The Role of the Notch Signaling Pathway during Postembryonic Retinal Neurogenesis in *Danio rerio*

by

Ru Yun Chiang

A thesis submitted in conformity with the requirements
for the degree of Master of Science

Department of Cell and Systems Biology
University of Toronto

© Copyright by Ru Yun Chiang 2015

The Role of the Notch Signaling Pathway during Postembryonic Retinal Neurogenesis in *Danio rerio*

Ru Yun Chiang

Master of Science

Department of Cell and Systems Biology
University of Toronto

2015

Abstract

The retina of teleost fish, such as zebrafish (*Danio rerio*), exhibits remarkable capacity for continuous neurogenesis throughout life – unlike mammalian retina. Postembryonic retinogenesis in zebrafish is mainly contributed by active stem/progenitor cells in ciliary marginal zone (CMZ). Notch signaling has been reported to regulate embryonic retinogenesis and Notch pathway components are expressed in adult zebrafish CMZ. Here I address the functions of Notch signaling during postembryonic retinogenesis in zebrafish. My research revealed that: (1) Notch signaling is activated in the transition zone of CMZ and differentiated retina; (2) CMZ cells do not exhibit Notch activity and do not require Notch signaling for maintenance; and (3) Notch signaling is required and sufficient for proper differentiation of Müller glia, cone photoreceptor and bipolar cells. These studies demonstrate how different levels of Notch activity regulate retinal stem cell behavior and provide insight into how to reactivate mammalian adult retinal stem cells *in vivo*.

Acknowledgments

I owe my deepest gratitude to my supervisor, Dr. Vincent Tropepe, for his continuous support and encouragement throughout the research. His excellent guidance and enthusiasm have motivated and helped me through the challenges of this thesis work. My graduate training in the Tropepe lab has allowed me to not only develop my research skills but also build my confidence to think critically and independently.

I express my sincere thanks to the members of my supervisory committee, Dr. Ian Scott and Dr. Tony Harris. The research would not have been possible without their continued support and inspiring suggestions.

I am extremely grateful to all my colleagues in the Tropepe and Bruce lab for their guidance, suggestions and friendships throughout the past few years. I would like to extend special thanks to Dr. Ashley Bruce for her knowledgeable guidance and suggestions on zebrafish and its husbandry. Gratitude is extended to other colleagues and friends from the Department of Cell and Systems Biology at the University of Toronto. In particular, I am gratefully indebted to Henry Hong and Audrey Darabie of the Imaging Facility, for their invaluable training and suggestions as well as their friendships. I am sincerely thankful to Ian Buglass, the Graduate Administrator at the Department of Cell and Systems Biology, for his patience and expertise.

I would like to acknowledge the Zebrafish International Resource Center for providing the *Tg(hsp70l:Gal4)* and *Tg(UAS:6xmyc-n1a-ICD)* fish lines; Dr. Jason Fish from the Toronto General Hospital for giving me the *Tg(Tp1bglob:eGFP)* fish line and Dr. Ajay Chitnis from the National Institutes of Health, the United States of America for sending me *notch1a* plasmid.

Heartfelt thanks are owed to my family for their infinite supports, motivations and patience. Thank you Mom and Dad for always believing in me and encouraging me to develop my interest and perform my best. I would not have been who I am without your endless love. I love you both.

Table of Contents

Acknowledgments iii

Table of Contents iv

List of Figures vii

Chapter 1 Introduction 1

1.1 General Introduction 1

1.2 Retinogenesis in vertebrates 2

1.2.1 Overview of vertebrate retinal structure 2

1.2.2 Molecular regulations of embryonic retinal development 4

1.2.3 Postembryonic retinal growth in vertebrates 6

1.2.4 Zebrafish as a model of postembryonic retinal neurogenesis 9

1.3 Zebrafish postembryonic retinal stem cell niches and Notch 9

1.3.1 Notch pathway components are expressed in the CMZ 9

1.3.2 Notch regulates Müller glia-derived retinal progenitors 10

1.4 Notch signaling pathway 11

1.4.1 Overview of the pathway 11

1.4.2 Notch regulates a variety of developmental processes 13

1.5 Notch pathway during vertebrate retinal development 14

1.5.1 RPC maintenance 14

1.5.2 Retinal cell fate determination 15

1.5.3 Notch and the cell cycle 16

1.6 Thesis objective and aims 16

Chapter 2 Methods and Materials 18

2.1 Fish care and use 18

2.1.1 Zebrafish husbandry and transgenic fish lines 18

2.1.2 Phenyl 2-thiourea (PTU) treatment 18

2.1.3 Notch1a-intracellular domain over-expression 19

2.1.4 Notch signaling pathway inhibition 19

2.1.5 Bromodeoxyuridine administration 19

2.1.6 Antisense morpholino injection 19

2.2 Cryosectioning 20

2.3 Immunohistochemistry 20

2.4 *in situ* Hybridization 21

2.5 Protein Electrophoresis and Western Blot 22

2.6 Imaging and Statistical Analyses 23

Chapter 3 Results 24

3.1 Notch pathway expression pattern in larval fish retina 24

3.1.1 Notch components are expressed in the postembryonic retina 24

3.1.2 Notch activity in the retina 26

3.2 Inhibiting the pathway in larval fish retina 30

3.2.1 Validating the effect of DAPT 30

3.2.2 Inhibited Notch activity does not affect RPC proliferation 35

3.2.3 Inhibited Notch activity alters the composition of new-born cells at the peripheral retina 37

3.3 Up-regulating the Notch pathway in larval zebrafish retina 45

3.3.1 Induced NICD expression 45

3.3.2 Induced Notch activity does not alter RPC proliferation in the CMZ 46

3.3.3 The composition of newborn cells is altered after Notch gain of function 50

Chapter 4 Discussion 63

4.1 Summary of the results 63

4.2 Notch-dependent cell fate determination during postembryonic retinogenesis 64

4.3 Müller glia, Notch activity and stemness 69

4.4 Numb as a candidate for inhibiting Notch activity in the CMZ 74

4.5 Conclusion 77

References 78

List of Figures

Figure 1. Lamination of the vertebrate retina. 3

Figure 2. The postembryonic retinogenesis in the normal and regenerating retina of teleost fish. 7

Figure 3. Overview of the Notch signaling pathway. 12

Figure 4. Expression patterns of Notch pathway components *notch1a* and *deltaC* on 5.5 dpf AB wild-type fish retina sections. 25

Figure 5. Expression patterns of Notch activity in 7.5 dpf (A, B, D) and 8 dpf (C) *Tg(Tp1bglob:eGFP)* as well as 5 dpf *Tg(her4.1:mCherry,Cre-ERT2)* (E, F) zebrafish retina were analyzed with immunohistochemistry. 28

Figure 6. Expression pattern of *her6*, a Notch-independent progenitor marker, on 5.5 dpf AB wild-type fish retina sections. 29

Figure 7. DAPT effectively decreases Notch activity in the postembryonic Müller glial cells. .. 32

Figure 8. The peripheral retinal cells (specifically Müller glia and photoreceptors) are more sensitive to the decreased Notch activity than their central counterparts. 33

Figure 9. The inhibition of Notch activity does not alter the RPC proliferation in the CMZ. 36

Figure 10. Inhibited Notch activity alters the composition of newborn retinal cells at the peripheral retina. 40

Figure 11. Notch activity is required for newborn cells to adopt a Müller glial cell fate and the inhibition of Notch activity decrease the proportion of Müller glial cells in the newborn retinal population. 41

Figure 12. Notch activity is required for proper differentiation of cone photoreceptors and the inhibition of Notch activity decreases the proportion of cone photoreceptors in the newborn retinal cohort. 42

Figure 13. The inhibition of Notch results in a noticeable, but not significant, increase of the bipolar cells in a cohort of postembryonically newborn retinal population. 44

Figure 14. Mis-expression patterns of the ectopic myc-labeled NICD in the 5.5 dpf *Tg(hsp70l:Gal4);(UAS:6xmyc-notch1a-ICD)* fish retina. 48

Figure 15. The induced Notch activity does not alter the RPC proliferation in the CMZ. 49

Figure 16. Forced expression of Notch1a-ICD disrupts the retinal lamination at the margin of postembryonic retina and more Müller glia are produced in the retina as clusters. An enhanced zn5signal is also observed in the Müller glia upon up-regulating Notch activity.... 52

Figure 17. Up-regulated Notch activity alter the composition of retinal cells at the peripheral retina at 7.5 dpf. 54

Figure 18. Notch signaling promotes the postembryonic retinal progenitors to adopt a Müller glial cell fate. 57

Figure 19. Up-regulated Notch signaling leads to overproduction of cone photoreceptors in the postembryonic zebrafish retina. 59

Figure 20. The induction of NICD leads to a decrease in the proportion of bipolar cells in the postembryonic newborn retinal cells. 61

Figure 21. Expression pattern of *numb* in 5.5 dpf AB wild-type zabrafish retinal sections. 76

Chapter 1
Introduction

1.1 General Introduction

The search and study of adult retinal stem cells, with the ultimate goal to deliver therapeutic solutions for recovering vision loss, is an emerging field of research. Specifically, the design of an endogenous retinal stem cell mobilization strategy includes stimulating the proliferation and differentiation of specific retinal cells and their functional incorporation into the damaged retina to restore vision loss. This regenerative strategy is possibly less invasive and less immuno-responsive, which is of great advantage compared with existing transplantation based therapies.

While in teleost fish remarkable capacities of postembryonic neurogenesis and regeneration have been reported, the adult mammalian retinal progenitor cells exhibit limited plasticity and proliferative ability. However, opportunities have come into view from recent studies that adult mammalian retinal stem cells, which are usually considered dormant, have the potential to react in response to signals like their counterparts in teleost fish. The studies of adult retinal stem cells in mammals have largely been focusing on how proliferation and differentiation are regulated in these cells and how they can be reprogrammed into a more proliferative state. The study of postembryonic retinal stem cells in model teleost fish, such as zebrafish, goldfish, or medaka, and their distinguishable proliferative ability may shed light on this process.

Two major types of postembryonic retinal stem cells in the fish retina have so far been reported: the retinal stem cell residing in the ciliary or circumferential marginal zone (CMZ), and Müller glia, which can be activated by a lesion to behave as stem cells. While these cells exhibit remarkable stem cell properties, the underlying mechanisms that regulate their proliferation and differentiation in the postembryonic retina are not well established. It has been considered that these stem cells reside in niches, especially those in the CMZ, that are under regulatory controls that are similar to the ones during embryonic retinal development. Notch signaling has been demonstrated to regulate embryonic stem cell behaviors in multiple aspects, from maintenance of the stem cell identity to cell fate decision control. The functional roles of this pathway in the postembryonic retinal stem cells, however, remain largely unresolved. Addressing this question is important for our understanding of how to “reactivate” the mammalian adult retinal stem cell

in that Notch signaling is crucial for the neurogenic activity of retinal stem cells not only in zebrafish, but also in the mammalian retina. This thesis aims to analyze the functions of the Notch signaling pathway during postembryonic retinogenesis in the zebrafish CMZ.

1.2 Retinogenesis in vertebrates

1.2.1 Overview of vertebrate retinal structure

The retina is a layered neural structure that converts external light into electrical and chemical signals and then transmits the impulses to the brain. In vertebrates, the morphology of the retina is highly conserved across species (Stenkamp, 2007). The well-organized vertebrate retina comprises three major cellular layers: the retinal ganglion cell layer (GCL), which is composed of ganglion cells and some amacrine cells; the inner nuclear layer (INL) consisting of bipolar cells, amacrine cells, horizontal cells and Müller glia; and the outer nuclear layer (ONL) formed by cell bodies of rod and cone photoreceptors (Fig 1). Dividing these cellular layers are two plexiform layers, which consist of networks of neuronal synapses.

In the visual pathway, the photoreceptors respond to the presence of light and synapse directly onto the bipolar cells, which in turn synapse to ganglion cells. In addition to the convergence and divergence of visual inputs through synaptic processes, the horizontal cells and amacrine cells also modify the signal by transmitting the information laterally. The axons of the ganglion cells form the optic nerve that exits the retina, followed by crossing at the optic chiasm and projecting to several other brain regions contralaterally and ipsilaterally.

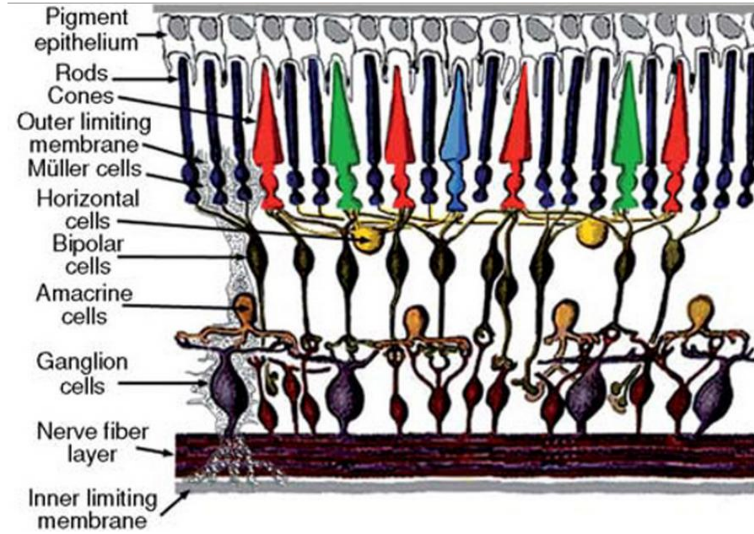


Figure 1. Lamination of the vertebrate retina. Diagram adapted from Stenkamp (2007). The highly conserved vertebrate retina contains three major cellular layers and two plexiform layers: the retinal ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL) and outer nuclear layer (ONL).

1.2.2 Molecular regulations of embryonic retinal development

During embryonic retinal development, different cell types are generated from a pool of multipotent retinal progenitor cells (RPCs) (Turner and Cepko, 1987; Turner et al., 1990; Wetts and Fraser, 1988). The RPCs possess stem cell like properties: a relatively undifferentiated cell identity, limited self-renewal, and the potential of giving rise to all seven retinal cell types mentioned above. As RPCs undergo various cell divisions, they become restricted in proliferative potential and specified in cell fate competence before their terminal divisions (Agathocleous and Harris, 2009). The progenitors give rise to different neurons and glia following an evolutionarily conserved sequence (Bassett and Wallace, 2012; Cepko, 2014). Birth dating studies have shown the overall order in rodents (Rapaport et al., 2004): ganglion cells are first to be generated, followed by the birth of horizontal cells, cone photoreceptors and amacrine cells. Rod photoreceptors, bipolar cells and Müller glia are formed at later stages. However, the genesis of these distinct retinal cells is not mutually exclusive at any given time.

The highly variable outcomes from the same pool of RPCs indicate that the RPCs might be genetically heterogeneous in terms of gene expression patterns (Blackshaw et al., 2004; Livesey et al., 2004; Trimarchi et al., 2008; Trimarchi et al., 2007) and thus result in different competency states (Cepko, 2014). Here I will briefly discuss some of the cellular and molecular factors that shape this process.

Retinal neurogenesis is regulated intrinsically in many ways since different cell types can arise from the same retinal environment at a same time. One of the regulating systems involves a network of transcription factors (TFs). For example, the retinal identity is defined by several eye-field transcription factors including retina and anterior neural fold homeobox 1 (Rax1), paired box gene 6 (Pax6), SIX homeobox 3 (Six3), optic Six gene 2 (Optx2) as well as many other TFs to form the presumptive eye field during early development (Zuber et al., 2003). While most of these TFs mark the retinal identity and stimulate RPC proliferation at early developmental stages, they may also take on different roles later. Rax1, for example, maintains the pluripotency of RPCs during retinal development but and is as well required for photoreceptor differentiation in zebrafish embryonic retina (Nelson et al., 2009). As retinogenesis progresses, further cell differentiation is shaped in a central-to-peripheral wave as TFs participate in permitting, inducing or preventing the generation of specific cell types.

Another intracellular regulating system is the control of cell cycle, especially G1 phase when cells exit the cycle and start to differentiate (Dyer and Cepko, 2001). Cell differentiation can be coupled with cell cycle through the cell cycle regulators, and the duration of cell cycle also affects differentiation. In addition, epigenetic changes including histone deacetylation, chromatin remodeling and asymmetric determinants inherited during cell division may also contribute to the differentiated fates of daughter cells (Katoh et al., 2012; Rhee et al., 2012).

Extrinsic pathways and environmental signals also participate in retinogenesis through mediating intrinsic regulation systems. Examples include, but are not limited to: Wnt pathway, Hedgehog (Hh) pathway, fibroblast growth factor (FGF) pathway and Notch pathway (Agathocleous and Harris, 2009). Studies on Wnt pathway suggest its multiple roles in retinal development including activating proneural TF expression, promoting proliferation and inhibiting differentiation of RPC, which might vary from species to species (Kubo and Nakagawa, 2008). The Hedgehog pathway also acts in the process, but its role appears to be more complex. The Hh signaling from retinal ganglion cells (RGCs) not only functions in structuring retinal organization, promoting cell cycle exit and accelerating cell cycle, but also regulates ganglion cell differentiation and even the guidance of RGC axons to the optic disc (Kolpak et al., 2005; Neumann and Nuesslein-Volhard, 2000; Wallace, 2008). The Notch pathway in retinal neurogenesis is particularly important for maintaining RPC identity and inhibiting neuronal differentiation (more details will be discussed later). The interaction of these networks within a cell, as well as their role in mediating communication between cells shape the complexity and diversity of their effects on retinogenesis. With time, more differentiated retinal cells are generated and they may also alter the RPC environment through secreting other signals (Hashimoto et al., 2006).

Nuclear migration and different orientations of cell division allow the new-born cells to be exposed to different microenvironments. Nuclear migrations are categorized into interkinetic nuclear migration (INM) in which the nuclei of neuroepithelial cells oscillate with cell cycle in an apical-basal manner, and nuclear translocation in which the soma of the postmitotic cells migrate, also along the apical-basal axis. The nuclear migrations, especially INM, may potentially contribute to the cell fate diversification as cells from the same clone are under the influence of diverse microenvironments (Baye and Link, 2008). In addition, INM may be linked to cell cycle progression and inhibiting INM leads to premature cell cycle exit and differentiation

(Agathocleous and Harris, 2009). In the retinal neuroepithelium, the cell body is on the apical side as the cell enters into G1 phase, and moves along to the basal side through G1 progression. The progenitor cell enters S phase at the basal side, and then, upon the entry of G2, migrates back to the apical side to complete mitosis (Buchman and Tsai, 2008; Del Bene, 2011). The orientation of division may allow the daughter cells to face different signals (Martins and Pearson, 2008), which can contribute to asymmetric cell fates (Poggi et al., 2005).

1.2.3 Postembryonic retinal growth in vertebrates

As retinal development progresses, a clone of RPC might be exhausted and all postmitotic daughter cells commit to a differentiated fate. However, other possibilities exist. In fish and amphibian retinas, some RPCs in the peripheral retina retain their proliferative ability beyond embryonic stages (Johns, 1977). These progenitors reside in the ciliary (or circumferential) marginal zone (CMZ) of the retina and contribute to the ongoing retinal growth under physiological and regenerative conditions during postembryonic stages (Fig 2). These RPCs also possess stem cell-like properties and, just like their embryonic counterparts, express a variety of eye-field transcription factors. In fact, the expression patterns of TFs in the amphibian retina have provided evidence for the heterogeneity of this RPC population: The most peripheral progenitors are less committed and express early eye-field transcription factors including *pax6*, *rx1* and *six3*, but not proneural TF. The more central progenitors become more committed and express more proneural genes such as *ath5* and *neuroD* (Casarosa et al., 2005; Perron and Harris, 2000; Perron et al., 1998).

In line with this, the proliferative activity of RPCs in the CMZ is spatially divided into five regions in *Xenopus* (Ohnuma et al., 2002). The most peripheral stem cells with lower expression of cell cycle activators divide slowly. Moving centrally to the next two regions, the expression of cell cycle activators are up-regulated and the RPCs here divide rapidly, in order to increase the rate of proliferation and expand the pool of RPCs. Proliferation is then down-regulated as cells move toward to the last two regions where the RPCs enter their last cell cycle and neuronogenesis and gliogenesis take place. The post-mitotic cells will ultimately differentiate into mature retinal cells and are added to the existing retinal circuitry.

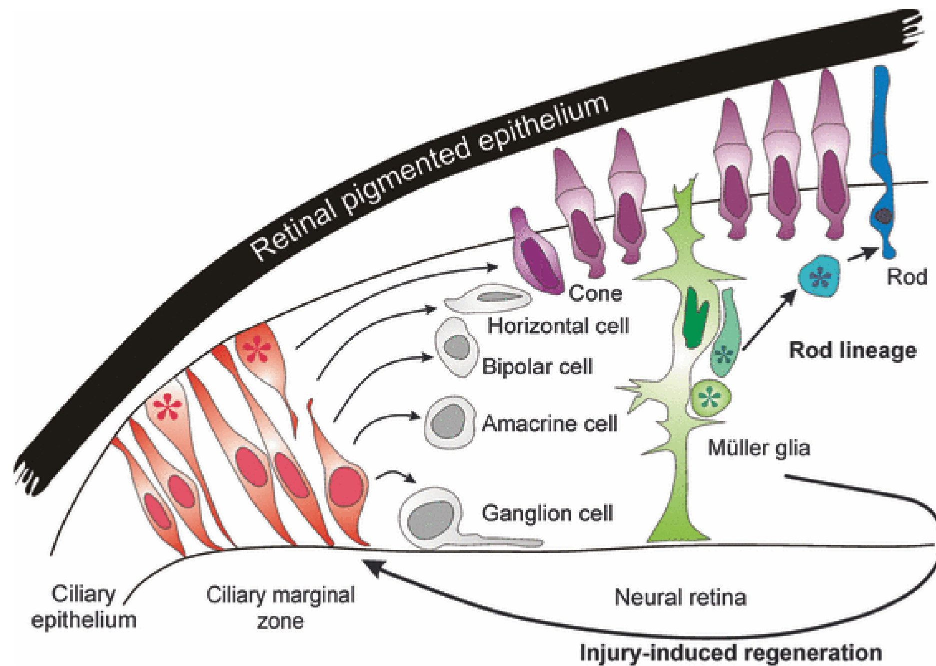


Figure 2. The postembryonic retinogenesis in the normal and regenerating retina of teleost fish. The multipotent retinal progenitor cells (RPCs, cells in red) reside in the ciliary (or circumferential) marginal zone (CMZ) are considered a remnant of the embryonic retinal stem and proliferating cells. They are proliferative and can give rise to a variety of retinal cells postembryonically: ganglion cells, amacrine cells, bipolar cells, horizontal cells, and cone photoreceptors. The Müller glia cells (cells in green) act as another contributor of RPC-like cells in the postembryonic retina. The glial cells can de-differentiate, proliferate and generate rod photoreceptors. While the CMZ cells are the main source of postembryonic retinal growth in zebrafish, all or most of the rod photoreceptors originate from the Müller glia-derived progenitors in the differentiated INL. The Müller glial cells are also important for retinal regeneration upon injury as they can de-differentiate and divide asymmetrically to generate a rapidly proliferating RPCs. The RPCs, together with the Müller glial cells, can create a regenerative niche and thus lead to the regeneration of damaged cells (Raymond et al., 2006). Modified from Adapted from Zupanc and Sirbulescu (2011).

In addition to the intrinsic factors mentioned above, the progenitors in the CMZ are under control of a number of signaling pathways, including but not limited to: Insulin-like Growth Factor (IGF) pathway, Hh pathway, Wnt pathway and Notch pathway (Locker et al., 2010). Together, a general spatial progression of neurogenesis is established in this postembryonic niche and the underlying molecular regulations are similar to the ones observed in the central retina during embryonic stages.

A CMZ-like marginal zone also exists in warm-blooded vertebrates. A periphery zone of proliferating cells is observed in the retina of hatched chicken. However the proliferative activity is transient and potency more limited (Fischer and Reh, 2000). In mouse retina, cells from the ciliary epithelium are demonstrated to possessed stem cell properties *in vitro*: the ability to self-renew and multipotency (Tropepe et al., 2000). More evidence arising from other mammals, including human, suggest the presence of cells with RPC identity in the mammalian retina (Coles et al., 2004; Gu et al., 2007; MacNeil et al., 2007; Xu et al., 2007a). Expression of TFs such as Pax6, Rx and Six3 are observed in the ciliary epithelium (Lord-Grignon et al., 2006; Martinez-Navarrete et al., 2008) and even linked to the maintenance of proliferation (Xu et al., 2007b). Yet these putative RPCs in mammals are usually quiescent or barely active *in vivo*, and their *in vivo* neurogenic potential is significantly constrained compared to their counterparts in fish and amphibians.

Another source of progenitor-like cells in the postembryonic vertebrate retina are the Müller glia cells (Fig 2). Radial glia function as support cells for the neurons, and are the only type of retinal glia derived from RPCs during embryonic development (Fischer and Bongini, 2010). Studies in several vertebrate classes have reported that Müller glia retain some RPC properties and are able to de-differentiate, proliferate and even give rise to retinal cells under certain conditions (Bernardos et al., 2007; Fischer and Bongini, 2010; Fischer and Reh, 2001; Karl et al., 2008). The molecular mechanisms underlying Müller glia-derived progenitors in response to injury has been reported (Lenkowski and Raymond, 2014). While the progenitor traits of Müller glia in most vertebrate classes are mainly induced by acute retinal damage and linked to retinal regeneration, Müller glia in teleost fish are well-characterized in their progenitor-like identity under physiological conditions. In fact, lineage-tracing studies in zebrafish have showed that Müller glial cells are the main source of rod lineage progenitors in the postembryonic fish retina

(Bernardos et al., 2007). Müller glial cells undergo de-differentiation and mitotically divide to produce Pax6^{+} neural progenitors in the INL. The expression of glial markers in these progenitors is down regulated as they translocate toward ONL and differentiate into rod photoreceptors. While CMZ cells are the main contributors of postembryonic retinal growth in zebrafish, all or most of the rod photoreceptors originate from the Müller glia-derived progenitors in the INL.

1.2.4 Zebrafish as a model of postembryonic retinal neurogenesis

Over the past decade, zebrafish has become a popular model organism for vertebrates. They are specifically favored for developmental research for their large number of offspring, external fertilization of embryos and rapid development (Fadool and Dowling, 2008). Specifically in the retina, the morphology, genesis and molecular controls of development in zebrafish are similar to that in other vertebrates including human (Fadool and Dowling, 2008; Stenkamp, 2007). In addition, an array of forward and reverse genetic approaches has been successfully applied to the fish system (Fadool and Dowling, 2008). Together with the active ongoing retinogenesis described above, the use of zebrafish as a genetic tool for studying postembryonic retinal development has become robust.

