zebrafish have a closer grouping of cell body diameters and dendritic field diameters compared to mammals (Kolb et al., 1981).

Response to Retinal Damage

Mammalian Response to Retinal Damage

Mammals do not spontaneously regenerate the retina in response to damage. They instead undergo a process known as reactive gliosis, which may initially be neuroprotective but has detrimental effects on the retina at later stages (Bringmann et al., 2006a; Bringmann et al., 2009; Reichenbach and Bringmann, 2010a). Retinal gliosis is typically characterized by upregulation of glial fibrillary acidic protein (GFAP), formation of glial scars, and loss of much of the support functions normally associated with MG (Fawcett and Asher, 1999; Honjo et al., 2000; Bringmann et al., 2006a).

It was previously thought that mammalian MG undergo reactive gliosis without any neurogenesis. However, it has been more recently recognized that mammalian MG have some limited neurogenic potential in response to damage. One study showed that neurotoxin N-methyl-D-aspartic acid (NMDA)-induced damage in mice could stimulate? limited MG dedifferentiation and generation of some new bipolar and rod cells (Ooto et al., 2004). Later studies showed further induction of MG dedifferentiation by intraocular injection of alpha-aminoadipate (AAA), glutamate, NMDA, or N-methyl-N-nitrosourea (MNU) (Ooto et al., 2004; Osakada et al., 2007; Karl et al., 2008).

While the above studies showed that certain types of damage could induce a limited regenerative response in mice, other strategies were found involving expression of transcription factors to reprogram the retina. One such factor was achaete-scute complex-like 1 (Ascl1), a member of the basic helix loop helix family of transcription factors. Ascl1 is normally expressed during retinal development and regulates proliferation of neural progenitor cells (Castro et al., 2011). However, Ascl1 is not normally expressed in the mouse retina in response to damage (Karl et al., 2008). Ascl1 has been shown to reprogram fibroblasts into neurons in culture (Vierbuchen et al., 2010). Expression of Ascl1 has also been shown to reprogram cultures of mouse MG to a progenitor cell state (Pollak et al., 2013a).

These findings led researchers to hypothesize that forced expression of Ascl1 in mammalian MG following damage might be sufficient to support regeneration. The transgenic mouse line Glast-CreER/tetO-Ascl1 allows for forced overexpression of Ascl1 in MG when treated with tamoxifen. This overexpression resulted in the formation of progenitors in neonatal mice after damage up to 15 days old, but the mice lost this regenerative potential after two weeks (Ueki et al., 2015). However, this same group was recently able to induce a limited proliferative response in adult mice with forced expression of Ascl1 after NMDA-induced damage with a histone deacetylase inhibitor treatment (Jorstad et al., 2017). This implies that by inhibiting mechanisms that limit the regenerative potential of MG, the proliferative potential can be stimulated in mammals, which could lead to potential therapeutic treatments to retinal damage. However, a recent study has highlighted a promising new approach. A study has shown that by transfecting a mouse retina with constructs with first *β-catenin* and then *otx2*, *crx*, and *nrl*, MG are capable of generating new rod photoreceptors (Yao et al., 2018). Wnt signaling has been shown to be promote retina regeneration in mammals (Osakada et al., 2007), while *otx2*, *crx*, and *nrl* are transcription factors that promote rod photoreceptor cell fate (Nishida et al., 2003; Hao et al., 2011; Ruzycki et al., 2018). This study then tested whether these new photoreceptors would be functional by using *Gnat1*^{rd17}:*Gnat2*^{cpfl3} double mutant mice, which lack photoreceptor-mediated light responses. The study found that these double mutant mice showed photoreceptor-mediated light responses after transfection of the different factors.

Zebrafish Response to Retinal Damage

Zebrafish are capable of regenerating multiple tissues, including heart (Poss et al., 2002; Zhang et al., 2013), fin (Nakatani et al., 2007; Thatcher et al., 2008; Knopf et al., 2011), spinal cord (Becker et al., 1997; Goldshmit et al., 2012), and the retina (Stenkamp, 2007). Retinal regeneration in teleost fish has been studied since initial findings in goldfish (Lombardo, 1968, 1972). Interestingly, the regeneration

does not stem from the ciliary marginal zone (CMZ), areas at the edges of the retina that are the main source of neurogenesis throughout the life of adult fish. Instead, clusters of proliferating cells are found in the center of the retina in the INL and ONL (Negishi et al., 1991; Hitchcock et al., 1992; Braisted et al., 1994; Otteson and Hitchcock, 2003). While these cells were initially thought to be rod precursors, later studies showed that these proliferating clusters of cells originate from the MG (Fausett and Goldman, 2006b; Bernardos et al., 2007a).

Fausett and Goldman generated a transgenic zebrafish that expressed GFP driven by the α1 tubulin promoter [Tg(*tuba1a:GFP*)] in which GFP expression in MG is dependent on retinal injury (Fausett and Goldman, 2006b) . Further study showed that, following damage, MG dedifferentiate to a multipotent state and give rise to progenitor cells (Bernardos et al., 2007a). MG-derived progenitors replace damaged photoreceptors after intense light damage (Bernardos et al., 2007a), as well as any other damaged cell type in the retina (Fausett and Goldman, 2006b).

Mechanisms of Müller Glia Reprogramming in Response to Damage

The mechanisms through which MG sense damage to the retina have not been completely identified or studied. An early damage signal is tumor necrosis factor α (TNF-α) which is released by dying neurons and has been proposed to induce MG dedifferentiation (Nelson et al., 2013). Another early factor, heparin-binding epidermal-like growth factor (HB-EGF), is rapidly upregulated in response to damage and regulates transcription of other early regeneration-associated genes such as *ascl1* and *pax6$_b$* (Wan et al., 2012). Insulin and insulin-like growth factor 1 (igf-1) have been shown to synergize with HB-EGF activity to stimulate MG progenitor formation (Wan et al., 2014). Leptin and interleukin 6 (IL6) family genes have also been shown to promote MG reprogramming via janus kinase and signal transducer and activator of transcription proteins (Jak/Stat) signaling, pathways that have also been

shown to regulate *ascl1* expression (Zhao et al., 2014). While the previously mentioned factors function early in retina regeneration, a recent study has proposed a model of intercellular signaling that acts earlier. γ-aminobutyric acid (GABA) levels have been shown to decrease after photoreceptor damage and have been proposed as a mechanism by which MG might sense retinal damage (Rao et al., 2017).

MG dedifferentiate to a stem cell-like state in response to damage and undergo asymmetric cell division to produce a daughter MG and a retinal progenitor cell (Bernardos et al., 2007a; Gorsuch and Hyde, 2013; Nagashima et al., 2013) . When MG reenter the cell cycle, various genes are activated indicative of both proliferation and dedifferentiation such as PCNA and cyclin B1 (Kassen et al., 2007); the transcription factor Ascl1 (Fausett et al., 2008b) ; and pluripotency genes such as Lin28, Oct4, Nanog, Klf4, Myc, Mycb, and Sox2 (Ramachandran et al., 2010b). After the first retinal progenitor cell (RPC) is produced, the RPC undergoes multiple rounds of replication to produce clusters of progenitor cells (Thummel et al., 2008b; Thummel et al., 2010). These clusters then migrate to damaged areas of the retina where they differentiate to produce new neurons for the retina (Gorsuch and Hyde, 2013; Nagashima et al., 2013). Studies show that the regenerated neurons in the zebrafish retina form new functional synaptic connections, and the zebrafish are capable of responding to visual stimuli (Mensinger and Powers, 1999; Fimbel et al., 2007; Sherpa et al., 2008). The current model of zebrafish retina regeneration is shown in **Figure 2**.

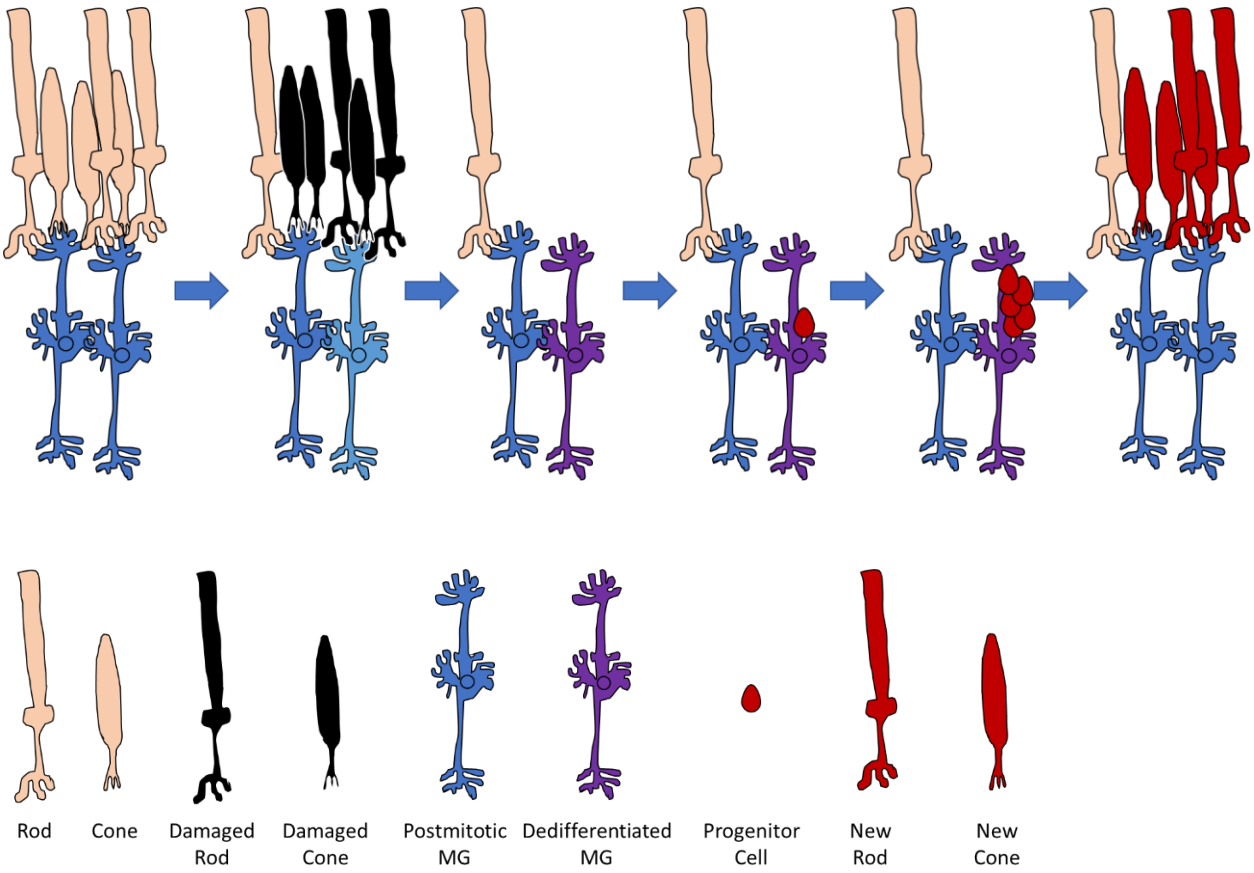

Figure 2. Schematic of zebrafish retina regeneration.

A model of retina regeneration in zebrafish. After cells in the retina are damaged, the MG dedifferentiates. The dedifferentiated MG then undergoes asymmetric cell division to produce a new MG and a progenitor cell. The progenitor cell then proliferates, the cluster of progenitors migrates, and these cells then differentiate into cells of the same type that were damaged.

Role of GABA in Regeneration

γ-aminobutyric acid (GABA) is a neurotransmitter, normally thought to be inhibitory. There are two main classes of GABA receptors: ionotropic ($GABA_A$ and $GABA_A$-ρ) and metabotropic ($GABA_B$). Ionotropic GABA receptors are pentamers comprised of one or more types of subunits. Ionotropic GABA receptors are ligand-gated Cl- ion channels, allowing Cl- ions to flow down the concentration gradient (Chebib and Johnston, 2000). $GABA_A$ receptors are formed from two α subunits, two β subunits, and one

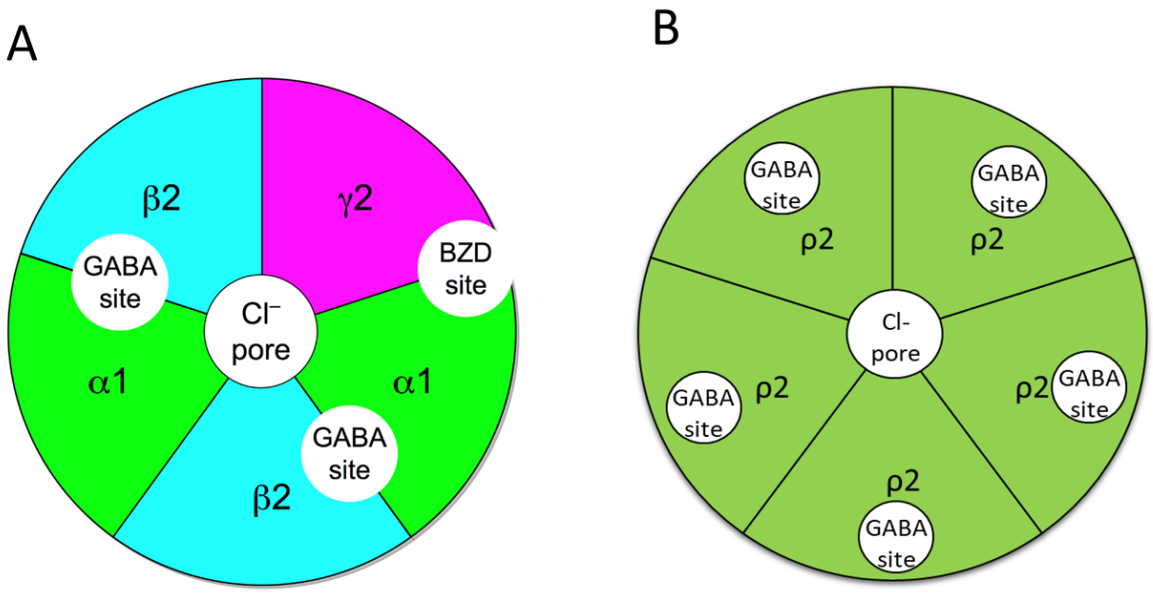

Figure 3. Ionotropic GABA receptor structure

An illustration of $GABA_A$ (**A**) and $GABA_A$-ρ (**B**) receptor structures. Both are pentameric and have GABA binding sites. $GABA_A$ receptors contain a benzodiazepine (BZD) binding site, absent on $GABA_A$-ρ receptors.

additional subunit, typically a γ subunit (**Figure 3A**) (Mortensen et al., 2011). $GABA_A$-ρ receptors are instead formed entirely of ρ subunits (**Figure 3B**), and are typically homopentamers though they can form heteropentamers (Enz and Cutting, 1998; Ogurusu et al., 1999). $GABA_B$ receptors, in contrast, are heterodimers and are G-protein coupled receptors that stimulate the opening of K+ ion channels (Wang and Lambert, 2000; Emson, 2007).

$GABA_A$-ρ receptors were originally identified as a pharmacologically distinct subtype of $GABA_A$ receptors, because they are insensitive to bicuculline and baclofen, antagonists of $GABA_A$ and $GABA_B$ receptors, respectively. They are also insensitive to many $GABA_A$ receptor modulators such as benzodiazepine (BZD) (Sivilotti and Nistri, 1991; Bormann and Feigenspan, 1995). While $GABA_A$-ρ receptors are expressed in many areas of the brain, they have a significant expression in the retina (Qian, 1995).

GABA has been previously shown to be involved in regeneration of stem cells in the mouse hippocampus (Song et al., 2012). Song *et al.* hypothesized a three-neuron network in which an active neuron releases glutamate, which is sensed by an interneuron that then releases the neurotransmitter GABA. GABA is then sensed by a glial cell, which is kept in a quiescent state. When this network is damaged, the decreased levels of glutamate lead to decreased GABA levels which cause activation of the glial cell leading to cell cycle activation and production of neuronal progenitor cells to replace the damaged area (Song et al., 2012).

A more recent study in zebrafish proposed a similar network in the retina. Rao *et al*. proposed that photoreceptors release glutamate which is sensed by horizontal cells, which then release GABA which is sensed by MG. In support of this model, the inhibition of glutamate or GABA signaling was sufficient to induce a proliferative response from MG (Rao et al., 2017).

microRNAs

microRNAs (miRNA) are small (approximately 22bp) double-stranded RNAs that are evolutionarily conserved and capable of post-transcriptionally regulating gene expression. Initially found in screens in *C. elegans* (Lee et al., 1993; Wightman et al., 1993), miRNAs were later found in *Drosophila*, humans, and plants (Reinhart et al., 2002; Ambros, 2003; Lagos-Quintana et al., 2003). While over 38,000 miRNAs have been identified on the miRNA registry (http://www.mirbase.org), the biological functions of many of these miRNAs have yet to be determined (Griffiths-Jones, 2006).

miRNAs function by base pairing with a complementary sequence on a target mRNA while associated with argonaute (AGO) proteins, collectively known as an RNA-induced silencing complex (RISC) (Khvorova et al., 2003; Schwarz et al., 2003). The RISC complex in animals typically binds to the 3'-Untranslated Region (UTR) of target mRNAs (Bartel, 2009). Pairing can either be perfect or imperfect, leading to different mechanisms of silencing. Perfect base pairing leads to cleavage of the mRNA by AGO2. Imperfect base pairing usually results in a bulge in the miRNA, preventing cleavage (Kiriakidou et al., 2004). Instead, the RISC complex remains bound to the mRNA and prevents translation (Ameres and Zamore, 2013). The most important sequence for target recognition is nucleotides 2-7, called the seed region. Binding of the seed region is generally considered the bare minimum necessary for the RISC complex to bind to the target mRNA, while the remaining nucleotides help with additional base pairing (Bartel, 2018).

miRNAs in Retina Regeneration

While many protein factors have been identified to regulate retina regeneration (as described above), the role of miRNAs is less understood. The first discovery of a miRNA regulating retina regeneration was made in zebrafish, through studies of the RNA-binding protein *lin-28*. *lin-28* expression

is upregulated 6 hours after retinal damage, leading to decreased expression of the miRNA *let-7* in retinal progenitors (Ramachandran et al., 2010b). *let-7* is a miRNA that targets several genes required for retina regeneration, and therefore the decrease in *let-7* levels allows retina regeneration to proceed. Interestingly, a recent study showed that this mechanism is conserved in mammals by overexpressing *lin-28*, resulting in decreased expression of *let-7*, which was sufficient to induce MG dedifferentiation in mice (Yao et al., 2016).

While *let-7* negatively regulates regeneration, it is clear that other miRNAs are required for regeneration to occur. Another recent study found that miRNAs globally are required for retina regeneration by showing that Dicer is required for retinal progenitor proliferation (Rajaram et al., 2014c). RNA sequencing (RNA seq) from whole zebrafish retina found 36 differentially regulated miRNAs (Rajaram et al., 2014c). Knockdown of several of these miRNAs, including miR-142b, 146a, 71, 27c and 31, was found to inhibit proliferation of retina progenitors (Rajaram et al., 2014c). In addition, *miR-203* was found to regulate progenitor cell proliferation via targeting of *pax6b* (Rajaram et al., 2014b).

Epigenetic Regulation of Retina Regeneration

Regulation of chromatin state (epigenetics) is essential in regulating gene expression (Barrero et al., 2010; Telese et al., 2013). Epigenetic modifications consist of DNA methylation and posttranslational modifications of histones.

In eukaryotic cells, DNA is organized into chromatin by packaging with histones. Four main histone proteins, H2A, H2B, H3, and H4 form an octameric structure wrapped by 146 bp of DNA, called a nucleosome (Kornberg, 1974; Kornberg and Thomas, 1974; Luger et al., 1997). Histones can be modified on the unstructured N-terminal tails that protrude out of the nucleosome (Kouzarides, 2007; Barrero et al., 2010). These modifications can induce changes in chromatin structure, through compaction or

relaxation, as well as act as sites for recruiting and stabilizing other proteins. The most common histone modifications are phosphorylation of serine and lysine residues, acetylation or methylation of arginine and lysine residues, and sumoylation and ubiquitylation of lysine residues. Furthermore, methylation can occur in mono-, di-, or trimethyl forms.

Histone modifications are not evenly distributed across the genome. Different modifications are associated with different genomic features (Liu et al., 2005; Pokholok et al., 2005; Ernst et al., 2011; Bernstein et al., 2012). Acetylation is mostly associated with regions that are transcriptionally active, while lysine methylation is associated with either euchromatin or heterochromatin based on which residues are methylated (Bernstein et al., 2007; Kouzarides, 2007; Campos and Reinberg, 2009). H3 lysine 4 (H3K4), H3 lysine 36 (H3K36), and H3 lysine 79 (H3K79) methylation are predominately associated with euchromatin. However, H3 lysine 9 (H3K9), H3 lysine 27 (H3K27) and H4 lysine 20 (H4K20) methylation are associated with heterochromatin. Histone acetylation reduces histone-DNA interactions via neutralization of positive charges, which is thought to drive euchromatin formation and increase accessibility of DNA for transcription (Hongs et al., 1993; Megee et al., 1995; Dion et al., 2005; Shogren-knaak et al., 2006; Zentner and Henikoff, 2013). In contrast, histone methylation recruits various proteins with different methyl binding domains. For example, H3K4 trimethylation (H3K4me3) facilitates transcription by recruiting CHD1, a chromatin remodeler (Gaspar-Maia et al., 2009).

Histone methylation patterns have been shown to change during early development (Mikkelsen et al., 2007; Vastenhouw et al., 2010; Gifford et al., 2013) and during generation of induced pluripotent stem cells (iPSCs) (Maherali et al., 2008; Mansour et al., 2012). It is therefore not surprising to find epigenetic regulators of MG reprogramming. DNA methylation in zebrafish retina regeneration has been previously studied, but interestingly, regeneration-associated genes such as Ascl1, HB-EGF, Lin28, Oct4, and Sox2 have low methylation levels in MG that do not change in response to damage (Powell et al., 2012; Powell et al., 2013). This suggests that these genes are poised for activation in MG. However,

these genes also had similarly low levels of methylation in mice (Powell et al., 2013), suggesting that DNA methylation levels are not why mammals have restricted regenerative potential. This leaves histone modifications as a potential regulator of these regeneration-associated genes. Recent studies in mice show that accessible chromatin in MG rapidly decreases with age, consistent with decreased regenerative potential (Ueki et al., 2015; Jorstad et al., 2017). However, specific histone modifications on regeneration-associated genes have yet to be identified.

Summary

The work in this dissertation focuses on characterizing factors involved in regulating the initiation of retina regeneration in zebrafish. Chapter II describes the role of $GABA_A$-ρ receptors in regulating retina regeneration. Chapter III describes how the miRNA *miR-216* regulates initiation of retina regeneration through targeting a methyltransferase. This dissertation demonstrates the varied methods through which the initiation of retina regeneration is regulated.

CHAPTER II

INHIBITION OF $GABA_A$-P RECEPTORS INDUCES RETINA REGENERATION IN ZEBRAFISH[1]

Matthew Kent, Nergis Kara, and James G. Patton

This chapter is a collaborative effort. Dr.Kara performed RNA seq on sorted MG from the *Tg(gfap:gfp)* transgenic line of zebrafish, analyzed the data, and calculated the Reads Per Kilobase of transcript, per Million mapped reads (RPKM) (**Table 1**).

[1] This work has been accepted for publication: Kent MR, Kara N, Patton JG (In Press) Inhibition of $GABA_A$-ρ receptors induces retina regeneration in zebrafish. Neural Regeneration Research.