1.3 Zebrafish postembryonic retinal stem cell niches and Notch

1.3.1 Notch pathway components are expressed in the CMZ

A complex network of intrinsic and extrinsic regulators in postembryonic CMZ has been briefly discussed above; specifically in zebrafish, a molecular profile of CMZ cells has been reported in 2-month-old zebrafish (Raymond et al., 2006). A variety of genes are expressed in the CMZ cells, which include *rx1*, *pax6*, *vsx2* and several Notch pathway components and its downstream targets. However little is known about the exact functions of the Notch pathway in this postembryonic niche. Based on the better-characterized roles of the pathway in the embryonic retina, it is widely considered that the Notch pathway is activated and responsible for the maintenance of multipotency and specification of retinal cell fate in the CMZ, recapitulating its

embryonic mechanisms (Raymond et al., 2006). In line with this, Ohnuma's *Xenopus* model indicates that Notch pathway genes are highly expressed in region 2 and 3 of the CMZ and regulate cell cycle exit and even cell fate decisions (Ohnuma et al., 2002). Yet in zebrafish their influences on the proliferation and differentiation of the CMZ population remain unclear; thus, whether the function of the Notch pathway is conserved in the postembryonic retina in vertebrates requires further testing.

1.3.2 Notch regulates Müller glia-derived retinal progenitors

In zebrafish, Notch signaling is also reported to play a functional role in the differentiated Müller glia to maintain the glial cells quiescent in the undamaged retina (Conner et al., 2014; Wan et al., 2012). In response to injury, Notch pathway regulates dedifferentiation and proliferation of Müller glia (Raymond et al., 2006). Repressing the pathway in undamaged retina with the γ-secretase inhibitor caused a subset of Müller glia to express dedifferentiation markers and reenter the cell cycle, suggesting that Notch pathway acts as a negative control of regeneration in zebrafish (Conner et al., 2014).

However, the pathway might possesses different functional roles in the avian retina. While studies from Ghai *et al.* have reported that, similar to the postembyronic zebrafish retina, the components of the Notch signaling pathway are expressed at low levels in the Müller glia of undamaged postnatal chick retina (Ghai et al., 2010), the signaling is upregulated during damage-induced postnatal retinal regeneration in chick (Hayes et al., 2007). The inhibition of Notch activity during early retinal regeneration showed that Notch activity may be necessary for Müller glia to de-differentiate, but the blockade of Notch activity after the progenitors have derived led to an enhanced production of newborn neurons (Hayes et al., 2007). Furthermore, the inhibition of Notch activity has been reported to prevent the fibroblast growth factor (FGF)/mitogen-activated protein kinase (MAPK)-induced proliferaltion in Müller glia (Fischer and Bongini, 2010; Ghai et al., 2010).

Despite the fact that the functions of the Notch signaling pathway in postembryonic retinal development remains unresolved, it is clear that the signaling is expressed in the normal, undamaged retina and is crucial for retinal regeneration in response to damage. In the following

sections, I will introduce the canonical Notch signaling pathway and how it influences vertebrate retinal development.

1.4 Notch signaling pathway

1.4.1 Overview of the pathway

John Dexter first described the Notch pathway in 1914 in mutant *Drosophila* with a notched wing phenotype (Louvi and Artavanis-Tsakonas, 2006). The Notch signaling pathway is a highly conserved intercellular signaling system in metazoans, which allows short-distance interactions between cells (Fig 3). The mechanism relies on direct interaction of the single-pass transmembrane Delta–Serrate–LAG2 (DSL) ligand proteins with a notch receptor on adjacent cells. In zebrafish, multiple DSL ligands (DeltaA, B, C and D; Delta-like4; and Jagged1a, 1b and 2) and Notch receptors (Notch1a, 1b, 2, 3 and Notch-like) have been identified (Clements and Traver, 2013). The Notch receptors require post-translational modifications inside the cell where the fucosylated Notch precursors are cleaved at their S1 site by a furin-like convertase to render a heterodimeric transmembrane protein before they are transported to the cell membrane (Kopan and Ilagan, 2009). Upon ligand binding, the Notch receptors are cleaved at the S2 site in the extracellular domain by the ADAM-family metalloproteases, followed by another cut at the S3 site in the transmembrane region by γ-secretase (Kopan and Ilagan, 2009). The proteolyses release the Notch intracellular domain (NICD), which enters the nucleus and, together with a CSL family transcription factor (CBF1 or RBPJ-κ in mammals, Suppressor of Hairless in *Drosophila*, and LAG-1 in *C. elegans*), activates the transcriptions of Notch target genes. The activation also requires a transcriptional co-activator Mastermind (MAM), which recognizes and stabilizes the NICD/CSL complex, and together the complex recruits histone acetyltansferases and other activators to initiate transcription (Fryer et al., 2004). In some cases, CSL may repress the transcription of some targets genes with ubiquitous co-repressors and/or histone deacetylases in the absence of NICD. The allosteric binding of NICD thus recruits co-activators and acts as a transcriptional switch of the expression of target genes (Andersson et al., 2011; Kopan and Ilagan, 2009).

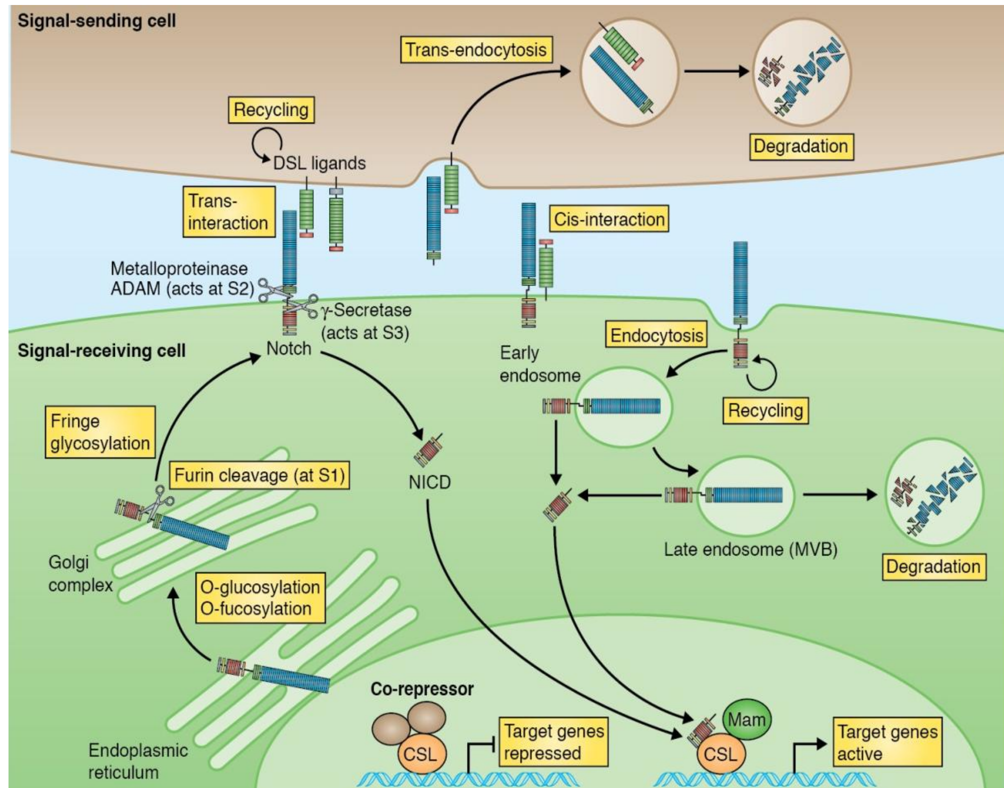


Figure 3. Overview of the Notch signaling pathway. Figure adapted from Hori et al. (2013). See text for details.

1.4.2 Notch regulates a variety of developmental processes

A broad spectrum of developmental events and diseases are regulated by the Notch pathway. Specifically for vertebrate neural development, the pathway regulates an array of cellular processes including neural and glial fate determination and differentiation, stem cell self-renewal, cell migration and cell death. In mature neural cells, Notch pathway is involved in synaptic plasticity, axon and dendrite growth and cell death (Louvi and Artavanis-Tsakonas, 2006). The versatility of the pathway relies not only on the pleiotropy of its downstream targets, but also on the ligand-receptor interactions (e.g., different ligand-receptor pairs, *trans*-activation and *cis*-inhibition) and a variety of modulations during signal transduction (e.g., Notch receptor modifications, endocytosis and trafficking of NICD) (Andersson et al., 2011; Guruharsha et al., 2012; Penton et al., 2012).

While here I focus on the functions of Notch signaling pathway in vertebrates, the invertebrate *Drosophila* model has been extensively studied over the past century and provided some valuable information on how the pathway regulates cell fate determinations. One of the best understood examples is the Notch-dependent binary cell fate decision of neural and epidermal cells in early *Drosophila* embryos (Cau and Blader, 2009; Heitzler and Simpson, 1991; Lewis, 1996; Parks et al., 1995). The Notch pathway is required to single out a neural precursor from a group of equipotent progenitors through lateral inhibition. Initially all of the precursor cells express proneural genes encoding basic helix-loop-helix (bHLH) transcriptional factors, which allow them to adopt a neural progenitor cell fate. However in the Notch signal-receiving cells, Notch activity upregulates the expression of the target gene family hairy and enhancer of split (Hes)/Hey that encode for transcriptional repressors of bHLH proteins (Jarriault et al., 1998; Jennings et al., 1994). The bHLH proteins not only promote neural differentiation but also promote the expression of Notch pathway ligands. Thus, Delta ligands in the signal-receiving cells are repressed by the NICD activation through proneural TFs and as a result the NICD activity in the original signal-sending cell is inhibited via this dynamic inhibitory feedback loop. This mechanism refines and amplifies the initial pattern, which allows a single cell within the progenitor group to downregulate its Notch activity (NICD activation) and retain its proneural gene expression. While this single cell commits to a neural cell fate, the neighboring cells with their neural cell fate inhibited by higher Notch activity give rise to an epidermal cell fate.

Emerging evidence from both vertebrate and invertebrate models has demonstrated that, other than its inhibitory role described above, Notch signaling pathway may have an instructive role, or act cooperatively with other factors, under other contexts. Hereafter I will only focus on the functions of the Notch signaling pathway during vertebrate retinal development.

1.5 Notch pathway during vertebrate retinal development

1.5.1 RPC maintenance

One of the most classical and major roles the Notch pathway performs in vertebrate retinal development is to maintain progenitor cells in an undifferentiated state. Evidence from several animal models has indicated that the pathway functions to prevent neuronal differentiation and maintain a pool of progenitors.

In Xenopus, *Xotch* expression is observed in the late embryonic retina and undifferentiated precursor cells in the CMZ. Continued activation of Notch signaling inhibits cell differentiation in the retina and maintains the progenitors undifferentiated during retinal development (Dorsky et al., 1997; Dorsky et al., 1995). Similarly, Henrique et al have demonstrated that Notch signaling controls a cell's choice between remaining as a progenitor and differentiating into a neuron in the embryonic chick retina. Nascent retinal neurons prevent the neighboring progenitors from entering differentiation via Notch-mediated lateral inhibition (Henrique et al., 1997). Studies on the developing mouse retina also show that inactivation of *Notch1* prior to the onset of cell differentiation leads to premature cell-cycle exit and neuronal specification (Yaron et al., 2006). Similarly, Riesenberg *et al.* have reported that in the prenatal mouse retina, removal of the transcription factor *Rbpjκ*, which interacts with NICD and is crucial for Notch signaling, results in reduced proliferation, premature neuronal differentiation and apoptosis (Riesenberg et al., 2009).

Interestingly, in zebrafish embryos, it has been reported that disrupted Notch activity does not deplete the pool of RPC (Bernardos et al., 2005). On the other hand, gain-of-function studies show that the misexpressed *Notch1a* leads to excessive and premature glial cell production, but also causes cells to remain undifferentiated in the developing zebrafish retina (Scheer et al., 2001).

1.5.2 Retinal cell fate determination

Another predominant role of the pathway during vertebrate retinogenesis is the regulation of cell fate determination. Notch pathway mediates the timing of cell birth and differentiation and is therefore instrumental for generating neuronal diversity in the vertebrate retina (Perron and Harris, 2000).

Notch pathway activation in this context is most related to the induction of Müller glial cell fate. As reported in the *Xenopus* and rodent developing retina, upregulated Notch signaling promotes the production of Müller glia (Dorsky et al., 1995; Furukawa et al., 2000). However, a growing body of evidence has provided insights into how the pathway influences the production of a variety of retinal cell types. Inactivation of *Notch1* or Notch downstream genes in the mouse retina leads to overproduction of photoreceptors (Riesenberg et al., 2009; Yaron et al., 2006). In contrast, knockdown of *Notch1* in the chick retina results in an excess of ganglion cells. In fact, ganglion cells are selected from a cluster of progenitor cells such that the number of ganglion cells produced is inversely related to the level of Notch activity (Austin et al., 1995; Nelson et al., 2006; Silva et al., 2003).

In zebrafish embryos, loss-of-function studies by Bernardos et al showed that the Notch pathway is required for proper retinal lamination as well as Müller glia and photorecptor differentiation. Inactivation of the pathway not only delays and disrupts photoreceptor differentiation, but also impairs the production of Müller glia (Bernardos et al., 2005). And as mentioned above, gain-of-function studies by Scheer *et al.* showed that Notch plays an instructive role in zebrafish retinal development since misexpressed *Notch1*a results in excessive and premature glial cells (Scheer et al., 2001). Similar trends of Notch-dependent cell fate choices are also reported at later developmental stages. Recent studies by Mizeracka *et al.* have demonstrated that conditional knockout of *Notch1* in the postnatal mouse retina results in an excess of rod photoreceptors at the expense of other cell types, Müller glia and bipolar cells in particular (Mizeracka et al., 2013a).

Taken together, these findings indicate that Notch pathway regulates multiple cell fate outcomes and is crucial to achieve proper cell-type composition. The findings suggest the influences of the pathway on cell fate specifications may be stage-specific, or even species-specific. Yet the underlying mechanisms of these processes and direct targets require further studies.

1.5.3 Notch and the cell cycle

Notch pathway also coordinates with the cell cycle and apical-basal polarity during retinal development through the interkinetic nuclear migration described above. Del Bene et al have demonstrated that in the zebrafish retinal neuroepithelium, the antineurogenic Notch activity is predominantly activated at the apical side (Del Bene et al., 2008). Previous observations have reported that the selection of postmitotic neuronal daughter cells from progenitors is linked to RPC apical-basal polarity (Baye and Link, 2007). In line with this, the expression of Notch pathway components and their effector genes are cell-cycle dependent (Cisneros et al., 2008), such that Notch activity reaches its maximum level during M phase (when RPC nuclei are located apically), and drops dramatically during S phase (when RPC nuclei are located basally). Together these findings suggest that in the retinal neuroepithelium, apical-basal Notch gradient and interkinetic nuclear migration allow the cell to be exposed to neurogenic versus proliferative signals, which in turn regulate cell-cycle exit.

1.6 Thesis objective and aims

Notch signaling during embryonic vertebrate retinal development has been extensively studied in various models to reveal several functional roles. In the embryonic retina, the signaling has been reported to exhibit stage-dependent functions: maintenance of an undifferentiated proliferative state in the retinal progenitors and regulating specific cell fate decisions in post-mitotic cells. However, our understanding of the role of Notch signaling in RPC proliferation/cell fate during postembryonic periods remains limited. The environment of the embryonic retina is remarkably different from the postembryonic retina. The central retina in the postembryonic period is filled with differentiated neurons, whereas the embryonic retina there are mostly proliferative RPCs. In some animals, proliferating retinal progenitors are sustained in specific stem cell niches in the postembryonic stages which allow for continuous retinal growth and regeneration upon injury. While it has been reported that Notch signaling is expressed in RPCs in the CMZ and Müller glia in the differentiated retina, little is known about the actual functions of Notch signaling during postembryonic retinal growth and regeneration. Whether Notch signaling functions in a similar manner in these niches as it does in early development has never been thoroughly examined.

The main purpose of my project is to analyze the functions of the Notch signaling pathway during postembryonic retinogenesis in zebrafish. To study this, the expression patterns of Notch pathway components and Notch activity were first analyzed in the postembryonic retina. Gain- and loss- of-function approaches were then performed to resolve the effects on proliferation and differentiation. Specifically, this thesis aims to address the following questions: (1) is Notch signaling required to maintain the proliferation status of RPCs in the postembryonic stem cell niche (i.e., CMZ); and (2) are the cell fate decisions of postembryonic RPCs regulated by Notch signaling in a similar manner as the embryonic RPCs. The results of my research advance our understanding of Notch regulation of proliferation and differentiation in the postembryonic vertebrate retina.

Chapter 2
Methods and Materials

2.1 Fish care and use

2.1.1 Zebrafish husbandry and transgenic fish lines

Adult fish and larvae beyond 6 day post-fertilization (dpf) were housed in a recirculating system (Aquaneering Inc.) under a 14-hour light/10-hour dark cycle at 28 ℃. All animal experiments were performed with the approval of the University of Toronto Animal Care Committee and Canadian Council for Animal Care (CCAC). In zebrafish, embryonic development ends at 3 dpf, followed by larval stages, which continue to ~30 dpf.

The AB wild-type fish, originally obtained from the Zebrafish International Resource Center (ZIRC), were bred from our fish facility. The *Tg(hsp70l:Gal4)* and *Tg(UAS:6xmyc-Notch1a-ICD)* were also acquired from ZIRC and maintained in our facility as separate breeding lines. The double transgenic fish *Tg(hsp70l:Gal4);(UAS:6xmyc-Notch1a-ICD)* were obtained from crossing the two fish lines and has been previously described (Scheer and Campos-Ortega, 1999; Scheer et al., 2001). The *Tg(Tp1bglob:eGFP)* line was a kind gift from Dr. Michael John Parsons (Johns Hopkins University, The United States) and Dr. Jason Fish (Toronto General Hospital, Canada) .

The embryonic development of the zebrafish retina starts at about 10 hour post-fertilization (hpf) from the anterior ectoderm and by the end of 3 day post-fertilization (dpf) the embryogenesis is completed and the laminar structure of retina is formed. In order to analyze the molecular controls of the postembryonic RPC niche, all of my experiments are conducted beyond 3.5 dpf hereafter.

2.1.2 Phenyl 2-thiourea (PTU) treatment

For whole-mount *in situ* hybridization and MG132 injection in larval eyes, pigment-less zebrafish were raised by exposing the embryos to 1-phenyl 2-thiourea (PTU, 0.003% in fish water) from ~15 hpf to desire stages to prevent pigment formation.

2.1.3 Notch1a-intracellular domain over-expression

The double transgenic fish *Tg(hsp70l:Gal4);(UAS:6xmyc-Notch1a-ICD)* were used for over-expressing Notch1a-ICD *in vivo*. Zebrafish were raised at 28.5 ℃ until needed. Heat-shocking was done by transferring the larvae into a 50 mL falcon tube and placed in a 37 ℃ water bath for 30 min every day during the over-expressing period. Following heat-shock, the fish were incubated back at 28.5 ℃ for at least 3 hours before sacrifice.

2.1.4 Notch signaling pathway inhibition

The γ-secretase inhibitor, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) has been used in zebrafish embryos to block Notch pathway by inhibiting intracellular proteolysis on the Notch receptors (Chapouton et al., 2010; Geling et al., 2002). Fish were raised in fish water supplemented with either DAPT (at the final concentration of 100 μM with 0.1% DMSO) or 0.1% DMSO starting from 3.5 dpf. Both treated water were refreshed daily until the fish were sacrificed at either 7.5 dpf or 20 dpf.

2.1.5 Bromodeoxyuridine administration

The DAPT- and DMSO-treated fish larvae at 5.5 dpf were triple-rinsed with fish water before bromodeoxyuridine (BrdU) treatment. To label proliferating cells in the S-phase of the cell cycle, BrdU was applied by exposing the fish to water containing 10 mM BrdU (with DMSO at a final concentration of 10%) for 20 min at 4℃. The larvae were then triple-rinsed and placed back in DAPT- and DMSO-treated water to grow until desire date. The protocol is adapted from Yamaguchi et al. (2005).

2.1.6 Antisense morpholino injection

Antisense morpholino oligonucleotides (MOs) were used to knock down *numb* and *numblike* in zebrafish embryos. The following antisense morpholinos published by Bresciani et al were available from Gene Tools, LLC and microinjected into zebrafish embryos (Bresciani et al.,

2010):

nb/nbl MO: 5'- CAGGCTCTGACGCAGCTTATTCATC-3'

nb MO1: 5'- CACACAGCAAAACTTACTTTTTTAA-3'

The *nb/nbl* MO was complementary to the sequence of AUG translation start site of both *numb* and *numblike*, allowing knockdown of both genes. The *nb* MO1 was complementary to the sequence of the eighth intron/exon junction of *numb*.

2.2 Cryosectioning

Zebrafish larvae were fixed with 4% paraformaldehyde in PBS for overnight at 4 ℃ and then stored in 100% methanol at -20 ℃ until used. Following fixation, the larvae were rinsed with PBT and cryoprotected with 30% sucrose in PBS overnight at 4 ℃. Fish were transferred into 30% sucrose in PBS/OCT (2:1, v/v) solution 3 hours prior embedding for sectioning. 16 µm sections were obtained using cryostat (Leica) and slides were stored at -80 ˚C until used.

2.3 Immunohistochemistry

For immunohistochemistry, larval zebrafish were fixed and cryosectioned as described. Sections used for BrdU immunostaining were pre-treated with 2N HCl at room temperature for 30 min to retrieve antigen and sequential immunohistochemistry was performed. Sections were re-hydrated in PBS for 10 min and blocked with 2% normal goat serum in PBT for 2 hours. Primary antibody in blocking solution was applied on sections either overnight at 4 ˚C or for 2 hours under room temperature. The slides were then washed extensively with PBT and incubated with secondary antibody for 1 hour at room temperature. Nuclei were counterstained with Hoechst (Sigma, 861405) before mounting the slides. The following primary antibodies were used: rabbit anti-PKCβ1 (1:200, Santa Cruz Biotechnology, Inc.); mouse anti-GS (1:500, Chemicon); rat anti-GFP (1:500, ChromoTek); mouse anti-PCNA (1:500, Zymed Laboratories); mouse anti-HuC/D (1:250, Molecular Probes); mouse anti-zpr1 (1:250, ZIRC); mouse anti-zn5 (1:250, ZIRC); rat anti-BrdU (1:250, AbD Serotec) and mouse anti-myc (1:500, Invitrogen). Secondary antibodies were used as following dilution: Cy2 (1:200, Jackson ImmunoResearch Laboratories, Inc.); Cy3

(1:500, Jackson ImmunoResearch Laboratories, Inc.); Cy5 (1:200, Jackson ImmunoResearch Laboratories, Inc.); and rabbit anti-GFP, Alexa 488 conjugated (1:500, Molecular Probes).

2.4 *in situ* Hybridization

For fluorescence *in situ* hybridization on cryosections, the slides were completely dried before use. The samples were re-hydrated in PBS for 30 min and re-fixed in 4% paraformaldehyde in PBS for 10 min. Rinsed slides were then incubated in pre-hybridization solution (50% Formamide, 5X SSC; 50 μg/ml Heparin; 0.1% Tween-20; 9.2 mM citric acid in ddH_2O) at 65 °C in a pre-warmed humid chamber for 45 min and left overnight with probes (in hybridization solution with 0.5 mg/mL torula RNA) at 65 °C. The slides were washed with 0.2X SSC for 1x 15 min, followed by 3x 30 min 0.05X SSC washes at 65 °C and incubated in Tris-NaCl-Tween buffer (TNT, pH 7.5 Tris-HCl, 0.1 M; 150 mM NaCl; 0.1% Tween-20 in ddH_2O) at room temperature. To quench the native peroxidase activities, the slides were treated with 2% H_2O_2 in TNT for 15 min at room temperature with gentle shake. For blocking, slides were triple-rinsed with TNT and then incubated in blocking solution TNB (0.5% Perkin-Elmer blocking powder in TN) for 3 hours at room temperature. Anti-digoxigenin-POD antibody (1:1000 in TNB, Roche) or anti-fluorescein-POD antibody (1:1000 in TNB, Roche) were pre-absorbed with zebrafish at desire stages and applied to the samples overnight at room temperature in a sealed box. The slides were then extensively washed with TNT the next day and incubated with Tyramide Signal Amplification (TSA) Diluent (PerkinElmer) for 30 min in the dark at room temperature for signal detection and amplification. The samples were then post-quenched with 2% H_2O_2 in TNT for 15 min and washed with 4x 15 min in PBT, and finally counterstained with Hoechst before mounting.

For whole-mount *in situ* colorimetric hybridization, the PTU-treated embryos were fixed with 4% paraformaldehyde in PBS for overnight at 4 °C and then stored in 100% methanol at -20 °C until used. Prior to hybridization, the embryos samples were gently re-hydrated through a series of PBT washes (from 0% to 100% in methanol) and digested with Proteinase K. Next, the samples were re-fixed in 4% paraformaldehyde in PBS for 20 min and rinsed with several PBT washes. Pre-hybridizations were done by incubating the embryos with hybridization solution at

65 ℃ in a pre-warmed humid chamber for 4 hours. For hybridization, labeled probes (in hybridization solution with 0.5 mg/mL torula RNA) were applied to the samples overnight at 65 ℃. The next day, the embryos underwent a series of washes with increasing concentrations of 2X SSC (from 25% to 100% in hybridization solution) at 65 ℃, followed by washes with increasing concentrations of PBT (from 0% to 100% in 0.2X SSC) at room temperature. For blocking, the embryos were incubated in blocking solution (2 mg/mL BSA; 2% normal goat serum in PBT) for two hours at room temperature with gentle agitation. Pre-absorbed anti-DIG or anti-fluorescein alkaline phosphatase-coupled Fab fragments antibodies (1:4000 in blocking solution, Roche) were applied overnight at 4 °C to label the probes. For colorimetric detection, the samples were extensively washed with PBT and incubated with NBT/BCIP solution (in AP buffer). The embryos were then washed with PBT and post-fixed with 4% paraformaldehyde in PBS overnight at 4 °C prior to mounting.

The following antisense RNA probes were used: *notch1a* (a gift from Dr. Ajay Chitnis; National Institute of Health, the United States), *deltaC*, *her6* and *numb* (Open Biosystems).

2.5 Protein Electrophoresis and Western Blot

Embryos and larvae were transferred into ice-cold fish water prior to dissections. For embryos, fish were truncated and only the heads (fraction anterior to the yolk) were used. For older fish, retinae were further collected from the heads. The samples were to transferred into ice-cold lysis buffer (20 mM MOPS, pH 7.2; 2 mM EGTA; 5 mM EDTA; protease inhibitor cocktail, Sigma; Halt phosphatase inhibitor cocktail, Pierce; 1% Triton X-100; and 1 mM dithiothreitol) and sonicated for several seconds on ice. The lysates were then centrifuged at 4 ℃ for 30 min at 12,000 rpm and the supernatants were collected and stored at -20 ℃ until use. Protein quantifications were done using Bradford assay.

For protein electrophoresis, 4X NuPAGE LDS sample buffer (Invitrogen) and 5% β-mercapthenol were added to the sample. The mixtures were heated to 72 ℃ for 10 min and then loaded onto a 4-12% Bis-Tris SDS Mini gel (Invitrogen) to perform electrophoresis according to the manufacturer’s instructions. The samples were transferred to a PVDF membrane (HyBond ECL, GE Healthcare Pharmacia Biotech). For Western blotting, membranes were blocked with

blocking solution (5% skim milk powder in TBST) for 1 hour at room temperature. Primary antibodies (in blocking solution) were applied to the membranes with gentle shake either overnight at 4 ℃ or 1 hour under room temperature. Membranes were then washed for 4x 10 min in TBST and labeled with secondary antibodies (in TBST). For detection, the membranes were washed 3x 10 min and incubated in ECL prime (Invitrogen) followed by film development. The following primary antibodies were used: mouse anti-myc (1:1000, Invitrogen); Mouse anti-actin (1:1000, Chemicon International, Inc.); and rat anti-GFP (1:1000, ChromoTek). Secondary antibodies used were: goat anti-mouse HRP; goat anti-rabbit HRP; and goat anti-rat HRP (1:5000, Jackson ImmunoResearch Laboratories, Inc.).

2.6 Imaging and Statistical Analyses

Images of whole-mount embryos were acquired from a Leica MZ16F dissecting microscope or a Leica DM4500B compound microscope. Images of sections were taken from Leica TCS SP5 II and SP8 Confocal Microscope. Z-stacks (at 1 μm intervals) of retinal sections were analyzed with Leica LAS AF software and Imaris. For immunohistochemistry analyses, raw data from two retinal sections at the center of retina (determined by the presence of optic nerve) were chosen and averaged in each fish. A total of five fish (10 retinal sections) were obtained from each group for statistical analyses. Statistical significances ($p \leq 0.05$) were determined by unpaired Mann-Whitney U test using Prism software (GraphPad).

Chapter 3
Results

3.1 Notch pathway expression pattern in larval fish retina

3.1.1 Notch components are expressed in the postembryonic retina

Previous studies have demonstrated that the following Notch pathway genes are expressed in the CMZ of 2-month-old zebrafish retina: *notch1a*, *notch1b*, *deltaC*, *her6* and *her2*. The expression patterns were reported to be relatively higher in the peripheral CMZ and decreased toward the more central CMZ (Raymond et al., 2006). Recent findings have also shown that *notch1a*, *notch1b*, *notch3* and *her6* mRNA are expressed in the INL in the adult zebrafish retina and that the pathway is required to repress Müller glia dedifferentiation and proliferation in the undamaged retina (Conner et al., 2014; Wan et al., 2012). These results, however, are in contrast with the observations in avian and rodent postembryonic retina in which Notch pathway components are expressed at low levels by Müller glia in undamaged retina and Notch signaling, acting downstream of FGF2/MAPK signaling, induces the proliferative activity of Müller glia in response to injury (Notch signaling influences neuroprotective and proliferative properties of mature Müller glia). Despite the conflicting functions reported in different vertebrate models, it seems that in the normal retina, Notch signaling is activated in Müller glia to some degree.

In order to examine Notch signaling in the larval zebrafish retina, some of the pathway components were examined using fluorescent *in situ* hybridization (FISH) at 5.5 dpf (Fig 4). At this stage, receptor *notch1a* (*n1a*) is expressed in the CMZ and the signal gradually decreases from peripheral to central retina (Fig 4A), suggesting that the Notch signaling can potentially be activated in the CMZ progenitor cells. In contrast, expression of the Notch ligand *deltaC* (*dlc*) is not observed in the CMZ, but at the transition zone of CMZ and differentiated retina, where RPCs become postmitotic and differentiate (Fig 4B). The signals of *n1a* and *dlc* expression are spatially contiguous and slightly overlap in the transition zone (Fig 4C), suggesting Notch signaling is activated at this zone and that *dlc*-*n1a* ligand-receptor interaction may be responsible for this activation specifically in the transition zone. In contrast, the data also suggest that while the CMZ cells may be competent for Notch signaling, ligands other than DeltaC may be required

at this location to activate Notch signaling. Besides the transition zone, *dlc* expression is also localized to the INL and GCL (Fig 4B). The INL signal may possibly activate Notch signals in the Müller glia. However, *n1a* expression is not observed here. Possibilities to explain these findings include low expression levels, which cannot be detected with FISH; different Notch receptors or targets are required and activated in the INL; or that the INL cells may not exhibit Notch activity.

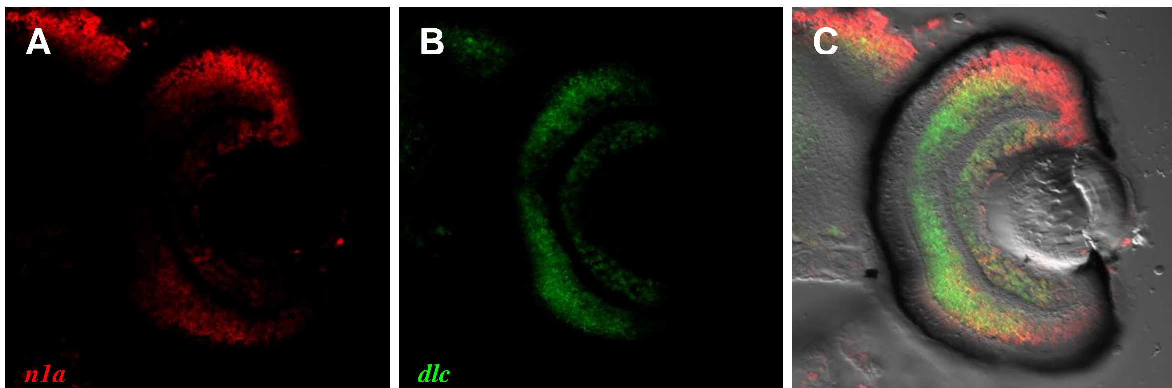


Figure 4. Expression patterns of Notch pathway components *notch1a* and *deltaC* on 5.5 dpf AB wild-type fish retina sections. (A) *notch1a* is expressed in the CMZ and the signal gradually decreases from periphery to central retina. (B) *deltaC* expression is not observed in the CMZ, but at the transition zone of CMZ and differentiated retina, as well as in the INL and GCL of the central retina. (C) The expression patterns of *notch1a* (red) and *deltaC* (green) overlap at the transition zone, where RPCs become postmitotic and differentiate.

3.1.2 Notch activity in the retina

To further investigate where Notch signal is activated, a Notch responsive transgenic fish line *Tg(Tp1bglob:eGFP)*^{um14} was examined for the activity of Notch in zebrafish larval retina. The *Tp1blgob* promoter consists of 6 copies of the Epstein Barr Virus terminal protein 1 (TP1) promoter (each TP1 promoter has two Rbp-Jκ binding sites) and the rabbit β-globin minimal promoter. As a result the expression of eGFP can serve as a direct readout for Notch activity (Parsons et al., 2009). Retinal sections of 7.5 dpf *Tg(Tp1bglob:eGFP)* fish were immunolabeled using GFP antibody and analyzed under a confocal microscope. Interestingly, no Notch activity is observed in the peripheral CMZ using this transgenic reporter line (Fig 5A, B). Instead, Notch signaling is activated specifically in the INL and the transition zone. Co-labeling these GFP-positive cells with immunohistochemistry (IHC) using glutamine synthetase (GS) (Fig 5D) and protein kinase C beta1 (PKCβ1) antibody (Fig 5C) reveals that activation of the Notch signal in all Müller glia (MG) cells (Fig 5D, white arrow), a subset of PKCβ1^{+} bipolar cells (Fig 5C, white arrow head) and some other GS^{-}PKCβ1^{-} cells (Fig 5C, yellow arrow). The MG and non-MG GFP-positive cells are different in morphology and MG seems to possess higher Notch activity. Upon closer examination at the periphery, GFP is expressed in the newborn cells (Fig 5C, red arrow) before they express cell-specific markers and gain their identities (Fig 5C, yellow arrow). This suggests that the Notch pathway could be involved in the decision of cell cycle exit and/or the process of cell fate determination in postmitotic cells. In contrast, no active Notch signaling is observed in the CMZ cells despite expression of receptor *n1a.*

Another transgenic fish line *Tg(her4.1:mCherry,Cre-ERT2)* has also been examined in our lab by Dr. Zachary Hall to reveal a similar expression pattern of a downstream Notch-responsive gene (Fig 5E, 5F). *Hairy-related 4, tandem duplicate 1* (*her4.1*) has been reported to be a zebrafish orthologue of mammalian hes5 and serves as a reporter for Notch signaling in adult zebrafish (Ganz et al., 2010; Kroehne et al., 2011). In the adult zebrafish brain, the expression of *her4.1* promoter-driven mCherry is restricted to ventricular cells that resemble radial glia, which can serve as neuronal progenitors in response to injury. Retinal sections from 5 dpf *Tg(her4.1:mCherry,Cre-ERT2)* fish retina has been analyzed to show that Notch signaling is activated in the differentiated retina, most predominantly Müller glia, but not in the uncommitted retinal stem cells/progenitors in the CMZ. The expression pattern is similar to the one in

Tg(Tp1bglob:eGFP) fish line, confirming the absence of active Notch in the postembryonic RPC. This phenomenon may be due to the spatial control of ligand expression and/or other regulations in the CMZ, which prevent the receptors from being activated in the RPCs.

The expression pattern of an alternative member of the zebrafish *her* family gene *her6* has also been examined in the postembryonic retina (Fig 6). Her6 is a zebrafish orthologue of the mammalian Hes1 (Chapouton et al., 2011) and has been reported to be expressed in the developing zebrafish diencephalon (Pasini et al., 2001) to regulate the progression of neurogenesis (Scholpp et al., 2009). However, previous studies on zebrafish have shown that the expression of *her6* may not always require Notch signaling. While the study by Pasini *et al.* demonstrates that *her6* transcription is under the control of the Notch pathway in the paraxial mesoderm of zebrafish (Pasini et al., 2004), another study by Hans *et al.* suggests that the transcription of *her6* may be independent of Notch activity (Hans et al., 2004). Here my *in situ* hybridization data shows that *her6* is expressed in the postembryonic CMZ, where Notch activity has been confirmed to be absent by the two Notch activity reporter fish lines *Tg(Tp1bglob:eGFP)* and *Tg(her4.1:mCherry,Cre-ERT2)*. Together these data reveal that *her6* may not be a downstream target of Notch activity in this context, suggesting the Notch-dependency of *her6* expression may be tissue- and/or stage-specific in zebrafish. It is possible that the expression of *her6* in the postembryonic CMZ is under the control of other upstream regulators and *her6* is a progenitor marker independent of Notch.

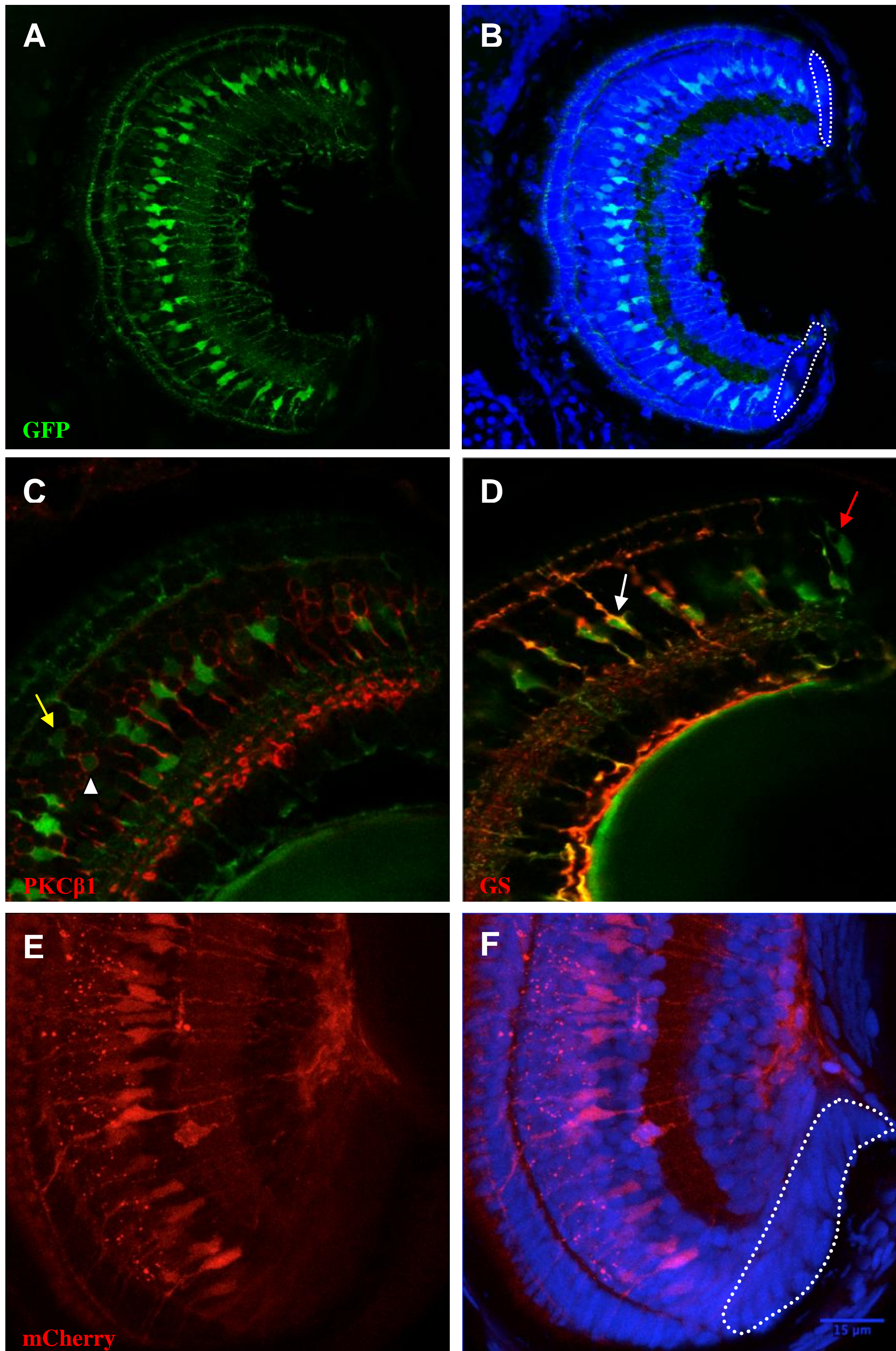


Figure 5. Expression patterns of Notch activity in 7.5 dpf (A, B, D) and 8 dpf (C) *Tg(Tp1bglob:eGFP)* as well as 5 dpf *Tg(her4.1:mCherry,Cre-ERT2)* (E, F) zebrafish retina were analyzed with immunohistochemistry. (A, B) Notch activity is observed specifically in the

differentiated INL and the transition zone (GFP, green; Hoechst, blue) of the CMZ (white dashed lines) and mature retina. (C, D) Dorsal periphery of the retina at higher magnification. Co-labeling GFP signal (green) with PKCβ1 antibody (C, red, bipolar cell marker) and GS antibody (D, red, Müller glia marker) shows that at least three different subtypes of GFP^{+} cells exist: Müller glia (MG) cells (white arrow), a subset of $PKC\beta1^{+}$ bipolar cells (white arrow head) and some other $GS^{-}PKC\beta1^{-}$ cells (yellow arrow). Among them MG cells seems to possess higher Notch activity. In addition, GFP expression at the transition zone demonstrated that Notch activity is activated in the newborn cells (red arrow) before they express cell-specific markers and gain their identities (yellow arrow). (E, F) *her4.1*, a downstream target of Notch signaling is expressed in the Müller glial cells but not in the retinal progenitor cells in CMZ (white dashed line) (figures kindly provided by Dr. Zachary Hall).

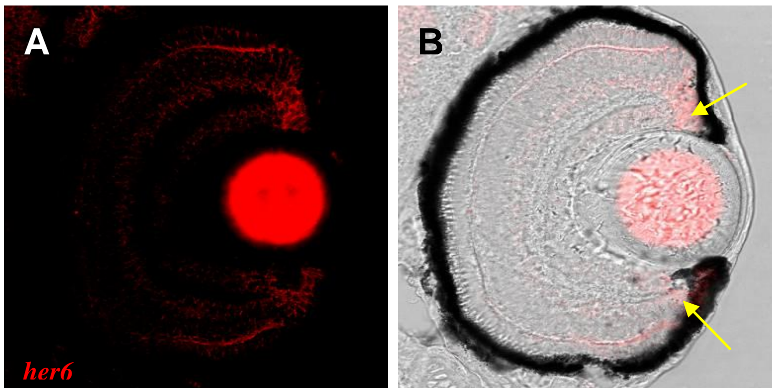


Figure 6. Expression pattern of *her6*, a Notch-independent progenitor marker, on 5.5 dpf AB wild-type fish retina sections. The CMZ is labeled with yellow arrows.

3.2 Inhibiting the pathway in larval fish retina

3.2.1 Validating the effect of DAPT

In order to analyze whether the Notch signaling pathway is required for postembryonic retinal development in terms of proliferation and differentiation, loss-of-function experiments were carried out. According to the previous studies by Bernardos et al, inhibition of Notch signaling with the γ-secretase inhibitor Compound E in the developing zebrafish retina results in reduced retina size, increased cell death, disrupted inner retina layers and failed differentiation for photoreceptors and Müller glia (Bernardos et al., 2005). As the release of functional Notch intracellular domain (NICD) upon ligand binding requires γ-secretase, the drug serves as a convenient method to inhibit Notch activity *in vivo*. Here, to examine the effect of downregulated Notch activity during postembryonic stages, the γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) was used to block Notch signaling *in vivo*. The drug has been reported to effectively inhibit Notch activity (Chapouton et al., 2010) and cause several phenotypes in zebrafish embryonic development including disrupted somite morphology and impaired neurogenesis (Geling et al., 2002).

In the loss-of-function experiments, the *Tg(Tp1bglob:eGFP)* fish were treated with 100 μM DAPT from 3.5 dpf and sacrificed on 7.5 dpf. Interestingly, Notch activity is not completely eliminated in the DAPT-treated retina (Fig 7A', B'). This finding is consistent with recent reports on adult fish using the same drug (Conner et al., 2014) and suggests that DAPT is a milder γ-secretase inhibitor under this context.

To further validate the effect of DAPT as a Notch inhibitor in the postembryonic retina, control and DAPT-treated retina were sectioned and mounted on the same slide, and the direct GFP intensities of the MG cells in the central retina were captured by Leica TCS SP8 confocal imaging and compared using Imaris (Fig 7). As expected, the GFP intensity mean of the MG cells from the central retina is significantly lower in the DAPT-treated fish (Fig 7C). The results confirm the function of DAPT as a Notch inhibitor in the retina and also suggest that the MG cells in the central retina might be less sensitive to DAPT comparing to their peripheral counterparts. This finding is consistent with the observation in chick retina, where peripheral MG cells are more plastic or less mature compared to the central ones (Fischer and Reh, 2003).

However, no significant reduction of the GFP intensity is observed in the GFP-positive non-MG cells. This may be due to the fact that GFP signals are too weak in these non-MG cell types posing a technical limitation on the analysis; or these non-MG cells are more resistant to the drug at the concentrations used.

Double-labeling immunohistochemistry experiments were then carried out on the 7.5 dpf *Tg(Tp1bglob:eGFP)* fish retina using GFP antibody (Fig 8, cells in green) and GS antibody (Fig 8, cells in red). While Notch activity is not fully diminished in the retina, the drug still effectively down-regulates it and several effects are observed. Firstly, significantly fewer GFP-positive MG cells in the whole retina are reported in the DAPT-treated retina (Fig 8E). The proportion of GFP^+ MG cells in the whole retina decreased by 37% (mean = 0.038 and 0.024 in DMSO- and DAPT-treated fish respectively) and the decrease is mainly observed at the retinal periphery (Fig 8A-D, A'-D'), but not in the central retina. The proportion of GFP^+ MG cells at the periphery dropped by 55% (mean = 0.038 and 0.017 in DMSO- and DAPT-treated fish respectively) while the ones in the central retina remained the same (mean = 0.039 and 0.037 in DMSO- and DAPT-treated fish respectively). In contrast, the other Notch-responsive non-MG cells were still observed at the peripheral retina (Fig 8C', white arrows), suggesting that these GFP^+ non-MG cells may not be as strongly influenced as Müller glia. However the actual proportions of these non-MG cells have not been quantified and thus it is unclear to what extent are they affected by Notch inhibition.

Secondly, the ONL is impaired in the DAPT-treated peripheral retina (Fig 8D', white bracket) and in some severe examples the entire ONL seems completely absent in the periphery. Thirdly, as described above, while there seemed to be no significant difference in the number of MG cells in the central retina (Fig 8E), the GFP intensity of these MG cells are significantly decreased in the DAPT-treated retina. The results suggest that the retinal cells (specifically Müller glia and the photoreceptors) at the periphery seem to be more sensitive to the down-regulated Notch activity than the cells in the central retina. The most likely reason for the disruption of MG and ONL cells at the periphery could be that the Notch pathway is required for the differentiation of Müller glia and photoreceptors at postembryonic stages, and the inhibition of Notch activity at the transition zone may disrupt the proper generation of these two cell types. Other possibilities include that the inhibited Notch activity will lead to the de-differentiation of Müller glia and the MG cells at the periphery are more plastic and likely to undergo a cell fate change, thus fewer

GS+ cells are observed at the peripheral retina. It is unclear, at this stage, how the inhibition of Notch activity affects CMZ proliferation, differentiation and even the maintenance of mature Müller glial cells and further analyses were carried out to resolve these questions.

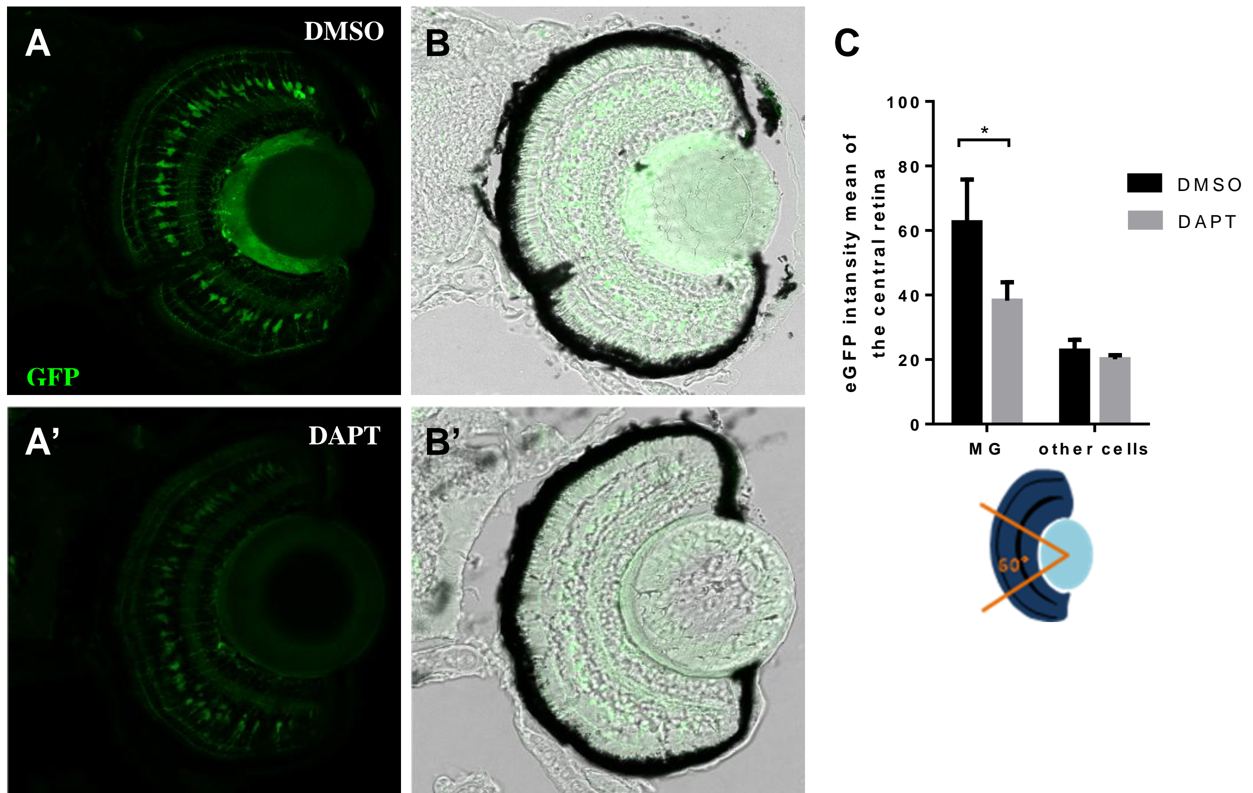


Figure 7. DAPT effectively decreases Notch activity in the postembryonic Müller glial cells. *Tg(Tp1bglob:eGFP)* fish were treated with either DMSO or 100 µM DAPT from 3.5 dpf, and sacrificed on 7.5 dpf. Control and DAPT-treated retina were sectioned and collected on the same slide, and their direct GFP intensities of the MG cells in the central retina were captured by Leica TCS SP8 confocal imaging and compared using Imaris. Overall the Müller glial cells in DMSO-treated retina (A,B) shows a higher eGFP activity, comparing to DAPT-treated retina (A’,B’). However the numbers of Müller glial cells in the central retina are similar between the two groups (see Fig 7 for more detail). (C) Quantification of the eGFP intensity mean in the MG and non-MG cells at 7.5 dpf. While eGFP activity is significantly decreased in the DAPT-treated retina, no significant intensity reduction is observed in the GFP-positive non-MG cells. Retinal sections (n = 3 for DMSO-treated group and n = 5 for DAPT-treated group) were analyzed with Imaris. Data indicate mean ± SD. Two-tailed MWU test.*, $P < 0.05$.