Abstract

A potential treatment for retinal diseases is to induce an endogenous Müller glia (MG)-derived regenerative response to replace damaged neurons. In contrast to mammalian MG, zebrafish MG are capable of mediating spontaneous regeneration. We seek to define the mechanisms that enable retina regeneration in zebrafish in order to identify therapeutic targets to induce mammalian retina regeneration. We previously used pharmacological and genetic methods to inhibit gamma aminobutyric acid A ($GABA_A$) receptors in undamaged zebrafish retinas and showed that such inhibition could induce initiation of retina regeneration, as measured by the dedifferentiation of MG and the appearance of MG-derived proliferating progenitor cells. Here, we show that inhibition of a pharmacologically distinct subset of $GABA_A$ receptors ($GABA_A$-ρ) can also induce retina regeneration. Dual inhibition of both GABA receptor subtypes led to enhanced retina regeneration. Gene expression analyses indicate that inhibition of $GABA_A$-ρ receptors induces a canonical retinal regenerative response. Our results support a model in which decreased levels of GABA, such as would occur after retinal cell death or damage, induce dedifferentiation of MG and the generation of proliferating progenitor cells during zebrafish retina regeneration.

Key Words: gamma aminobutyric acid; morpholino; Müller glia; neurotransmitter; regeneration; retina; stem cells; zebrafish

Introduction

The health and overall economic consequences of vision loss, whether due to injury or disease, is significant with 2014 estimates of annual costs by Prevent Blindness of $145 billion, a number that will certainly increase as life expectancy increases (Wittenborn JS, 2014). This has led to a concerted effort to identify therapies to restore loss of vision or reduce the effects of degenerative retinal disorders. One current treatment involves intravitreal injection of either stem cells or retinal precursor cells, but these treatments are not yet capable of fully restoring vision (Barber et al., 2013; Hanus et al., 2016; Gonzalez-Cordero et al., 2017; Stern et al., 2018). Recent gene therapy approaches have been used for delivery or overexpression of factors to induce retina regeneration or to restore expression of defective genes, but in certain cases this approach is limited to those diseases where the exact defective gene is known (Jorstad et al., 2017; Russell et al., 2017; Yao et al., 2018). An alternative to these approaches is to induce damaged or diseased retinas to undergo regeneration using resident adult stem cells. Zebrafish retinas undergo spontaneous retina regeneration in response to damage (Wan and Goldman, 2016). In contrast, the mammalian retina does not naturally regenerate, more often responding to damage via reactive gliosis (Bringmann et al., 2006b). Intriguingly, the adult stem cell that is responsible for regeneration in zebrafish (Müller glia; MG)(Bernardos et al., 2007b) is present in the mammalian retina, but for unknown reasons, MG-derived regeneration is blocked in mammals, possibly related to the hippo pathway (Rueda et al., 2019). In fish, MG respond to damage by dedifferentiation, asymmetric division, and the generation of proliferating neuronal progenitor cells which can then migrate and differentiate into any lost retinal cell type (Wan and Goldman, 2016).

Gamma aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the central nervous system (Roberts and Kuriyama, 1968; Farrant and Nusser, 2005). Beyond its role in synaptic transmission, GABA has recently been shown to regulate neural stem cell activation in the mouse hippocampus (Catavero et al., 2018). We previously showed that inhibition of $GABA_A$ receptors can

activate MG-derived stem cell proliferation during the initial stages of retina regeneration in zebrafish (Rao et al., 2017). We hypothesized that normal GABA levels maintain MG quiescence, but that disruption of the GABA levels, whether by damage, disease or pharmacological inhibition, activates MG leading to dedifferentiation, asymmetric division, production of proliferating progenitor cells, and overall induction of a regenerative response.

We previously showed that injection of the $GABA_A$ antagonist gabazine led to MG-derived proliferation in undamaged fish retinas (Rao et al., 2017). However, a second class of GABA receptors, $GABA_A$-ρ (Blarre et al., 2014; Alexander et al., 2017)(originally referred to as $GABA_C$ receptors (Drew et al., 1984)), is also expressed in the retina (Cutting et al., 1991; Cutting et al., 1992; Boue-Grabot et al., 1998). Whether $GABA_A$-ρ receptors might also be involved in regulating retina regeneration is unknown. The $GABA_A$-ρ receptor was first discovered as a subtype of the $GABA_A$ receptor family that is insensitive to the $GABA_A$ receptor antagonist bicuculline (Drew et al., 1984). $GABA_A$-ρ receptors have a similar structure to $GABA_A$ receptors in that they are both pentameric ionotropic ligand-gated ion channels (Connolly et al., 1996; Enz and Cutting, 1998; Ogurusu et al., 1999). However, the two types of receptors differ in the subunits that form the functional receptor. $GABA_A$ receptors consist of two α subunits, two β subunits and a fifth subunit, most commonly a γ subunit. In contrast, the $GABA_A$-ρ receptors consist of five ρ subunits, of which there are three main types, typically homomeric (Connolly et al., 1996). This difference in subunit composition results in $GABA_A$-ρ receptors being insensitive to $GABA_A$ allosteric modulators such as benzodiazepines. While the ρ subunits were first discovered in mammals, they are also prevalent in zebrafish (Connaughton et al., 2008). The most abundant ρ subunit in the zebrafish retina is ρ2a, with ρ1 and ρ3a showing significantly lower expression and even lower levels of ρ2b (Cocco et al., 2017). In contrast to the $GABA_A$ receptor, $GABA_A$-ρ receptors display a more sustained response to activation (Bormann and Feigenspan, 1995) which could therefore better sustain retina

regeneration. Here, we sought to test whether $GABA_A$-ρ receptors are involved in regulating initiation of retina regeneration in zebrafish.

Materials and Methods

Zebrafish Lines and Maintenance

Zebrafish were used in accordance with Vanderbilt's Institutional Animal Care and Use Committee (Protocol M1800200; approved 1/29/2019, expires 1/29/2022). Zebrafish used in this study include Tg(*gfap*:GFP)^{mi2001} (Bernardos and Raymond, 2006) which marks mature MG and Tg(*tuba1a*:GFP)(Fausett and Goldman, 2006a) which marks dedifferentiated MG. All fish were maintained at 28.5°C in a 14:10 hour light:dark cycle. All fish used were between 5 and 8 months old, and were a random mix of males and females. A total of 296 zebrafish were used in this study: 73 Tg(*gfap*:GFP)^{mi2001} zebrafish and 223 Tg(*tuba1a*:GFP) zebrafish.

Drug and Morpholino Injections

Pharmacological inhibitors and morpholinos were injected into the vitreous as described (Rao et al., 2017). Drugs included gabazine (S106, Sigma-Aldrich, St. Louis, MO, USA) and (1,2,5,6-Tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA) (1040, Tocris, Minneapolis, MN, USA). Briefly, zebrafish were anesthetized in 0.016% tricaine and a small incision was made in the sclera using a sapphire blade. A blunt-end 30-gauge needle was inserted into the vitreous and 0.5 µL of drug (15, 20, 25, and 35 nmol) or morpholino (0.75 nmol) were injected into the vitreous, the amounts of which are indicated in the respective figure legends. Fish were then placed in recovery tanks for the times listed in

each experiment. For 5-ethynyl-2´-deoxyuridine (EdU) injections, 20 µL of a 10 mM solution of EdU was administered via intraperitoneal injection as described by Kinkel et al. (Kinkel et al., 2010).

Morpholino Electroporation

Morpholinos (0.75 nmol) with a 3'-lissamine tag (Gene Tools, Philomath, OR, USA) were injected into the vitreous with or without drugs. 3 hours after injection, injected eyes were electroporated (75 V/pulse, two pulses, 1-second intervals between pulses). Fish were allowed to recover for the times indicated. Morpholinos used in this study were *Gabrr2a* MO1 (5'-AGT AGT GGC GCA GAT ATA ATG TCA T-3'), *Gabrr2a* MO2 (5'-TCG GCC TCA TAG TGA AGT CAT GAT C-3'), *ascl1a* MO1 (5'-ATC TTG GCG GTG ATG TCC ATT TCG C-3')(Cau and Wilson, 2003), *ascl1a* MO2 (5'-AAG GAG TGA GTC AAA GCA CTA AAG T-3')(Cau and Wilson, 2003), and a standard control morpholino (5'-CCT CTT ACC TCA GTT ACA ATT TAT A-3').

Immunohistochemistry and TUNEL Labeling

Zebrafish were euthanized in 0.08% tricaine and treated eyes were removed and fixed in 4% paraformaldehyde overnight at 4°C. Eyes were then washed with 5% sucrose in PBS and then cryoprotected with 30% sucrose overnight at 4°C. Eyes were then transferred to a solution of 2 parts optimal cutting temperature compound (OCT) and 1 part 30% sucrose for 3 hours before moving to 100% OCT for 30 minutes. Eyes were then embedded in OCT for cryosectioning. Slides were rehydrated in PBS, then incubated in 10mM sodium citrate at 95°C. Sections were then blocked in 3% donkey serum, 0.1% Triton X-100 in PBS. Antibodies used were mouse anti-proliferating cell nuclear antigen (PCNA) (1:500; ab29, Abcam, Cambridge, MA, USA) and rabbit anti-green fluorescent protein (GFP) (1:500; TP401, Torrey Pines Biolabs, Secaucus, NJ, USA) diluted in antibody solution (1% donkey serum,

0.05% Tween-20 in PBS) overnight at 4°C. Slides were washed with PBS, then secondary antibodies were applied: anti-mouse cy3 (1:100) and anti-rabbit Alexa fluor 488 (AF488) (Jackson Immuno Research, West Grove, PA, USA) with TO-PRO-3 (1:1000, Thermo Fisher Scientific, Waltham, MA, USA) in antibody solution described above for 2 hours at room temperature. Slides were washed, dried, and coverslipped with Vectashield (Vector Laboratories, Inc, Burlingame, CA, USA). PCNA-positive cells were counted in the inner nuclear layer across the entirety of retinal sections. Two non-consecutive sections were counted and averaged for each eye. Images for immunofluorescence staining were taken using a META Zeiss LSM 510 Meta confocal microscope. Optical slice thickness is 0.44µm. Images shown are stacks unless otherwise noted. The number of slices per stack are indicated in the respective figure legends. Fiji ImageJ software 4.13 was used to process images (Schindelin et al., 2012).

TUNEL labeling was done using the *in situ* cell death detection kit, TMR Red (12156792910, Roche Life Science, Indianapolis, IN, USA) to detect apoptotic cells. EdU detection was done using the Click-iT EdU Alexa Fluor 555 Imaging Kit (C10338, Thermo Fisher Scientific) according to the manufacturer's instructions prior to immunohistochemistry. TUNEL-positive cells were counted across the entirety of retinal sections, in all layers of the retina. Each data point is from a single eye and is an average of counts from two-nonconsecutive sections, with each fish providing only one eye. The total number of fish used are indicated in the respective figure legends.

Fluorescent Activated Cell Sorting

Fluorescent activated cell sorting (FACS) was used to isolate GFP^+ cells from Tg(*gfap*:GFP)^{mi2001} zebrafish retinas, either undamaged or 24 hours after TPMPA injection, using BD FACSAria III (BD Biosciences, San Jose, CA, USA) in the VUMC Flow Cytometry Shared Resource. Retinas were dissociated according to Rajaram et al. (Rajaram et al., 2014b) with the following changes. Retinas were dissected

and collected in Leibovitz L-15 media (21083-027, Thermo Fisher Scientific). Retinas were then treated with 1mg/ml hyaluronidase (H3884, Sigma-Aldrich) rocking at room temperature for 15 minutes. Dead cells were detected via propidium iodide. A total of 24 retinas were collected and pooled from 12 adult fish per sorting.

Quantitative Reverse Transcription PCR

RNA was collected from sorted cells using TRIzol-LS (10296028, Thermo Fisher Scientific). Taqman small RNA assays (Thermo Fisher Scientific) were used to perform quantitative reverse transcription PCR (RT-qPCR) for *let-7a*. Briefly, 5 µL RNA was used per RT, which was then diluted 1:2. 1.33µL of the diluted cDNA was used in a 10 µL qPCR reaction in technical triplicates. qPCR reactions were done in 384-well plates using the Bio-Rad CFX384 Real-time System (Bio-Rad Laboratories). The reactions were normalized to a U6 snRNA control. qPCR of mRNAs was done by first treating RNA with DNase (TURBO DNA free kit, AM1907, Thermo Fisher Scientific) and converted to cDNA using AccuScript High-Fidelity 1st Strand cDNA Synthesis Kit (Cat # 200820; Agilent Technologies, Stratagene, La Jolla, CA, USA). qPCR was performed using SYBR Green (Bio-Rad Laboratories). All qPCR primers spanned exon-exon junctions (Integrated DNA Technologies, Inc, Coralville, IO, USA). The reactions were normalized to 18S rRNA levels. qPCR reactions were done in 384-well plates using the Bio-Rad CFX384 Real-time System. Analysis was done using the ΔΔCt method (Livak and Schmittgen, 2001). The following primers were used for qRT-PCR: *18S* Forward (TTACAGGGCCTCGAAAGAGA); *18S* Reverse (AAACGGCTACCACATCCAAG); *ascl1* Forward (TGAGCGTTCGTAAAAGGAAACT); *ascl1* Reverse (CGTGGTTTGCCGGTTTGTAT); *insm1a* Forward (CCGGGACTTACGAGACACAT); *insm1a* Reverse (GGCTGGGAAGCACTGGTTTA); *sox2* Forward (GAAAAACAGCCCGGACCGCATGAGACC); *sox2* Reverse (GTCTTGGTTTTCCTCCGGGGTCTGTATTTG); *dkk1b* Forward (AATGACCCTGACATGATTCAGC); *dkk1b* Reverse (AGGCTTGCAGATTTTGGACC).

In Situ Hydbridization

in situ hybridization of Gabrr2a and Gabrg2 was performed using RNAScope (Advanced Cell Diagnostics, Newark, CA, USA). Hybridizations were performed according to manufacturer's instructions for fixed frozen tissues on cryosections from Tg(*gfap*:GFP)^{mi2001} retinas, with the following changes. After creating a hydrophobic barrier around the tissue sections, RNAScope Protease III was applied for only 20 minutes rather than 30 minutes. After applying the HRP blocker solution, immunohistochemistry was performed according to ACDBio's instructions, using rabbit anti-GFP, and anti-rabbit AF488 and TOPRO-3 from above.

Statistical Analysis

Two tailed Student's *t*-tests were used when comparing two sample means, a one-way ANOVA was used to compare multiple means, and Fisher's LSD test was used for qRT-PCR analysis with ΔCt values. Each data point represents an average of two separate counts per eye. For each count, only the inner nuclear layer was counted. Eyes that were damaged were not used for analysis. Damage was indicated by disrupted morphology or high amounts of proliferation. For in-situ hybridization analysis, a Costes' image randomization and evaluation of Pearson's coefficient was performed using the JACoP plugin for ImageJ across all optical slices per z-stack, each comprising between 68 and 70 optical slices (Costes et al., 2004; Bolte and Cordelieres, 2006).

Results

Inhibition of $GABA_A$-ρ Receptors Induces Proliferation in the Undamaged Retina

Our lab has previously shown that inhibition of $GABA_A$ receptor signaling induces spontaneous proliferation in an undamaged zebrafish retina (Rao et al., 2017). However, the ρ2a subunit of the

$GABA_A$-ρ receptor is also expressed in the whole retina (Cocco et al., 2017) and in purified MG by RNAseq (**Table 1**). Thus, we tested whether pharmacological inhibition of $GABA_A$-ρ receptors would also induce proliferation. For this, we injected the $GABA_A$-ρ receptor antagonist TPMPA (Murata et al., 1996; Ragozzino et al., 1996) into undamaged eyes from Tg*(1016tuba1a:gfp)* transgenic fish and assessed proliferation using immunostaining against PCNA or by direct incorporation of EdU. The Tg*(1016tuba1a:gfp)* transgenic line specifically marks dedifferentiated MG and MG-derived neural

Gene name	Subunit name	Average RPKM values
Gabrr1	$GABA_A$-ρ subunit ρ1	9.665615
Gabrr2a	$GABA_A$-ρ subunit ρ2a	30.42483939
Gabrr2b	$GABA_A$-ρ subunit ρ2b	0.231149438
Gabrr3a	$GABA_A$-ρ subunit ρ3a	10.65283172
Gabrr3b	$GABA_A$-ρ subunit ρ3b	15.82386383
Gabra1	$GABA_A$ subunit α1	39.81043
Gabrb2	$GABA_A$ subunit β2	16.69505
Gabrg2	$GABA_A$ subunit γ2	36.93309

Table 1 Average RPKM values of GABA subunits from sequencing of sorted Müller glia.

Retinas were collected from undamaged Tg(*gfap:gfp*) adult fish. Tg(*gfap:gfp*) transgenic zebrafish express GFP in Müller glia driven by the glial fibrillary acidic protein promoter (Bernardos and Raymond, 2006). Fluorescence activated cell sorting was used to enrich for populations of Müller glia from undamaged Müller glia. RNA was isolated from the cell pools and RNA-seq was performed. Read data from undamaged retinas are shown as reads per kilobase of transcript per million mapped reads (RPKM). GABA: Gamma aminobutyric acid.

progenitors in actively regenerating retinas (Fausett and Goldman, 2006a). TPMPA is a commercially available competitive antagonist of the $GABA_A$-ρ receptor with only minimal effects on $GABA_A$ or $GABA_B$ receptors (Ragozzino et al., 1996). Intravitreal injection of TPMPA induced a significant increase in $PCNA^+$ cells compared to control PBS injections (**Figure 4**). Similar increases were detected using incorporation of EdU (**Figure 5**). Induction of DNA replication was dose dependent up to 25 nmol, but we noticed a decrease in the number of PCNA positive cells at higher concentrations (**Figure 6**). This is consistent with

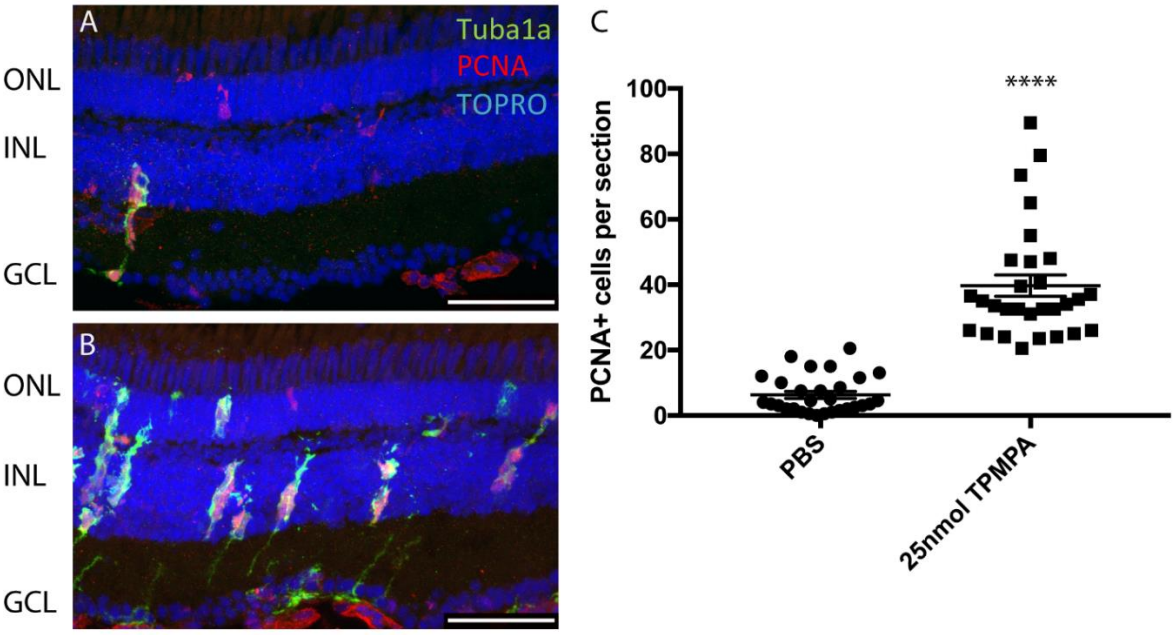

Figure 4. Inhibition of the $GABA_A$-ρ receptor in the absence of retina damage results in a MG-derived proliferative response.

Tg(1016tuba1a:gfp) zebrafish were intravitreally injected with either 1× PBS (A) or 25 nmol of the $GABA_A$-ρ receptor inhibitor TPMPA (B), and then allowed to recover for 48 hours before sectioning. Immunostaining was performed with antibodies against PCNA or GFP to monitor DNA replication or dedifferentiation of Müller glia, respectively. Green–tuba1a:GFP; red–PCNA; blue–TO-PRO-3 (A,B). Scale bar: 50 µm. Number of optical slices: 53 (A) and 55 (B). GCL: ganglion cell layer; INL: inner nuclear layer;

ONL: outer nuclear layer. (C) Quantification of $PCNA^+$ cells. Each data point is from a separate eye and is an average of two sections, counting all $PCNA^+$ cells in the inner nuclear layer. Two-tailed Student's t-tests were used to test for significance. Error bars indicate the mean ± SEM. N = 29. ****$p = 1.12 \times 10^{-13}$, vs. Ctl-MO. GABA: Gamma aminobutyric acid.

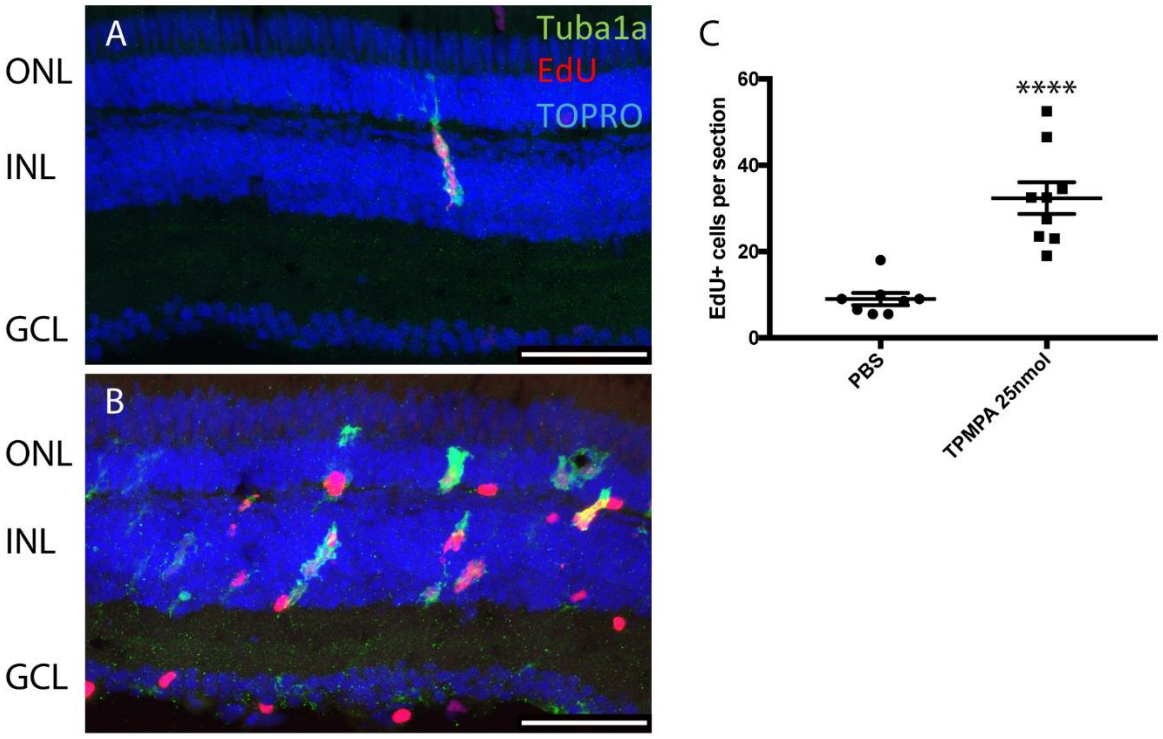

Figure 5 Inhibition of the $GABA_A$-ρ receptor in the absence of damage results in a proliferative response.

Tg*(1016tuba1a:gfp)* zebrafish were intravitreally injected with either PBS (**A**) or 25 nmol of the $GABA_A$-ρ receptor inhibitor TPMPA (**B**). After 3 hours, fish were given an intraperitoneal injection of 20 µL of 10 mM EdU with a second intraperitoneal injection of EdU after 24 hours. Eyes were collected 48 hours after the TPMPA injection and sectioned, followed by immunostaining with antibodies against GFP to monitor dedifferentiation of MG and detection of EdU incorporation using the Click-iT EdU labeling kit to monitor DNA replication. Scale bar: 50 µm. Green–*tuba1a*:GFP; red–EdU; blue–TO-PRO-3 (**A,B**). ONL: outer nuclear layer. INL: inner nuclear layer. GCL: ganglion cell layer. Number of optical slices: 47 (**A**) and

45 (**B**). **(C)** Quantification of EdU^{+} cells. Each data point is a separate eye and is an average of two sections, counting all PCNA^{+} cells in the inner nuclear layer. Two-tailed Student's *t*-tests were used to test for significance. Error bars are the mean ± SEM. n = 9. ****$p = 4.7 \times 10^{-5}$ vs. PBS.

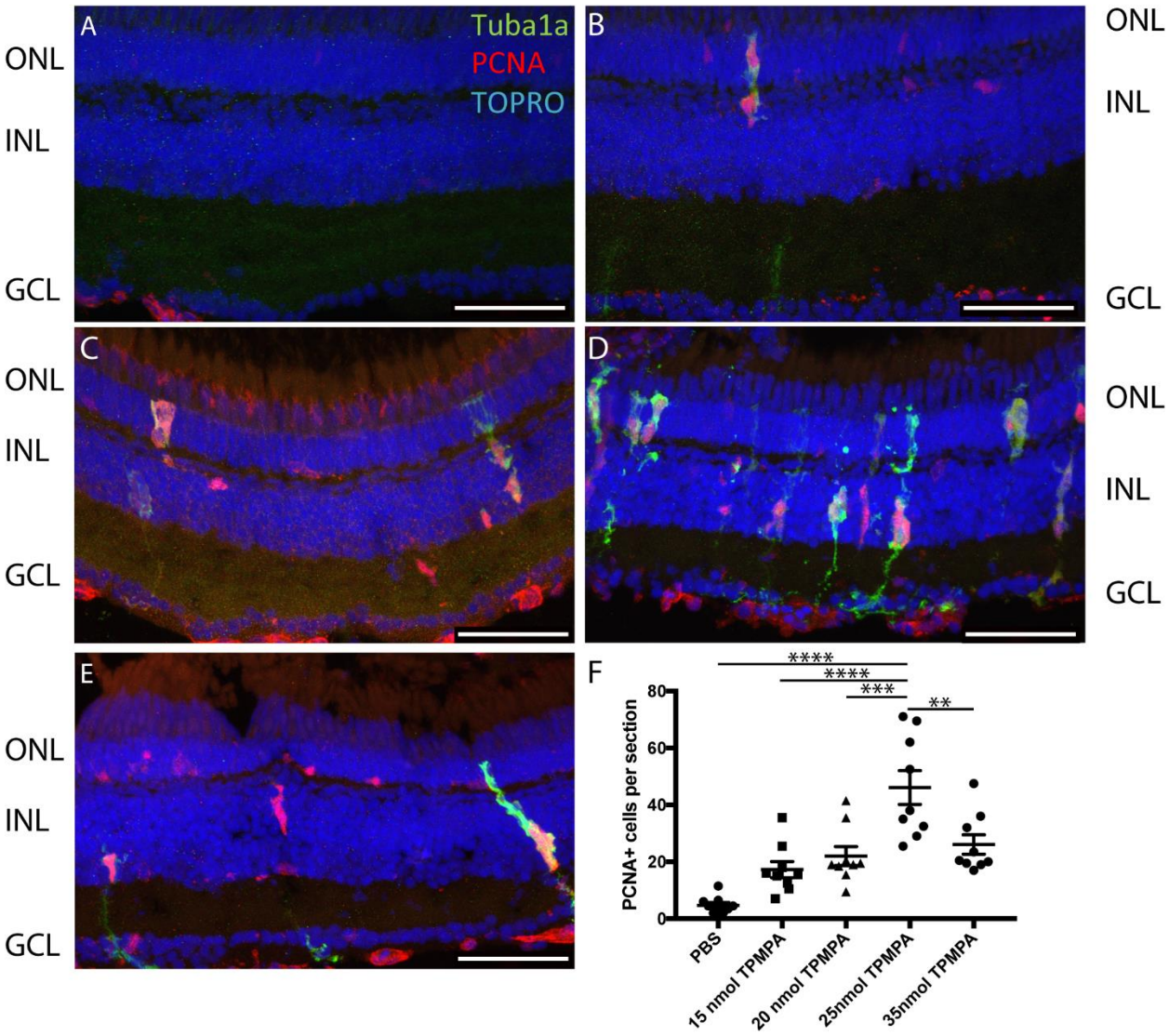

Figure 6 Dose dependent inhibition of the GABA$_A$-ρ receptor.

Tg*(1016tuba1a:gfp)* zebrafish were intravitreally injected with either 1× PBS (**A**) or 15 nmol of the GABA$_A$-ρ receptor inhibitor TPMPA (**B**)**,** 20 nmol TPMPA (**C**), 25 nmol TPMPA (**D**), or 35 nmol TPMPA (**E**). Fish were allowed to recover for 48 hours before sectioning and immunostaining with antibodies against

PCNA or GFP to monitor DNA replication or dedifferentiation of Müller glia, respectively. ONL: outer nuclear layer. INL: inner nuclear layer. GCL: ganglion cell layer. Scale bar: 50 µm. Green–*tuba1a*:GFP; red–PCNA; blue–TO-PRO-3 (**A-E**). Number of optical slices: 49 (**A**), 45 (**B**), 49 (**C**), 44 (**D**), and 53 (**E**). **(F)** Quantification of $PCNA^+$ cells. Each data point is from a separate eye and is an average of two sections, counting all $PCNA^+$ cells in the inner nuclear layer. One-way analysis of variance with Tukey's multiple comparison tests were used to test for significance. Error bars are the mean ± SEM. n = 9. ****$p = 8.4 \times 10^{-9}$ (PBS vs. 25 nmol TPMPA); ****$p = 1.8 \times 10^{-5}$ (15 nmol TPMPA vs. 25 nmol TPMPA); ***$p = 0.0003$ (20 nmol TPMPA vs. 25 nmol TPMPA); ****$p = 0.0025$ (35 nmol TPMPA vs. 25 nmol TPMPA).

a specific effect of TPMPA, especially because it has been reported that TPMPA can act as an agonist at higher concentrations (Ragozzino et al., 1996). Importantly, all *tuba1a*-GFP^+ cells co-localized with $PCNA^+$ cells indicating that the proliferative cells were derived from MG (**Figure 4**). To ensure that the proliferation we observed was not an indirect consequence of cell death due to application of TPMPA, we utilized TUNEL staining which showed no difference in the number of apoptotic cells between the PBS-injected eyes and the TPMPA-injected eyes (**Figure 7**). Combined with earlier work, the data indicate that impaired GABA signaling can activate MG and induce a regenerative response in undamaged zebrafish retinas.

While TPMPA is >100-fold more potent against $GABA_A$-ρ receptors compared to $GABA_A$ receptors (Murata et al., 1996), it was possible that under the conditions of intravitreal injection, the effect of TPMPA could have been due to unexpected inhibition of $GABA_A$ receptors. To complement the TPMPA experiments, we tested whether knocking down the ρ2a subunit (GABRR2a), the most abundant

ρ subunit in the undamaged retina (**Table 1**), would also be sufficient to induce a proliferative response. For this, we independently electroporated two different antisense morpholinos targeting the ρ2a subunit into undamaged retinas (**Figure 8**). Compared to control morpholino injections, injection of the

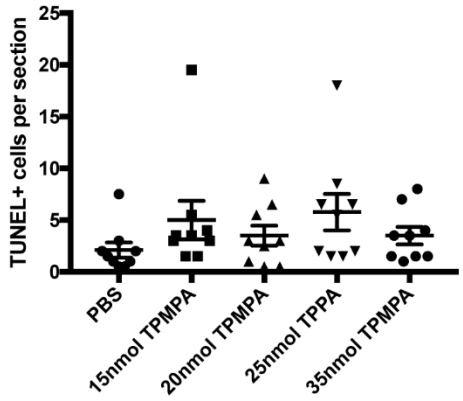

Figure 7 Inhibition of the GABA$_A$-ρ receptor does not result in increased apoptosis.

Tg*(1016tuba1a:gfp)* zebrafish were intravitreally injected with the indicated amounts of either PBS or the GABA$_A$-ρ receptor inhibitor TPMPA. After injection, fish were allowed to recover for 48 hours followed by analysis of apoptotic cells using TUNEL staining. Each data point is a separate eye and is an average of two sections, counting all TUNEL$^+$ cells in the retina section. One-way analysis of variance with Tukey's multiple comparison tests were used to test for significance. Error bars are shown as the mean ± SEM. n = 10 for PBS, n = 9 for 15 nmol TPMPA, n = 9 for 20 nmol TPMPA, and n = 8 for 25 nmol TPMPA.

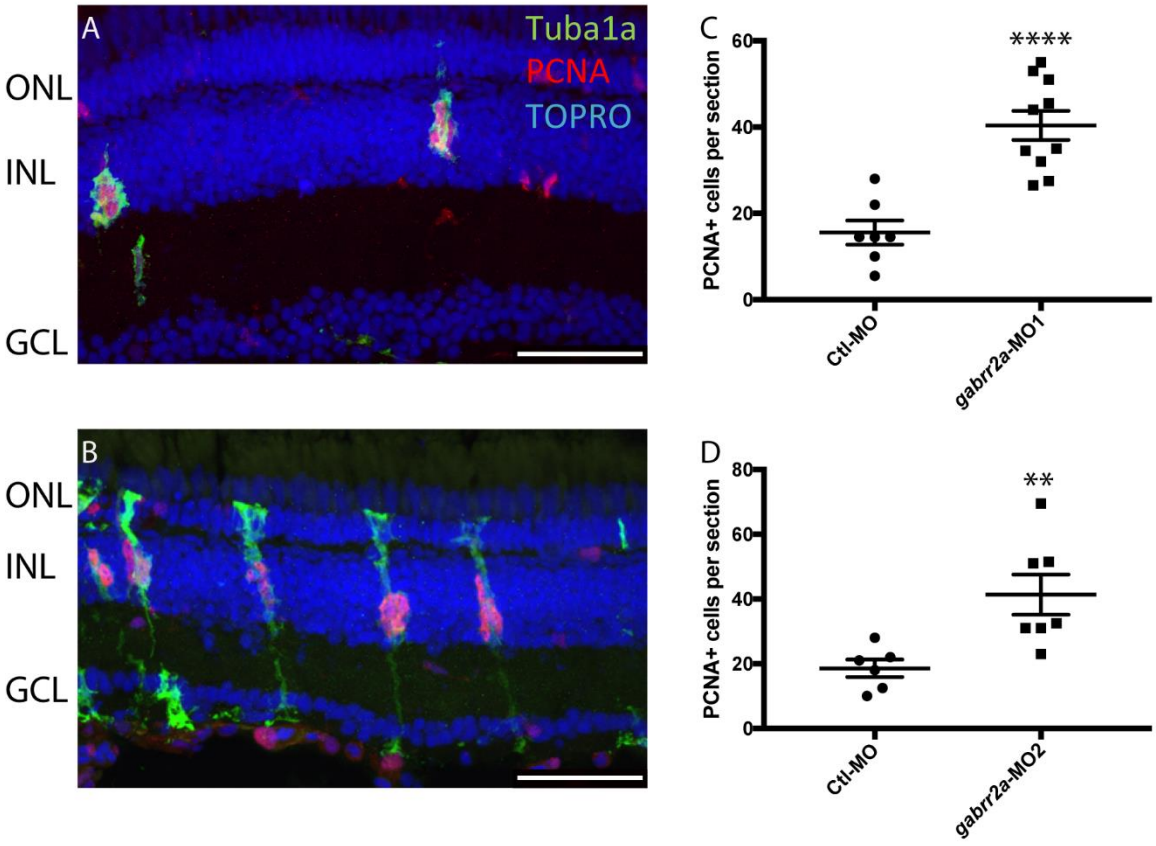

Figure 8 Inhibition of $GABA_A$-ρ receptors via antisense morpholino injections in the absence of damage results in a proliferative response.

Tg*(1016tuba1a:gfp)* zebrafish were intravitreally injected with 0.75 nmol of either a control morpholino (Ctl-MO; **A**) or one of two independent antisense morpholinos targeting the gabrr2A subunit of the $GABA_A$-ρ receptor (*gabrr2a*-MO1 (**B, C**) and *gabrr2a*-MO2 (**D**)) and then allowed to recover for 3 hours. Injected eyes were then electroporated and the fish were allowed to recover for 72 hours before sectioning. Immunostaining was performed with antibodies against PCNA or GFP to monitor DNA replication or dedifferentiation of MG, respectively. Scale bars: 50 µm in **A** and **B.** Green–*tuba1a*:GFP; red–PCNA; blue–TO-PRO-3 (**A,B**). Quantification of $PCNA^+$ cells compared to control morpholinos with either *gabrr2a*-MO1 (**C**) or *gabrr2a*-MO2 (**D**). n = 7 for Ctl-MO and n = 10 for Gabrr2a-MO (**C**). n = 6 for Ctl-MO and n = 7 for Gabrr2a-MO2 (**D**). Each data point is from a separate eye and is an average of two

sections, counting all PCNA$^+$ cells in the inner nuclear layer. Two-tailed Student's *t*-tests were used to test for significance. Error bars indicate the mean ± SEM. ****p =8.7 × 10^{-5} (**C**), **p =0.0089 (**D**), vs. Ctl-MO. ONL: outer nuclear layer. INL: inner nuclear layer. GCL: ganglion cell layer. Number of optical slices: 52 (**A**) and 46 (**B**).

two different morpholinos targeting the ρ2a subunit induced significantly higher proliferation (**Figure 8C and D**). Again, all *tuba1a*-GFP$^+$ cells co-localized with PCNA indicating that antisense inhibition of the $GABA_A$-ρ receptor induces a MG-based regenerative response.

Inhibition of $GABA_A$-ρ Signaling Induces a Regenerative Response

To further test whether inhibition of $GABA_A$-ρ receptors induces a bona fide regenerative response, we combined TPMPA injections with antisense inhibition of *ascl1a* (**Figure 9**). Ascl1a is a transcription factor that is required for MG-derived retina regeneration (Fausett et al., 2008a; Ramachandran et al., 2010a; Brzezinski et al., 2011; Ramachandran et al., 2011; Pollak et al., 2013b; Ueki et al., 2015; Wohl and Reh, 2016). The prediction is that if TPMPA is inducing a bona fide regenerative response, loss of Ascl1a should reduce the proliferation observed after injection of TPMPA. As shown in **Figure 9**, after knockdown of *ascl1a*, we observed a significant decrease in the amount of proliferation compared to co-injection of TPMPA and a control morpholino (**Figure 9E and F**).

Lastly, to confirm that TPMPA-induced proliferation is indeed activating the canonical retina regeneration pathway, we used qRT-PCR on purified MG to determine if specific regeneration-associated genes are differentially expressed after treatment with TPMPA. Previous work has shown

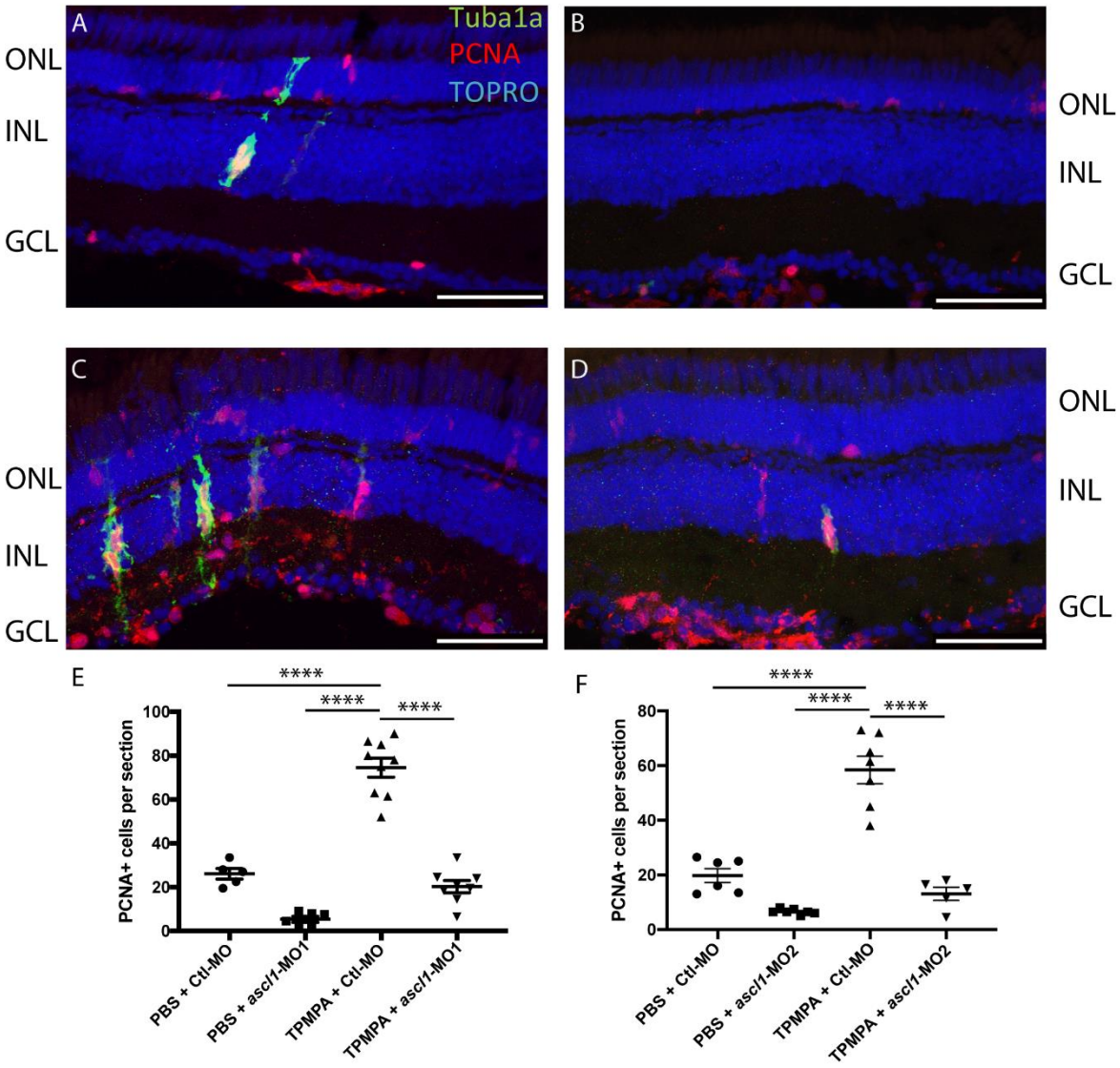

Figure 9 Inhibition of ascl1a blocks TPMPA induced-proliferation.

Tg*(1016tuba1a:gfp)* zebrafish were intravitreally injected with either 1× PBS, 25 nmol of the $GABA_A$-ρ receptor inhibitor TPMPA, 0.75 nmol of control morpholino (Ctl-MO), 0.75 nmol *ascl1a*-MO targeting *ascl1a*, or combinations thereof. Fish were allowed to recover for 3 hours prior to electroporation, and an additional 45 hours before sectioning. (**A)** PBS/Ctl-MO co-injection. (**B)** PBS/Ascl1-MO1 co-injection. (C) 25 nmol TPMPA/Ctl-MO co-injection. (**D)** 25 nmol TPMPA/*ascl1*-MO1 co-injection. Immunostaining was performed with antibodies against PCNA or GFP to monitor DNA replication or dedifferentiation of

MG, respectively. Scale bars: 50 µm. Green–*tuba1a*:GFP; red–PCNA; blue–TO-PRO-3 (**A-D**). ONL: outer nuclear layer. INL: inner nuclear layer. GCL: ganglion cell layer. Number of optical slices: 41 (**A**), 46 (**B**), 46 (**C**), and 47 (**D**). (**E, F)** Quantification of PCNA+ cells, with either *ascl1*-MO1 (**E**), or *ascl1*-MO2 (**F**). n = 5 for PBS/Ctl-MO, n = 6 for PBS/Ascl1-MO1, n = 9 for TPMPA/Ctl-MO, and n = 8 for TPMPA/Ascl1-MO1 (**E**). n = 7 for PBS/Ctl-MO, n = 10 for PBS/Ascl1-MO2, n = 8 for TPMPA/Ctl-MO, and n = 8 for TPMPA/Ascl1-MO2 (**F**). Each data point is from a separate eye and is an average of two sections, counting all $PCNA^{+}$ cells in the inner layer. One-way analysis of variance with Tukey's multiple comparison tests were used to test for significance. Error bars are the mean ± SEM. (E) ****$p = 5.8 \times 10^{-9}$ (PBS/Ctl-MO vs TPMPA/Ctl-MO); ****$p = 1.2 \times 10^{-12}$ (PBS/*ascl1*-MO1 vs. TPMPA/Ctl-MO); ****$p = 3.7 \times 10^{-11}$ (TPMPA/Ctl-MO vs TPMPA/*ascl1*-MO1). (**F)** ****$p = 1.5 \times 10^{-7}$ (PBS/Ctl-MO vs. TPMPA/Ctl-MO); ****$p = 4 \times 10^{-10}$ (PBS/*ascl1*-MO2 vs. TPMPA/Ctl-MO); ****$p = 2.45 \times 10^{-8}$ (TPMPA/Ctl-MO vs. TPMPA/*ascl1*-MO2).

that retina regeneration in zebrafish results in an increase in the expression of *ascl1a*, *insm1a*, and *sox2*, and decreased expression of *dkk1b* and *let-7a*. Like Ascl1a, Insm1a and Sox2 are transcription factors that are required for retina regeneration (Ramachandran et al., 2012; Gorsuch et al., 2017). Downregulation of Dkk1b, a negative regulator of Wnt signaling, is also required for regeneration (Ramachandran et al., 2011) as is downregulation of *Let-7a*, a miRNA that represses several mRNAs encoding factors required for retina regeneration, including Ascl1a (Ramachandran et al., 2010a). For these experiments, we used the Tg*(GFAP:GFP)*^{mi2001} zebrafish line in which MG expression of GFP is controlled by the GFAP (glial fibrillary acid protein) promoter (Bernardos and Raymond, 2006; Nagashima et al., 2013). After damage, expression of the GFP reporter and/or GFAP is increased in the zebrafish retina (Vihtelic et al., 2006; Bernardos et al., 2007b; Lenkowski et al., 2013; Lenkowski and

Raymond, 2014; Sifuentes et al., 2016). Thus, retinas from either undamaged fish or fish injected with TPMPA were dissociated and sorted to enrich for GFP^+ cells (**Figure 10**). RNA was isolated and qRT-PCR was performed to determine fold changes in expression between undamaged and TPMPA injected GFP^+ cells. We observed significantly increased levels of expression of *ascl1a* and *insm1a* and slightly upregulated expression of *sox2* (**Figure 10**). We also detected significantly decreased expression levels of *dkk1b* and *let-7a*. These results support the hypothesis that inhibition of $GABA_A$-ρ receptors by TPMPA induces expression of factors consistent with a canonical regenerative response in zebrafish.