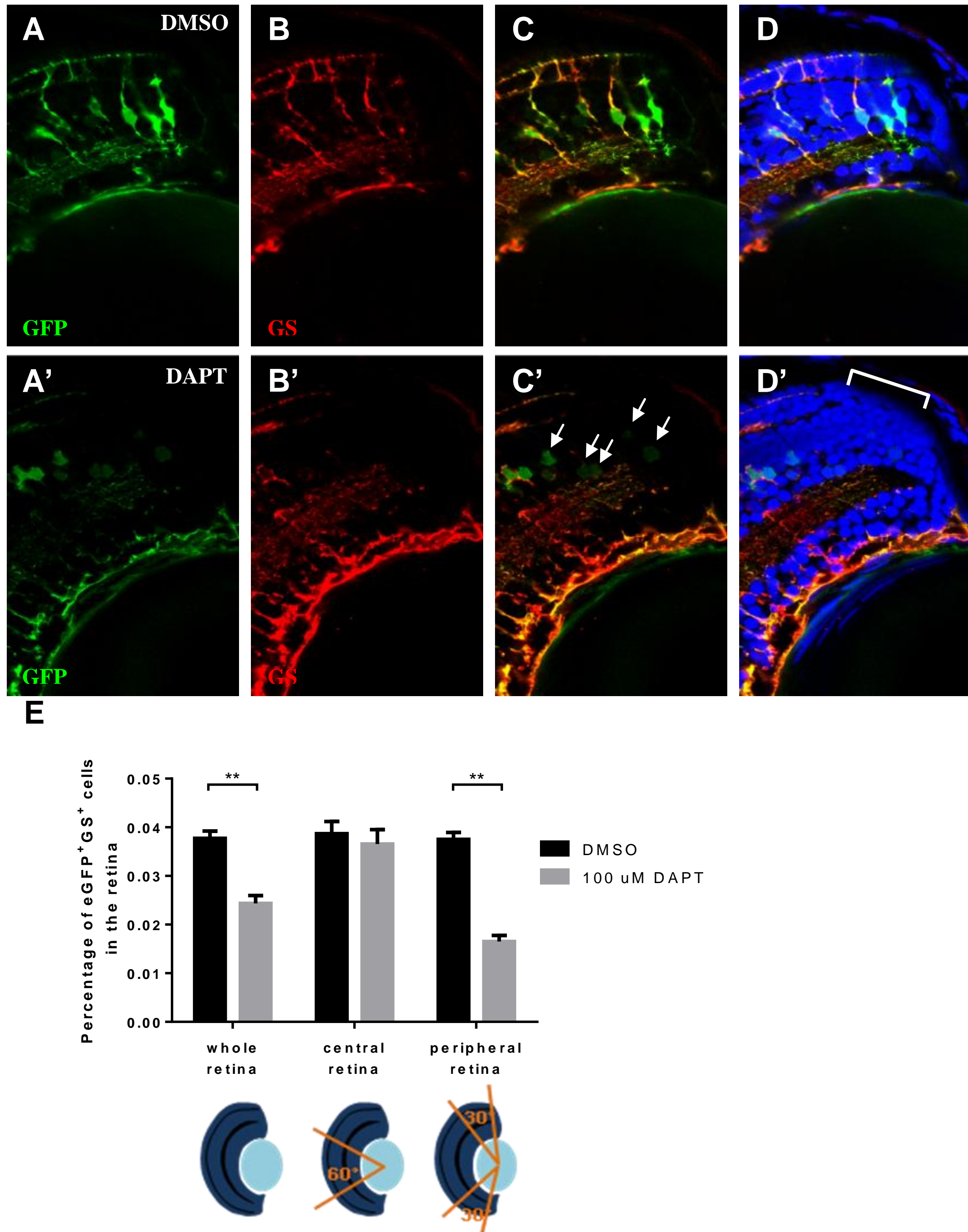


Figure 8. The peripheral retinal cells (specifically Müller glia and photoreceptors) are more sensitive to the decreased Notch activity than their central counterparts. (A-D, A'-D') *Tg(Tp1bglob:eGFP)* fish were treated with either DMSO or 100 µM DAPT from 3.5 dpf, and sacrificed on 7.5 dpf. The eGFP signals (green) were co-labeled with GS antibody (red, Müller glia marker) and Hoechst (blue) to confirm cell identity. Only dorsal peripheral retina is

presented here. While the other Notch-responsive non-MG cells are still observed at the periphery (C', white arrows), fewer GFP$^+$ MG cells are located here. In addition, differentiation of ONL cells seems to be impaired in the DAPT-treated retina as well (D', white bracket). (E) Significantly fewer GFP$^+$ MG cells in the DAPT-treated retina were observed and the decrease is mainly contributed by the loss of MG cells at the retinal periphery. In contrast, for the other Notch-responsive non-MG cells, no obvious decrease in number is observed. Retinal sections ($n = 5$) were analyzed with Imaris. Data indicate mean ± SD. Two-tailed MWU test.**, $P < 0.01$.

3.2.2 Inhibited Notch activity does not affect RPC proliferation

Notch signaling has been reported to be critical for maintenance of the RPC identity in many vertebrate models. However, Notch activity is not observed in the CMZ as discussed above. To explore whether the pathway actually exhibits a similar function on RPC proliferation in the postembryonic zebrafish retina, AB wild-type fish were treated with either DMSO or 100 μM DAPT from 3.5 dpf, and sacrificed on 7.5 dpf. I then examined the retina with IHC using PCNA antibody, a marker for proliferating RPCs (Fig 9, cells in red), to examine the percentage of proliferating $PCNA^+$ CMZ cells in the whole retina. Interestingly, no significant difference is reported between the control (Fig 9A, 9B) and DAPT-treated retina (Fig 9A', 9B') (Fig 9C, mean = 0.049 and 0.051 respectively). The result is in line with previous finding that blocking Notch activity in zebrafish embryonic development does not deplete the mitotically active retinal progenitor pools (Bernardos et al., 2005). Along with the fact that there is no Notch activity at the most peripheral margin, it is possible that Notch signaling is not required for RPC maintenance in zebrafish postembryonic CMZ, but rather plays an important role in cell-fate control.

In addition, no obvious changes of PCNA-positive cells is observed in the differentiated retina (data not shown), suggesting that the decreased Notch activity does not alter the proliferation of Müller glia under this context. A recent report showed that compared to DAPT a different γ-secretase inhibitor, RO4929097, caused a significant increase in the number of PCNA-positive INL cells, suggesting that the inhibition of Notch activity in the zebrafish retina could induce Müller glia proliferation under certain conditions (Conner et al., 2014). However my results here suggest that the inhibited Notch activity with DAPT (e.g., validated through reporter expression analyses above) does not alter the RPC proliferation and nor does it induce the de-differentiation of mature Müller glia under this context.

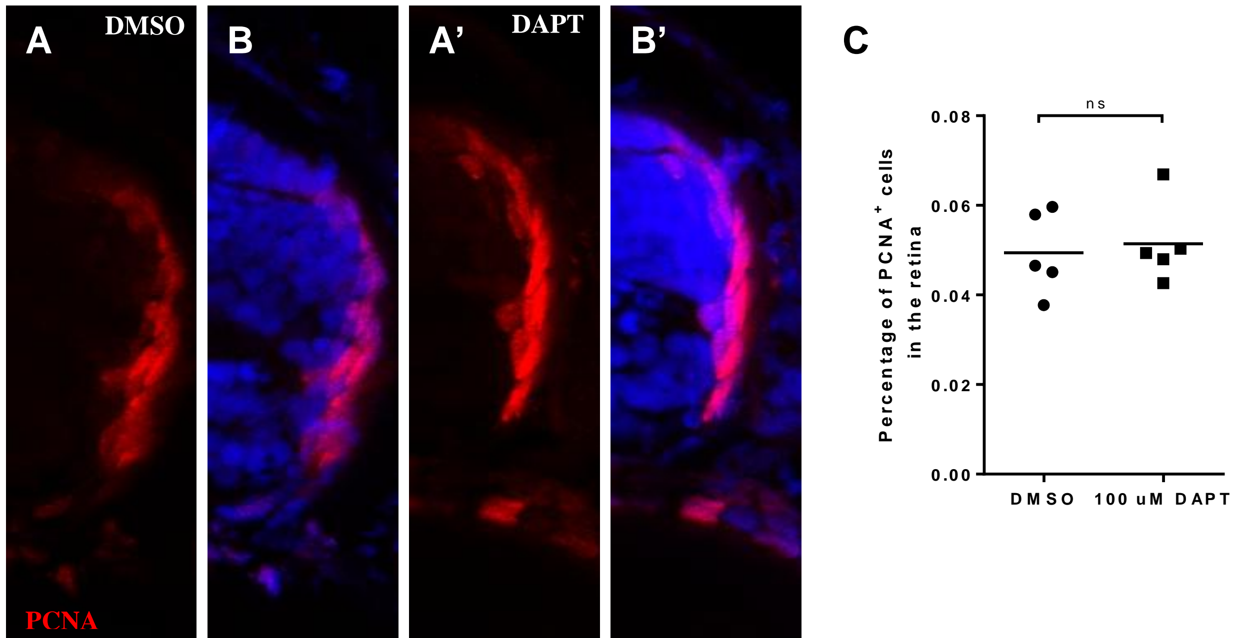


Figure 9. The inhibition of Notch activity does not alter the RPC proliferation in the CMZ. AB wild-type fish were treated with either DMSO or 100 µM DAPT from 3.5 dpf, and sacrificed on 7.5 dpf (A, B, A', B'). PCNA antibody labeled proliferative cells (red) in the dorsal CMZ. Hoechst, blue. Qualitatively, the decreased Notch activity does not affect RPC proliferation between the DMSO-treated (A, B) and DAPT-treated retina (A', B'). (C) Quantitatively, no significant difference of the percentage of PCNA-positive cells in the whole retina is observed between the control (mean = 0.049) and DAPT-treated groups (mean = 0.051). Retinal sections (n = 5) were analyzed with Imaris. Data indicate mean ± SD. Two-tailed MWU test.

3.2.3 Inhibited Notch activity alters the composition of new-born cells at the peripheral retina

To understand how down-regulated Notch activity affects cell fate determination during postembryonic stages, IHC using cell marker antibodies were carried out on 7.5 dpf DMSO- and DAPT-treated AB wild-type fish (Fig 10). For ganglion cells and amacrine cells, HuC/D (ganglion and amacrine cell marker, Fig 10A, B, A', B', cells in red) and zn5 antibody (ganglion cell marker, Fig 10C,D,C',D', cells in red) were used to examine if the inhibition of the Notch pathway altered the differentiation of ganglion and amacrine cells. Comparing the signal labeling of these two cell markers at the peripheral retina quantitatively shows no apparent difference between the control (A-D) and DAPT-treated fish (A'-D'). However, detailed BrdU pulse-chase quantifications on these two cell types were not performed (see below). Thus the possibility of Notch activity playing a role in a ganglion cells and amacrine cell differentiation should not be completely excluded.

For outer nuclear layer cells, the differentiation of these cells at the periphery is impaired in the DAPT-treated as described above (Fig 8D). Here I utilized the zpr1 antibody to label the cone photoreceptor (Fig 10E, F. E', F', cells in red) and the IHC results demonstrate that the postembryonic differentiation of cone photoreceptor is severely affected by the inhibition of Notch (Fig 10F', white bracket).

For inner nuclear layer cells, fewer Müller glial cells are observed at the peripheral retina as described above in the DAPT-treated fish using the GS antibody, suggesting that Notch activity is required for the proper differentiation of MG cells (Fig 8). Bipolar cells were also examined using PKCβ1 antibody (Fig 10G, H, G', H', cells in red). Qualitatively, the bipolar cells seem to extend and replace the missing ONL (Fig 10H', yellow bracket), yet it is unclear whether this phenotype is due to the actual increase in the number of new-born bipolar cells.

In order to further investigate (1) if the inhibited Notch activity truly disrupts the postembryonic cell fate determination and (2) what are that the actual changes in cell composition under this context, BrdU pulse-chase experiments were conducted to track the newborn retinal cells and their cell fates. However, only the Müller glia, cone photoreceptor and bipolar cells were further analyzed here as they appeared to be more affected by the altered Notch activity. The AB wild-

type fish were treated with 100 μM DAPT from 3.5 d to 10.5 d, and then maintained until 20 d (or 23 d) before sacrifice. BrdU was applied on 5.5 d for 20 min and by 20 d (or 23 d) the cohort of newborn cells labeled by BrdU has differentiated and integrated into the mature retina (protocol adapted from Yamaguchi et al. (2005)). Double-labeling IHC using cell specific markers and BrdU antibody were performed on the retinal sections to examine the proportion of each cell type among these BrdU-traced newborn cells (Fig 11-13).

Newborn Müller glial cells were co-labeled using GS antibody (Fig 11, cells in red) and BrdU antibody (Fig 11, cells in cyan). The results report a significant decrease ($p = 0.0079$, MWU test) of the GS^+BrdU^+ cells in the $BrdU^+$ cohort in the DAPT-treated fish (Fig 11E). The percentage of Müller glial cells in the a newborns has dropped from 4.1% in the control to1.3% in the DAPT-treated fish, suggesting that Notch pathway is required for postembryonic newborn cells to adopt a Müller glial cell fate. Co-labeling of BrdU (Fig 12, cells in cyan) with zpr1 antibody (Fig 12, cells in red) to identify newly differentiated cone photoreceptors also reveals a significantly smaller share of the $zpr1^+$ $BrdU^+$ cells in the $BrdU^+$ population ($p = 0.0079$, MWU test) (Fig 13E). The percentage of cone photoreceptor in the newborn cohort has decreased from 13.3% in the control to 4.1% in the DAPT-treated fish. These results suggest that the Notch pathway is required for the proper cell fate determination of Müller glia and cone photoreceptors in order to achieve a balanced cell composition of the newborn cells in the postembryonic retina. It is also interesting to discover that, as the fish are no longer exposed to DAPT beyond 10.5 d, the production of Müller glia and cone photoreceptors are resumed and the retina seem to develop normally. This suggests that the effects of DAPT as a Notch inhibitor may be transient and that only the cells in the transition zone which are in their last few cycles or have just become post-mitotic are sensitive to the change in Notch activity.

In contrast, double-labeling the newborn $BrdU^+$ cells (Fig 13, cells in cyan) with PKCβ1 antibody (Fig 13, cells in red) showed a noticeable increase in the $PKC\beta1^+BrdU^+$ ratio of the $BrdU^+$ population. While qualitatively an increasing trend is observed (from 29.3% in the control to 38.3% in the DAPT-treated fish), the difference is however not significant ($p = 0.22$) (Fig 12E). Further analyses with an increased sample size may be required to confirm whether there is an actual increase in the number of $PKC\beta1^+$ bipolar cells under the inhibition of Notch.

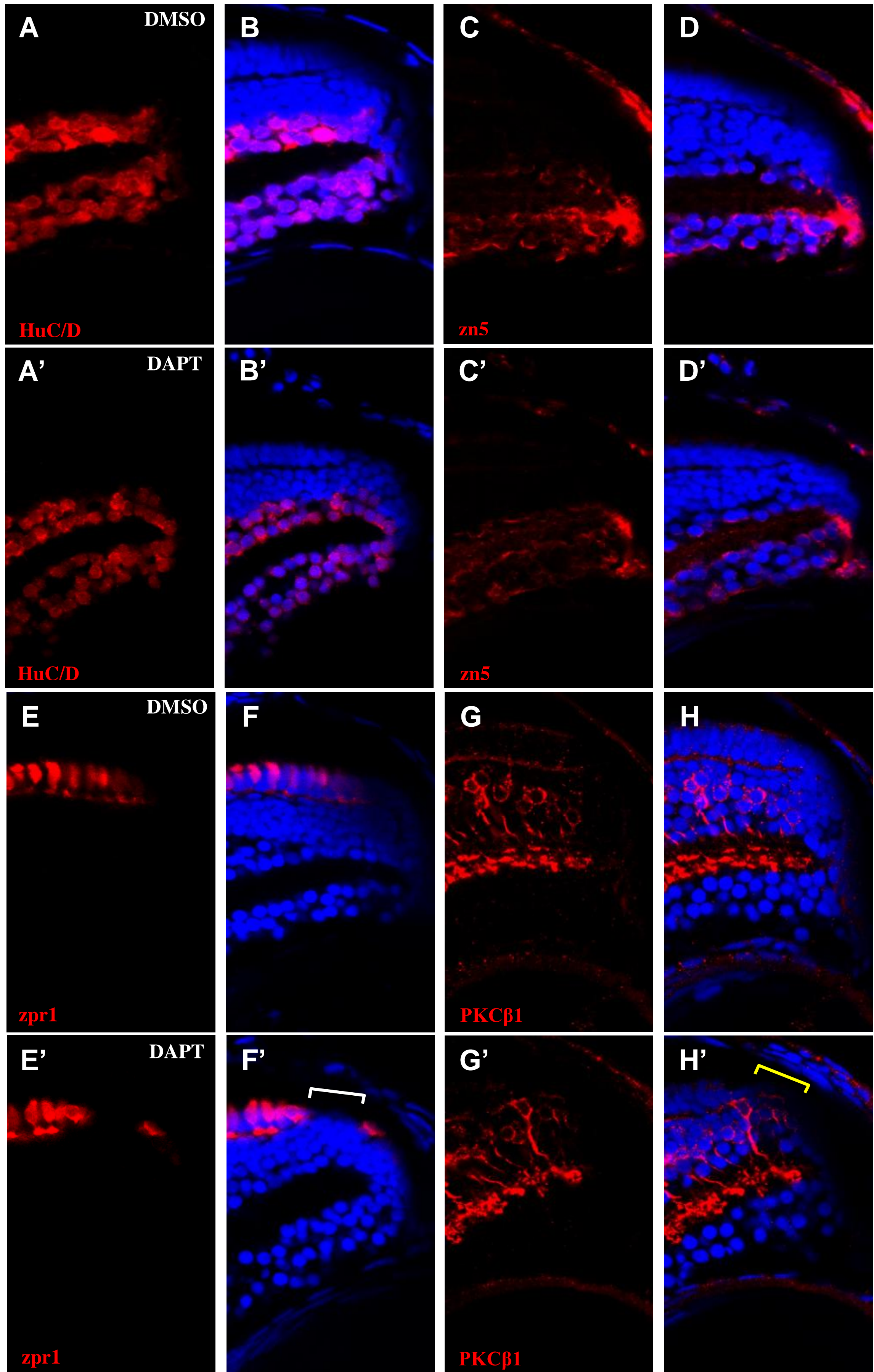

A
DMSO
HuC/D
B
C
zn5
D
A'
DAPT
HuC/D
B'
C'
zn5
D'
E
DMSO
zpr1
F
G
PKCβ1
H
E'
DAPT
zpr1
F'
G'
PKCβ1
H'

Figure 10. Inhibited Notch activity alters the composition of newborn retinal cells at the peripheral retina. AB wild-type fish were treated with either DMSO (A-H) or 100 μM DAPT (A'-H') from 3.5 dpf, and sacrificed on 7.5 dpf. Hoechst, blue. (A, B, A' B') HuC/D antibody (red, marker for ganglion and amacrine cells) was used to label ganglion cells and amacrine cells and no apparent difference between the control (A, B) and DAPT-treated fish (A', B') is observed. (C,D,C',D') Zn5 antibody (red, ganglion cell marker) was used to label ganglion cells and the similar expression patterns were observed between control (C, D) and DAPT-treated fish (C', D'). (E, F. E', F') Zpr1 antibody (red, marker for double-cone photoreceptor) was utilized to label the cone photoreceptor and the results show that the cone photoreceptor layer is severely affected by the inhibition of Notch (F', white bracket). (G, H, G', H') Bipolar cells were labeled using PKCβ1 antibody (red, bipolar cell marker). Qualitatively, the bipolar cells seem to extend and replace the missing ONL (H', yellow bracket).

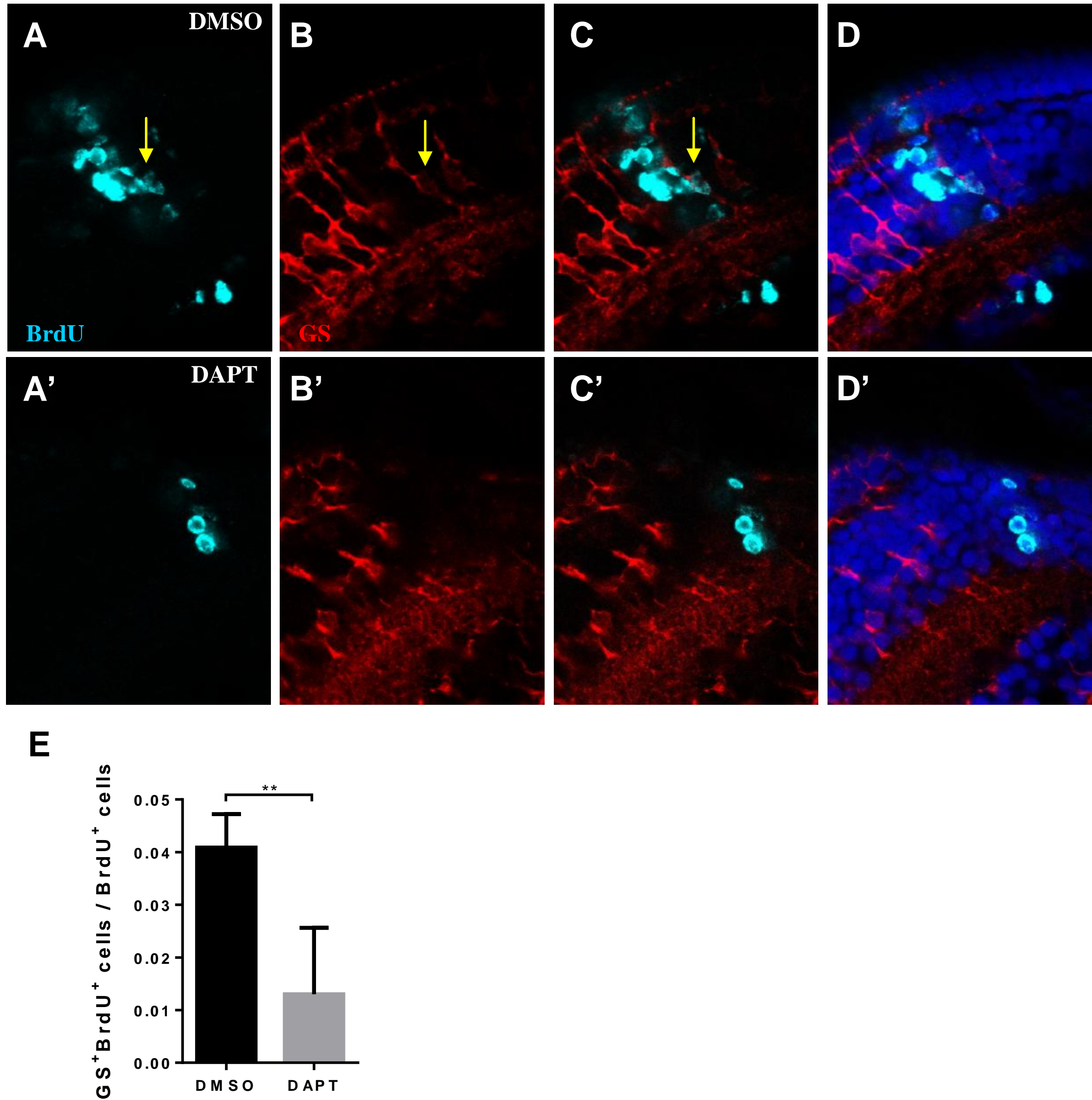


Figure 11. Notch activity is required for newborn cells to adopt a Müller glial cell fate and the inhibition of Notch activity decrease the proportion of Müller glial cells in the newborn retinal population. Double-labeling IHC using GS antibody (red, Müller glia marker) and BrdU antibody (cyan) were performed on the 20 dpf retinal sections. (A-D) Müller glia are differentiated properly in the DMSO-treated retina (yellow arrow). (A'-D') Müller glial cells fail to differentiated in the DAPT-treated retina. (E) A significant decrease ($p = 0.0079$, MWU test) of the GS^+BrdU^+ cells in the $BrdU^+$ cohort is reported in the DAPT-treated fish. Retinal sections ($n = 5$) were and analyzed with Imaris. Data indicate mean ± SD. Two-tailed MWU test. **, $P < 0.01$.

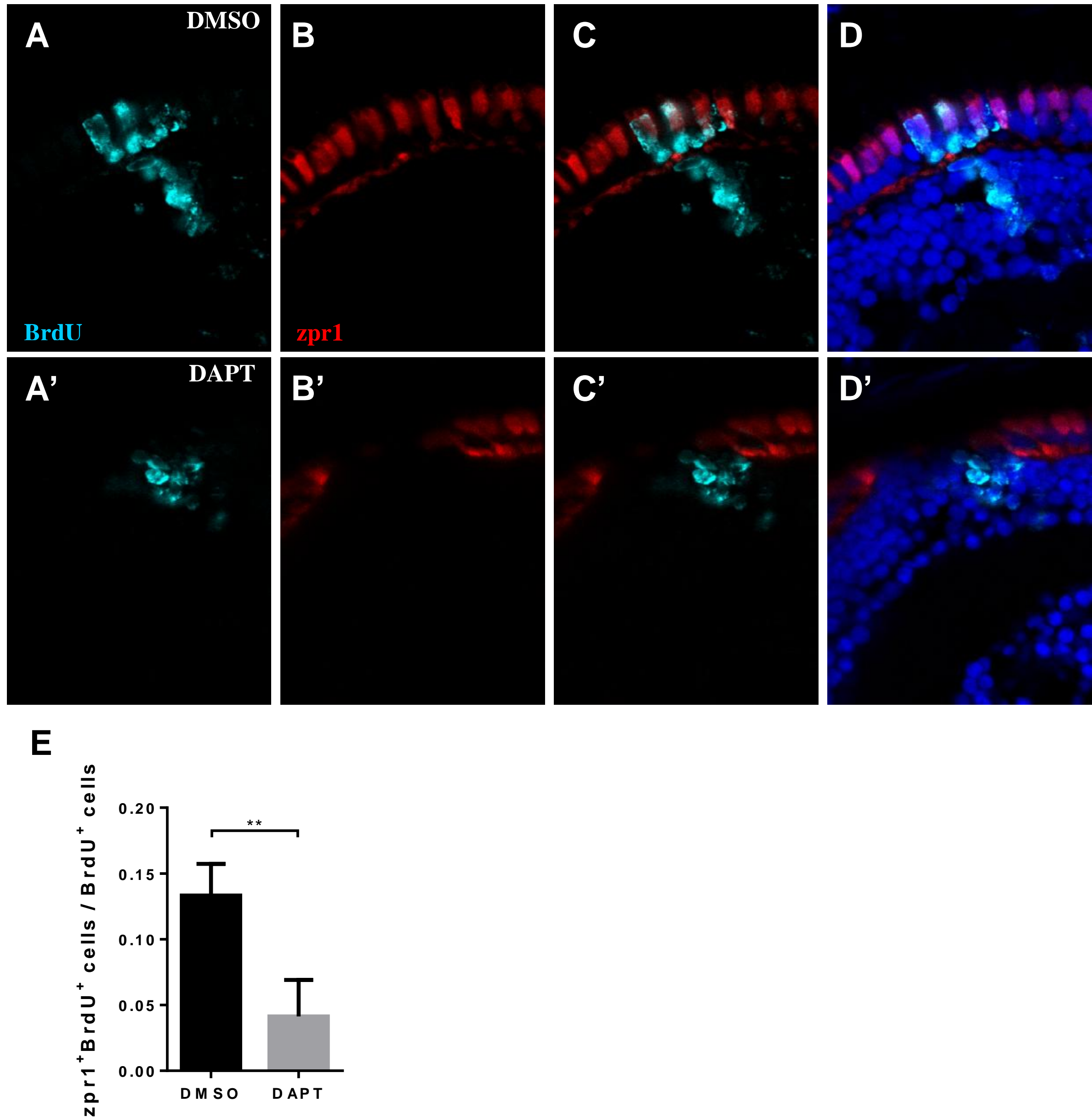


Figure 12. Notch activity is required for proper differentiation of cone photoreceptors and the inhibition of Notch activity decreases the proportion of cone photoreceptors in the newborn retinal cohort. The newborn cone photoreceptors were co-labeled with BrdU (cyan) and zpr1 antibody (red, double-cone photoreceptor marker) on the 20 dpf retinal sections. (A-D) Under DMSO treatment, the cone photoreceptors can differentiate postembryonic from the CMZ. (A'-D') With the inhibition of Notch activity, cone photoreceptors fail to differentiate and the little (or none) of the postembryonically newborn cells are zpr1-positive. (E) The data reveals a significantly smaller share of the zpr1^{+} BrdU^{+} cells in the BrdU^{+} population ($p = 0.0079$, MWU test) in the DAPT-treated fish. The percentage of cone photoreceptor in the newborn cohort has

decreased from 13.3% in the control to 4.1% in the DAPT-treated fish. Retinal sections (n = 5) were analyzed with Imaris. Data indicate mean ± SD. Two-tailed MWU test. **, $P < 0.01$.