$GABA_A$-ρ Receptors are Localized to the Inner and Outer Nuclear Layers

If detection of reduced GABA levels is mediated by MG, GABA receptors should be expressed in MG. By RNAseq of undamaged retinas using the Tg*(GFAP:GFP)*^{mi2001} transgenic line, we found that $GABA_A$ and $GABA_A$-ρ receptor subunits are indeed expressed in MG (**Table 1**). Besides transcriptomic analysis, we also tested whether $GABA_A$-ρ receptors co-localize with MG. We previously used immunostaining to show close association between $GABA_A$ receptors on MG processes flanking horizontal cell processes (Rao et al. 2017). Because antibodies against the zebrafish $GABA_A$-ρ receptors are not available, we used *in situ* hybridization to localize RNAs encoding both the ρ2a and γ2 subunits of the $GABA_A$-ρ and $GABA_A$ receptors, respectively. RNA transcripts encoding ρ2a subunits (gabrr2a) were detected in the outer plexiform layer, the inner nuclear layer, and cell bodies of the outer nuclear layer (**Figure 11**). $GABA_A$-ρ receptors are known to be expressed in both horizontal and bipolar cells, (Qian and Dowling, 1993; Fletcher et al., 1998; Lopez-Chavez et al., 2005), but we also detected ρ2a transcripts associated with MG processes (**Figure 11**). To better determine whether ρ2a subunits co-localize with MG processes, we used the Image J plug-in JACoP (Bolte and Cordelieres, 2006) to evaluate the extent of co-localization and also applied Costes' image randomization and evaluation of

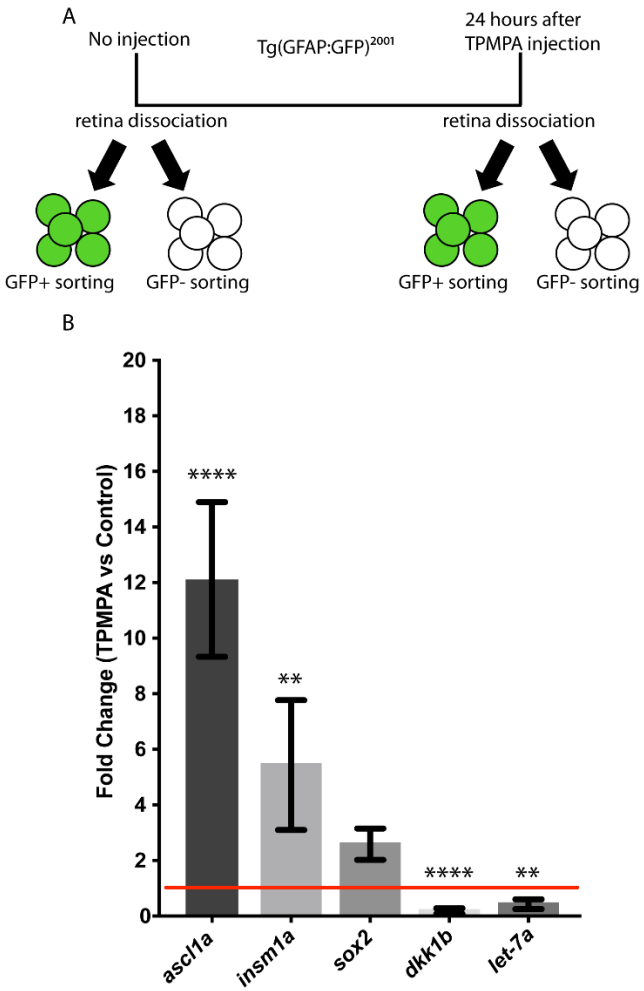

Figure 10 Inhibition of the GABAA-ρ receptor induces gene expression changes consistent with canonical retina regeneration.

(**A)** Retinas from Tg*(GFAP:GFP)*^{mi2001} zebrafish expressing GFP in Müller glia were dissected, dissociated, and fluorescence sorted to obtain GFP$^-$ and GFP$^+$ cells without injection of the $GABA_A$-ρ receptor inhibitor TPMPA or 24 hours after 25 nmol TPMPA injection. (**B)** qRT-PCR was performed on RNA from GFP$^+$ and GFP$^-$ pools and fold changes in expression were determined for the indicated mRNAs. Fold changes are displayed as $2^{-\Delta\Delta Ct}$. The red line indicates no change in expression. Fisher's least significant difference tests were used to analyze fold change expression of the indicated RNAs in TPMPA-injected retinas compared to the uninjected retinas. Error bars are shown as the mean ± SEM. n = 3 biological replicates, each with 3 technical triplicates. ****$p = 3.3 \times 10^{-6}$ (Ascl1a); **$p = 0.0037$ (Insm1a); ****$p = 5.4 \times 10^{-6}$ (dkk1b); **$p = 0.0056$ (let-7a).

Pearson's coefficient on three sets of optical slices (single slice shown in **Figure 11B**) from one of which the Z stack in **Figure 11** was generated. This analysis resulted in an average Pearson's coefficient of 0.133667. For these analyses, Pearson's coefficients can range from 1 (perfect correlation) to -1 (no correlation). Thus, there is a positive correlation for co-localization between RNA transcripts encoding ρ2a subunits with MG processes. The correlation is weak, but the resulting *P*-value is 1.0, meaning high confidence (> 95%) that the colocalization is not due to random chance.

Additionally, we used *in situ* hybridization to localize the γ2 subunit (gabrg2), which showed that RNA transcripts encoding the γ2 subunit are broadly expressed across the retina (**Figure 12**). Closer examination of the merged image revealed that puncta corresponding to γ2 subunits are detectable in retinal layers containing both MG cell bodies and processes, consistent with previous immunostaining. Combined, immunostaining, RNA localization, and RNAseq support the hypothesis that MG processes are in position to sense GABA levels in the retina.

Synergistic Activation of Regeneration by Simultaneous Inhibition of $GABA_A$-ρ and $GABA_A$ Receptors

Individually, inhibition of $GABA_A$-ρ (**Figures 4 and 8**) or $GABA_A$ receptors (Rao et al., 2017) is sufficient to induce proliferation as part of a canonical retina regenerative response. However, since both receptors are associated with MG processes, overall detection of GABA levels by MG could be mediated by both receptors. If true, combined inhibition of both receptors should synergize to activate MG during regeneration. To test this, we co-injected gabazine, a $GABA_A$ receptor antagonist, and TPMPA. We observed a significant increase in the number of proliferating PCNA+ cells compared to either treatment alone or the PBS control treatment (**Figure 13**).

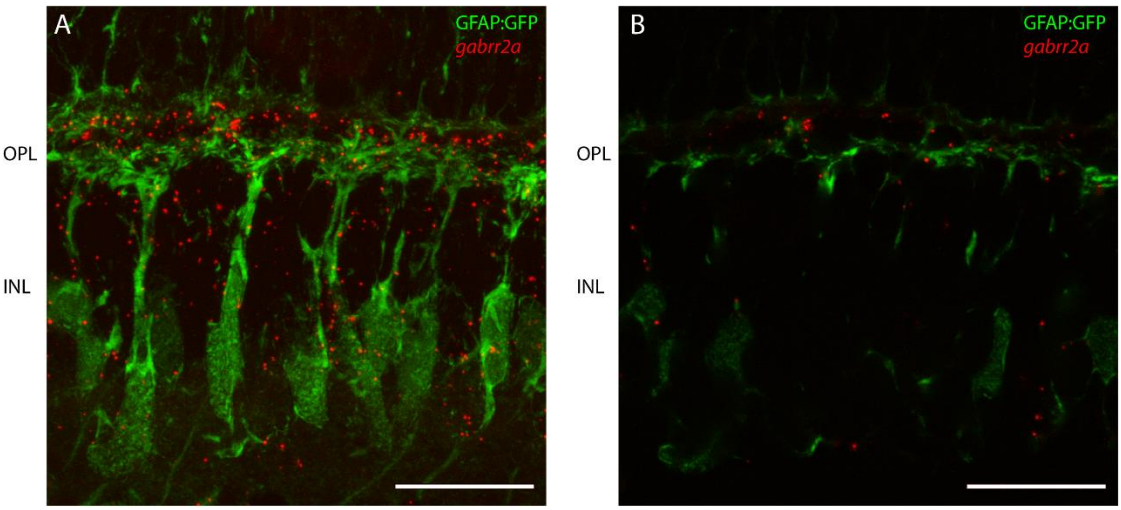

Figure 11 Localization of GABAA and GABAA-ρ transcripts via in situ hybridization.

Retinas from Tg(*GFAP*:GFP)^{mi2001} zebrafish expressing GFP in Müller glia were immunostained with antibodies against GFP to mark Müller glia. *In situ* hybridization was performed on the same sections using probes against the *gabrr2a* subunit of the $GABA_A$-ρ receptor (*gabrr2a*). (**A)** Z-stack formed from 69 optical slices. Optical slice thickness is 0.439 µm. (**B)** Representative optical slice from the z-stack in **A**. Scale bars: 20 µm. INL: Inner nuclear layer; OPL: outer plexiform layer. Green–*GFAP*:GFP; red–*gabrr2a* (**A,B**).

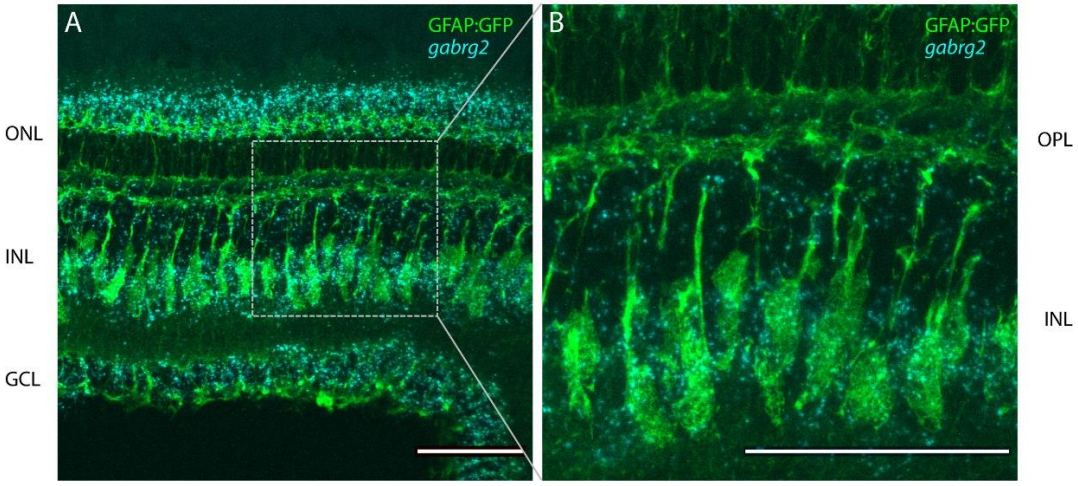

Figure 12 Localization of transcripts encoding the γ2 subunit of GABAA receptors.

Representative image of a retina from the Tg*(GFAP:GFP)*^{mi2001} transgenic line immunostained with GFP antibody to mark Müller glia (green) and probed for GABA receptor γ2 subunit (*gabrg2*) (cyan) expression by *in situ* hybridization. (**A)** High magnification image taken with a 40× objective. Outlined area is magnified and shown in **B**. Scale bars: 50 µm. GCL: Ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer; OPL: outer plexiform layer.

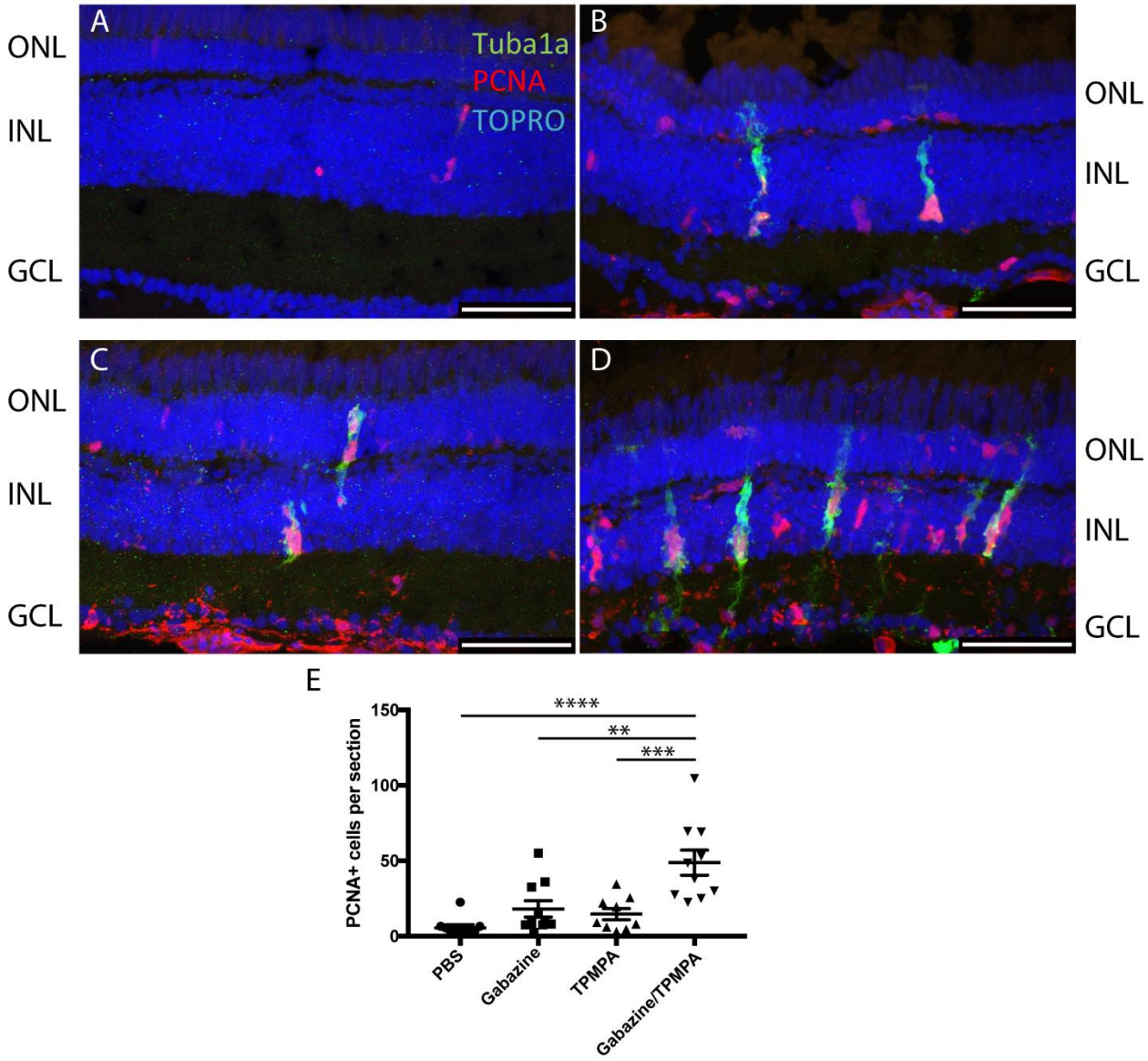

Figure 13 Dual inhibition of GABAA and GABAA-ρ receptors enhances proliferation.

Tg*(1016tuba1a:gfp)* zebrafish were intravitreally injected with either 1× PBS (**A**), 6.25 nmol of the $GABA_A$ antagonist gabazine (**B**), 12.5 nmol of the $GABA_A$-ρ receptor inhibitor TPMPA (**C**), or both (**D**). Fish were allowed to recover for 48 hours before sectioning and immunostaining with antibodies against PCNA or GFP to monitor DNA replication or dedifferentiation of Müller glia, respectively. GCL: Ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer. Scale bars: 50 µm. Green–*tuba1a*:GFP; red–PCNA; blue–TO-PRO-3 (**A-D**). Number of optical slices: 37 (**A**), 49 (**B**), 48 (**C**), and 61 (**D**). (**E**)

Quantification of $PCNA^+$ cells. Each data point is a separate eye and is an average of two sections, counting all $PCNA^+$ cells in the inner nuclear layer. One-way analysis of variance with Tukey's multiple comparison tests were used to test for significance. Error bars are the mean ± SEM. n = 10 PBS, n = 10 Gabazine, n = 9 TPMPA, and n = 10 TPMPA/Gabazine. ****$p = 1.2 \times 10^{-5}$ (PBS vs. Gabazine/TPMPA), **p = 0.0016 (Gabazine vs. Gabazine/TPMPA); ***p = 0.0006 (TPMPA vs. Gabazine/TPMPA).

Discussion

We previously showed that inhibition of $GABA_A$ receptors can induce retina regeneration in adult undamaged retinas (Rao et al., 2017). Here, we extend that work to show that inhibition of $GABA_A$-ρ receptors can induce a similar regenerative response. Several lines of evidence support that the induction of proliferation that we observe is mediated by MG in a canonical regenerative pathway. First, the transcription factor Ascl1 must be activated during retina regeneration in both fish and mice (Fausett et al., 2008a; Brzezinski et al., 2011; Jorstad et al., 2017) and knockdown of *ascl1* blocks the effects of inhibition of $GABA_A$-ρ receptors. Second, we observed the expected activation of *ascl1* and *insm1a* (Ramachandran et al., 2012) following inhibition of $GABA_A$-ρ receptors, and we also observed reduced levels of *dkk1b* and *let-7a* (Ramachandran et al., 2010a; Ramachandran et al., 2011). We did not observe significant activation of *sox2,* but this seems to be more related to timing. Sox2 is normally activated during regeneration, reaching its peak by 31 hrs of light damage (Gorsuch et al., 2017) whereas we examined *sox2* levels only 24 hr after inhibition of $GABA_A$-ρ receptors. Given the significant differential expression of the other factors, it is likely that *sox2* would show higher expression if tested at a later timepoint. Altogether, immunostaining, (Rao et al., 2017) *in situ* hybridization, and RNAseq support the idea that MG are positioned to respond to reduced levels of GABA and induce activation of

MG (**Figure 14**). Normally, this cascade would be induced after retinal injury, but our experiments show that simply mimicking the loss of GABA in an undamaged retina can induce regeneration.

Direct vs Indirect Effects of GABA Inhibition

Because multiple cell types express GABA receptors in the retina, it remains possible that the effects we observe are not directly due to sensing of decreased GABA levels by MG. This could explain the increase in PCNA+ cells in the ganglion cell layer, as GABA receptors have been found in retinal ganglion cells (Popova, 2015). Inhibition of $GABA_A$ and $GABA_A$-ρ receptors on bipolar cells (Connaughton et al., 2008) could result in excess glutamate leading to excitotoxic damage (Olney, 1982) and MG-derived proliferation. Excess glutamate could also activate AMPA receptors on MG, leading to an influx of Ca^{2+} (Zhang et al., 2019) and subsequent proliferation (Pinto et al., 2015). The Ca2+ activated protein CAPN5 is upregulated in MG after damage (Coomer and Morris, 2018); excess glutamate activating AMPA receptors on MG could be responsible for upregulation of CAPN5 and other Ca2+ activated proteins during regeneration. Lastly, it is also possible that damage induced by injection could be causing cell death leading to induction of a regenerative response, as opposed to regeneration as a consequence of inhibition of GABA signaling. We did not observe increased levels of apoptosis as measured by TUNEL assays, but it remains formally possible that damage leading to necrosis or

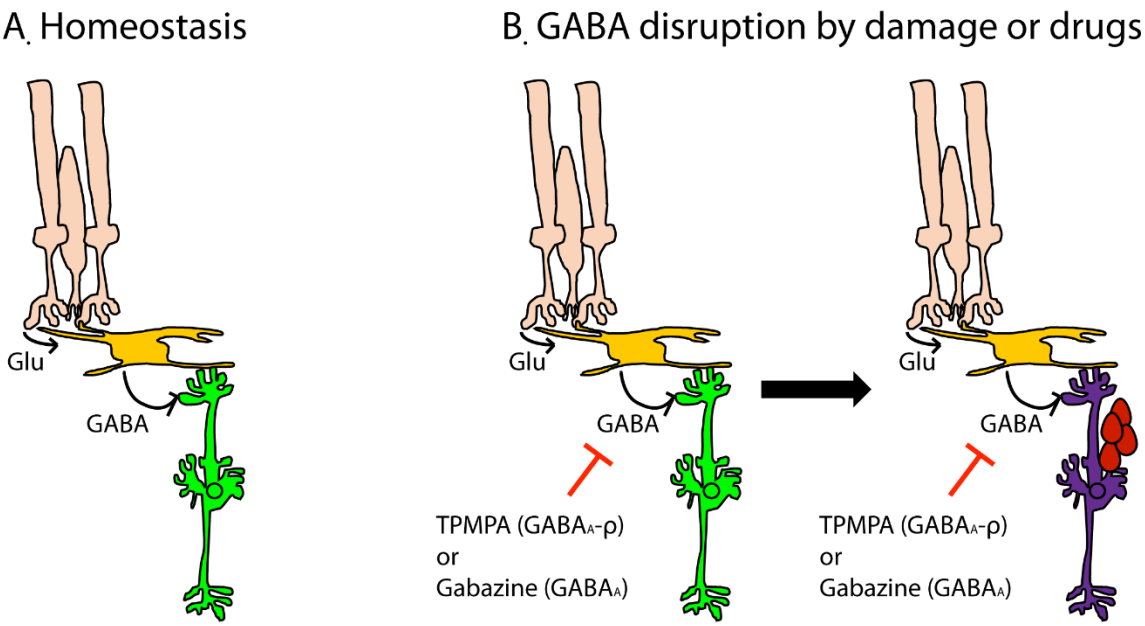

Figure 14 Model of GABA receptor inhibition-induced proliferation.

(A) Model of neuronal circuit maintaining Müller glia quiescence. Photoreceptors (taupe) normally release glutamate which is sensed by Horizontal cells (yellow) which release GABA that is then sensed by Müller glia (green) to remain quiescent. **(B)** Inhibiting $GABA_A$-ρ receptors via the $GABA_A$-ρ receptor inhibitor TPMPA and/or $GABA_A$ receptors via gabazine mimics disruption of the normal circuit as would occur after damage or disease. In this model, Müller glia sense decreased levels of GABA, dedifferentiate (purple) and generate proliferating progenitor cells (red) as part of a regenerative response. Pharmacological blocking of GABA receptors induces a regenerative response in the absence of damage. GABA: Gamma aminobutyric acid; gabazine: $GABA_A$ antagonist.

autophagy could also result in initiation of regeneration. Definitive testing of the model that GABA levels are directly sensed by MG awaits generation of transgenic zebrafish lines with inducible, MG-specific knockouts of these receptors.

There also remains the possibility that the effects of TPMPA are not entirely due to inhibition of $GABA_A$-ρ receptors. While TPMPA is a highly selective antagonist of $GABA_A$-ρ receptors, it is also a weak antagonist of $GABA_A$ receptors and an even weaker agonist of $GABA_B$ receptors (Ragozzino et al., 1996). It is therefore formally possible that the effects of TPMPA we observe could be mostly due to inhibition of $GABA_A$ receptors. However, the morpholino knockdown of $GABA_A$-ρ subunit ρ2a argues against this possibility and provides further support that the effect of TPMPA is through inhibition of $GABA_A$-ρ receptors. In addition, the dual inhibition of $GABA_A$ and $GABA_A$-ρ receptors provide further support that the effects are synergistic and not solely due to effects on $GABA_A$ receptors. This synergistic effect is likely due to more GABA receptors being inhibited. As shown, both here and in our previous work (Rao et al., 2017), higher doses of the inhibitors increase the resulting number of PCNA+ cells.

GABA Receptors and Adult Neurogenesis

Neural stem cell activity in the mouse hippocampus has been proposed to be regulated by sensing of non-synaptic GABA levels (Chell and Frisen, 2012; Song et al., 2012; Catavero et al., 2018). Our proposed activation of MG (**Figure 13**) is very similar to that proposed by Song et al. (Song et al., 2012) although we do not have evidence of long range GABAergic inputs (Bao et al., 2017) which would not seem to be necessary in the retina. Interestingly, $GABA_B$ receptors have been proposed to play a role in adult neurogenesis in the mouse hippocampus (Giachino et al., 2014). We have no evidence thus far for an involvement in G-protein coupled $GABA_B$ receptors in regulating activation of MG in the zebrafish retina. Supporting our work in the retina, inactivation of $GABA_A$ receptors was shown to inhibit proliferation of cultured progenitor cells from adult mouse retina (Wang et al., 2019). Thus, despite some differences, there appears to be a an evolutionarily conserved mechanism involving stem cell activity and the sensing of GABA levels. A major question, then, is how loss of GABA signaling mechanistically induces regeneration in the zebrafish retina. $GABA_A$ and $GABA_A$-ρ are both ionotropic

receptors that selectively transport Cl^- ions either into or out of the cell depending on membrane potential. Recently, reduced levels of intracellular Cl^- were found to induce tumor necrosis factor α in endothelial cells (Yang et al., 2012). Tumor necrosis factor α has been shown to be involved in the early stages of retina regeneration (Nelson et al., 2013). It is therefore possible that loss of GABA signaling after retina damage results in reduced intracellular Cl^-, which then leads to an upregulation of tumor necrosis factor α.

As above, excess glutamate in the retina can be excitotoxic (Olney, 1982) which could be due to TPMPA acting on bipolar cells. While our previous work showed that inhibiting glutamate receptors in the retina leads to MG-derived proliferation (Rao et al., 2017), inhibiting AMPA receptors in an injury model involving injection of $CoCl_2$ led to reduced proliferation, an apparent neuroprotective effect (Medrano et al., 2018). While other explanations are possible, these seemingly contradictory results could simply be due to a $CoCl_2$-mediated injury response, whereas we blocked AMPA receptors in undamaged retinas.

GABA and β-cell Regeneration

Beyond the mouse hippocampus and the zebrafish retina, GABA levels can drive pancreatic α cells to a β cell fate (Ben-Othman et al., 2017; Li et al., 2017). Intriguingly, the role of GABA in this case, both in adult mice and cultured cells, is the opposite of what we observe in the retina in that increased GABA levels or administration of indirect agonists of the $GABA_A$ stimulated increased numbers of α cells derived from glucagon secreting β cells. Even though the mechanism of action of GABA is the opposite, the common finding is that altered GABA signaling can activate regeneration.

Conclusion

We have shown that inhibiting $GABA_A$-ρ receptors is sufficient to induce a regenerative response in the zebrafish retina in the absence of damage and that inhibiting both $GABA_A$ $GABA_A$-ρ receptors simultaneously produces a synergistic effect. It will be important to determine if this effect is directly mediated through MG, but, together, our results suggest a novel approach to induce a regenerative response in the mammalian retina.

CHAPTER III

THE MIR-216A-DOT1L REGULATORY AXIS IS NECESSARY AND SUFFICIENT FOR MÜLLER GLIA REPROGRAMMING DURING RETINA REGENERATION[2]

Nergis Kara, Matthew R. Kent, Dominic Didiano, Kamya Rajaram, Anna Zhao, Emily R. Summerbell, and James G. Patton

This chapter is a collaborative effort to which I demonstrated that the H3K79 methyltransferase, Dot1l, is significantly upregulated after damage in the zebrafish retina (**Figure 18B**), that Dot1l expression is significantly lower after electroporation of a *miR-216a* mimic (**Figure 18E**), that Dot1l expression is significantly higher after electroporation of a morpholino targeting *miR-216a* (**Figure 22C, D**), that the small molecule inhibitor GSK3β can rescue the loss of proliferation from knockdown of Dot1l (**Figure 25B, C**), and that the loss of proliferative response seen due to *miR-216a* mimic or *dot1l*-MO electroporation is not due to loss of MG (**Figure 16**).

[2] This work has been published: Kara N, Kent MR, Didiano D, Rajaram K, Zhao A, Summerbell ER, Patton JG (2019) The miR-216a-Dot1l Regulatory Axis Is Necessary and Sufficient for Muller Glia Reprogramming during Retina Regeneration. Cell Rep 28:2037-2047 e2034.

Abstract

Unlike the adult mammalian retina, Müller glia (MG) in the adult zebrafish retina are able to dedifferentiate into a 'stem cell'-like state and give rise to multipotent progenitor cells upon retinal damage. We show that *miR-216a* is downregulated in MG after constant intense light lesioning and that *miR-216a* suppression is necessary and sufficient for MG dedifferentiation and proliferation during retina regeneration. *miR-216a* targets the H3K79 methyltransferase Dot1l which is upregulated in proliferating MG after retinal damage. Loss-of-function experiments show that Dot1l is necessary for MG reprogramming and mediates MG proliferation downstream of *miR-216a*. We further demonstrate that *miR-216a* and Dot1l regulate MG-mediated retina regeneration through canonical Wnt signaling. Together, our study reports a novel regulatory mechanism upstream of Wnt signaling during retina regeneration and provides potential targets for enhancing regeneration in the adult mammalian retina.

Introduction

A promising strategy to restore impaired vision due to degenerative retinal disorders is to induce endogenous repair mechanisms to regenerate lost cell types. Unfortunately, mammals are unable to spontaneously regenerate retinal neurons and instead, damage often induces reactive gliosis (Bringmann et al., 2009). However, retinal damage in teleost fish, including zebrafish, initiates a robust spontaneous regenerative response that restores both retinal structure and function (Goldman, 2014). Given that the cells and structure of the retina are highly conserved among vertebrates, understanding the molecular mechanisms that allow zebrafish to spontaneously regenerate damaged retinas is key to develop novel therapeutic strategies for retinal damage and disease in humans.

In zebrafish, Müller glia (MG) are the source of regenerated neurons in the retina (Fausett and Goldman, 2006b; Bernardos et al., 2007a). After injury, MG dedifferentiate, undergo asymmetric cell division, and generate a population of proliferating neuronal progenitor cells (Thummel et al., 2008b; Ramachandran et al., 2010b; Nagashima et al., 2013). MG-derived neural progenitors are able to differentiate into any of the lost retinal cell types and fully restore visual function in the zebrafish retina. Understanding the major cellular events and identifying key differentially expressed genes is a current focus of much research, but the precise molecular mechanisms that regulate retina regeneration remain largely unknown (Goldman, 2014; Lenkowski and Raymond, 2014; Rajaram et al., 2014b).

Micro RNAs (miRNAs) are a family of highly conserved small noncoding RNAs that post-transcriptionally regulate gene expression and play important roles in many cellular processes during development and regeneration (Wienholds and Plasterk, 2005; Zhao and Srivastava, 2007; Thatcher and Patton, 2010). We recently showed that the major miRNA processing enzyme, Dicer, is required for retina regeneration in zebrafish and profiled dynamic miRNA expression patterns in the retina during regeneration induced by constant intense light damage (Rajaram et al., 2014c). Here, we report that *miR-216a* acts a gatekeeper for MG reprogramming, maintaining MG in a quiescent state in undamaged

retina. *miR-216a* suppression is necessary and sufficient for MG dedifferentiation and proliferation. We identify the disruptor of telomeric silencing-1-like (Dot1l) as a *bona fide* target of *miR-216a* and demonstrate that the *miR-216a*/Dot1l regulatory axis mediates initiation of retina regeneration through the Wnt/β-catenin pathway. Previous studies in multiple species have revealed that Dot1l is able to regulate transcription of Wnt-target genes by directly interacting with T-cell factor (TCF)/β-catenin complexes (Mahmoudi et al., 2010; Mohan et al., 2010; Castaño Betancourt et al., 2012). Our work uncovers for the first time a requirement for Dot1l downstream of *miR-216a* during MG dedifferentiation, proliferation, and retina regeneration.

Results

miR-216a is Suppressed in Dedifferentiated MG During Early Retina Regeneration

We previously demonstrated a general requirement for the Dicer-dependent miRNA biogenesis pathway during retina regeneration induced by constant intense light damage in adult zebrafish (Rajaram et al., 2014c). *miR-216a* belongs to a highly conserved miRNA family with previously characterized functions in gliogenesis during retina development (Olena et al., 2015). We sought to test whether *miR-216a* might also regulate reprogramming of MG during retina regeneration. First, we determined the expression levels of *miR-216a* in quiescent and proliferating MG, as well as non-MG cells in the retina. We used fluorescence activated cell sorting (FACS) to isolate GFP^+ quiescent MG from undamaged Tg*(gfap:gfp)* retinas, GFP^+ dedifferentiated MG after 45 hours intense light damage using Tg*(1016tuba1a:gfp)* retinas (Bernardos and Raymond, 2006; Fausett and Goldman, 2006b), and GFP^- cells from both sorts (non-MG) **(Figure 15A).** GFAP is expressed in quiescent MG; the Tg*(1016tuba1a:gfp)* transgenic line specifically marks dedifferentiated MG and MG-derived neural progenitors in actively regenerating retinas (Fausett and Goldman, 2006b). We chose 45 hours post

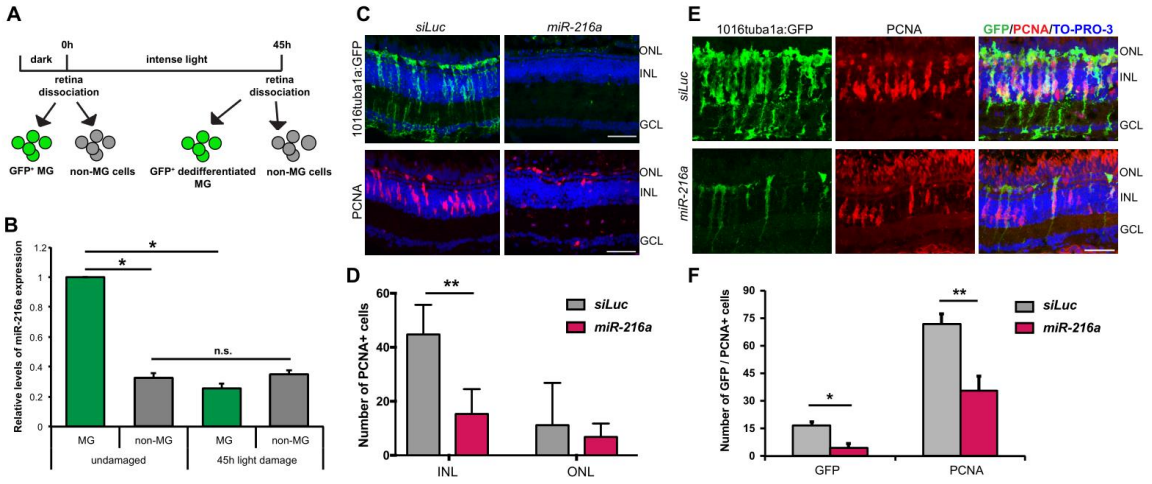

Figure 15 Suppression of miR-216a is required for Müller Glia dedifferentiation and proliferation during retina regeneration.

(**A**) Schematic for post-mitotic and dedifferentiated MG sorting. Adult zebrafish were dark adapted for 2 weeks and then exposed to constant intense light lesioning for 45 hours. For post-mitotic MG isolation, GFP$^+$ cells were sorted from dark adapted *Tg(gfap:gfp)* retinas. For dedifferentiated MG isolation, GFP$^+$ cells were isolated from 45 hr light lesioned *Tg(1016tuba1a:GFP)* retinas. (**B**) Fold changes in *miR-216a* levels in FACS-purified MG were determined by qPCR. *miR-216a* is enriched in post-mitotic MG (GFP$^+$) from undamaged retinas in *Tg(gfap:gfp)* fish. After 45h of light damage, *miR-216a* is down-regulated ~5 fold in dedifferentiated MG (GFP$^+$) in *Tg(1016tuba1a:gfp)* fish. *miR-216a* expression did not change in non-MG cells (GFP$^-$) during regeneration. Data are from five independent experiments with three technical replicates of qPCR. MG were purified from 18 and 20 light damaged fish in each experiment. Error bars represent the SEM. * $p<0.05$, ** $p<0.01$ using Two-way ANOVA with Fisher's LSD *post-hoc* test. (**C**) Control miRNA (siRNA against luciferase; *siLuc*) or *miR-216a* was injected and electroporated into the left eyes of Tg(*1016tuba1a*:gfp) zebrafish before intense light exposure (0h). After 45h, retinas were collected, sectioned and immunostained using antibodies against GFP and PCNA. Nuclei were counterstained with TOPRO (blue). *miR-216a* gain-of-function abolished *tuba1a*:GFP transgene expression and significantly reduced the number of INL PCNA$^+$ proliferating cells. (**D**) Quantification of

PCNA$^+$ cells in the INL and ONL. Error bars represent the SEM (n=5-6 fish); **, p<0.01 using Student's t-test. (**E**) Overexpression of *miR-216a* reduced the number of GFP$^+$/PCNA$^+$ proliferating progenitor cells after 60 hrs intense light damage using Tg(*1016tuba1a*:gfp) zebrafish. (**F**) Quantification of total GFP$^+$ and PCNA$^+$ cells. Error bars represent the SEM (n=10 fish); * p<0.03 , ** p<0.003 using Student's t-test. ONL, Outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar 50um.

light damage to focus on initial reprogramming of MG when MG undergoes first round of division between 35-52 hours of intense light damage (Rajaram et al., 2014a). Quantitative real-time PCR (qPCR) analysis showed that *miR-216a* is expressed at significantly higher levels in quiescent MG compared to the non-MG cell population in undamaged retinas **(Figure 15B)**. After damage, however, *miR-216a* was significantly down regulated in dedifferentiated MG in regenerating retinas compared to quiescent MG. When we compared non-MG cell populations in undamaged and intense light damaged retinas, we did not detect any significant changes in expression levels of *miR-216a* indicating that *miR-216a* expression is more highly expressed in MG than other retinal cell types. Of note, we discovered that *miR-216b* is also expressed in MG and undergoes a significant decrease in expression after damage. *miR-216b* differs from *miR-216a* at two positions, one of which is in the seed region, meaning it targets a different set of mRNAs. Based on our prior work and because we identified Dot1l as a target of *miR-216a* and not *miR-216b*, we focus here on *miR-216a*.

miR-216a Suppression is Required for MG Dedifferentiation and Proliferation

To test whether *miR-216a* suppression is required for MG dedifferentiation and proliferation during retina regeneration, we performed over expression analysis of *miR-216a*. We injected and

electroporated *miR-216a* mimics or a control miRNA intravitreally into the eye of Tg*(tuba1a:gfp)* transgenic fish before intense light damage (0h) and assessed the effects on MG dedifferentiation and proliferation at 45h of light exposure **(Figure 15C)**. While there were numerous GFP^+ dedifferentiated MG in control miRNA injected retinas at 45h of light damage, there was a striking absence of GFP^+ dedifferentiated MG in *miR-216a* overexpressing retinas **(Figure 15C)**. We then analyzed the effect of *miR-216a* overexpression on MG proliferation using proliferating cell nuclear antigen (PCNA) as a marker of DNA replication. Compared to control miRNA overexpressing retinas which had clusters of $PCNA^+$ cells in the inner nuclear layer (INL), excess *miR-216a* resulted in significantly decreased numbers of proliferating MG **(Figure 15C, D)**. Proliferation of rod progenitor cells in the outer nuclear (ONL) was not affected **(Figure 15C, D)**.

We then analyzed the effects of *miR-216a* overexpression on neural progenitor cell proliferation (**Figure 15F**). At 60 hours of intense light exposure, there were significantly less dedifferentiated MG marked by GFP, as well as decreased numbers of $PCNA^+$ proliferating progenitors in the INL of *miR-216a* overexpressing Tg(*tuba1a:GFP*) retinas compared to control (**Figure 15G**). This suggests that the inhibitory effects of *miR-216a* overexpression on the proliferation of MG and MG-derived progenitors are observed at later stages of regeneration, consistent with the model that suppression of *miR-216a* is a critical step in both the initiation of MG dedifferentiation and the generation of proliferating progenitor cells during retina regeneration. To ensure that the effects we observed were not indirectly a consequence of unexpected apoptosis due to overexpression of *miR-216a*, we conducted TUNEL assays. We did not observe any changes in apoptotic cells between control and *miR-216a* overexpressed retinas (**Figure 16**).

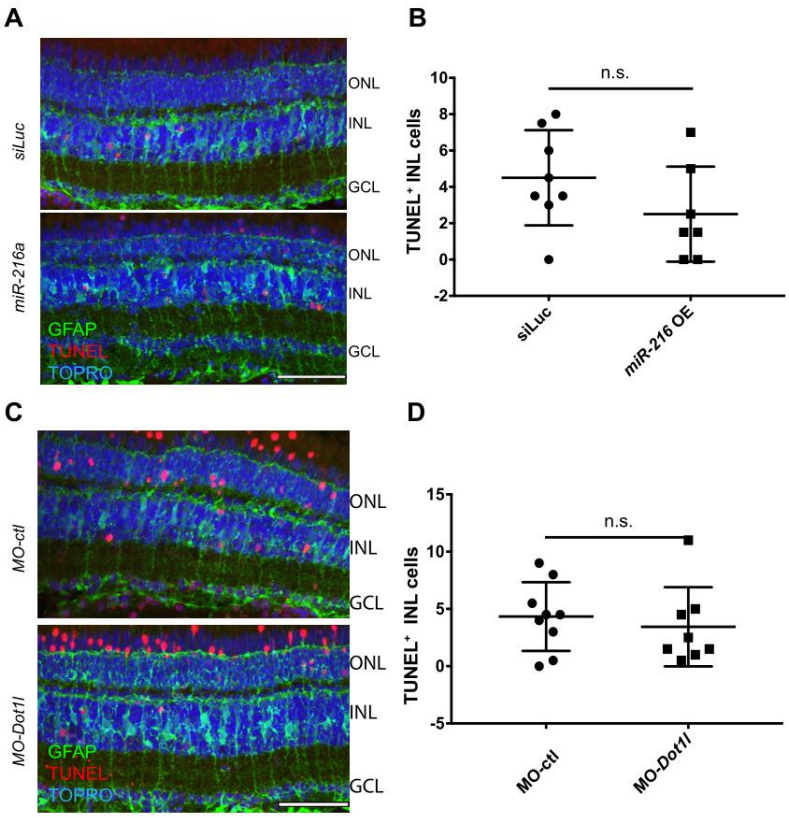

Figure 16 miR-216a mimic and Dot1l morpholino injections do not lead to increased apoptosis in the retina.

(A) Control miRNA or *miR-216a* or **(C)** control morpholinos or Dot1l morpholinos were injected and electroporated into the left eyes of Tg*(gfap:GFP)* zebrafish before intense light exposure (0h). After 45h, retinas were collected, sectioned and TUNEL labeling was performed along with anti-GFP immunostaining. Nuclei were counterstained with TOPRO (blue). **(B, D)** TUNEL-positive cells in the INL were quantified. Data represent mean +/- SEM, n=7-9 fish. Two-tailed, Mann–Whitney *U* test is performed. ONL, Outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar 50um.

Dot1l is a Direct Target of miR-216 In Vivo

Suppression of *miR-216a* in dedifferentiated MG predicts an upregulation of mRNA targets that carry *miR-216a* binding sites in their 3' untranslated regions (UTR). To investigate the molecular mechanism through which *miR-216a* regulates MG reprogramming, we used the target prediction algorithm TargetScanFish and identified 61 potential target genes based on spatiotemporal expression patterns using RNA-seq transcriptome analysis in purified MG before and after intense-light lesion (manuscript in preparation). We further narrowed the list down to those with validated upregulation by qRT-PCR and those containing at least two miRNA recognition elements (MRE) for *miR-216a*. Dot1l emerged as a strong candidate target of *miR-216a* and potential regulator of retina regeneration, since it was previously shown that it functions as an activator of canonical Wnt dependent transcription in zebrafish (Mahmoudi et al., 2010) and canonical Wnt activation is necessary for MG dedifferentiation and proliferation during retina regeneration (Ramachandran et al., 2011).

There are 3 MREs for *miR-216a* in the 3'UTR of *dot1l* mRNA (**Figure 17A**). To test whether *miR-216a* directly targets *dot1l*, we performed GFP reporter assays in embryos. We cloned the *dot1l* 3'UTR downstream of the GFP coding sequence and *in vitro* transcribed reporter mRNAs. We then injected the reporter mRNAs, either alone or with co-injected *miR-216a,* mimics into 1-cell stage zebrafish embryos. At 24 hours post fertilization (hpf), GFP expression levels were significantly lower in the embryos co-injected with *miR-216a* compared to those injected with only the reporter mRNA (**Figure 17B, C**). This indicates that *miR-216a* can directly target the 3'UTR of *dot1l* mRNAs. To test whether this targeting is via the MREs predicted by the algorithm, we mutated the *miR-216a* seed sites in all three MRE sites. Reporter assays using the mutated reporter construct did not show any changes in the levels of GFP fluorescence upon co-injection with *miR-216a* (**Figure 17D, E**). This result shows that *dot1l* mRNAs can be targeted by *miR-216a* through the indicated MREs.

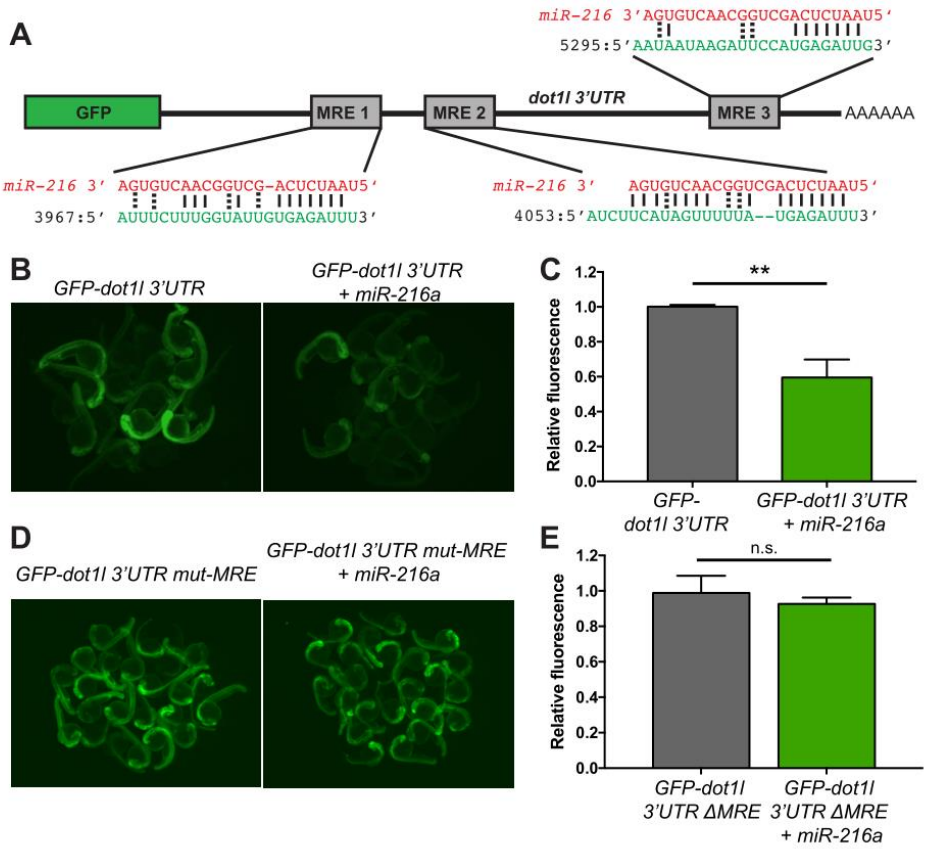

Figure 17 Dot1l is a direct target of miR-216a.