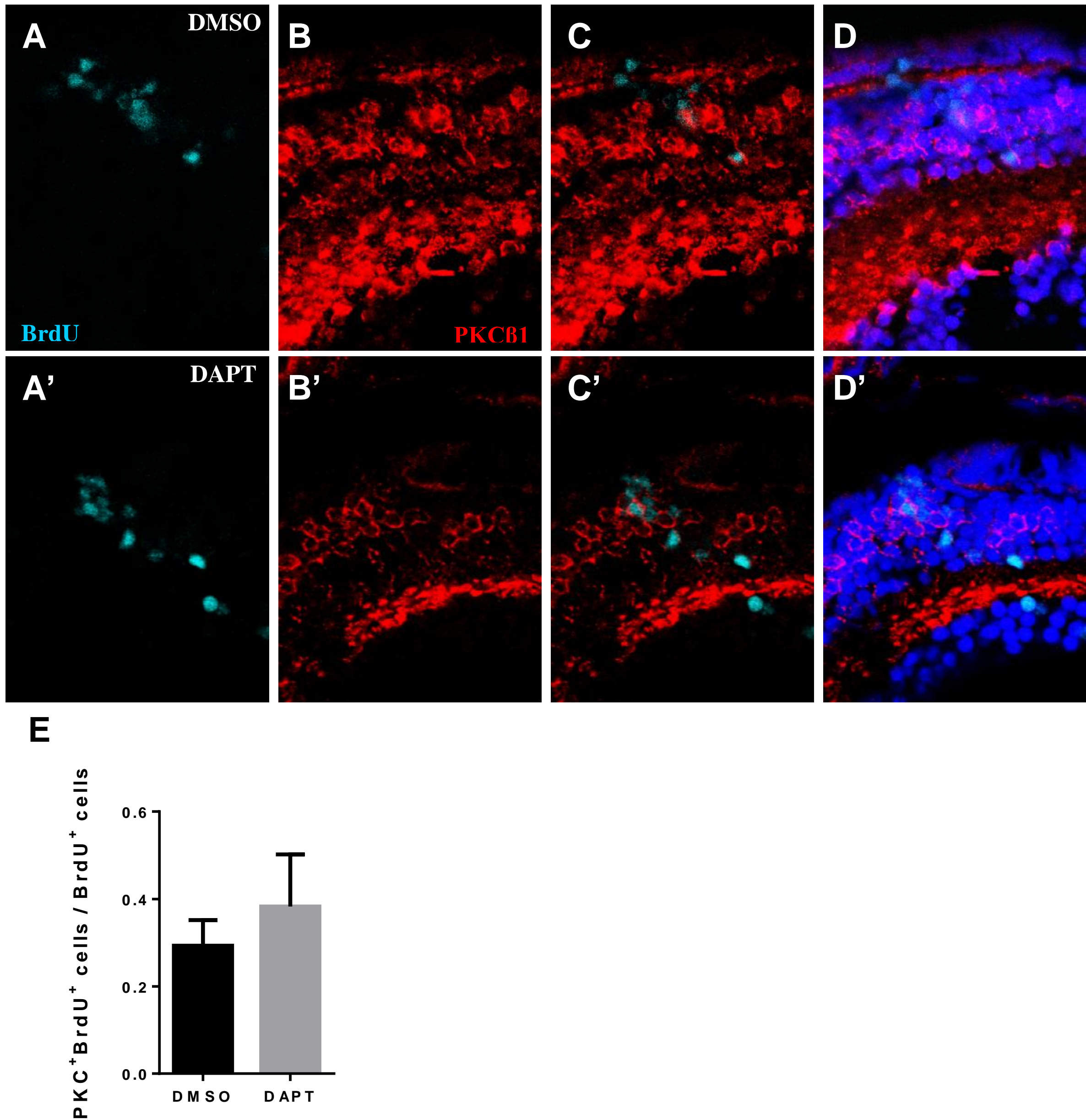


Figure 13. The inhibition of Notch results in a noticeable, but not significant, increase of the bipolar cells in a cohort of postembryonically newborn retinal population. The newborn bipolar cells were double-labeled with BrdU$^+$ cells (cyan) and PKCβ1 antibody (red, bipolar cell marker) on 23 dpf retinal section. Qualitatively, an increasing trend is observed between the DMSO-treated (A-D) and DAPT-treated retina (A'-D'). (E) The means are 29.3% in the control and 38.3% in the DAPT-treated fish, and the difference is however not significant ($p = 0.22$). Retinal sections ($n = 5$) were analyzed with Imaris. Data indicate mean ± SD.

3.3 Up-regulating the Notch pathway in larval zebrafish retina

3.3.1 Induced NICD expression

Previous gain-of-function analyses of Notch activity in the postnatal rodent retina have suggested that NICD stimulates proliferation while at the same time promotes Müller glia cell fate. It is interesting to test whether the upregulated Notch signaling has similar effects on the postembryonic zebrafish retina. In order to study this, heatshock-controlled mis-expression of Notch1a- intracellular domain (NICD) is performed by crossing two transgenic fish lines, *Tg(hsp70l:Gal4)* and *Tg(UAS:6xmyc-notch1a-ICD)* and heatshocking the double transgenic fish to induce the NICD expression (Scheer and Campos-Ortega, 1999; Scheer et al., 2001). Previous studies using these double transgenic fish *Tg(hsp70l:Gal4);(UAS:6xmyc-notch1a-ICD)* demonstrated that the mis-expressed NICD in zebrafish embryos leads to an excessive and premature glial cell production in the embryo, while at the same time maintains other RPCs in an undifferentiated state. To examine if the ectopically induced NICD affects the postembryonic retina in a similar way, I utilized the same double transgenic fish to perform the gain-of-function experiments.

While the double transgenic fish line has been widely used to study up-regulated Notch activity in the zebrafish embryos, the myc-NICD mis-expression pattern has never been reported in the postembryonic retina. In order to examine and confirm if a similar protocol could be performed on older fish, the double transgenic fish were divided into three groups and each received a 37℃ heatshock for 30 minutes at different time points, and all of them were sacrificed on 5.5 dpf (Fig 14). The first group was heatshocked at 4 dpf (Fig 14, A-E), the second group at 5 dpf (Fig 14, A'-E') and the last group at three hours prior to sacrifice at 5.5 dpf (Fig 14, A"-E"). IHC were then carried out using the anti-myc antibody (cells in red) on retinal sections. Interestingly, induced myc-NICD is broadly expressed in ONL, INL and the transition zone as early as 3 hours post-heatshock. However, it is expressed at very low levels (or absent) in most cells of the GCL and the peripheral CMZ (Fig 14B-E). With increasing time post-heatshock, the myc-NICD expression is depleted from the periphery (Fig 14B, C, B',C',B'',C''). In contrast, the signals in the ONL and INL of the central retina are similar between each group and persists for at least 1.5 d (Fig 14D, E, D', E', D", E"). It is noticeable that the myc-NICD protein in the central retina is either stably expressed at 1.5 day post-heatshock or has a slower turn-over rate. A previous study

by Scheer *et al.* has also reported a comparable trend at embryonic stages, in which the embryos were heatshocked at 24 hpf and fixed at 54 hpf and the myc-NICD staining is the strongest in the central retina and appears weaker at the margin (Scheer et al., 2001). However at 54 hpf the layers are not yet formed and all retinal cells in the central retina are myc-positive.

It is unclear why myc-NICD expression is not observed in the CMZ. It may be possible that the 30 minutes heatshock pulse is unable to induce myc-NICD expression specifically in these cells, or that the expression levels are too low to be detected here. Other possibilities include that the ectopically expressed myc-NICD proteins in these cells may not be detectable due to aggregation or proteins being packed in inclusion bodies, or that the myc-NICD proteins have a higher turn-over rate in these cells and thus cannot be captured within the time frames used. In addition, the fact that newborn retinal cells are generated and contributed to the existing retina from the CMZ should not be overlooked. The myc-positive cells may be "pushed away" from the transition zone by the newborn cells that escaped heat-shock induction. This, however, may not be the main cause due to the fact that the depletion of myc-NICD expression at the periphery is considerably faster than the production of new retinal cells.

Despite the fact that ectopic myc-NICD is not reported in the most peripheral CMZ, the double transgenic fish may still serve as a good model to study postembryonic RPC differentiation. All the following gain-of-function experiments were carried out by heatshocking the fish every day at 37℃ for 30 minutes until the desire day to ensure a sufficient level of myc-NICD is achieved at the transition zone.

3.3.2 Induced Notch activity does not alter RPC proliferation in the CMZ

As described above, ectopic expression of myc-NICD at the marginal CMZ is either extremely labile or is not inducible. Thus, it was predicted that the proliferation of RPCs should not be affected in double transgenic larvae after heatshock. In order to investigate this, the fish received a 30-miunute heatshock pulse at 37℃ everyday starting from 3.5 dpf, and then were sacrificed on 7.5 dpf. The retinal sections were labeled with PCNA antibody to examine the percentage of proliferating PCNA$^+$ CMZ cells in the whole retina (Fig 15, cells in red) and no significant

difference is reported between the siblings (Fig 15A, B) and the double transgenic fish (Fig 15A', B') (Fig 15C, mean = 0.041 and 0.044 respectively).

In addition, while the ectopic myc-NICD can be expressed in the differentiated INL, the number of PCNA-positive cells in the differentiated retina is not altered in the double transgenic fish (data not shown). While the results suggest that the up-regulated Notch activity is not sufficient to turn the mature Müller glia into mitotically active cells, detail examination (e.g. double-labeling of the myc-positive cells with GS antibody) is required to confirm whether the ectopic myc-NICD is truly expressed in the Müller glia.

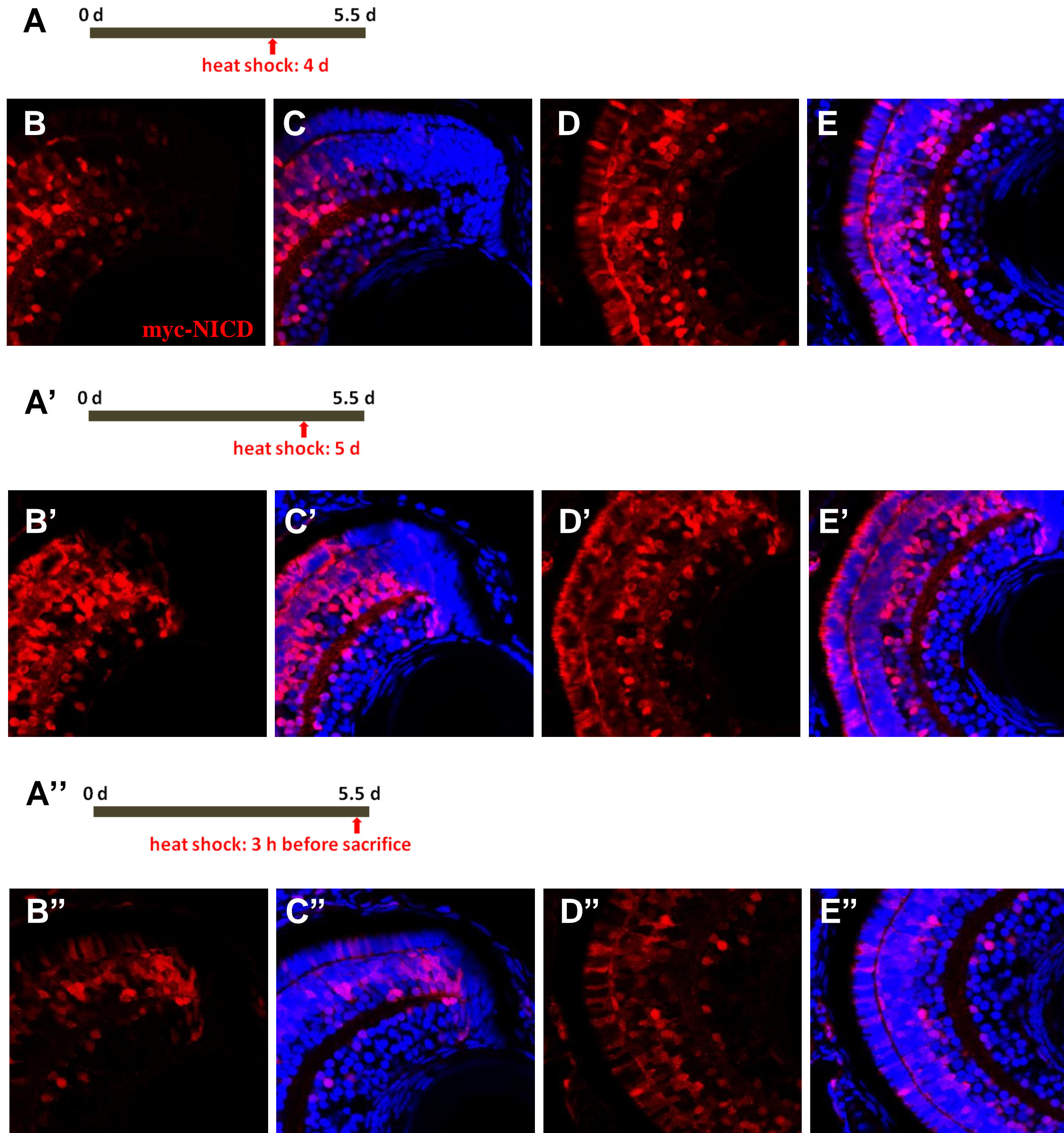


Figure 14. Mis-expression patterns of the ectopic myc-labeled NICD in the 5.5 dpf *Tg(hsp70l:Gal4);(UAS:6xmyc-notch1a-ICD)* peripheral retina (B-B", C-C") and central retina (D-D", E-E"). (n≧5) (A-A") Schemes for heatshock experimental designs. IHC were carried out using the anti-myc antibody (cells in red) on retinal sections. (B-B", C-C") Induced myc-NICD is expressed in the ONL and INL and the signals persist for at least 1.5 d. However, the myc-NICD expression expressed at very low levels (or absent) in most cells of the GCL and the peripheral retina (D-D", E-E") and the signals here are depleted over time.

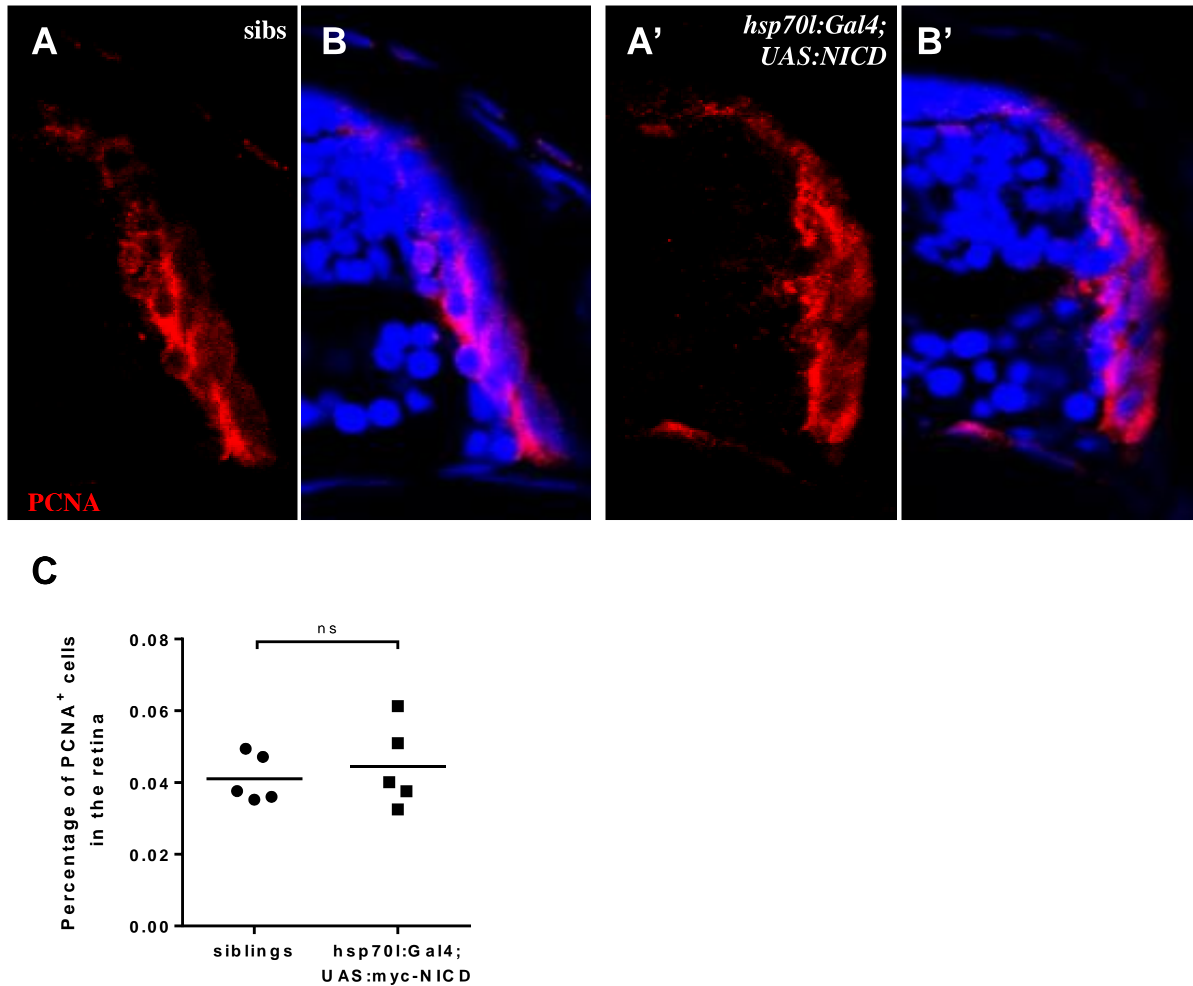


Figure 15. The induced Notch activity does not alter the RPC proliferation in the CMZ. The proliferating cells were labeled with PCNA antibody (cells in red). Te fish were heatshocked once a day at 37℃ for 30 miuntes until sacrifice on 7.5 dpf. No significant difference is reported between the 7.5 dpf siblings (A, B) and double transgenic fish (A', B'). (C) The numbers of proliferating CMZ cells are not different between the siblings and *Tg(hsp70l:Gal4);(UAS:6xmyc-notch1a-ICD)* retina (mean = 0.041 and 0.044 respectively). Retinal sections (n = 5) were analyzed with Imaris. Data indicate mean ± SD. Two-tailed MWU test.

3.3.3 The composition of newborn cells is altered after Notch gain of function

As described above, the Notch gain-of-function was carried out by heatshocking the double transgenic fish daily from 3.5 dpf to ensure sufficient myc-NICD is expressed at the transition zone. In order to understand how up-regulated Notch activity alters the composition of retinal cell during postembryonic stages, the double transgenic fish and their siblings were sacrificed at 7.5 dpf (or 8 dpf) and immunohistochemistry experiments were conducted using cell specific antibodies to examine cell identity (Fig 16, 17).

In the double transgenic fish, the lamination is reported to be disrupted in the retina (Fig 16A', white brackets; 16C', yellow bracket). While the relative apical-basal positions of different cell types seem to remain the same, the outer plexiform layer no longer exists and the INL and ONL are merged. These disruptions, however, are not seen throughout the whole retina, but only exist as pulses in the periphery. The fact that heatshock pulses are applied once a day and the level of myc-NICD at the peripheral retina decrease over time may be the reason for this. It is possible that the organized structure and proper cell differentiation may resume in between the daily heatshock intervals.

Interestingly, more Müller glia are observed to locate at these pulses as clusters (Fig 16A', white brackets). The Müller glial cells in the siblings are usually well aligned with intervals in between, with the soma located near the center of INL. In the heatshock-induced double transgenic fish, the soma of the MG cell within the pulses seem more apical than the soma of other MG cell. The finding suggests that the induced NICD is sufficient for newborn cells to adopt a Müller glial cell fate, which is consistent with the results from other experiments in which Notch pathway promotes gliogenesis (Dorsky et al., 1995; Georgi and Reh, 2011; Scheer et al., 2001).

For ganglion and amacrine cells, HuC/D immunostaining (Fig 17, cells in red) on the 7.5 dpf retina shows no obvious difference in the number of these cells between double transgenic fish (Fig 17A', B') and their siblings (Fig 17A, B). It is demonstrated that with the lamination being disrupted, HuC/D^{+} cells are sometimes, though rarely, located at a more apical position (Fig 17', white arrow). In contrast, zn5-positive cells were abnormally present in the INL (Fig 16C', yellow bracket) in the double transgenic fish. Zn5 antibody recognizes activated leukocyte cell

adhesion molecule a (alcama) protein and is known to label retinal ganglion cells and the optic nerve in the zebrafish retina. However, the zn5-positive cells in the INL seem spindle-shaped and thus do not look like ganglion cells by morphology. Also, while most of these zn5-labeled INL cells cluster at the periphery, especially at the disruptions where MG cell present as groups, they are not confined to the newborn cells, which are just added to the differentiated retina. Instead, the expression of these zn5-positive cells in the INL expands toward the central retina. These findings suggest that instead of these cells being newborn ganglion cells, which are displaced in the INL, zn5 expression might be turned on in some INL cells as a result of increased Notch activity. It is very likely that these HuC/D^{-}zn5^{+} positive cells are actually Müller glial cells, according to the morphology, clustered position and the hypothesis that Müller glial cells at the periphery are more sensitive to altered Notch activity than those in the central retina. Yet the exact identities of these cells remain to be fully determined, and the reason and consequence of alcama misexpression in them still need to be resolved. Interestingly, in recent studies, the adhesion molecule alcama has been reported to be a novel marker of multipotent retinal stem cells, such as injury-induced Müller glia cells (Nagashima et al., 2013).

For cone photoreceptors, immunostaining with zpr1 antibody on 7.5 dpf retina reveals that while cone photoreceptors seem to be formed properly at the periphery, the ONL is disrupted in the double transgenic fish (Fig 17C, D, C’, D’, white bracket). However, it is unclear at this stage whether the ratio of newborn cone photoreceptor is altered as a result of up-regulated Notch activity. Also, the zpr1 staining throughout the whole retina seems impaired, but this phenomenon should be uncoupled with the differentiation of cone photoreceptors at the transition zone.

For bipolar cells, immunolabeling with PKCβ1 antibody on 7.5 dpf retina were conducted and fewer bipolars cells are observed specifically at the disruptions where outer plexiform layer no longer exists and the INL and ONL are merged (Fig 17E, F, E’, F’, yellow bracket). Detailed quantification of the proportion of bipolar cells in the postembryonic newborn retinal cells was carried out and will be discussed later in this section.

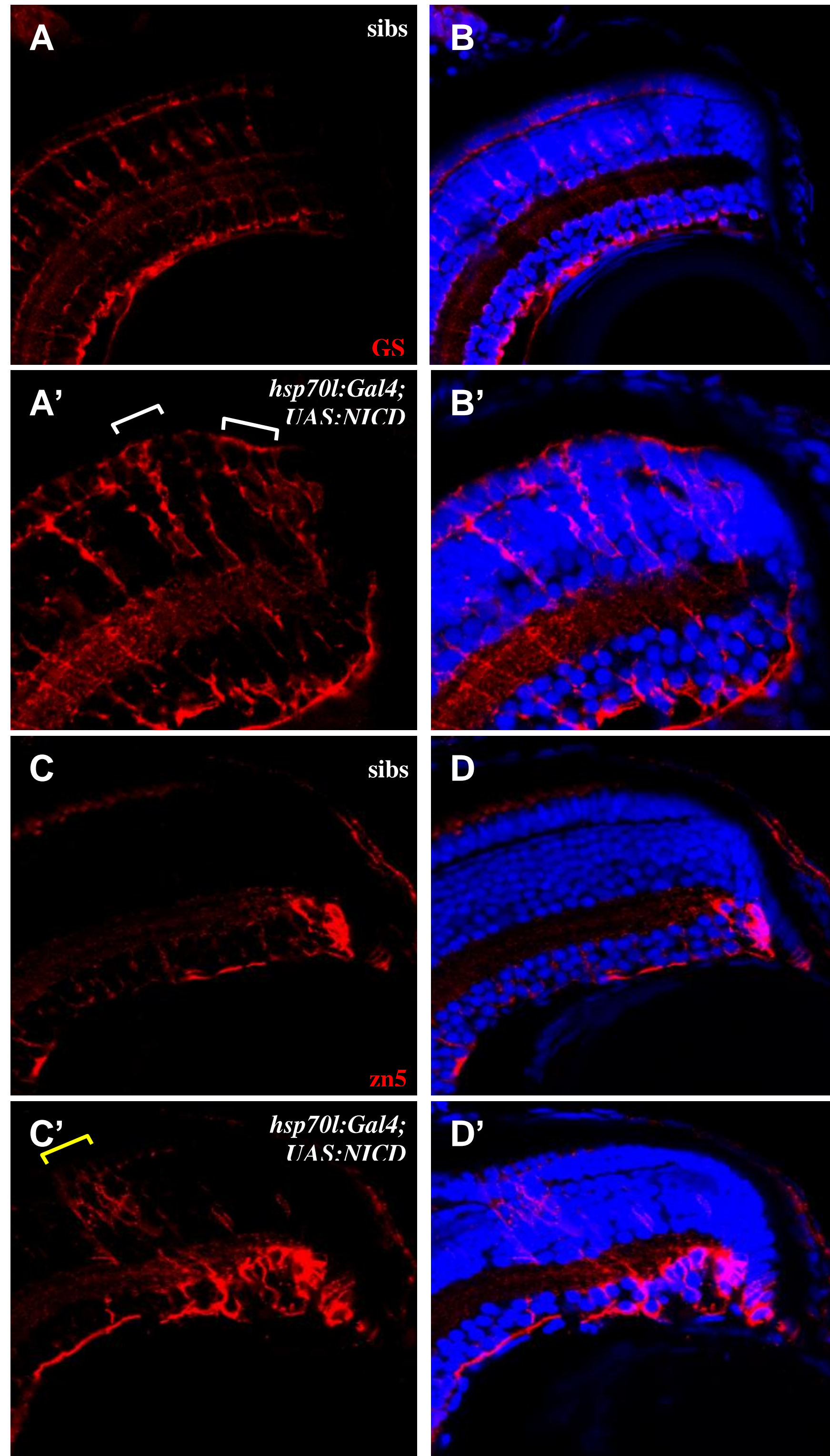


Figure 16. Forced expression of Notch1a-ICD disrupts the retinal lamination at the margin of postembryonic retina and more Müller glia are produced in the retina as clusters. An enhanced zn5signal is also observed in the Müller glia upon up-regulating Notch activity. Notch gain-of-

function was conducted by heatshocking the fish daily from 3.5 dpf to 7.5 dpf (or 8 dpf) to ensure sufficient myc-NICD is expressed at the transition zone. (A, B, A’, B’) Immunostaining using GS antibody (red, Müller glia marker) shows increased Müller glia in the double transgenic retina (A’, B’) compared with their siblings (A, B). The overproducted Müller glia locates in the retina as clusters, specifically in the regions where lamination is disrupted (white brackets). (C, D, C’, D’) Immunostaining using zn5 antibody (red, marker for ganglion cell and retinal stem cell) reveals an up-regulated zn5signal in the Müller glia of double transgenic fish (C’, D’, yellow bracket) compared with their siblings (C, D) (see text for details).