(**A**) Schematic of the reporter mRNA consisting of the coding sequence of GFP fused to the *dot1l* 3'UTR. Three predicted miRNA recognition elements (MREs) are indicated. Predicted base-pairing between MREs (shown in green) and the *miR-216a* sequence (shown in red). (**B**) Embryos injected at the one-cell stage with 100pg of *GFP-dot1l 3'-UTR*, with or without 100pg of *miR-216a* were examined for GFP expression at 1 dpf. GFP expression was apparent in embryos injected with *GFP-dot1l 3'-UTR* but was reduced in embryos co- injected with *miR-216a*. (**C**) Quantification of relative fluorescence in 1dpf embryos injected with GFP reporter only or co-injected with *miR-216a* and the GFP reporter. (**D**) 1 dpf old embryos injected at the one-cell stage with 100pg of *GFP-dot1l 3'-UTR* carrying mutations in all *miR-216a* MREs. Embryos were injected with the mutant reporter, either alone or co-injected with *miR-216a*. (**E**) Quantification of relative fluorescence in 1dpf embryos injected with mutant GFP reporter alone, or with co-injection of *miR-216a*.

To determine whether Dot1l is expressed in a manner consistent with regulation by *miR-216a*, we analyzed mRNA expression levels of *dot1l* in quiescent MG using Tg(*gfap:gfp*) retinas and in dedifferentiated MG using Tg(*tuba1a:gfp*) retinas (**Figure 18A**). qRT/PCR analysis of RNA from sorted cell populations showed a ~2-fold upregulation of *dot1l* transcripts in dedifferentiated MG compared to post-mitotic MG (**Figure 18B**). No changes in *dot1l* expression were observed in other retinal cells (GFP^- cells). We also investigated whether *miR-216a* is able to target endogenous *dot1l* in the retina during regeneration by injection and electroporation of *miR-216a* mimics into the dorsal retina of fish exposed to 24h of constant intense light damage (**Figure 18C**). Increased *miR-216a* levels (**Figure 18D**) led to a significant decrease (~95%) in endogenous *dot1l* mRNA levels, as shown by qRT/PCR analysis (**Figure 18E**). We also showed co-localization of Dot1l with PCNA by performing immunohistochemistry on Tg(*tuba1a:gfp*) fish after 51h of light exposure (**Figure 19)**. Despite weak signals with available antibodies, we observed punctate nuclear localization of Dot1l protein in *tuba1a*:GFP^+/$PCNA^+$ dedifferentiated and proliferating MG. Collectively, our data support the hypothesis that *miR-216a* regulates *dot1l* in the adult retina.

Dot1l is Necessary for Proliferation During Retina Regeneration

Given that excess *miR-216a* inhibited dedifferentiation and proliferation of MG, we hypothesized that Dot1l is required for the early phases of retina regeneration. To analyze the loss-of-function of Dot1l during retina regeneration, we first generated Dot1l null alleles by CRISPR/Cas9. However, homozygous mutants displayed embryonic lethality similar to homozygous Dot1l mice (Jones et al., 2008). Thus, we knocked down Dot1l in adult zebrafish retinas by injecting and electroporating previously characterized morpholinos (MOs) against *dot1l* into the retina prior to intense light damage (Mahmoudi et al., 2010) (**Figure 20A**). We used the Tg(*tuba1a:GFP*) transgenic line to assess the dedifferentiation of MG, cell cycle re-entry, and proliferation of MG-derived progenitors. At 45h of

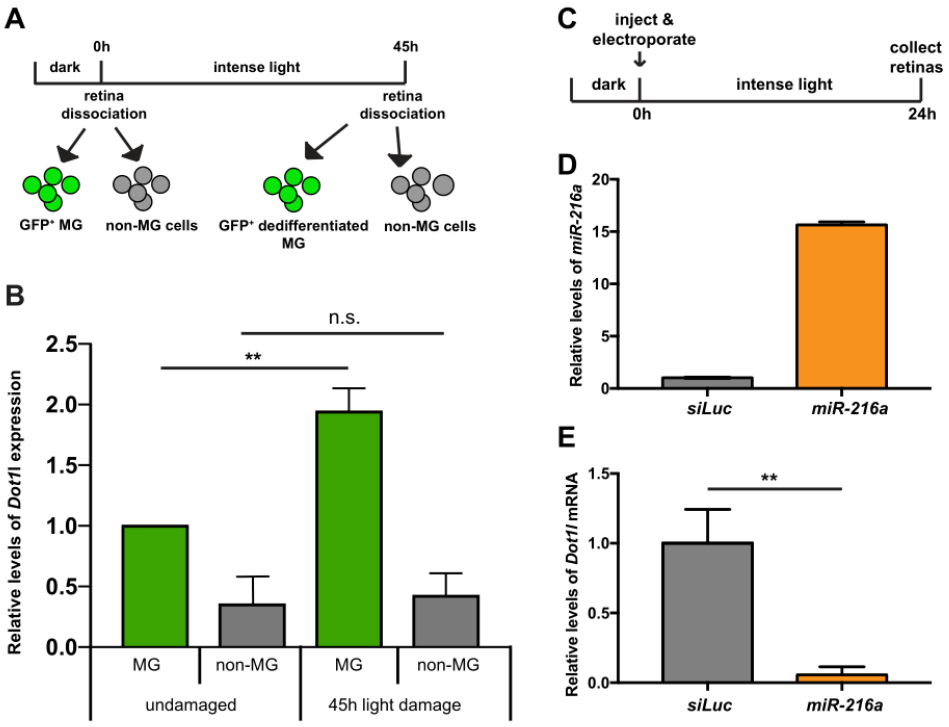

Figure 18 miR-216 targets Dot1l in the retina during photoreceptor regeneration.

(**A**) For post-mitotic MG isolation, GFP$^+$ cells were sorted from dark adapted undamaged *Tg(gfap:gfp)* retinas. For dedifferentiated MG isolation, GFP$^+$ cells were isolated from 45 hr light lesioned *Tg(1016tuba1a:GFP)* retinas. (**B**) Fold changes in *dot1l* levels in FACS-purified MG were determined by qPCR. After 45h of light damage, *dot1l* is up-regulated in dedifferentiated MG (GFP$^+$) in *Tg(1016tuba1a:gfp)* fish. *Dot1l* expression did not change in non-MG cells (GFP$^-$) during regeneration. Data represent the mean +/- SEM from 15 undamaged fish and dedifferentiated MG were purified from 18 light damaged fish. (**C**) Experimental scheme to test effects of *miR-216a* overexpression on *dot1l* levels. Wild-type adult zebrafish were dark adapted and then either control miRNA or *miR-216a* was injected and electroporated into the left eyes before intense light exposure. After 24 hours of light exposure, retinas were dissected for RNA isolation. (**D**) Fold changes in *miR-216a* levels in control miRNA (siRNA against luciferase; *siLuc*) or *miR-216a* mimic electroporated retinas were quantified by qPCR. *miR-216a* levels were upregulated by 15-fold in *miR-216a* mimic injected retinas compared to

controls. Data represent the mean +/- SEM from 6 retinas. (**E**) Fold changes in *dot1l* levels in *siLuc* or *miR-216* mimic electroporated retinas were quantified by qPCR. After 24h of light damage, *dot1l* was downregulated in *miR-216a* overexpressing retinas ~20 fold. Data represent mean +/- SEM from 3 independent experiments. 6 retinas were pooled for RNA isolation in each experiment. ** $p<0.01$ (Student's t-test), $p=0.0093$. Scale bar 50um.

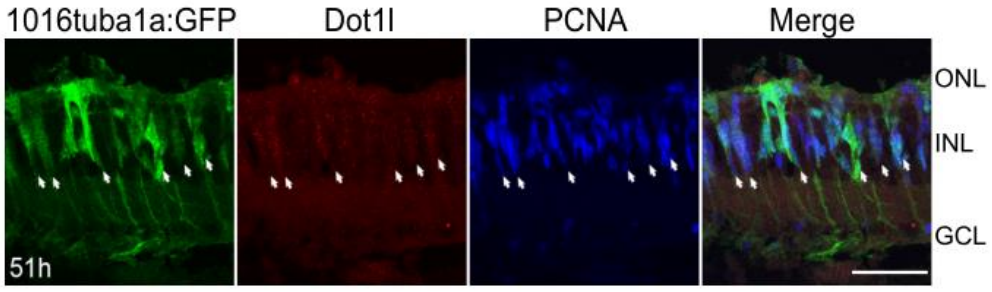

Figure 19 Dot1l is expressed in proliferating MG after photoreceptor loss.

Dot1l (red), GFP, and PCNA (blue) immunostaining after 51 hr intense light lesioning in Tg(1016*tuba1a*:gfp) retinas. Dot1l is expressed in proliferating MG. Arrows indicate $GFP^+/PCNA^+$ dedifferentiated MG that express Dot1l.

constant intense light damage, we detected significantly less dedifferentiated MG, as well as decreased numbers of PCNA+ proliferating progenitors in retinas electroporated with the *dot1l* MO, compared to injection of a control MO (**Figure 20B, C**). To test for specificity and possible off target effects of MOs, we first used a second *dot1l* morpholino (MO-*dot1l*-2) and again assessed the effect of *dot1l* knock-down on regenerating retinas after intense light damage (**Additional Figure 21A**). *dot1l*-MO-2 injected

retinas also displayed significantly reduced numbers of PCNA+ proliferating neural progenitors at 45h of light damage. Further, we showed that the effects of the Dot1l morpholino on MG proliferation are dose-dependent, arguing in favor of specificity **(Figure 20D)**. Lastly, we showed that the increase in MG proliferation and dedifferentiation upon *dotl1* morpholino injection was not a consequence of unrelated apoptosis since TUNEL staining showed no significant difference in cell death compared to control morpholinos (**Figure 16C, D)**.

To test whether the requirement for Dot1l is through its histone methyltransferase activity, we used a small molecule inhibitor of Dot1l catalytic activity that competitively binds to the S-adenosylmethionine-binding pocket of Dot1l (iDot1l; EPZ004777) (Daigle et al., 2011). Compared to control vehicle injected retinas, intravitreal injection of iDot1l led to a significant reduction in the number of dedifferentiated MG, as well as the number of proliferating progenitors in the INL after 45h of intense light damage **(Figure 20E, F**). The effects of the Dot1l inhibitor were dose dependent **(Figure 20G).** Together, the knockdown experiments and pharmacologic inhibition experiments argue that Dot1l is required for MG reprogramming acting as an epigenetic modifier required for dedifferentiation and proliferation of MG-derived neural progenitors during retina regeneration.

Suppression of miR-216a is Sufficient for Retina Regeneration Through Targeting Dot1l

Next, we investigated whether *miR-216a* down-regulation is sufficient to drive MG-dedifferentiation and the formation of neural progenitors. To test this, we suppressed the *miR-216a* levels by injection and electroporation of antisense *miR-216a* morpholinos (MO-*216a*) in undamaged Tg(*tuba1a:gfp*) retinas (**Figure 22A**). At 51 hours post injection (hpi), we showed that *miR-216a* suppression significantly increased the *dot1l* mRNA and protein levels in the retinas **(Figure 22B-D)**. Then, we assessed whether loss of *miR-216a* function results in the initiation of a regenerative response

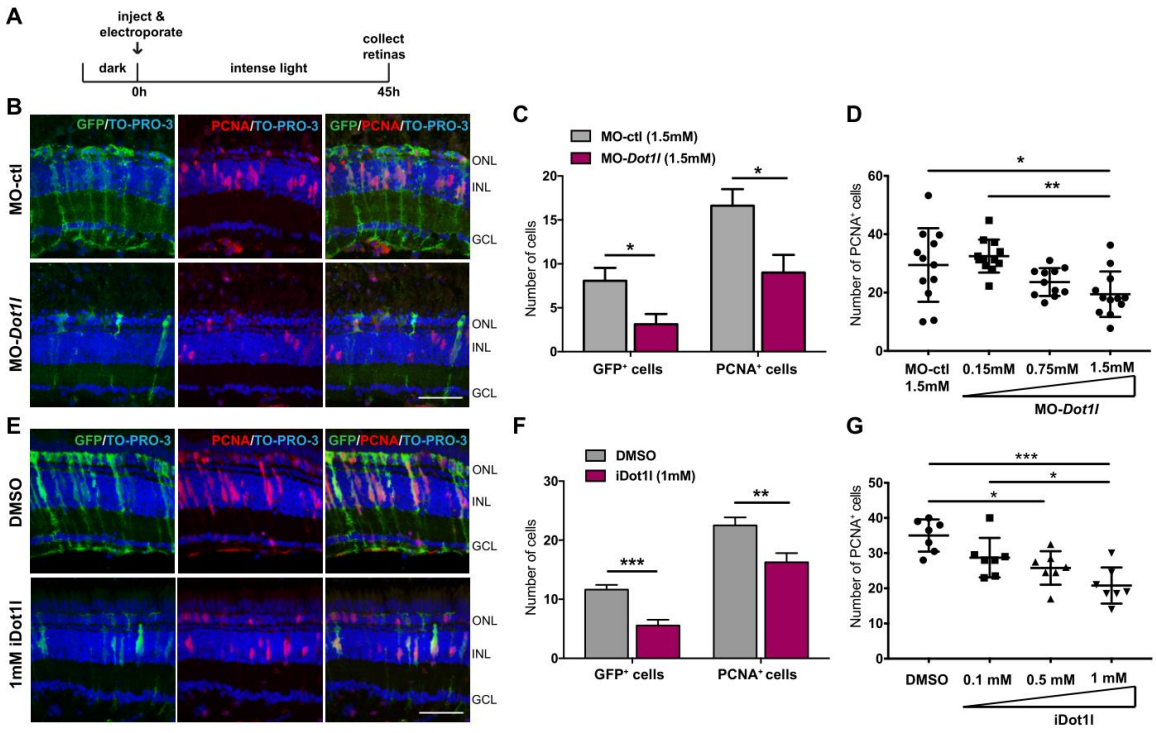

Figure 20 Dot1l is required for MG dedifferentiation and proliferation during retina regeneration.

(**A**) Control morpholinos (MO) or *dot1l* MOs were injected and electroporated into the left eyes of Tg(*1016tuba1a*:gfp) zebrafish before intense light exposure (0h). After 45h, retinas were collected, sectioned and immunostained using antibodies against GFP, PCNA. Nuclei were counterstained with TOPRO (blue). (**B**) Dot1l loss-of-function reduced *tuba1a*:GFP transgene expression and the number of INL PCNA$^+$ proliferating cells. (**C**) Quantification of GFP$^+$ dedifferentiated MG and PCNA$^+$ proliferating progenitors in MO-ctl and MO-*dot1l* electroporated retinas. Data represent mean +/- SEM, n= 5-6 fish; **, p<0.01 by two-tailed, Mann–Whitney *U* test. (**D**) Dose response to MO-*dot1l*. Increasing amounts of MO-*dot1l* morpholinos were injected and analyzed as in B and C. Each data point represents an individual fish; *<0.05, **<0.01, ***<0.001 by one way Anova. (**E**) Control vehicle (DMSO) or iDot1l (Dot1l inhibitor- EPZ004777) were injected intravitreally into the left eyes of Tg(*1016tuba1a*:gfp) zebrafish before intense light exposure (0h). After 45h, retinas were collected, sectioned and immunostained using antibodies against GFP, PCNA. Nuclei were counterstained with TOPRO (blue). (**F**)

Quantification of total GFP$^+$ and PCNA$^+$ cells. Data represent mean +/- SEM, n=10 fish; *p=0.0111; ****$p < 0.0001$ by two-tailed, Mann–Whitney *U* test. (**G**) Dose response to iDot1l. Increasing amounts of the Dot1l inhibitor were injected and analyzed as in E and F. Each data point represents an individual fish; *<0.05, **<0.01, ***<0.001 by one way Anova. ONL, Outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar 50um.

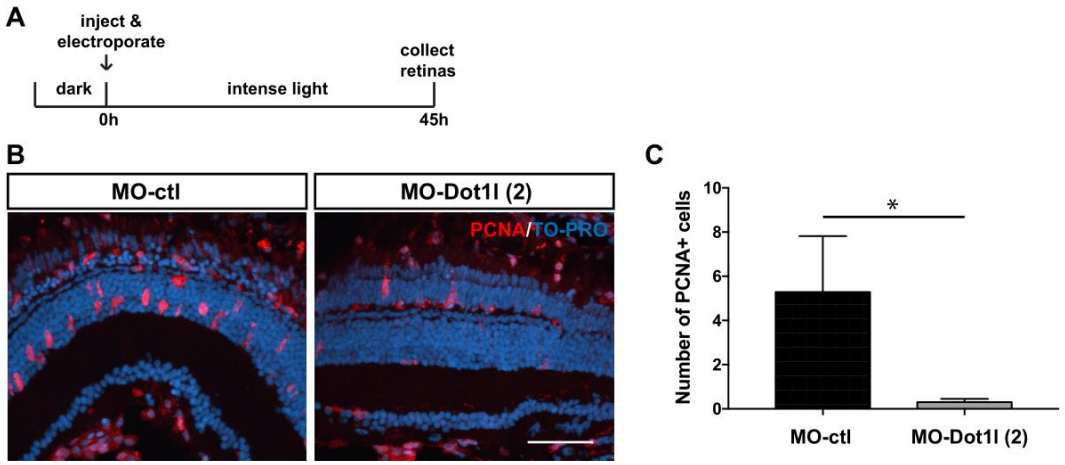

Figure 21 dot1l knock-down by a second independent morpholino injection inhibits MG proliferation during retina regeneration.

(**A**) Control morpholinos or a second independent *dot1l* morpholino (*dot1l*-MO-2) were injected and electroporated into the left eyes of Tg(*1016tuba1a*:gfp) zebrafish before intense light exposure (0h). After 45h, retinas were collected, sectioned and immunostained using antibodies against GFP, PCNA. Nuclei were counterstained with TOPRO (blue). (**B**) *dot1l* loss-of-function reduced the number of INL PCNA$^+$ proliferating cells. (**C**) Quantification of PCNA$^+$ proliferating progenitors in MO-ctl and MO-*dot1l* electroporated retinas. Data represent mean +/- SEM, n= 7-10 fish; *, p<0.05 by two-tailed Student's t-test. ONL, Outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar 50um.

similar to what happens after retinal damage. While we did not detect any dedifferentiated GFP$^+$ MG in control morpholino injected retinas, *miR-216a* suppression resulted in a significant increase in the number of dedifferentiated MG in undamaged Tg(*tuba1a:gfp*) retinas (**Figure 22E, F**). We also detected significantly higher numbers of proliferating PCNA$^+$ cells in *miR-216a* suppressed retinas compared to control morpholino injected retinas (**Figure 22E, G**). By showing colocalization of PCNA$^+$ and *tuba1a*-GFP$^+$ cells, we confirmed that proliferating cells upon *miR-216* suppression are dedifferentiated MG **(Figure 22H)**.

Our data are most consistent with a model whereby *miR-216a* suppression stimulates MG proliferation through targeting *dot1l*. If true, co-suppressing *dot1l* in the presence of MO-*216a* should block MG proliferation. To test this, we combined intravitreal injection and electroporation of both *dot1l* and *miR-216a* morpholinos in uninjured Tg(*tuba1a:gfp*) retinas. Compared to control retinas, we no longer observed a significant increase in the number of dedifferentiated and proliferating MG at 51 hours post injection after double knockdown of both *dot1l* and *miR-216a* (**Figure 22E-G**). This result demonstrates that *miR-216a* must be downregulated to activate Dot1l during retina regeneration and that Dot1l is required for the initiation of MG proliferation.

Wnt/β-catenin Signaling is Required Downstream of miR-216a/Dot1l During Retina Regeneration

Dot1l is a histone methyltransferase responsible for H3K79me3 modification associated with gene activation (Feng et al., 2002; Shanower et al., 2005; Jones et al., 2008) (Van Leeuwen et al., 2002; Steger et al., 2008). Dot1l serves as a co-activator of Wnt/β-catenin signaling (Mahmoudi et al., 2010; Mohan et al., 2010) which is known to be activated during retina regeneration and is required for the formation of MG-derived progenitors (Ramachandran et al., 2011; Meyers et al., 2012). Since *miR-216a* suppression is necessary for MG-dependent retina regeneration, we wanted to test whether *miR-216a*

suppression is also required for the activation of Wnt/β-catenin signaling. First, we assessed β-catenin accumulation in MG after light damage since β-catenin accumulation as a result of Wnt signaling activation is necessary for MG dedifferentiation and proliferation (Ramachandran et al., 2011). We injected and electroporated *miR-216a* mimics or control miRNAs in the retinas of adult wild-type fish prior to intense light damage (**Figure 23A**). At 51h of light damage, β-catenin accumulation was clearly observed in MG associated with $PCNA^+$ neural progenitors in control retinas. However, no β-catenin accumulation was observed in *miR-216a* overexpressing retinas (**Figure 23B**). These results support the hypothesis that the *miR-216a*/Dot1l regulatory pathway regulates retina regeneration through canonical Wnt signaling. To activate Wnt signaling, we pharmacologically stabilized β-catenin using a glycogen synthase kinase-3β (GSK3β) inhibitor and confirmed that at the concentration tested, the inhibitor is able to induce MG proliferation in undamaged retinas as expected (Ramachandran et al., 2011) (**Figure 24**). We then tested whether the proliferation defects after knockdown of Dot1l could be rescued by activation of Wnt signaling. We intravitreally injected either the GSK3β inhibitor or control vehicle (DMSO) in the presence of either control MOs or *dot1l* MOs prior to intense light damage (**Figure 25A**). At 51h of light damage, *dot1l* depletion resulted in significantly decreased numbers of $PCNA^+$ proliferating progenitors compared to control retinas (**Figure 25B-C**). However, co-injection of *dot1l* MOs and the GSK3β inhibitor showed no defects in the number of neural progenitors. Next, we tested whether in undamaged retinas Dot1l is required for induction of MG proliferation through activation of Wnt/β-catenin signaling (**Figure 25D**). While stabilization of β-catenin by injection of the GSK3β inhibitor induced MG proliferation, Dot1l inhibition by co-injection of the Dot1l inhibitor led to no significant increase in MG proliferation. This indicates that Dot1l regulates MG proliferation through Wnt/ β-catenin signaling during intense light damage induced retina regeneration.

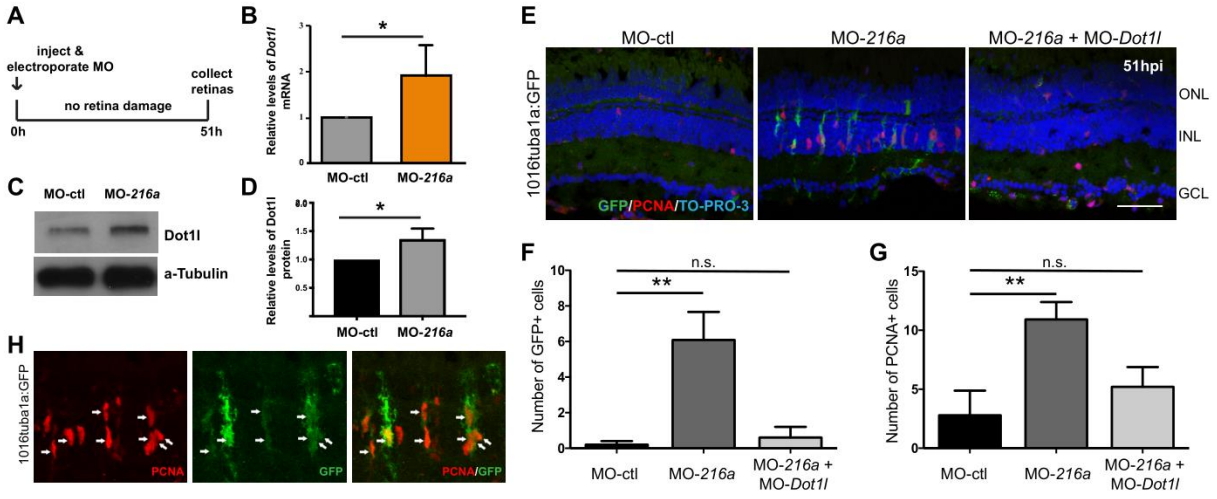

Figure 22 miR-216a suppression stimulates MG dedifferentiation and proliferation in the uninjured retina through regulating Dot1l.

(**A**) Undamaged *Tg(1016tuba1a:gfp)* zebrafish were injected and electroporated with control MOs (n=5), *miR-216a* MOs (n=6), or both (n=5). Either eyes for immunostaining or retinas for expression analysis were collected at 51 hours post-injection (hpi). (**B**) Fold changes in *dot1l* levels in MO-ctl or MO-*216a* electroporated retinas were quantified by qPCR. At 51hpi, *dot1l* was significantly upregulated in *miR-216a* overexpressing retinas ~2 fold. Data represent mean +/- SEM, *p<0.05 using Student's t-test, p=0.0365. (**C**) Representative western blot for Dot1l and α-tubulin in control and *miR-216a* MO injected retinas at 51hpi. (**D**) Quantification for the relative levels of Dot1l in *miR-216a* MO injected retinas compared to control. (**E**) *miR-216a* MOs significantly increased the number of $PCNA^+$ and GFP^+ cells in the INL, while there was no significant difference between *miR-216*a MO + *dot1l* MO co-injected eyes and control eyes. (**F**) Quantification of GFP^+ dedifferentiated MG and (**G**) $PCNA^+$ proliferating progenitors in MO-ctl, MO-*216a* and MO-*216a* + MO-*dot1l* electroporated retinas. (**H**) Representative retinal sections showing colocalization of *tuba1a*-GFP^+ and $PCNA^+$ cells in *miR-216* MO injected undamaged retinas at 51 hpi. Data represent mean +/- SEM. ** $p < 0.01$, PCNA p-value=0.0098 and GFP p=0.0039 by one-way ANOVA with Dunnett's multiple comparisons test. ONL, Outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar 50um.

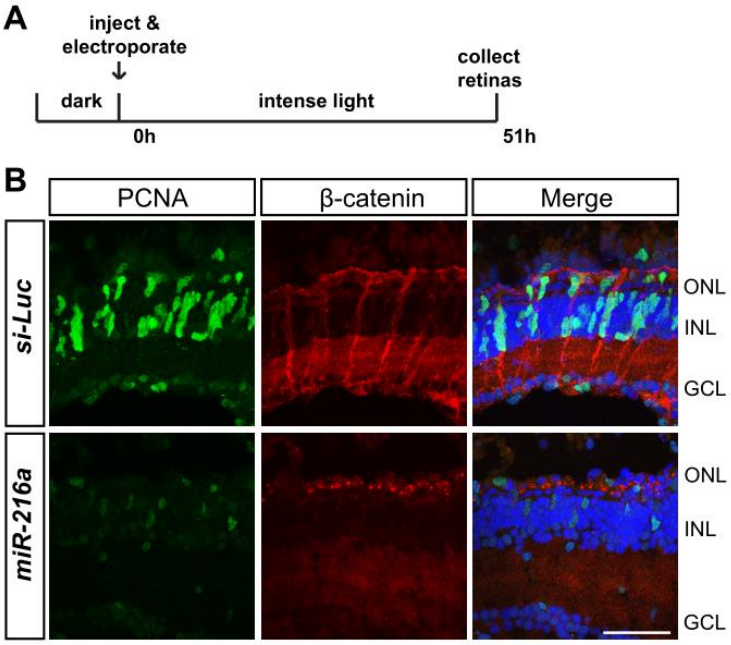

Figure 23 miR-216a gain-of-function impairs β-catenin accumulation in MG after intense light damage.

(**A**) Control miRNA or *miR-216a* was injected and electroporated into the left eyes of wild-type zebrafish before intense light exposure (0h). After 45h, retinas were collected, sectioned and immunostained using antibodies against β-catenin (red), PCNA (green). Nuclei were counterstained with TOPRO (blue). (**B**) β-catenin colocalized with PCNA+ proliferating MG after 45h of intense light damage. β-catenin accumulation was not detected in *miR-216a* overexpressing retinas.

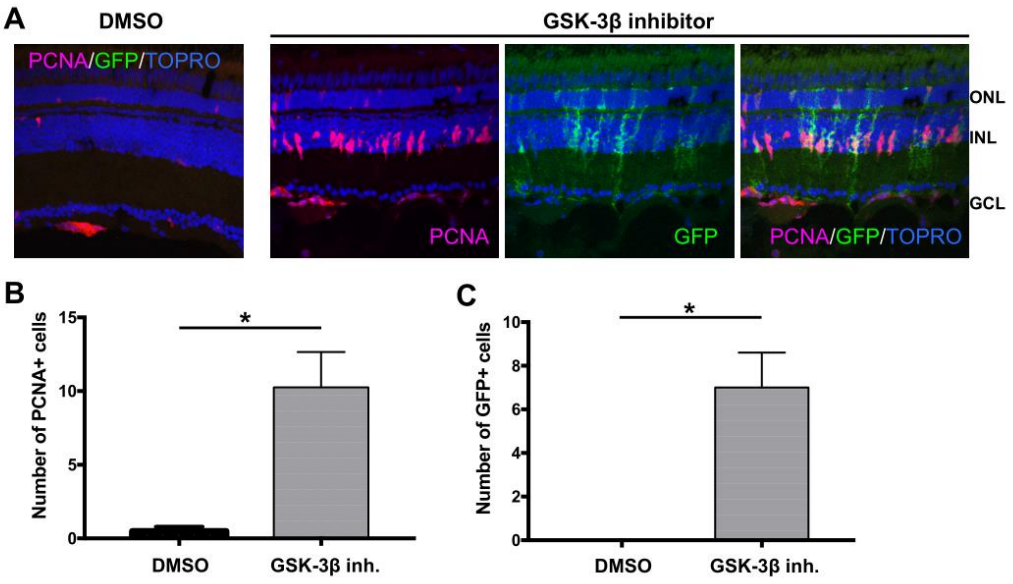

Figure 24 β-catenin stabilization stimulates MG dedifferentiation and proliferation.

(**A**) Tg(*1016tuba1a:gfp*) adult fish were intravitreally injected with 1mM GSK-3β inhibitor (n=8) or control vehicle (DMSO) (n=4). Eyes were collected 51h post injection and sectioned retinas were immunostained using antibodies against GFP for dedifferentiated MG and PCNA for proliferating progenitors. Nuclei were counterstained with TOPRO (blue). (**B**) Quantification of $PCNA^+$ proliferating progenitors and (**C**) GFP^+ dedifferentiated MG in control vehicle and GSK-3β inhibitor injected retinas. Data represent mean +/- SEM, n= 4-8 fish; *, $p<0.05$ by two-tailed, Mann–Whitney *U* test. ONL, Outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

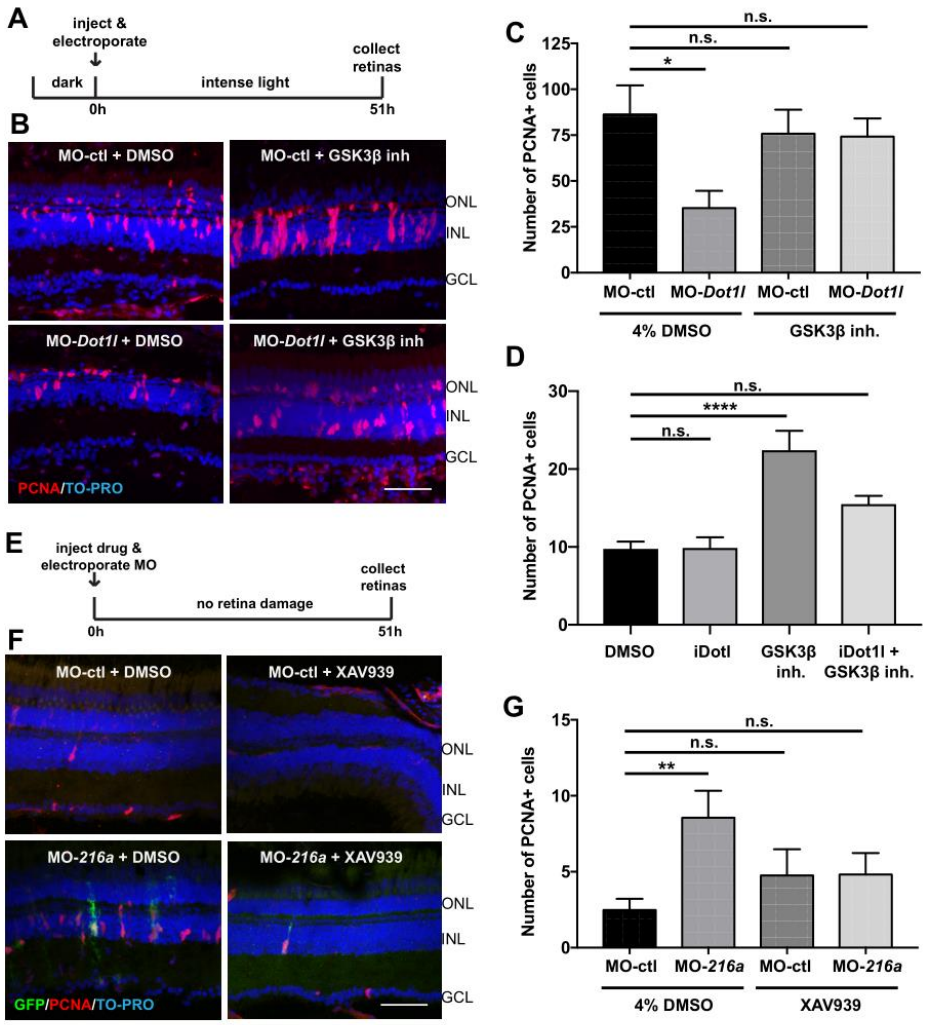

Figure 25 miR-216a and Dot1l regulate retinal regeneration through the Wnt/ß-catenin pathway.

(**A**) Control morpholinos or Dot1l morpholinos were injected and electroporated into the left eyes of wild-type zebrafish, followed by either 4% DMSO or GSK3β-inhibitor (1mM) before intense light exposure (0h). Eyes were collected after 51 hours of intense light lesion and immunostained using antibodies against PCNA. Nuclei were counterstained with TOPRO (blue). (**B**) *dot1l* MOs significantly decreased the number of $PCNA^+$ cells in the INL, while there was no significant difference between *dotl1l* MO + GSK3β-inhibitor co-injected eyes and control eyes. (**C**) Quantification of $PCNA^+$ proliferating progenitors in MO-ctl+DMSO, MO-*dot1l*+DMSO, MO-ctl+ GSK3β-inhibitor and MO-*dot1l* + GSK3β-inhibitor electroporated retinas. Activation of Wnt signaling rescued the decrease in the number of

proliferating progenitors upon *dot1l* knockdown after 51 hours of intense light lesion. Data represent mean +/- SEM, n=9-11 fish. * $p < 0.05$, p-value=0.0167 (MO-ctl+DMSO vs MO-*dot1l*+DMSO) by one-way ANOVA with Dunnett's multiple comparisons test. (**D**) GSK3β-inhibitor alone, Dot1l inhibitor (iDot1l) alone or the combination was injected into the left eyes of Tg*(1016tuba1a:GFP)* zebrafish with DMSO alone was used as a control. 51 hours post injection, eyes were collected for PCNA immunostaining. GSK3β-inhibitor alone induced MG proliferation in undamaged eyes while co-injection of the Dot1l inhibitor led to no significant changes in number of proliferating MG. (**E**) Control morpholino or *miR-216a* morpholino was injected and electroporated into the left eyes of Tg*(1016tuba1a:GFP)* zebrafish, followed by either 4%DMSO or XAV939(10μM). Eyes were collected at 51 h post-injection and immunostained using antibodies against GFP for dedifferentiated MG and PCNA for proliferating progenitors. Nuclei were counterstained with TOPRO (blue). (**F**) Suppression of *miR-216a* by MO-*216a* injection stimulates MG proliferation, however upon co-injection with XAV939, no significant increase in the number of proliferating progenitors was detected. (**G**) Quantification of $PCNA^+$ proliferating progenitors in MO-ctl+DMSO, MO-*216a*+DMSO, MO-ctl+ XAV939 and MO-*216a* + XAV939 electroporated retinas. Inhibition of Wnt signaling reversed the increase in the number of progenitors upon *miR-216a* knockdown after 51 hours of intense light lesion. Data represent mean +/- SEM, n=18-21 fish. ** $p < 0.01$, p-value=0.0089 (MO-ctl+DMSO vs MO-*216*+DMSO) by one-way ANOVA with Dunnett's multiple comparisons test. ONL, Outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar 50um.

Finally, we investigated whether *miR-216a* depletion in undamaged retinas would induce MG proliferation through modulating Wnt/β-catenin signaling (**Figure 25E**). We pharmacologically inhibited canonical Wnt signaling by injecting XAV939, a tankyrase inhibitor, which stabilizes Axin and stimulates β-catenin degradation (Huang et al., 2009). We injected either XAV939 (10μM) or control vehicle (DMSO) along with co-injection of either control or *miR-216a* MO into undamaged retinas. Intravitreal injection of XAV939 at 10μM concentration was previously shown to be sufficient to prevent injurydependent β-catenin accumulation in MG (Ramachandran et al., 2011). As above, *miR-216a* morpholino injection in undamaged retinas caused spontaneous proliferation of MG as detected by the presence of significantly higher numbers of PCNA+ proliferating cells in the INL at 51hpi (**Figure 25F, G**). However, co-injection of XAV939 and MO-*216a* suppressed the increase in the number PCNA+ proliferating cells compared to control MOs or DMSO injected retinas. These results demonstrate that Wnt/β-catenin signaling is required for spontaneous MG proliferation initiated by depletion of *miR-216a*.

Discussion

MG dedifferentiation and re-entry into the cell-cycle are key events during retina regeneration. In zebrafish, MG are capable of eliciting a robust spontaneous regenerative response upon damage, while in mammals, MG lack this ability and typically become reactive and undergo hypertrophy (Bringmann et al., 2006a). Understanding the molecular mechanisms of MG activation during regeneration is necessary to develop therapeutic strategies for retinal diseases in humans. Here, we identified a novel chromatin mediated mechanism regulating initiation of retina regeneration in adult zebrafish. We show that suppression of *miR-216a* in MG is required for dedifferentiation and proliferation upon constant intense light damage leading to de-repression of the H3K79 methyltransferase Dot1l which is required for regeneration. Furthermore, we demonstrate that *miR-*

216a and Dot1l regulate MG activation through Wnt/β-catenin signaling. Together, our data provide a novel mechanism through which *miR-216a* serves a gatekeeper for MG dedifferentiation and proliferation by suppressing Dot1l during retina regeneration.

Although many individual miRNAs have been identified that regulate cell fate in development and disease, only a few miRNAs have been shown to be functionally involved in modulating retina regeneration (Ramachandran et al., 2010b; Rajaram et al., 2014c; Rajaram et al., 2014b). We show that *miR-216a* is expressed in quiescent MG in the adult zebrafish retina and must be repressed to allow MG dedifferentiation and re-entry into cell cycle. Expression of *miR-216a* does not change in non-MG cells of the retina. Interestingly, *miR-216a* was first reported to modulate retinal gliogenesis by targeting *snx5* (sorting nexin 5) during development (Olena et al., 2015). *miR-216a* is suppressed in the central retina to allow MG specification through activation of Notch signaling. In that model, *miR-216a* targets *snx5* (sorting nexin 5) to block association of the Notch ligand Delta and prevent Delta endocytosis and thereby regulate Notch signaling. Those experiments predict that *miR-216a* functions in cells containing the Notch ligand Delta. Interestingly, we did not detect any significant changes in *snx5* expression levels in FACS-purified MG populations before and after intense light damage (data not shown). In addition, a previous study showed that while Notch receptors are present in proliferating neural progenitors, Notch ligands were detected in cells adjacent to the proliferating progenitors during retina regeneration (Wan et al., 2012). Therefore, *miR-216a* plays distinct roles during development versus regeneration regulating distinctly different targets.

Previous reports have shown that it is possible to stimulate MG proliferation in undamaged zebrafish retinas through manipulation of various factors such as tumor necrosis factor α (TNFα)(Nelson et al., 2013), GSK-3β (Ramachandran et al., 2011), leptin, interleukin-6 (IL-6)(Zhao et al., 2014) and γ-aminobutyric acid (GABA)(Rao et al., 2017). Although the interdependency of these factors is still to be determined, they hold great promise as therapeutic agents to induce a regenerative response in

mammals. Here, we show that *miR-216a* is an endogenous inhibitor of retina regeneration and suppression of *miR-216a* is sufficient to induce a regenerative response in the absence of damage. This suggests that *miR-216a* might be upstream of the many signaling pathways that are required for MG proliferation. Indeed, stimulation of MG proliferation upon suppression of *miR-216a* was blocked by co-suppression of canonical Wnt signaling (**Figure 25F, G**). In addition, excess *miR-216a* levels resulted in decreased β-catenin accumulation in MG after intense light damage (**Figure 23**). These findings suggest that *miR-216a* serves as an inhibitory factor in quiescent MG that needs to be suppressed to turn on canonical Wnt signaling and allow MG to dedifferentiate and proliferate.

It is perhaps not surprising that epigenetic modifications accompany cell fate changes in MG during retina regeneration. Analysis of DNA methylation profiles in quiescent MG and MG-derived progenitors have shown that many pluripotency and regeneration-associated genes are hypomethylated during zebrafish retina regeneration (Powell et al., 2013). Interestingly, in mouse MG, these genes are also hypomethylated, suggesting that DNA methylation is not an epigenetic barrier for retina regeneration in mammals. Accessible chromatin in mouse mature MG decreases relatively rapidly, coincident with the loss of neurogenic capacity in postnatal development (Ueki et al., 2015; Jorstad et al., 2017). This suggests that changes in histone modifications may underlie MG reprogramming during retina regeneration in zebrafish but the precise chromatin modifying enzymes have remained unknown. Our data support the hypothesis that the H3K79 methyltransferase Dot1l is required for MG dedifferentiation and proliferation and is regulated by *miR-216a*. First, 3'UTR reporter assays showed that *miR-216a* targets the 3'UTR of *dot1l* (**Figure 17**). Second, we showed that Dot1l is upregulated in dedifferentiated MG compared to post-mitotic MG after 45hours of constant intense light damage (**Figure 18A-C**). Third, morpholino knockdown and inhibition of Dot1l H3K79 methyltransferase activity showed that Dot1l upregulation is required for MG activation during early regeneration (**Figure 20B-E**).

Lastly, suppression of *miR-216* alone is sufficient to stimulate a regenerative response in the undamaged retina, while co-suppressing Dot1l prevented the MG proliferation.

Previous studies in multiple species have revealed that Dot1l is the only methyltransferase that catalyzes the histone H3-lysine 79 (H3K79) mono-, di-, and trimethylation (Feng et al., 2002; Shanower et al., 2005; Jones et al., 2008) (Van Leeuwen et al., 2002; Steger et al., 2008). Dot1l-mediated H3K79 methylation is associated with the transcription of Wnt-target genes, which is mediated by TCF transcription factors and the co-activator β-catenin (Clevers, 2006; Mahmoudi et al., 2010; Mohan et al., 2010; Castaño Betancourt et al., 2012). A direct interaction between Dotl1l and β-catenin-dependent TCF4 complexes was identified in zebrafish intestinal stem cells, as well as in mouse small intestinal crypts (Mahmoudi et al., 2010). The hypothesis is that recruitment of Dot1l to Wnt target genes by β-catenin leads to H3K79 methylation and preferential activation of transcription of Wnt target genes. The interaction of Dot1l with β-catenin was confirmed in Drosophila embryos by demonstrating the presence of β-catenin within Dot1L-containing protein complexes and a requirement for H3K79me3 in regulating Wnt target genes (Mohan et al., 2010). Additionally, in a genome-wide association study (GWAS), a Dot1l polymorphism was linked to reduced risk for osteoarthritis. Further it was shown that Dot1l interacts with Tcf4 in articular chondrocytes and is required for Wnt-dependent chondrogenesis (Castaño Betancourt et al., 2012). In zebrafish, retinal damage including both intense light and mechanical damage leads to activation of Wnt/β-catenin signaling in MG (Ramachandran et al., 2011; Meyers et al., 2012). In this study, we show that canonical Wnt signaling is required along with Dot1l activity during MG dedifferentiation and proliferation. Our data show that activation of Wnt signaling by stabilizing β-catenin alleviates defects due to Dot1l knockdown during retina regeneration (**Figure 25A-C**). In addition, while β-catenin stabilization is able induce MG proliferation in the absence of retinal damage, inhibiting Dot1l methyltransferase activity by co-injecting the Dot1l inhibitor significantly reduced the number of proliferating MG (**Figure 25D**).

Despite the experiments cited above, it remains controversial whether Dot1l solely mediates its effects via association with TCF4 transcription factors and β-catenin. It has also been reported to associate with the c-Myc-p300 complex to activate epithelial-mesenchymal transition regulators (Cho et al., 2015). Because Wnt activation is required for retina regeneration (Osakada et al., 2007; Ramachandran et al., 2011; Sanges et al., 2013; Gallina et al., 2016; Yao et al., 2016; Yao et al., 2018), and because we have shown that loss of Dot1l inhibits retina regeneration, we favor the hypothesis that in MG during retina regeneration, Dot1l association with TCF4 transcription factors and β-catenin activates Wnt genes. It remains possible that Dot1l mediated H3K789me3 modifications might track with basal RNA Polymerase II transcription and show enrichment of H3K79me3 modifications on activated Wnt genes. It is also possible that the activation of Wnt genes by Dot1l is MG-specific.

We used morpholinos to knock down expression of both *miR-216a* and Dot1l. There have been concerns raised about the concordance between mutant and morpholino-induced phenotypes in zebrafish (Stainier et al., 2015). To ensure specificity, we used multiple morpholinos, and morpholinos that have been previously published. However, most directly, we used suppression/rescue experiments with combinations of morpholinos. The ability to suppress the effects of morpholinos against *miR-216a* by co-injection of morpholinos against *dot1l* is the best evidence of specificity, regardless of how many different morpholinos are used. In addition, we used pharmacologic inhibitors to complement the Dot1l morpholino experiments and obtained nearly identical results. For Dot1l, we also created CRISPR/Cas9 mutants but, unfortunately, they are embryonic lethal. We are now attempting to create MG conditional knockouts of Dot1l.

Our experiments focused on *miR-216a* due to previous work and because we identified *dot1l* as a target of *miR-216a*. However, *miR-216b* is also repressed during retina regeneration. Because *miR-216b* has a different seed sequence, it targets a different set of mRNAs (it does not target *dot1l*). Of note, our morpholino based experiments deplete both *miR-216a* and *miR-216b* but all the

overexpression and rescue experiments were only done with *miR-216a*. It will be interesting to identify and test targets of *miR-216b* and to determine the mechanisms that underlie repression of both miRNAs because they are produced from the same polycistronic transcript.