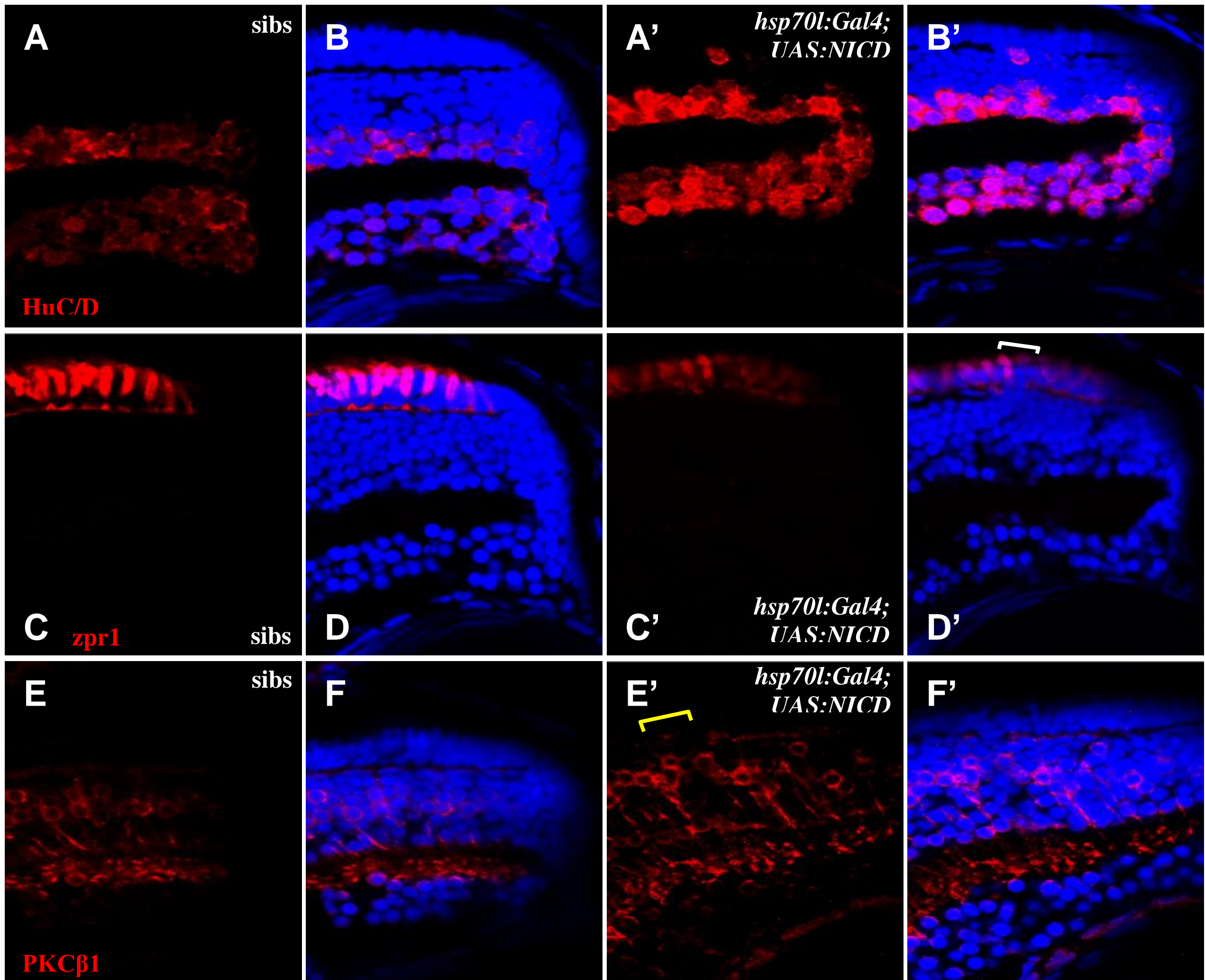
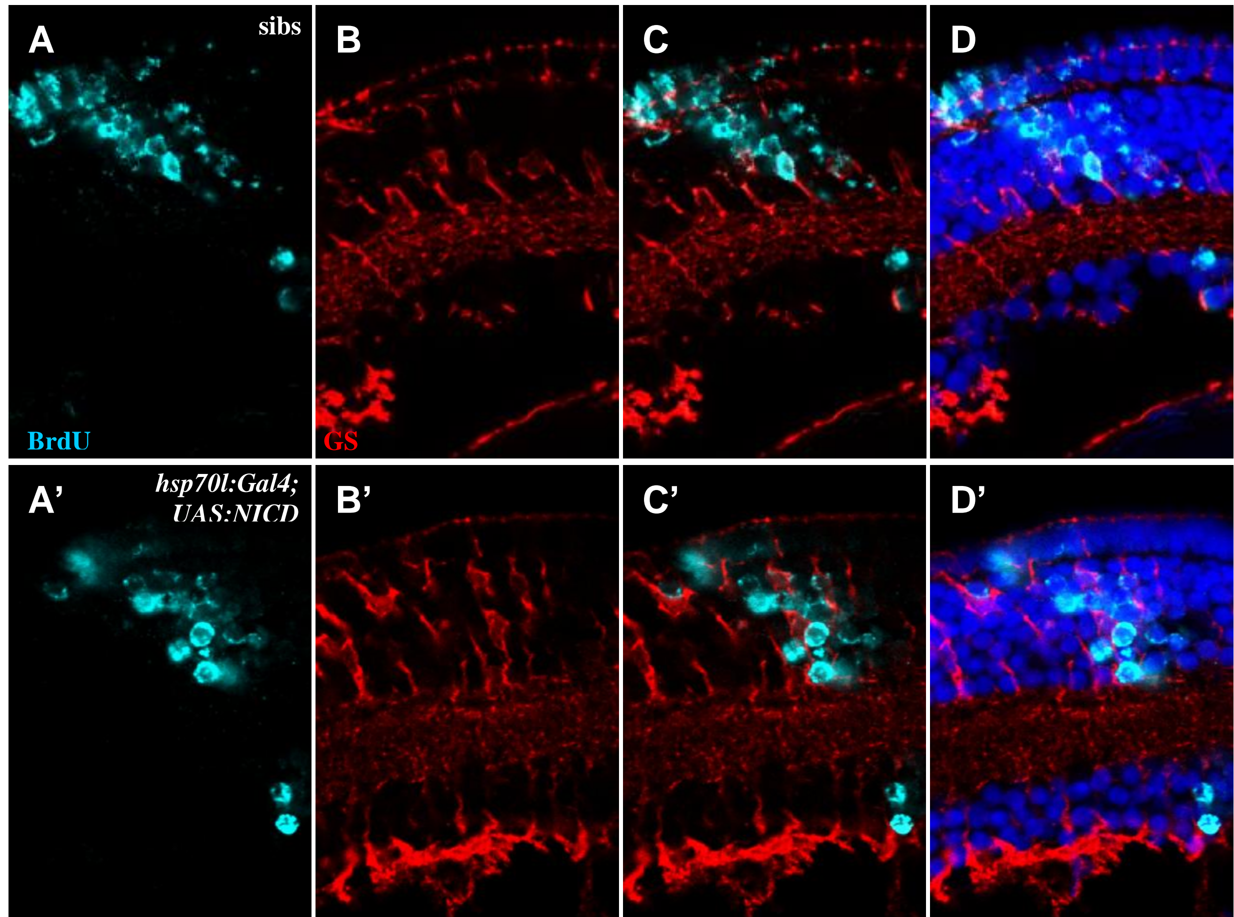


Figure 17. Up-regulated Notch activity alter the composition of retinal cells at the peripheral retina at 7.5 dpf. (A, B, A', B') HuC/D immunostaining (red, marker for ganglion and amacrine cells) shows no apparent difference between the double transgenic fish (A', B') and their siblings (A, B), suggesting no obvious changes in the differentiation of ganglion cells and amacrine cells. However, while the lamination is disrupted in the double transgenic fish (A', B'), a single HuC/D^{+} cell is sometimes, though rarely, located at a more apical position. (C, D, C', D') For cone photoreceptors, immunostaining with zpr1 antibody (red, double-cone photoreceptor marker) shows that while cone photoreceptors seem to be formed properly at the periphery, the ONL is sometimes disrupted in the double transgenic fish (C', D', white bracket) compared to their siblings (C, D). (E, F, E', F') Immunolabelings with PKCβ1 antibody (red, bipolar cell marker) were conducted to show that fewer bipolars cells exist specifically at the disruptions

where outer plexiform layer no longer exists and the INL and ONL are merged in the double transgenic fish (E', F', yellow bracket) compared to their siblings (E, F).

To further investigate how the induced Notch signaling modulates postembryonic cell fate determination and thus the composition of newborn retinal cells under this context, BrdU pulse-chase experiments were conducted. Likewise, only the Müller glia, cone photoreceptor and bipolar cells were further analyzed here as they appeared to be more disrupted by the myc-NICD induction. The fish were heatshocked at 37℃ for 30 minutes from 3.5 dpf to 8 dpf, and then maintained until 23 dpf before sacrifice. BrdU was applied on 5.5 dpf to track the newborn cohort. The $BrdU^+$ cells were double-labeled with cell specific markers to confirm the identities of the BrdU-traced newborn cells (Fig 18-20).

Co-labeling of the $BrdU^+$ cells (Fig 18, cells in cyan) with GS antibody (Fig 18, cells in red) shows that within these BrdU-traced newborn cells, the proportion of GS^+BrdU^+ cells is significantly increased in the double transgenic fish ($p = 0.0079$, MWU test) (Fig 18E). The percentage of MG cells in the newborn cohort has increased from 4.0% in the siblings (Fig 18A-D) to 6.3% in the double transgenic retina (Fig 18A'-D'). The finding indicates that the induced Notch activity is sufficient to promote the Müller glial cell fate in the postembryonic retina. Studies on Notch gain-of-function in the embryonic zebrafish retina and other postembryonic vertebrate retinas also reported similar results that Notch signaling promotes Müller glial cell fate determination (Dorsky et al., 1995; Georgi and Reh, 2011; Scheer et al., 2001). For newborn cone photoreceptors, colabeling $BrdU^+$ (Fig 19, cells in cyan) with zpr1 antibody (Fig 19, cells in red) reveals a significant increase of the proportion of $zpr1^+BrdU^+$ cells in the $BrdU^+$ population in the double transgenic fish ($p = 0.0317$, MWU test) (Fig 19E). The cone photoreceptor population has increased from 10.8% in the siblings (Fig 19A-D) to 15.5% in the double transgenic fish (Fig 19A'-D'), indicating that the induction of Notch also promotes cone photoreceptor cell fate. In contrast, double-labeling the newborn $BrdU^+$ cells (Fig 20, cells in cyan) with PKCβ1 antibody (Fig 20, cells in red) shows a significant decrease of the $PKC\beta1^+BrdU^+$ ratio in the $BrdU^+$ population. ($p = 0.0317$, MWU test) (Fig 20E). The ratio of bipolar cells in the $BrdU^+$ cohort has decreased by 26.6% from the siblings (mean = 30.1%) to double transgenic (mean = 22.1%) retina. Together my data suggest that the cell fate control of Müller glia, cone photoreceptor and bipolar cells are regulated by Notch signaling. Mis-expressing NICD in postembryonic zebrafish retina gives rise to a higher proportion of Müller glia and cone photoreceptors, at the expense of PKCβ1-positive bipolar cells in the newborn cohort.



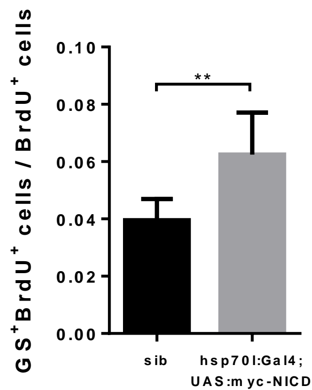
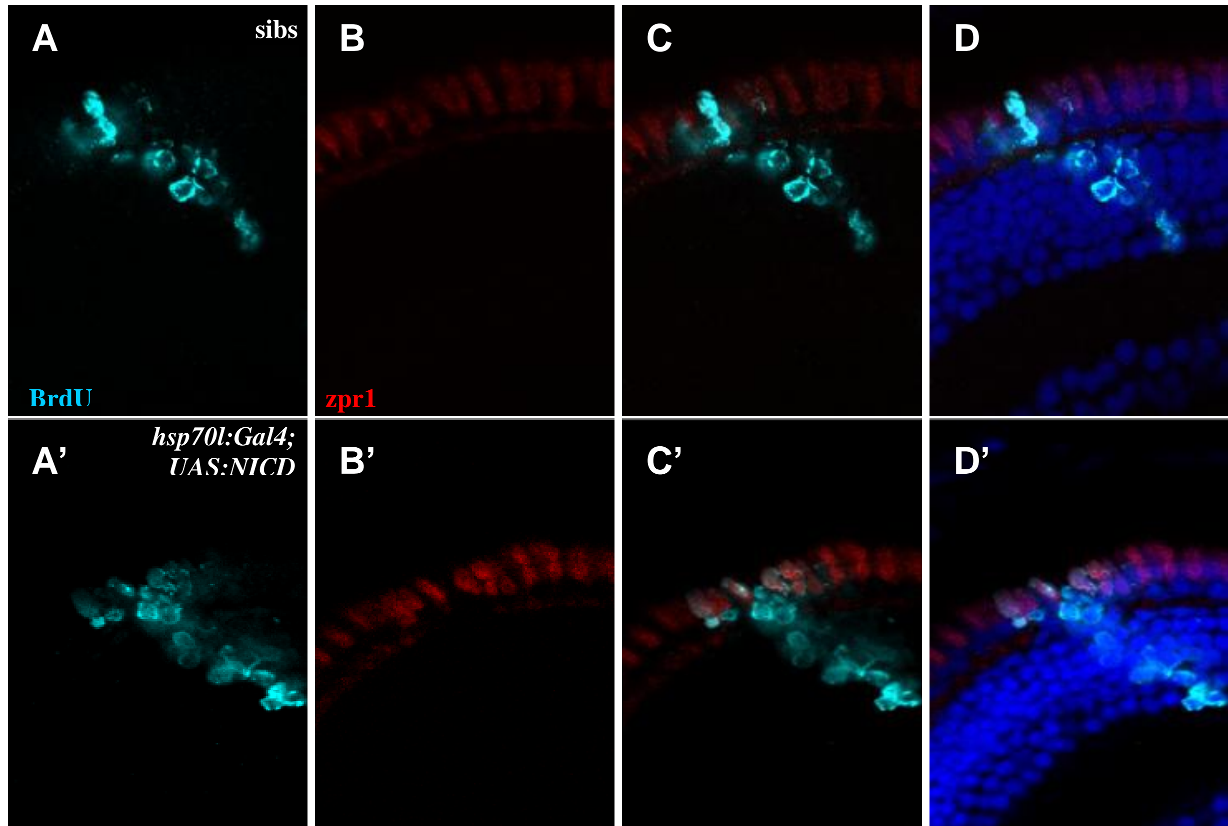


Figure 18. Notch signaling promotes the postembryonic retinal progenitors to adopt a Müller glial cell fate. *Tg(hsp70l:Gal4);(UAS:6xmyc-notch1a-ICD)* fish and their siblings receive a 30-minute heatshock at 37°C for 30 minutes from 3.5 dpf to 8 dpf, and then maintained until 23 dpf before sacrifice. BrdU was applied on 5.5 dpf to track the newborn cohort. IHC using GS (red, Müller glia marker) and BrdU antibody (cyan) were performed on the retinal sections to double

label the newborn Müller glia. Hoechst, cells in blue. (A-D) Müller glia are differentiated properly in the siblings retina. (A'-D') Overproduction of Müller glia is observed in the retina of double transgenic fish. (E) A significant increase ($p = 0.0079$, MWU test) of the $GS^{+}BrdU^{+}$ cells in the newborn cohort is reported in the NICD mis-expressing retina. Retinal sections ($n = 5$) were analyzed with Imaris. Data indicate mean ± SD. Two-tailed MWU test. **, $P < 0.01$.



E

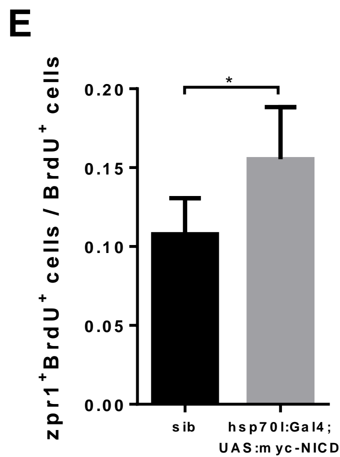
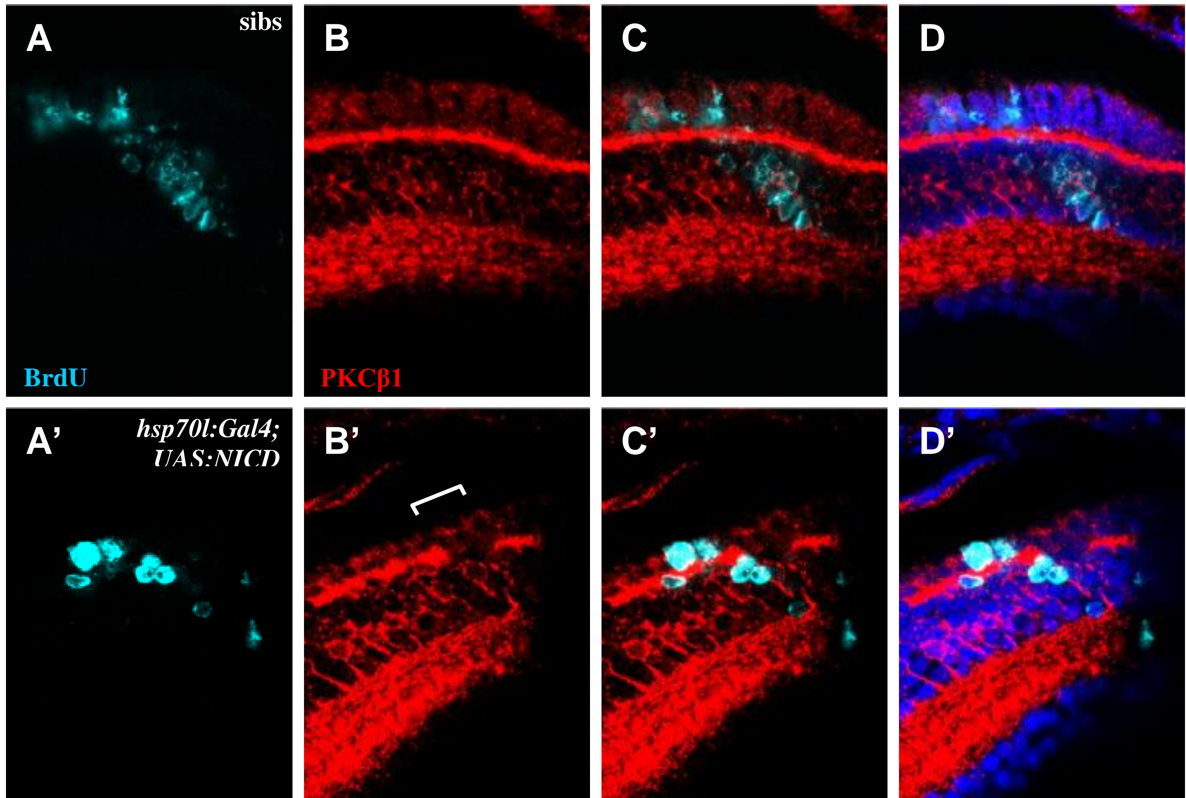


Figure 19. Up-regulated Notch signaling leads to overproduction of cone photoreceptors in the postembryonic zebrafish retina. *Tg(hsp70l:Gal4);(UAS:6xmyc-notch1a-ICD)* fish and their siblings receive a 30-minute heatshock at 37℃ for 30 minutes from 3.5 dpf to 8 dpf, and maintained until 23 dpf before sacrifice. BrdU was applied on 5.5 dpf. IHC using zpr1 (red, marker for double-cone photoreceptor) and BrdU antibody (cyan) were performed on the retinal sections to double label the newborn cone photoreceptors. Hoechst, cells in blue. (A-D) Cone

photoreceptors are differentiated properly in the sibling retina. (A'-D') The number of newborn cone photoreceptor is increased in the retina of double transgenic fish. (E) A significant increase ($p = 0.0317$, MWU test) of the zpr1^{+}BrdU^{+} cells in the newborn cohort is reported in the NICD mis-expressing retina. Retinal sections ($n = 5$) were analyzed with Imaris. Data indicate mean ± SD. Two-tailed MWU test. *, $P < 0.05$.



E

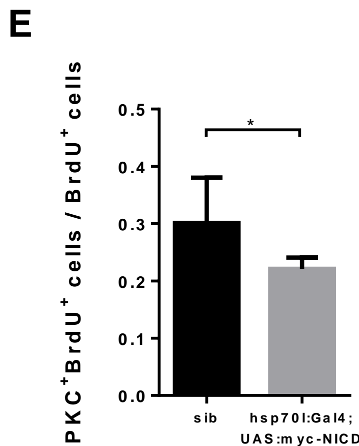


Figure 20. The induction of NICD leads to a decrease in the proportion of bipolar cells in the postembryonic newborn retinal cells. *Tg(hsp70l:Gal4);(UAS:6xmyc-notch1a-ICD)* fish and their siblings receive a 30-minute heatshock at 37°C for 30 minutes from 3.5 dpf to 8 dpf, and maintained until 23 dpf before sacrifice. BrdU was applied on 5.5 dpf. IHC using PKCβ1 (red, bipolar cell marker) and BrdU antibody (cyan) were performed on the retinal sections to double label the newborn bipolar cells. Hoechst, cells in blue. (A-D) Bipolar cells are differentiated properly in the siblings retina. (A'-D') Fewer bipolar cells are observed in the retina of double transgenic fish. (E) A significant decrease ($p = 0.0317$, MWU test) of the PKCβ1$^+$BrdU$^+$ cells in

the newborn cohort is reported in the NICD mis-expressing retina. Retinal sections (n = 5) were analyzed with Imaris. Data indicate mean ± SD. Two-tailed MWU test. *, $P < 0.05$.

Chapter 4
Discussion

4.1 Summary of the results

The goal of this project was to determine the functional roles of Notch signaling during postembryonic retinogenesis in zebrafish. My data show that despite the fact that CMZ cells express *Notch1a* receptor and are apparently competent for Notch signaling, no Notch activity is reported in this region. Notch signaling is activated predominantly in the INL cells, Müller glia and some bipolar cells specifically, and the transition zone between the CMZ and differentiated peripheral retina, where it regulates cell fate decisions. Down-regulating Notch activity results in postembryonic newborn cells unable to adopt Müller glia and cone photoreceptor cell fates, but differentiate disproportionately into bipolar cells. On the other hand, increased Notch activity leads to a higher proportion of newborn Müller glia and cone photoreceptor cells, at the expense of bipolar cells. These findings are consistent with the observations in other studies of vertebrate retinogenesis that Notch signaling promotes Müller glia cell fate in the embryonic retina (Dorsky et al., 1995; Georgi and Reh, 2011; Scheer et al., 2001). In contrast, inactivation of Notch activity in the mouse retina results in an excess of rod photoreceptors (Mizeracka et al., 2013a) whereas here the transient inhibition of Notch signaling results in fewer cone photoreceptors.

Although the Notch pathway is required for cell fate determination, it seems that the pathway is not required for RPC maintenance in the CMZ as the Notch pathway is not activated at the peripheral CMZ and the size of the RPC population remains unaltered after up- or down-regulating Notch signaling. While the *n1a* receptor expression pattern suggests the competence for Notch activation in CMZ cells, the underlying mechanisms that inhibit Notch activity here remain unresolved. This observation is in line with recent evidence in the embryonic zebrafish retina, in which downregulated Notch activity does not deplete the pool of RPC (Bernardos et al., 2005). However, this finding is in direct contradiction to the observations in other vertebrate models where Notch pathway regulates proliferation and is required for the maintenance of stem cell-like properties (Dorsky et al., 1997; Dorsky et al., 1995); (Henrique et al., 1997); (Riesenberg et al., 2009; Yaron et al., 2006).

The data presented here contribute insight into how neurogenesis in a postembryonic retinal stem cell niche is regulated by Notch signaling. As the expression patterns of Notch pathway genes and the functional role of Notch signaling varies somewhat in different vertebrate models, it is interesting to investigate whether a similar mechanism can explain the varying phenotypic results, or that different underlying regulations exist in different vertebrates. Here I will compare my results to the some of the findings from the literature, draw some postulations according to the data and discuss some open questions. In particular, (1) how cell fate control of RPC is regulated by Notch signaling, (2) the possible functions of the continuing Notch activity in postembryonic Müller glia; and (3) the possible reasons why Notch activity is absent in the postembryonic zebrafish CMZ will be addressed.

4.2 Notch-dependent cell fate determination during postembryonic retinogenesis

Notch signaling in vertebrate retinal development has been extensively studied in different models to reveal several functional roles. Specifically for cell fate determination, while various studies have reported the functions of Notch pathway in regulating cell diversity, it is not fully understood when Notch signaling influences cell fate choices during postembryonic stages. My data here report that in the postembryonic retina, Notch activity is not observed in the peripheral CMZ, but exists in the transition zone of CMZ and differentiated retina, suggesting that Notch signaling is activated in (1) progenitor cells during their last few cycles and/or (2) postmitotic cells that have just exited the cell cycle. In addition, while the exposure to Notch inhibitor DAPT leads to decreased production of Müller glia and cone photoreceptors, the proper differentiation of these cells can be resumed when DAPT is no longer applied. The myc-NICD mis-expressing retina showed a similar pattern whereby only a specific subgroup of RPCs are sensitive to Notch induction. The disproportionate differentiation of Müller glia, cone photoreceptor and bipolar cells only exist as a “burst” in the retina, specifically in the region where outer plexiform layer disappears and the ONL and INL merge. As the level of myc-NICD decreases over time between the daily heatshock intervals, the proper formation of lamination and composition of newborn cells resume between the pulses. Together these data suggest that Notch pathway regulates cell fate determination only in the late progenitors and/or newly postmitotic cells, and manipulating

Notch activity in these cell can result in a permanent effect, in terms of cell fate choice, in the newborn retinal cells. In line with this, a previous study on the postnatal mouse retina has demonstrated that the conditional knockout of Notch1 leads to a biased cell fate in the newly postmitotic retinal cells (Mizeracka et al., 2013a). However, it is unclear how this temporal control of Notch activity is achieved. In zebrafsih, while my data suggest that the stem cells/early progenitor cells may be as competent for Notch activity as the late progenitors, the actual time frame of activated Notch signaling is not determined in the postembryonic RPCs. The *deltaC* and *notch1a* expression patterns suggest that the activation of Notch signaling may be, in part, spatially regulated through the different transcriptional levels of Notch ligand and receptor in different progenitors. However, other intrinsic mechanisms may also exist to regulate Notch activation here. Future study exploring the temporal and spatial controls of Notch activity may provide insight into the functions of Notch pathway in RPC differentiation.

The fact that Notch signaling is activated at the transition zone of the uncommitted CMZ and differentiated retina in larval zebrafish retina suggests its functional role in cell fate determination during postembryonic retinal development. However, while Notch signaling has been widely reported to regulate cell fate control of retinal progenitors in many vertebrate embryos, it is less understood in the postembryonic retinogenesis. Here my data addressed this question by examining how postembryonic retinal progenitors react to the alteration of Notch activity in the zebrafish retina.

The inhibition of Notch signaling has been reported to result in an overproduction of early cell types. In the developing *Xenopus* retina, progenitors mis-expressing Delta1, which leads to Notch inhibition through negative feedback, produce more ganglion cells at an early developmental stage (Dorsky et al., 1997). Similar trends have been observed in the chick retina where retinal progenitors that mis-express the dominant-negative construct of Delta1, which blocks the Delta-Notch signaling within the same cell, give rise to a higher number of ganglion and amacrine cells (Henrique et al., 1997). Parallel to this, inhibition of Notch expression in the embryonic chick retina also increases the number of ganglion cells (Austin et al., 1995). Together with the facts that reduced proliferation, premature cell exit and increased neuronal differentiation are observed under the inhibition of Notch in vertebrate retina (Dorsky et al., 1997; Dorsky et al., 1995; Riesenberg et al., 2009), these observations lead to the hypothesis that Notch signaling is required to block cell differentiation and the removal of Notch allows the cell to

adopt an early-born cell fate (Jadhav et al., 2006). In contrast, here my data suggest no obvious effects on the early born cell (ganglion and amacrine cells) fate determination upon up- and down-regulating Notch activity in the postembryonic zebrafish retina. This finding is in line with previous studies that, despite the disrupted retinal organization, ganglion cells can differentiate and locate near the inner retina under the inhibition of Notch in zebrafish embryos (Bernardos et al., 2005). Together with the observation that CMZ proliferation is not affected by the alteration of Notch activity, it is possible that Notch signaling may not be required nor sufficient to suppress progenitor cells from exiting cell cycle and adopting an early born cell fate in zebrafish and the influence of Notch signaling on ganglion cell fate control may be species-specific.

The differentiation of cone photoreceptors has also been reported to be Notch-dependent in some vertebrate models. During the early development of the mouse retina, the inhibition of Notch1 leads to a remarkable increase in the number of cone photoreceptors, at the expense of other cell types (Jadhav et al., 2006). In fact, Notch1 has been reported to suppress cone photoreceptor fate in the developing mouse retina (Yaron et al., 2006). In line with this, the study on developing *Xenopus* retina also reveals a dramatically enhanced cone photoreceptor cell fate in the Delta1 mis-expressing progenitors (Dorsky et al., 1997). The loss of function experiments on zebrafish embryos reveal that the inhibition of Notch also impairs the differentiation of photoreceptors (Bernardos et al., 2005). Here my data showed that in the zebrafish retina, the postembryonic newborn cells are unable to adopt cone photoreceptor cell fate under the inhibition of Notch signaling. On the other hand, increased Notch activity leads to a higher proportion of newborn cone photoreceptor cells. It is unclear why the inhibition of Notch gives rise to opposing outcomes, in terms of cone photoreceptor fate control, in different vertebrates. While underlying mechanisms of how Notch signaling regulate photoreceptor differentiation need to be further determined, differences between species may also exist.

In addition, Notch signaling has been shown to regulate bipolar cell fate determination by previous studies. Deletion of Notch1 in postnatal mouse retina promotes rod photoreceptor production at the expanse of bipolar and Müller glia cells (Jadhav et al., 2006). Similar result has been reported that upon the removal of Notch1 in newly postmitotic mouse retina, fewer bipolar cells are generated (Mizeracka et al., 2013a). However, my data here show an opposing trend that the inhibition of Notch signaling during postembryonic zebrafish retinal development gives rise to more bipolar cells while over-expression of Notch1a results in fewer bipolar cells in the

newborn cohort. Again the Notch regulation of bipolar cell fate may be species-specific, yet gain- and loss-of-function experiments should be conducted on zebrafish of other ages to confirm this. Still, the reasons why, and how, the differentiation of various cell types are controlled by the same signaling pathway, during similar developmental stages in the same species, remain unresolved. One possible reason is that varying levels of Notch activity in retinal progenitors may give rise to different outcomes. It will be interesting the investigate if the level of Notch activity in late progenitors and newly postmitotic cell is correlated with different cell fate choice.

Unlike the varying outcomes of other retinal cell fate controls reported above, the Notch regulation of Müller glia cell fate determination has been revealed to be more consistent among different vertebrates. During embryonic stages, Notch signaling has been described to promote Müller glial cell fate and gain-of-function studies reveal the production of excessive Müller glia in *Xenopus* (Dorsky et al., 1995), zebrafish (Scheer et al., 2001) and mouse embryos (Georgi and Reh, 2011). In line with this, similar trends have been reported in the postnatal mouse that forced expression of Notch pathway components promotes retinal progenitors to adopt a Müller glial cell fate (Furukawa et al., 2000) and the inhibition of Notch leads to a decrease in Müller glia production (Jadhav et al., 2006; Mizeracka et al., 2013a). These data have suggested a model that prolonged Notch activity in the retinal progenitors results in the adoption of Müller glial cell fate during both embryonic and postembryonic retinal development in vertebrates. In addition, a genome-wide microarray analysis has demonstrated that Notch signaling is required to maintain the glial cell fate in the postmitotic cells during a certain postnatal period (Nelson et al., 2011). In postembryonic zebrafish retina, a recent study has reported that Notch signaling is required to repress dedifferentiation and proliferation in undamaged retina in adult zebrafish (Conner et al., 2014). My data here also demonstrate that Notch signaling is continuously activated in mature Müller glia. Together these findings suggest that Notch signaling may exhibit two distinct roles in the vertebrate retina: (1) regulating the decision of glial and non-glial cell fate choice in the uncommitted progenitors and (2) maintaining glial properties in the postmitotic Müller glia, at least during some certain postembryonic stages. Yet detailed mechanisms of how the controls of gliogenesis and neurogenesis are determined by the regulation of Notch remain unclear.

It is interesting that while multiple retinal cell types require Notch signaling for proper differentiation, the pathway is particularly important for the maintenance of Müller glial identity.

What do these glial cells become upon the removal of Notch? Specifically in zebrafish, as noted above, Notch signaling is required to repress dedifferentiation and proliferation in Müller glia and the inhibition of Notch signaling has been described to permit Müller glia of regenerative activities (Conner et al., 2014). However, besides serving as a main source of stem cell/progenitor cell during retinal regeneration, it has also been reported that the rod photoreceptor lineage comes from Müller glia in the differentiated postembryonic retina under normal conditions (Bernardos et al., 2007). Lineage-tracing study shows that Müller glial cells in the central retina are the main source of rod-lineage progenitors in zebrafish as they undergo de-differentiation and mitotically divide to produce Pax6-positive neural progenitors, which translocate toward ONL and differentiate into rod photoreceptors. However previous studies have not addressed how the activity of Notch during this process is regulated. While it is unclear whether Notch activity is as well down-regulated in Müller glia as they generate rod progenitors in the uninjured growing zebrafish retina, findings from rodent retina have provided insight into the relation of Notch signaling and rod photoreceptor differentiation. During the postembryonic stages in the mouse retina, Notch signaling has been shown to inhibit rod photoreceptor fate and the removal of Notch leads to an overproduction of rod photoreceptors at the expense of Müller glia and bipolar cells (Jadhav et al., 2006; Mizeracka et al., 2013a). In line with this, misexpression of activated Notch1 and Hes1 in the postnatal rat retina gives rise to cells that are positive for Müller glia markers while in the control retina most cells develop into normal rod photoreceptor cells (Furukawa et al., 2000). However, it should be noted that rod photoreceptors account for around 97% of the photoreceptor population in the ONL in mouse retina (Carter-Dawson and LaVail, 1979). In addition to these data, microarray study of single cells also reveals that the expression of *NeuroD1*, a proneural bHLH transcription factor, which induces photoreceptor fate, is upregulated in the *Notch1* conditional knockout retina (Mizeracka et al., 2013b). Together the results have demonstrated the crucial role of Notch signaling to inhibit rod photoreceptor differentiation in the rodent retina.

Interestingly, recent studies on mammalian retina have reported that isolated Müller glia from adult mouse and human retina are able to differentiate into rod photoreceptors *in vitro* (Giannelli et al., 2011). The authors have also shown that the inhibition of Notch pathway in mouse Müller glia leads to a noticeable reduction in gliogenesis and an increase in neurogenesis, most importantly photoreceptor formation. It is interesting to hypothesize that a similar mechanism

may be regulating the rod photoreceptor lineage in both rodent and zebrafish: Notch signaling exhibits a stage-specific effect of on cell fate determination: during early development, Notch signaling is required to suppress neuronal cell fate (early born cell fates) and maintain the cells at an undifferentiated state (progenitor cells in rodents and Müller glia in zebrafish); whereas at later developmental stages, Notch signaling inhibits them from adopting rod photoreceptor fate and the inhibition of Notch is required to activate their neurogenic potential. However, this hypothesis raises several questions including: (1) is down-regulating Notch signaling crucial in zebrafish Müller glia as they generate the rod lineage progenitors, specifically under normal undamaged conditions; (2) what are the regulating mechanisms that inhibit the progenitors and Müller glia from becoming rods; and (3) what are the regulators that direct and distinguish these cells to a specific neuronal cell fate (rod photoreceptor and/or other neurons)? Investigating the underlying mechanisms may provide insight into the differentiation and regeneration of rod photoreceptor upon injury to possibly restore vision loss in human retina.

4.3 Müller glia, Notch activity and stemness

The regeneration ability of Müller glia is considerably robust in teleost fish compared with other vertebrate animals (reviewed by (Fischer and Bongini, 2010). Retinal injury in fish results in de-differentiation of Müller glia, followed by cell cycle entry and one self-renewing asymmetric division to give rise to a rapidly dividing progenitor that generates the proliferating regeneration cluster. In contrast, the mammalian Müller glia have restricted ability to regenerate in response to injury. It is of great interest to resolve the underlying mechanisms that regulates the varying regeneration ability of Müller glia in different vertebrates, which can potentially enable us to understand how to enhance retinal regeneration the human retina.

The activation of Müller glia during postembryonic retina regeneration has been associated with the Notch signaling pathway in several vertebrate models. However, conflicting observations are reported. In postnatal chick retina, it has been reported that Notch signaling is activated in Müller glia in response to injury and the inhibition of Notch reduces the potential of Müller glia to become proliferating progenitors (Ghai et al., 2010; Hayes et al., 2007). In the rodent retina, it has been reported that Müller glia are capable of proliferating and expressing retinal stem cell markers in response to damage, but their regeneration ability *in vivo* is strictly limited (Karl et al.,

2008). Similarly, the expression of Notch pathway components are increased (Karl et al., 2008; Wan et al., 2012) when the retina is injured. Together the data indicate that Notch signaling is up-regulated in chick and rodent retina in order to stimulate Müller glia in the damaged retina. However, studies on zebrafish retina reveal that Notch signaling may act very differently under similar conditions. In fact, a recent study has reported that in the intact, undamaged zebrafish retina, Notch signaling is required to repress dedifferentiation and proliferation in Müller glia (Conner et al., 2014). In the injured zebrafish retina, Notch signaling also suppresses the process by restricting the number of injury-activated Müller glia. Interestingly, while the pathway acts to inhibit the regeneration ability of Müller glia, the components of Notch pathway are actually induced by injury (Wan et al., 2012). The reason why Notch pathway seems to exhibit opposing roles in different vertebrates is unclear due to the fact that a detailed mechanism of how it regulates retinal regeneration is yet to be resolved.

Investigation of Notch activity in the undamaged retina among different animals may shed light on the functional roles of Notch in response to damage. In this thesis I report that in the postembryonic zebrafish retina, a relatively higher Notch activity is observed in the Müller glia. In contrast, study on chick retina shows that the components of Notch pathway are expressed at low levels in the undamaged chick retina, with the peripheral retina exhibiting higher expression levels compared to the central retina (Ghai et al., 2010). The same study has also reported that the inhibition of Notch activity in the Müller glia before injury enhances neuronal survival, suggesting that the low level of Notch signaling in the Müller glia decreases the neuroprotective properties of these glia in the postnatal chick retina.

In the mouse retina, a genome-wide analysis of Müller glia differentiation has been conducted on by Nelson *et al.* to reveal that components of the Notch pathway are highly expressed in the presumptive Müller glia during postnatal retinal development (Nelson et al., 2011). The continuing expression of Notch signaling for almost a week after these cells become postmitotic is hypothesized to stabilize and maintain the Müller glial fate, by preventing them from differentiating into other cell fates. During this period their glial identity is labile, and the expression levels of proneural transcription factors steadily decrease while the levels of other gliogenic TFs increase. After this period, however, Notch signaling decreases and the Müller glial identity is no longer Notch-dependent. It is interesting that, during the period that Müller glia are “immature” and their glial identity labile, these glial cells react to the alteration of Notch

activity in the same manner that Müller glia in the zebrafish retina do. The inhibition of Notch has been reported to result in loss of glial markers and induction of Ascl1a, a critical factor for Müller glial proliferation, in both undamaged zebrafish adult retina (Conner et al., 2014) and the “immature” postnatal mouse retina (Nelson et al., 2011).

Comparing current data from different vertebrate models suggest that the level of Notch activity may be associated with: (1) Müller glia identity; and (2) the proliferative ability of Müller glia. I hypothesize that the stem cell properties of Müller glia may be regulated by Notch activity: During retinal development, Notch signaling is activated in the newly post-mitotic cells to promote a Müller glial cell fate. The newborn Müller glia, which have a higher Notch activity, may be less committed and more plastic. The higher Notch activity at this stage is required to maintain the Müller glia identity thus these cells are sensitive to changes in Notch activity. This high-Notch activity status may be the terminal state for normal retinal development in the zebrafish, which means that the “mature” Müller glia in the postembryonic fish retina are less committed and more plastic than the mature Müller glia in other vertebrates with a diminished regenerative capacity. In contrast, the Müller glia in other vertebrates continue to become more committed and more “glial” as gliogenesis takes place. Glial markers increase and Notch activity decreases during the process and the mature Müller glia in these animals are more committed and less plastic. As noted above, components of Notch pathway are expressed at low levels in the postnatal chick retina (Ghai et al., 2010). In line with this, the expression of Notch pathway components in the postnatal mouse retina starts to decline after they reach their peak around postnatal day 10 (Nelson et al., 2011) and the glial identity is no longer regulated by Notch at later stages.

With this model, Notch activity may regulate stem cell properties of Müller glia in a way that lower Notch activity (e.g. in the chick and rodent retina) allows Müller glia to act more protective to the neurons and thus increase their survival rates, but at the same time dampens the stemness of Müller glia and decreases the potential damage-induced proliferation. Müller glia with higher Notch activity (e.g. Müller glia in zebrafish) may be more stem cell-like and less glia-like, and perhaps less neuroprotective in response to injury. This may be the reason why Notch signaling seems to have different functional roles in the Müller glia of different animals. In the zebrafish retina, Notch signaling is required to repress dedifferentiation and proliferation in Müller glia and the inhibition of Notch stimulates regenerative activities (Conner et al., 2014).

A possible reason for this is that Notch signaling, similar to that in the immature rodent Müller glia, is required to maintain glial identity. In other words, Notch activity may need to be down-regulated in these glia in order to proliferate and re-enter mitotic cycle upon injury. In contrast, the avian and rodent Müller glia are likely less stem cell-like and more glia-like, which means they have limited ability to react as stem cell/progenitor. Notch activity is reported to be up-regulated after injury in the Müller glia (Hayes et al., 2007; Karl et al., 2008) and a possible reason may be that an increase in Notch activity is required to convert glia into a less glial and less mature status in order for them to lose glial properties and gain stemness before they can act like stem cell/progenitor. However, the fact that Müller glia in these vertebrate are unable to do so *in vivo* suggests that tissue environment may impose limits on how much stemness they can acquire.

To investigate this hypothesis, critical mechanisms need to be resolved. It would be important to examine if, and how, Notch signaling is associated with the glial identity of Müller glia. In addition, how Notch activity is eventually down-regulated as Müller glia become mature in mammalian retina may shed light on the possibility to restore Notch activity and "reactivate" these glia in response to injury. While data suggest that Notch activity may be crucial for the regulation of stem cell and glial properties in Müller glia, other mechanisms are required for proper regeneration. The study on intact, undamaged zebrafish retina shows that the inhibition of Notch signaling using a more potent γ-secretase inhibitor RO4929097 in the undamaged retina upregulates Ascl1a and Stat3 expression, which are crucial for Müller glial proliferation, and as a result, more proliferating ($PCNA^+$) Müller glia cells are observed (Conner et al., 2014). Ascl1a has been reported to participate in Müller glia reprogramming and retinal regeneration, as well as the proliferation in embryonic stem cells (Ramachandran et al., 2010).The loss of Notch signaling alone, however, is not sufficient to promote neuronal lineage specification as the proliferating neural progenitors cannot differentiate and eventually undergo cell death. Coinjection of RO4929097 and exogenous TNFα, an identified damage signal, results in a synergistic increase in the number of committed neuronal progenitor cell clusters, which mimics the damage- activated mechanism reported in the retina. The data suggest that down-regulated Notch signaling alone is not sufficient for proper regeneration and other regulating mechanisms, such as damage signals, are critical for the progenitor cells to commit to neuronal fates. This

injury-gated regulation may be important so that the regeneration only occurs when an actual cell loss exists.

The level of Notch activity has been linked to the transition of quiescent and active states in radial glia. Previous studies have shown that the high levels of Notch signaling in the endocrine progenitor cells and adult telencephalon neural stem cells in quiescence and the inhibition of Notch leads to the re-entry of cell cycle in both cases (Chapouton et al., 2010; Ninov et al., 2012). Specifically in the ventricular zone of adult zebrafish, radial glia/progenitors at three different states are reported: (1) nondividing radial glial cells expressing radial glia markers but not PCNA; (2) dividing radial glial cells expressing both glia marker and PCNA; and (3) committed progenitors that express PCNA in the absence of radial glia markers (Chapouton et al., 2010). The state I cells are considered progenitors maintained in quiescence by high Notch signaling, while the state II cells are considered the dividing progenitors and state III the committed progenitors in the process of becoming neurons. The authors demonstrate that Notch induction can convert dividing progenitors into quiescence, whereas the inhibition of Notch promotes proliferation and subsequent commitment. In line with this, my data has revealed that the induction of Notch leads to an up-regulated expression of alcama, an identified stem cell marker, in the Müller glia, while PCNA is not induced. The adhesion molecule, alcama, is recently reported to be a multipotent retinal stem cell marker in the CMZ and injury-induced Müller glia cells of adult zebrafish (Nagashima et al., 2013).

It would be important to resolve: (1) the relative levels of Notch activity in Müller glia at different states; (2) if the level of Notch activity regulates the transition of quiescent and active states in Müller glia similar to the way it does in the radial glia in ventricular zone; and (3) whether this high-Notch activity, PCNA-negative Müller glia status resembles the proliferation state of the state I nondividing radial glial cells in telencephalon. This hypothesis may explain why the induction of myc-NICD in the zebrafish larval retina, while converting Müller glia into a more stem cell-like status (e.g. expressing stem cell marker), maintains the glia at a quiescent state (e.g. not expressing PCNA) in the undamaged retina. The answer to these questions may be beneficial to our understanding of how retinal regeneration is initiated as the fact that Müller glia are often considered dormant and injury-induced events are important to reprogram them. Future study of these mechanisms underlying Müller glia regulation may advance our understanding of

retinal regeneration and shed light on new possibilities for the treatment of retinal diseases in humans.

4.4 Numb as a candidate for inhibiting Notch activity in the CMZ

The Notch pathway has been widely reported to regulate retinal development in vertebrates and one of its predominate roles is to maintain RPC identity. In the embryos of other vertebrate models, the continued activation of Notch has been shown to inhibit the progenitor cells from differentiation (Dorsky et al., 1997; Dorsky et al., 1995) and the inactivation of Notch has been demonstrated to reduce RPC proliferation, as well as promote premature cell exit and neuronal differentiation (Riesenberg et al., 2009). However, in the embryonic zebrafish retina, disrupted Notch signaling does not deplete the proliferating RPC pool (Bernardos et al., 2005). Consistent with this, my data shows that in the postembryonic zebrafish retina, no endogenous Notch activity is observed in the peripheral CMZ and relatively low levels in the ganglion cell layer. In addition, the mis-expression patterns of myc-NICD shows that the myc-NICD induction is broadly expressed in the retina except the peripheral CMZ and most cells in the ganglion cell layer. However, Notch receptor gene *n1a* is transcribed in the peripheral CMZ, suggesting that the CMZ cells are competent for Notch activation. Together these findings reveal that Notch pathway may not be required for the RPC maintenance in zebrafish and that possible post-transcriptional or post-translational regulatory mechanisms may exist to strictly control the Notch activity in the CMZ (and perhaps GCL). A well known inhibitor of Notch, Numb, is reported to inhibit Notch activity in *Drosophila* and mammals (Couturier et al., 2013; McGill et al., 2009). This cell-fate determinant is reported to be partitioned unequally into two daughter cells during asymmetric cell division. By promoting Notch degradation through endosomal sorting in one cell but not another, Numb creates an unequal and directional Notch signaling, and thus different cell fates between the two cells.

In zebrafish embryos, *numb* is broadly expressed in the central nervous system (Niikura et al., 2006). It is reported that during teleost embryogenesis, knockdown of *numb* and *numblike* could cause hematopoietic defects while overexpression results in malformation of optic cup and even cyclopia (Bogdanovic et al., 2012; Niikura et al., 2006). My preliminary FISH data shows that zebrafish *numb* is expressed postembryonically in the CMZ and GCL at 5.5 dpf (Fig 21).

Therefore I hypothesize that Numb is playing an essential role here to inhibit Notch activity and the failure of mis-expressing myc-NICD in these regions may be due to Notch being rapidly degraded by Numb. Also, based on this hypothesis, I would expect that it is possible to sensitize the CMZ to Notch activity by down-regulating Numb.

In order to knockdown Numb in the CMZ, a published antisense morpholino targeting both zebrafish *numb* and *numblike* (Bresciani et al., 2010) was microinjected into the *Tg(Tp1bglob:eGFP)* zebrafish embryos to determine if the inhibition of Numb can lead to a higher Notch activity. However, my preliminary Western blot data (not shown here) shows that the levels of eGFP protein exhibit no difference between the morphants and uninjected fish. One possible reason could be that the eGFP protein is too stable so that the differences are overlooked; thus the effect should be more prominent using the *hsp70l:Gal4;UAS:myc-NICD* fish. However the normal morpholinos are injected at 1 to 2 cell stage, which not only inevitably affects the embryonic retinal development, but also wears out at later stages. For the purpose of analyzing whether Numb has a functional role in the postembryonic zebrafish retina, one could attempt to microinject the larvae using the cardiac ventricular method (Konantz and Antos, 2014) to achieve the temporal control of gene knockdown at postembryonic stages in the hsp70l:Gal4;UAS:myc-NICD double transgenic fish.

An alternative approach to block the Numb-mediated Notch degradation at the CMZ would be using a proteosome inhibitor. Previous studies have shown that Numb promotes the ubiquitination of Notch receptors and MG132, a proteosome inhibitor, has been reported to inhibit the ubiquitin proteosome pathway in zebrafish embryos (Yogev et al., 2013). Together with the morpholino method, a slower turnover rate of NICD or even mis-expression at the most peripheral CMZ is expected using the hsp70l:Gal4;UAS:myc-NICD fish model. This would confirm the essential role of Numb at this stem cell niche, which is likely to be inhibiting the Notch activity and preventing the RPCs from adopting a certain cell fate too early. However, my preliminary Western blot data has failed to confirm the proteosome inhibiting function of MG132 in both zebrafish embryos and larvae. The use of other proteosome inhibitors (e.g., chlorpromazine and cytochalasin B, drugs that have been reported to selectively inhibit clathrin-mediated endocytosis; and nystatin and methyl-β-cyclodextrin which targets caveolae-dependent endocytosis (Hussain et al., 2011)) may be able to confirm the hypothesis that Numb-mediated endosomal sorting is the reason for the lack of Notch activity and mis-expressed myc-NICD in

the postembryonic zebrafish CMZ and provide insight into the strict regulations of Notch activity in zebrafish retinal stem cells. Further experiments investigating why, and how, Notch signaling is required to maintain retinal stem cell characters in other vertebrate models but not in zebrafish may be critical to answer why active retinal stem cells/progenitors are found in the postembryonic teleosts *in vivo* but are absent from other warm-blooded vertebrates including human.