Here, we report the discovery of a novel MG reprogramming mechanism driven by the H3K79 methyltransferase Dot1l that is under the regulation of *miR-216a. miR-216a* is repressed in response to photoreceptor loss in adult zebrafish, thereby de-repressing *dot1l*. Retinitis pigmentosa and age-related macular degeneration involve photoreceptor dysfunction that eventually leads to loss of vision. Previously, mouse retina regeneration could be induced in young mice (~2 weeks) by overexpression of the transcription factor Ascl1 and co-administration of a histone deacetylase inhibitor trichostatin-A (Jorstad et al., 2017). It will be interesting to test whether Dot1l plays a role in additional chromatin modifications in mammalian MG and whether suppressing *miR-216a* can induce mammalian MG proliferation and neural progenitor production.

Methods

Zebrafish Husbandry and Adult Zebrafish Light Lesioning

Wild-type (AB)(Walker, 1999), T*g(1016tuba1a:gfp)*(Fausett and Goldman, 2006b), *Tg(gfap:gfp)*^{mi2001} *(Bernardos and Raymond, 2006)* lines were maintained at 28.5^{0}C on a 14:10 hour light:dark cycle. All experiments with zebrafish were performed with the approval of the Vanderbilt University Institutional Animal Care and Use Committee. Adult zebrafish used for the experiments were between 5-12 months old. Constant intense light lesioning to induce cone and rod photoreceptor cell death was performed as previously described (Rajaram et al., 2014a). Briefly, adult fish were dark adapted for 14 days, transferred to clear tanks placed between two fluorescent lights with light intensity at ~20,000 lux for 16h-3days. The tank temperature was maintained at 30- 33°C.

Fluorescence Activated Cell Sorting (FACS)

FACS was used to isolate GFP$^+$ and GFP$^-$ cells from the retinas of undamaged Tg(*gfap*:gfp)^{mi2001} and Tg(1016*tuba1a*:gfp) fish using BD FACSAria III (BD Biosciences) at the VUMC Flow Cytometry Shared Resource. Retinas were dissociated as previously described (Rajaram et al., 2014b) with the following changes. After the retinas were dissected, they were collected in Leibovitz L-15 media (ThermoFisher #21083-027) and treated with 1mg/ml hyaluronidase (Sigma #H3884) at room temperature for 15 minutes on a rocker. Cells from the dissociated retinas were stained with propidium iodide to detect dead cells. 12 adult fish were used for each FACS experiment. As a quality control, sorted GFP$^+$ cells were re-analyzed to check the purity of the cell population by re-sorting.

Western Blots

Protein was collected from whole retinas by dissociation in 1x RIPA buffer with 1x SDS (Life Technologies) for 3 hours at 4°C followed by centrifugation at 12,000 rpm for 20 minutes. Protein concentrations were determined using BCA assays (BIO-RAD). 10µg of protein was separated on 12% MINI-PROTEAN TGX® pre-cast gels (BIO-RAD) and transferred to PVDF membranes using the Trans-Blot® Turbo Transfer System (BIO-RAD). Membranes were blocked in 5% milk in 1x TBS-T at room temperature for 1 hour. Primary antibodies against Dot1l (1:300; ab228766, Abcam) and α-tubulin (1:10,000; ab15246, Abcam) were incubated at 4°C overnight. Secondary anti-rabbit antibodies (1:50,000; 7074S, Cell Signaling Technologies) were incubated in 5% milk in 1x TBS-T at room temperature for 1 hour. For imaging, SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) was incubated with the blots for 60 seconds before exposure.

RT-PCR

Total RNA was isolated from FAC-sorted cells using TRIzol-LS (ThermoFisher # 10296028). Taqman small RNA assays (Life Technologies) were used to perform qRT-PCR of the indicated miRNAs. 5ng of total RNA was used per RT reaction and 1.33µl of 1:2 diluted resultant cDNA was used in 10µl qPCR reaction in technical triplicates. qPCR reactions were conducted in either 96-well plates using Bio-Rad CFX96 Real-time system or in 384-well plates using Bio-Rad CFX384 Real-time System. All quantifications were normalized to an endogenous U6 snRNA control. Fold changes were calculated using the ΔΔC(t) method, where Δ = C(t)miRNA − C(t)U6 snRNA, and ΔΔC(t) = ΔC(t)condition1 − ΔC(t) condition2, and FC = 2−ΔΔC(t). Taqman probe #: U6 snRNA: 001973; hsa-miR-216a:002220. For RT-PCR of mRNAs, RNA was DNase treated (TURBO DNAfree kit ThermoFisher #AM1907), converted to cDNA using Maxima first strand cDNA synthesis kit (Thermo Scientific) and qPCR was performed using SYBR Green (Biorad). All qPCR primers spanned exon-exon junctions (IDT). miRNA realtime PCR was

perfomed using Taqman probes as per the manufacturer's instructions (Life Technologies). Relative RNA expression during regeneration were determined using the ΔΔCt method and normalized to 18s rRNA levels and U6 snRNA levels for mRNAs and miRNAs respectively. Real time PCR was performed on a Biorad CFX 96 Real time system. The following primer sequences were used: dot1l-qpcr-fp: 5'-CATGATGCTGCACACGAAAT-3'; dot1l-qpcr-rp: 5'-TCTCGAAGCTCTTGGTGTCA-3'; 18srRNA-qpcr-fp: 5'-ACGCGAGATGGAGCAATAAC-3'; 18srRNA-qpcr-rp: 5'-CCTCGTTCATGGGAAACAGT-3'.

Plasmid Construction and Embryo Injections

The *dot1l* 3'UTR was amplified from cDNA by PCR with the following primers: dot1l-3'utr-fp: 5'-AGACTTGAATTCCCTTCCAGGAACTGAGTTTAACC-3' dot1l-3'utr-rp: 5'-AGTCTGCTCGAGCAGCTCCACAGGTAAATGATCC-3'. The 3'UTR was cloned downstream of the GFP coding sequence in the PCS2+ vector. miRNA recognition elements (MREs) within the *dot1l* 3'UTR were deleted using the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene). mRNAs were *in vitro* synthesized from linearized constructs using mMESSAGE mMACHINE® SP6 Transcription Kit (Life Technologies). *In vitro* transcribed RNA was purified by NucAway™ Spin Columns (Life Technologies). For reporter assays, GFP-dot1l-3'UTR mRNA was injected at 100pg/embryo concentration either alone or with a synthetic *miR-216a* duplex (Dharmacon) at 100pg/embryo.

Morpholino and miRNA Mimic Injection & Electroporation

Lissamine tagged morpholinos (MOs) (Gene Tools) were injected intravitreally and electroporated into adult zebrafish eyes prior to light lesioning as described (Thummel et al., 2006). The following 3'-Lissamine-tagged MOs were used: Gene Tools standard control MO: 5'-

CCTCTTACCTCAGTTACAATTTATA-3'; *Dot1* MO: 5'-CCCAGCTATACACACAAAAAGCAGC-3'; Dot1l MO-2: 5'AAGAGAACATTTCTCACCTCCTGGT-3'; *miR-216a* MO: 5'-TCACAGTTGCCAGCTGAGATTA-3'.

Duplex mature miRNAs (Thermo scientific) were injected and electroporated into eyes prior to start of light lesioning as previously described (Rajaram et al., 2014b). Double stranded mature miRNAs were synthesized with 3'- UU overhangs for the following target sequences: *miR-216*: 5'-UAAUCUCAGCUGGCAACUGUGAUU-3' control (*siLuc*): 5'-AAAAACAUGCAGAAAAUGCUG-3' Electroporation was performed using the Gene Pulser Xcell^{TM} Electroporation Systems (Biorad).

Pharmacological Treatment

To stimulate Wnt signaling, GSK-3β Inhibitor I (Calbiochem; CAS 327036-89-5) was intravitreally injected at 1mM in 4%DMSO. Wnt signaling was blocked by the tankyrase inhibitor/axin stabilizing agent XAV939 (Cayman chemical; 10mM stock in DMSO) intravitreally injected at 10µM in 4%DMSO. For catalytic inhibition of Dot1l, EPZ004777 (Calbiochem; CAS 1338466-77-5) was intravitreally injected.

Immunohistochemistry

Adult zebrafish eyes were collected and fixed in either 4% paraformaldehyde at 4^0C overnight or a fixant containing 9 parts 95% ethanol:1 part 37% formaldehyde (for dot1l IHC), cryoprotected in 30% sucrose/1X PBS before embedding. 10-12 micron sections were obtained using a cryostat (Leica), collected on charged Histobond slides (VWR), dried and stored at -80°C. For IHC, slides were warmed to room temperature, rehydrated in 1X PBS and blocked (3% Donkey serum, 0.1% TritonX-100 in 1X PBS) for 1–2h at room temperature before incubating with primary antibodies overnight at 4^0C. The following primary antibodies were used: mouse anti-PCNA monoclonal antibody (1:500, Sigma), anti-PCNA polyclonal antibody (1:500, Abcam), rabbit anti-GFP polyclonal antiserum (1:1000, Torrey Pines

Biolabs), mouse anti-β-catenin antibody (1:500, BD Bioscience) and rabbit anti-dot1l polyclonal antibody (1:200, Bethyl labs). After primary antibody incubation, sections were washed and incubated with secondary antibody and nuclear stain TOPRO 3 (1:1000, Invitrogen) at room temperature. Secondary antibodies were donkey anti-mouse AF488 (1:200), donkey anti-mouse AF647 (1:200), donkey anti-mouse Cy-3 (1:100), donkey anti-rabbit Cy3 (1:100) and donkey anti-rabbit AF488 (1:200)(Jackson Immuno). Slides were washed, dried and coverslipped with Vectashield (Vector labs). Antigen retrieval was performed for β-catenin and PCNA IHC as previously described (Rajaram et al., 2014b).

Imaging and Image Processing

For imaging of immunofluorescent staining, a META Zeiss LSM 510 Meta confocal microscope was used. Images were processed using ImageJ software 4.13. Fluorescence intensity measurements for the GFP reporter assays were done using ImageJ software (Gavet and Pines, 2010). For each image, "integrated density", "area" and "mean gray value" GFP^+ region, as well as background, were measured. Corrected fluorescence intensity of the selected region was calculated according to the formula: "Corrected fluorescence intensity= Integrated Density - (Area of selected region * mean fluorescence of background)". Data were represented as a mean of corrected fluorescence intensity for each experimental condition and statistical analyses were performed using a two-tailed Student's t-test. For immunostaining and cell quantification, only retina sections that comprised optic nerves were used. All cell counts were done in the central-dorsal retina, at a linear distance of ~300 microns from the optic nerve. Cells from one to four sections were quantified and averaged from each eye. In all figures, data are represented as mean +/- standard error of the mean (s.e.m). Significance was calculated either by the non-parametric Mann–Whitney U test or one-way ANOVA with Dunnett's multiple comparisons test for cell quantifications.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Summary of Results

Research presented in this dissertation identified novel regulatory functions of $GABA_A$-ρ receptors and *miR-216a* in retina regeneration in zebrafish. For $GABA_A$-ρ receptors, I discovered that inhibition of these receptors can induce a proliferative response in the zebrafish retina. $GABA_A$-ρ receptors were found on MG cell processes, providing further evidence that GABA acts directly on MG. In addition, I found that $GABA_A$ and $GABA_A$-ρ receptors act in a synergistic manner to regulate the initiation of retina regeneration. For *miR-216* and Dot1l, the H3K79 methyltransferase, Dot1l, is significantly upregulated after damage in the zebrafish retina and seems to activate regeneration through the Wnt cascade, consistent with the finding that the GSK-3β inhibitor is sufficient to rescue proliferation after Dot1l knockdown. Importantly, the loss of proliferation due to *miR-216a* mimics or *dot1l*-MO electroporation is not due to loss of MG. Together, the studies in this thesis demonstrate two novel factors that regulate initiation of retina regeneration in zebrafish.

Discussion and Future Directions

Mammalian vs Teleost Fish Response to Retinal Damage

Mammals have a quite different response to retinal damage compared to teleost fish, such as zebrafish. While zebrafish undergo spontaneous regeneration, mammals undergo a process called reactive gliosis. MG hypertrophy and upregulation of GFAP after retinal damage are early indicators of retinal stress and MG reactivity (Bignami and Dahl, 1979; Grosche et al., 1995; Lewis and Fisher, 2003; Xue et al., 2010). This is also accompanied by upregulation and release of neurotrophic factors by MG, such as FGF-2, as well as phagocytosis of blood-derived proteins (Bringmann and Wiedemann, 2012).

While this process is initially neuroprotective, slowing further retinal cell death, persistent gliosis is detrimental to retinal health. This will eventually lead to loss of normal MG function and further retinal cell death (Bringmann et al., 2006a; Ganesh and Chintala, 2011).

Persistent gliosis can either be non-proliferative or proliferative. Non-proliferative gliosis is associated with mild retinal damage, and results in continued MG hypertrophy, upregulation of GFAP, and downregulation of glutamine synthetase. Glutamine synthetase is necessary for glutamate recycling, and loss of this function leads to retinal neuron excitotoxicity (Bringmann and Wiedemann, 2012). Proliferative gliosis is associated with severe retinal damage. This type of response results in loss of normal homeostatic function and unregulated MG proliferation caused by inactivation of the cell cycle regulator cyclin-dependent kinase inhibitor *Cdkn1b* (Dyer and Cepko, 2000; Vazquez-Chona et al., 2011). This proliferation of MG often results in mislocalized retinal neurons, which contribute to form a glial scar that can severely compromise vision (Bringmann and Wiedemann, 2009; Lewis et al., 2010).

In contrast to the mammalian response, teleost fish such as zebrafish undergo spontaneous regeneration in response to retinal damage. Following damage, the MG will dedifferentiate and undergo asymmetric cell division to form a new progenitor cell. The progenitor cell will then undergo multiple rounds of division to form clusters or progenitor cells, which will then migrate to the damaged area before differentiating into new retinal neurons (Bernardos and Raymond, 2006; Fimbel et al., 2007; Fausett et al., 2008b; Thummel et al., 2008a).

Recent studies have looked at the initial response to retinal damage in zebrafish. Interestingly, zebrafish show signs of undergoing reactive gliosis in the early stages of responding to damage with upregulation of GFAP and early neuroprotective activity (Thomas et al., 2016). This study found that by further restricting MG proliferation that persistent reactive gliosis occurs in zebrafish, resulting in MG hypertrophy, continued upregulation of GFAP, and increased retinal neuroprotection. This early reactive

gliosis response in zebrafish suggests that mammals and teleost fish do not have distinctly separate responses to damage, but rather both have the capacity to become reactive and to act as stem cells for regeneration.

The next step in investigating this process is to determine what blocks mammalian retinas from transitioning to a controlled proliferative state after initiating a reactive gliosis response. A recent study found that the Hippo pathway repression of the transcription cofactor YAP blocks mammalian MG reprogramming and proliferation (Rueda et al., 2019). This study found that by knocking out elements of the Hippo pathway (Lats1 and Lats2) and inducing transgenic expression of YAP in MG in mice, the MG significantly upregulated cyclin D1, dedifferentiated, and began proliferating progenitor-like cells.

Despite the significant findings of these studies, it is still unknown whether the progenitor-like cells generated in mammalian retina regeneration studies are capable of correctly differentiating to replace damaged cells. It is likely, given the endogenous mammalian response to retinal damage, that further intervention would be required outside of repressing the Hippo pathway. This is because with another recent study, despite inducing formation of progenitor cells by transfecting the retina of a blind mouse with a β-catenin construct to activate wnt signaling, further factors were required to induce the progenitor cells to differentiate to the correct cell fate (Yao et al., 2018).

$GABA_A$-ρ Receptors

The work presented here suggests that GABA directly acts on MG through GABA receptors. Both pharmacological inhibition as well as morpholino-mediated knockdown of $GABA_A$-ρ receptors was sufficient to induce a proliferative response. Importantly, this response is not due to damage resulting from the inhibitor, as shown by *TUNEL* stain (**Figure 7**). *In situ* experiments show that $GABA_A$-ρ receptors are actually found on MG, previously thought to be mainly found on bipolar and horizontal cells

(Feigenspan et al., 1993; Qian and Wilusz, 1993; Lukasiewicz, 1996). This presents two distinct possibilities. It is possible that the colocalization shown by the *in situ* is not real. In addition, the presence of RNA transcripts does not mean that the protein is actually expressed. However, it is also possible that the difference in detection via *in situ* could be due to the increased sensitivity and signal amplification from the newer technology of RNAScope (ACDBio), and that the colocalization is real.

A key remaining question is whether inhibition of GABA receptors is detected directly by MG. GABA receptors in the retina are thought to mediate signals along the phototransduction pathway (Lukasiewicz and Shields, 1998). It is therefore possible that inhibition of GABA signaling is predominantly blocking these signals, which are then sensed by MG. However, previous work by (Rao et al., 2017) provides some evidence that MG are capable of directly mediating GABA signals Electroporation of a dominant negative γ2 GABA subunit under control of the GFAP promoter, a glial-specific promoter, led to increased proliferation. The work in this dissertation provides additional evidence supporting this hypothesis. Both *in situ* hybridization experiments and sequencing of sorted MG show that MG transcribe RNA encoding the various GABA subunits.

An important question moving forward is if GABA levels are directly sensed by MG. In the future, generation of zebrafish-specific antibodies against various GABA receptor subunits will allow for a detection of protein levels, as opposed to RNA expression studies used here. Ideally, a transgenic conditional knockdown will be generated to block expression of GABA receptors only in MG As the ρ2a subunit is the most abundant ρ subunit in the retina (Cocco et al., 2017), and a previous study showed the potential effectiveness of a dominant negative γ2 subunit (Rao et al., 2017), targeting these two subunits should inhibit both $GABA_A$-ρ and $GABA_A$ receptors, respectively. While I have shown here that regeneration-associated genes are upregulated after inhibition of $GABA_A$-ρ receptors (**Figure 10**), it is important moving forward to understand the immediate effects on MG of GABA signaling inhibition. If the hypothesis of direct mediation of GABA signaling by MG is correct, the most immediate effect on

MG is predicted to be an upregulation of TNFα. A recent study has shown that a decrease in cytoplasmic Cl^- ions results in upregulation of TNFα (Yang et al., 2012), which is also one of the earliest signals in retina regeneration (Nelson et al., 2013). Generation of a transgenic zebrafish line expressing a Cl^- specific channelrhodopsin under control of the GFAP promoter would allow for MG-specific activation of these ion channels and provide an excellent test of the hypothesis. If our proposed model is correct, activation of channelrhodopsins during light-induced retina damage should delay or even prevent MG dedifferentiation.

miR-216a and Dot1l

The work presented in this dissertation suggests that *miR-216a* regulates MG dedifferentiation through targeting the H3K79 methyltransferase Dot1l. GFP reporter assays showed that *miR-216a* targets the 3'-UTR of Dot1l (**Figure 17**), and upregulation of *miR-216a* decreased the levels of Dot1l (**Figure 18E**). qPCR analysis of Dot1l showed it is upregulated in dedifferentiated MG (**Figure 18B**), and knockdown of Dot1l was sufficient to inhibit the MG proliferative response to damage (**Figure 20**).

Dot1l and Epigenetic Regulation of MG Dedifferentiation

During development, lineage-specific epigenetic modifications lead to eventual fixation of lineage fate as pluripotent cells differentiate. This presents major roadblocks in cell reprogramming, in that fully differentiated cells only inefficiently reprogram to a more stem cell-like state (Takahashi and Yamanaka, 2006; Wernig et al., 2007; Zhou et al., 2008). However, manipulating chromatin modifying-enzymes can increase the efficiency of reprogramming cells (Pasque et al., 2011; Onder et al., 2012; Rais et al., 2013). It is therefore likely that epigenetic changes such as observed with Dot1l are involved in MG reprogramming.

No differences in DNA methylation have been detected across pluripotency- and regeneration-associated genes between postmitotic and dedifferentiated MG (Powell et al., 2012; Powell et al., 2013). Ascl1a, Hb-egfa, lin28, Oct4 and Sox2 all show low levels of DNA methylation in both zebrafish and mice (Powell et al., 2013). This suggests that DNA methylation does not play a role in the decreased regenerative potential in mammals. Instead, it seems likely that histone and chromatin epigenetic modifications are involved in retina regeneration.

Because H3K79 methylation is associated with euchromatin and active transcription (Feng et al., 2002; Shanower et al., 2005; Jones et al., 2008; Steger et al., 2008), it is possible that this methylation is required for expression of various genes associated with dedifferentiation of MG. Previous studies show that Dot1l activity is associated with activation of Wnt-target genes via interaction with TCF transcription factors and β-catenin (Clevers, 2006; Mahmoudi et al., 2010; Mohan et al., 2010; Castaño Betancourt et al., 2012). Dot1l was found to form a complex with Tcf4 and β-catenin in zebrafish and mice (Mahmoudi et al., 2010). The current hypothesis is that β-catenin recruits Dot1l to wnt-target genes. Dot1l would then methylate H3K79 at wnt target genes, providing a more transcriptionally available region.

The work in this dissertation shows that Wnt signaling acts downstream of Dot1l during retina regeneration. The inhibitor GSK3β, an activator of Wnt signaling, was sufficient to rescue proliferation after knockdown of Dot1l (**Figure 25**). While this result supports the hypothesis that Dot1l is an upstream positive regulator of Wnt signaling during retina regeneration, further work is necessary to understand how regulation of H3K79 levels by Dot1l affects MG during retina regeneration. Dot1l was shown to be necessary for regeneration (**Figure 20**). However, wnt signaling in the absence of Dot1l is sufficient to partially rescue the proliferative response during light damage (**Figure 25**). This suggests that β-catenin is able to induce transcription of wnt target genes without Dot1l activity. From this, I hypothesize that these wnt target genes are already primed for transcription. Given the results here,

these genes are likely kept in a state where they are transcriptionally available. I further hypothesize that Dot1l further increases the availability of wnt target genes for transcription. Given that Dot1l was shown to be necessary for regeneration, I would also hypothesize that Dot1l either helps stabilize the β-catenin/Tcf4 complex or helps destabilize the β-catenin destruction complex. Due to its interaction with β-catenin and Tcf4, I would more strongly support that it stabilizes the β-catenin/Tcf4 complex.

One important experiment for further study is the generation of a conditional knockout of Dot1l in MG. This would allow for specific targeting of MG and give concrete evidence that the interaction of *miR-216a* and Dot1l is directly mediated in MG. Generation of zebrafish-specific Dot1l antibodies would also allow for chromatin immunoprecipitation (ChIP) experiments, specifically ChIP-sequencing. Another potential approach to this would be to use CRISPR to insert an epitope tag attached to Dot1l. These would provide information on which genes are specifically affected by Dot1l-mediated histone modification. Based on the work in this dissertation, it is likely that Dot1l acts on Wnt-target genes. However, it is also likely that Dot1l acts to methylate histones associated with other genes upregulated early in retina regeneration, such as *ascl1*.

GABA and miR-216

The work presented here presents further evidence for the hypothesis that GABA is an initial regulator for retina regeneration, while *miR-216* acts as a regulator for chromatin remodeling prior to MG reprogramming. In this hypothesized model, loss of GABA signaling to MG leads to an upregulation of TNFα. This increase in TNFα expression leads to an eventual decrease in *miR-216* expression, which directly leads to an increase in Dot1l expression. Increased Dot1l expression then results in H3K79 methylation of wnt-target genes, leading to MG reprogramming and retina regeneration.

Conclusion

In conclusion, the work presented here identified two distinct mechanisms driving initiation of retina regeneration. Inhibition of $GABA_A$-ρ receptors was shown to inhibit retina regeneration in a model that seems consistent with a very early step in regeneration disrupting a key neuronal network that controls stem cell quiescence. In addition to GABA levels controlling the initial signals that lead to the initiation of regeneration, miRNAs were shown to regulate Dot1l. Dot1l must be activated during retina regeneration in a model consistent with activation of Wnt signaling.

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