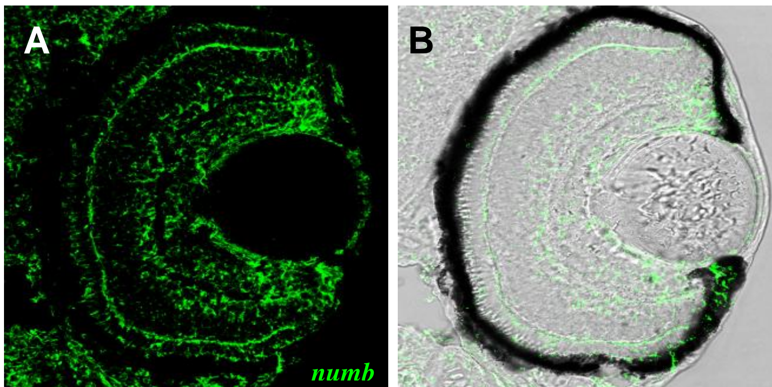


Figure 21. Expression pattern of *numb* in 5.5 dpf AB wild-type zabrafish retinal sections. (A, B) zebrafish *numb* is expressed postembryonically in the peripheral CMZ and GCL.

4.5 Conclusion

The significant proliferation and regeneration ability in the postembryonic zebrafish retina has become an interesting and important topic to study. While various studies have revealed that Notch signaling is one of the key regulators of proliferation and differentiation in the embryonic vertebrate retina, very little is understood about the signaling at the postembryonic stages. For the first time, this thesis reports that the proliferative ability of RPCs in the postembryonic zebrafish retinal stem cell niche, the CMZ, is surprisingly Notch-independent, and possible regulatory mechanisms are discussed. Notch activity, however, is up-regulated in late-progenitors/newly post-mitotic cells for cell fate decision controls of postembryonic newborn retinal cells. Relatively higher Notch activity is also reported in the fish Müller glia and a hypothesized model is discussed in respect of the level of Notch activity and proliferative status of these glial cells. Together the thesis provides insight into our understandings of RPCs and Müller glia behavior in the postembryonic retina, and how Notch signaling modulates these behaviours.

References

Agathocleous, M., and Harris, W.A. (2009). From progenitors to differentiated cells in the vertebrate retina. Annual review of cell and developmental biology *25*, 45-69.

Andersson, E.R., Sandberg, R., and Lendahl, U. (2011). Notch signaling: simplicity in design, versatility in function. Development *138*, 3593-3612.

Austin, C.P., Feldman, D.E., Ida, J.A., Jr., and Cepko, C.L. (1995). Vertebrate retinal ganglion cells are selected from competent progenitors by the action of Notch. Development *121*, 3637-3650.

Bassett, E.A., and Wallace, V.A. (2012). Cell fate determination in the vertebrate retina. Trends in neurosciences *35*, 565-573.

Baye, L.M., and Link, B.A. (2007). Interkinetic nuclear migration and the selection of neurogenic cell divisions during vertebrate retinogenesis. The Journal of neuroscience : the official journal of the Society for Neuroscience *27*, 10143-10152.

Baye, L.M., and Link, B.A. (2008). Nuclear migration during retinal development. Brain research *1192*, 29-36.

Bernardos, R.L., Barthel, L.K., Meyers, J.R., and Raymond, P.A. (2007). Late-stage neuronal progenitors in the retina are radial Muller glia that function as retinal stem cells. The Journal of neuroscience : the official journal of the Society for Neuroscience *27*, 7028-7040.

Bernardos, R.L., Lentz, S.I., Wolfe, M.S., and Raymond, P.A. (2005). Notch-Delta signaling is required for spatial patterning and Muller glia differentiation in the zebrafish retina. Developmental biology *278*, 381-395.

Blackshaw, S., Harpavat, S., Trimarchi, J., Cai, L., Huang, H., Kuo, W.P., Weber, G., Lee, K., Fraioli, R.E., Cho, S.H.*, et al.* (2004). Genomic analysis of mouse retinal development. PLoS biology *2*, E247.

Bogdanovic, O., Delfino-Machin, M., Nicolas-Perez, M., Gavilan, M.P., Gago-Rodrigues, I., Fernandez-Minan, A., Lillo, C., Rios, R.M., Wittbrodt, J., and Martinez-Morales, J.R. (2012). Numb/Numbl-Opo antagonism controls retinal epithelium morphogenesis by regulating integrin endocytosis. Developmental cell *23*, 782-795.

Bresciani, E., Confalonieri, S., Cermenati, S., Cimbro, S., Foglia, E., Beltrame, M., Di Fiore, P.P., and Cotelli, F. (2010). Zebrafish numb and numblike are involved in primitive erythrocyte differentiation. PloS one *5*, e14296.

Buchman, J.J., and Tsai, L.H. (2008). Putting a notch in our understanding of nuclear migration. Cell *134*, 912-914.

Carter-Dawson, L.D., and LaVail, M.M. (1979). Rods and cones in the mouse retina. I. Structural analysis using light and electron microscopy. The Journal of comparative neurology *188*, 245-262.

Casarosa, S., Leone, P., Cannata, S., Santini, F., Pinchera, A., Barsacchi, G., and Andreazzoli, M. (2005). Genetic analysis of metamorphic and premetamorphic Xenopus ciliary marginal zone. Developmental dynamics : an official publication of the American Association of Anatomists *233*, 646-651.

Cau, E., and Blader, P. (2009). Notch activity in the nervous system: to switch or not switch? Neural development *4*, 36.

Cepko, C. (2014). Intrinsically different retinal progenitor cells produce specific types of progeny. Nature reviews Neuroscience *15*, 615-627.

Chapouton, P., Skupien, P., Hesl, B., Coolen, M., Moore, J.C., Madelaine, R., Kremmer, E., Faus-Kessler, T., Blader, P., Lawson, N.D*., et al.* (2010). Notch Activity Levels Control the Balance between Quiescence and Recruitment of Adult Neural Stem Cells. Journal of Neuroscience *30*, 7961-7974.

Chapouton, P., Webb, K.J., Stigloher, C., Alunni, A., Adolf, B., Hesl, B., Topp, S., Kremmer, E., and Bally-Cuif, L. (2011). Expression of hairy/enhancer of split genes in neural progenitors and neurogenesis domains of the adult zebrafish brain. The Journal of comparative neurology *519*, 1748-1769.

Cisneros, E., Latasa, M.J., Garcia-Flores, M., and Frade, J.M. (2008). Instability of Notch1 and Delta1 mRNAs and reduced Notch activity in vertebrate neuroepithelial cells undergoing S-phase. Molecular and cellular neurosciences *37*, 820-831.

Clements, W.K., and Traver, D. (2013). Signalling pathways that control vertebrate haematopoietic stem cell specification. Nature reviews Immunology *13*, 336-348.

Coles, B.L., Angenieux, B., Inoue, T., Del Rio-Tsonis, K., Spence, J.R., McInnes, R.R., Arsenijevic, Y., and van der Kooy, D. (2004). Facile isolation and the characterization of human retinal stem cells. Proceedings of the National Academy of Sciences of the United States of America *101*, 15772-15777.

Conner, C., Ackerman, K.M., Lahne, M., Hobgood, J.S., and Hyde, D.R. (2014). Repressing notch signaling and expressing TNFalpha are sufficient to mimic retinal regeneration by inducing Muller glial proliferation to generate committed progenitor cells. J Neurosci *34*, 14403-14419.

Couturier, L., Mazouni, K., and Schweisguth, F. (2013). Numb localizes at endosomes and controls the endosomal sorting of notch after asymmetric division in Drosophila. Current biology : CB *23*, 588-593.

Del Bene, F. (2011). Interkinetic nuclear migration: cell cycle on the move. The EMBO journal *30*, 1676-1677.

Del Bene, F., Wehman, A.M., Link, B.A., and Baier, H. (2008). Regulation of neurogenesis by interkinetic nuclear migration through an apical-basal notch gradient. Cell *134*, 1055-1065.

Dorsky, R.I., Chang, W.S., Rapaport, D.H., and Harris, W.A. (1997). Regulation of neuronal diversity in the Xenopus retina by Delta signalling. Nature *385*, 67-70.

Dorsky, R.I., Rapaport, D.H., and Harris, W.A. (1995). Xotch inhibits cell differentiation in the Xenopus retina. Neuron *14*, 487-496.

Dyer, M.A., and Cepko, C.L. (2001). Regulating proliferation during retinal development. Nature reviews Neuroscience *2*, 333-342.

Fadool, J.M., and Dowling, J.E. (2008). Zebrafish: a model system for the study of eye genetics. Prog Retin Eye Res *27*, 89-110.

Fischer, A.J., and Bongini, R. (2010). Turning Muller glia into neural progenitors in the retina. Molecular neurobiology *42*, 199-209.

Fischer, A.J., and Reh, T.A. (2000). Identification of a proliferating marginal zone of retinal progenitors in postnatal chickens. Developmental biology *220*, 197-210.

Fischer, A.J., and Reh, T.A. (2001). Muller glia are a potential source of neural regeneration in the postnatal chicken retina. Nature neuroscience *4*, 247-252.

Fischer, A.J., and Reh, T.A. (2003). Potential of Muller glia to become neurogenic retinal progenitor cells. Glia *43*, 70-76.

Fryer, C.J., White, J.B., and Jones, K.A. (2004). Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover. Molecular cell *16*, 509-520.

Furukawa, T., Mukherjee, S., Bao, Z.Z., Morrow, E.M., and Cepko, C.L. (2000). rax, Hes1, and notch1 promote the formation of Muller glia by postnatal retinal progenitor cells. Neuron *26*, 383-394.

Ganz, J., Kaslin, J., Hochmann, S., Freudenreich, D., and Brand, M. (2010). Heterogeneity and Fgf dependence of adult neural progenitors in the zebrafish telencephalon. Glia *58*, 1345-1363.

Geling, A., Steiner, H., Willem, M., Bally-Cuif, L., and Haass, C. (2002). A gamma-secretase inhibitor blocks Notch signaling in vivo and causes a severe neurogenic phenotype in zebrafish. EMBO reports *3*, 688-694.

Georgi, S.A., and Reh, T.A. (2011). Dicer is required for the maintenance of notch signaling and gliogenic competence during mouse retinal development. Dev Neurobiol *71*, 1153-1169.

Ghai, K., Zelinka, C., and Fischer, A.J. (2010). Notch signaling influences neuroprotective and proliferative properties of mature Muller glia. The Journal of neuroscience : the official journal of the Society for Neuroscience *30*, 3101-3112.

Giannelli, S.G., Demontis, G.C., Pertile, G., Rama, P., and Broccoli, V. (2011). Adult human Muller glia cells are a highly efficient source of rod photoreceptors. Stem cells *29*, 344-356.

Gu, P., Harwood, L.J., Zhang, X., Wylie, M., Curry, W.J., and Cogliati, T. (2007). Isolation of retinal progenitor and stem cells from the porcine eye. Molecular vision *13*, 1045-1057.

Guruharsha, K.G., Kankel, M.W., and Artavanis-Tsakonas, S. (2012). The Notch signalling system: recent insights into the complexity of a conserved pathway. Nature reviews Genetics *13*, 654-666.

Hans, S., Scheer, N., Riedl, I., v Weizsacker, E., Blader, P., and Campos-Ortega, J.A. (2004). her3, a zebrafish member of the hairy-E(spl) family, is repressed by Notch signalling. Development *131*, 2957-2969.

Hashimoto, T., Zhang, X.M., Chen, B.Y., and Yang, X.J. (2006). VEGF activates divergent intracellular signaling components to regulate retinal progenitor cell proliferation and neuronal differentiation. Development *133*, 2201-2210.

Hayes, S., Nelson, B.R., Buckingham, B., and Reh, T.A. (2007). Notch signaling regulates regeneration in the avian retina. Developmental biology *312*, 300-311.

Heitzler, P., and Simpson, P. (1991). The choice of cell fate in the epidermis of Drosophila. Cell *64*, 1083-1092.

Henrique, D., Hirsinger, E., Adam, J., Le Roux, I., Pourquie, O., Ish-Horowicz, D., and Lewis, J. (1997). Maintenance of neuroepithelial progenitor cells by Delta-Notch signalling in the embryonic chick retina. Current biology : CB *7*, 661-670.

Hori, K., Sen, A., and Artavanis-Tsakonas, S. (2013). Notch signaling at a glance. Journal of cell science *126*, 2135-2140.

Hussain, K.M., Leong, K.L., Ng, M.M., and Chu, J.J. (2011). The essential role of clathrin-mediated endocytosis in the infectious entry of human enterovirus 71. The Journal of biological chemistry *286*, 309-321.

Jadhav, A.P., Mason, H.A., and Cepko, C.L. (2006). Notch 1 inhibits photoreceptor production in the developing mammalian retina. Development *133*, 913-923.

Jarriault, S., Le Bail, O., Hirsinger, E., Pourquie, O., Logeat, F., Strong, C.F., Brou, C., Seidah, N.G., and Isra l, A. (1998). Delta-1 activation of notch-1 signaling results in HES-1 transactivation. Molecular and cellular biology *18*, 7423-7431.

Jennings, B., Preiss, A., Delidakis, C., and Bray, S. (1994). The Notch signalling pathway is required for Enhancer of split bHLH protein expression during neurogenesis in the Drosophila embryo. Development *120*, 3537-3548.

Johns, P.R. (1977). Growth of the adult goldfish eye. III. Source of the new retinal cells. The Journal of comparative neurology *176*, 343-357.

Karl, M.O., Hayes, S., Nelson, B.R., Tan, K., Buckingham, B., and Reh, T.A. (2008). Stimulation of neural regeneration in the mouse retina. Proceedings of the National Academy of Sciences of the United States of America *105*, 19508-19513.

Katoh, K., Yamazaki, R., Onishi, A., Sanuki, R., and Furukawa, T. (2012). G9a histone methyltransferase activity in retinal progenitors is essential for proper differentiation and survival of mouse retinal cells. The Journal of neuroscience : the official journal of the Society for Neuroscience *32*, 17658-17670.

Kolpak, A., Zhang, J., and Bao, Z.Z. (2005). Sonic hedgehog has a dual effect on the growth of retinal ganglion axons depending on its concentration. The Journal of neuroscience : the official journal of the Society for Neuroscience *25*, 3432-3441.

Konantz, J., and Antos, C.L. (2014). Reverse genetic morpholino approach using cardiac ventricular injection to transfect multiple difficult-to-target tissues in the zebrafish larva. Journal of visualized experiments : JoVE.

Kopan, R., and Ilagan, M.X. (2009). The canonical Notch signaling pathway: unfolding the activation mechanism. Cell *137*, 216-233.

Kroehne, V., Freudenreich, D., Hans, S., Kaslin, J., and Brand, M. (2011). Regeneration of the adult zebrafish brain from neurogenic radial glia-type progenitors. Development *138*, 4831-4841.

Kubo, F., and Nakagawa, S. (2008). Wnt signaling in retinal stem cells and regeneration. Development, growth & differentiation *50*, 245-251.

Lenkowski, J.R., and Raymond, P.A. (2014). Muller glia: Stem cells for generation and regeneration of retinal neurons in teleost fish. Progress in retinal and eye research *40*, 94-123.

Lewis, J. (1996). Neurogenic genes and vertebrate neurogenesis. Current opinion in neurobiology *6*, 3-10.

Livesey, F.J., Young, T.L., and Cepko, C.L. (2004). An analysis of the gene expression program of mammalian neural progenitor cells. Proceedings of the National Academy of Sciences of the United States of America *101*, 1374-1379.

Locker, M., El Yakoubi, W., Mazurier, N., Dullin, J.P., and Perron, M. (2010). A decade of mammalian retinal stem cell research. Archives italiennes de biologie *148*, 59-72.

Lord-Grignon, J., Abdouh, M., and Bernier, G. (2006). Identification of genes expressed in retinal progenitor/stem cell colonies isolated from the ocular ciliary body of adult mice. Gene expression patterns : GEP *6*, 992-999.

Louvi, A., and Artavanis-Tsakonas, S. (2006). Notch signalling in vertebrate neural development. Nature reviews Neuroscience *7*, 93-102.

MacNeil, A., Pearson, R.A., MacLaren, R.E., Smith, A.J., Sowden, J.C., and Ali, R.R. (2007). Comparative analysis of progenitor cells isolated from the iris, pars plana, and ciliary body of the adult porcine eye. Stem cells *25*, 2430-2438.

Martinez-Navarrete, G.C., Angulo, A., Martin-Nieto, J., and Cuenca, N. (2008). Gradual morphogenesis of retinal neurons in the peripheral retinal margin of adult monkeys and humans. The Journal of comparative neurology *511*, 557-580.

Martins, R.A., and Pearson, R.A. (2008). Control of cell proliferation by neurotransmitters in the developing vertebrate retina. Brain research *1192*, 37-60.

McGill, M.A., Dho, S.E., Weinmaster, G., and McGlade, C.J. (2009). Numb regulates post-endocytic trafficking and degradation of Notch1. The Journal of biological chemistry *284*, 26427-26438.

Mizeracka, K., DeMaso, C.R., and Cepko, C.L. (2013a). Notch1 is required in newly postmitotic cells to inhibit the rod photoreceptor fate. Development *140*, 3188-3197.

Mizeracka, K., Trimarchi, J.M., Stadler, M.B., and Cepko, C.L. (2013b). Analysis of gene expression in wild-type and Notch1 mutant retinal cells by single cell profiling. Developmental dynamics : an official publication of the American Association of Anatomists *242*, 1147-1159.

Nagashima, M., Barthel, L.K., and Raymond, P.A. (2013). A self-renewing division of zebrafish Muller glial cells generates neuronal progenitors that require N-cadherin to regenerate retinal neurons. Development *140*, 4510-4521.

Nelson, B.R., Gumuscu, B., Hartman, B.H., and Reh, T.A. (2006). Notch activity is downregulated just prior to retinal ganglion cell differentiation. Developmental neuroscience *28*, 128-141.

Nelson, B.R., Ueki, Y., Reardon, S., Karl, M.O., Georgi, S., Hartman, B.H., Lamba, D.A., and Reh, T.A. (2011). Genome-wide analysis of Muller glial differentiation reveals a requirement for Notch signaling in postmitotic cells to maintain the glial fate. PloS one *6*, e22817.

Nelson, S.M., Park, L., and Stenkamp, D.L. (2009). Retinal homeobox 1 is required for retinal neurogenesis and photoreceptor differentiation in embryonic zebrafish. Developmental biology *328*, 24-39.

Neumann, C.J., and Nuesslein-Volhard, C. (2000). Patterning of the zebrafish retina by a wave of sonic hedgehog activity. Science *289*, 2137-2139.

Niikura, Y., Tabata, Y., Tajima, A., Inoue, I., Arai, K., and Watanabe, S. (2006). Zebrafish Numb homologue: phylogenetic evolution and involvement in regulation of left-right asymmetry. Mechanisms of development *123*, 407-414.

Ninov, N., Borius, M., and Stainier, D.Y. (2012). Different levels of Notch signaling regulate quiescence, renewal and differentiation in pancreatic endocrine progenitors. Development *139*, 1557-1567.

Ohnuma, S., Hopper, S., Wang, K.C., Philpott, A., and Harris, W.A. (2002). Co-ordinating retinal histogenesis: early cell cycle exit enhances early cell fate determination in the Xenopus retina. Development *129*, 2435-2446.

Parks, A.L., Turner, F.R., and Muskavitch, M.A. (1995). Relationships between complex Delta expression and the specification of retinal cell fates during Drosophila eye development. Mechanisms of development *50*, 201-216.

Parsons, M.J., Pisharath, H., Yusuff, S., Moore, J.C., Siekmann, A.F., Lawson, N., and Leach, S.D. (2009). Notch-responsive cells initiate the secondary transition in larval zebrafish pancreas. Mechanisms of development *126*, 898-912.

Pasini, A., Henrique, D., and Wilkinson, D.G. (2001). The zebrafish Hairy/Enhancer-of-split-related gene her6 is segmentally expressed during the early development of hindbrain and somites. Mechanisms of development *100*, 317-321.

Pasini, A., Jiang, Y.J., and Wilkinson, D.G. (2004). Two zebrafish Notch-dependent hairy/Enhancer-of-split-related genes, her6 and her4, are required to maintain the coordination of cyclic gene expression in the presomitic mesoderm. Development *131*, 1529-1541.

Penton, A.L., Leonard, L.D., and Spinner, N.B. (2012). Notch signaling in human development and disease. Seminars in cell & developmental biology *23*, 450-457.

Perron, M., and Harris, W.A. (2000). Determination of vertebrate retinal progenitor cell fate by the Notch pathway and basic helix-loop-helix transcription factors. Cellular and molecular life sciences : CMLS *57*, 215-223.

Perron, M., Kanekar, S., Vetter, M.L., and Harris, W.A. (1998). The genetic sequence of retinal development in the ciliary margin of the Xenopus eye. Developmental biology *199*, 185-200.

Poggi, L., Vitorino, M., Masai, I., and Harris, W.A. (2005). Influences on neural lineage and mode of division in the zebrafish retina in vivo. The Journal of cell biology *171*, 991-999.

Ramachandran, R., Reifler, A., Parent, J.M., and Goldman, D. (2010). Conditional gene expression and lineage tracing of tuba1a expressing cells during zebrafish development and retina regeneration. The Journal of comparative neurology *518*, 4196-4212.

Rapaport, D.H., Wong, L.L., Wood, E.D., Yasumura, D., and LaVail, M.M. (2004). Timing and topography of cell genesis in the rat retina. The Journal of comparative neurology *474*, 304-324.

Raymond, P.A., Barthel, L.K., Bernardos, R.L., and Perkowski, J.J. (2006). Molecular characterization of retinal stem cells and their niches in adult zebrafish. BMC developmental biology *6*, 36.

Rhee, K.D., Yu, J., Zhao, C.Y., Fan, G., and Yang, X.J. (2012). Dnmt1-dependent DNA methylation is essential for photoreceptor terminal differentiation and retinal neuron survival. Cell death & disease *3*, e427.

Riesenberg, A.N., Liu, Z., Kopan, R., and Brown, N.L. (2009). Rbpj cell autonomous regulation of retinal ganglion cell and cone photoreceptor fates in the mouse retina. The Journal of neuroscience : the official journal of the Society for Neuroscience *29*, 12865-12877.

Scheer, N., and Campos-Ortega, J.A. (1999). Use of the Gal4-UAS technique for targeted gene expression in the zebrafish. Mechanisms of development *80*, 153-158.

Scheer, N., Groth, A., Hans, S., and Campos-Ortega, J.A. (2001). An instructive function for Notch in promoting gliogenesis in the zebrafish retina. Development *128*, 1099-1107.

Scholpp, S., Delogu, A., Gilthorpe, J., Peukert, D., Schindler, S., and Lumsden, A. (2009). Her6 regulates the neurogenetic gradient and neuronal identity in the thalamus. Proceedings of the National Academy of Sciences of the United States of America *106*, 19895-19900.

Silva, A.O., Ercole, C.E., and McLoon, S.C. (2003). Regulation of ganglion cell production by Notch signaling during retinal development. Journal of neurobiology *54*, 511-524.

Stenkamp, D.L. (2007). Neurogenesis in the fish retina. International review of cytology *259*, 173-224.

Trimarchi, J.M., Stadler, M.B., and Cepko, C.L. (2008). Individual retinal progenitor cells display extensive heterogeneity of gene expression. PloS one *3*, e1588.

Trimarchi, J.M., Stadler, M.B., Roska, B., Billings, N., Sun, B., Bartch, B., and Cepko, C.L. (2007). Molecular heterogeneity of developing retinal ganglion and amacrine cells revealed through single cell gene expression profiling. The Journal of comparative neurology *502*, 1047-1065.

Tropepe, V., Coles, B.L., Chiasson, B.J., Horsford, D.J., Elia, A.J., McInnes, R.R., and van der Kooy, D. (2000). Retinal stem cells in the adult mammalian eye. Science *287*, 2032-2036.

Turner, D.L., and Cepko, C.L. (1987). A common progenitor for neurons and glia persists in rat retina late in development. Nature *328*, 131-136.

Turner, D.L., Snyder, E.Y., and Cepko, C.L. (1990). Lineage-independent determination of cell type in the embryonic mouse retina. Neuron *4*, 833-845.

Wallace, V.A. (2008). Proliferative and cell fate effects of Hedgehog signaling in the vertebrate retina. Brain research *1192*, 61-75.

Wan, J., Ramachandran, R., and Goldman, D. (2012). HB-EGF is necessary and sufficient for Muller glia dedifferentiation and retina regeneration. Dev Cell *22*, 334-347.

Wetts, R., and Fraser, S.E. (1988). Multipotent precursors can give rise to all major cell types of the frog retina. Science *239*, 1142-1145.

Xu, H., Sta Iglesia, D.D., Kielczewski, J.L., Valenta, D.F., Pease, M.E., Zack, D.J., and Quigley, H.A. (2007a). Characteristics of progenitor cells derived from adult ciliary body in mouse, rat, and human eyes. Investigative ophthalmology & visual science *48*, 1674-1682.

Xu, S., Sunderland, M.E., Coles, B.L., Kam, A., Holowacz, T., Ashery-Padan, R., Marquardt, T., McInnes, R.R., and van der Kooy, D. (2007b). The proliferation and expansion of retinal stem cells require functional Pax6. Developmental biology *304*, 713-721.

Yamaguchi, M., Tonou-Fujimori, N., Komori, A., Maeda, R., Nojima, Y., Li, H., Okamoto, H., and Masai, I. (2005). Histone deacetylase 1 regulates retinal neurogenesis in zebrafish by suppressing Wnt and Notch signaling pathways. Development *132*, 3027-3043.

Yaron, O., Farhy, C., Marquardt, T., Applebury, M., and Ashery-Padan, R. (2006). Notch1 functions to suppress cone-photoreceptor fate specification in the developing mouse retina. Development *133*, 1367-1378.

Yogev, O., Williams, V.C., Hinits, Y., and Hughes, S.M. (2013). eIF4EBP3L acts as a gatekeeper of TORC1 in activity-dependent muscle growth by specifically regulating Mef2ca translational initiation. PLoS biology *11*, e1001679.

Zuber, M.E., Gestri, G., Viczian, A.S., Barsacchi, G., and Harris, W.A. (2003). Specification of the vertebrate eye by a network of eye field transcription factors. Development *130*, 5155-5167.

Zupanc, G.K., and Sirbulescu, R.F. (2011). Adult neurogenesis and neuronal regeneration in the central nervous system of teleost fish. The European journal of neuroscience *34*, 917-929.